FAK and Pyk2 Regulate the Intracellular Signaling Networks Required for Integrin-mediated Migration and Phagocytosis by Macrophages

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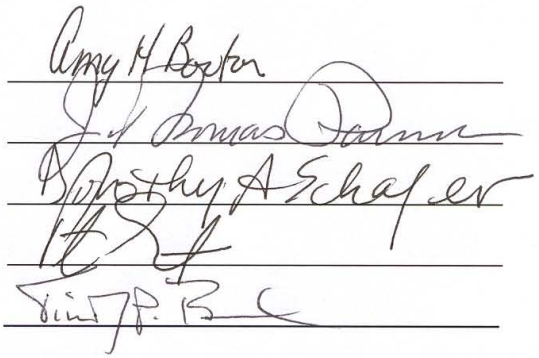

Abstract

As an essential component of the immune system, macrophages are capable of a diverse range of functions including integrin-mediated adhesion to and extravasation from blood vessels, chemotaxis, phagocytosis and the release of cytokines. The FAK family of kinases consists of two closely related protein tyrosine kinases, FAK and Pyk2, which function as important regulators of integrin-mediated signaling. The generation of myeloid-specific conditional FAK knockout mice has allowed us to examine the complex interplay between FAK, Pyk2 and other downstream effectors involved in integrin signaling in primary macrophages. In this thesis, I demonstrate that macrophages derived from these mice display significant motility defects *in vitro* and that the loss of FAK *in vivo* results in reduced levels of inflammatory monocyte infiltration to sites of inflammation. These data indicate that by disrupting FAK in monocyte/macrophages, such that the ability of these cells to migrate is inhibited, the macrophage-specific host response to inflammation can be altered. These results have significant consequences when considered within the context of chronic inflammatory diseases and cancer. Importantly, we establish that the invasive capacity of tumor cells co-cultured with $FAK^{-/-}$ macrophages is reduced and provide preliminary *in vivo* evidence indicating that tumor outgrowth may be delayed in myeloid-specific conditional FAK knockout mice.

Further investigation of the signaling pathways controlling macrophage motility reveals that migration is regulated by two distinct pathways: one dependent on the expression of FAK and Pyk2, and the other pathway dependent on the linkage between

α4-integrin and the adapter protein paxillin. I have also examined the host signaling networks involved in the integrin-mediated phagocytosis of the enteropathogen *Yersinia pseudotuberculosis*, demonstrating that simultaneous expression of the two Yersinia adhesins, invasin and YadA, stimulates two independent pathways in which FAK and Pyk2 perform distinct, non-redundant functions required for bacterial internalization. In summary, the studies presented here shed light on the molecular signaling involved in macrophage motility and phagocytosis, and contribute to a more comprehensive understanding of the regulation of the actin cytoskeleton in cells of the myeloid lineage.

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Abbreviations

BMM	**Bone marrow macrophage**
bFGF	**Basic fibroblast growth factor**
BHK	**Baby hamster kidney**
BSA	**Bovine serum albumin**
Cas	**Crk associated substrate**
CCD	**Charged-coupled device**
COPD	**Chronic obstructive pulmonary disease**
CR	**Complement receptor**
CSF-1	**Colony stimulating factor-1**
CSF-1R	**Colony stimulating factor-1 receptor**
ECM	**Extracellular matrix**
EGF	**Epidermal growth factor**
EGFR	**Epidermal growth factor receptor**
ERK	**Extracellular related kinase**
FAK	**Focal adhesion kinase**
FAT	**Focal adhesion targeting**
FBS	**Fetal bovine serum**
FERM	**Four point one-ezrin-radixin-moesin**
FN	**Fibronectin**
FRET	**Fluorescence resonance energy transfer**
FRNK	**FAK related non-kinase**

GAP	**Guanine-nucleotide activating factor**
GEF	**Guanine-nucleotide exchange factor**
GEMM-CFU	**Granulocyte-erythrocyte-megakaryocyte-macrophage-colony forming unit**
GFP	**Green fluorescent protein**
GM-CFU	**Granulocyte-macrophage-colony forming unit**
GM-CSF	**Granulocyte-macrophage-colony stimulating factor**
ICAM	**Intracellular adhesion molecule**
IF	**Immunofluorescence**
IFN	**Interferon**
IgSF	**Immunoglobin superfamily**
IL	**Interleukin**
IPTG	**Isopropyl-beta-D-thiogalacopyranoside**
LFA-1	**Leukocyte functional antigen-1**
LPS	**Lipopolysaccharide**
LysM	**Lysozyme M**
MAPK	**Mitogen activated protein kinase**
M-CFU	**Macrophage-colony forming unit**
MEF	**Mouse embryo fibroblast**
MLC	**Myosin light chain**
MLCK	**Myosin light chain kinase**
MMP	**Matrix metalloproteinase**
MOI	**Multiplicity of infection**

MS	**Multiple sclerosis**
MT1-MMP	**Membrane-tethered 1 matrix metalloproteinase**
MTOC	**Mitotic organizing center**
NHS	**N-Hydroxysuccinimide**
NF-κB	**Nuclear factor-κB**
NO	**Nitric oxide**
OM	**Outer membrane**
PBS	**Phosphate buffered saline**
PDGF	**Platelet derived growth factor**
PDGFR	**Platelet derived growth factor receptor**
PI3K	**Phosphoinositol 3 kinase**
PIP_2	**Phosphatidylinositol(3,4,5)triphosphate**
PKA	**Protein kinase A**
PRNK	**Pyk2 related non-kinase**
PSI	**Plexin-semaphorin-integrin**
PTK	**Protein tyrosine kinase**
Pyk2	**Proline rich tyrosine kinase 2**
RNAi	**RNA interference**
ROCK	**Rho kinase**
ROI	**Reactive oxygen intermediate**
RSV	**Rous sarcoma virus**
RTK	**Receptor tyrosine kinase**
SFK	**Src family kinase**

TAM	**Tumor-associated macrophage**
TG	**Thioglycollate**
TGF	**Transforming growth factor**
TH1	**T helper 1**
TH2	**T helper 2**
TIRF	**Total internal reflection fluorescence**
TLR	**Toll-like receptor**
TNF	**Tumor necrosis factor**
VEGF	**Vascular endothelial growth factor**
WASP	**Wiskott Aldrich syndrome protein**
WT	**Wildtype**
YOPs	**Yersinia outer proteins**

Acknowledgements

First, I would like to acknowledge my mentor, Dr. Amy Bouton. When I began working in Amy's lab in the summer of 2002, the lab contained three graduate students and an undergraduate "scrubbie." Fast-forward to the summer of 2007, and the lab has grown to support nine graduate students, two full-time technicians, an undergraduate "scrubbie " and various rotating students. I truly believe this growth is a reflection of both the phenomenal mentorship provided by Amy, and the quality of research accomplished in the lab. More than anything, she has set an example of the type of scientist I hope to become in the future, and I can say without reservation that my graduate school experience has been exceptional under her guidance. I have also been extremely fortunate to have spent the last several years working with an extraordinary group of talented colleagues. To the members of the "pathogenesis group," Michelle Abshire, Brianne Ray and Kat Schorenberg, and former lab members Drs. Kris Hudson and Tasha Mitchell-Torok, thank you for your continual scientific input to this project. I would also like to thank Randy Schrecengost, Huy Ta and Tasha Schuh, as well as the newest members, Michael Guerrero and Michael Gutknecht, for making the lab a great place to study and work. Finally, the lab would not run as smoothly as it does without the exceptional efforts of our technicians Keena Thomas and Stacey Donelan.

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Chapter 1: Introduction

1.1. Overview.

The primary function of the immune system is to protect the host from infectious agents in the environment (Chaplin, 2006). While the innate immune system recognizes molecular patterns common to many types of pathogens (e.g. bacterial lipopolysaccharide, LPS), the adaptive immune system is capable of responding to unique microbial structures. Cells of the myeloid lineage, including macrophages, neutrophils and dendritic cells (DCs), play a major role in the maintenance of tissue homeostasis and are key effectors of immunological defense. In particular, macrophages are large, highly motile phagocytic cells that are critically involved in the establishment of innate immunity as well as the cytokine-mediated regulation of the acquired immune response (Unanue and Allen, 1987; Medzhitov and Janeway, 1998). This chapter will focus on macrophage development as well as the intracellular signaling networks that are required for macrophages to perform a diverse range of integrin-mediated functions, including chemotactic migration and bacterial phagocytosis. Specific emphasis will be placed on the roles played by focal adhesion kinase (FAK) and proline rich tyrosine kinase 2 (Pyk2), both of which function as important regulators of integrin and adhesion signaling.

1.2. Macrophage differentiation and maturation.

Macrophages represent a heterogeneous population of mononuclear phagocytes. The generation of these cells takes place in sequential stages and is controlled by multiple transcription factors, which are either constitutively expressed or induced at certain stages

of cell differentiation (Tenen *et al.*, 1997). In the presence of interleukin (IL)-1, IL-3 and/or IL-6, pluripotent myeloid cells (granulocyte-erythrocyte-megakaryocyte-macrophage-colony forming units: GEMM-CFUs) give rise to progenitors of granulocytes and macrophages, known as granulocyte-macrophage colony forming units (GM-CFUs) (Figure 1.1) (Valledor *et al.*, 1998). Further stimulation with IL-3, as well as the growth factors granulocyte-macrophage colony stimulating factor (GM-CSF) and colony stimulating factor-1 (CSF-1) promote the proliferation of these myeloid-lineage cells and ultimately their differentiation into monocytic precursors.

1.2.1. Lysozyme M (LysM) expression.

Lysozyme is an antibacterial enzyme involved in host defense (Cross *et al.*, 1988). In contrast to humans, which express one lysozyme gene (Peters *et al.*, 1989), the murine genome encodes for two lysozyme genes: the M gene, specific for myeloid cells and the P gene, specific for Paneth cells located in the small intestine (Cross and Renkawitz, 1990). Although lysozyme protein can be found in most tissues, studies at the cellular level have revealed high levels of lysozyme specifically in phagocytic cells, including macrophages and granulocytes as well as Paneth cells (Cross *et al.*, 1988). More recently, analyses examining the blood and bone marrow of mice in which a gene expressing enhanced green fluorescent protein (EGFP) was "knocked-in" to the *lysM* locus, have revealed that myelomonocytic cells (monocyte/macrophages and neutrophils), were fluorescent positive, while other cell lineages were EGFP-negative (Faust *et al.*, 2000). From these and other studies, LysM is likely expressed at the granulocyte/monocyte precursor stage of hematopoietic differentiation (see asterisk,

Figure 1.1). Consequently, *lysM* promoter activity may be used to successfully direct transgene expression specifically in cells of the myeloid lineage (Clausen *et al.*, 1999).

1.2.2. Colony stimulating factor.

As a key regulator of macrophage differentiation, CSF-1 promotes the early transition of GM-CFUs into monocyte precursors cells, and the maturation of blood monocytes into fully functional tissue macrophages (Figure 1.2) (for reviews, see Stanley *et al.*, 1997; Valledor *et al.*, 1998; Pixley and Stanley, 2004). At the molecular level, CSF-1 is a disulfide-linked homodimer that binds and stabilizes the CSF-1 receptor (CSF-1R), a tyrosine kinase encoded by the proto-oncogene *c-fms*. Ligation of the CSF-1R results in receptor autophosphorylation and activation in *trans,* leading to rapid stimulation of cytoskeletal remodeling, gene transcription and protein translation (Yeung and Stanley, 2003). The importance of CSF-1 and the CSF-1 receptor to macrophage differentiation is underscored by the fact that mice lacking the *CSF-1* gene (osteopetrotic, $Csf1^{op}$) or mice that are nullizygous for the CSF-1R ($Csf1r^{-/-}$) have severely decreased numbers of macrophages and bone-resorbing osteoclasts (Wiktor-Jedrzejczak *et al.*, 1990; Yoshida *et al.*, 1990; Cecchini *et al.*, 1994).

Circulating blood monocytes give rise to a variety of tissue-resident macrophages throughout the body (Gordon and Taylor, 2005). These cells also form a pool of mononuclear phagocytes available to respond to various inflammatory or immunological stimuli. Differentiation into mature macrophages occurs in peripheral tissues and is characterized by an increase in cell size, lysosomal and mitochondrial content and enhanced energy metabolism (Valledor *et al.*, 1998). In addition to CSF-1, the

Figure 1.1. Differentiation of stem cells into monocyte/macrophages. In the bone marrow, IL-1, IL-3 and IL-6 induce heteromitosis in the stem cell. This gives rise to a new stem cell and a pleuripotent myeloid cell (GEMM-CFU). In the presence of IL-1 and/or IL-3, GEMM-CFUs commit to becoming progenitors of both macrophages and granulocytes (GM-CFUs). The growth factor GM-CSF promotes proliferation of these myeloid precursor cells while M-CSF induces the proliferation and differentiation of GM-CFUs into monocytic precursor cells (M-CFUs). Promonocytes, which display limited phagocytic capability, are the first detectable cells arising from the terminal monocytic stage. Bone marrow-derived monocytes enter tissues via peripheral blood vessels where they differentiate into macrophages and establish a resident population of cells. The growth factors involved at each stage are indicated. Asterisk indicates probable LysM expression. Curved arrows indicate the points where derivations to other lineages are generated (adapted from Valledor *et al.*, 1998).

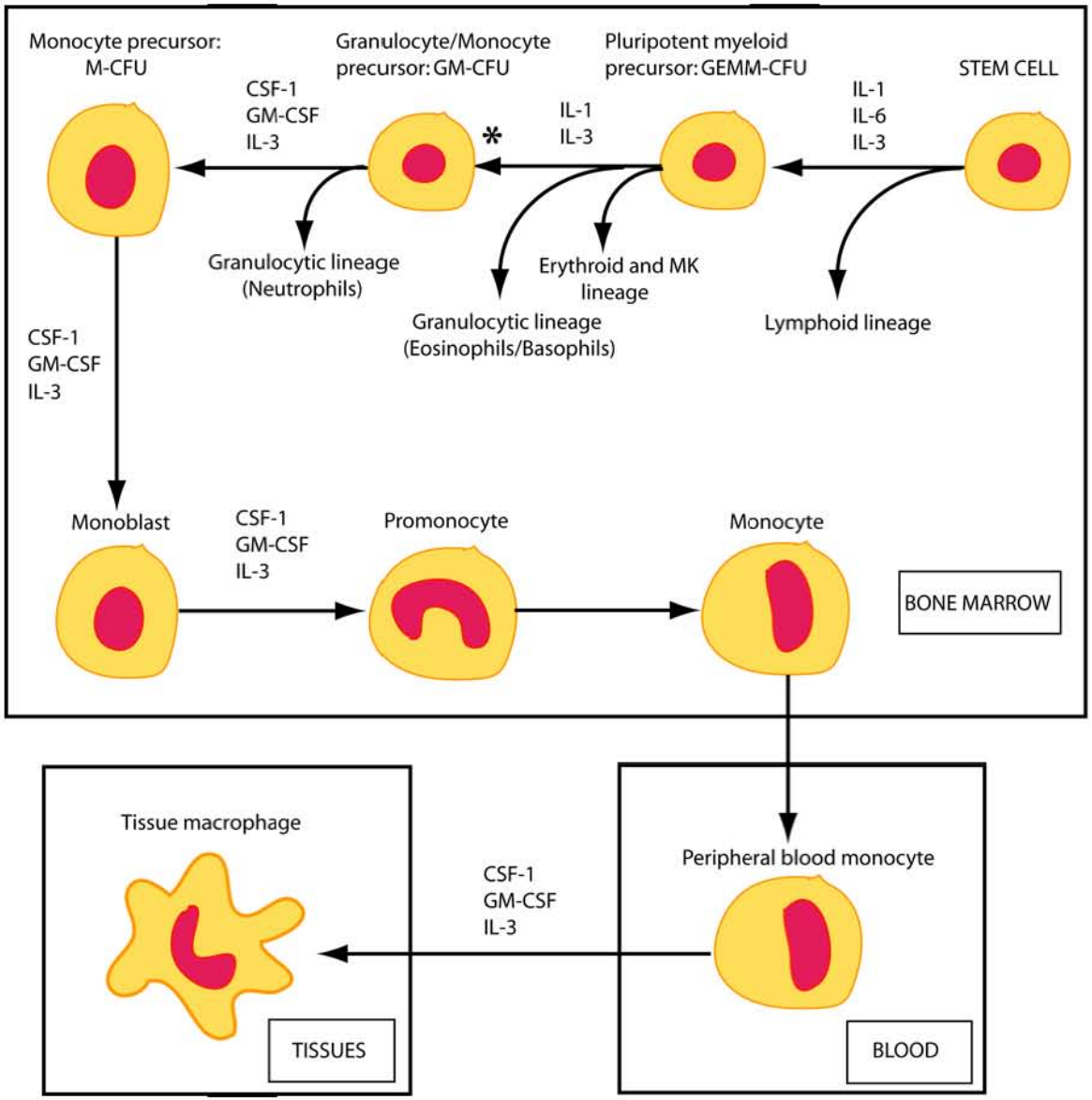
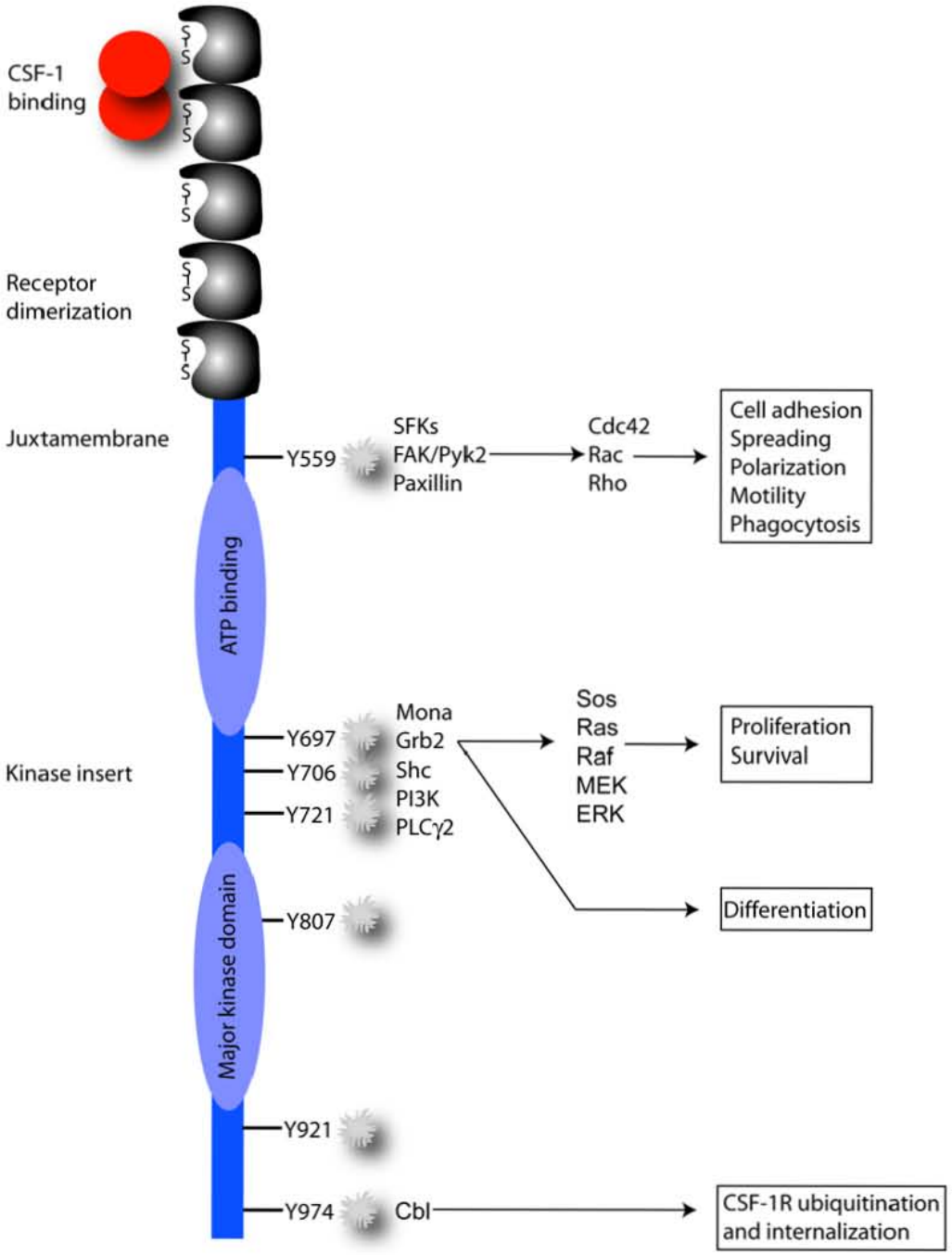

Monocyte precursor:
M-CFU
Granulocyte/Monocyte
precursor: GM-CFU
Pluripotent myeloid
precursor: GEMM-CFU
STEM CELL
CSF-1
GM-CSF
IL-3
*
IL-1
IL-3
IL-1
IL-6
IL-3
Granulocytic lineage
(Neutrophils)
Erythroid and MK
lineage
Granulocytic lineage
(Eosinophils/Basophils)
Lymphoid lineage
CSF-1
GM-CSF
IL-3
Monoblast
CSF-1
GM-CSF
IL-3
Promonocyte
Monocyte
BONE MARROW
Tissue macrophage
CSF-1
GM-CSF
IL-3
Peripheral blood monocyte
TISSUES
BLOOD

Figure 1.2. CSF-1 receptor structure and binding partners. The CSF-1R contains an extracellular ligand-binding domain, a transmembrane domain and an intracellular tyrosine kinase domain that is interrupted by a kinase insert domain. CSF-1 is a disulfide linked homodimer that stabilizes CSF-1R dimerization and facilitates receptor *trans* autophosphorylation. In the murine response to CSF-1, seven tyrosine residues are known to be phosphorylated upon receptor ligation. Phosphorylation of most of these tyrosine residues creates binding sites for signaling molecules that contain specific phospho-tyrosine-binding domains. Although the precise signaling pathways that connect the activated CSF-1R with direct effectors of actin cytoskeleton remodeling have not been delineated, Src family kinases (SFKs), paxillin and Pyk2 have been identified as important regulators of CSF-1-dependent macrophage adhesion and motility. The signaling pathways involved in the regulation of macrophage proliferation also remain unclear but may include the activation of extracellular-related kinase (ERK). Phosphoinositol 3 kinase (PI3K) is involved in CSF-1 related macrophage survival but not proliferation. Finally, differentiation has primarily been studied in the context of committed macrophage progenitors and likely involves multiple signaling pathways (adapted from Pixley and Stanley 2004).


CSF-1
binding
Receptor
dimerization
Juxtamembrane
Y559
SFKs
FAK/Pyk2
Paxillin
Cdc42
Rac
Rho
Cell adhesion
Spreading
Polarization
Motility
Phagocytosis
ATP binding
Kinase insert
Y697
Y706
Y721
Mona
Grb2
Shc
PI3K
PLCγ2
Sos
Ras
Raf
MEK
ERK
Proliferation
Survival
Differentiation
Major kinase domain
Y807
Y921
Y974
Cbl
CSF-1R ubiquitination
and internalization

stimulation of cell adhesion molecules also provide external signals required for the activation of transcription factors, such as AP-1 and NF-κB, involved in the maturation of monocytes (Bianchi *et al*., 2000; Shi *et al*., 2001; Shi and Simon, 2006). Macrophage function is predominately tissue-specific despite the common origin of these cells in the bone marrow; hence splenic macrophages are responsible for the phagocytosis of apoptotic red blood cells while Langerhans cells in the skin internalize and present antigen to proximal lymphocytes. Taken together, myelopoiesis is a complex process in which temporal and lineage-specific gene expression promote the generation of a heterogeneous population of macrophages essential for development and tissue homeostasis.

1.3. Macrophage function.

1.3.1. Leukocyte trafficking.

Leukocyte trafficking is the broad physiological process whereby cells of the immune system sense and respond to chemotactic stimuli by emigrating toward the source of inflammation. The recruitment of mononuclear phagocytes to sites of infection or tissue injury is mediated by the expression of cell adhesion molecules that are induced on the surface of local blood vessel endothelium. Chemoattractant distress signals released by cells in damaged tissues trigger initial intravascular leukocyte adhesion to endothelial cells and establish a gradient for interstitial leukocyte migration and positioning in inflamed tissues (Luster *et al*., 2005). The up-regulation of P- and E-selectins on activated endothelium initiates endothelium-leukocyte interactions by weakly

binding to sulfated sialyl-Lewis^X oligosaccharides expressed on the surface of circulating leukocytes. L-selectin, expressed by leukocytes, recognizes and binds to similarly modified sugars on the surface of inflamed endothelial cells. All selectins share highly conserved N-terminal C-type lectin domains followed by regulatory epidermal growth factor (EGF)-like domains (Alon and Dustin, 2007). These reversible "capture" interactions give rise to leukocyte rolling, a process that can last from seconds to minutes depending on leukocyte type and the target endothelial bed (Ley, 2002; McEver, 2002). When rolling cells encounter chemokines, the activating signal induces rapid activation of β2 (αLβ2/leukocyte functional antigen-1/LFA-1) and/or α4 integrins (α4β1/very late antigen-4/VLA-4 and α4β7), which assume extended conformations and bind tightly to endothelial immunoglobin superfamily members (intracellular adhesion molecules/ICAMs) (Kinashi, 2007). Once arrested, leukocytes polarize and move by diapedesis across the venule wall. Successful extravasation requires localized degradation of the underlying endothelial basement membrane and the activation of extracellular matrix (ECM)-binding integrins (αMβ2/Mac-1 and α5β1/VLA-5). Monocytes responding to chemokine gradients in the proximity of inflamed or damaged tissues mature into tissue macrophages and are responsible for the phagocytosis of invading pathogens, presentation of antigen, clearance of cellular debris and the promotion of healing. Thus, the generation of a regulated immune response is dependent on the ability of cells such as monocyte/macrophages to migrate in response to integrated chemical signals (Baggiolini, 1998; Jones, 2000).

1.3.2. Cell migration.

Myeloid leukocytes comprise the first line of host defense against infection. Consequently, the directed migration of these cells in response to perturbations in tissue homeostasis as described above is an essential component of their function. The initial response of an isolated tissue macrophage to a migration stimulus is to polarize and extend protrusions in the form of broad lamellipodia and spike-like filopodia in the direction of locomotion (Calle *et al*., 2006). These protrusive structures are stabilized by adhesion to the ECM and their formation is regulated by the dynamic reorganization of the actin cytoskeleton and tubulin-based microtubules (Jones, 2000; Worthylake and Burridge, 2001). Motility also requires the disassembly of adhesions at the trailing edge of a moving cell, allowing for uropod detachment and retraction. In contrast to fibroblasts and other mesenchymal-derived cell types, leukocytes are highly deformable and, because of the lack of stable focal contacts and the absence of actin stress fibers, move with high velocities (2-30 μm/hr) (Friedl *et al*., 1998). Leukocytes have the ability to sense a wide variety of chemoattractants, including bacterial components, leukotrienes, complement factors, cytokines and chemokines that function as migratory guides within tissues (Foxman *et al*., 1997; Foxman *et al*., 1999; Turner *et al*., 1999). The stimulus to migrate is transduced through both G-protein coupled receptors and receptor tyrosine kinases.

1.3.3. Polarization and protrusion formation.

In order to migrate, cells must first establish polarity, or a “front” and a “rear.” Cdc42 is a member of the Rho family of small GTPases that also include Rac and Rho

proteins. This molecule influences cell polarity by either restricting the region in which lamellipodia can form and/or by orienting the Golgi apparatus and microtubule organizing center (MTOC) toward the leading cell edge (Etienne-Manneville and Hall, 2002; Rodriguez *et al*., 2003; Srinivasan *et al*., 2003). In fibroblasts, recent evidence points to a role for FAK in similarly regulating the formation of a leading edge by coordinating integrin signaling and directing the reorientation of cellular organelles (Tilghman *et al*., 2005). PI3Ks have also been extensively implicated in driving the initial steps of cell polarization and migration (Sotsios and Ward, 2000; Vanhaesebroeck *et al*., 2001; Stephens *et al*., 2002). Chemokinetic signaling through G-protein coupled receptors results in the early activation of target enzymes like PI3Ks. These enzymes generate phosphatidylinositol(3,4,5) triphosphate (PIP_3) and $PI(3,4)P_2$, both of which are key signaling molecules that become concentrated at the leading edge of chemotaxing cells. There are four catalytic subunits of class I PI3Ks, α, β, γ and δ, of which PI3Kγ is primarily expressed in hematopoietic cells. Macrophages derived from PI3Kγ–null mice exhibit significant polarization and chemotactic defects associated with an inability to migrate persistently in one direction (Jones *et al*., 2003). In addition, pharmacological inhibition of PI3K activity results in a loss of adhesion structures and cell protrusions (Wheeler *et al*., 2006a).

Lamellipodia formation also requires the localization of activated Rac to the leading edge of migrating cells. Mammals have three highly homologous Rac genes, *Rac1*, *Rac2* and *Rac3*, of which *Rac1* and *Rac2* are expressed in leukocytes. Rac functions as a morphological switch that cycles between a GTP-bound (active) state and a GDP-bound (inactive) state. The GTP/GDP binding cycle is regulated by the interaction

of Rac with guanine-nucleotide exchange factors (GEFs) that catalyze the exchange of GDP for GTP, and GTPase activating proteins (GAPs) that enhance the hydrolysis of GTP to GDP. Several Rac GEFs are activated by derivatives of PI3K signaling and the local delivery of Rac-specific GEFs likely promotes the activation of Rac at the periphery (Welch *et al*., 2003). Integrin engagement also leads to Rac activation and membrane targeting (Del Pozo *et al*., 2000). In particular, α4 integrins (α4β1/VLA-4 and α4β7), which are prominently expressed on leukocytes, are able to confine Rac activity to the cell anterior during cell migration (Kummer and Ginsberg, 2006). α4 integrin phosphorylation only occurs at the leading edge of migrating cells due to the precise spatial localization of protein kinase A (PKA). PKA-mediated phosphorylation prevents the binding of the cytoskeletal adaptor paxillin to the integrin cytoplasmic tail, which allows active Rac to localize to the leading cell edge and promote protrusion formation. However, at other sites around the cell periphery, α4 integrins tightly associate with paxillin. This interaction recruits Rac GAPs to the sides and rear of migrating cells, thereby inhibiting Rac activity and the subsequent generation of stable lamellipodia at these locations.

One of the major functions of Rac is to induce actin polymerization in lamellipodia and membrane ruffles by regulating Wiskott-Aldrich syndrome proteins (WASP/WAVE) and the Arp2/3 complex. Rac stimulates lamellipodia formation by activating WASP/WAVE. Members of the WASP family localize with Arp2/3 complexes at the sites of existing actin filaments, facilitating the generation of branched daughter filaments and the generation of a protruding force (Welch, 1999; Cory and Ridley, 2002). WASP-mediated actin polymerization is required for macrophage migration, as the loss

of this protein results in a failure to generate adhesion structures and reduced motility (Jones *et al.*, 2002). Conversely, despite the established role of Rac in the production of lamellipodia, this molecule is not required for efficient macrophage migration (Wells *et al.*, 2004). Rac1-deficient macrophages exhibit altered cell morphologies, appearing less well spread and significantly more elongated than wildtype (WT) control cells. However, these cells migrate normally in response to chemotactic stimuli. The deletion of other Rac isoforms, including Rac2, also has little effect on macrophage chemotaxis, with Rac1/2$^{-/-}$ cells able to migrate further and faster than WT cells under certain conditions (Wheeler *et al.*, 2006b). The loss of Rac1, but not Rac2 did affect the ability of cells to invade through 3-dimensional substrates, indicating that Rac1 may specifically contribute to invasion.

1.3.4. Adhesion disassembly and rear retraction.

Integrin-mediated adhesions at the base of a protrusion disassemble as new adhesions form nearer the leading edge in migrating cells (Webb *et al.*, 2002). In the case of macrophages, adhesion structures are often concentrated towards the front of polarized cells (Calle *et al.*, 2006). The role of FAK in adhesion turnover is supported by studies showing that FAK-null fibroblasts exhibit migration defects associated with the formation of stable adhesion structures (Ilic *et al.*, 1995; Ren *et al.*, 2000). It has been hypothesized that FAK may control focal adhesion disassembly by down-regulating Rho activity. The inhibition of Rho in FAK$^{-/-}$ fibroblasts causes these cells to resemble WT cells whereas the over-expression of activated Rho in normal fibroblasts induces the FAK$^{-/-}$ phenotype (Ren *et al.*, 2000). Alternatively, there is evidence supporting a model

in which activated FAK and c-Src promote the localization and activation of Rac at the leading edge, resulting in adhesion turnover at the cell periphery. It is currently unknown whether FAK is similarly involved in the turnover of the adhesion structures found in macrophages.

The process of retraction may be mediated by several inter-related signaling pathways involving c-Src, FAK/Pyk2, Rho and myosin II. The interaction between myosin II and actin filaments generates contractile forces localized at sites of adhesion (Ridley *et al*., 2003). Myosin activity, which is regulated by myosin light chain (MLC) phosphorylation, results in increased contractility and the transmission of tension to adhesion sites. The phosphorylation of myosin is mediated by myosin light chain kinase (MLCK) or Rho kinase (ROCK), which in turn is regulated by GTP-bound Rho. In macrophages, signaling through Pyk2 has been implicated in uropod retraction since Pyk2-deficient macrophages were unable to generate the contractile force necessary for cell migration (Okigaki *et al*., 2003). Because Rho activation has been shown to be critical for the contraction of lamellipodia, the decreased contractility observed in $Pyk2^{-/-}$ macrophages could be due to decreased levels of Rho activity in these cells.

1.3.5. Phagocytosis.

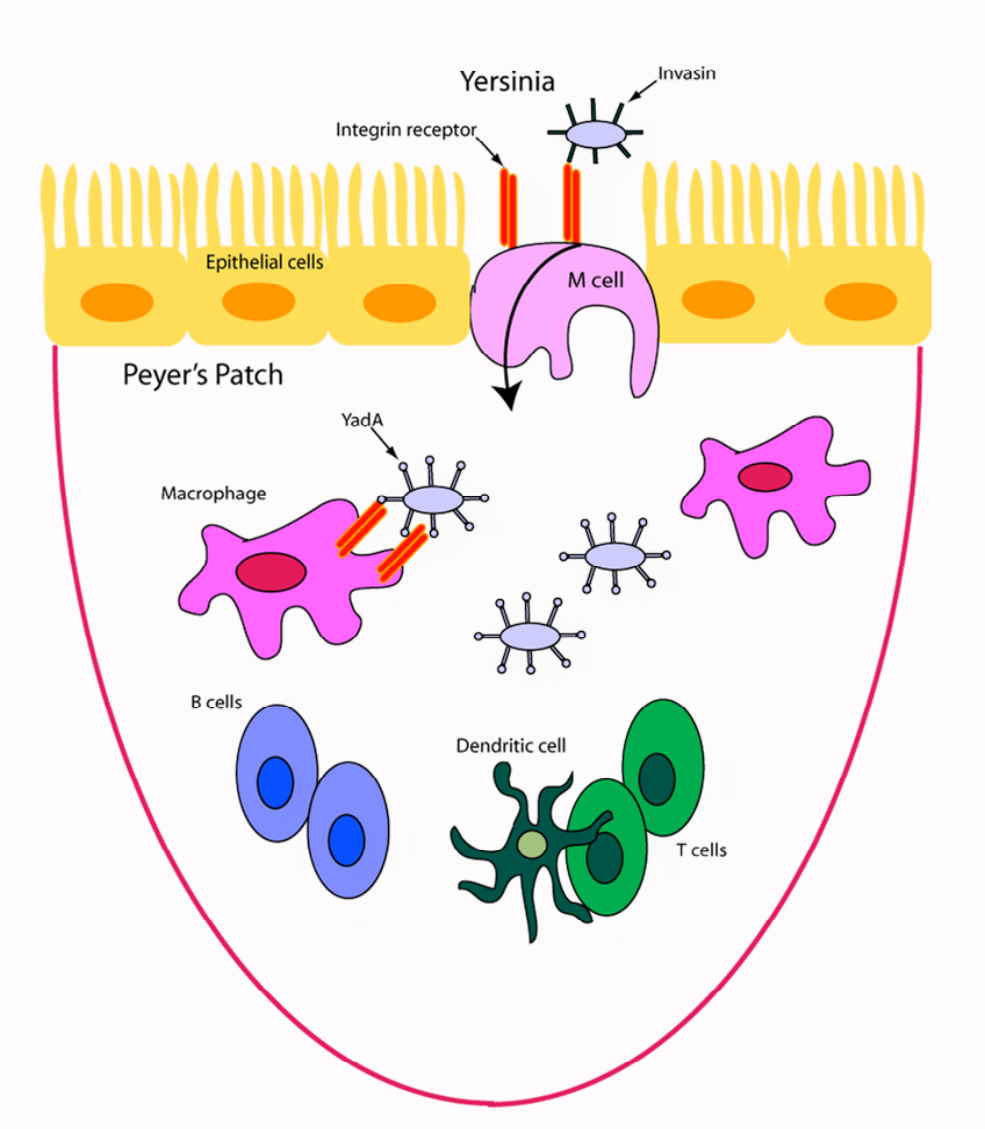
Phagocytosis is the uptake of macromolecules and large particles accompanied by actin-based cytoskeletal changes. The phagocytic process begins when particles bind to specific cell surface receptors, triggering the clustering of receptors within the membrane at the site of particle contact (Kwiatkowska and Sobota, 1999). The receptors involved in mediating phagocytosis may be specifically located on professional phagocytes, such as

Fc and complement receptors (CRs), or more generally expressed on a wide variety of cell types, as is the case for integrin receptors. Most cells, professional phagocytes and non-phagocytic cells included, have the basic machinery required for the uptake of extracellular material (Aderem and Underhill, 1999). Actin polymerization leads to membrane extension outward to surround the particle, resulting in engulfment. Phagocytic receptors present on professional phagocytes, such as macrophages, also have the ability to mediate cellular adherence to substrates such as ECM components (May and Machesky, 2001). For example, adhesion mediated through CR3 is coupled to the appearance of modified focal adhesions at CR-associated phagosomes (Allen and Aderem, 1996). Thus, phagocytosis can involve the recruitment of many of the same components associated with focal adhesion structures, among them FAK and Pyk2.

1.3.6. Yersinia pathogenesis

The enteropathogenic yersiniae (*Y. pseudotuberculosis* and *Y. enterocolitica*) cross the intestinal epithelium and gain access to subepithelial regions of the gut through M cells, specialized epithelial cells located over aggregated lymphoid follicles (Peyer's patches) (Figure 1.3). Initial adherence to M cells is mediated by the Yersinia-encoded invasin protein, an outer membrane adhesin that binds with high affinity to β1 integrin receptors present on the surface of M cells (Isberg *et al.*, 1987; Leong *et al.*, 1990). The interaction between dimerized invasin and clustered β1 integrin receptors results in the activation of cellular signaling cascades important for the regulation of the actin cytoskeleton. The signal generated by invasin-host binding induces the formation of a

Figure 1.3. Establishment of *Y. pseudotuberculosis* infection. Initial host-pathogen contact is mediated through high affinity interactions between the Yersinia invasin protein and integrin receptors present on the surface of M cells. Contact between invasin and integrin promotes the uptake and translocation of bacteria across the epithelial cell layer and into the underlying Peyer's Patch. Upon entry into the subepithelial layer, *Y. pseudotuberculosis* is capable of injecting a number of anti-phagocytic and cytotoxic effector molecules that help promote pathogen colonization. In the uncompromised host, Yersinia infections are effectively eliminated by the overwhelming presence of host macrophages.

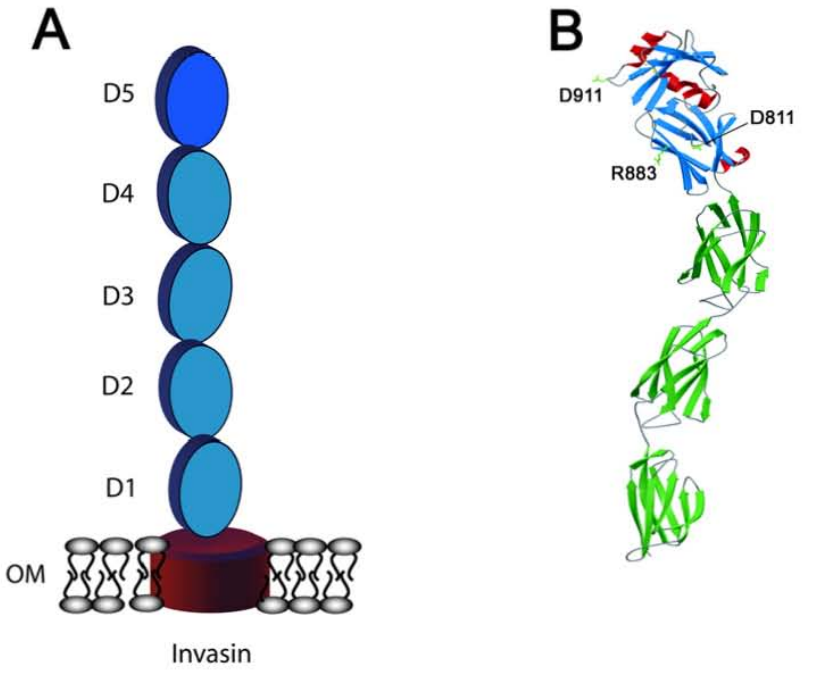

Yersinia
Invasin
Integrin receptor
Epithelial cells
M cell
Peyer's Patch
YadA
Macrophage
B cells
Dendritic cell
T cells

phagocytic cup that allows for the internalization and translocation of whole bacteria through M cells and into the underlying Peyer's patches. Upon entry into the lymphoid tissues, expression of invasin is down-regulated while expression of YadA, a second non-pilus associated adhesin is up-regulated (El Tahir and Skurnik, 2001; Nagel *et al*., 2001). YadA mediates close contact with the leukocytes in the Peyer's patch facilitating the injection of Yersinia outer membrane proteins (Yops) into the host cell cytoplasm via a Type III secretion system. The delivery of Yop effector proteins directly antagonizes the phagocytic process and enables enteropathogenic Yersinia species to evade phagocytosis by host macrophages. The ability of *Y. pseudotuberculosis* to establish an infection is therefore dependent upon two spatially and temporally separated integrin-mediated events. Initial adhesion between the bacterially-expressed invasin protein and β1 integrin receptors located on M cells results in the transcytosis of *Y. pseudotuberculosis* across the intestinal epithelium, while the second adhesion event, involving YadA and possibly other adhesins, serves to prevent further phagocytosis by macrophages in the lymphoid tissues.

1.3.7. Adhesin-mediated uptake *in vitro* and *in vivo*.

The Yersinia-expressed invasin protein is composed of five immunoglobin-superfamily-like domains (D1-D5; Figure1.4A). Amino terminal domain 1 anchors the molecule to the bacterial outer membrane, while distal domains 4 and 5 are required for binding to β1 integrin receptors. Invasin-mediated adherence to mammalian cells alone is

Figure 1.4. Domain structure of invasin and YadA. A. Schematic model of the structure of invasin. Invasin is composed of five extracellular domains (blue) that extend approximately 180Å from the bacterial outer membrane (OM). Domains 1-4 adopt folding topology related to the eukaryotic immunoglobin superfamily (IgSF), while domain 5 folding topology is similar to that of C-type lectin domains. Extensive interactions between domains 4 and 5 create a superdomain that is required for integrin binding. The N-terminal residues (red) anchor invasin in the OM (Hamburger *et al.*, 1999). **B.** Ribbon structure of the extracellular domains of invasin. Domains 1-4 are composed of β-sheets while domain 5 is made up of α-helices and β-sheets. Residues specifically implicated in integrin binding include Asp911, Asp811 and Arg883. **C.** Schematic model of the structure of YadA. YadA is a homotrimeric collagen binding molecule composed of a globular head domain, a rigid stalk and a C-terminal anchor (Hoiczyk, 2000). **D.** Stereo picture of the trimeric YadA head domain. Individual monomers are represented by different colors (Nummelin *et al.*, 2004).


A
D5
D4
D3
D2
D1
OM
Invasin
B
D911
D811
R883
C
Head
Stalk
Anchor
OM
YadA
D

not sufficient to promote uptake. Rather multiple integrin receptors must bind to invasin in a circumferential zipper-like formation, a process that eventually leads to internalization. Invasin self-association presumably aids integrin receptor multimerization and clustering, resulting in the extension of membrane protrusions and the internalization of bacteria (Dersch and Isberg, 1999).

The efficient uptake of both invasin-coated beads and intact, Yop-deficient *Y. pseudotuberculosis* into epithelial cells requires FAK (Alrutz and Isberg, 1998; Bruce-Staskal and Bouton, 2001). Inhibition of FAK activity with dominant-interfering molecules prevents efficient internalization, but not adherence of invasin-expressing *Escherichia coli*, suggesting a defect in completion of the phagocytic cup (Alrutz and Isberg, 1998). In FAK-null fibroblasts challenged with *Y. pseudotuberculosis*, full bacterial internalization rarely occurs, possibly as a result of the inability to extend membrane protrusions at the local site of adherence (Bruce-Staskal *et al.*, 2002). The association between the adaptor molecules Crk-associated substrate (Cas) and Crk has been shown to be important during integrin-mediated processes (Klemke *et al.*, 1998). Previous studies have indicated that the infection of HeLa cells expressing endogenous FAK results in the formation of Cas-Crk signaling complexes, and that signaling downstream of Crk is essential for efficient Yersinia uptake (Weidow *et al.*, 2000). However, the uptake of Yersinia can be mediated via FAK-independent signaling pathways. The over-expression of Cas in FAK-deficient fibroblasts is able to restore Yersinia internalization back to the levels observed in FAK-expressing cells. Expression of dominant-negative Crk in FAK-null cells was observed to significantly inhibit Cas-dependent *Y. pseudotuberculosis* uptake (Bruce-Staskal *et al.*, 2002). In this case,

disruption of Cas-Crk complexes may be affecting the downstream signaling molecule Rac, resulting in reduced Rac activity and the loss of membrane protrusions. Indeed, expression of dominant-negative Rac1 inhibited Cas-dependent Yersinia uptake in FAK-deficient mouse embryo fibroblasts (MEFs) (Bruce-Staskal *et al*., 2002). Together these data indicate that the Cas-Crk-Rac1 pathway, which has been shown previously to function during Yersinia uptake in HeLa cells, can also function independently of FAK in MEFs to promote the internalization of *Y. pseudotuberculosis*.

The extracellular environment has the potential to play a critical role in dictating bacteria-host cell interactions. Extensive work in our lab has focused on the signaling networks required for Yersinia uptake by macrophages. In the presence of low levels of ECM components, invasin expression was found to be a potent inducer of integrin-dependent phagocytosis, coincident with the robust and persistent activation of signaling molecules involved in actin remodeling, including FAK and Pyk2 (Hudson *et al*., 2005). In addition to invasin, YadA is capable of inducing Yersinia internalization by macrophages. YadA covers the bacterial surface by forming a capsule-like structure containing a globular head domain, an intermediate stalk and a C-terminal outer membrane anchor (Figure 1.4B) (Hoiczyk *et al*., 2000). Although YadA cannot directly bind β1 integrins, this adhesin can cluster integrin receptors through its ability to cross-link integrin-bound ECM. In the presence of higher levels of ECM, invasin function was inhibited while YadA-mediated signaling and phagocytosis were enhanced. Through the use of *Y. pseudotuberculosis* strains that lack invasin and/or YadA, we have been able to elucidate the mechanisms through which FAK and Pyk2 become activated during

infection as well as the functional requirement for these kinases during phagocytosis (Hudson *et al*., 2005).

Systemic *Y. pseudotuberculosis* infections are characterized by bacterial dissemination to the liver, spleen and lungs. Several studies examining Yersinia infections *in vivo* have utilized invasin- and YadA-deficient strains of *Y. pseudotuberculosis* to determine how these adhesins contribute to the colonization and establishment of infection. Recent work from our lab indicates that the expression of invasin and/or YadA results in lower colonization levels of these strains in the liver of infected mice compared to a strain deficient for both adhesins (Hudson and Bouton, 2006). This may be because *inv*/*yadA* mutants, by virtue of their low ability to adhere to host cells, are able to avoid clearance by the innate immune response. However, *Y. pseudotuberculosis* strains expressing YadA were able to colonize and proliferate in the lungs. Lung tissue contains a complex array of extracellular matrix components that may enhance YadA-mediated adherence, thus providing this strain with a colonization advantage compared to strains deficient for this adhesin.

1.4. Signaling.

1.4.1. FAK and Pyk2.

The focal adhesion protein tyrosine kinase family consists of FAK and Pyk2, two non-receptor tyrosine kinases sharing approximately 45% sequence identity and 65% sequence similarity. The activation of FAK is associated with tyrosine phosphorylation at six sites including Y397 and Y407 in the N-terminal domain, Y576 and Y577 in the

kinase domain activation loop and Y861 and Y925 in the C-terminal domain (Figure 1.5) (Schlaepfer *et al*., 1999). Four of the phosphorylation sites (Y397, 576, 577 and 925) are conserved at analogous positions in Pyk2 (Y402, 597, 880 and 881) (Avraham *et al*., 2000) (Figure 1.6). The FAK autophosphoylation site, Y397, is located outside of the kinase domain and provides a docking site for SH2-containing molecules, such as the Src family kinases (SFKs). The FAK N-terminal region harbors a band four point one-ezrin-radixin-moesin (FERM) homology domain that helps integrate signals from growth factor receptors, including the epidermal growth factor receptor (EGFR) and the platelet-derived growth factor receptor (PDGFR) (Schlaepfer *et al*., 2004). Null mutations in murine FAK genes are embryonic lethal and FAK-deficient MEFs contain numerous mature focal adhesions (Furuta *et al*., 1995; Ilic *et al*., 1995). As discussed previously, FAK is hypothesized to be a mediator of focal adhesion disassembly since the absence of FAK results in inhibited focal adhesion turnover and reduced levels of cell migration in fibroblasts (Ilic *et al*., 1997; Webb *et al*., 2004).

FAK is ubiquitously expressed in most tissue and cell types, and is activated primarily in response to integrin-mediated ECM adhesion events. Focal adhesion targeting (FAT) sequences located within the C-terminal domain of FAK help mediate the localization of this molecule to integrin-dependent focal adhesions (Hildebrand *et al*., 1993) In contrast, the expression of Pyk2 is more varied, with the highest levels of this molecule occurring in cells of the central nervous system and in cells of hematopoietic lineage. Pyk2 can become activated by a diverse range of stimuli depending on cell type, including β1 integrin ligation in B cells and β2 integrin ligation in osteoclasts (Salgia *et al*., 1996; Duong and Rodan, 2000). Phosphorylation of Pyk2 can also occur

Figure 1.5. Binding sites for FAK-associated proteins. The N-terminal FERM domain is important for signal transduction from growth factor receptors such as the EGFR and PDGFR. The FAK C-terminal FAT domain binds the cytoskeletal proteins paxillin and talin and mediates FAK localization to integrin-rich focal adhesions. SH3-containing molecules such as Cas bind to proline-rich regions also located in the C-terminus. Phosphorylation of FAK at Y397 creates high affinity SH2 binding sites for protein tyrosine kinases (PTKs) and the adapter protein Shc, whereas phosphorylation at Y925 promotes the SH2-mediated binding of the Grb2 adaptor protein. FAK phosphorylation within the kinase domain activation loop at Y576/577 promotes maximal catalytic activity (adapted from Schlaepfer *et al.*, 2004).

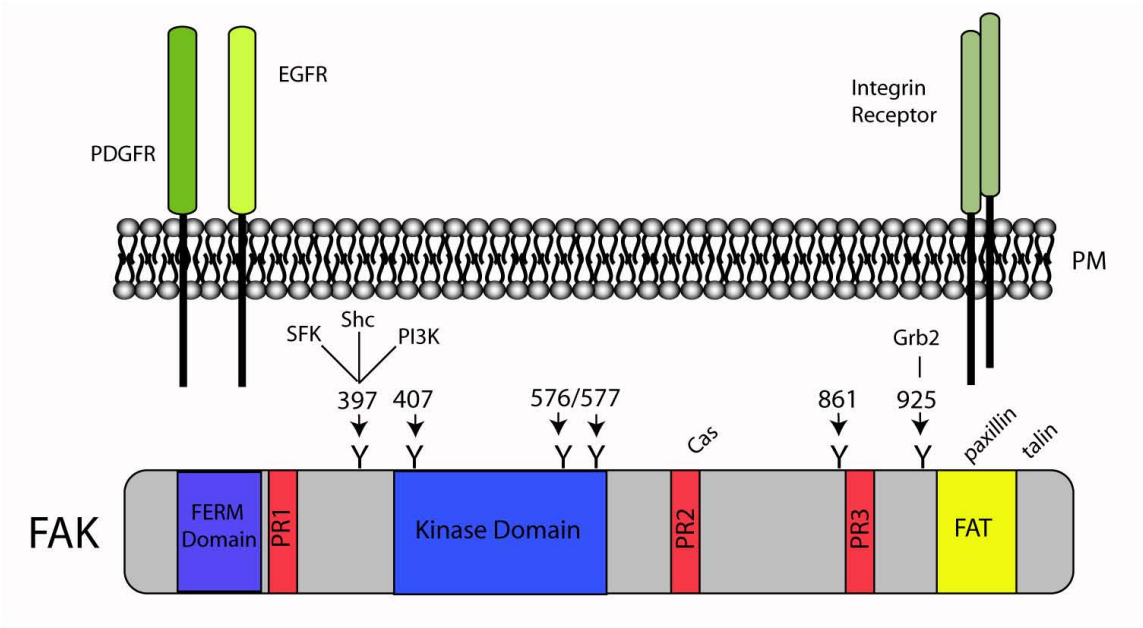
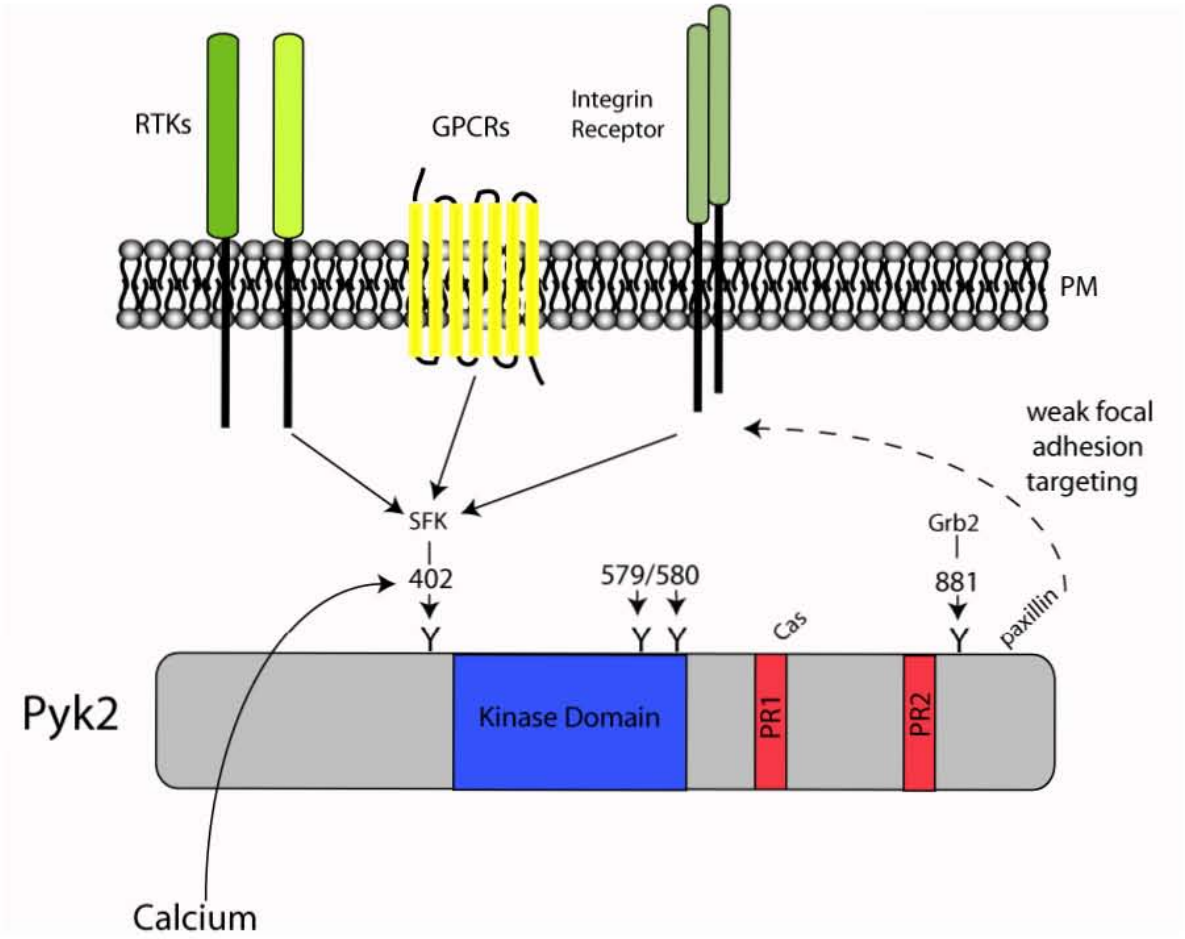

EGFR
PDGFR
Integrin
Receptor
PM
SFK
Shc
PI3K
Grb2
397
407
576/577
861
925
Cas
paxillin
talin
FAK
FERM
Domain
PR1
Kinase Domain
PR2
PR3
FAT

Figure 1.6. Binding sites for Pyk2-associated proteins. Pyk2 can be activated by a variety of extracellular signals mediated by RTKs, G-protein coupled receptors (GPCRs) and integrin receptors. Pyk2 is also activated by signals that elevate intracellular calcium levels. Phosphorylation at Y402 creates an SH2 binding site for SFKs and potentially the p85 subunit of PI3K. C-terminal proline rich domains create binding sites for SH3-containing molecules such as Cas. Paxillin binding sequences allow Pyk2 to bind paxillin, an adaptor molecule important for the recruitment and activation of other proteins involved in focal adhesion formation. Although Pyk2 can become activated in response to integrin ligation, this protein only weakly localizes at focal adhesions, remaining concentrated in the perinuclear region of the cell. Solid lines represent Pyk2 activating events; dotted line represents weak localization to integrin receptor cytoplasmic tails.


RTKs
GPCRs
Integrin
Receptor
PM
weak focal
adhesion
targeting
SFK
Grb2
402
579/580
881
paxillin
Cas
Pyk2
Kinase Domain
PR1
PR2
Calcium

independently of integrin involvement, such as during the activation of T cell receptors on mature T lymphocytes and in response to intracellular increases in calcium levels (Avraham *et al*., 1995; Tokiwa *et al*., 1996; Wange and Samelson, 1996). The C-terminus of Pyk2 shares the least amount of homology with the corresponding domain in FAK, lacking the FAT sequences that allow for direct interactions with β-integrin cytoplasmic tails (Klingbeil *et al*., 2001; Schaller, 2001). As a result, Pyk2 exhibits a more diffuse intracellular expression pattern and is mainly concentrated within the perinuclear region of the cell (Ohba *et al*., 1998).

In macrophages, the function of FAK is controversial, as several groups have detected this molecule in human neutrophils, monocytes and monocytic cell lines (Gotoh *et al*., 1995; Fernandez *et al*., 1997; Kume *et al*., 1997), while other investigators have failed to visualize FAK expression in human and mouse macrophages (Roach *et al*., 1997; De Nichilo *et al*., 1999). It has therefore been assumed that Pyk2, which is expressed in hematopoietic cells, plays a more significant signaling role in phagocytic cells. It is now clear that both FAK and Pyk2 are expressed in primary murine macrophages and monocyte cell lines, and that these kinases become activated in response to integrin ligation and stimulation with growth factors.

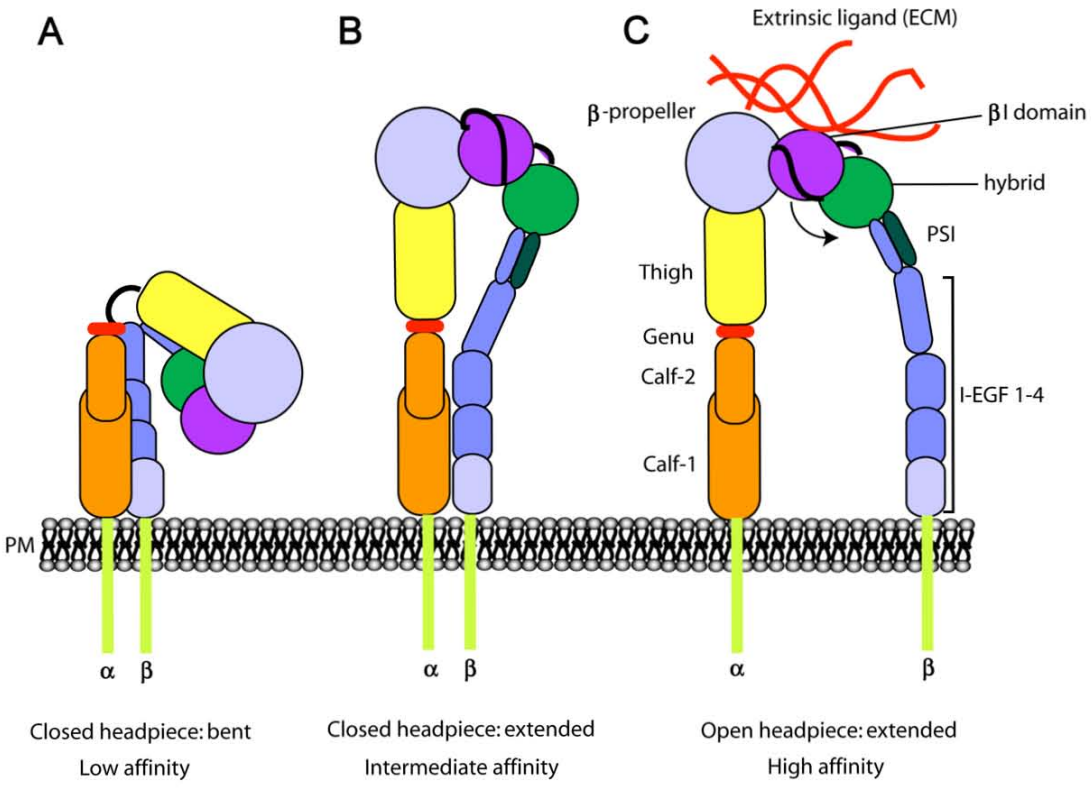
1.4.2. Integrin receptor activation.

Integrins are heterodimeric membrane-spanning receptors composed of α and β subunits that mediate cell-cell and cell-matrix interactions. These receptors exist in multiple conformation states reflecting their level of activation: receptors assuming a "bent" or closed conformation represent a physiologically low affinity state, whereas

priming and receptor ligation induce large-scale conformational changes indicative of a high affinity state (Figure 1.7) (Takagi *et al*., 2002; Kim *et al*., 2003; Takagi *et al*., 2003). Integrin activation is precipitated by the recruitment of the cytoskeletal molecule talin to the cytoplasmic domain of the integrin β subunit (Tadokoro *et al*, 2000; Calderwood *et al*., 2004). The interaction between talin and the β tail physically forces a separation between the α and β integrin subunits resulting in a series of conformational changes (Ginsberg *et al*., 2005). This "inside-out" mechanism of signaling allows for high affinity ligand binding.

Once in the high affinity state, contact between integrin receptors and their cognate matrix ligand induces receptor clustering and the recruitment of "outside-in" signaling intermediates. Non-receptor protein tyrosine kinases and various adaptor molecules are brought in close proximity to the cytoplasmic domains of ligated integrins to form large multiprotein complexes that are connected, either directly or indirectly, to the actin cytoskeleton (Adams, 2002). Mature focal adhesions reversibly anchor the actin cytoskeleton to the ECM and function as a monitor of intra- and extracellular tension (Wehrle-Haller and Imhof, 2002). In fibroblasts, ligated integrins, signaling in part through FAK, affect actin cytoskeleton dynamics via the stimulation of pathways that regulate the small GTPases Cdc42, Rac and Rho. These proteins act as molecular switches controlling the actin-based processes of filopodia, lamellipodia and stress fiber formation respectively (Tapon and Hall, 1997).

Figure 1.7. Integrin architecture and conformational changes associated with affinity regulation. Integrins, such as the αVβ3 integrin pictured, contain two non-covalently associated α and β subunits with large extracellular domains, single spanning transmembrane domains and short cytoplasmic domains. The integrin β subunit contains several domain inserts: the β I domain (purple) is inserted into the hybrid domain (green), which in turn is inserted into the plexins, semaphorins and integrin (PSI) domain (dark green). The β I domain directly binds extrinsic ligands. **A**. The bent confirmation represents the physiologically low affinity state. **B-C**. Priming and ligand binding induce large-scale conformational rearrangements allowing for integrin extension (adapted from Luo and Springer, 2006).


A
B
C
Extrinsic ligand (ECM)
β-propeller
βI domain
hybrid
PSI
Thigh
Genu
Calf-2
Calf-1
I-EGF 1-4
PM
α
β
α
β
α
β
Closed headpiece: bent
Low affinity
Closed headpiece: extended
Intermediate affinity
Open headpiece: extended
High affinity

1.4.3. Integrin signal transduction in macrophages.

In addition to their role regulating cell adhesion, integrins transduce intracellular signals that promote the rearrangement of the actin cytoskeleton, cell migration, proliferation and cell survival (Berton and Lowell, 1999). These intracellular signals collaborate with signals transduced from growth factor receptors, cytokine receptors and other transmembrane receptors to regulate multiple anchorage-dependent cellular properties. Macrophages express a specific repertoire of integrins consisting primarily of members of the β1, β2, β3 and β7 families (Table 1.1). Integrin engagement in these cells triggers the tyrosine phosphorylation of a number of proteins including the cytoskeletal-associated proteins paxillin, tensin and cortactin; the mitogen activated kinases ERK1/2; phopholipase Cγ2, Vav and Cbl; and the SFKs Fgr and Syk.

In the canonical model of integrin signaling developed for fibroblasts, the clustering of integrins and the association of the cytoskeletal proteins talin and paxillin results in the formation of focal adhesion complexes. FAK localizes to sites of integrin clustering and undergoes a conformational change that induces autophosphorylation at Y397, creating a high affinity binding site for the SFKs. (for reviews, see (Hanks *et al.*, 2003; Parsons, 2003; Schlaepfer *et al.*, 2004). Active c-Src bound to Y397 subsequently phosphorylates Y576 and Y577 in the kinase domain of FAK, an event that is essential for maximal FAK kinase activity and activation (Calalb *et al.*, 1995; Hanks *et al.*, 2003; Cox *et al.*, 2006). In its role as a scaffolding protein, FAK recruits other signaling molecules, such as Cas, to clustered integrin receptors. Src-mediated Cas phosphorylation leads to the recruitment of a Crk family adaptor molecule and the activation of the small GTPases Rac and Cdc42 (Cox *et al.*, 2006; Natarajan *et al.*, 2006).

Table 1.1. Integrin receptors expressed by phagocytic cells (adapted from Berton and Lowell, 1999).

INTEGRINS	LIGANDS
β1	
α4β1 (CD49d/CD29; VLA-4)	Fibronectin, VCAM-1
α5β1 (CD49e/CD29; VLA-5)	Fibronectin
α6β1 (CD49f/CD29; VLA-6)	Laminin
β2	
αLβ2 (CD11a/CD18; LFA-1)	ICAMs-1-2-3
αMβ2 (CD11b/CD18; CR3; Mac-1)	ICAMs-1-2, C3bi, Fibrinogen, Factor X, LPS, b-glucan, Heparin, Neutrophil inhibitory factor (NIF), Elastase
αXβ2 (CD11c/CD18; gp 150/95)	Fibrinogen, LPS, C3bi
αDβ2 (CD11d/CD18)	ICAM-3
β3	
αvβ3 (CD51/CD61)	Vitronectin, Enactin and other RGD- and KGAGDV containing ECM proteins
β7	
α4β7	MadCam-1, VCAM-1, Fibronectin

In macrophages, integrin-mediated signaling pathways are incompletely defined. Several lines of evidence suggest that the Src family of kinases play a dominant role during adhesion signaling. First, macrophages derived from mice deficient in the SFKs Hck, Fgr and Lyn manifest poor tyrosine phosphorylation of downstream targets, reduced rates of motility and delayed Fcγ-mediated phagocytosis (Suen *et al.*, 1999; Fitzer-Attas *et al.*, 2000). Secondly, the actin cytoskeletal structure and subcellular localization of paxillin, tensin and talin are altered in Hck-Fgr double knockout macrophages. Pyk2 is also involved in integrin-mediated signal transduction, as Pyk2-deficient macrophages exhibit reduced levels of Rho and PI3K activity and impaired migration (Okigaki *et al.*, 2003). Despite the overwhelming evidence implicating FAK as a key regulator of adhesion signaling, the role of FAK in macrophages has not been examined in depth primarily because the expression of this molecule has remained questionable. Since it is now clear that FAK is indeed expressed in macrophages, these cells provide an excellent model system in which to study the complex interplay between FAK, Pyk2 and other downstream effectors involved in integrin-mediated signaling events.

1.4.4. Cross-talk between integrins and growth factor receptors.

Synergistic interactions between integrins and growth factor receptors can occur at membrane proximal and distal sites (for review, see Ross, 2004). A number of growth factor receptor tyrosine kinases have been shown to associate in complex with integrin receptors, including the receptors for PDGF, EGFR and vascular endothelial growth factor (VEGFR) with αvβ3 integrin (Schneller *et al.*, 1997; Moro *et al.*, 1998; Soldi *et*

al., 1999). Co-localization in plasma membrane lipid rafts may provide the cellular platform needed to facilitate the integrin-growth factor receptor interactions (Leitinger and Hogg, 2002). Once in close proximity, the activation of signaling cascades by integrins may lead to growth factor receptor dimerization and phosphorylation. As previously described, FAK is recruited to the cytoplasmic tail of β integrin subunits and directs the assembly of downstream signaling complexes. FAK is also recruited to activated growth factor receptors, such as EGFR, where it is proposed to function as a receptor proximal component of both integrin and growth factor receptor signaling (Sieg *et al*., 2000). Thus, cooperativity between growth factors and integrins may be regulated in part by the ability of integrins to organize the cytoskeleton as well as direct the translocation of signaling components of both pathways. Alternatively, prior growth factor receptor ligation may enhance "inside-out" integrin signaling by modulating integrin affinity for ligand and receptor clustering (Ross, 2004). Recent work has shown that events initiated by non-integrin receptors can stimulate the conversion of the integrin from a low to a high activation state (Kim *et al*., 2003).

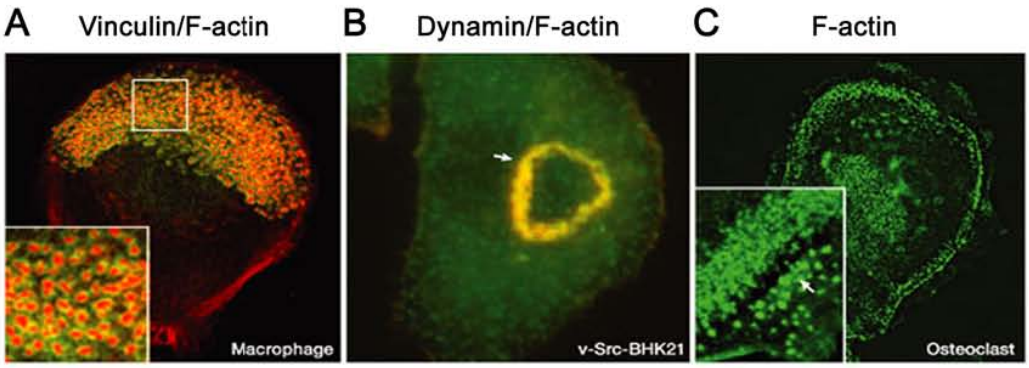
As mentioned previously, CSF-1 is a pleiotrophic growth factor that regulates macrophage survival, proliferation, spreading and motility, as well as monocyte-macrophage differentiation. CSF-1 binding results in receptor dimerization and the rapid phosphorylation of tyrosine residues thereby creating binding sites for SH2 containing molecules (Figure 1.2) (Sengupta *et al*., 1988). Macrophages undergo major morphological changes in response to CSF-1 including lamellipodia formation, dorsal ruffling, polarization and chemotaxis toward the source of CSF-1 (Boocock *et al*., 1989; Webb *et al*., 1996; Allen *et al*., 1997). Both integrin-mediated signaling and CSF-1R-

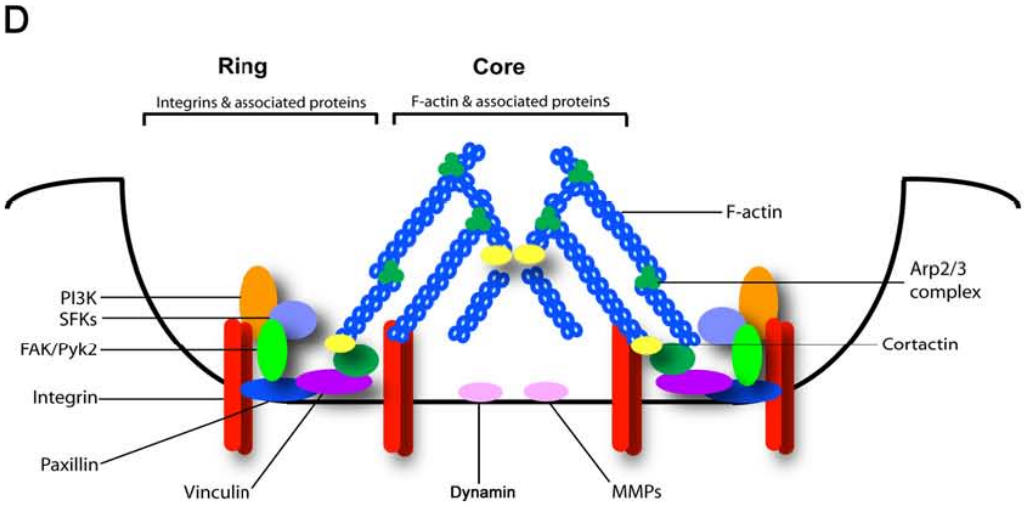
mediated signaling are required for these processes. Cross-talk between integrins and the CSF-1R has already been described in myeloid lineage-derived osteoclasts in which the CSF-1R was found to rapidly co-localize with αvβ3 integrin at the motile region of the cell membrane during osteoclast migration (Faccio *et al.*, 2003). While it seems likely that CSF-1R signaling enhances integrin-mediated signaling events in macrophages, the mechanism of cross-talk, and whether FAK and/or Pyk2 function at the intersection of these two pathways, remains to be elucidated.

1.4.5. Macrophage adhesion structures

Cell-substrate adhesions, in the form of focal complexes, focal adhesions or larger fibrillar adhesions, mediate the attachment of stationary and migrating cells to the underlying substratum (for review, see Geiger *et al.*, 2001). Importantly, cell contact with surrounding matrix physically links the extracellular environment to the intracellular actin cytoskeleton. Podosomes are highly dynamic adhesion structures found in motile cell types including macrophages and dendritic cells. (Linder *et al.*, 1999; Burns *et al.*, 2001; Destaing *et al.*, 2003). Individual podosomes, which can last for approximately 2-12 minutes, assemble into clusters or coalesce into rings and/or peripheral belts depending on cell type (Figure 1.8A-C). Structurally, podosomes consist of a core of F-actin and actin-associated proteins surrounded by a ring of integrins and integrin-associated proteins (Figure 1.8D). Typical core components include F-actin, and the actin regulating molecules Wiskott-Aldrich Syndrome protein (WASP), Arp2/3 and cortactin. Adhesion mediators such as paxillin, vinculin, talin, PI3K, FAK and/or Pyk2 associate with the outer ring structure (Linder and Aepfelbacher, 2003; Buccione *et al.*, 2004).

Figure 1.8. Podosome structures. A. Filamentous (F)-actin (red) and vinculin (green) staining of an actively motile macrophage. Numerous punctate spots that are rich in F-actin and other cytoskeletal components are significant features of podosomes in these cells. Vinculin surrounds the F-actin core (see inset panel for magnified image). **B.** Cultured Rous sarcoma virus (RSV)-transformed baby hamster kidney (BHK) 21 cells have large, prominent, ring shaped podosomes (see arrow) that contain F-actin (red) and the endocytic motor protein dynanin-2 (green). **C.** Cultured RAW 264.7 macrophages that have been differentiated into osteoclasts in culture and stained for F-actin (green). Note the formation of the peripheral podosomal belt. Inset panel shows individual podosomes (see arrow). Images in panels A, B and C adapted from Buccione *et al*., 2004. **D.** Individual podosomes are composed of a core of F-actin and actin-associated proteins embedded in a ring of integrin and integrin-associated proteins. Typical core components are F-actin, the Arp2/3 complex, dynamin and cortactin, whereas adhesion mediators such as paxillin, vinculin, talin and the kinases PI3K and FAK/Pyk2 preferentially associate with the ring structure (adapted from Linder and Kopp, 2005).


A
Vinculin/F-actin
Macrophage
B
Dynamin/F-actin
v-Src-BHK21
C
F-actin
Osteoclast


D
Ring
Core
Integrins & associated proteins
F-actin & associated proteins
F-actin
Arp2/3
complex
Cortactin
PI3K
SFKs
FAK/Pyk2
Integrin
Paxillin
Vinculin
Dynamin
MMPs

Podosomes may play an accessory role in cell migration. Although podosomes can form along the entire ventral surface of quiescent cells, these structures will localize to the leading edge of cells polarized in response to a chemokine gradient. The dynamic nature of podosomes implies that these organelles may help transfer tension along the substrate, which then enables the cell to pull on the lamellipodium as it extends and advances forward (Buccione *et al*., 2004) Consistent with this hypothesis, lost or altered podosome formation in macrophages is often associated with migration defects (Jones *et al*., 2002; Wheeler *et al*., 2006b). In addition to linking the cell membrane to solid surfaces, podosomes may also govern tissue invasion and matrix remodeling by controlling the degradation of ECM and the activation of matrix metalloproteinases (MMPs) (Gimona and Buccione, 2006). Indeed, a number of MMPs have been observed to localize within the inner F-actin core of podosomes, namely membrane-type 1-MMP (MT1-MMP) and the soluble gelatinases MMP2 and MMP9 (Wymann and Arcaro, 1994; Hooshmand-Rad *et al*., 1997). It has recently been shown that macrophages produce proteinases and are capable of direct proteolysis of extracellular matrix through podosomal structures (Yamaguchi *et al*., 2006).

Similar to podosomes, invadopodia also represent cell surface structures with proteolytic capability. However, invadopodia are typically associated with invasive tumor or transformed cells and have been defined as actin rich protrusions emanating from the ventral surface of invasive cells rather than adhesive structures (Linder, 2007). Podosomes and invadopodia also differ with respect to their numbers and size. Cells can form dozens of podosomes that range from 0.5-1 μm in diameter and 0.2-0.4 μm in depth. In contrast, cells maintain only a few invadopodia, but these structures can have a

diameter of up to 8 μm and extend several micrometers deep (Buccione *et al*., 2004; McNiven *et al*., 2004). Despite these differences, similarities in composition and architecture exist between these two structures and it has been proposed that invadopodia may arise from podosomal precursor structures (Linder and Aepfelbacher, 2003; Buccione *et al*., 2004).

1.5. Macrophages and disease.

1.5.1. Chronic inflammatory diseases.

Small numbers of leukocytes reside in resting tissues. In response to inflammatory cues, these numbers are massively augmented by the recruitment of cells from the circulation (Martin and Leibovich, 2005). The presence of cytokines and microbial products profoundly and differentially affect the function of mononuclear phagocytes (Mantovani *et al*., 2004). It has now been recognized that macrophages play a key role in polarized innate and adaptive immunity by promoting the orientation of adaptive immune responses in a type I (T cell helper type I: Th1) or type II (T cell helper type 2; Th2) direction (Gordon, 2003; Mosser, 2003). The Th1 inflammatory response is characterized by the infiltration of interferon-γ (IFN-γ) secreting $CD4^+$ and $CD8^+$ effector T cells. IFN-γ alone or in concert with microbial products (e.g. LPS) or cytokines (e.g. tumor necrosis factor; TNF) induces the "classical" mode of macrophage activation. Classically activated macrophages, designated M1, exhibit an elevated capacity to present antigen and produce high levels of IL-12, IL-23, toxic intermediates (nitric oxide; NO) and reactive oxygen intermediates (ROIs) (Mantovani *et al*., 2004). In general, M1

macrophages are potent effector cells integrated within Th1-mediated responses, which function to kill microorganisms and tumor cells and produce copious amounts of proinflammatory cytokines. In contrast, macrophages exposed to immune complexes and LPS are characterized by an IL-10^{high}, IL-12^{low} phenotype. These "alternatively" activated cells, or M2 macrophages, promote Th2 responses, playing a role in immunosupression, parasite killing, matrix deposition and tissue remodeling. M2 macrophages have also been associated with the promotion of tumor growth.

During the final resolution phase of an inflammatory reaction, leukocytes that have been recruited to a region of injury either disperse or undergo apoptosis. The fate of macrophages is not entirely clear, but it is likely that many eventually die, while others egress into the surrounding tissues (Bellingan *et al*., 1996; Burman *et al*., 2005). However, the robust protective response that is initiated during an inflammatory reaction can also be deleterious to host tissues due to the release of cytotoxic mediators that destroy otherwise healthy host cells along with target infectious agents (Martin and Leibovich, 2005). The Th1 inflammatory response involving M1 macrophages is thought to play an important pathophysiological function in a multitude of prominent human diseases, including type I diabetes mellitus, Crohn's disease, multiple sclerosis (MS), rheumatoid arthritis, atherosclerosis and chronic obstructive pulmonary disease (COPD) (for review, see Luster *et al*., 2005). During COPD for example, the inflammatory response to irritants such as cigarette smoke is greatly amplified, resulting in a significant influx of macrophages into the lungs and alveolar spaces. The excessive inflammation caused by the release of cytokines, chemokines and proteases leads to fibrosis of the small airways and ultimately, irreversible damage to the lung parenchyma (Barnes,

2004). Interfering with trafficking molecules on Th1 cells/or activated macrophages has been shown to be beneficial in many of these chronic disorders. Indeed, blocking α4 integrin receptor function prevents leukocyte trafficking and is thus efficacious in the treatment of both Crohn's disease and MS, although it can lead to increased susceptibility to infection (Ghosh *et al*., 2003; Miller *et al*., 2003). Alternatively, inhibition of intracellular signaling pathways involved in cell migration may ultimately have similar therapeutic applications in the prevention and/or treatment of chronic inflammatory diseases. For example, the treatment of cells with small molecular inhibitors of FAK (PF-228) that specifically target FAK catalytic activity, exhibited reduced levels of cell migration and focal adhesion turnover (Slack-Davis *et al*., 2007). The importance of FAK in the regulation of processes like cell migration implies that PF-228 and similar inhibitor compounds may function as effective anti-inflammatory agents.

1.5.2. Cancer.

Hematopoietic cells are recruited to most solid tumors, with tumor-associated macrophages or TAMs, constituting the majority of the tumor mass (Pollard, 2004). The persistent recruitment of macrophages and other immune cells under conditions of chronic inflammation is thought to establish a microenvironment that is conducive to the initiation of malignancy (Condeelis and Pollard, 2006). The continual presence of tissue-damaging reactive oxygen and nitrogen species may facilitate malignant changes within the local epithelium (Pollard, 2004; Condeelis and Pollard, 2006). During tumor progression, TAMs acquire a skewed M2 macrophage phenotype and express protumoral functions (Mantovani *et al*., 2004). Thus, while macrophages may initially be involved in

mounting anti-tumor immune responses, these cells play an active role in several aspects of tumor cell metastasis, including migration/invasion through tissues, entrance and exit from the vasculature, and the stimulation of tumor growth at metastatic sites.

Recent studies have demonstrated that macrophages and tumor cells co-migrate in mammary tumors and that the invasion of these cell types through ECM components is a cooperative event (Wyckoff *et al*., 2004). During the early stages of tumorigenesis, macrophages are associated with the break down of the basement membrane, whereas in later stage tumors, macrophages are found predominantly at the invasive front (Pollard, 2004). This suggests that tumor cells are able to exploit the normal tissue remodeling capabilities of macrophages, allowing them to infiltrate surrounding stroma (Lin *et al*., 2001; Pollard, 2004; Wyckoff *et al*., 2004). In addition, cancer cells secrete CSF-1 to attract macrophages, and once recruited, macrophages are induced to produce EGF to stimulate tumor growth (Goswami *et al*., 2004). Consequently, high levels of TAMs correlate with poor prognosis in many types of cancer (Leek *et al*., 1996; Goede *et al*., 1999). Similarly, the overexpression of CSF-1 or the CSF-1 receptor is associated with a poor prognosis for breast, endometrial and ovarian cancers (Lin *et al*., 2001). It is now clear that interactions occurring between tumor cells and macrophages result in tumor progression. Thus, inhibition of macrophage recruitment to tumor sites may represent a novel mechanism for cancer treatment.

1.6. Research objectives and significance.

Monocyte/macrophages play a key role in the establishment and maintenance of tissue homeostasis. The plasticity and versatility of macrophages reflects the ability of

these cells to respond to a diverse range of microenvironmental signals. As a critical component of the innate immune system, the main functions of macrophages involve integrin-mediated adhesion to and extravasation from blood vessels, chemotaxis, phagocytosis and the release of cytokines and chemokines. FAK and Pyk2 lie at the convergence of integrin adhesion, signaling and the actin cytoskeleton. While the roles of FAK and/or Pyk2 have been studied extensively during integrin-mediated signaling events in other cell types, the potential function of these molecules (particularly FAK) in macrophages has remained largely ignored. Therefore, by investigating FAK and Pyk2 in macrophages, we have the unique opportunity to (i) examine the interplay between these endogenously expressed molecules and other downstream effectors during common macrophage functions such as cell migration and phagocytosis, and (ii) determine how the loss or reduced expression of these molecules from cells of myeloid lineage affects the host response to pathological processes.

The generation of myeloid-specific conditional FAK knockout mice has allowed us to begin characterizing the role of FAK in primary macrophages. We have been able to establish that the loss of FAK from bone marrow macrophages (BMMs) results in significantly impaired migration toward a variety of chemotactic agents as well as reduced invasion through ECM components (Chapter 2). These data strongly suggest that the loss of FAK from macrophages causes a generalized defect in the cell migration machinery rather than an inability to sense and/or respond to a specific migration stimulus. We further show that the reduced expression of paxillin, but not Pyk2 causes an additional decrease in the invasive capacity of $FAK^{-/-}$ macrophages, indicating for the

first time that macrophage migration requires both a FAK/Pyk2-dependent pathway as well as a paxillin-dependent pathway (Chapter 3).

In response to inflammation, macrophages are among the first cells to mobilize at sites of injury and infection. However, the induction of an inflammatory response in myeloid-specific conditional FAK knockout mice resulted in impaired mononuclear leukocyte recruitment *in vivo*. These results imply that by targeting FAK in leukocytes, such that the ability of these cells to migrate is inhibited, the host response to inflammation can be altered. The overall significance of these findings are two-fold: first, the *in vitro* data confirm that loss of FAK activity results in impaired migration validating the potential of this molecule as an effective drug target and second, the *in vivo* studies performed suggest that the specific inhibition of FAK in monocyte/macrophages may result in the faster resolution of inflammatory reactions, less recruitment of these cells to solid tumors and ultimately reduced metastasis to distant sites.

Equally important for the resolution of inflammation and infection is the ability of macrophages to recognize and eliminate pathogens. The second component of this thesis (Chapter 5) is designed to further our current understanding of the host cell signaling networks involved in the integrin-mediated internalization of bacteria. Both FAK and Pyk2 have been implicated in the uptake of *Y. pseudotuberculosis* by macrophages and in this study we investigated the mechanisms of activation and the functional requirement for these kinases during phagocytosis. A panel of *Y. pseudotuberculosis* strains that differentially express the adhesins invasin and YadA were used to infect J774A.1 cells in which FAK and/or Pyk2 expression was reduced. We found that bacterial strains simultaneously expressing invasin and YadA activated non-redundant FAK and Pyk2

signaling pathways required for Yersinia internalization. FAK activation was found to be sufficient for the phagocytosis of bacteria expressing invasin alone, and Pyk2 was sufficient when YadA was expressed in the absence of invasin. These studies significantly extend previous work from our lab by finding that the nature of the external signal dictates whether FAK and Pyk2 function coordinately to activate distinct pathways, or independently, to induce the cytoskeletal changes needed for phagocytosis.

In summary, the results presented in this thesis make an important contribution to our understanding of the signaling pathways involved in the regulation of common macrophage functions. The migration of primary macrophages requires signaling via a FAK/Pyk2-dependent pathway whereas FAK and Pyk2 become independently activated and may therefore function within separate pathways during *Yersinia* phagocytosis. Despite the fact that both migration and Yersinia internalization are integrin-mediated processes, the discrepancies in pathway requirement may reflect differences between primary cells and a macrophage-like cell line, or the utilization of alternative integrin receptors to perform different functions.

Chapter 2:

Regulation of Lamellipodial Persistence, Adhesion Turnover and Motility in Macrophages by Focal Adhesion Kinase

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2.1. Abstract.

Macrophages are a critical component of the innate immune system that play a key role during development, tissue remodeling after injury, or infection. In this report, we investigate how FAK and the related kinase Pyk2 integrate adhesion signaling and growth factor receptor signaling to regulate diverse macrophage functions. Inducible loss of FAK from primary bone marrow-derived macrophages in conditional knockout mice or by small interfering RNAs significantly impaired chemotaxis and reduced invasion through extracellular matrix components. While decreased Pyk2 expression also diminished the invasive capacity of macrophages, the combined loss of FAK and Pyk2 had no greater effect than the loss of either molecule alone, indicating that both kinases function within the same pathway to promote invasion. The defect in motility exhibited by FAK-deficient macrophages was associated with elevated protrusive activity at the cell periphery, altered adhesion dynamics, and a marked inability to form stable lamellipodia necessary for directional locomotion. The significance of these findings was substantiated by the fact that monocyte recruitment *in vivo* to sites of inflammation was impaired in the absence of FAK. Collectively, our analyses demonstrate an essential function of FAK as a regulator of macrophage adhesion, chemotaxis, invasion, and infiltration into inflamed tissues.

2.2. Introduction.

The ability of macrophages to quickly respond to diverse extracellular cues allows these cells to function as important mediators of innate and adaptive immunity. In

response to migratory stimuli, macrophages polarize and extend broad lamellipodia and spike-like filopodia in the direction of the chemotactic gradient (Calle *et al.*, 2006). Formation of these protrusive structures is controlled by the dynamic reorganization of the actin cytoskeleton and tubulin-based microtubules (Jones, 2000; Worthylake and Burridge, 2001). These structures are subsequently stabilized by integrin-mediated adhesions with the ECM. Members of the FAK family, which includes FAK and Pyk2, are critical integrators of these and other processes involved in cell motility. Through its function as a kinase and signaling scaffold, FAK has been shown to regulate focal adhesion turnover and migration in fibroblasts (Hanks *et al.*, 2003; Parsons, 2003; Schlaepfer *et al.*, 2004), while Pyk2 is a known regulator of macrophage motility (Okigaki *et al.*, 2003). However, the biological function of FAK and the molecular interplay between FAK and Pyk2 in highly motile cell types such as macrophages has been largely unexplored. FAK was initially considered to be absent or expressed at low levels in monocyte/macrophages (Lin *et al.*, 1994; De Nichilo and Yamada, 1996). It is now clear that FAK is indeed present in this cell lineage (Kume *et al.*, 1997; Okigaki *et al.*, 2003; Rovida *et al.*, 2005), allowing for investigation into its role during macrophage migration and the immune response to inflammation.

During migration, FAK coordinates lamellipodial formation and the turnover/disassembly of focal adhesions (Webb *et al.*, 2004; Tilghman *et al.*, 2005). Focal adhesions are highly dynamic structures that form at sites of membrane contact with the ECM and are associated with a dense network of bundled actin stress fibers (Zaidel-Bar *et al.*, 2004; Vicente-Manzanares *et al.*, 2005). A critical role for FAK during cell migration is highlighted by the fact that fibroblasts derived from FAK-null mice

migrate poorly in response to chemotactic and haptotactic factors, and contain exceptionally large and stable focal adhesions (Ilic *et al*., 1995). In contrast to fibroblasts, macrophages form small focal complexes rather than large focal adhesions, and produce fine actin cables rather than the stress fibers observed in cells of mesenchymal derivation (Pixley and Stanley, 2004; Pixley *et al*., 2005). Macrophage-substrate contact also gives rise to podosomes, which are believed to play a role in adhesion, motility, matrix remodeling, and invasion (Calle *et al*., 2006). The formation of focal complexes and/or podosomes, as well as the lack of actin stress fibers, is consistent with the rapid motile response required of these cells.

In macrophages, integrin-dependent signaling can be enhanced by the presence of cytokines and growth factors (Schneller *et al*., 1997; Kiosses *et al*., 2001; Faccio *et al*., 2003). CSF-1 is a pleiotrophic myeloid lineage-specific growth factor that stimulates cell survival, proliferation and monocyte-macrophage differentiation (for reviews, see Stanley *et al*., 1997; Pixley and Stanley, 2004). It also functions as a potent macrophage chemoattractant (Boocock *et al*., 1989). Macrophages undergo significant morphological changes in response to CSF-1, including lamellipodia formation, dorsal ruffling, polarization, and CSF-1-directed chemotaxis (Boocock *et al*., 1989; Webb *et al*., 1996; Allen *et al*., 1997). CSF-1 receptor activation results in the activation of the small GTPases Rac1 and Cdc42, which contribute to membrane ruffling and cell polarization (Cox *et al*., 1997; Kraynov *et al*., 2000). Recent data suggest that FAK is also important in establishing a proper leading edge and maintaining the polarity of moving cell (Tilghman *et al*., 2005).

To examine the role of FAK in primary macrophages, we have generated myeloid-specific conditional FAK knockout mice. We show that macrophages derived from these mice display significant motility defects, coincident with elevated protrusive activity at the cell periphery, reduced adhesion turnover, and a marked inability to form stable lamellipodia necessary for directional locomotion. While reduced expression of Pyk2 also resulted in motility defects, the combined loss of both FAK and Pyk2 had no additional consequence above what was observed in the absence of either molecule alone. The effects of FAK deletion on macrophage functions *in vitro* corresponded with decreased infiltration of FAK-null inflammatory monocytes into sites of inflammation *in vivo*. These data provide for the first time genetic evidence that FAK is critically involved in the regulation of macrophage motility, findings that have profound implications considering the physiological importance of macrophages for the control of infection and the maintenance of tissue homeostasis.

2.3 Materials and methods.

Generation of myeloid-specific conditional FAK knockout mice

Mice homozygous for *cre* recombinase under the control of the myeloid-specific Lysozyme M (LysM) promoter were purchased from the Jackson Laboratory (Stock # 004781, Bar Harbor, ME) and have been described previously (Clausen *et al.*, 1999). To generate myeloid-specific conditional FAK knockout mice and their control littermates, mice homozygous at the *fak* locus ($FAK^{fl/fl}$) (Beggs *et al.*, 2003) were crossed with mice homozygous for *cre* at the *lysM* locus ($LysM^{cre/cre}$). Mice heterozygous at both loci were

backcrossed to generate LysM$^{wt/wt}$-FAK$^{fl/fl}$ and LysM$^{wt/cre}$-FAK$^{fl/fl}$ mice, and the controls LysM$^{wt/wt}$–FAK$^{wt/wt}$ and LysM$^{wt/cre}$–FAK$^{wt/wt}$.

Isolation of bone marrow macrophages (BMMs)

BMMs were isolated from 6-8 week old mice by flushing femurs and tibias with phosphate buffered saline (PBS) containing 0.5% bovine serum albumin (BSA) and 1% 0.5M EDTA. Cells were magnetically labeled using anti-CD11b microbeads (Milteny Biotec, Auburn, CA) and positively selected on an MS MACS column as per the manufacturer's instructions. Cells were seeded onto bacterial plates and cultured in Alpha Minimal Essential Medium (α-MEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 10% CMG 14-12 cell conditioned medium as a source of CSF-1 (kindly provided by Dr. Gregory Longmore, Washington University, St. Louis, MO) and 100 U/ml penicillin and 100 U/ml streptomycin (Gibco BRL, Life Technologies, Rockville, MD). Media was replaced every 3-4 days until confluent.

Genotyping of mice and analysis of Cre-mediated recombination

Animals were routinely genotyped from tail DNA and subjected to PCR analysis. The following primers were used for PCR of the FAK locus: P1 5'-GAG AAT CCA GCT TTG GCT GTT G-3' and GenoRV 5'-GAA TGC TAC AGG AAC CAA ATA AC-3'. This primer set generates 290 bp (WT) and 400 bp (FAK-*flox*) fragments. To determine the status of the LysM locus, the following primers were used: LysM1 5'-CTT GGG CTG CCA GAA TTT CTC-3', LysM2 5'-TTA CAG TCG GCC AGG CTG AC-3', and Cre8 5'-CCC AGA AAT GCC AGA TTA CG-3'. This primer set generates 350 bp (WT)

and 700 bp (Cre) fragments. To check for Cre-mediated recombination in BMMs, DNA was isolated from macrophages and subjected to PCR with the following primers: LoxP 5'-GAC CTT CAA CTT CTC ATT TCT CCC-3' and GenoRV listed above. The amplified PCR products consisted of a WT (1.4 kb), FAK-*flox* (1.6 kb) and Cre-mediated recombined fragment (327 bp). All PCR fragments were separated on 1.5% agarose gels.

Antibodies and reagents

Polyclonal FAK C-20 was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). A monoclonal antibody (mAb) recognizing phospho-ERK1/2, fibronectin (FN) and collagen I were all obtained from Sigma-Aldrich (St. Louis, MO). MAbs recognizing Pyk2 and Rac1 were purchased from BD Biosciences (Bedford, MA). A polyclonal anti-ERK1/2 antibody was purchased from Cell Signaling (Beverly, MA). Fluoroscein isothiocyanate (FITC)-conjugated rat anti-mouse CD45 (clone 30-F11), PE Cy7-conjugated rat anti-mouse CD11b (clone M1/70) and 7AAD were purchased from eBiosciences (San Diego, CA), PE-conjugated rat anti-mouse GR-1 (clone RB6-8C5), allophycocyanin (APC)-conjugated CD11b (clone M1/70.5), PE conjugated anti-mouse F4/80 (clone BM8) and rat anti-mouse CD16/32 antibodies (clone FCR-4G8) were purchased from Caltag laboratories (Burlingame, CA). Texas Red (TR)-conjugated phalloidin was purchased from Molecular Probes (Eugene, OR). Horseradish peroxidase (HRP)-conjugated sheep anti-mouse Ig and HRP-conjugated donkey anti-rabbit antibodies were purchased from Amersham Pharmacia Biotech (Piscataway, NJ). CSF-1 was purchased from PeproTech, Inc. (Rocky Hill, NJ). Stromal cell derived factor-1α

(SDF-1α) and macrophage chemoattractant protein-1 (MCP-1) were purchased from R and D systems (Minneapolis, MN).

Transfection procedures

siGenome SmartPool siRNAs targeting murine FAK (100 nmol) and Pyk2 (100 nmol) and non-specific siControl (100 nmol) were purchased from Dharmacon Inc. (Lafayette, CO). Transfection of green fluorescent protein (GFP)-vinculin (1 μg) and siRNAs into BMMs was achieved by nucleofection as per the manufacturer's instructions using the mouse macrophage nucleofector kit (Amaxa Corporation, Gaithersburg, MD).

Time-lapse video microscopy and analysis

To study cell spreading in response to CSF-1, $1X10^5$ cells were seeded on 35 mm bacterial dishes and starved of CSF-1 overnight. The following day, cell images were collected for 5 minutes before the addition of exogenous CSF-1 (120 ng/ml) using a Nikon Diaphot with a JVC video camera every 15 seconds for a total of 45 minutes. To quantitate protrusive behavior, kymographs of cells from the videos were generated using ImageJ to create time space plots. Briefly, a minimal intensity projection was performed for each movie. Four lines of interest were drawn at 90° angles along the perimeter of the cell. Kymographs obtained from each region of interest allow for the visualization of individual protrusive and retracting areas. Protrusion distance and persistence was measured using the segmented line tool in ImageJ (NIH) and the data were exported to excel for statistical analysis.

To study random cell migration, $1X10^5$ cells were seeded onto FN-coated (10 μg/ml) 35 mm Bioptechs delta-T dishes (Fisher Scientific) designed for live cell imaging and starved of CSF-1 overnight. The following day, the media was changed to Leibovitz L-15 media (Gibco) containing 10% FBS and 120 ng/ml CSF-1. Time-lapse movies were made by capturing images every 5 minutes for 2.5 hours using a Nikon TE200 inverted microscope with a 20X DIC objective and a Bioptechs heated stage. Images were captured with a Hamamatsu ORCA camera and compiled using OpenLab software (Improvision Inc., Lexington, MA). For analysis, each cell in the first frame was tracked for the entire time-lapse sequence and the distance traveled was measured in OpenLab.

To study adhesion dynamics by total internal reflective fluorescence (TIRF) microscopy, $1X10^5$ cells expressing GFP-vinculin were seeded onto FN-coated (2 μg/ml) 35 mm glass bottomed dishes in CCM1 medium (Hyclone, Logan, UT). Prior to filming, exogenous CSF-1 (120 ng/ml) was added and images were captured using an Olympus 1x70 inverted microscope with a 60X objective. TIRF images were captured with a cooled charged-coupled device (Retiga Exi; Qimaging).

Quantification of adhesion dynamics

The fluorescent intensity of individual adhesions from cells expressing GFP-vinculin was measured over time as follows: images were acquired every 5 seconds using Metamorph software (Molecular Devices Corp., Downingtown, PA). Adhesions located at the cell periphery and/or protruding edge were selected for analysis. ImageJ software was then applied to the entire image stack to subtract the background fluorescent intensity and to correct for overall photobleaching. The incorporation of vinculin into adhesions was

linear on a semilogarithmic plot of the fluorescent intensity as a function of time. The apparent rate constants for formation of vinculin-containing adhesions were determined from the slope of these graphs. Similarly, semilogarithmic graphs of the decrease in fluorescent intensity plotted as a function of time were also linear. From these plots, rate constants for the disassembly of vinculin from adhesions could be determined from the slope. For each rate constant determination, measurements were obtained for 3-5 individual adhesions on 8-10 cells.

Quantification of cell adhesive area

For analysis of cell adhesive area, approximately $2X10^5$ cells were seeded onto FN-coated (10 μg/ml) coverslips and incubated for 24 hours at 37°C. Cells were then starved of CSF-1 overnight or left in complete (CSF-1-containing) media. Where indicated, cells were re-stimulated with CSF-1 (120 ng/ml) for 20 minutes and then fixed and stained for F-actin. Cells were visualized through a Nikon TE2000-E Eclipse fluorescence microscope and photographed with an ORCA CCD camera controlled by OpenLab software (Improvision, Inc., Lexington, MA). To quantify cell area, digitized images acquired by immunofluorescence microscopy were analyzed with ImageJ.

Migration and invasion assays

For chemotaxis assays, the lower chamber of a modified Boyden chamber (6.5 mm, 8.0-μm Transwell Costar membrane, Corning International, Corning, NY) was pre-incubated for 2 hours with α-MEM and one of the following chemoattractants: CSF-1 (120 ng/ml), SDF-1α (100 nM) or MCP-1 (100 nM). $5x10^4$ WT and $FAK^{-/-}$ BMMs previously starved

of CSF-1 and serum overnight were loaded into the top chamber in CSF-1-free media and allowed to migrate toward each chemoattractant for 4 hours at 37°C. For invasion, the top and bottom of Biocoat invasion chambers (24-wells, 8.0-μm, growth factor reduced Matrigel matrix, BD Biosciences, Bedford, MA) were pre-incubated in CSF-1-free media for 2 hours. The media in the bottom chamber was then changed to include CSF-1 (120 ng/ml) and cells were loaded as described above and allowed to invade through the Matrigel toward CSF-1 for 24 hours at 37°C. Following migration or invasion, non-migratory cells were removed from the top of the membrane using cotton swabs. The underside of each membrane was fixed, stained using the Diff-Quik staining set (Dade Behring, Newark, DE) and mounted onto coverslips using Cytoseal 60 (Richard Allen Scientific, Kalamazoo, MI). For migration assays, the number of cells migrated in 10 random fields was determined using light microscopy. For invasion assays, the total number of cells invading after 24 hours were counted.

Thioglycollate-induced peritonitis

8-9 week old mice were administered 1 ml of 4% thioglycollate broth (Sigma Adlrich, St. Louis, MO) intraperitoneally. At various time points, mice were euthanized by carbon dioxide exposure and peritoneal cavities were flushed with 5 mls PBS containing 0.5% BSA and 1% 0.5M EDTA. Cells recovered from peritoneal lavage were suspended in red blood cell (RBC) lysis buffer (eBioscience, San Diego, CA) for 3 minutes on ice, counted with a hemocytometer and analyzed by flow cytometry.

Positive cell selection and flow cytometry

Cells flushed from the peritoneal cavities of mice were treated with RBC lysis buffer prior to magnetic labelling with anti-CD11b or anti-GR-1 microbeads (Milteny Biotec, Auburn, CA) and positively selected on an MS MACS column as per the manufacturer's instructions. Following positive selection, 10^6 cells were incubated for 25 minutes on ice with fluorophore-conjugated antibodies recognizing the cellular antigens (CD45, GR-1, F4/80 and CD11b) diluted in PBS containing 0.5% BSA and 0.05% sodium azide (PBA). Cells were also stained with the viability dye 7AAD or DAPI. Prior to staining, Fc receptors were blocked with anti-CD16/32 (1 μg per 10^6 cells) antibodies. Following staining, cells were washed twice, resuspended in PBA, and gated cells (based on live cells) were analyzed for fluorescence staining on a FACScalibur system™ (Becton Dickinson, San Jose, CA).

GTP-Rac1 pull downs and immunoblotting

Unless indicated otherwise, BMMs were starved of CSF-1 overnight before re-stimulation the following day with cytokine (120ng/ml). Cells were rinsed twice with PBS and lysed in modified RIPA (50 mM Tris, 150 mM NaCl, 1% Igepal CA-630, 0.5% deoxycholate) containing protease and phosphate inhibitors (100 μM leupeptin, 1 mM phenylmethylsulfonyl fluoride, 0.15 unit/ml aprotinin, 1 mM vanadate) as previously described (Kanner *et al*., 1989). Protein concentrations were determined with the BCA Assay kit (Pierce, Rockford, IL). Active GTP-bound Rac pull-down assays were performed with a Rac activation kit according to the manufacturer's instructions (Upstate Biotechnology, Lake Placid, NY). For immunoblotting, 6 μg of total cell lysate were

resolved by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were transferred to nitrocellulose membranes and immunoblotted as previously described (Burnham *et al.*, 2000; Weidow *et al.*, 2000). Proteins were detected by HRP-conjugated anti-mouse or anti-rabbit Ig followed by enhanced chemiluminescence (ECL) (Millipore Corporation, Billerica, MA). To quantify changes in levels of protein phosphorylation, densitometry was performed (Molecular Dynamics). Band intensities were quantified by ImageQuant 5.0 (Molecular Dynamics, Inc.) and values for phosphorylated proteins were divided by those for total protein and expressed relative to values obtained for un-stimulated cells.

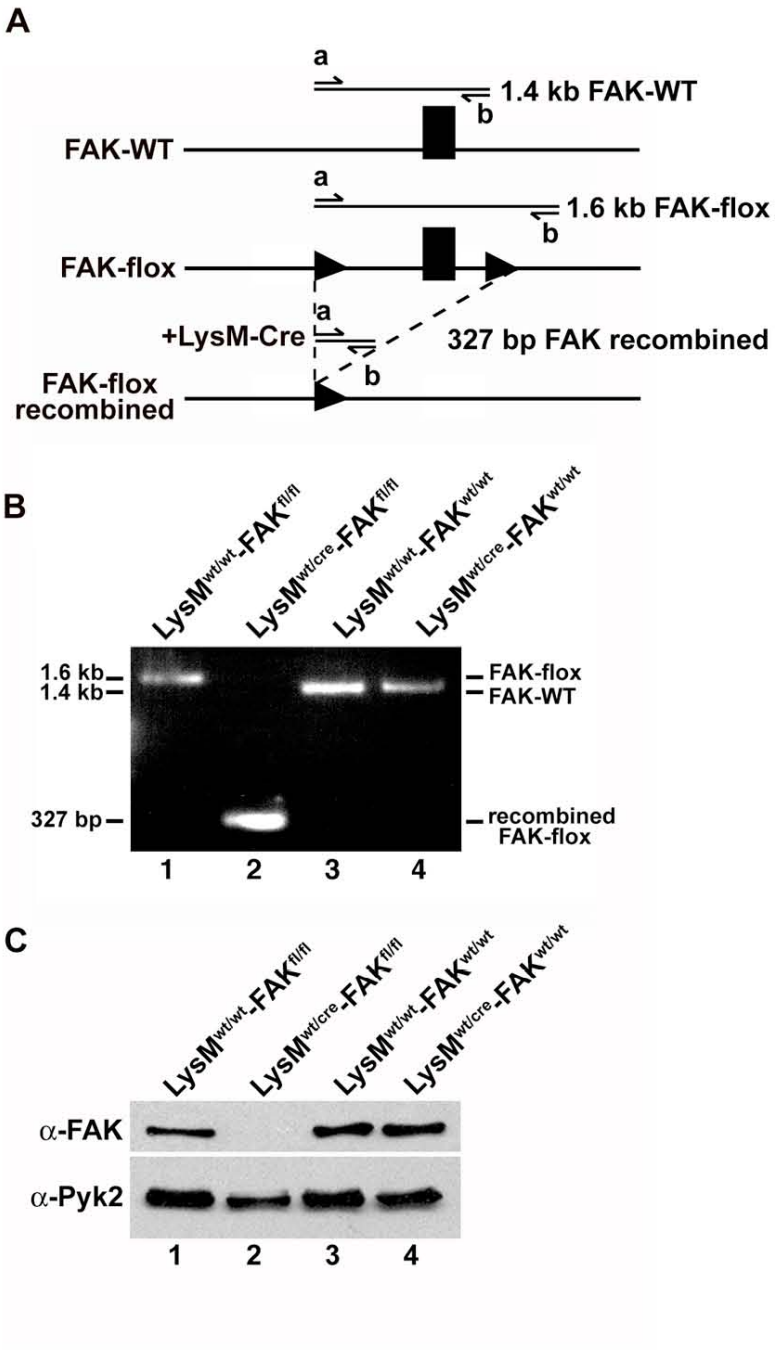
Statistical analysis

A two-sample t-test assuming unequal variance was used to determine statistical significance between condition means with a significance level of ≤ 0.05.

2.4. Results.

Generation of myeloid-specific conditional FAK knock-out mice. The generation of myeloid-specific conditional FAK knock-out mice has facilitated the study of FAK in primary macrophages. Mice harboring a *floxed*-FAK allele (Beggs *et al.*, 2003) were crossed with mice expressing Cre recombinase under the control of the myeloid lineage-specific Lysozyme M (LysM) promoter (Clausen *et al.*, 1999). Recombination catalyzed by Cre recombinase results in excision of the floxed target exon (Figure 2.1A). FAK sequences from bone marrow macrophages isolated from $LysM^{wt/cre}$-$FAK^{fl/fl}$ mice were efficiently deleted during recombination (Figure 2.1B) and FAK protein expression levels

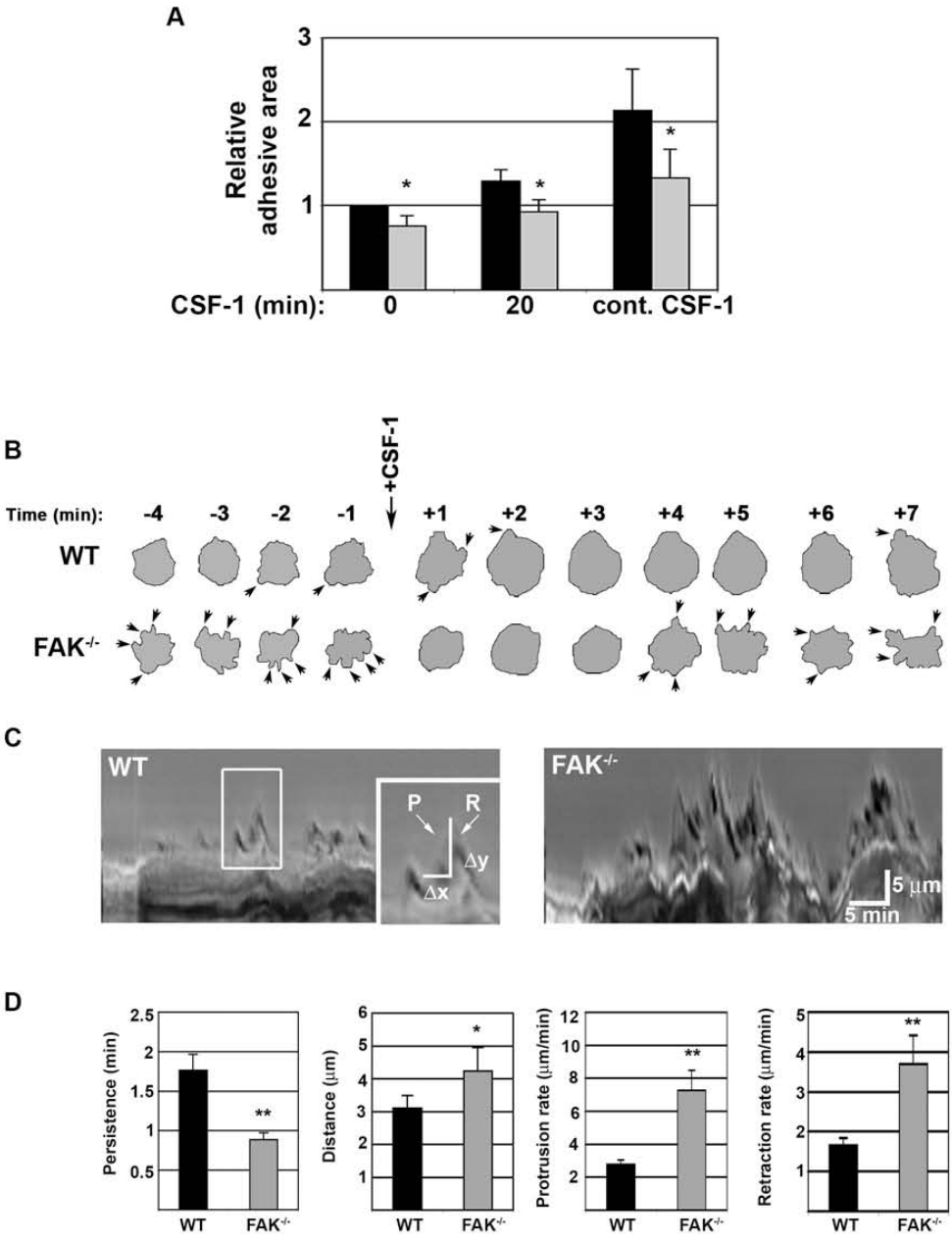
Figure 2.1. Generation of myeloid-specific conditional FAK knock-out mice. A. Schematic diagram of the floxed-FAK locus and the structure of the FAK locus after LysM-driven Cre-mediated recombination. The second kinase domain exon of FAK (filled box) is flanked by loxP sites (filled triangles). Primers (short arrows) and PCR products (lines) are shown above each allele. **B.** PCR of DNA isolated from BMMs using the primers LoxP ("a") and GenoRV ("b") to distinguish the FAK-flox allele (1.6 kB, lane 1), the recombined locus (327 bp, lane 2) and the FAK-WT locus (1.4 kB, lanes 3-4). **C.** Immunoblot analysis of total FAK (top panel) and Pyk2 (lower panel) expression in BMMs isolated from $LysM^{wt/wt}/FAK^{fl/fl}$ and $LysM^{wt/cre}/FAK^{fl/fl}$ mice (lanes 1 and 2, respectively), and $LysM^{wt/wt}/FAK^{wt/wt}$ and $LysM^{wt/cre}/FAK^{wt/wt}$ (lanes 3-4).


A
a
1.4 kb FAK-WT
b
FAK-WT
a
1.6 kb FAK-flox
b
FAK-flox
a
+LysM-Cre
327 bp FAK recombined
b
FAK-flox
recombined
B
LysMwt/wt-FAKfl/fl
LysMwt/cre-FAKfl/fl
LysMwt/wt-FAKwt/wt
LysMwt/cre-FAKwt/wt
1.6 kb
1.4 kb
FAK-flox
FAK-WT
327 bp
recombined
FAK-flox
1
2
3
4
C
LysMwt/wt-FAKfl/fl
LysMwt/cre-FAKfl/fl
LysMwt/wt-FAKwt/wt
LysMwt/cre-FAKwt/wt
α-FAK
α-Pyk2
1
2
3
4

were significantly reduced compared to FAK levels observed in BMMs isolated from LysM$^{wt/wt}$-FAK$^{fl/fl}$ littermates (Figure 2.1C, top panel). Unlike FAK-null MEFs, which exhibit upregulated levels of Pyk2 (Ilic *et al.*, 1995), both wildtype (WT) and FAK-deficient (FAK$^{-/-}$) BMMs expressed equivalent levels of Pyk2 (lower panel). BMMs were also extracted from control mice (LysM$^{wt/wt}$-FAK$^{wt/wt}$ and LysM$^{wt/cre}$-FAK$^{wt/wt}$) to determine if the insertion of flox sites or the expression of cre recombinase affected protein expression levels. There was no detectable difference in the expression of FAK or Pyk2 between WT and control macrophages (Figure 2.1C, compare lanes 3 and 4 to lane 1).

The loss of FAK promotes elevated protrusive activity at the cell periphery and altered adhesion dynamics. Adherent BMMs represent a highly heterogeneous population of cells that can vary significantly in their shape and size. Despite this variability, the mean adhesive area exhibited by FAK$^{-/-}$ macrophages plated onto fibronectin-coated coverslips was consistently reduced regardless of whether cells were cultured in the absence of CSF-1 overnight (Figure 2.2A), stimulated with CSF-1 for 20 minutes, or grown in the continual presence of CSF-1 (second and third data sets, respectively). To gain a better understanding of how the loss of FAK in macrophages affects cell spreading in response to CSF-1 stimulation, WT and FAK$^{-/-}$cells were examined by time-lapse video microscopy (see Movie 1). Prior to the addition of CSF-1, FAK$^{-/-}$ macrophages were observed to extend and retract numerous, short-lived protrusions, resulting in a highly irregular peripheral edge. These structures were evident upon examination of individual FAK$^{-/-}$ BMMs taken at one minute intervals (Figure 2.2B). Immediately after CSF-1 stimulation, both WT and FAK$^{-/-}$cells exhibited

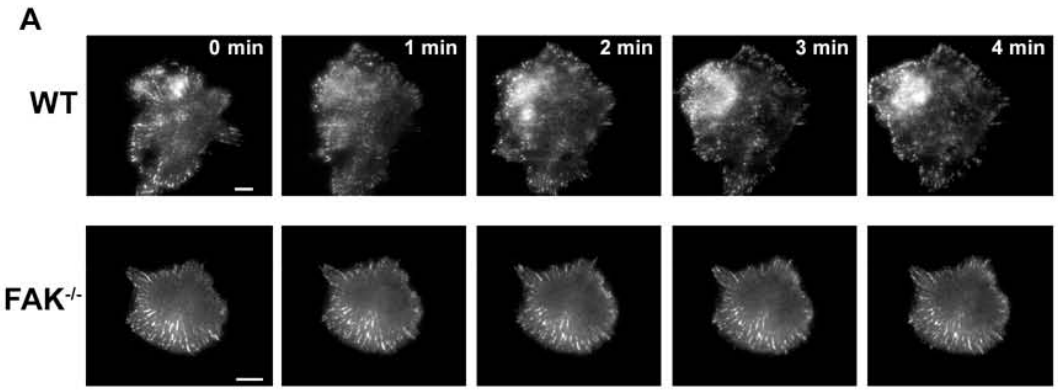
Figure 2.2. FAK$^{-/-}$ macrophages exhibit elevated protrusive behavior in response to CSF-1. A. WT (black bars) and FAK$^{-/-}$ (gray bars) macrophages were fixed and stained for F-actin and the total area of adhesion was measured with ImageJ. Results shown are the mean ± the standard error of mean (S.E.M.) and represent approximately 150 cells from each population over 5 separate experiments. Asterisks indicate a statistically significant difference from the mean at ≥95% confidence level relative to CSF-1 starved WT cells. **B.** The periphery of representative WT and FAK$^{-/-}$ macrophages taken from time-lapse video microscopy were outlined at time points before and after CSF-1 stimulation. Arrows indicate protrusions. **C.** Sample kymographs from WT and FAK$^{-/-}$ cells are shown. Boxed area highlights protrusions extending from the cell periphery and are enlarged in the right-hand inset. "P" indicates protrusion, "R" indicates retraction. **D.** Protrusion persistence (Δx), distance (Δy), protrusion rate ($\Delta y/\Delta x$) and retraction rate ($-\Delta y/\Delta x$) were determined for each cell examined. The data represent the mean ± S.E.M. of 10 cells (4 parameters of membrane activity per cell) from each population over 3 separate videos. Asterisks indicate values significantly different from WT cells (*P<0.05, **P<0.001).

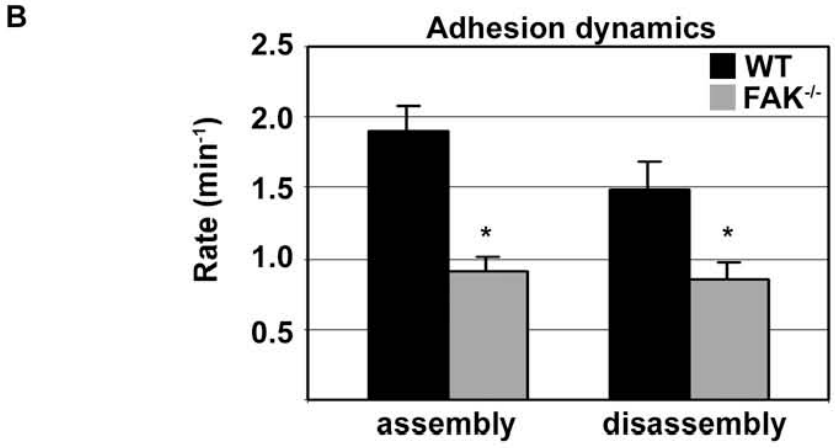

A
Relative adhesive area
3
2
1
*
*
*
CSF-1 (min):
0
20
cont. CSF-1
B
+CSF-1
Time (min):
-4
-3
-2
-1
+1
+2
+3
+4
+5
+6
+7
WT
FAK-/-
C
WT
P
R
Δy
Δx
FAK-/-
5 μm
5 min
D
Persistence (min)
2.5
2
1.5
1
0.5
**
WT
FAK-/-
Distance (μm)
6
5
4
3
2
1
*
WT
FAK-/-
Protrusion rate (μm/min)
12
10
8
6
4
2
**
WT
FAK-/-
Retraction rate (μm/min)
5
4
3
2
1
**
WT
FAK-/-

circumferential spreading and fewer discrete protrusions. However, by 4 minutes after the addition of CSF-1, increased protrusive activity was again evident in FAK-deficient macrophages. To compare the protrusive activity of WT and $FAK^{-/-}$ BMMs, these time-lapse videos were analyzed by kymography, a technique that allows for the quantitation of protrusion persistence (Δx), protrusion distance (Δy), protrusion rate ($\Delta x/\Delta y$), and retraction rate ($-\Delta x/\Delta y$) (Figure 2.2C). Protrusions formed by $FAK^{-/-}$ BMMs were shorter-lived, lasting approximately 1 minute before retraction, compared to the 1.7 minute life span of WT protrusions (Figure 2.2D). $FAK^{-/-}$ protrusions also extended further than those produced by WT cells (4 ± 0.37 μm vs. 3 ± 0.71 μm respectively). Thus, the protrusion and retraction rates calculated for $FAK^{-/-}$ macrophages (7.2 ± 1.2 μm/min and 3.7 ± 0.71 μm/min, respectively) were significantly faster than those observed for WT cells (2.7 ± 0.27 μm/min and 1.6 ± 0.18 μm/min, respectively). Collectively, these data show that macrophages deficient for FAK expression exhibit high levels of activity at the cell periphery, characterized by the rapid formation and retraction of small protrusions. These data show that, while $FAK^{-/-}$ BMMs are capable of forming lamellipodia, they may not be able to establish functional adhesions to stabilize the protrusions.

To determine whether FAK is involved in the regulation of adhesion formation and/or disassembly in macrophages, WT and $FAK^{-/-}$ BMMs were transfected with GFP-vinculin and examined by TIRF-based video microscopy (Figure 2.3A and Movie 2). GFP-vinculin localized to prominent peripheral adhesion structures in both WT and $FAK^{-/-}$ cells. To quantify adhesion turnover, the kinetics of adhesion formation and disassembly was determined for vinculin by integrating the fluorescent intensity in individual adhesions over time. The rate of adhesion formation was reduced 2-fold in the

Figure 2.3. FAK regulates adhesion assembly and disassembly in macrophages. A. TIRF-based video microscopy was used to examine adhesion formation and turnover in macrophages. GFP-vinculin localizes in adhesion structures observed in both WT and $FAK^{-/-}$ macrophages. Scale bars = 10 μm. **B.** Quantitative analysis of adhesion turnover. Adhesions from approximately 8-10 cells were examined (3-5 adhesions per cell). Asterisks indicate a statistically significant difference from the mean at ≥95% confidence level.


A
0 min
1 min
2 min
3 min
4 min
WT
FAK-/-

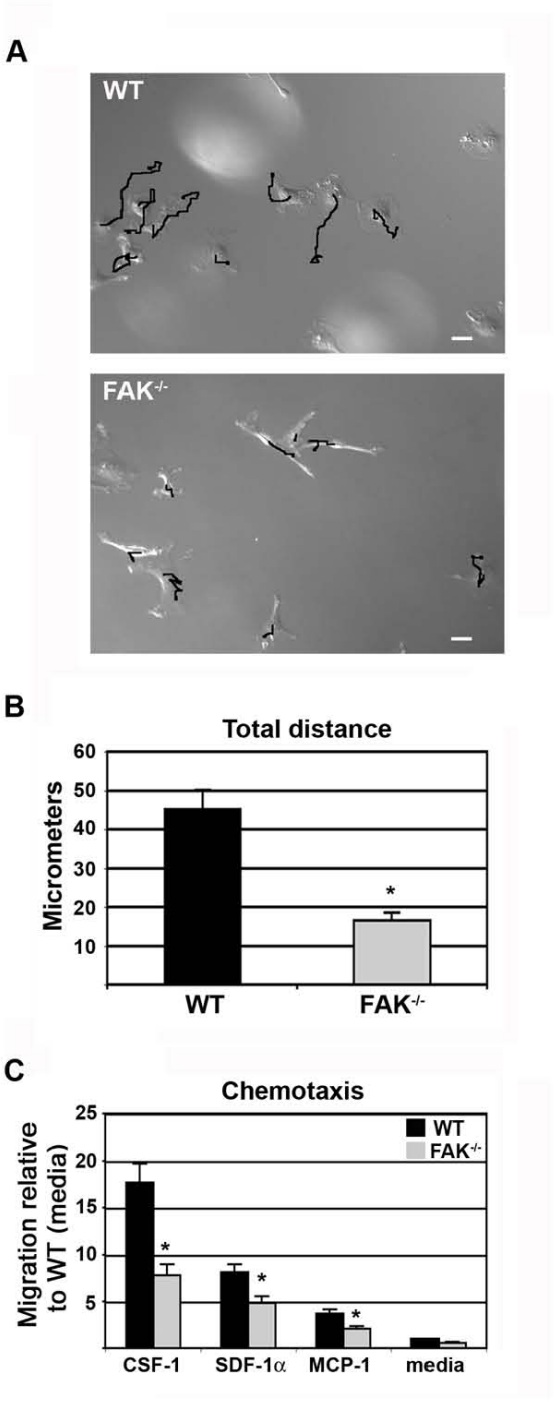

B
Adhesion dynamics
WT
FAK-/-
Rate (min-1)
2.5
2.0
1.5
1.0
0.5
*
*
assembly
disassembly

absence of FAK (1.9 ± 0.17 min^{-1} and 0.91 ± 0.19 min^{-1} for WT and $FAK^{-/-}$ cells, respectively; Figure 2.3B). Similarly, adhesion disassembly in $FAK^{-/-}$ BMMs (0.85 ± 0.11 min^{-1}) was also impaired compared to WT cells (1.48 ± 0.21 min^{-1}). These results are consistent with the role of FAK as a dynamic mediator of adhesion turnover.

$FAK^{-/-}$ BMMs exhibit a generalized defect in cell migration and invasion. Since FAK-deficient BMMs displayed poor lamellipodial stabilization in combination with altered adhesion dynamics, live cell imaging was used to examine chemokinesis, or random migration, in the presence of CSF-1 (see Movie 3). WT macrophages stimulated with CSF-1 were observed to spread, extend lamellipodia, and move in a directed fashion throughout the course of the 2.5 hour video analysis (Figure 2.4A). In contrast, $FAK^{-/-}$ BMMs rarely migrated more than 15 μm from their point of origin compared to 45 μm for WT cells (Figure 2.4B). To investigate whether FAK is required for directed cell migration in addition to random motility, WT and FAK-deficient macrophages were analyzed for their ability to migrate toward CSF-1 in a Boyden chamber assay. The loss of FAK inhibited CSF-1-induced motility by 53% (Figure 2.4C). The CSF-1-induced motility of BMMs extracted from $LysM^{wt/wt}$-$FAK^{wt/wt}$ and $LysM^{wt/cre}$-$FAK^{wt/wt}$ mice was identical to that observed for $LysM^{wt/wt}$-$FAK^{fl/fl}$ BMMs (data not shown), demonstrating that the presence of flox sites in the absence of Cre, or the expression of Cre in the absence of a floxed allele, had no effect on migration.

To determine whether the migration defect toward CSF-1 was specific for this cytokine, we next examined the ability of WT and $FAK^{-/-}$ BMMs to migrate toward stromal cell-derived factor-1α (SDF-1α) and macrophage chemoattractant protein-1

Figure 2.4. FAK$^{-/-}$ BMMs exhibit impaired CSF-1-dependent motility. A. Time-lapse video microscopy was used to examine cell movement in response to CSF-1. WT and FAK$^{-/-}$ BMMs were starved of CSF-1 overnight before re-stimulation with CSF-1 (120 ng/ml) for 2.5 hours. See Movie 3 in Supplemental Material. Migration tracks overlay the final still image taken upon completion of the video. Scale bar = 10 μm. **B.** The total distance traveled for WT (black bar) and FAK$^{-/-}$ (gray bar) BMMs was determined as described in Materials and Methods. The data represent the mean ± S.E.M. of approximately 30 cells from each population over 3 separate videos. Asterisks indicate a statistically significant difference from the mean at ≥95% confidence level. **C.** WT (black bars) and FAK$^{-/-}$ cells (gray bars) were starved of CSF-1 overnight before seeding onto Boyden chambers. Cells were then allowed to migrate toward CSF-1 (120 ng/ml), SDF-1α (100 nM) or MCP-1 (100 nM) for 4 hours at 37°C. The number of migrated cells was determined and expressed relative to WT migration toward media alone. The data represent the mean ± S.E.M. for 4-6 separate experiments. Asterisks indicate a statistically significant difference from WT migration (media) at ≥95% confidence level.


A
WT
FAK-/-
B
Total distance
Micrometers
60
50
40
30
20
10
*
WT
FAK-/-
C
Chemotaxis
Migration relative to WT (media)
25
20
15
10
5
WT
FAK-/-
*
*
*
CSF-1
SDF-1α
MCP-1
media

(MCP-1). These factors signal through chemokine receptors, which are structurally and mechanistically distinct from the receptor tyrosine kinase, CSF-1R. Migration of FAK$^{-/-}$ BMMs toward SDF-1α and MCP-1 was reduced 42% and 46%, respectively, compared to WT BMMs (Figure 2.4C), indicating that the defect in chemotaxis exhibited by FAK$^{-/-}$ macrophages is not restricted to CSF-1.

Invasion through 3-dimensional extracellular matrices may require alternative signaling pathways to those regulating migration over 2-dimensional substrates (Wells *et al*., 2004). To determine the requirement for FAK during this process, WT and FAK$^{-/-}$ BMMs were seeded onto Matrigel-coated Boyden chambers and allowed to invade toward CSF-1 for 24 hours. The invasive capacity of FAK$^{-/-}$ BMMs was decreased by 60% compared to WT macrophages (Figure 2.5). Taken together, these data indicate that the absence of FAK in macrophages results in a generalized motility defect affecting chemotaxis, random migration, and invasion through a 3-dimensional matrix.

FAK$^{-/-}$ macrophages exhibit high basal levels of activated Rac1. We next examined whether the loss of FAK affected the activity of molecules involved in CSF-1-induced signaling. Specifically, the small GTPase Rac1 has been shown to regulate actin polymerization during lamellipodia formation in macrophages (Allen *et al*., 1997; Wells *et al*., 2004). Rac1 activity was measured in WT and FAK$^{-/-}$ BMMs that were starved of cytokine overnight and then stimulated with CSF-1 for 0-30 minutes. While the kinetics of activation were similar between the cell types, with peak Rac activity at approximately 1 minute of CSF-1 treatment, basal Rac1-GTP levels were 9-fold higher in FAK-deficient macrophages than in WT cells (Figure 2.6A, top panel, compare lanes 1 and 6). The

Figure 2.5. FAK$^{-/-}$ BMMs are impaired in their ability to invade through 3-dimensional matrices. A. WT and FAK$^{-/-}$ BMMs were starved of CSF-1 overnight before seeding onto Matrigel-coated Boyden chambers. Cells were allowed to invade toward CSF-1 (120 ng/ml) for 24 hours at 37°C. Upon completion of the assay, the total number of cells that successfully invaded was determined. The data represent the mean ± S.E.M. for 3 separate experiments. Asterisks indicate a statistically significant difference from the mean at ≥95% confidence level.

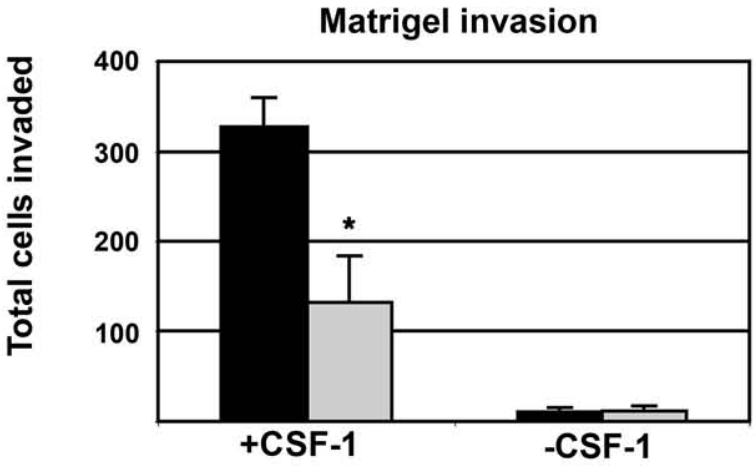
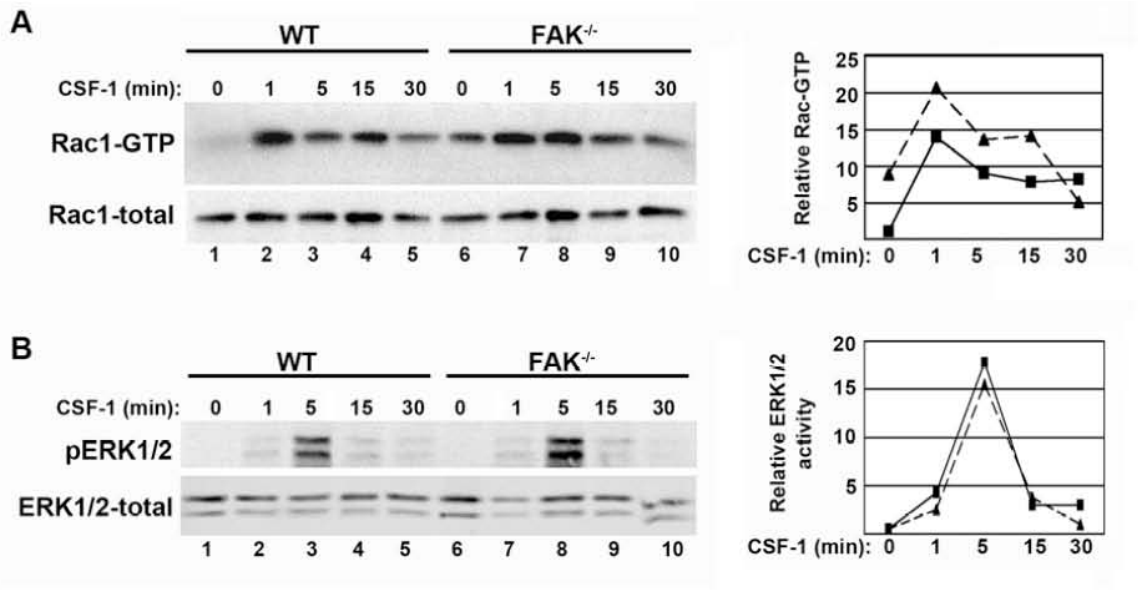

Matrigel invasion
Total cells invaded
400
300
200
100
*
+CSF-1
-CSF-1

Figure 2.6. Analysis of CSF-1-induced signaling in WT and FAK$^{-/-}$ BMMs. Cells were plated onto tissue culture plastic and starved of CSF-1 overnight before stimulation with CSF-1 (120 ng/ml) for 0-30 minutes. **A.** GTP-bound Rac1 was isolated from lysates by incubation with Pak-1 binding domain (PBD) agarose. Bound proteins (top panel) and total Rac1 (lower panel) were detected by immunoblotting with Rac1 antibodies. Relative band intensities are displayed in graph form to the right. **B.** Cellular proteins immunoblotted with antibodies recognizing phospho-ERK1/2 (top panel) and total ERK1/2 (lower panel). Relative band intensities were determined using densitometry and are displayed in graph form to the right. (WT, solid line-square; FAK$^{-/-}$, dashed line-triangle). Each immunoblot represents multiple independent experiments.

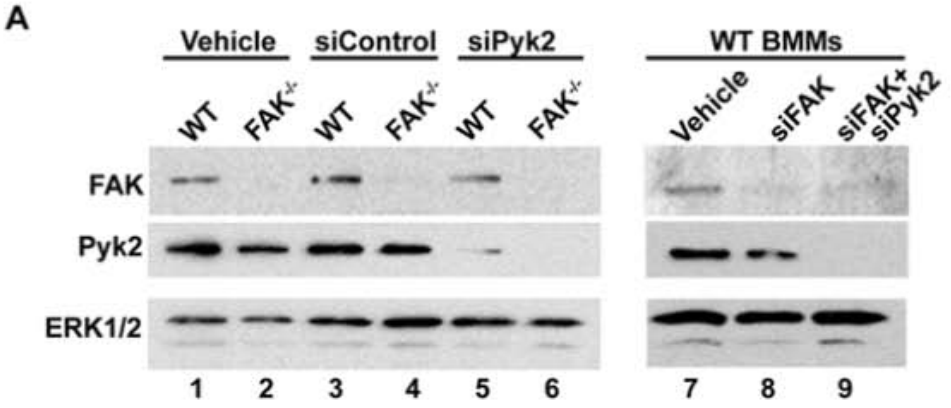

A
WT
FAK-/-
CSF-1 (min): 0 1 5 15 30 0 1 5 15 30
Rac1-GTP
Rac1-total
1 2 3 4 5 6 7 8 9 10
Relative Rac-GTP
25
20
15
10
5
CSF-1 (min): 0 1 5 15 30
B
WT
FAK-/-
CSF-1 (min): 0 1 5 15 30 0 1 5 15 30
pERK1/2
ERK1/2-total
1 2 3 4 5 6 7 8 9 10
Relative ERK1/2 activity
20
15
10
5
CSF-1 (min): 0 1 5 15 30

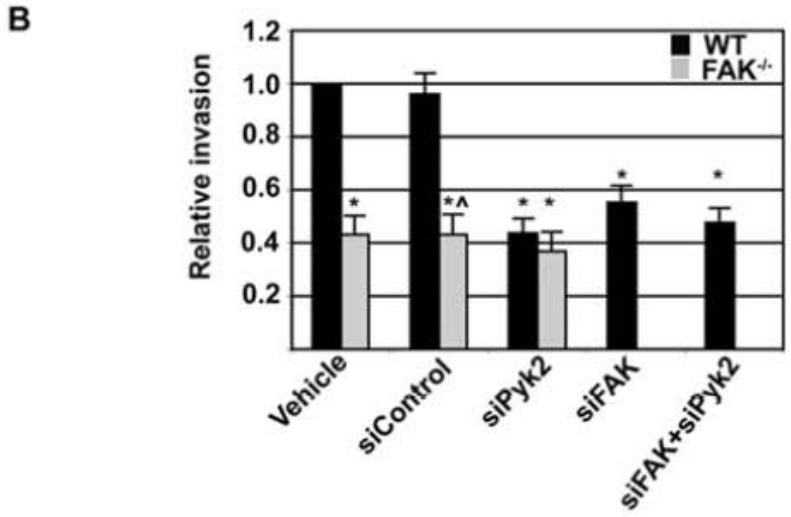
overall CSF-1-dependent increase in Rac1 activity was not as great in FAK$^{-/-}$ BMMs as in WT BMMs (2.2-fold compared to 13.9-fold), largely due to the abnormally high basal activity in these cells. In contrast, the activation of ERK1/2 occurred with nearly identical amplitude and kinetics in both cell types, peaking after 5 minutes of CSF-1 stimulation (Figure 2.6B).

Pyk2 and FAK coordinately regulate macrophage invasion. The data presented thus far support a role for FAK in the regulation of macrophage motility. However, it was important to confirm that the defects exhibited by FAK$^{-/-}$ BMMs were directly caused by the loss of FAK. As an alternative to genetic deletion of *fak*, BMMs isolated from control mice were treated with small interfering RNA duplexes (siRNAs) targeting FAK. This resulted in a 90% reduction in FAK expression (Fig. 2.7A, lane 8). Control and siRNA-treated BMMs were seeded onto Matrigel-coated Boyden chambers and allowed to invade toward CSF-1 overnight. BMMs treated with siRNAs targeting FAK exhibited significantly reduced invasion relative to WT BMMs treated with vehicle or control siRNAs (Figure 2.7B, black bars). These data lend further support for an essential role of FAK in CSF-1 induced macrophage motility.

While the loss of FAK from primary BMMs via both genetic deletion and RNA interference (RNAi) resulted in a significant reduction in invasion, residual invasion was still evident under both conditions (see Figures 2.4C and 2.7B). This would suggest that a second, FAK-independent pathway, is also involved in the regulation of macrophage motility. Macrophages derived from Pyk2$^{-/-}$ mice exhibit altered cell polarization and diminished contractility associated with reduced chemokine-induced motility (Okigaki *et*

Figure 2.7. Macrophage invasion requires Pyk2 and FAK expression. A. WT and $FAK^{-/-}$ BMMs were treated with vehicle (H_2O), siControl, siPyk2 and/or siFAK. 48 hours post-siRNA transfection, cells were lysed and immunoblotted for total FAK and Pyk2 (top panels) and ERK1/2 (lower panel). **B.** Vehicle and siRNA-treated cells were starved of CSF-1 overnight before seeding onto Matrigel-coated Boyden chambers. WT (black bars) and $FAK^{-/-}$ (gray bars) BMMs were then allowed to invade toward CSF-1 (120 ng/ml) for 24 hours at 37°C. The data represent the mean ± S.E.M. for 4-6 separate experiments. Asterisks indicate a statistically significant difference from vehicle-treated WT cells at ≥95% confidence level; ^ indicate a statistically significant difference from WT siControl-treated cells at ≥95% confidence level.


A
Vehicle
siControl
siPyk2
WT BMMs
WT
FAK-/-
WT
FAK-/-
WT
FAK-/-
Vehicle
siFAK
siFAK+ siPyk2
FAK
Pyk2
ERK1/2
1
2
3
4
5
6
7
8
9


B
Relative invasion
1.2
1.0
0.8
0.6
0.4
0.2
WT
FAK-/-
Vehicle
siControl
siPyk2
siFAK
siFAK+siPyk2

al., 2003). To more definitively assess the relationship between FAK and Pyk2 during macrophage invasion, siRNA oligonucleotides were used to reduce Pyk2 expression by approximately 85-95% in both WT and $FAK^{-/-}$ BMMs (Figure 2.7A, middle panel, lanes 5-6). This treatment had no effect on FAK expression in WT cells (top panel, lane 5). As before, the invasive capacity of $FAK^{-/-}$ BMMs treated with vehicle or siControl was reduced by 55% compared to WT macrophages (Figure 2.7B, gray bars). Invasion was similarly decreased by 55% in siPyk2-treated WT cells (3^{rd} data set, black bar). Importantly, reduced expression of Pyk2 in $FAK^{-/-}$ BMMs did not cause an additional reduction in invasion over the effect of FAK deletion alone (gray bar). Combined knockdown of both FAK and Pyk2 from WT cells by siRNA also decreased invasion by a similar level. These data indicate that both FAK and Pyk2 contribute to the regulation of macrophage invasion in response to CSF-1, and that they appear to function within the same pathway.

FAK regulates recruitment of monocyte/macrophages to sites of inflammation *in vivo*. To assess the role of FAK during leukocyte recruitment to sites of inflammation *in vivo*, mice were injected intraperitoneally with 4% thioglycollate (TG) to induce an inflammatory response. The peritoneal cavities of $LysM^{wt/wt}$-$FAK^{fl/fl}$ (phenotypically WT) mice and myeloid lineage-specific conditional FAK knockout mice were lavaged 8, 16, and 72 hours post-injection and the exudates analyzed by flow cytometry using markers to distinguish between resident macrophages, infiltrating monocytes, and neutrophils (Melnicoff *et al*., 1989; Chan *et al*., 1998). Resident macrophages are characterized by high CD11b and F4/80 expression and low expression of GR-1. In contrast, GR-1 is

upregulated in infiltrating inflammatory monocytes, while F4/80 expression is reduced in this model of inflammation (Table 2.1 and Figure 2.8). Neutrophils were characterized by the high expression of GR-1. In the resting peritoneum, equivalent numbers of resident macrophages were extracted from both mouse genotypes (Figure 2.9A). After 16 hours, twice as many infiltrating CD11b positive cells were recovered from the peritoneum of WT mice compared to conditional knockout mice. By 72 hours post-TG treatment, CD11b-positive cells continued to infiltrate the peritoneum of WT mice, while the accumulation of these cells was delayed in the knockout animals. This impaired recruitment was not true of all leukocyte populations, however, as both WT and conditional knockout animals exhibited similar recruitment of GR-1 positive cells in response to TG (Figure 2.9B).

To confirm that *fak* was deleted from the CD11b-positive population of cells isolated from the peritoneum of TG-stimulated conditional FAK knockout mice, cells harvested from these mice were positively selected for CD11b surface expression and the extent of Cre-mediated recombination was examined by PCR. CD11b-positive cells lavaged from FAK-deficient mice exhibited high levels of Cre-mediated recombination compared to cells obtained from TG-stimulated WT mice (Figure 2.9C, compare lanes 5 and 6) or cultured WT BMMs (lane 1). Resident cells harvested from unstimulated conditional FAK knockout animals also exhibited Cre-mediated recombination compared to cells taken from WT littermates (compare lanes 3 and 4). The high levels of recombination seen in FAK knockout mice corresponded to a concomitant loss of FAK expression from elicited and resident cells (Figure 2.9D, top panel, lane 6 and 4, respectively).

Table 2.1. Phenotype of resident and infiltrating cell subsets. Expression level of the indicated surface antigens were measured have been assigned symbols that represent no expression (-) and increasing amounts of expression (+, ++, +++) based on flow cytometric analysis.

Population	A	B	C
Antigen	**'Resident macrophages'**	**'Inflammatory monocytes'**	**'Neutrophils'**
CD45	**+++**	**+++**	**+++**
CD11b	**+++**	**++**	**+**
F4/80	**+++**	**+**	**-**
GR-1 (Ly6G+Ly6C)	**-**	**++**	**+++**

Figure 2.8. Characterization of surface markers on resident and infiltrating cells. A. CD11b positive selection of cell exudates from unstimulated and TG-stimulated mice. CD11b positive cells extracted from unstimulated mice are characterized by the robust surface expression of CD11b and F4/80 (left panel). These cells make up the resident macrophage population. Cells extracted from the peritoneum of TG-stimulated mice express reduced amounts of CD11b and F4/80. Both epitopes are down regulated on infiltrating mononuclear phagocytes in this model of inflammation (right panel). **B.** Further characterization of resident and infiltrating cells. Population "A" stains positively for CD11b and negatively for GR-1. This population is predominantly visible in unstimulated mice and represents resident macrophages (left panel). During TG-stimulation, cells expressing CD11b and moderate levels of GR-1 (population "B") and cells expressing moderate levels of CD11b and high levels of GR-1 (population "C") are detected (right panel). Population B represents inflammatory monocytes, while population C represents neutrophils.

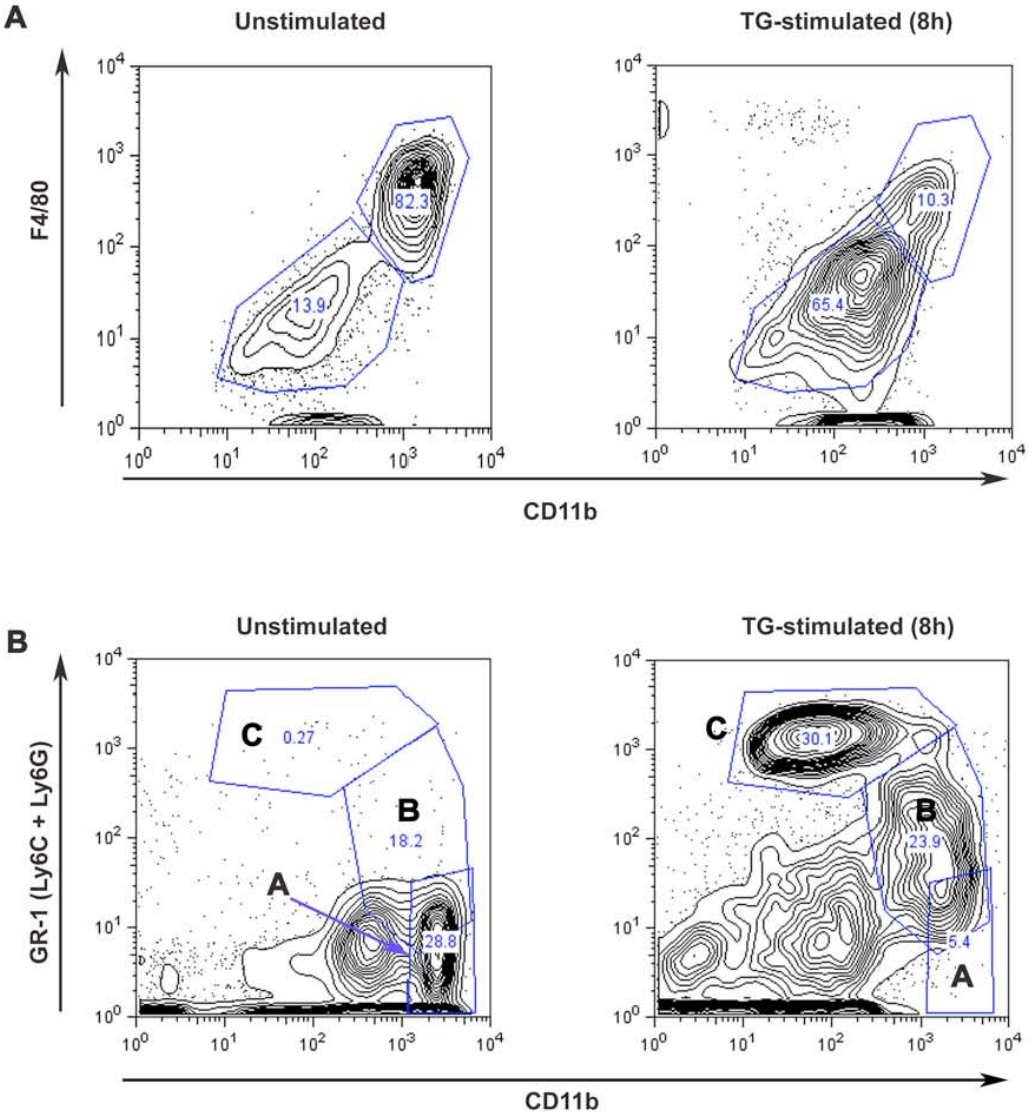
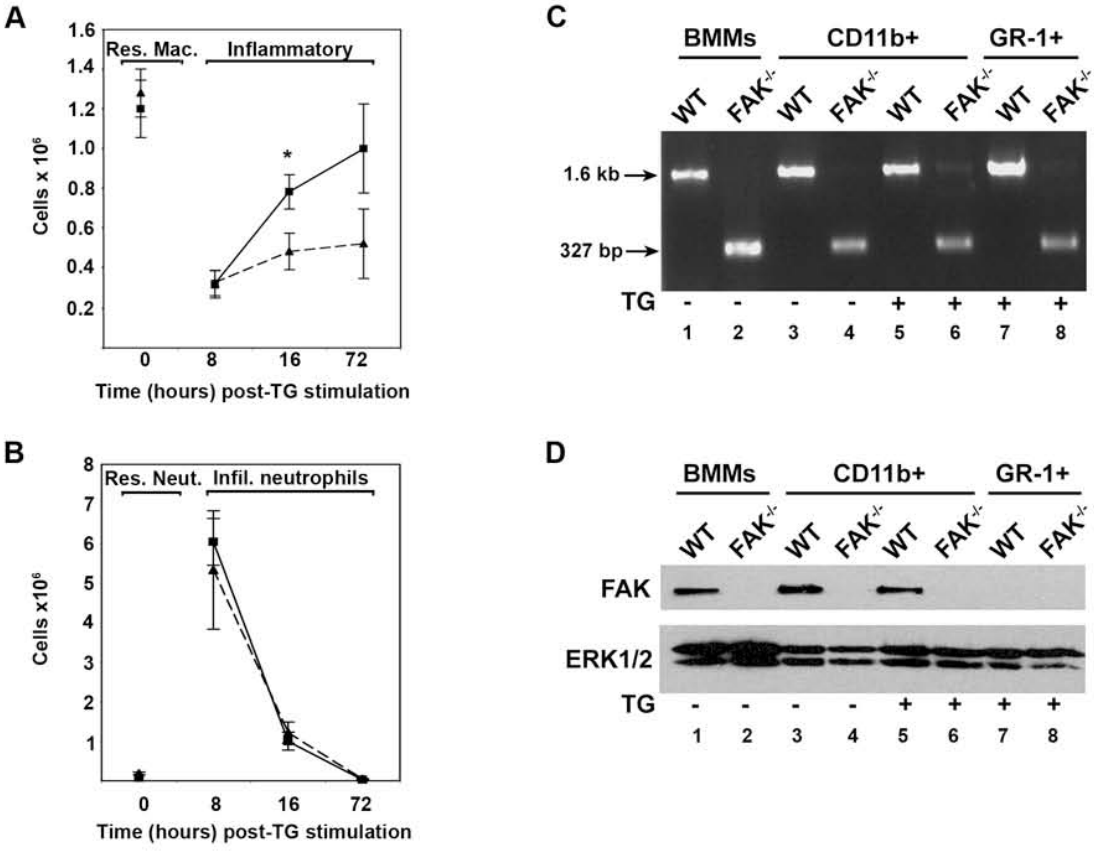

A
Unstimulated
TG-stimulated (8h)
F4/80
CD11b
82.3
13.9
10.3
65.4
B
Unstimulated
TG-stimulated (8h)
GR-1 (Ly6C + Ly6G)
CD11b
C
0.27
B
18.2
A
28.8
C
30.1
B
23.9
5.4
A

Figure 2.9. The recruitment of CD11b positive cells is impaired in conditional FAK knockout mice during thioglycollate challenge. WT and $FAK^{-/-}$ animals were injected intraperitoneally with 4% thioglycollate, and lavage fluid was collected at the indicated timepoints. To examine resident populations of cells, lavage fluid was also collected from the peritoneal cavities of unstimulated mice. Total cell numbers were obtained by hemocytometer and expression of CD11b and GR-1 was determined by flow cytometry. Data are expressed as cell numbers based on the percentage of cells designated as resident/inflammatory monocytes (**A**) or neutrophils (**B**) (see also Table 2.1 and Figure 2.8). WT, solid line-square; $FAK^{-/-}$, dashed line-triangle. Data are the means ± S.E.M. from 4 mice per time point (2 experiments). Asterisks indicate a statistically significant difference from the mean at ≥95% confidence level. **C.** PCR products of DNA isolated from cultured BMMs (lanes 1-2), CD11b-positively selected cells from unstimulated (lanes 3-4) or TG-stimulated mice (lanes 5-6), and GR-1 positively-selected cells from TG-stimulated mice (lanes 7-8) to distinguish the FAK-flox allele (1.6 kB) and the recombined locus (327 bp). **D.** Cellular proteins from cultured BMMs, CD11b positively-selected cells from unstimulated (lanes 3-4) or TG-stimulated mice (lanes 5-6) and GR-1 positively-selected cells from TG-stimulated mice (lanes 7-8) were immunoblotted with antibodies recognizing FAK (top panel) and total ERK1/2 (lower panel).


A
Res. Mac.
Inflammatory
Cells x 10^6
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
*
0
8
16
72
Time (hours) post-TG stimulation
B
Res. Neut.
Infil. neutrophils
Cells x10^6
8
7
6
5
4
3
2
1
0
8
16
72
Time (hours) post-TG stimulation
C
BMMs
CD11b+
GR-1+
WT
FAK-/-
1.6 kb
327 bp
TG
- - - - + + + +
1 2 3 4 5 6 7 8
D
BMMs
CD11b+
GR-1+
WT
FAK-/-
FAK
ERK1/2
TG
- - - - + + + +
1 2 3 4 5 6 7 8

The infiltration kinetics of GR-1 positive neutrophils was similar between the two mouse genotypes. (Figure 2.9B). This was somewhat surprising since LysM-Cre mediated recombination is reported to occur with approximately 99% efficiency in neutrophils (Clausen *et al.*, 1999). To determine whether *fak* was similarly deleted in GR-1-expressing cells, GR-1-positive cells were harvested from TG-stimulated WT and conditional FAK knockout animals and examined for Cre-mediated recombination by PCR. GR-1 positive cells from the conditional knockout mice exhibited efficient Cre-mediated recombination (Figure 2.9C, compare lanes 7 and 8). However, we were unable to detect FAK protein in GR-1 positive cells obtained from either WT or FAK-deficient mice, indicating that FAK is not normally expressed in neutrophils (Figure 2.9D, lanes 7-8). Thus, neutrophil infiltration was most likely not affected by the deletion of *fak* because FAK is not expressed in these cells. This suggests that the recruitment of neutrophils to sites of inflammation occurs via a FAK-independent mechanism.

2.5. Discussion.

As critical effectors of the innate immune system, macrophages utilize adhesion signaling to accomplish many essential cellular functions, including adhesion to and extravasation from blood vessels, chemotaxis, and phagocytosis. In this study, we demonstrate for the first time that FAK plays a critical role in processes involving macrophage adhesion and motility *in vitro*. We also show that disruption of FAK-dependent adhesion pathways in macrophages *in vivo* results in an attenuated inflammatory response, marked by reduced monocyte/macrophage infiltration into sites of inflammation. Collectively, these results provide a framework for examining the

signaling pathways controlling both FAK-dependent and FAK-independent based motility.

Regulation of macrophage motility by FAK family kinases. CSF-1 is a macrophage chemoattractant that interacts with its cognate receptor tyrosine kinase and is produced endogenously by activated endothelial cells and tissue fibroblasts. In this report, we demonstrate that $FAK^{-/-}$ BMMs were significantly impaired in their ability to migrate toward CSF-1. The chemotaxis of FAK-deficient macrophages toward SDF-1α and MCP-1, chemokines that signal through different sub-classes of heptahelical G protein-coupled receptors (CXCR4 and CCR2 respectively), was similarly reduced. These results suggest that the deficiencies in macrophage chemotaxis observed in $FAK^{-/-}$ macrophages may be a consequence of a fundamental breakdown in the cell migration machinery rather than an inability to respond to specific migratory stimuli. This conclusion is supported by data showing that macrophages lacking FAK exhibited impaired invasion through Matrigel. The essential contribution of FAK to macrophage function was further confirmed by the finding that knockdown of FAK expression via siRNA treatment inhibited the invasive capacity of macrophages to the same extent as did genetic deletion of this molecule.

To delineate the mechanism(s) underlying the migratory defects observed in FAK-deficient macrophages, we first compared the morphology and actin dynamics of WT and $FAK^{-/-}$ BMMs. CSF-1 induces monocyte/macrophage spreading, polarization, and extension of lamellipodia (Boocock *et al*., 1989; Webb *et al*., 1996; Jones, 2000). FAK-deficient macrophages were observed to continuously extend and retract numerous

short-lived protrusive structures. The inability of $FAK^{-/-}$ macrophages to form broad lamellipodia is consistent with a role for FAK in regulating and/or maintaining a leading edge during migration. This is in accordance with previous studies showing that FAK plays a central role in organizing and propagating signals required for directional migration in fibroblasts (Tilghman *et al*., 2005).

Although $FAK^{-/-}$ BMM migration and invasion were significantly impaired relative to control cells, the loss of FAK did not result in the complete abolishment of migratory activity, indicating that FAK-independent mechanisms also regulate macrophage motility. The genetic deletion of other signaling molecules such as Pyk2 and PI3K-γ from macrophages induces a phenotype similar to $FAK^{-/-}$ BMMs, characterized by formation of multidirectional lamellipodia, reduced polarization, and diminished migration (Jones *et al*., 2003; Okigaki *et al*., 2003). However, Pyk2 does not appear to be responsible for the residual invasive/migratory activity exhibited by FAK-deficient cells since the invasive/migratory capacity of $FAK^{-/-}$ macrophages was not further impaired by a loss of Pyk2. Nonetheless, depletion of Pyk2 from WT BMMs had an inhibitory effect on invasion/migration similar to that observed in the absence of FAK, suggesting that FAK and Pyk2 may regulate macrophage motility through a single pathway. We are currently investigating whether PI3K-γ or other known regulators of cell motility contribute to the FAK-independent pathway(s) controlling migration of macrophages.

FAK regulation of macrophage membrane protrusions and adhesions. An essential step in cell migration is the formation of lamellipodia at the leading edge, a process that is regulated, in part, by the small GTPase Rac1 (Ridley *et al*., 2003; Webb *et al*., 2004;

Moissoglu and Schwartz, 2006). The presence of active Rac1 at the leading edge of fibroblasts facilitates lamellipodia stabilization and promotes adhesion turnover within these protruding regions (Kraynov *et al*., 2000; Nayal *et al*., 2006). In this report, we show that FAK-deficient macrophages contain elevated basal levels of GTP-Rac1. These data, taken together with the observation that $FAK^{-/-}$ BMMs are highly protrusive, suggest that FAK may be directly involved in the local regulation of Rac1 activity at the leading edge. FAK may also indirectly modulate the localization of active Rac by regulating RhoA, which is reported to function in a mutually antagonistic manner with Rac1 under some circumstances (Pestonjamasp *et al*., 2006; Filippi *et al*., 2007). For example, in highly motile neutrophils, RhoA inhibits the formation of lateral pseudopodia by suppressing Rac1 activity at the sides and rear of migrating cells (Pestonjamasp *et al*., 2006; Filippi *et al*., 2007). Conversely, membrane-localized Rac1 downregulates RhoA function at the leading edge, a process that promotes the formation of lamellipodia and stimulates migration. FAK may function in macrophages in a similar fashion to enhance RhoA activity at lateral positions along the cell periphery, thus effectively downregulating Rac1 activity in these regions. This is consistent with previous studies showing that FAK is able to directly interact with p190RhoGEF, a RhoA-specific exchange factor that induces Rho activation (Zhai *et al*., 2003). Interestingly, evidence from $Pyk2^{-/-}$ macrophages indicates that these cells exhibit reduced RhoA activity coupled to defects in contractility and migration (Okigaki *et al*., 2003). Clearly, the regulation of macrophage migration by these molecules is complex. Future studies will focus on determining how FAK interacts with Rho family GTPases to promote cell motility.

It is of note that, despite the established role for Rac1 in the formation of lamellipodia, this molecule is not required for efficient macrophage migration (Wells *et al*., 2004). Rac1-deficient macrophages exhibit altered cell morphologies, appearing less spread and significantly more elongated than control cells. However, these cells migrate normally in response to chemotactic stimuli. Deletion of other Rac isoforms, including Rac2, also has little effect on macrophage chemotaxis. Interestingly, Rac1/2$^{-/-}$ cells were observed to migrate farther and faster than WT cells under certain conditions (Wheeler *et al*., 2006b). Nonetheless, high levels of GTP-Rac1 in fibroblasts correlated with a lack of motility and adoption of either a rounded non-ruffling, or a flattened, highly ruffled morphology (Pankov *et al*., 2005).

FAK also plays an important role in the regulation of adhesion dynamics. By inducing adhesion disassembly in fibroblasts, FAK signaling is postulated to interrupt the maturation of adhesions, thus allowing frequent adhesion turnover as the membrane protrudes and the cell advances (Webb *et al*., 2004). Based on the abnormally stable adhesion structures observed in FAK$^{-/-}$ macrophages, FAK may perform a similar function in these cells. However, the larger, more mature focal adhesions present in fibroblasts are not evident in BMMs (Pixley and Stanley, 2004). Moreover, unlike fibroblasts, FAK is required for both adhesion assembly as well as disassembly in macrophages. Irrespective of the mechanism, the stabilization of adhesions in the absence of FAK is likely to negatively impact cell migration due to an inability to turnover adhesions and recycle resident proteins.

Regulation of macrophage functions associated with the inflammatory response by FAK. Macrophages play a central role in the inflammatory process, releasing cytokines that control key events in the initiation, resolution and repair processes of inflammation (Henderson *et al*., 2003). Neutrophils are rapidly recruited into sites of acute infection and are the principal cell type during the initial influx of infiltrating leukocytes (Issekutz and Movat, 1980). However, monocyte/macrophages replace neutrophils as the predominant infiltrating population within 16 hours of an inflammatory reaction (Henderson *et al*., 2003). The induction of an inflammatory response in myeloid-specific conditional FAK knockout mice resulted in the delayed recruitment of inflammatory CD11b positive monocyte/macrophages relative to control mice, whereas the recruitment of GR-1 positive neutrophils occurred with identical kinetics in both mouse genotypes. That the floxed-*FAK* allele underwent recombination in neutrophils from conditional FAK knockout mice is not surprising since efficient LysM-driven Cre-mediated excision of floxed target genes has been reported in this cell type (Clausen *et al*., 1999). However, because FAK protein is not endogenously expressed in this cell lineage, the genetic deletion of *FAK* had no effect on neutrophil recruitment. These findings underscore the importance of FAK during recruitment of macrophages for which FAK-dependent mechanisms of migration are essential, and suggest that other FAK-independent mechanisms of motility are employed in cell lineages that do not typically express FAK.

Based on these data, we suggest that it may be possible to alter the macrophage-specific host response to inflammation by targeting FAK in the monocyte/macrophage lineage. This has significant consequences when considered within the context of

diseases in which the accumulation of macrophages contributes to disease progression, such as chronic inflammatory diseases and cancer. Adhesion signaling in immune cells has already been established as a viable therapeutic target. For example, antibodies that block α4 integrin functions on the surface of T-helper 1 cells or activated macrophages have been shown to be beneficial for treating both Crohn's disease and multiple sclerosis (Ghosh *et al*., 2003; Miller *et al*., 2003). Similar approaches might be useful for treating solid tumors, which are often found to contain macrophages, since high numbers of tumor-associated macrophages correlate with a poor prognosis(Pollard, 2004). Recent studies using small molecular inhibitors of FAK, which specifically target FAK catalytic activity, have shown that cell migration and focal adhesion turnover are inhibited by the drug (Slack-Davis *et al*., 2007). While further studies will be required to elucidate the FAK-dependent and independent signaling pathways required for macrophage migration, the data presented herein highlight potential therapeutic applications involving the inhibition of FAK activity in macrophages

Chapter 3:

α4–integrin-paxillin signaling promotes macrophage invasion independent of FAK

Katherine A. Owen, Keena S. Thomas and Amy H. Bouton

3.1. Abstract.

The FAK family of kinases regulate cell adhesion, migration and proliferation in a variety of cell types. The importance of FAK during macrophage migration is underscored by the fact that genetic deletion of this molecule from macrophages results in significantly impaired motility. Previously, we demonstrated that targeting Pyk2 expression using RNAi resulted in impaired macrophage invasion, while the combined loss of FAK and Pyk2 had no additional effect on macrophage invasion through 3-dimensional matrices. In this report, we show that, unlike Pyk2, the reduced expression of the adapter protein paxillin in FAK-deficient macrophages, or the treatment of these cells with a blocking α4-integrin antibody further inhibited the invasive capacity of macrophages. Combined treatment with siRNAs targeting paxillin expression and an antibody blocking α4-integrin function did not cause an additional reduction in the invasive capacity of macrophages over either treatment alone. These results indicate that macrophages utilize a FAK/Pyk2-dependent and an α4-integrin-paxillin-dependent pathway to promote an efficient motile response to CSF-1.

3.2. Introduction.

In response to integrin ligation, signaling transducers such as FAK become activated and are able to trigger a variety of distinct cellular responses. Integrin receptors do not possess intrinsic catalytic activity, thus, integrin-mediated signaling events are promoted by interactions with other receptors, and by the recruitment and clustering of

intracellular signaling molecules at integrin cytoplasmic tails. As a non-receptor tyrosine kinase, FAK is able to associate with integrins and growth factor receptors through the recruitment of other signaling molecules to the cytoplasmic domains of these receptors. The formation of signaling complexes at these sites of interaction relays information about the extracellular matrix to the actin cytoskeleton, a process that can initiate cell adhesion, spreading, polarization and migration. Through the conditional ablation of FAK from cells of myeloid lineage, we have been able to demonstrate the importance of FAK for macrophage motility (Chapter 2). While the loss of FAK from macrophages clearly impairs the ability of cells to migrate during chemotaxis, the motile response of these cells is never completely abolished, suggesting that FAK-independent pathways are also involved in the regulation of macrophage migration.

CSF-1 is a macrophage specific growth factor that supports the survival, proliferation and differentiation of mononuclear phagocytes (Pixley and Stanley, 2004). This molecule also functions as a powerful macrophage chemoattractant. The effects of CSF-1 are mediated via autophosphorylation of the CSF-1R and the subsequent recruitment of intracellular signaling molecules. The precise signaling pathways that connect the activated CSF-1R to the actin cytoskeleton have not been defined, but molecules such as the FAK family member Pyk2 and the cytoskeleton-associated proteins paxillin and vinculin have been shown to be involved in CSF-1-induced spreading and adhesion. Pyk2 is also involved in the regulation of motility in myeloid-lineage cells, as Pyk2-deficient macrophages were found to be impaired in their ability to migrate in response to inflammatory stimuli (Okigaki *et al.*, 2003).

Integrins coordinate spatial signaling events essential for cell polarity and directed migration. The α4 subfamily of integrins, which include α4β1 and α4β7, are predominantly expressed on leukocytes, neural crest cells and skeletal muscle (Berton and Lowell, 1999). Ligation of the α4-integrinsubunit dramatically enhances cell migration by tightly regulating the ability of the α4 cytoplasmic tail to associate with the adapter protein paxillin (Chan *et al*., 1992; Liu *et al*., 1999; Liu and Ginsberg, 2000). The spatial regulation of α4-paxillin interactions contributes to the suppression of lamellipodia at the sides and rear of a cell, but not at the leading edge (Goldfinger *et al*., 2003). Leukocyte homing to inflamed tissue is a crucial first step toward the resolution of inflammation and is dependent upon α4-paxillin mediated migration. While FAK, Pyk2 and paxillin are endogenously expressed in macrophages, the signaling pathways regulating macrophage migration and invasion remain poorly defined, and it is currently unclear whether these molecules function within separate or interdependent pathways to promote cell motility.

Using RNAi to reduce levels of paxillin and antibodies designed to block α4-integrin function in primary macrophages derived from myeloid lineage-specific conditional FAK knockout mice, we have begun to assess the role of these host molecules during CSF-1 induced macrophage invasion. We show here that macrophage motility is regulated by two distinct pathways: one dependent on the expression of FAK and Pyk2, and the other dependent on α4-integrin and paxillin. Previously, we have demonstrated that reduced Pyk2 expression results in lower levels of macrophage invasion, whereas the combined loss of FAK and Pyk2 did not cause an additional decrease in the ability of these cells to invade 3-dimensional substrates (Chapter 2).

However, depletion of paxillin expression in $FAK^{-/-}$ BMMs nearly abolished macrophage invasion, suggesting that paxillin functions independently of FAK/Pyk2 during this process. In addition, blocking α4-integrin function had essentially the same effect on invasion as targeting paxillin expression. Taken together, the studies presented herein describe for the first time a detailed analysis of the signaling pathways controlling macrophage motility in response to CSF-1 stimulation.

3.3. Materials and methods.

Cell culture. Primary bone marrow macrophages were isolated from control and conditional FAK knockout mice as described previously (Chapter 2).

Antibodies and reagents. Polyclonal FAK C-20 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). mAbs recognizing paxillin were purchased from BD Biosciences (San Diego, CA). A phospho-specific antibody recognizing paxillin Y118 was purchased from BioSource international (Camarillo, CA). A blocking mAb recognizing α4-integrin was purchased from Chemicon (Temecula, CA). PE-conjugated anti-CD115 (CSF-1R) was purchased from eBiosciences (San Diego, CA). Purified rat IgG was purchased from Lampire Biological Laboratories (Pipersville, PA). Anti-CD16/32 and PE-conjugated anti-rat IgG were purchased from Caltag (Burlingame, CA). HRP-conjugated sheep anti-mouse Ig and HRP-conjugated donkey anti-rabbit antibodies were purchased from Amersham Pharmacia Biotech (Piscataway, NJ). CSF-1 was purchased from PeproTech, Inc. (Rocky Hill, NJ).

Immunoprecipitation and Immunoblotting. Immunoprecipitations, immunoblotting and densitometry were performed as described previously (Burnham *et al*., 1996 and Chapter 2).

Invasion assays. Invasion assays were performed as described previously (Chapter 2). Where indicated, blocking α4-integrin (10 μg/ml) or IgG control (10 μg/ml) antibodies were added to the top and bottom chambers. The total number of cells invading after 24 hours were counted.

Flow cytometry. Approximately $1x10^6$ BMMs were labeled with anti-α4-integrin antibody (1:50) followed by secondary staining with PE-conjugated anti-rat IgG (1:500), or PE-conjugated anti-CD115 (1:200). As a control, cells were stained with PE-conjugated anti-rat IgG (secondary) alone. Prior to staining, Fc receptors were blocked with anti-CD16/32 for 10 minutes on ice. Macrophages were washed 3 times after each staining step and resuspended in buffer containing PBS, 0.5% BSA and 0.05% sodium azide (Sigma, St. Louis, MO) before analysis on a FACs-Calibur™ system (Becton Dickinson, San Jose, CA). Data were analyzed with FloJo version 6.4.1 (TreeStar, Ashland, OR).

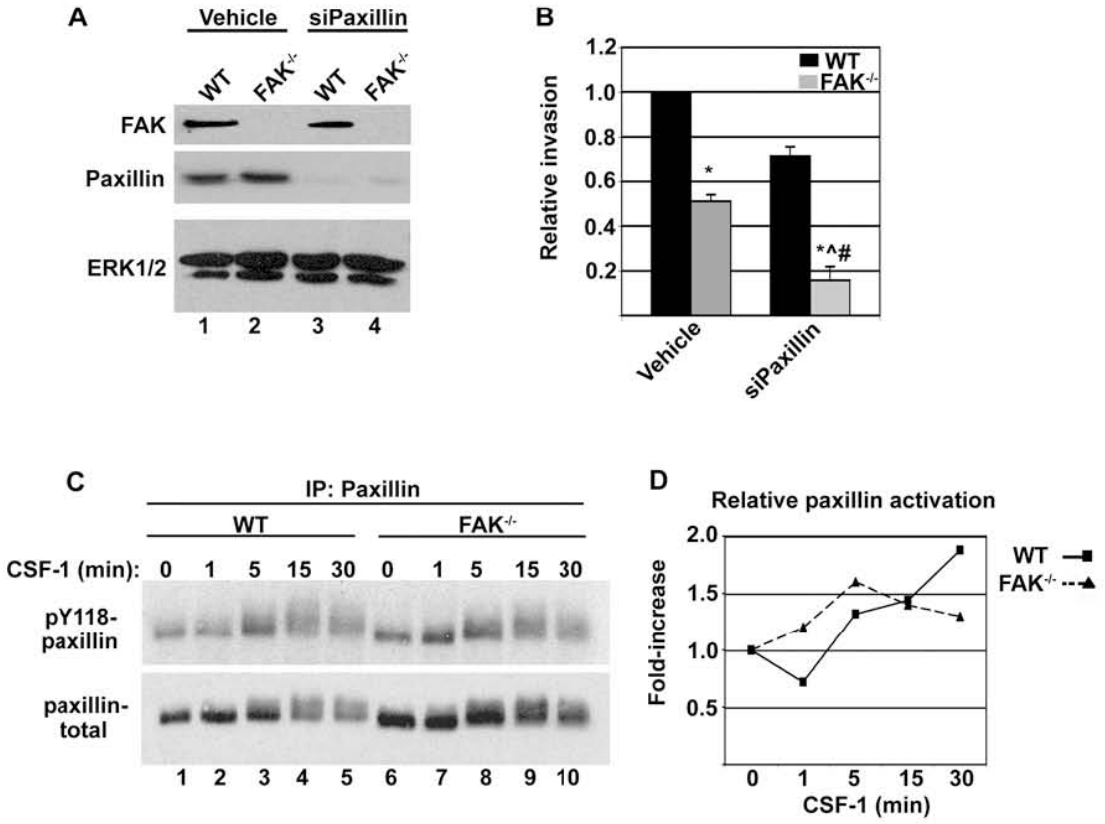
siRNA transfection. Transfection of siGenome SmartPool siRNAs targeting murine FAK (100 nmol) and paxillin (300 nmol) (Dharmacon Inc., Lafayette, CO) was achieved by nucleofection as previously described (Chapter2).

Statistical analysis. A two-sample t-test assuming unequal variance was used to determine statistical significance between condition means with a significance level of ≤ 0.05.

3.4. Results and Discussion.

Paxillin is required for macrophage invasion. Previous work from our lab has established that FAK and Pyk2 account for approximately 50% of the invasive capacity of macrophages. We therefore explored whether paxillin was responsible for the residual level of invasion observed by $FAK^{-/-}$ BMMs. To determine if paxillin is required for CSF-1-induced macrophage invasion, WT and $FAK^{-/-}$ cells were treated with vehicle or siRNAs targeting paxillin before seeding onto matrigel-coated Boyden chambers for 24 hours. Transfection with siPaxillin resulted in the reduced expression of paxillin by approximately 80-90% in both cell types (Fig. 3.1A; middle panel, lanes 3-4) without affecting FAK expression in WT cells (top panel, lane 3). Treatment with vehicle did not affect paxillin expression (middle panel, lanes 1-2) or expression of the non-targeted proteins ERK1/2 (lower panel). As expected, invasion of $FAK^{-/-}$ BMMs treated with vehicle was reduced by 50% compared to WT cells (Fig. 3.1B). Knockdown of paxillin in WT and $FAK^{-/-}$ cells resulted in a 30% and 93% decrease in invasion, respectively. It is notable that the reduced expression of paxillin in $FAK^{-/-}$ cells caused an additional reduction in invasion over the effect of FAK deletion alone. From these results, we propose that paxillin promotes macrophage invasion via a FAK-independent pathway. In

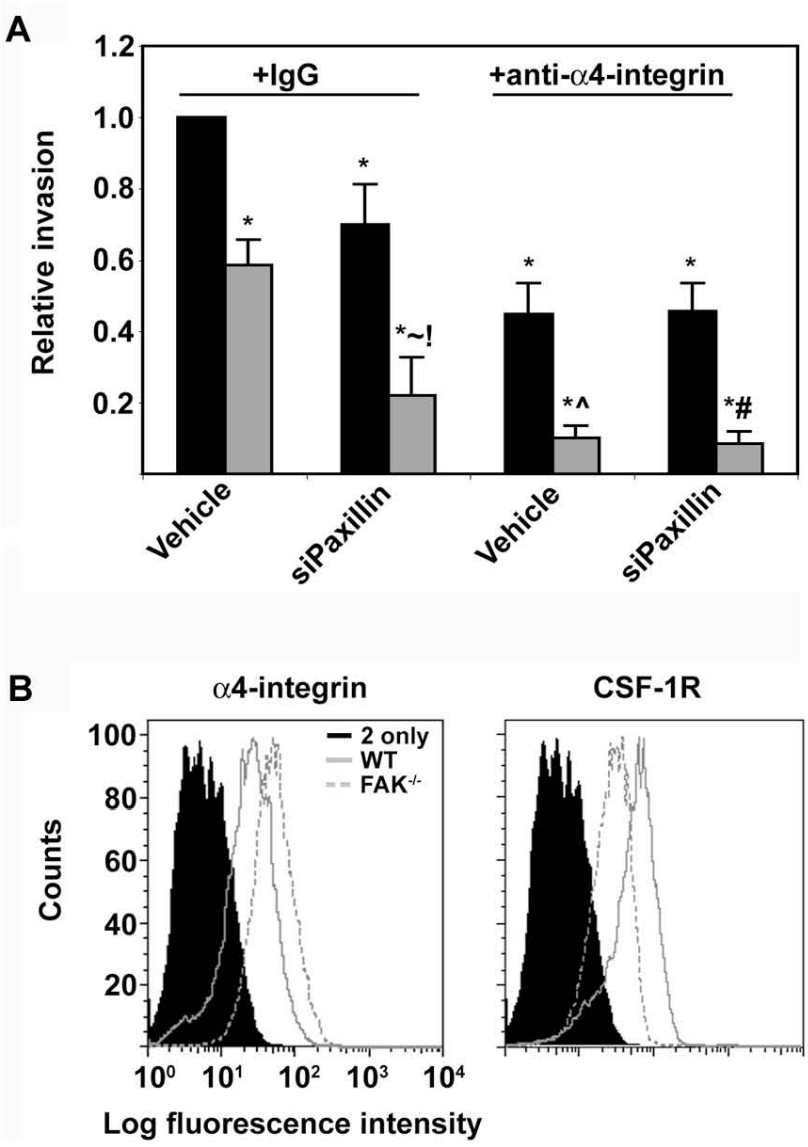
Figure 3.1. Paxillin contributes to macrophage invasion. A. WT and FAK$^{-/-}$ BMMs were treated with vehicle (H_2O) or siPaxillin. 72 hours post-siRNA transfection, cells were lysed and immunoblotted for total FAK and paxillin (top panels) and ERK1/2 (lower panel). **B.** Vehicle and siRNA-treated cells were starved of CSF-1 overnight before loading onto matrigel-coated Boyden chambers. WT (black bars) and FAK$^{-/-}$ (gray bars) BMMs were then allowed to invade toward CSF-1 (120 ng/ml) for 24 hours at 37°C. The data represent the mean ± S.E.M. for 4 separate experiments. Asterisks indicate a statistically significant difference from vehicle-treated WT cells at ≥95% confidence level; ^ indicate a statistically significant difference from vehicle-treated FAK$^{-/-}$ cells at ≥95% confidence level; # indicate a statistically significant difference from siPaxillin-treated WT cells at ≥95% confidence level. **C.** Cells were plated onto tissue culture plastic and starved of CSF-1 overnight before stimulation with CSF-1 (120 ng/ml) for 0-30 minutes. Paxillin immune complexes were generated from lysates, separated by SDS-PAGE, and immuoblotted with phosphospecific antibodies recognizing phosphorylated Y118 on paxillin (top panel). The blot was stripped and probed for total paxillin (lower panel)**. D**. Relative (relative to WT "0" or FAK$^{-/-}$ "0") band intensities are displayed in graph form to the right (WT, solid line-square; FAK$^{-/-}$, dashed line-triangle). Each immunoblot represents multiple independent experiments.


A
Vehicle
siPaxillin
WT
FAK-/-
WT
FAK-/-
FAK
Paxillin
ERK1/2
1 2 3 4
B
Relative invasion
1.2
1.0
0.8
0.6
0.4
0.2
WT
FAK-/-
*
*^#
Vehicle
siPaxillin
C
IP: Paxillin
WT
FAK-/-
CSF-1 (min): 0 1 5 15 30 0 1 5 15 30
pY118-paxillin
paxillin-total
1 2 3 4 5 6 7 8 9 10
D
Relative paxillin activation
Fold-increase
2.0
1.5
1.0
0.5
WT
FAK-/-
0 1 5 15 30
CSF-1 (min)

further support of this hypothesis, paxillin activity was not greatly affected by the loss of FAK, since CSF-1-induced stimulation of WT and FAK-deficient macrophages did not reveal any major differences in paxillin phosphorylation intensity or kinetics between the cell types (Fig. 3.1C and D).

α4-integrin-mediated invasion requires paxillin but not FAK. α4-integrin-mediated cell migration is essential for embryogenesis, hematopoiesis, lymphocyte homing, and the recruitment of leukocytes to sites of inflammation (Springer, 1994; Butcher *et al*., 1999). The binding of paxillin directly to the cytoplasmic tail of α4-integrin has been shown to be important for leukocyte migration (Liu *et al*., 1999; Nishiya *et al*., 2005). We therefore hypothesized that paxillin-mediated invasion, occurring independently of FAK and/or Pyk2 activity, may involve signaling via the α4-integrin receptor. To determine whether α4-integrin promotes macrophage invasion in response to CSF-1, WT and $FAK^{-/-}$ BMMs were transfected with vehicle or siPaxillin before treatment with blocking α4 antibodies or isotype IgG control antibodies. These cells were then allowed to invade through matrigel-coated Boyden chambers for 24 hours. Treatment with control IgG had no detectable effect on the pattern of invasion observed previously: vehicle-treated $FAK^{-/-}$ BMMs were 40-50% less efficient at invasion and the depletion of paxillin from these cells resulted in a further reduction in invasion, while the invasion of siPaxillin-treated WT macrophages was inhibited approximately 30% (Fig. 3.2A). Treatment with the blocking α4-integrin antibody impaired the ability of vehicle-treated WT cells to invade through matrigel by 50%, whereas the invasive capacity of $FAK^{-/-}$ BMMs, similarly treated with blocking antibody, was nearly abolished (Fig. 3.2A). WT cells concomitantly

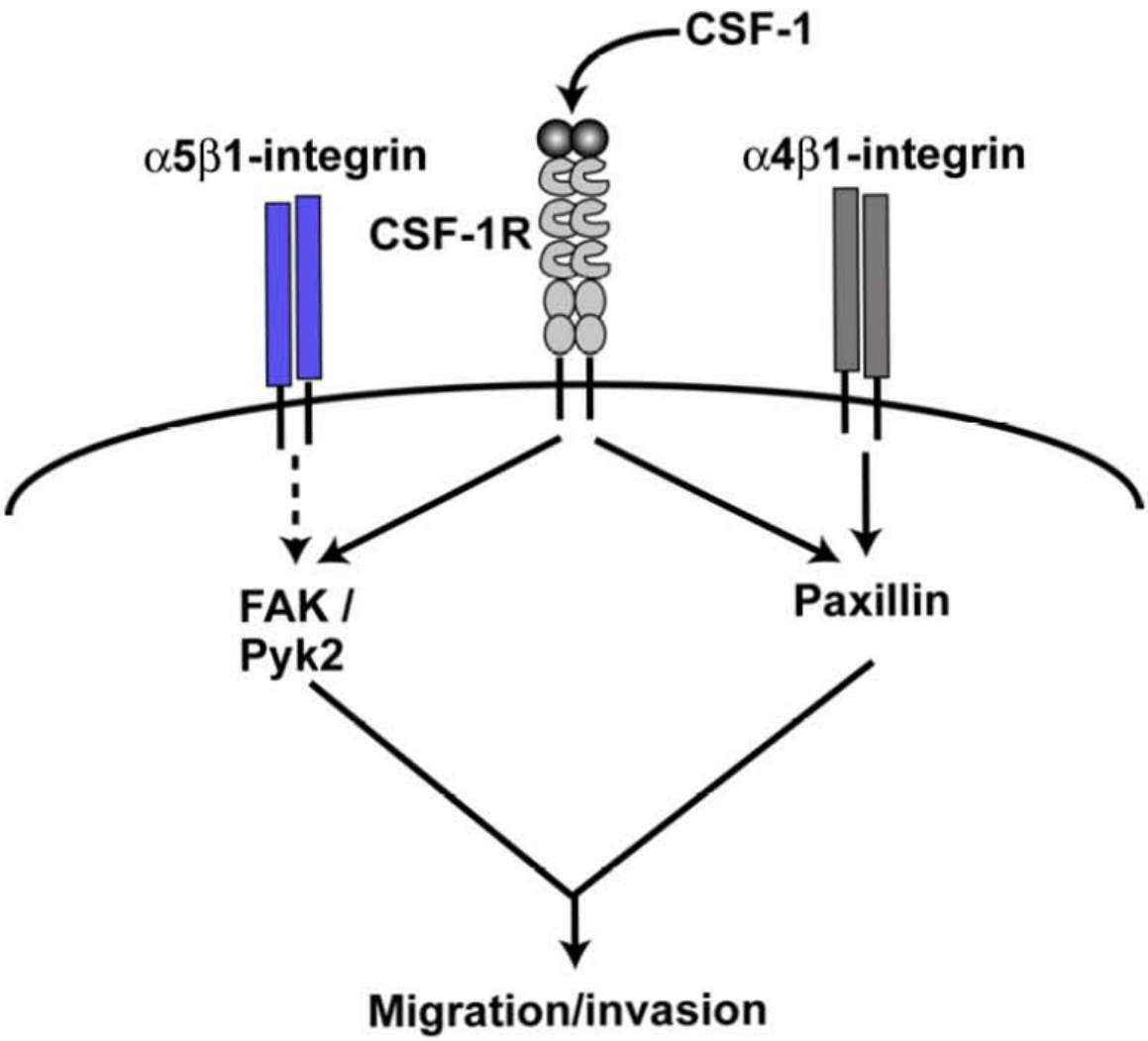
Figure 3.2. Blockade of α4-integrin signaling inhibits macrophage motility. A. WT and $FAK^{-/-}$ BMMs were treated with vehicle (H_2O) or siPaxillin. 48-hours post-siRNA transfection, vehicle and siRNA-treated cells were starved of CSF-1 overnight before loading onto matrigel-coated Boyden chambers containing anti-α4-integrin antibodies (10 μg/ml) or isotype control IgG (10 μg/ml). WT (black bars) and $FAK^{-/-}$ (gray bars) BMMs were then allowed to invade toward CSF-1 (120 ng/ml) for 24 hours at 37°C. The data represent the mean ± S.E.M. for 3 separate experiments. Asterisks indicate a statistically significant difference from vehicle-treated, IgG-treated WT cells, ~ indicate a statistically significant difference from vehicle-treated, IgG-treated $FAK^{-/-}$ cells, ! indicate a statistically significant difference from siPaxillin-treated, IgG-treated WT cells, ^ indicate a statistically significant difference from vehicle-treated, α4-integrin-treated WT cells and # indicate a statistically significant difference from siPaxillin-treated, α4-integrin-treated WT at ≥95% confidence level. **B.** Flow cytometry analyses of endogenous α4-integrin and CSF-1R expression in WT (solid gray line) and $FAK^{-/-}$ BMMs. (dashed gray line). Staining with PE-conjugated anti-rat secondary control antibody is indicated by the filled peaks.


A
Relative invasion
1.2
1.0
0.8
0.6
0.4
0.2
+IgG
+anti-α4-integrin
*
*
*
*~!
*
*^
*
*#
Vehicle
siPaxillin
Vehicle
siPaxillin
B
α4-integrin
CSF-1R
2 only
WT
FAK-/-
Counts
100
80
60
40
20
10⁰ 10¹ 10² 10³ 10⁴
Log fluorescence intensity

treated with both the blocking α4-integrin antibody and siPaxillin showed a 50% reduction in invasion, while the invasive capacity of FAK$^{-/-}$ cells was inhibited by approximately 90%. Overall, invasion in the presence of the blocking α4-integrin antibody was not found to be significantly different from the levels of invasion observed in siPaxillin-treated cells. These data indicate that an α4-integrin-paxillin pathway promotes efficient CSF-1 induced invasion independent of FAK activity. To confirm that the reduced levels of invasion observed in the presence of the blocking α4-integrin antibody was not due to altered receptor expression, the surface expression of α4-integrin and the CSF-1R (CD115/c-fms) on WT and FAK$^{-/-}$ BMMs was examined by flow cytometry (Fig. 3.2B). Both cell types express roughly equivalent levels of α4-integrin and the CSF-1R on their surface.

This work has allowed us to generate a model for macrophage invasion through 3-dimensional substrates (Fig. 3.3). We propose that FAK and Pyk2 function within the same pathway to regulate macrophage invasion toward CSF-1. Invasion also requires a separate α4-integrin-paxillin linkage. This is based on data showing that the reduced expression of paxillin in FAK-deficient macrophages almost entirely abolished the ability of these cells to invade. Furthermore, the functional blockade of α4-integrin signaling had essentially the same effect on invasion as depleting paxillin expression in WT and FAK$^{-/-}$ cells. Signaling events initiated by the CSF-1R may have the ability to alter integrin receptor function by modulating integrin activation states. CSF-1-mediated paxillin phosphorylation, occurring independent of FAK expression, may effect conformational changes required to convert the α4-integrin from a low to a high activation state. There are numerous examples where cooperation between growth factors

Figure 3.3. Model describing the signaling pathways involved in the promotion of CSF-1-induced macrophage invasion. Our results support a model in which CSF-1-induced motility requires a FAK/Pyk2-dependent pathway and an α4-integrin-paxillin-dependent pathway. In the presence of ligand, CSF-1R activation induces the activation of FAK and Pyk2. FAK/Pyk2-dependent motility may require signaling via the α5β1-integrin receptor. Paxillin activation also occurs in response to CSF-1, but is independent of FAK activity. Paxillin phosphorylation and recruitment to the α4β1 cytoplasmic tail may ultimately promote the conformational changes necessary for α4β1-integrin receptor activation, and suggests a level of coopertivity between growth factor receptors and integrins. Solid lines indicate known pathways, dotted lines represent hypothetical pathways.


CSF-1
α5β1-integrin
CSF-1R
α4β1-integrin
FAK /
Pyk2
Paxillin
Migration/invasion

and integrins is able to induce significant changes in cellular signaling pathways (for review, see Chan *et al.*, 2006). In this way, synergistic interactions between the CSF-1R and α4-integrins may be required to promote the motility of macrophages.

While our findings suggest a link between CSF-1R ligation and the activation of FAK and Pyk2, we have not yet established that these molecules lie directly downstream of CSF-1R signaling. It is therefore possible that, during CSF-1 stimulation, FAK and Pyk2 are themselves activated in response to integrin receptor ligation. In a recent report (Hsia *et al.*, 2005), signaling via α4β1- and α5β1-integrins in fibroblasts activates motility promoting pathways through distinct receptor-proximal connections. However, in their model of cell migration, FAK and Pyk2 were fundamentally linked to α5β1-integrin function whereas α4β1-integrin-mediated motility did not require paxillin. Currently, the contribution of α5β1-integrin to macrophage migration is unknown, yet the possibility exists that some level of CSF-1-induced migration is occurring in response to α5β1 ligation. Compared to macrophages, which endogenously express α4 integrins, the fibroblasts used by Hsia *et al* to examine α4β1- and α5β1-signaling pathways were genetically manipulated to express a functional α4β1 integrin receptor, which may account for some of the discrepancies between our data. Alternatively, the differential requirement for paxillin during α4β1-mediated signaling may reflect the fact that, as a highly motile cell type, macrophages utilize a divergent motility-promoting signaling pathway.

Integrin-mediated and CSF-1R-mediated signaling have been shown to be important in the regulation of macrophage spreading, motility and adhesion structure formation (Boocock *et al.*, 1989; Pixley *et al.*, 2001; Caveggion *et al.*, 2003). α4-

integrins in particular, strongly promote cell migration through their association with paxillin (Goldfinger *et al*., 2003). Integrin receptors signal through a number of different pathways, and extensive studies have been performed to show that integrins and growth factor receptors are able to interact in a collaborative manner (Miyamoto *et al*., 1996; Ross, 2004). The results presented here provide evidence for a novel mechanism involving both FAK-dependent and independent signaling pathways required for efficient macrophage motility. However, it is still unclear if the requirements for a FAK/Pyk2-dependent and an α4-integrin-paxillin-dependent pathway during CSF-1-induced invasion are specific for this growth factor, or if other chemoattractant receptors require a similar signaling mechanism.

Chapter 4:

Conditional deletion of FAK from cells of the myeloid lineage may confer resistance to tumor invasiveness and growth

Katherine A. Owen, Fiona J. Pixley, Michael Gutknecht, Elizabeth Thompson, Victor H. Englehard and Amy H. Bouton

4.1. Abstract.

Macrophages are among the first cells to arrive at sites of infection and tissue wounding. These cells produce numerous cytokines and chemokines that recruit other immune cells to damaged tissues, and produce growth factors, angiogenic factors and proteases that facilitate healing. However, tumors have been observed to subvert the normal developmental roles of macrophages to promote their own growth and metastatic potential. FAK is a ubiquitously expressed non-receptor tyrosine kinase that plays an important role in the regulation of macrophage migration, chemotaxis and phagocytosis. Using macrophages derived from myeloid-specific conditional FAK knockout mice, we have examined how the loss of FAK expression from macrophages affects tumor cell behavior. Preliminary data suggest that $FAK^{-/-}$ macrophages are unable to promote the invasion of tumor cells through matrix, and this deficiency may be associated with an inability to secrete invasion-promoting factors. We also provide preliminary *in vivo* evidence indicating that tumor outgrowth may be delayed in the myeloid lineage-specific conditional FAK knockout mice. Although further experimentation needs to be completed, the results presented here suggest that altering macrophage function through the loss of FAK expression may modulate tumor behavior.

4.2. Introduction.

The tumor microenvironment plays an important role in the progression and metastasis of solid tumors. Chemoattractants, growth factors, ECM composition and the presence of infiltrating leukocytes modulate the invasive capacity of tumor cells within the tumor microenvironment (Mueller and Fusenig, 2004). Hematopoietic cells, including circulating monocytes, are recruited to solid tumors by growth factors and chemokines, which are often produced by the tumor cells themselves. Macrophages are key mediators of the innate immune system, functioning as sentinels involved in the organization of immune defenses and the coordination of tissue repair processes, matrix remodeling and angiogenesis (Coussens and Werb, 2002). It is now clear that macrophages can also function within the tumor microenvironment to promote tumor growth at distal metastatic sites.

Tumor-associated macrophages (TAMs) are induced to adopt trophic roles similar to those seen during development and tissue repair upon recruitment to the tumor microenvironment (Elgert *et al.*, 1998; Ohm and Carbone, 2001; Condeelis and Pollard, 2006). The presence of immunosuppressive factors, such as transforming growth factor-β1 (TGF-β1) and CSF-1, attenuates the ability of macrophages and DCs to respond to and present tumor antigens (Gorelik and Flavell, 2001; Condeelis and Pollard, 2006). In addition, TAMs are also important producers of soluble mediators required for growth and angiogenesis, including EGF, basic fibroblast growth factor (bFGF), VEGF, IL-1, IL-8 and tumor necrosis factor-α (TNF-α) (Condeelis and Pollard, 2006). These factors

promote the proliferation and migration of endothelial cells, matrix remodeling and the formation of new blood vessels, as well as the recruitment of additional leukocytes.

FAK plays a crucial role in mediating signal transduction pathways initiated either at sites of cell attachment or at growth factor receptors. Activation of FAK leads to a number of cell biological processes, including cell attachment, migration, invasion, proliferation and survival (for review, see van Nimwegen and van de Water, 2007). Multiple reports describe the expression and activity of FAK in primary and metastatic human tumor tissue with the majority of studies showing enhanced expression of FAK mRNA and/or protein in a variety of human cancers including squamous cell carcinoma of the larynx (Aronsohn *et al*., 2003, invasive colon and breast (Owens *et al*, 1995) and malignant melanoma (Kahana *et al*., 2002). Given the important role of FAK in processes such as tumorigenesis, metastasis and survival signaling, this molecule has been regarded as a potential target in the development of anti-cancer drugs.

FAK regulates a number of macrophage-specific functions such as chemotaxis and migration (Chapter 2) as well as phagocytosis (Chapter 5). It has recently been shown that monocyte recruitment to sites of inflammation is significantly delayed in myeloid lineage specific conditional FAK knock out mice (Chapter 2). These data indicate that, by targeting FAK in monocyte/macrophages such that the ability of these cells to migrate is inhibited, the macrophage-specific host response to inflammation can be altered. Since many tumors, as they become invasive, are abundantly populated by macrophages, we became interested in determining whether the loss of FAK from macrophages could affect the progression of tumor malignancy.

Here, we provide evidence that macrophages derived from myeloid lineage-specific conditional FAK knockout mice are unable to promote the invasion of carcinoma cells through a collagen matrix. One of the pro-tumorigenic functions of macrophages involves the production of proteases such as urokinase-type plasminogen activator (uPA), MMPs and cathepsins, which breakdown basement membranes and allow for tumor cell invasion of the surrounding stroma (Condeelis and Pollard, 2006). However, we were unable to detect any defects in the ability of $FAK^{-/-}$ macrophages to form podosomal rosettes, structures typically enriched in proteolytic components, or in the ability of these cells to degrade fibronectin. Rather, our results suggest that these cells may be deficient in the production or secretion of soluble invasion-enhancing factors. Finally, we demonstrate that the deletion of FAK from cells of myeloid lineage may ultimately confer a delay in the establishment and/or initiation of tumor growth in an *in vivo* mouse model.

4.3. Materials and methods.

Cell culture and bacterial strains. MTLn3-GFP cells were cultured in α-MEM with 5% FBS. BAC1.2F51.2F2 cells were cultured in α-MEM with 10% FBS and 36 ng/ml human recombinant CSF-1 (Chiron Corp, Emeryville, CA). Primary bone marrow macrophages were isolated from control (phenotypically WT) and conditional FAK knockout (conditional $FAK^{-/-}$) mice as described previously (Chapter 2). All cells were grown in the presence of 100 U/ml penicillin and 100 U/ml streptomycin (Gibco BRL

Life Technologies; Rockville, MD). The growth conditions for the pYV-cured *Yersinia pseudotuberculosis* adhesin mutant strain used in these studies are detailed in Chapter 5.

Generation of myeloid-specific conditional FAK knockout mice and the isolation of BMMs. The generation of myeloid-specific conditional FAK knockout mice and their control littermates, and the isolation of BMMs has been described previously (Chapter 2).

Antibodies and reagents. An antibody recognizing IκBα was a kind gift from Dr. M. Mayo (University of Virginia, Charlottesville, VA). Alexa Fluor 488-phalloidin was purchased from Molecular Probes (Eugene, OR). FITC-conjugated rabbit anti-mouse Ig and TR-phalloidin were purchased from Jackson Immunoresearch Laboratories, Inc. (West Grove, PA). HRP-conjugated sheep anti-mouse Ig and HRP-conjugates donkey anti-rabbit Ig were purchased from Amersham Pharmacia Biotech (Picastaway, NJ). A monoclonal antibody recognizing paxillin was purchased from Cell Signaling Technology (Beverly, MA). CSF-1 was purchased from Peprotech, Inc. (Rocky Hill, NJ). FN and collagen I were purchased from Sigma Aldrich (St. Louis, MO). N-Hydroxysuccinimide (NHS)-fluorescein was purchased from Pierce (Rockford, IL).

Immunoblotting. Briefly, WT and $FAK^{-/-}$ BMMs were infected with the adhesin-deficient *Y. pseudotuberculosis* ($I^{-}Y^{-}$) at a multiplicity of infection (MOI) of 200:1, rinsed twice with PBS, and lysed in modified RIPA (50 mM Tris, 150 mM NaCl, 1% Igepal CA-630, 0.5% deoxycholate) containing protease and phosphate inhibitors (100 μM leupeptin, 1

mM phenylmethylsulfonyl fluoride, 0.15 unit/ml aprotinin, 1 mM vanadate) as previously described (Kanner et al., 1989). Immunoblotting was performed as previously described (Burnham *et al*., 2000; Weidow *et al*., 2000 and Chapter 2).

Collagen invasion assay (adapted from Goswami et al., 2005). MTLn3-GFP cell were plated on a 35-mm MaTek Dish in the presence of BAC1.2F51.2F5 cells or BMMs derived from control or conditional FAK knockout mice in 2 mls α-MEM with 10% FBS and 36 ng/ml CSF-1. After 16 hours, cells were overlaid with a 750- to 1000-μm layer of collagen I, which was allowed to gel for 90 minutes before the addition of 1 ml α-MEM with 10% chemically defined lipid mix and insulin-transferrin-selenium, both of which were purchased from Gibco BRL Life Technologies (Rockville, MD). After 24 hours, the assay was fixed in 4% formaldehyde and analyzed by confocal microscopy: optical z-sections were taken and the absorbance at 20 μm was determined.

Matrix degradation assay. Glass coverslips were first coated with 100 μg/ml of type I collagen overnight at 4°C. The collagen layer was over-coated with NHS-fluorescein (Pierce, Rockford, IL) initially dissolved in 50 ul N,N-Dimethyl Formamide (Sigma-Aldrich, St. Louis, MO) and then brought to a final concentration of 33 μg/ml in sodium carbonate (pH 9) for 15 minutes at room temperature. Coverslips were washed twice with sodium carbonate and once with PBS before coating with a final layer of FN (20 μg/ml). $2x10^5$ BMMs were plated onto the coverslips for 4 hours at 37°C. Cells were fixed with 4% paraformaldehyde in PBS for 20 minutes and permeabilized for 2-3 minutes with 0.4% Triton X-100 in PBS before staining with TR-phalloidin. Cell were visualized by

immunofluorescence (IF). To quantify degradation, areas cleared of NHS-fluorescein in 10 randomly selected fields were measured with ImageJ.

Mouse KC enzyme-linked immunosorbant assay (ELISA). Clarified culture supernatants collected from 24 hour *Y. pseudotuberculosis* infections were diluted 1:40. Supernatants were analyzed for mouse KC protein using a Quantikine mouse KC ELISA kit (R and D Systems; Minneapolis, MN), as directed by the manufacturer.

Microscopy. For IF, BMMs were plated onto fibronectin-coated (10 μg/ml) coverslips overnight at 37°C. Cells were fixed with 4% paraformaldehyde in PBS for 20 minutes and permeabilized for 2-3 minutes with 0.4% Triton X-100 in PBS before staining with TR-phalloidin and an anti-paxillin antibody, followed by FITC-conjugated rabbit anti-mouse Ig. All incubations were at room temperature for 30 minutes. Cells were viewed with a Nikon fluorescence microscope and photographed with a cooled CCD camera controlled by Openlab software. For TIRF microscopy, BMMs were treated as described above, except that after permeabilization, cells were stained with Alexa Fluor 488-phalloidin. Cells were viewed with a Nikon TE2000 inverted microscope and TIRF images were collected with a Retiga 1300i CCD camera controlled by QED imaging software (Media Cybernetics, Inc., Silver Spring, MD).

Tumor outgrowth and metastasis studies. Five control and five conditional FAK knockout mice (8-12 weeks old) were injected subcutaneously with $1x10^5$ B16F1 melanoma cells, obtained from the American Type Tissue Collection (Mannassas, VA) in

0.2 ml sterile saline. Mice were monitored daily and tumor growth was measured with calipers and recorded every other day. When tumor growth reached or exceeded 250 mm^3, mice were sacrificed. To examine metastasis, five control and five conditional FAK knockout mice (8-12 weeks old) were injected intravenously via the tail vein with $1x10^5$ B16F1 melanoma cells. After 18 days, mice were sacrificed and tissues were examined for the presence of lesions.

Statistical methods. Statistical analysis was performed using raw, non-normalized data. A two-sample *t*-test, assuming unequal variance was used to determine statistical significance between condition means with a significance level of ≤ 0.05.

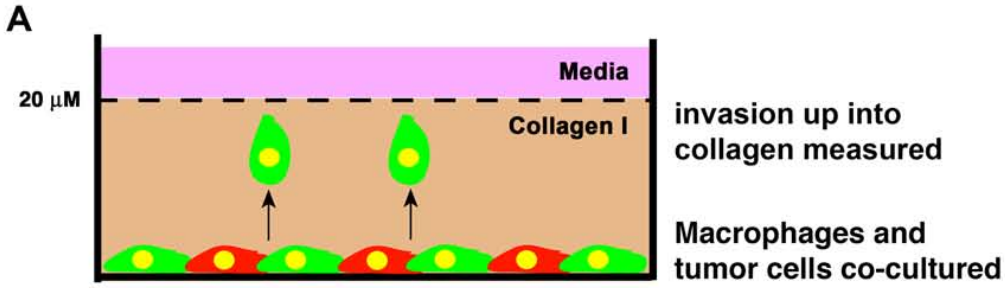
4.4. Results.

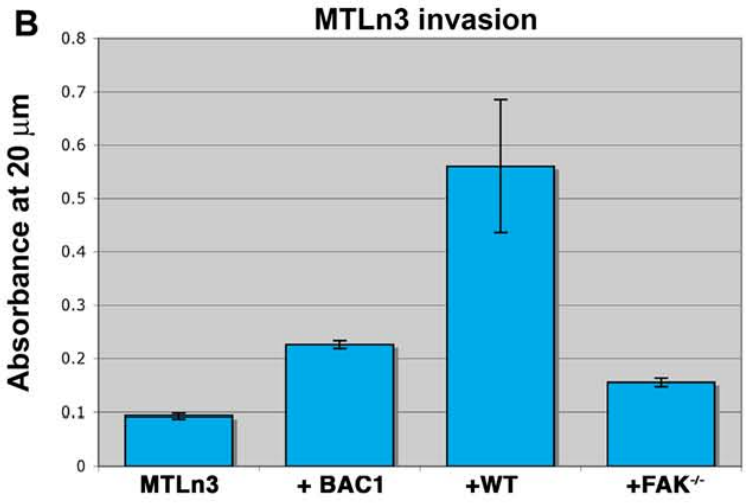
$FAK^{-/-}$ macrophages are unable to promote the invasion of carcinoma cells into a collagen matrix. Previous studies have established that tumor cell motility and invasion can be enhanced in the presence of macrophages (Goswami *et al.*, 2005). Chemotactic factors released by macrophages into the tumor microenvironment can stimulate tumor cell invasion and ultimately the metastasis of cancer to distant sites in the body. Additionally, it has been hypothesized that macrophage-mediated ECM degradation and basement membrane breakdown can facilitate the release of tumor cells into surrounding tissues (Pollard, 2004). To identify differences in the ability of primary WT and $FAK^{-/-}$ bone marrow macrophages to affect the invasiveness of carcinoma cells, we performed co-culture experiments using GFP-tagged MTLn3 mammary carcinoma cells and either

the BAC1.2F51.2F5 (BAC1) macrophage cell line or primary WT and FAK$^{-/-}$ BMMs. To simulate a breast tumor microenvironment, co-cultures of GFP-expressing carcinoma cells and macrophages were overlaid with a collagen I gel (Fig. 4.1A shows a schematic representation of the experimental setup). After 24 hours, the proportion of carcinoma cells invading at least 20 μm into the collagen matrix was determined by taking optical sections with a confocal microscope as described in the Materials and Methods. When MTLn3 cells were co-cultured with BAC1 cells, the absorbance at 20 μm increased 2.2-fold over the absorbance levels observed when MTLn3 cells were cultured alone (Fig. 4.1B). When carcinoma cells were cultured with primary WT BMMs, the proportion of invading MTLn3 cells increased approximately 5.5-fold over the culture of carcinoma cells alone. However, FAK$^{-/-}$ BMMs stimulated MTLn3 invasion to a much lesser extent than did WT cells, as the absorbance at 20 μm was elevated only 1.5-fold over cells cultured in the absence of macrophages (compare bar 1 with bar 4). These data indicate that FAK$^{-/-}$ macrophages are deficient in promoting the invasion of carcinoma cells into a 3-dimensional collagen matrix.

WT and FAK$^{-/-}$ BMMs make podosomes that coalesce into ring-like structures. Podosomes are highly dynamic, actin rich adhesion structures that link the cell membrane to solid surfaces (see Chapter 1). These structures also govern tissue invasion and matrix remodeling by controlling the focal degradation of the ECM and the activation of MMPs (Gimona and Buccione, 2006). Macrophages are important producers of proteases and it has been noted that elevated numbers of TAMs coincide with the breakdown of basement membrane and tumor cell egress (Arnott *et al*., 2002; Lin *et al*., 2002). The proteolytic

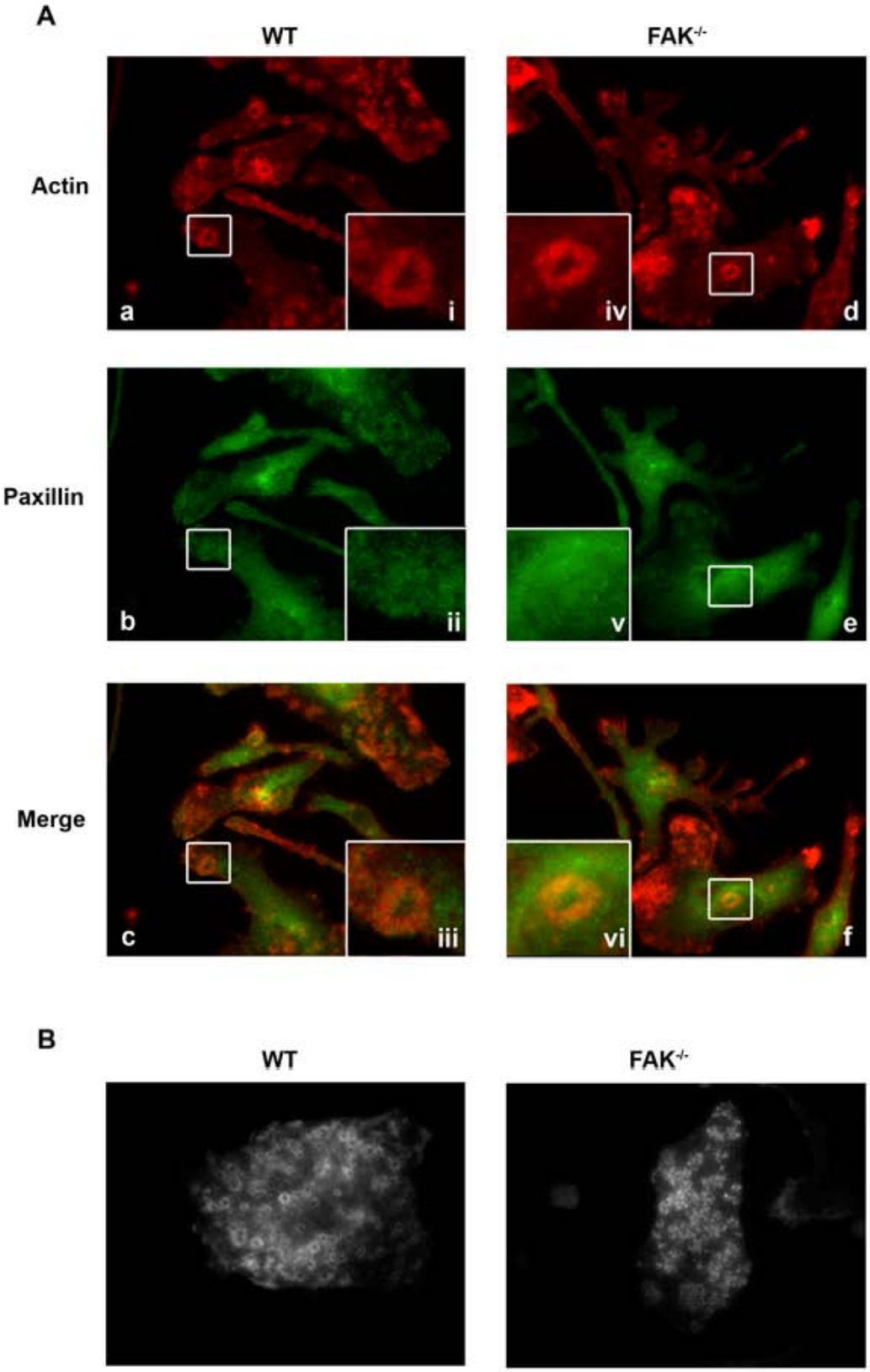
Figure 4.1. WT macrophages promote the invasion of carcinoma cells into a collagen matrix. A. Diagram of the experimental set-up. Cells were plated on the bottom of a MatTek dish and allowed to adhere before overlaying with a collagen gel. After 24 hours, the proportion of cells that invaded into the collagen was determined using a laser scanning microscope to take optical sections. The dotted line represents a distance of 20 μm above the bottom of the dish. Cells above this point are scored as invasive cells. Figure adapted from Goswami et al., 2005. **B.** Absorbance of invasive (above 20 μm) GFP-MTLn3 carcinoma cells plated alone or in the presence of macrophages. Data are from 2 independent experiments performed by Dr. Fiona Pixley.


A
Media
20 μM
Collagen I
invasion up into
collagen measured
Macrophages and
tumor cells co-cultured


B
MTLn3 invasion
Absorbance at 20 μm
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
MTLn3
+ BAC1
+WT
+FAK-/-

activity associated with TAMs may create a "portal" through which tumor cells invade tissues (Pollard, 2004). Since podosomes are associated with matrix degradation, one possible explanation for the inability of $FAK^{-/-}$ BMMs to promote carcinoma cell invasion is that the loss of FAK may result in reduced and/or altered podosome formation. To test this hypothesis, WT and $FAK^{-/-}$ BMMs were plated onto fibronectin-coated coverslips in the presence of CSF-1 to promote podosome formation. Twenty-four hours later, cells were fixed and stained for F-actin and paxillin, two well established components of podosomes, and examined by immunofluorescence (IF) (Fig. 4.2A). Large, actin rich ring-like structures or rosettes were observed in both WT and FAK-deficient macrophages (compare panels a and d). These structures were composed of multiple individual podosomes (see insets i and iv). In general, paxillin did not appear to co-localize with these ring-like structures (see merged panels c and f). While this was somewhat surprising, it may be that paxillin is a more prominent component of podosomes found in other cell types, such as osteoclasts (Luxenburg *et al*., 2006). To confirm that the structures visible in the IF images were present on the ventral surface of cells and therefore in contact with the substratum, WT and $FAK^{-/-}$ BMMs were plated onto fibronectin-coated coverslips, stained for actin and then examined by TIRF microscopy (Fig 4.2B). TIRF microscopy has the advantage of illuminating distances limited to 100-200 nm from the coverslip surface, thus serving as a powerful tool to study cell-substrate contact. Actin-rich rings and plaques were observed in both WT and $FAK^{-/-}$ BMMs, indicating that these structures likely form adhesive contact with the underlying substrate. Similarly, no difference was detected in the ability of either cell type to make podosomes or form rosettes when the cells were first starved and then re-stimulated with

Figure 4.2. Podosome rosette structures can be detected in WT and FAK$^{-/-}$ BMMs. **A.** Macrophages were plated onto FN-coated coverslips and allowed to adhere overnight in media containing CSF-1. Pictures represent fluorescence images (600x magnification) of macrophages stained for actin and paxillin (panels a-f). Enlarged areas (insets i-vi) were used to more closely examine rosette structures. **B.** WT and FAK$^{-/-}$ BMMs stained for actin were examined by TIRF microscopy.

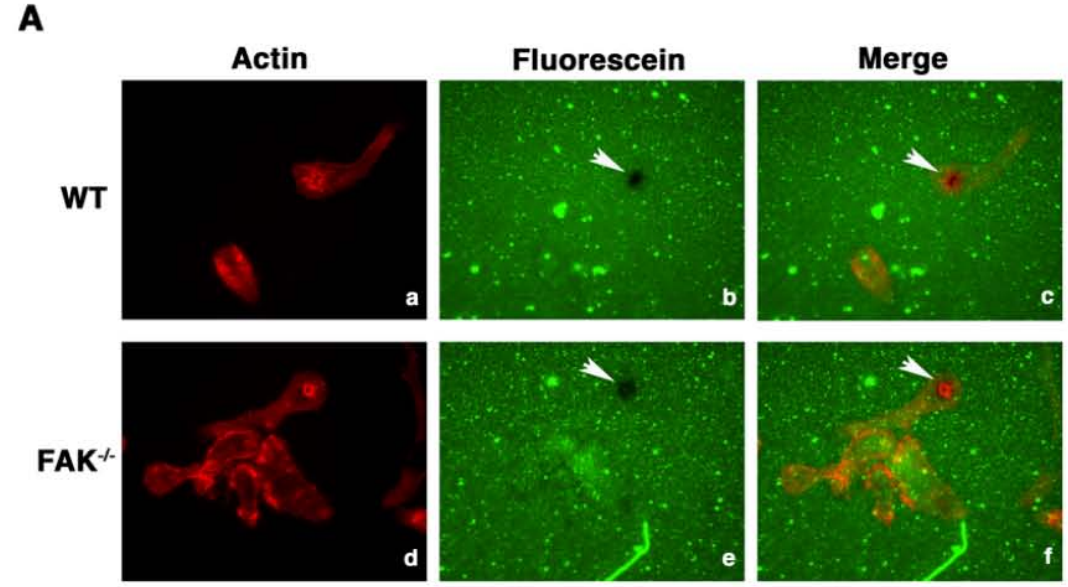

A
WT
FAK-/-
Actin
Paxillin
Merge
a
i
iv
d
b
ii
v
e
c
iii
vi
f
B
WT
FAK-/-

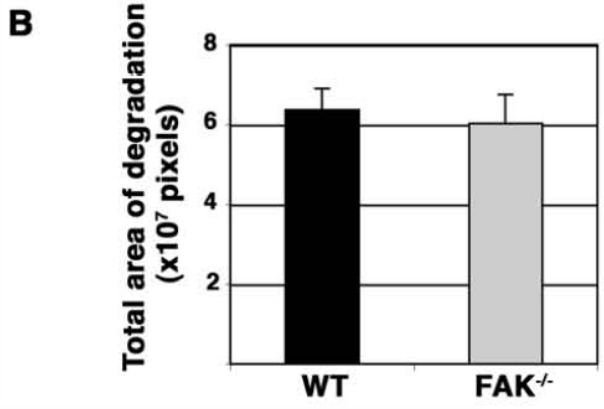
CSF-1 (data not shown). These results suggest that the inability of FAK-deficient macrophages to promote carcinoma cell invasion is not due to the absence or delayed formation of podosomal adhesion structures in these cells.

Proteolytic activity is unaltered in FAK$^{-/-}$ BMMs. Podosomes are not only important for macrophage adhesion to underlying substrate, but they are also enriched in proteases and thus play a significant role in the degradation of ECM. Therefore, the possibility remained that, while FAK$^{-/-}$ BMMs are capable of making podosomes and rosette structures, these cells may nonetheless exhibit defective proteolytic ability. To test this hypothesis, WT and FAK$^{-/-}$ cells were plated onto coverslips coated with fluorescein and overlayed with fibronectin. Four hours later, they were fixed and stained for F-actin (Fig. 4.3A). Both cell types were observed to form podosomal rosettes (see panels a and d). Moreover, WT and FAK$^{-/-}$ BMMs were able to degrade through the fibronectin and the underlying fluorescein layer, resulting in a cleared area of fluorescence (panels b and e, see white arrows) in the region of the rosette structure (panels c and f, see white arrows). No difference was detected in the total area of degradation associated with WT cells compared to FAK$^{-/-}$ cells (Fig. 4.3B). While these results confirm earlier reports demonstrating that matrix degradation coincides with podosome aggregation (Yamaguchi *et al.*, 2006), they suggest that FAK is not essential for the formation of podosomal rosettes or the proteolysis of ECM.

Solid tumor growth is delayed in conditional FAK knockout mice. Since macrophages derived from conditional FAK knockout mice were unable to stimulate the

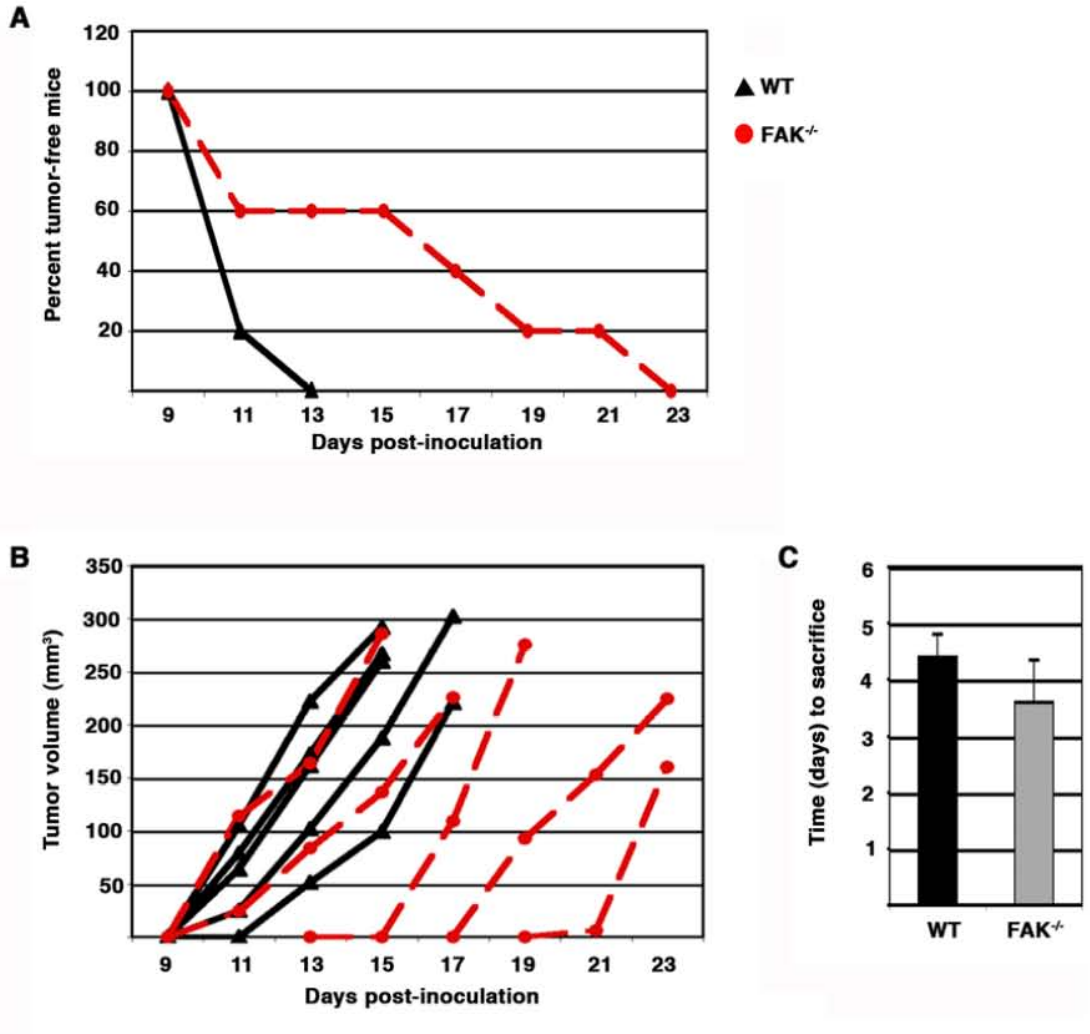
Figure 4.3. WT and FAK$^{-/-}$ macrophage degrade extracellular matrix with equal ability. A. WT and FAK$^{-/-}$ BMMs were starved of CSF-1 overnight before plating onto coverslips coated with fluorescein and overlayed with fibronectin (prepared as described in Materials and Methods) in media containing CSF-1. After 4 hours, cells were fixed and stained for actin (panels a and d). Degradation of ECM results in a cleared area of fluorescein (panels b and e, white arrows). Merged images are shown in panels c and f. **B.** Areas of ECM degradation were measured in 10 randomly selected fields using ImageJ. Data shown are from 3 independent experiments.


A
Actin
Fluorescein
Merge
WT
FAK-/-
a
b
c
d
e
f


B
Total area of degradation (x10^7 pixels)
8
6
4
2
WT
FAK-/-

invasion of carcinoma cells *in vitro* (Fig. 4.1B), we postulated that *in vivo* tumor growth and metastasis in these mice might be inhibited or delayed. To test this hypothesis, control and myeloid lineage-specific conditional FAK knockout mice were injected subcutaneously (s.c.) with B16F1 melanoma cells and tumor outgrowth was measured over the course of three weeks. Mice were sacrificed and tumors harvested when the tumors had reached or exceeded 250 mm^3. Tumor growth was first detected by 11 days post-inoculation in 4/5 control animals and 2/5 conditional FAK knockout animals (Fig. 4.4A). By day 13, tumors were detected in the remaining control animal, while the conditional FAK knockout animals did not exhibit tumor growth until days 17, 19 and 21 respectively. However, once tumors were detected, the time until sacrifice (4.4 days for the control mice compared to 3.6 days for the $FAK^{-/-}$ mice) was similar, suggesting that once tumor growth was initiated, tumor growth rates were not significantly different in the two mouse genotypes (panels B and C). To determine how FAK expression in cells of myeloid lineage impacts tumor metastasis, WT and conditional FAK knockout animals were injected intravenously (i.v.) via the tail vein with B16F1 melanoma cells. Eighteen days post-injection, mice were sacrificed and the lungs, liver and spleens were examined for lesions. In general, very few lung metastases were visible, and there did not appear to be a significant difference in the establishment of tumor growth in the lungs of either mouse type (12.2 vs. 10 lesions counted in the lungs of WT and $FAK^{-/-}$ animals respectively, data not shown). Upon examination of the livers, 2 out of 5 WT mice harbored multiple large lesions (between 8-17 masses each),

Figure 4.4. Delayed tumor outgrowth in myeloid lineage-specific conditional FAK knockout mice. Five mice of each genotype were injected subcutaneously with $1x10^5$ B16F1 melanoma cells. Tumor growth was measured using calipers and mice were monitored over the course of three weeks. When tumor volume reached approximately 250 mm^3, mice were sacrificed and the tumors were harvested. Tumor-free survival (**A**), tumor outgrowth (**B**) and the average number of days it took for tumors, once detected, to reach 250 mm^3 (**C**). Black solid line-triangles represent control mice; red dashed line circles represent conditional FAK knockout mice.

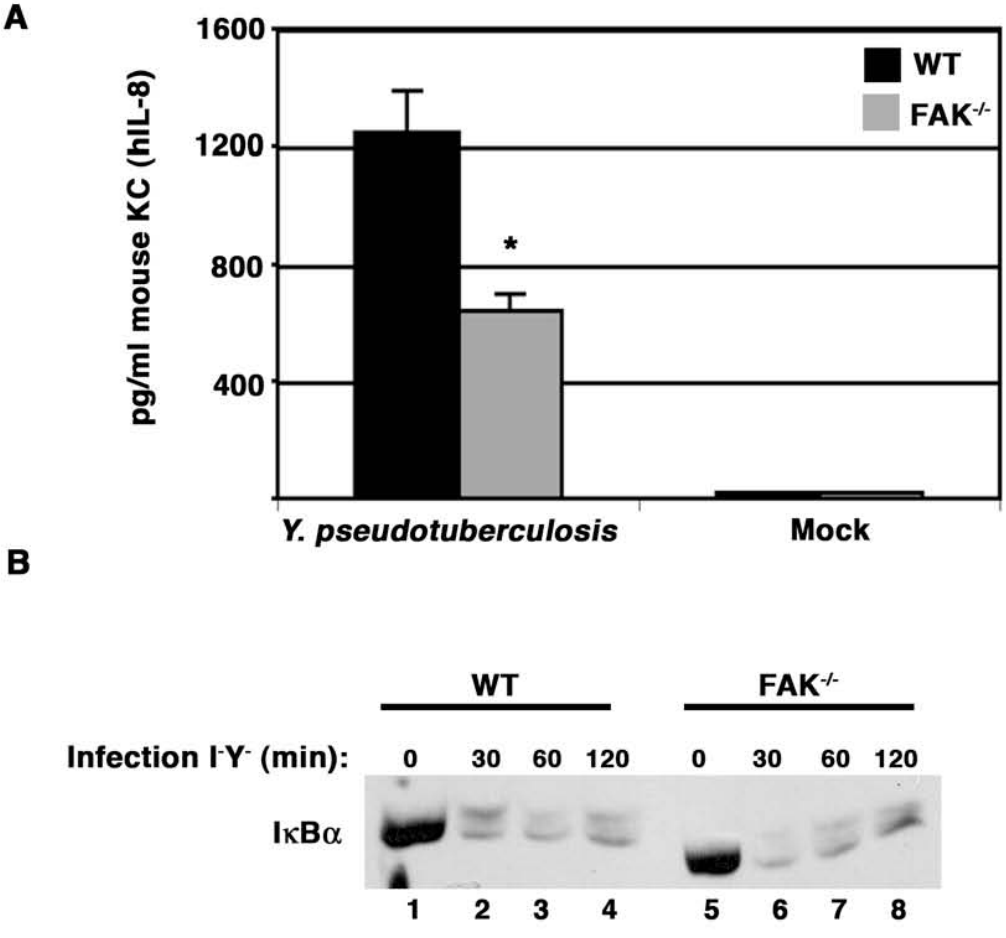

A
120
100
80
60
40
20
Percent tumor-free mice
9
11
13
15
17
19
21
23
Days post-inoculation
WT
FAK-/-
B
350
300
250
200
150
100
50
Tumor volume (mm3)
9
11
13
15
17
19
21
23
Days post-inoculation
C
6
5
4
3
2
1
Time (days) to sacrifice
WT
FAK-/-

whereas 1 out of 5 FAK$^{-/-}$ animals contained visible lesions (4 smaller masses; data not shown). The spleens of all mice appeared free of lesions.

The induction of mouse KC (hIL-8) expression is significantly reduced in FAK-deficient macrophages. The secretion of soluble factors, including cytokines, chemokines and growth factors within the tumor microenvironment has been reported to stimulate the migratory and invasive potential of tumor cells (Condeelis and Pollard, 2006). An alternative explanation for the inability of FAK$^{-/-}$ cells to promote the invasion of carcinoma cells is that the production and/or secretion of invasion-inducing factors requires FAK activity. To investigate whether FAK is involved in chemokine production, we examined the ability of WT and FAK$^{-/-}$ macrophages to produce the inflammatory chemokine mouse KC (the homolog of human IL-8: hIL-8) in response to infection with a Gram-negative bacterium, *Yersinia pseudotuberculosis*. The strain used in this study was deficient for the two major adhesins, invasin and YadA, commonly expressed by this bacterium. Therefore KC production is probably due to the macrophage response to bacterial LPS. After a 24-hour infection period, the supernatants were collected and analyzed by ELISA for the presence of mouse KC. FAK$^{-/-}$ BMMs were found to produce significantly less mouse KC, exhibiting a 50% reduction in chemokine secretion compared to WT infected cells (Fig 4.5A). Treatment of cells with Hank's buffered saline solution (HBSS, mock treatment) did not induce the production of KC ("mock").

Under the conditions described above, bacterial infection likely initiates KC production via a toll-like receptor 4 (TLR-4) signaling pathway. TLR-4 is a host recognition receptor that binds distinct pathogen-associated molecular patterns such as

Figure 4.5. FAK$^{-/-}$ BMMs produce reduced levels of mouse KC (hIL-8) after bacterial challenge. A. BMMs were cultured in the presence of an adhesin-deficient *Yersinia pseudotuberculosis* strain for 24 hours. Supernatants from infected or mock-treated cells were analyzed for KC secretion by ELISA. Data shown are from 3 independent experiments. Asterisks indicate statistically significant differences from WT infected cells at a confidence level greater than 95%. **B.** Bacterial challenge induces the degradation of the IκBα subunit. WT and FAK$^{-/-}$ BMMs were infected for the indicated periods of time with the adhesin-deficient *Y. pseudotuberculosis* strain (MOI 200) and subjected to immunoblot analysis. Lysates were separated by SDS-PAGE, transferred to nitrocellulose, and immunoblotted with antibodies recognizing IκBα. Preliminary data; immunoblot analysis represents one experiment.


A
1600
1200
800
400
pg/ml mouse KC (hIL-8)
WT
FAK-/-
*
Y. pseudotuberculosis
Mock
B
WT
FAK-/-
Infection I-Y- (min):
0 30 60 120 0 30 60 120
IκBα
1 2 3 4 5 6 7 8

bacterial LPS. Stimulation with LPS leads to the phosphorylation of IκB proteins bound to nuclear factor-kB (NF-κB) complexes. Phosphorylation of IkB results in IkB ubiquitination and degradation, a process that frees NF-κB complexes, allowing them to translocate to the nucleus and induce the expression of genes involved in the inflammatory response such as IL-8. To determine if the loss of FAK results in defective signaling downstream of TLR-4, we examined the degradation of the regulatory IκBα subunit of NF-κB during Yersinia infection (Fig. 4.5B). After 30 minutes of infection with adhesin-deficient *Y. pseudotuberculosis*, the expression of IκBα was reduced by half in both WT and FAK-deficient cells compared to the IκBα levels observed in uninfected cells (compare lanes 1 and 2; 5 and 6). IκBα expression was consistently reduced throughout the entire time course of infection for both WT and $FAK^{-/-}$ cells. These data show that, while the loss of FAK significantly affects the ability of these cells to produce the inflammatory chemokine mouse KC, this defect is not a consequence of impaired TLR-4 signaling to IκBα.

4.5. Discussion.

Tumor cell motility and invasion is required for tumors to metastasize from their site of origin. Chemotactic factors found in the tumor microenvironment can promote the motility and invasive capacity of tumor cells and facilitate metastasis. Recent evidence points to a role for tumor associated macrophages in the production of the soluble factors necessary to sustain tumor growth. Similarly, TAMs within the tumor microenvironment have the ability to produce proteases that degrade basement membrane to facilitate tumor

cell entry into the surrounding stroma. It is therefore not surprising that a strong correlation exists between macrophage abundance and poor prognosis for a number of different cancers.

Previous studies from our lab have established an important role for the non-receptor tyrosine kinase FAK in the regulation of macrophage migration, chemotaxis and phagocytosis (see Chapters 2, 3 and 5). Through the use of mice in which FAK expression is specifically deleted from the monocyte/macrophage lineage, we have the unique opportunity to determine if tumor cell behavior is altered in the presence of $FAK^{-/-}$ macrophages. In the preliminary studies presented here, we demonstrate that the invasive capacity of tumor cells co-cultured with $FAK^{-/-}$ macrophages is reduced. We also provide preliminary *in vivo* evidence suggesting that tumor outgrowth may be delayed in myeloid lineage-specific conditional FAK knockout mice. Although further experimentation is needed, the results presented here indicate that tumor behavior can be modulated through alterations in macrophage-specific adhesion signaling.

While the role of FAK in cell migration, proliferation and survival is well established, it was unclear what effect the loss of FAK would have on the ability of macrophages to promote tumor cell invasion. We found that, compared to their WT counterparts, $FAK^{-/-}$ macrophages were unable to promote efficient carcinoma cell invasion through a 3-dimensional collagen matrix (Fig. 4.1). We hypothesize that this could be due to reduced protease secretion, leading to a reduced ability to degrade matrix. Recently, induced MMP-9 expression by lung macrophages and endothelial cells in response to primary tumors has been linked to the invasion of lung tissues by tumor cells (Hiratsuka *et al.*, 2002). MMP-9, MMP-2, membrane tethered-1 matrix metalloproteinase

(MT1-MMP) and other proteases associated with matrix degradation are associated with macrophage podosomes, structures involved in adhesion and matrix remodeling (Yamaguchi *et al*., 2006). However, examination of WT and $FAK^{-/-}$ BMMs did not reveal any difference in the ability of these cells to form podosomes or the rosette structures typically associated with matrix degradation (Fig. 4.2). Moreover, there was no difference in the area of ECM proteolysis associated with $FAK^{-/-}$ cells compared to WT cells (Fig. 4.3).

TAMs located in solid tumors help establish an environment that enhances the survival, proliferation and migration of primary tumor cells. Based on our previous data showing that $FAK^{-/-}$ BMMs exhibited impaired recruitment in response to inflammatory stimuli *in vivo* (Chapter 2), and that these cells are unable to promote tumor cell invasion *in vitro* (Fig. 4.1), we hypothesized that tumor onset in conditional FAK knockout mice may be delayed, and the incidence of metastasis reduced. Although these experiments need to be repeated before we are able to draw major conclusions, our preliminary data suggest that tumor outgrowth was inhibited in the conditional knockout animals. There are several possible explanations for these results. First, tumor outgrowth may require macrophage accumulation to reach a "critical density" within the tumor microenvironment to facilitate growth. Delayed recruitment of monocyte/macrophage in the conditional FAK knockout animals due to migratory defects may therefore inhibit tumor onset. Second, the loss of FAK may impair other macrophage functions, such as the ability to secrete tumor-promoting growth factors. Thus, macrophages in the conditional knockout animals that have successfully infiltrated the tumor microenvironment might not be able to produce high enough levels of tumor-promoting

cytokines and other factors to support tumorigenesis. This hypothesis is partially supported by the fact that $FAK^{-/-}$ macrophages were significantly impaired in their ability to release KC cytokine in response to bacterial LPS. Based on data showing that the NF-κB pathway is activated equivalently following *Y. pseudotuberculosis* infection of WT and $FAK^{-/-}$ BMMs, it does not appear that reduced KC production is due to altered TLR signaling. This suggests that $FAK^{-/-}$ macrophages may, in fact, be defective in KC synthesis and/or secretion. Nonetheless, further analyses are needed to specifically determine if FAK regulates cytokine and/or growth factor production affecting TAM function.

With respect to the metastatic potential of tumor cells in this model, our results remain inconclusive. Mice were administered tumor cells intravenously and therefore tumor growth should have initiated in the lungs before seeding to other sites, such as the liver and spleen. Unexpectedly, we observed relatively few lesions in the lungs of either the control or the conditional knockout mice. The metastatic lesions that formed were more prominent in the livers of mice. While the lesions that formed in the livers of 2/5 WT mice were larger and more numerous that the smaller lesions found in the liver of 1/5 conditional FAK knockout animal, it is unclear from these results whether or not the loss of FAK from macrophages delays metastasis. Nonetheless, the results presented in this preliminary report provide an exciting framework for future experimentation.

The importance of FAK as a regulator of normal cellular function is underscored by the number of cancers reported to have alterations in FAK expression and/or activity (Gabarra-Niecko *et al.*, 2003; McLean *et al.*, 2005; Slack-Davis *et al.*, 2007). Recent reports indicate that pharmacological inhibition of FAK function of normal and cancer

cells results in decreased cell migration and reduced adhesion turnover (Slack-Davis *et al.*, 2007). Macrophages associated with tumors are not themselves malignant, and therefore are much less likely to develop drug resistance (Condeelis and Pollard, 2006). The use of pharmacological agents aimed at inhibiting selected macrophage functions, such as migration or the secretion of growth factors, may be efficacious in preventing or delaying tumor progression. Inhibitors of FAK activity have recently been described and have already entered human clinical testing for the treatment of cancer (Slack-Davis *et al.*, 2007 and Pizer analysts meeting, 2006). Future studies designed to address how FAK contributes to macrophage function may pave the way for the next generation of chemotherapeutic agents specifically targeting FAK activity in this cell type.

Chapter 5:

The differential expression of *Yersina pseudotuberculosis* adhesins determines the requirement for FAK and/or Pyk2 during bacterial phagocytosis by macrophages

Katherine A. Owen, Keena S. Thomas and Amy H. Bouton

5.1. Abstract.

Phagocytosis of *Yersinia pseudotuberculosis* by macrophages is initiated by interactions between host cell integrin receptors and the bacterial adhesins, invasin and YadA. The non-receptor protein tyrosine kinases, FAK and Pyk2, have been implicated in this process. In this study, we investigated the mechanisms of activation and functional requirements for these kinases during phagocytosis. A panel of Yersinia strains that differentially express invasin and YadA were used to infect cells in which FAK and/or Pyk2 expression was reduced by RNAi. Bacterial strains that simultaneously express invasin and YadA activated FAK and Pyk2 signaling pathways that perform non-redundant functions required for Yersinia internalization. In contrast, FAK activation was found to be sufficient for phagocytosis of bacteria expressing invasin alone, and Pyk2 activation was sufficient when YadA was expressed in the absence of invasin. Based on these data, we suggest that the activation states of FAK and Pyk2, as well as the subsequent signaling events that lead to phagocytosis, are differentially regulated through the unique mechanisms of integrin engagement utilized by invasin and YadA. These findings lend insight into the molecular events that control bacterial phagocytosis as well as other integrin-based processes such as cell adhesion and migration.

5.2. Introduction.

The ability of the innate immune system to respond to extracellular pathogens is essential for the resolution of infection. *Y. pseudotuberculosis* is an enteropathogen that has adapted to survive and grow in the lymphoid tissues of the intestinal tract. This has been facilitated by the acquisition of a virulence plasmid that encodes a Type III secretion apparatus and numerous antiphagocytic and cytotoxic effector molecules that are injected into host cells upon close contact with the pathogen (for reviews, see Cornelis, 2002a, b). In spite of this potent virulence mechanism, *Y. pseudotuberculosis* infections are effectively eliminated in the uncompromised host by the overwhelming activity of macrophages and other cells that contribute to the innate immune response.

Macrophages are equipped with multiple receptors involved in the recognition and binding of pathogens, including TLRs, Fc receptors, lectins, scavenger receptors, and integrins. Under physiological conditions, *Y. pseudotuberculosis* expresses two major adhesins, invasin and YadA, that are capable of interacting with integrin receptors. While invasin binds directly to integrin receptors, YadA binds indirectly through an ECM bridge (Dersch and Isberg, 1999; Isberg *et al.*, 2000; Eitel and Dersch, 2002; Heise and Dersch, 2006). However, the signaling pathways that are activated upon host cell-pathogen contact and are required for bacterial uptake remain unclear. FAK and its related family member Pyk2 represent two non-receptor PTKs that are endogenously expressed in macrophages and have been implicated in the Yersinia internalization process (Alrutz and Isberg, 1998; Weidow *et al.*, 2000; Bruce-Staskal *et al.*, 2002; Hudson *et al.*, 2005).

During infection, both FAK and Pyk2 become activated in macrophages (Hudson *et al*., 2005), a biologically important cell lineage involved in the clearance of natural Yersinia infections. Therefore, macrophage cell lines provide an excellent system with which to investigate whether these endogenously expressed molecules have overlapping or distinct activities required for Yersinia internalization. Using RNAi to reduce endogenous levels of FAK and/or Pyk2, we have begun to assess the role of these host molecules in macrophages during *Y. pseudotuberculosis* infections. The use of *Y. pseudotuberculosis* invasin and YadA adhesin mutants has also allowed us to investigate the signaling cascades initiated upon host cell-pathogen contact. We show here that efficient internalization of Yersinia strains simultaneously expressing invasin and YadA involves two pathways in which FAK and Pyk2 perform distinct, non-redundant activities. Infections conducted with an invasin-only expressing Yersinia strain revealed that, while this strain was capable of activating both FAK and Pyk2, invasin-induced FAK activation was sufficient for phagocytosis. Infection with a YadA-only expressing strain in the presence of exogenous FN induced Pyk2 activation in the absence of FAK and bypassed the uptake requirement for FAK. These results suggest a model whereby Yersinia internalization requires FAK and Pyk2 activity when both invasin and YadA compete for integrin receptors. However, in the absence of competition between these adhesins, invasin-dependent activation of FAK or YadA/ECM-dependent activation of Pyk2 are sufficient for integrin-mediated bacterial uptake. The studies presented herein describe for the first time signaling pathways involving unique roles for FAK and Pyk2 that have important biological implications for host-pathogen interactions as well as other integrin-dependent processes such as cell adhesion and migration.

5.3. Materials and methods.

Cell culture. J774A.1 murine macrophages were purchased from the American Type Tissue Culture Collection (Rockville, MD). Cells were maintained in DMEM supplemented with 10% heat-inactivated FBS and 100 U/ml penicillin and 100 U/ml streptomycin. All cells were grown at 37°C in 7.5% CO_2. All media, FBS, and antibiotics were obtained from Gibco BRL Life Technologies (Rockville, MD).

Bacterial strains and growth conditions. The *Y. pseudotuberculosis* strain Yp17/pVector expresses the chromosomally-encoded invasin protein as well as the virulence plasmid (pYV)-encoded YadA protein. This strain is a derivative of *Y. pseudotuberculosis* strain YP17 (*yopE*/*yopH*) containing the control vector pMMB67EH (Black and Bliska, 1997; Weidow *et al*., 2000). The four adhesion mutant strains (Inv^+YadA^+, Inv^+YadA^-, Inv^-YadA^+ and Inv^-YadA^-) were generated from *Y. pseudotuberculosis* strains YP137 and YP202 (an *inv* mutant of YP137 kindly provided by Dr. Ralph Isberg, Tufts University, Boston, MA) by introduction of a control vector (pMMB67EH) or a related plasmid encoding YadA under the control of a lactose-inducible promoter (pYadA) (Hudson *et al*., 2005). Each of these strains lacks the virulence plasmid pYV. The growth conditions specific for this strain have been described previously (Black and Bliska, 1997; Weidow *et al*., 2000; Bruce-Staskal *et al*., 2002). For the YP137 and YP202 mutants, YadA-encoding plasmids were maintained through the addition of 100 μg/ml ampicillin to the media and YadA was induced by the addition of 0.5 M isopropyl-beta-D-

thiogalactopyranoside (IPTG). Invasin-negative bacteria were grown in the presence of kanamycin 50 μg/ml). Under these growth conditions, invasin and YadA were expressed at levels comparable to the expression of these proteins on Yp17/pVector (Hudson *et al*., 2005 and K. Husdon, pers. comm.)

Antibodies and reagents. Polyclonal FAK C-20, FITC-conjugated anti-β1 integrin and mAb Myc (9E10) antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). FLAG M5 mAb and FN were obtained from Sigma. A mAb recognizing Pyk2 was purchased from BD Biosciences. Phosphospecific FAK-Y397 and Pyk2-Y402 antibodies were purchased from BioSource International (Camarillo, CA). Polyclonal Yersinia antibodies were kindly provided by James Bliska (SUNY-Stony Brook, NY) and have been previously described (Black and Bliska, 2000). PE-conjugated anti-CD11b antibodies and purified rat IgG (isotype control) antibodies were purchased from Caltag (Burlingame, CA). FITC-conjugated goat anti-rabbit immunoglobulin (Ig), FITC-conjugated goat anti-mouse Ig and TR-conjugated goat anti-rabbit Ig were all purchased from Jackson Immunoresearch Laboratories, Inc. (West Grove, PA). Cascade blue-conjugated goat anti-mouse antibodies were purchased from Molecular Probes (Eugene, OR). HRP-conjugated sheep anti-mouse Ig and HRP-conjugated donkey anti-rabbit antibodies were purchased from Amersham Pharmacia Biotech (Piscataway, NJ). TransIT-TKO transfection reagent was obtained from Mirus (Madison, WI). Fugene 6 transfection reagent was purchased from Roche Molecular Biochemicals (Indianapolis, IN).

RNA preparation and transfection. Twenty-one nucleotide complementary RNAs were synthesized, annealed and deprotected by Dharmacon, Inc. (Lafayette, CO). The siRNA sequence targeting murine FAK (5'-AAGCTAGTGACGTGTGGATGT-3') corresponds to nucleotides 1913-1931, and the siRNA targeting murine Pyk2 (5'-AAAGCCTCTGTGACCCGTCTA-3') corresponds to nucleotides 177-195 of the coding sequence. Non-specific duplex siRNA (siControl) was also purchased from Dharmacon, Inc. Approximately 18 hours before siRNA transfection, $3.2x10^5$ J774A.1 macrophages were seeded into 6-well plates. Cells were transfected with 40 nM of RNA duplexes using 16 μl of *Trans*IT TKO transfection reagent per well. Expression levels of FAK and Pyk2 were measured by immunoblotting and immunofluorescence 72 hours post-siRNA transfection. For *Y. pseudotuberculosis* infections, cells were replated onto FN-coated coverslips 48 hours post–siRNA transfection and allowed to incubate overnight prior to infection with *Y. pseudotuberculosis*.

Flow cytometry analysis. Approximately $1x10^6$ J774A.1 macrophages pre-treated with siRNAs (described above) were dual-labeled with FITC-conjugated anti-β1 integrin antibodies (1:100) and PE-conjugated anti-CD11b antibodies (1:100) for 30 minutes at room temperature. Prior to staining, Fc-receptors were blocked for 10 minutes with rat IgG antibodies. Macrophages were washed twice and resuspended in buffer containing PBS, 0.5% BSA and 0.5% 1M EDTA before analysis on a FACscalibur™ system (Becton Dickinson, San Jose, CA). Data were analyzed with FloJo version 4.5 (TreeStar Inc. Ashland, OR).

Plasmids and transfection. The pcDNA3-2AB plasmid encoding FLAG-tagged full length chicken FAK was generously provided by J.T. Parsons (University of Virginia, Charlottesville, VA), and pCMV encoding Myc-tagged rat Pyk2 was kindly provided by W.C. Xiong (University of Alabama at Birmingham, Birmingham, AL). The pRK5 plasmid encoding Myc-tagged Cas-ΔSH3 has been previously described (Burnham *et al.*, 2000; Harte *et al.*, 2000). All plasmid constructs were transfected according to the manufacturer's instructions using FuGene 6 transfection reagent (6 μl per 1 μg DNA) 24 hours post-siRNA transfection. The media used for transfection did not contain antibiotics.

Bacterial uptake assay. Prior to infection, $5x10^5$ cells were washed twice with PBS, and antibiotic-free media was added. Cells were infected for 1-2 hours at an MOI of approximately 20, unless otherwise indicated, at 37°C in 7.5% CO_2. Cells were washed 3 times with PBS between all subsequent steps. Cells were fixed in 3% paraformaldehyde for 20 minutes at room temperature (RT) and blocked in 10% BSA/PBS before immunostaining. The immunostaining procedure is adapted from Heesemann and Laufs (Heesemann and Laufs, 1985) and has been described previously in Bruce-Staskal *et al.*, 2002. Briefly, cells were treated with the polyclonal anti-Yersinia antibody SB349 (1:500) followed by FITC-conjugated goat anti-rabbit Ig (1:500). Cells were permeablized with 0.4% Triton X-100 before staining a second time with SB349 (1:500) followed by TR-conjugated goat anti-rabbit Ig (1:500). Therefore, internalized bacteria appear red and external bacteria appear yellow/green. All antibody incubation steps were

performed for 30 minutes at RT. Cells were viewed with a Nikon Eclipse E800 fluorescence microscope (Nikon Inc., Melville, NY) with a CCD camera controlled by Openlab software for scientific imaging from Improvision (Improvision Inc., Lexington, MA). Bacterial uptake was determined for each experiment using the following calculation: Percent uptake = internalized yersiniae (stained only with TR)/total macrophage-associated yersiniae (stained with FITC and TR) x 100. Since the total number of bacteria per cell was similar under all conditions for all strains except Inv$^-$ YadA$^-$, determination of the percentage of adhered bacteria that become internalized is an accurate reflection of the number of internalized bacteria.

Immunofluorescence. Cells previously transfected with either siRNA or vehicle were replated onto FN-coated coverslips and allowed to adhere overnight. Cells were then fixed with 3% paraformaldehyde for 20 minutes at RT, washed 3 times with PBS, and permeabilized with 0.4% Triton X-100 in PBS for 2 minutes. Cells were washed 3 times between all subsequent steps. All antibodies were diluted in PBS containing 2% BSA, and all incubations were at RT for 1 hour. Each coverslip was double-stained for FAK (FAK C-20, 1:1000) and Pyk2 (mAb, 1:600), followed by incubation with FITC-conjugated goat anti-rabbit Ig (1:750) and TR-conjugated goat anti-mouse Ig (1:750). To identify macrophages expressing ectopic protein, cells were treated with mAb FLAG (1:1000) or mAb Myc (1:1000) followed by incubation with Cascade blue-conjugated goat anti-mouse Ig (1:750). Cells were viewed with a Nikon Eclipse E800 fluorescence microscope (Nikon Inc., Melville, NY) with a CCD camera controlled by Openlab

software for scientific imaging from Improvision (Improvision Inc., Lexington, MA). When obtaining pictures of stained cells, equivalent exposure times were maintained throughout each individual experiment.

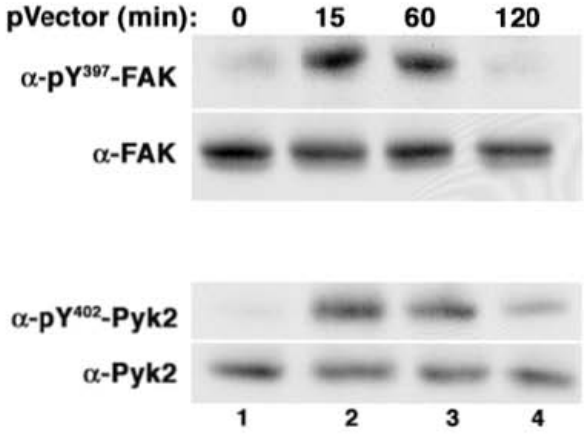
Immunoprecipitation and immunoblotting. J774A.1 macrophages were rinsed twice with PBS and lysed in modified RIPA (50 mM Tris, 150 mM NaCl, 1% Igepal CA-630, 0.5% deoxycholate) containing protease and phosphate inhibitors (100 μM leupeptin, 1 mM phenylmethylsulfonyl fluoride, 0.15 unit/ml aprotinin, 1 mM vanadate) as previously described (Kanner *et al.*, 1989a). Immunoprecipitations and immunoblotting were performed as previously described (Burnham *et al.*, 2000; Weidow *et al.*, 2000 and Chapter 2).

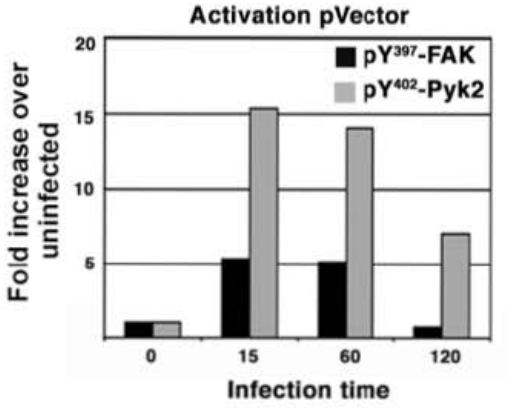
Statistical methods. Data obtained from bacterial uptake assays were analyzed as described previously (Bruce-Staskal *et al.*, 2002). To control for variability related to repetitions of experiments, two-way analysis of variance (ANOVA) was performed on the percentage of internalized bacteria. A two-sample t-test assuming unequal variance was used to determine statistical significance between condition means with a significance level of ≤ 0.05.

5.4. Results.

FAK and Pyk2 become activated in J774A.1 macrophages during *Y. pseudotuberculosis* infection. Receptor-mediated phagocytosis requires the activation of numerous signaling molecules. Proteins involved in the assembly of focal adhesion complexes, such as talin, vinculin, paxillin, and FAK, have been shown to play a role in phagocytosis (Allen and Aderem, 1996; Critchley, 2000). To confirm previous data showing that FAK and Pyk2 become activated during infection of macrophages (Hudson *et al.*, 2005), J774A.1 murine macrophages were infected for 0-120 minutes with *Y. pseudotuberculosis* strain Yp17/pVector. This strain expresses the adhesins invasin and YadA, but lacks the antiphagocytic and cytotoxic effector molecules YopE and YopH, that contribute to bacterial persistence during natural infections (Black and Bliska, 1997). Activation of FAK and Pyk2 was measured by immunoblotting cell extracts with phospho-specific antibodies directed against the respective autophosphorylation sites in FAK (phospho-tyrosine 397 (pY397)) and pY402 in Pyk2. In the J774A.1 cell line, maximal activation of both proteins (5-fold and 15-fold respectively) was observed 15 minutes post-infection (Fig. 5.1, top panels). By 2 hours, FAK activity returned to basal levels, while Pyk2 activation remained elevated. No change in total FAK and Pyk2 expression was observed over the course of infection in the J774A.1 cells (bottom panels). These data indicate that FAK and Pyk2 undergo temporal activation in response to the Yp17/pVector Yersinia strain, and suggest that these molecules may play a role during bacterial internalization in macrophages.

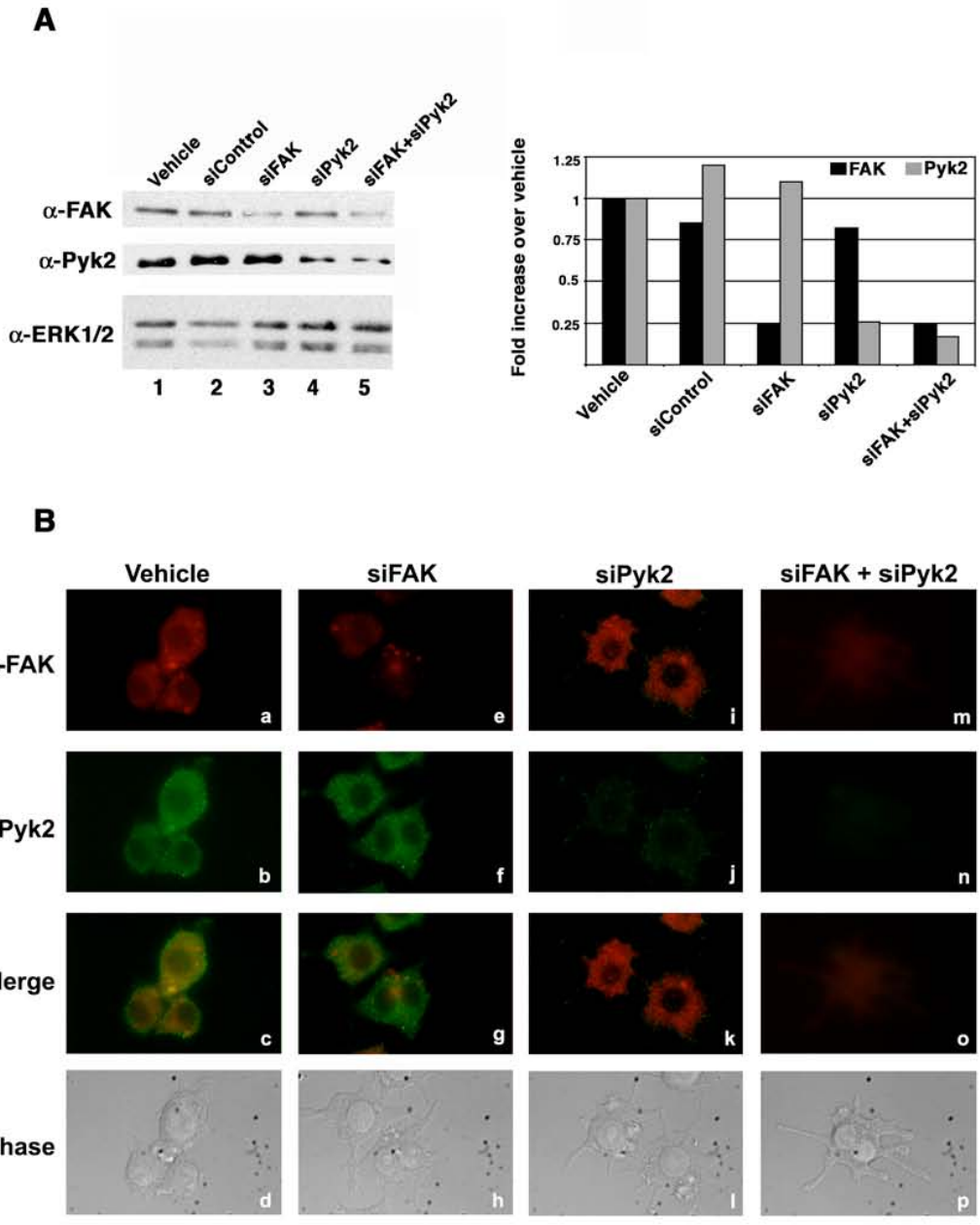
Figure 5.1. FAK and Pyk2 exhibit similar activation kinetics upon Yersinia infection of J774A.1 macrophages. J774A.1 macrophages were infected for the indicated periods of time with *Y. pseudotuberculosis* strain Yp17/pVector (MOI 200) and subjected to immunoblot analysis. Lysates were separated by SDS-PAGE, transferred to nitrocellulose, and immunoblotted with antibodies recognizing the autophosphorylation site of FAK and Pyk2 (upper panels) or total FAK and Pyk2 (bottom panels). Band intensities were quantitated by densitometry and are presented relative to uninfected cells in graph form. Each immunoblot represents multiple independent experiments.


pVector (min): 0 15 60 120
α-pY397-FAK
α-FAK
α-pY402-Pyk2
α-Pyk2
1 2 3 4


Activation pVector
pY397-FAK
pY402-Pyk2
Fold increase over uninfected
20
15
10
5
0 15 60 120
Infection time

RNAi independently reduces the expression of FAK or Pyk2. Expression of the dominant-interfering molecules FRNK (FAK-related non-kinase) or PRNK (Pyk2-related non-kinase) in macrophages has been shown to significantly impair the ability of cells to internalize bacteria upon Yersinia infection (Bruce-Staskal *et al*., 2002) and unpublished data). However, this does not shed light on the specific roles of FAK and Pyk2 during Yersinia uptake, since FRNK has been reported to inhibit the activity of both FAK and Pyk2 (Du *et al*., 2001). To more definitively assess the role of these molecules in the Yersinia uptake process, RNAi was used with the goal of independently reducing FAK and Pyk2 expression. Transfection of J774A.1 macrophages with siRNA duplexes directed toward FAK or Pyk2 resulted in reduced expression of the targeted molecule without affecting the expression of the heterologous molecule (Fig. 5.2A). Thus, introduction of siFAK efficiently knocked down FAK protein levels (lane 3, top panel) without affecting Pyk2 (lane 3, middle panel), while introduction of siPyk2 reduced Pyk2 expression (lane 4, middle panel) without affecting FAK (lane 4, top panel). Transfection of siFAK and siPyk2 duplexes together effectively reduced expression of both proteins to approximately the same levels exhibited upon transfection of the individual siRNAs (lane 5). In each case, FAK and Pyk2 expression was reduced by approximately 75% in siRNA-treated cells, as measured by densitometry. Importantly, transfection of a non-specific siRNA duplex (siControl) did not significantly reduce FAK or Pyk2 expression (lane 2). Expression of the non-targeted proteins, ERK1/2, was largely unaffected by siRNA treatment (bottom panel). IF confirmed the effect of siRNA on FAK and Pyk2 expression levels. Vehicle-treated cells exhibited robust FAK (red) and Pyk2 (green) staining (Fig. 5.2B, panels a-c), while approximately 90% of cells treated with siFAK

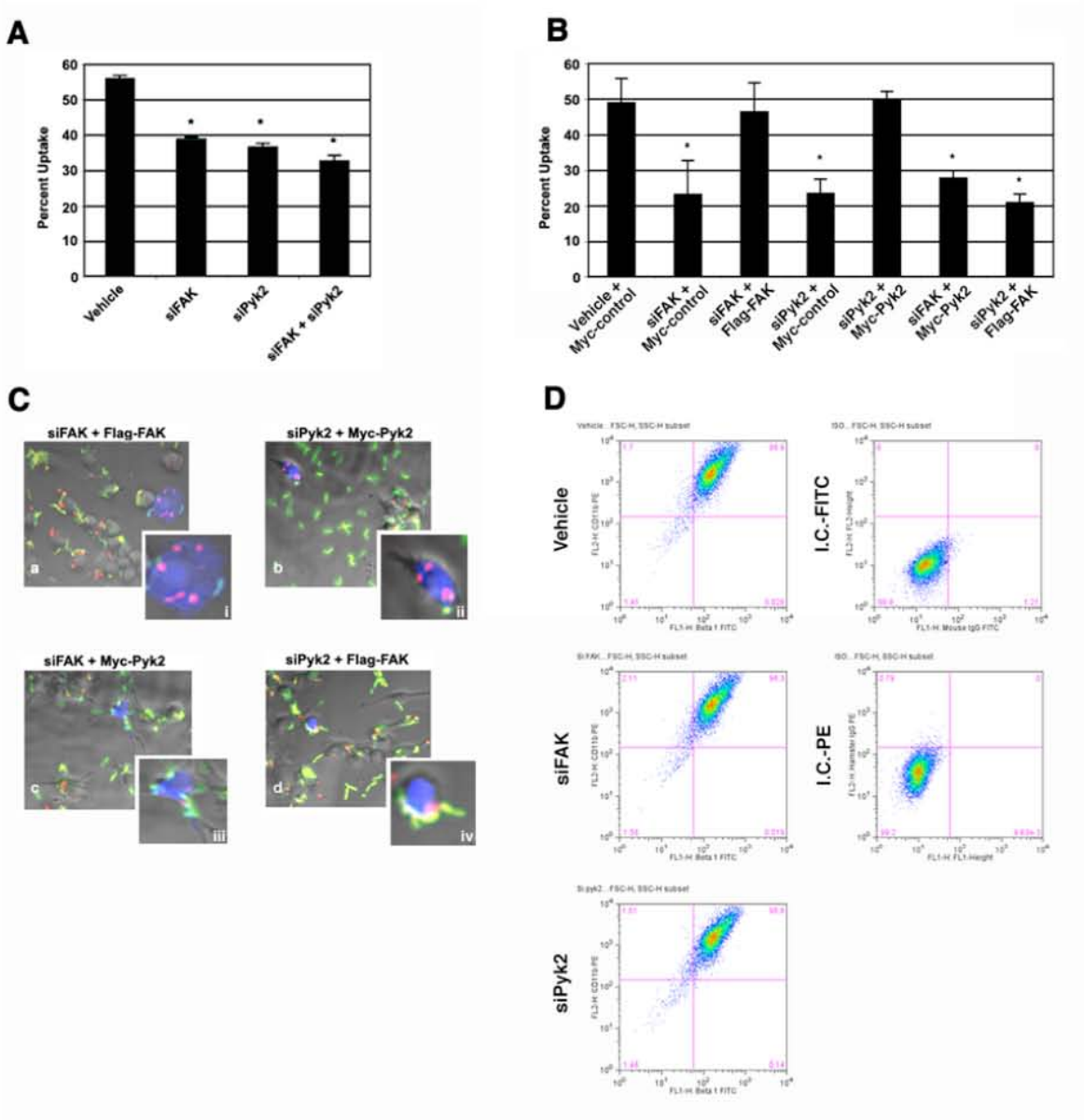
Figure 5.2. RNAi independently reduces the expression of FAK or Pyk2. A. Cell lysates from J774A.1 macrophages treated with either vehicle (H_2O) or the designated siRNAs were immunoblotted for total FAK, Pyk2 or the non-targeted proteins ERK1/2. Band intensities were quantitated by densitometry and presented relative to vehicle-treated cells under each set of lanes and in graph form. **B.** J774A.1 macrophages transfected with either vehicle or siRNAs were plated onto glass coverslips, fixed and probed for total FAK (red) and Pyk2 (green). Merged and phase images are also presented.


A
Vehicle
siControl
siFAK
siPyk2
siFAK+siPyk2
α-FAK
α-Pyk2
α-ERK1/2
1 2 3 4 5
Fold increase over vehicle
1.25
1
0.75
0.5
0.25
FAK
Pyk2
Vehicle
siControl
siFAK
siPyk2
siFAK+siPyk2
B
Vehicle
siFAK
siPyk2
siFAK + siPyk2
α-FAK
α-Pyk2
Merge
Phase
a
b
c
d
e
f
g
h
i
j
k
l
m
n
o
p

demonstrated significantly reduced level of FAK and maintained Pyk2 expression at levels comparable to vehicle-transfected cells (panels e-g). Cells transfected with siPyk2 showed a corresponding loss of Pyk2 expression when compared to vehicle-treated cells, whereas FAK expression remained normal (panels i-k). Co-transfection of siFAK and siPyk2 together resulted in the reduced expression of both molecules (panels m-o). In no case did treatment with siRNA appear to affect cellular morphology (panels d, h, l and p).

Both FAK and Pyk2 play a role in the uptake of Yersinia into macrophages. To determine whether the uptake of *Y. pseudotuberculosis* required FAK and/or Pyk2, J774A.1 murine macrophages were transfected with siFAK and/or siPyk2 duplexes, infected with *Y. pseudotuberculosis* 3 days post-transfection, and examined for their ability to internalize bacteria. The percentage of internalized bacteria was determined for each condition using a double-labeling IF assay (Weidow *et al*., 2000; Bruce-Staskal *et al*., 2002). Vehicle-transfected macrophages were found to internalize an average of 56% of the attached bacteria, while cells transfected with either siFAK or siPyk2 were observed to internalize 39% and 36% respectively (Fig. 5.3A). The coordinated reduction of both FAK and Pyk2 also resulted in significantly impaired Yersinia uptake, as cells co-transfected with siFAK and siPyk2 RNA duplexes were found to internalize only 33% of attached bacteria. The reduction in bacterial internalization exhibited by all three of the siRNA-treated cell populations was statistically significant relative to vehicle-treated cells or cells transfected with siControl. However, simultaneous treatment with siFAK and siPyk2 was not found to cause a synergistic decrease in internalization levels.

Figure 5.3. FAK and Pyk2 are required for the efficient uptake of *Y. pseudotuberculosis*. A. J774A.1 macrophages were treated with vehicle (H_2O) or the designated siRNA. Seventy-two hours post-transfection, macrophages were infected for 2 hours with Yp17/pVector (MOI 20) and processed for the bacterial uptake assay (see Materials and Methods). B and C. Twenty-four hours post-siRNA transfection, cells were transfected with plasmids encoding a Myc-control, full-length Flag-FAK or Myc-Pyk2. After a further 48 hours (72 hours post-siRNA transfection), cells were infected with Yp17/pVector (MOI 20) for 2 hours and processed for immunofluorescence to measure bacterial uptake. **B.** The percentage of internalized *Y. pseudotuberculosis* observed in infected macrophages treated with the designated siRNAs and expressing either Flag-FAK or Myc-Pyk2 is presented as the average of 6 independent experiments. Under conditions of ectopic protein expression, between 50 and 75 Myc- or Flag-stained (blue) cells were scored for Yersinia internalization. Error bars indicate standard deviation from the mean. Asterisks indicate statistically significant differences from vehicle-treated, vector-control cells at a confidence level greater than 95%. **C.** Pictures represent overlayed fluorescence images (400X magnification) of infected macrophages. Intracellular bacteria appear red, extracellular bacteria appear yellow/green and cells expressing ectopic protein appear blue. Enlarged images of Myc- or Flag-stained (blue) cells are presented in the lower quadrant (insets i-iv). **D.** J774A.1 macrophages transfected with either vehicle (H_2O) or siRNA were double-stained for total β_1 integrin and CD11b expression. Untreated cells were stained with rat isotype control antibodies conjugated to either FITC or PE. Cells were then analyzed by flow cytometry.


A
Percent Uptake
60
50
40
30
20
10
0
Vehicle
siFAK
siPyk2
siFAK + siPyk2
B
Percent Uptake
60
50
40
30
20
10
0
Vehicle + Myc-control
siFAK + Myc-control
siFAK + Flag-FAK
siPyk2 + Myc-control
siPyk2 + Myc-Pyk2
siFAK + Myc-Pyk2
siPyk2 + Flag-FAK
C
siFAK + Flag-FAK
siPyk2 + Myc-Pyk2
siFAK + Myc-Pyk2
siPyk2 + Flag-FAK
a
b
c
d
i
ii
iii
iv
D
Vehicle
I.C.-FITC
siFAK
I.C.-PE
siPyk2

To verify that the defect in *Y. pseudotuberculosis* internalization exhibited by siRNA-treated cells was due to the specific knock-down of FAK or Pyk2, these molecules were re-expressed following siRNA transfection. Twenty-four hours post-siRNA treatment, cells were transfected with plasmids encoding either a Myc-tagged protein shown previously to have no effect on Yersinia uptake (Myc-CasΔSH3; Bruce-Staskal *et al.*, 2002 and data not shown), Flag-tagged FAK or Myc-tagged Pyk2. Cells were infected 48 hours later (a total of 72 hours post-siRNA treatment) with *Y. pseudotuberculosis* (Yp17/pVector) and then immunostained to allow detection of the Myc or Flag epitopes as well as internalized and external bacteria (Fig. 5.3A). cDNAs encoding chicken FAK and rat Pyk2 were used for these studies because they contain at least 2 base pair differences from the murine sequences targeted by the siRNAs. Reconstitution of FAK in siFAK-treated cells restored Yersinia uptake efficiencies to levels similar to control cells (48%) and reexpression of Pyk2 in siPyk2-treated cells rescued Yersinia uptake efficiencies to approximately 50% (Fig. 5.3B). This is clearly evident by IF, where siRNA-treated cells that stained positively for the expression of Flag-FAK or Myc-Pyk2 (blue) were found to be associated with a greater number of internalized bacteria (red) than cells in the same field that did not express these molecules and thus had reduced levels of FAK or Pyk2 (Fig. 5.3C, top panels, see insets i-ii). To confirm that these differences in internalization were not due to altered β1 integrins expression in siRNA-treated cells, surface expression of β1 integrins in vehicle-, siFAK and siPyk2-treated J774A.1 macrophages was examined by flow cytometry. There was no difference in β1 integrin under any of these conditions, as approximately 97% of cells stained positive for both β1 integrin and the macrophage marker CD11b regardless of

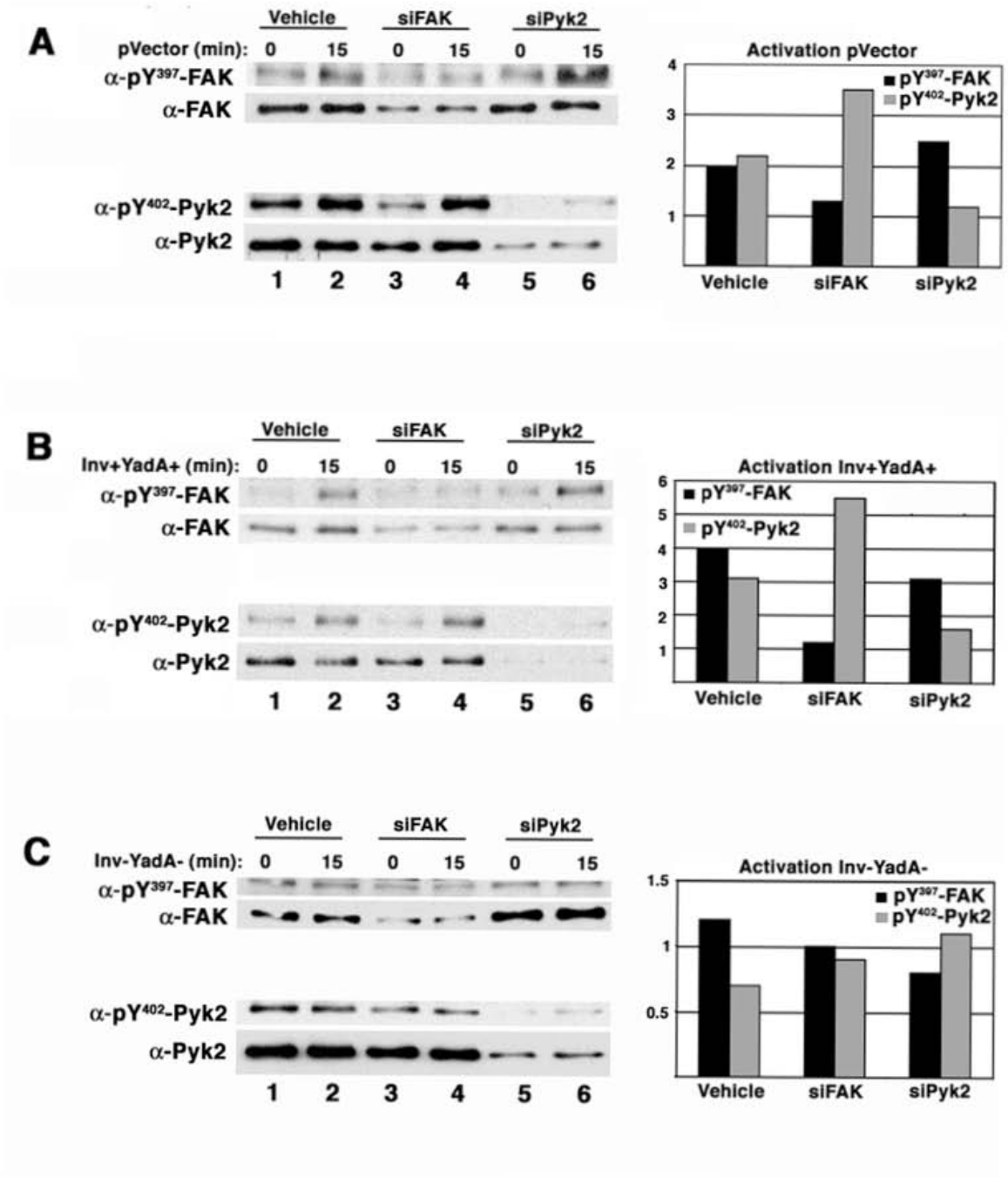
treatment (Fig. 5.3D). While these data confirm that decreases in bacterial uptake were likely to be the direct effect of reduced FAK and/or Pyk2 expression, the residual uptake exhibited under conditions of FAK and/or Pyk2 knockdown (Fig. 5.3A and B) may have arisen through an integrin- and/or invasin/YadA-independent pathway. In fact, a strain lacking expression of both invasin and YadA (Inv^-YadA^-) was internalized to some degree by both vehicle- and siRNA-treated cells (Fig. S5.1A, red bacteria), suggesting that such a pathway(s) does exist. However, because this strain was markedly defective in adherence to J774A.1 cells (Fig. S5.1B and Hudson *et al.*, 2005), it was not possible to accurately quantify the efficiency of bacterial internalization for this strain.

Because FAK and Pyk2 are structurally related, we postulated that they might perform redundant functions during the process of Yersinia internalization. If this were the case, then the defect in uptake exhibited by siFAK- or siPyk2-treated cells should not only be rescued by expression of the targeted protein, but also by expression of the heterologous molecule. However, ectopic expression of Flag-FAK in siPyk2-treated cells or Myc-Pyk2 in siFAK-treated cells was unable to restore Yersinia uptake levels (Fig. 5.3B, last two bars; Fig 5.3C, insets iii-iv). Taken together, these data indicate that functions associated with both FAK and Pyk2 contribute to the ability of macrophages to internalize *Y. pseudotuberculosis*, and that these functions do not appear to be redundant.

FAK and Pyk2 function within independent pathways during infection with Yersina strains co-expressing invasin and YadA. To determine the hierarchy of FAK and Pyk2 signaling during Yersinia infection, we examined how the reduced expression of one molecule via RNAi potentially affected the activation state of the heterologous molecule

in the presence of both Yersinia adhesins. Macrophages were treated with vehicle or the designated siRNA and infected for 15 minutes on day 3 post-siRNA transfection with *Y. pseudotuberculosis* strains that express both invasin and YadA (Yp17/pVector or Inv^+YadA^+) (Fig. 5.4 A and B). The activation status of FAK and Pyk2 was determined by immunoblotting with the phospho-specific antibodies recognizing phosphorylated Y397 on FAK and phosphorylated Y402 on Pyk2. Macrophages expressing reduced total levels of FAK contained less activated FAK upon infection with Yp17/pvector compared to vehicle-treated infected cells (1.3-fold increase in infected cells compared to a 2.0-fold increase; Fig. 5.4A, top panel, compare lane 4 with lane 2). Similarly, Pyk2 activation was reduced in cells treated with siPyk2 (1.2-fold increase in activation compared to a 2.2-fold increase in vehicle-treated infected cells; Fig. 5.4A, bottom panel, compare lane 6 with lane 2). However, Pyk2 activation was readily apparent, and even elevated, under conditions of reduced FAK expression (3.5-fold increase in infected cells compared to 2.2-fold increase in vehicle-treated, uninfected cells; Fig. 5.4A, bottom panel, compare lane 4 with lane 2). Depletion of Pyk2 similarly had no deleterious effect on FAK activation (2.5-fold increase over uninfected cells, compared to a 2.0-fold increase in vehicle-treated infected cells; Fig. 5.4A, top panel, compare lane 6 with lane 2). An analogous activation pattern was observed in siRNA-treated cells infected with the Inv^+YadA^+ Yersinia strain (Fig. 5.4B). Both FAK and Pyk2 became activated upon infection, and phosphorylation levels were not decreased by loss of the heterologous molecule. In fact, Pyk2 activation was again elevated above the level seen in vehicle-treated cells when FAK expression was reduced (5.5-fold increase over uninfected cells, compared to a 3.2-fold increase in vehicle-treated infected cells; Fig. 5.4B, lower panels,

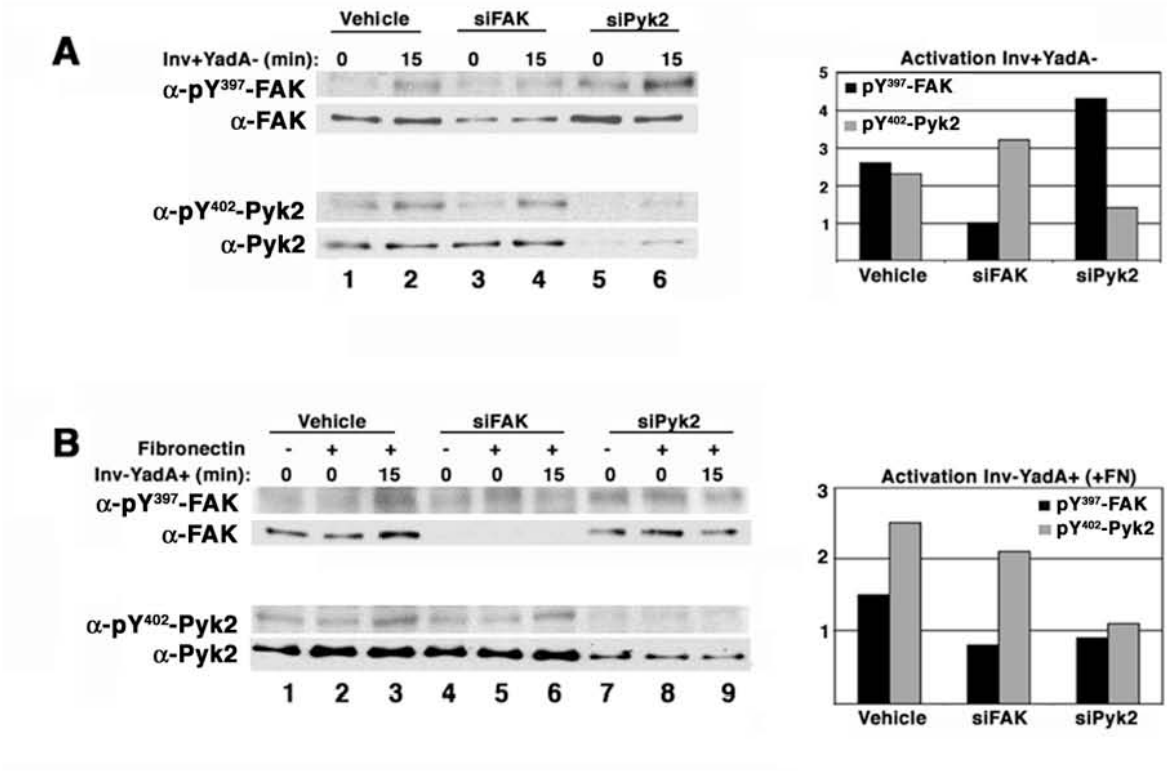
Figure 5.4. FAK and Pyk2 function independently during infection with *Y. pseudotuberculosis* strains expressing both invasin and YadA. J774A.1 macrophages were transfected with either vehicle (H_2O) or siRNAs. On day 3 post-transfection, cells were infected for 0 or 15 minutes with the *Y. pseudotuberculosis* strains Yp17/pVector (**A**), Inv^+YadA^+ (**B**) or Inv^-YadA^- (**C**) at an MOI of 200. Whole cell lysates were immunoblotted for Y397 and total FAK (top panels) or Y402 and total Pyk2 (bottom panels). Phosphorylation data were quantitated by densitometry to determine the amount of activated FAK and/or Pyk2 relative to basal levels in uninfected cells (lanes 1, 3 and 5). Relative band intensities are depicted in graph form to the right. Each immunoblot is representative of multiple experiments.


A
Vehicle
siFAK
siPyk2
pVector (min): 0 15 0 15 0 15
α-pY397-FAK
α-FAK
α-pY402-Pyk2
α-Pyk2
1 2 3 4 5 6
Activation pVector
pY397-FAK
pY402-Pyk2
Vehicle
siFAK
siPyk2
B
Vehicle
siFAK
siPyk2
Inv+YadA+ (min): 0 15 0 15 0 15
α-pY397-FAK
α-FAK
α-pY402-Pyk2
α-Pyk2
1 2 3 4 5 6
Activation Inv+YadA+
pY397-FAK
pY402-Pyk2
Vehicle
siFAK
siPyk2
C
Vehicle
siFAK
siPyk2
Inv-YadA- (min): 0 15 0 15 0 15
α-pY397-FAK
α-FAK
α-pY402-Pyk2
α-Pyk2
1 2 3 4 5 6
Activation Inv-YadA-
pY397-FAK
pY402-Pyk2
Vehicle
siFAK
siPyk2

compare lane 2 with lane 4). Taken together, these results indicate that, in the presence of both invasin and YadA, FAK and Pyk2 become independently activated and may therefore function within separate pathways. Importantly, the majority of FAK and Pyk2 phosphorylation can be attributed to the presence of these adhesins since infection with the non-invasive control strain, $Inv^{-}YadA^{-}$ did not result in appreciable activation of either FAK or Pyk2 (Fig. 5.4C).

FAK and Pyk2 activation is regulated by the differential expression of Yersinia adhesin*s*. Previous studies by our lab have determined that the phagocytic signaling cascade initiated upon Yersinia-host cell contact is differentially activated by invasin and YadA (Hudson *et al*., 2005). Therefore, we were interested in examining the individual contribution of each adhesin to the activation status of FAK and Pyk2. While invasin is capable of binding integrin receptors directly, YadA is thought to interact with integrins via an ECM bridge (Heise and Dersch, 2006); for review, see (El Tahir and Skurnik, 2001). Macrophages were treated with vehicle or the designated siRNAs before infection with an invasin-only expressing Yersinia strain ($Inv^{+}YadA^{-}$) or a YadA-only expressing strain ($Inv^{-}YadA^{+}$) (Fig. 5.5A and B, respectively). Infection with $Inv^{+}YadA^{-}$ induced an activation pattern that was similar in many respects to that observed during infection with strains coexpressing invasin and YadA. For example, in siFAK-treated cells, Pyk2 activation became elevated to a greater extent than was seen in infected vehicle-treated cells, but was nearly undetectable in siPyk2-treated cells (Fig. 5.5A, bottom panel). One notable difference was that FAK activation became highly elevated under conditions of depleted Pyk2 following infection with this strain, exhibiting a 4.3-fold increase over

Figure 5.5. FAK and Pyk2 become differentially activated in the presence of invasin or YadA. J774A.1 macrophages were transfected with vehicle (H_2O) or siRNAs. On day 3 post-transfection cells were infected for 0 or 15 minutes with *Y. pseudotuberculosis* expressing invasin alone (Inv^+YadA^-) (**A**) or YadA alone (Inv^-YadA^+) (**B**). For Inv^-YadA^+ infections, cells were pre-treated for 30 minutes with 200 μg/ml FN before infection. Whole cell lysates were subjected to immunoblot analysis and phosphorylation data were quantitated as described for Fig. 5.4. Phosphorylation data were analyzed by densitometry to determine the fold-increase of activated FAK or Pyk2 relative to basal levels in uninfected cells (A) or in cells cultured in the presence of FN (B). Relative band intensities are depicted in graph form to the right. Each immunoblot is representative of multiple experiments.


A
Vehicle
siFAK
siPyk2
Inv+YadA- (min): 0 15 0 15 0 15
α-pY397-FAK
α-FAK
α-pY402-Pyk2
α-Pyk2
1 2 3 4 5 6
Activation Inv+YadA-
pY397-FAK
pY402-Pyk2
Vehicle
siFAK
siPyk2
B
Vehicle
siFAK
siPyk2
Fibronectin - + + - + + - + +
Inv-YadA+ (min): 0 0 15 0 0 15 0 0 15
α-pY397-FAK
α-FAK
α-pY402-Pyk2
α-Pyk2
1 2 3 4 5 6 7 8 9
Activation Inv-YadA+ (+FN)
pY397-FAK
pY402-Pyk2
Vehicle
siFAK
siPyk2

uninfected cell compared to a 2.6-fold increase in vehicle-treated cells (top panel, compare lanes 6 and 2).

For the $Inv^{-}YadA^{+}$ strain, macrophages were pretreated with FN prior to infection because YadA-mediated signaling was found to be significantly enhanced in the presence of increasing amounts of ECM components (Hudson *et al*., 2005). Under these conditions, the addition of supplemental FN did not significantly activate either FAK or Pyk2 in the absence of infection (Fig. 5.5B, lanes 1-2). Following infection with the $Inv^{-}YadA^{+}$ strain, FAK was slightly activated (top panels, lanes 2-3), and this was completely abrogated when Pyk2 expression was knocked down (lanes 8-9). In contrast, Pyk2 activation was observed in both vehicle- and siFAK-treated cells (bottom panels; 2.5- and 2.1-fold increase over uninfected cells respectively). Unlike the strains expressing both adhesins, however, Pyk2 activation in response to $Inv^{-}YadA^{+}$ infection was not significantly enhanced under conditions of reduced FAK (bottom panels, compare lane 6 with lane 3). Together these data show that, while invasin is capable of activating both FAK and Pyk2, YadA/FN preferentially activates Pyk2.

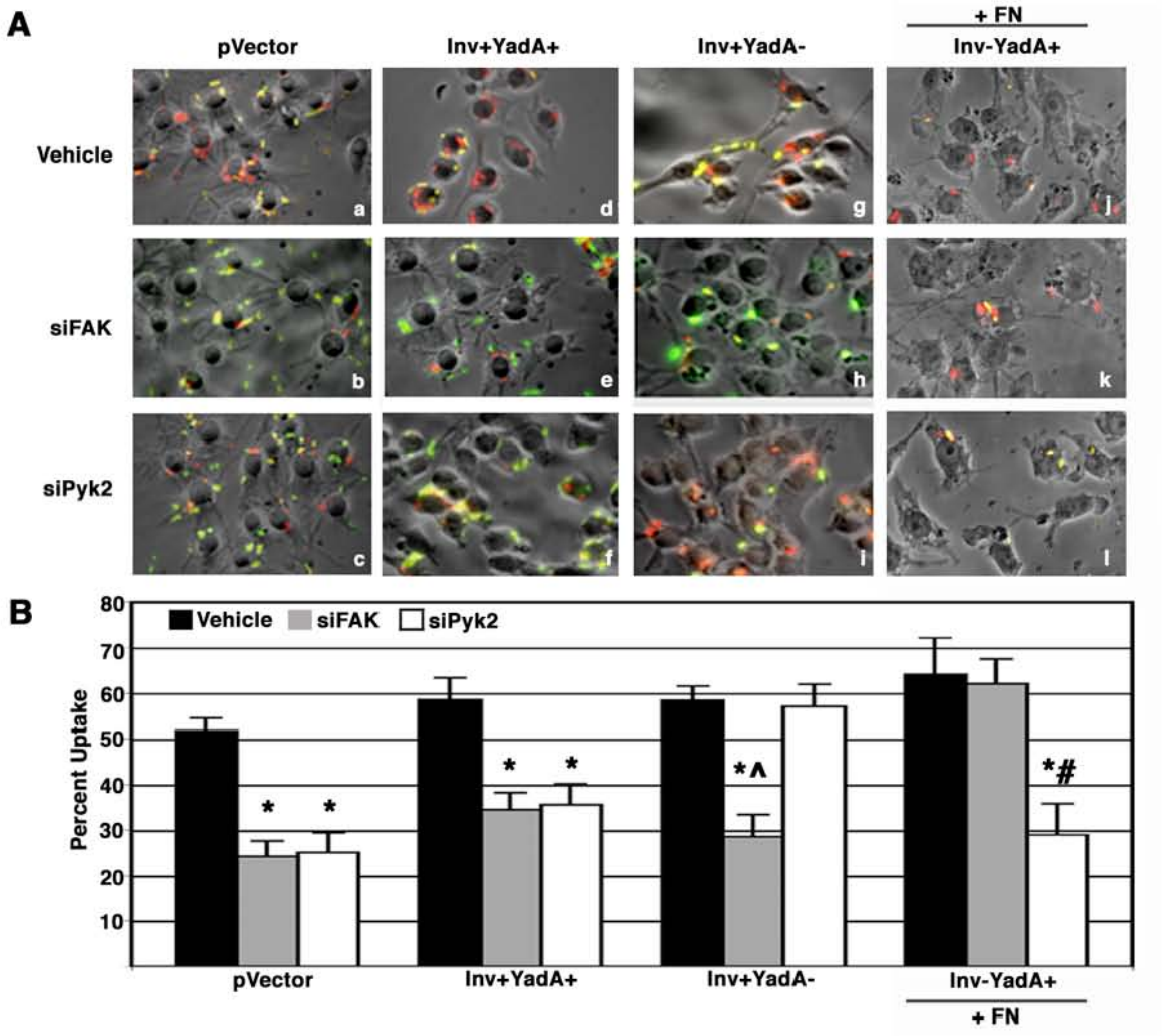
Adhesin expression determines the molecular requirements for Yersinia uptake. As differential adhesin expression was observed to induce distinct activation patterns for FAK and Pyk2, we were interested in determining the molecular requirements for efficient Yersinia internalization under these circumstances. Macrophages were treated with vehicle or the designated siRNAs for 72 hours before a 1-hour infection with Yp17/pVector or the adhesin mutants, and assessed for their ability to take up bacteria.

As demonstrated previously (Fig. 5.3A), Yersina strains co-expressing invasin and YadA (Yp17pVector or $Inv^{+}YadA^{+}$) require the presence of both FAK and Pyk2 for efficient internalization (Fig. 5.6). However, macrophages infected with the $Inv^{+}YadA^{-}$ strain required only FAK for efficient phagocytosis; siFAK-treated cells internalized 29% of cell-associated bacteria compared to vehicle-treated cells (59% uptake) or siPyk2-treated cells (56% uptake). These results were observed in spite of the fact that the $Inv^{+}YadA^{-}$ strain is capable of activating both FAK and Pyk2 (Fig. 5.5A, lanes 1-2). Conversely, internalization of the YadA-only expressing strain, in the presence of supplemental FN, required Pyk2 but not FAK, as siPyk2-treated cells internalized 30% of attached bacteria compared to vehicle-treated cells (63% uptake) or siFAK-treated cells (60% uptake). Collectively, these data demonstrate that adhesin expression dictates the molecular requirements for phagocytosis.

5.5. Discussion.

This study provides insight into how the FAK family of PTKs contributes to the process of Yersinia internalization. We have shown previously that co-expression of invasin and YadA results in competition for integrin binding (Hudson *et al*., 2005). Two types of signals are initiated under these conditions, one arising from high affinity interactions between invasin and β1 integrins and the other from YadA/ECM-induced integrin clustering. Our current data show that when invasin and YadA compete for the same β1 integrin receptors, independent functions of both FAK and Pyk2 are required to generate the signals necessary for bacterial uptake. However, when one mechanism of

Figure 5.6. The molecular requirements for bacterial uptake are determined by the differential expression of Yersinia adhesins. A. J774A.1 macrophages were treated with vehicle (H_2O) or the designated siRNAs. Seventy-two hours post-siRNA transfection, cells were infected with *Y. pseudotuberculosis* strains Yp17pVector, Inv^+YadA^+, Inv^+YadA^-, and Inv^-YadA^+ (MOI 20) for 1 hour and then processed for the bacterial uptake assay. Thirty minutes prior to infection with the Inv^-YadA^+ strain, macrophages were pre-treated with 200 μg/ml FN. Intracellular bacteria appear red and extracellular bacteria appear yellow/green. **B.** The percentage of internalized *Y. pseudotuberculosis* observed in infected macrophages treated with vehicle or siRNA is presented as the average of 4-6 experiments. Error bars indicate standard deviation from the mean; asterisks indicate statistically significant differences from vehicle-treated cells at a confidence level greater than 95%, ^ indicate statistically different differences from siPyk2-treated cells at a confidence level greater than 95% and # indicate statistically different differences from siFAK-treated cells at a confidence level greater than 95%.


A
pVector
Inv+YadA+
Inv+YadA-
+ FN
Inv-YadA+
Vehicle
siFAK
siPyk2
a
b
c
d
e
f
g
h
i
j
k
l
B
Vehicle
siFAK
siPyk2
Percent Uptake
80
70
60
50
40
30
20
10
*
*
*
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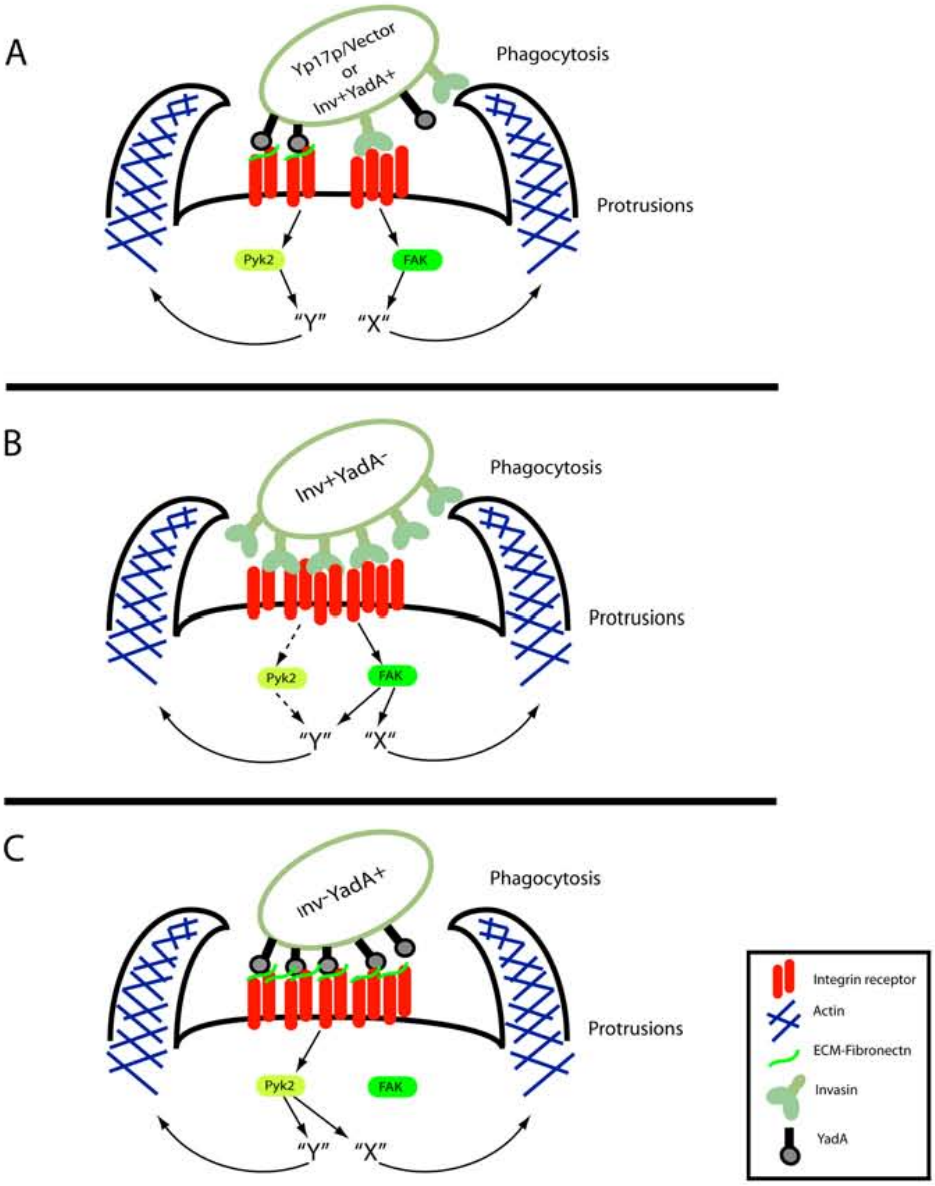
*^
*#
pVector
Inv+YadA+
Inv+YadA-
Inv-YadA+
+ FN

integrin engagement predominates, as is the case when cells are infected by the $Inv^{+}YadA^{-}$ strain or by the $Inv^{-}YadA^{+}$ strain in the presence of exogenous ECM, the requirement for these kinases is altered. For example, in the absence of competition for β1 integrin binding by YadA/ECM, invasin-dependent activation of FAK is sufficient to promote signaling functions required for bacterial internalization that are normally performed by FAK as well as Pyk2 (Fig. 5.6B). Similarly, Pyk2 activation by YadA/ECM in the absence of competing inputs from invasin can override the requirement for FAK. Work is currently underway to determine whether these competing activities involve catalytic and/or the adapter/scaffolding functions of these kinases.

Adhesin-driven bacterial internalization: a model

When both invasin and YadA are expressed on the bacterial surface, we propose that FAK and Pyk2 can play independent and non-redundant functions required for phagocytosis. This is supported by data showing that 1) FAK and Pyk2 exhibit similar activation kinetics in response to infection with a strain that expresses both invasin and YadA (Fig. 5.1); 2) specific reduction of either FAK or Pyk2 coincided with a marked inhibition in *Y. pseudotuberculosis* internalization and re-expression of the targeted molecule restored Yersinia uptake efficiencies (Fig. 5.3); and 3) the combined reduction of both FAK and Pyk2 was not more deleterious for Yersinia internalization than was reduction of either molecule individually (Fig. 5.3A). An alternative possibility is that the FAK and Pyk2 signaling pathways may be interdependent, and that one pathway becomes up-regulated when the other is inactivated. This is supported by the finding that

Figure 5.7. Model describing the signaling pathways initiated in the presence of invasin and/or YadA leading to efficient *Y. pseudotuberculosis* uptake. Solid arrows reflect activation and requirement for bacterial phagocytosis while dotted arrows reflect activation but no requirement phagocytosis.


A
Yp17p/Vector
or
Inv+YadA+
Phagocytosis
Protrusions
Pyk2
FAK
"Y"
"X"
B
Inv+YadA-
Phagocytosis
Protrusions
Pyk2
FAK
"Y"
"X"
C
Inv-YadA+
Phagocytosis
Protrusions
Pyk2
FAK
"Y"
"X"
Integrin receptor
Actin
ECM-Fibronectn
Invasin
YadA

Pyk2 activation was enhanced under conditions of reduced FAK (Figs. 5.4 and 5.5). However, FAK and Pyk2 do not perform redundant functions during phagocytosis of bacterial strains expressing both invasin and YadA since overexpression of the heterologous molecule could not rescue the defect in bacterial uptake exhibited by siFAK- or siPyk2-treated cells (Figs. 5.3B and 5.3C).

Based on this evidence, we propose that the combined expression of invasin and YadA on the bacterial surface results in the activation of phagocytic signaling pathways via two distinct mechanisms of integrin engagement. Invasin engages β1 integrins directly through high affinity interactions (Isberg *et al.*, 2000; Wiedemann *et al.*, 2001), while YadA binds to integrins through an ECM bridge (El Tahir and Skurnik, 2001). Our data suggest that invasin potently activates FAK as well as Pyk2, while YadA/ECM activates Pyk2. In the presence of both adhesins, the activation of FAK and Pyk2-dependent pathways combine to provide the molecular signals necessary for efficient bacterial internalization ("X" and "Y," respectively) (Fig. 5.7A). However, when invasin is expressed in the absence of YadA (Inv^+YadA^-), we suggest that high-affinity interactions that occur between invasin and β1 integrins contribute to potent receptor activation as well as clustering, ultimately leading to recruitment of FAK to the cytoplasmic integrin tail. We speculate that activation of FAK under these conditions is sufficient to activate both the "X" and "Y" components of the phagocytic signaling pathway, thus allowing bacterial internalization to occur independently of Pyk2 activity (Fig. 5.7B). While invasin is also capable of activating Pyk2 under these circumstances (Fig. 5.5A), this molecule is not recruited to integrin cytoplasmic tails upon stimulation

(Avraham *et al*., 2000) and our data show that it is not required for internalization of the $Inv^{+}YadA^{-}$ strain (Fig. 5.6).

In the absence of invasin ($Inv^{-}YadA^{+}$), FAK is activated relatively poorly compared to Pyk2 (Fig. 5.5B). This may be because YadA/ECM does not bind β1 integrins with the same affinity as does invasin (Heise and Dersch, 2006). Nonetheless, the expression of multiple YadA molecules on the bacterial surface is likely to induce significant integrin clustering, which can also lead to activation of downstream signals. Uptake of the $Inv^{-}YadA^{+}$ strain occurred independently of FAK (Fig. 5.6), suggesting that the Pyk2 pathway is sufficient to generate the full complement of events required for phagocytosis when integrin engagement is mediated predominantly by YadA/ECM (Fig. 5.7C).

The unique factors/pathways downstream of FAK and Pyk2 that are collectively required to promote bacterial internalization are currently unknown. Studies using fibroblasts and other cell types that do not normally express Pyk2 suggest some redundancy in function for these two related kinases (Avraham *et al*., 2000). However, there is some evidence that these proteins have distinct functions in cells/tissues that normally express both proteins (macrophages, osteoclasts, vascular smooth muscle cells, neuronal tissues, and intestinal epithelial cells) (Kang *et al*., 2004; Orr and Murphy-Ullrich, 2004; Jiang *et al*., 2006). Our work is the first to show distinct and non-redundant functions for these two proteins in macrophages.

There are several candidate molecules that might function as unique downstream effectors of either FAK or Pyk2. For example, the process of "zipper-like" phagocytosis utilized by macrophages to internalize Yersinia generally requires activation of the small

GTPase Rac1, and Rac1 has been shown to be activated in response to *Y. pseudotuberculosis* infections (Hudson *et al.*, 2005; Wong and Isberg, 2005b, a). Previous work has also shown that tyrosine phosphorylation of Cas following *Y. pseudotuberculosis* infection in epithelial cells can trigger the formation of Cas/Crk complexes (Weidow *et al.*, 2000). Cas/Crk signaling can lead to activation of Rac1 (Chodniewicz and Klemke, 2004), an event shown to be required for bacterial internalization (Weidow *et al.*, 2000). Preliminary evidence from our lab suggests that Yersinia-dependent phosphorylation of Cas is abolished in siPyk2-treated, but not siFAK-treated, cells (data not shown). This suggests that Cas/Crk/Rac1 may be a component of the Pyk2 pathway. However, there is evidence that invasin-coated beads can also activate Rac1, suggesting that the involvement of Rac1 may be more complex (Alrutz *et al.*, 2001). A second candidate molecule is PI3K, which has been shown to be required for YadA-mediated phagocytosis in HEp2 cells (Dersch and Isberg, 1999; Isberg *et al.*, 2000; Eitel and Dersch, 2002; Eitel *et al.*, 2005; Heise and Dersch, 2006). Studies are currently under way to determine if PI3K activity is required downstream of one or both of these kinases. Finally, newly emerging data suggest that contractile forces produced by microtubules help to regulate membrane protrusions (Elbaum *et al.*, 1999; Vicente-Manzanares *et al.*, 2005). We are currently investigating whether this regulation is mediated preferentially by FAK or Pyk2.

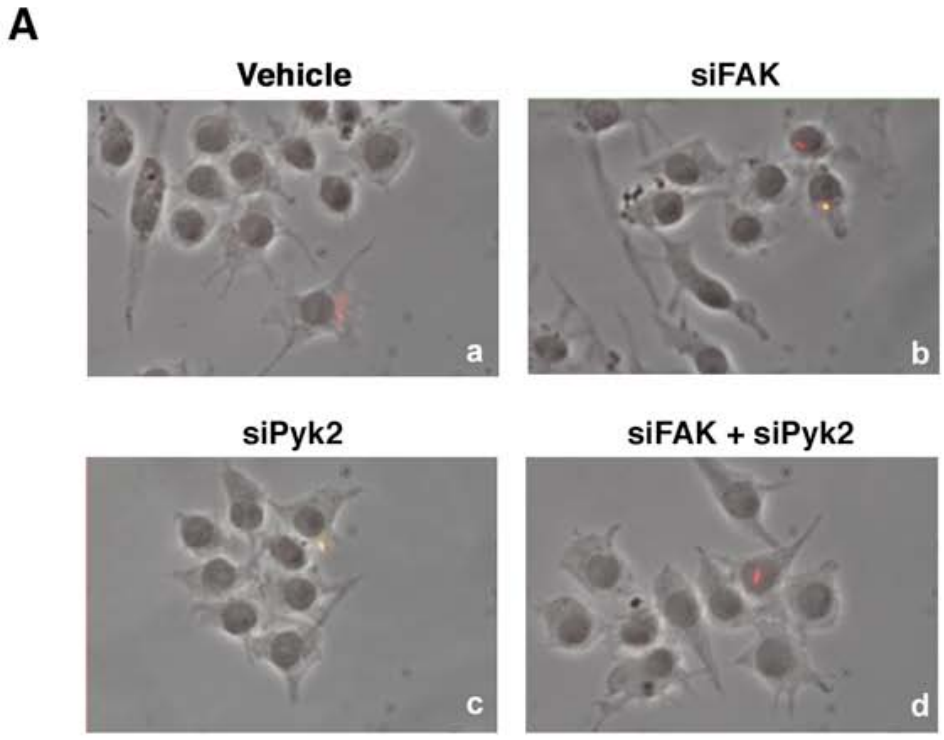
The biological significance of FAK- and Pyk2-dependent pathways

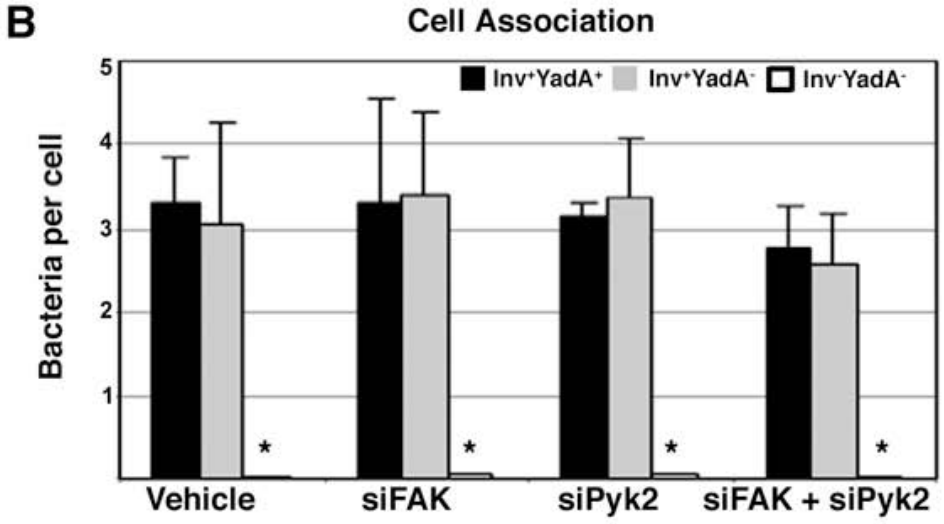
The ability to modulate the activation state and subsequent downstream signaling of FAK and Pyk2 through distinct mechanisms of integrin engagement has significant

physiological relevance to a number of biological processes. During a typical infection, *Y. pseudotuberculosis* breaches the epithelial cell barrier in the intestine to colonize underlying lymphoid tissues, and in rare instances, disseminate to other tissues (for review, see Brubaker, 1991). The fate of the pathogen in these varied environments is dependent on 1) the expression of antiphagocytic and cytotoxic effector molecules, several of which target FAK and Pyk2 signaling pathways (Fallman and Gustavsson, 2005; Viboud and Bliska, 2005); 2) the relative expression of invasin and YadA on the bacterial surface; and 3) the ECM composition in the tissue milieu. We suggest that, at any given time and place, these factors together control the relative activation levels of FAK and Pyk2 and ultimately, the extent of phagocytosis. A similar argument can be made for cell adhesion and migration, in that the composition and presentation of the ECM vary greatly in different tissue environments. We propose that migration can be controlled by these factors, as well as growth factor/cytokine/chemokine gradients, through regulated activation of the FAK and Pyk2 pathways.

In summary, by investigating the mechanism of Yersinia uptake by macrophages, which endogenously express both FAK and Pyk2, we have been able to define unique roles for these molecules in the process of phagocytosis. Depending on the nature of the external signal, we have found that FAK and Pyk2 can function coordinately to activate distinct pathways, or independently, to induce the cytoskeletal changes needed for phagocytosis. Future studies will help to formulate a more cohesive picture of how and where these pathways interact and thus gain better insight into the dynamics of host-pathogen interactions as well as other integrin-dependent activities.

Supplementary Figure S5.1. Adhesin-deficient *Y. pseudotuberculosis* adhere poorly to macrophages. A. J774A.1 macrophages were treated with vehicle (H_2O) or the designated siRNAs. Seventy-two hours post-siRNA transfection, cells were infected with Inv^-YadA^- bacteria (MOI 20) for 1 hour and then processed for the bacterial uptake assay as described in Experimental Procedures. **B.** Vehicle and siRNA-treated J774A.1 macrophages were infected and processed as described in panel A and the number of macrophage- associated bacteria per cell was determined for each strain. Data shown are the mean of 4-6 experiments. Error bars indicate standard deviation from the mean; asterisks indicate statistically significant deviation relative to both the Inv^+YadA^+ strain and the Inv^+YadA^- strain at a confidence level greater than 95%.


A
Vehicle
siFAK
siPyk2
siFAK + siPyk2
a
b
c
d


B
Cell Association
Inv+YadA+
Inv+YadA-
Inv-YadA-
Bacteria per cell
5
4
3
2
1
*
Vehicle
siFAK
siPyk2
siFAK + siPyk2

Chapter 6:

Summary and Perspectives

6.1. Overview.

Macrophages are found in almost every tissue of the body where they participate in numerous cellular functions including immune defense and normal tissue development. The goals of this research were (i) to define the requirement for FAK and Pyk2 activity during common macrophage functions and (ii) to determine whether these molecules maintain unique or overlapping roles in response to the integrin-dependent processes of migration and phagocytosis. In this thesis, we show that FAK and Pyk2 function within the same signaling pathway to promote cell migration, however, these molecules can also function independently during bacterial internalization. Given the heterogeneity of macrophages, the differential requirements for FAK and Pyk2 in these biological activities may reflect the versatility of these cells as they respond to a diverse array of environmental cues. The following discussion will provide a summary of the data presented and the major conclusions drawn thus far, as well as introduce some compelling new areas for future research.

6.2. FAK and Pyk2 in macrophage migration.

While FAK function has been extensively studied in other cell systems, this is the first body of work to directly assess the role of FAK in primary macrophages. The generation of myeloid-specific conditional FAK knockout mice has allowed us to examine how FAK, Pyk2, and other molecules involved in the regulation of the actin cytoskeleton promote chemotaxis and migration. Monocytes originating in the bone marrow are released into the peripheral blood where they circulate for several days before entering tissues and replenishing resident populations (Gordon and Taylor, 2005). Pro-

inflammatory, metabolic and immune stimuli elicit the recruitment of monocytes to peripheral sites (Van Furth *et al.*, 1973). The loss of FAK from primary bone marrow macrophages resulted in compromised chemotaxis and reduced invasion *in vitro*. The failure of $FAK^{-/-}$ macrophages to migrate and invade also correlated with a deficiency in inflammatory infiltration *in vivo*. Based on these findings, we concluded that the defects exhibited by macrophages in the absence of FAK may be the result of a generalized breakdown in the cell migration machinery rather than an inability to detect migratory stimuli or degrade matrix components.

Further characterization of the FAK-deficient macrophage phenotype revealed that these cells form numerous transitory protrusions along the cell periphery. Examination of these cells suggests that protrusions extending away from the cell body are unable to form stable contact with substrate. Consistent with these results, we observed altered adhesive contact turnover in the absence of FAK. While FAK is known to be a major regulator of focal adhesion disassembly in fibroblasts (Webb *et al.*, 2004), in this thesis, we demonstrate that FAK is involved in the assembly as well as the disassembly of adhesive contacts in macrophages. The requirement for FAK in both processes may reflect the fact that macrophages, as a highly motile cell type, need a constant supply of recycled proteins coalescing into small adhesion structures to maintain migratory velocity. Dysregulation of the small GTPase Rac1, which is an established inducer of membrane protrusions (Nobes and Hall, 1995), may also contribute to the elevated protrusive activity occurring at the plasma membrane in $FAK^{-/-}$ cells. Active Rac1 concentrated at the leading cell edge promotes the formation of lamellipodia required for polarization and migration. Interestingly, $FAK^{-/-}$ BMMs exhibit high basal

levels of Rac activity. Thus, we might envision that in FAK-deficient macrophages, activated Rac, distributed along the cell periphery, instead of confined to the leading edge, may account for the formation of numerous protrusive structures, rather than a single leading lamellipodia in these cells.

However, it is still unclear why the loss of FAK results in high levels of basal Rac activity. Rac can be activated by integrin signaling (DeMali and Burridge, 2003) and, along with its family member, Cdc42, is involved in membrane dynamics and remodeling (Takenawa *et al*., 2001). Recently, Yano et al., (2004) reported that HeLa cells treated with siRNAs targeting FAK expression exhibited aberrant protrusive behavior associated with impaired migration. Further examination of the protrusive structures observed in those cells revealed high expression of GTP-Rac. It was suggested that FAK may be required to down regulate Rac activity within the non-motile regions of the membrane, such as the sides and rear of the cell. If this is also the case in macrophages, we might predict that expression of RacV12, a GTP-hydrolysis mutant, would induce protrusive behavior in WT cells, whereas expression of RacT17N, a GTP binding mutant, might suppress the formation of these structures in $FAK^{-/-}$ cells. The use of other techniques, such as fluorescence resonance energy transfer (FRET), could also be used to examine the activity of Rac1 in situ. The transfection of Rac-expressing plasmids suitable for the detection of a FRET specific for Rac1 activation would allow us to determine differences in the localization of active Rac in WT and $FAK^{-/-}$ macrophages.

$FAK^{-/-}$ BMMs exhibited significant impairments in migration and invasion, yet the loss of FAK did not result in the complete abolishment of migratory activity. While the invasive capacity of WT macrophages expressing reduced levels of Pyk2 was

inhibited to the same degree as that observed in $FAK^{-/-}$ BMMs, depleting Pyk2 expression in FAK-deficient cells did not cause an additional decrease in invasion. These results indicate that FAK and Pyk2 may be functioning within the same pathway during CSF-1-induced cell migration. In contrast to Pyk2, the reduced expression of paxillin from $FAK^{-/-}$ BMMs resulted in an almost total loss of invasive ability. Our data also indicates that the requirement for paxillin may be regulated by α4 integrin receptors. We therefore hypothesize that macrophage invasion requires the combined activities of a FAK/Pyk2-dependent pathway and a α4 integrin-paxillin dependent pathway. These results conflict with the integrin signaling model recently proposed by Hsia *et al.*, (2005) which implies that α4β1 integrin-mediated motility occurs independently of paxillin activity, whereas α5β1-mediated motility depends on a FAK-paxillin linkage. However, compared to macrophages, which endogenously express α4 integrins, the fibroblasts used in this report were genetically manipulated to express a functional α4β1 integrin receptor, which may account for some of the discrepancies between our data. Alternatively, the differential requirement for paxillin during α4β1-mediated signaling may reflect the fact that, as a highly motile cell type, macrophages utilize a divergent motility-promoting signaling pathway.

Experiments are currently underway to determine which receptor initiates signaling via the FAK/Pyk2 pathway in macrophages. One potential candidate is α5β1 integrin, which is considered to be the classical fibronectin receptor. The use of fibronectin fragments that specifically bind to either the α5β1 receptor (recombinant FN (9-11) or the α4β1 receptor (FN-CS-1) will allow us to further define the signaling pathways initiated upon ligation of these receptors in macrophages. If the α5β1 receptor

functions upstream of the FAK/Pyk2-dependent pathway, we predict that the motility of $FAK^{-/-}$ macrophages stimulated with the α5β1 receptor specific recombinant FN (9-11) fragment would be completely inhibited. In contrast, stimulation of FAK-deficient BMMs with the α4β1 receptor specific FN-CS-1 fragment might result in migration equivalent to that observed in WT cells since this pathway potentially functions independently of FAK expression. It is likely however, that Rac1 is a common downstream target of both pathways and further investigation is required to determine how signaling via the integrins α4β1 and α5β1 modulate Rac activity and the intracellular localization of this molecule.

One issue that has not yet been addressed is how the loss of FAK affects Rho activity in macrophages. The activation of Rac and PI3K at the leading cell edge promotes and maintains the formation of lamellipodia, while RhoA- and myosin-dependent pathways determine the trailing edge (Xu *et al.*, 2003). To establish polarity, these pathways are mutually suppressive such that RhoA activity is downregulated at the cell front, and Rac is downregulated at the back and sides. FAK has been shown to be required for the transient downregulation of Rho, presumably by increasing the phosphorylation of p190RhoGAP (Arthur *et al.*, 2000; Ren *et al.*, 2000; Arthur and Burridge, 2001). Although the mechanisms have not been extensively explored, FAK can also promote RhoA activity through the binding of p190RhoGEF. The elevated Rac activity observed in FAK-deficient macrophages may, in part, be due to the fact that overall RhoA activity is lower in these cells and thus, Rac is not appropriately suppressed. This hypothesis is consistent with evidence from $Pyk2^{-/-}$ macrophages, which exhibit reduced RhoA activity coupled to defects in contractility and migration. Based on

these data, as well as our own data showing that FAK and Pyk2 function within the same pathway, we hypothesize that a similar alteration in RhoA activity would occur in the absence of FAK.

6.3. Integrin-mediated phagocytic signaling.

The other aspect of macrophage function that has been extensively investigated in this thesis is the signaling pathway(s) governing integrin-mediated phagocytosis. The establishment of a productive *Yersina pseudotuberculosis* infection relies on the ability of the bacteria to translocate across the intestinal epithelium and evade phagocytosis by immune cells present in the sub-epithelial lymphoid tissues. *Y. pseudotuberculosis* express two major adhesins, invasin and YadA, capable of interacting with integrin receptors on host cells. While invasin binds directly to β1 integrin receptors, YadA binds indirectly through ECM components. Through their ability to promote interactions with professional phagocytes as well as epithelial cells, invasin and YadA have the potential to contribute to the establishment and clearance of Yersinia infections (Wiedemann *et al.*, 2001; Grosdent *et al.*, 2002). The study of how these adhesins interact with host receptors on macrophages has allowed us to propose a model in which invasin potently activates FAK and Pyk2 but only requires FAK for bacterial uptake, while YadA/ECM activates and requires Pyk2 for Yersinia internalization.

Prior to the results presented in this thesis, it was shown that FAK and the adapter protein Cas were involved in uptake of a Yersinia strain expressing both invasin and YadA, and that the overexpression of Cas was sufficient to promote efficient bacterial internalization by FAK-null MEFs (Bruce-Staskal *et al.*, 2002). These studies provided

an initial framework in which to investigate the molecular nature and regulation of signaling networks contributing to the phagocytic process. More recent studies in our lab using *inv* and *yadA* mutant *Y. pseudotuberculosis* strains allowed us to investigate how these adhesins were able to activate signaling pathways and promote bacterial uptake by macrophages (Hudson *et al.*, 2005). Importantly, Hudson *et al.*, (2005) demonstrated that the efficiency of adhesin-mediated phagocytic responses was dependent on both the relative expression of invasin and YadA as well as the expression of ECM in the cellular microenvironment. Building on these past reports, we present data here indicating that the activation states of FAK and Pyk2, as well as the subsequent signaling events leading to phagocytosis, are differentially regulated through the unique mechanisms of integrin engagement utilized by invasin and YadA.

Here, we have determined that in the presence of both invasin and YadA, the activation of FAK and Pyk2 combine to provide the molecular signals necessary for efficient internalization. In the absence of YadA however, invasin-mediated FAK activation is sufficient to induce phagocytosis in a Pyk2-independent manner. Conversely, uptake of the YadA-only expressing Yersinia strain, in the presence of supplemental fibronectin, was able to occur independently of FAK expression, suggesting that the Pyk2 pathway is sufficient to generate the full complement of events required for phagocytosis.

Despite the progress made, many questions remain regarding how these signaling pathways function to promote internalization, and future studies are needed to determine the downstream molecular signals needed for phagocytosis. Preliminary data support a role for paxillin and Cas functioning within the Pyk2-dependent internalization pathway,

as the phosphorylation of both molecules was significantly reduced under conditions of depleted Pyk2 expression in macrophages. While these results are consistent with previous reports showing that tyrosine phosphorylation of Cas following *Y. pseudotuberculosis* infection of epithelial cells was able to promote the formation of Cas/Crk complexes (Weidow *et al.*, 2000), it is unclear what role, if any, is played by Cas and/or paxillin during Yersinia internalization. We have also not been able to identify any unique downstream molecular effectors within the FAK-dependent pathway. Since bacterial phagocytosis requires major rearrangements of the actin cytoskeleton, Rac1 may function downstream of either the FAK- or Pyk2-dependent pathways, although it is equally likely to function as a common target of both pathways. Future experiments examining Rac activity during the infection of macrophages expressing reduced amounts of FAK or Pyk2 will help elucidate which pathway(s) require small GTPase activity.

6.4. Foundations for future studies.

From these studies and others reported in the literature, it is clear that FAK participates in a multitude of events linked to cell motility and directionality. The finding that inflammatory monocyte recruitment in the conditional FAK knockout mice was delayed indicates that the loss of FAK expression in macrophages induces a fundamentally immunosuppressive response to inflammation. It is therefore of note that during neoplastic transformation, the recruitment of macrophages in numerous cancer types has been shown to create an environment permissive for tumor cell growth and migration. Thus, we would predict that, by restricting the ability of macrophages to infiltrate solid tumors, we might observe delayed tumor progression. Studies are currently

underway to address the role of FAK in macrophages during tumor initiation and metastasis (some of which are detailed in Chapter 4). Through the use of control and conditional FAK knockout mice, we will be able to assess the extent of macrophage recruitment to sites of tumor growth in each mouse genotype. Studies examining the tumor microenvironment will also provide a better understanding of the cytokines, chemokines and growth factors produced, and how these agents affect macrophage interactions with tumor cells. While the role of FAK in cell migration cannot be overstated, the possibility exists that FAK regulates, either directly or indirectly, processes unrelated to motility. For example, it is unclear whether the loss of FAK affects the ability of macrophages to polarize and/or adopt the M1 "classically activated" or M2 "alternatively activated" phenotype. M1 macrophages are characterized by the increased production of pro-inflammatory cytokines, increased antigen presentation and increased tumor-killing and microbicidal activity, whereas M2 cells are primarily involved in tissue repair and are generally thought of as tumor-promoting. By treating cells with either IFNγ to initiate the M1 phenotype, or IL-4 to induce M2 activation, we will be able to determine (i) if FAK-deficient macrophages are able to polarize, (ii) whether these cells preferentially acquire one phenotype over another, and (iii) whether polarization affects tumor progression *in vivo*.

Based on the results presented in this thesis, we would also predict that because FAK is involved in multiple immune functions, mice containing FAK-deficient macrophages are more likely to exhibit compromised clearance of bacterial infections. Using the Yersinia adhesin mutants in a model of systemic infection, we have previously attempted to examine bacterial colonization of the liver, spleen and lungs of control and

conditional FAK knockout mice. However, experiments testing this hypothesis have failed to definitively determine whether the loss of FAK from macrophages actually affects the dissemination of Yersinia to multiple organs. The difficulties encountered while performing these experiments have arisen because levels of bacterial colonization may be dependent on both tissue-specific microenvironments as well as the bacterial factors that regulate interactions with host phagocytes. Characterization of immune cell infiltration may prove to be a better indicator of clinical outcome. Microabscesses have been shown to form in the livers of mice infected with *Y. pseudotuberculosis*, and the resolution of these lesions is dependent upon invasin and YadA expression (Hudson and Bouton, 2006). If our hypothesis is correct, abscesses are likely to persist longer in the livers of conditional FAK knockout mice, and these lesions would be associated with fewer infiltrating monocytes. We would also be in position to examine the infiltration of other cell types, such as neutrophils and dendritic cells, to Yersinia-induced lesions to identify any defects in the recruitment of these immune cells.

The issues addressed here underscore the dichotomy between cancer prevention on one hand, and the need to mount an effective immune response against invading pathogens on the other. Studies performed in this thesis and elsewhere suggest the possibility of targeting macrophage function as a means of treating cancer in the future. However, tipping the balance too far in favor of suppressing macrophage activity may ultimately have a deleterious affect with respect to our ability to fight disease.

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