The motility of blood monocytes is orchestrated by the activity of cell-surface integrins, which translate extracellular signals into cytoskeletal changes to mediate adhesion and migration. *Toxoplasma gondii* is an intracellular parasite that infects migratory cells and enhances their motility, but the mechanisms underlying *T. gondii*–induced hypermotility are incompletely understood. We investigated the molecular basis for the hypermotility of primary human peripheral blood monocytes and THP-1 cells infected with *T. gondii*. Compared with uninfected monocytes, *T. gondii* infection of monocytes reduced cell spreading and the number of activated β1 integrin clusters in contact with fibronectin during settling, an effect not observed in monocytes treated with lipopolysaccharide (LPS) or *Escherichia coli*. Furthermore, *T. gondii* infection disrupted the phosphorylation of focal adhesion kinase (FAK) at tyrosine 397 (Tyr-397) and Tyr-925 and of the related protein proline-rich tyrosine kinase (Pyk2) at Tyr-402. The localization of paxillin, FAK, and vinculin to focal adhesions and the colocalization of these proteins with activated β1 integrins were also impaired in *T. gondii*–infected monocytes. Using time-lapse confocal microscopy of THP-1 cells expressing enhanced GFP (eGFP)–FAK during settling on fibronectin, we found that *T. gondii*–induced monocyte hypermotility was characterized by a reduced number of enhanced GFP-FAK–containing clusters over time compared with uninfected cells. This study demonstrates an integrin conformation–independent regulation of the β1 integrin adhesion pathway, providing further insight into the molecular mechanism of *T. gondii*–induced monocyte hypermotility.

Monocytes are migratory cells that circulate in the blood and home to sites of infection or injury by extravasating from the bloodstream into tissue (1). This process involves monocyte traversal of the vascular endothelium and the underlying basement membrane and extracellular matrix (ECM) (2). Cell adhesion and migration are mediated by the activity of cell surface adhesion molecules called integrins, which link extracellular signals to intracellular changes in the actin cytoskeleton (2).

Integrin signaling is initiated by the binding of integrins to ECM or cellular ligands and results in the coordinated formation and disassembly of focal adhesions, the connections that anchor a cell to its substrate. Integrins are noncovalently associated α/β heterodimeric glycoproteins, and each subunit is usually composed of a short, intracellular tail domain, a membrane-spanning helix, and a large ectodomain (3). Inactive integrins are expressed on the cell surface in a low-affinity, bent structure, but can undergo conformational changes to expose the extracellular ligand-binding site upon activation based on talin interactions within the tail domain of the β subunit of the integrin (4, 5). Monocytes regulate this inside-out signaling through the activity of chemokine-activated G protein–coupled receptors (6). Ligand binding to the activated integrins induces receptor clustering and triggers an intracellular signaling cascade. During β1 integrin interactions with fibronectin, integrin clustering recruits structural and signaling proteins to form the focal adhesion complex, which connects integrins to the actin cytoskeleton, thereby establishing a focal adhesion (7).

One component of the focal adhesome is the ubiquitously expressed cytoplasmic tyrosine kinase focal adhesion kinase (FAK) (8–10). As demonstrated by its many roles in embryonic development, cancer progression, and anti-apoptotic pathways (11–12), FAK is a critical signaling protein, consisting of three major domains: a FERM (4.1, ezrin, radixin, moesin) domain, kinase domain, and focal adhesion targeting (FAT) domain. FAK is also a structural protein composed of two proline-rich regions, and it can bind to nascent adhesome components, such as talin (13) and paxillin (14), as well as actin restructuring proteins, such as Arp2/3 (15) and RACK1 (16). In response to β1 integrin clustering, FAK is autophosphorylated at tyrosine residue 397 (p-FAK Tyr-397) to initiate binding with Src via its SH2 and SH3 domains (17). The formation of the FAK-Src complex results in additional phosphorylation of both proteins, leading to maximal downstream effects on actin cytoskeleton.

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1 The abbreviations used are: ECM, extracellular matrix; FAK, focal adhesion kinase; SH, Src homology; Pyk2, proline-rich tyrosine kinase; DCs, dendritic cells; PE, phycoerythrin.
remodeling (18). FAK null cells are characterized by a defect in focal adhesion disassembly and motility (19).

Monocytes also express a related tyrosine kinase called proline-rich tyrosine kinase (Pyk2, also known as CADTK, RAFTK, CAKβ), which is 45% identical and 66% similar to FAK (20). The two proteins have a similar domain structure and the same relative positioning of their phosphorylation sites (21). Interestingly, alternative splicing of Pyk2 in monocytes results in an isoform that lacks 42 amino acids between the proline-rich regions of the C terminus, resulting in a protein that is 5 kDa smaller (20). The monocyte isoform of Pyk2 localizes to lamellipodia, and disrupting its activity reduces monocyte cell spreading and motility (22). Notably, targeting Pyk2 to focal contacts can rescue the motility of cells deficient in FAK (23).

Toxoplasma gondii is an obligate intracellular parasite capable of infecting and replicating within nucleated cells of warm-blooded animals, and infection of humans can cause severe tissue damage in organs such as the brain and eye (24). Monocytes are recruited to sites of T. gondii infection where they can phagocytose and degrade the parasite or become infected themselves (25). One proposed mechanism for T. gondii dissemination within an infected host is through parasite invasion of migratory leukocytes, such as monocytes or dendritic cells (DCs). In this model, an infected cell can act as a Trojan horse for T. gondii in the bloodstream or tissues (26). Several studies have demonstrated that T. gondii infection of monocytes (27, 28), neutrophils (29), natural killer (NK) cells (30), and DCs (31–34) induces a hypermotility phenotype in these cells.

We have previously reported that T. gondii–infected monocytes exhibit an impairment of integrin clustering, and in conditions of shear stress, infected cells roll and crawl on vascular endothelium at higher velocities and over greater distances than uninfected monocytes (27, 28). In the current study, we have investigated the downstream consequences of reduced β1 integrin clustering on the activation of FAK and Pyk2 and in focal adhesion formation in T. gondii–infected monocytes. We found that infected monocytes formed fewer paxillin-, FAK-, and vinculin-containing focal adhesions and had reduced phosphorylation of FAK Tyr-397 and Tyr-925 and Pyk2 Tyr-402 than did uninfected monocytes during adhesion to fibronectin. Additionally, enhanced GFP (eGFP)–FAK–expressing monocytes infected with T. gondii exhibited decreased colocalization of eGFP-FAK and β1 integrins compared with uninfected cells. Furthermore, real-time imaging of human monocytes during cell settling revealed increased motility and fewer eGFP-FAK clusters in infected cells compared with uninfected cells. These findings indicate that the hypermotility of T. gondii–infected monocytes is associated with an impairment in the extent of focal adhesion formation downstream of β1 integrin signaling.

**Results**

**Clustering of activated β1 integrins is reduced in T. gondii–infected monocytes settled on fibronectin**

The conformational activation of integrins allows for high-affinity interactions with extracellular ligand to mediate cell crawling and arrest (35). We investigated the effect of T. gondii infection on the ability of activated β1 integrins to cluster, the initial step in focal adhesion formation. THP-1 monocytes were mock infected with media alone or infected with GFP-expressing Type II T. gondii for 4 h and settled on fibronectin for 30 min. The cells were imaged at the plane of contact with the fibronectin after staining with a monoclonal antibody that specifically recognizes the activated conformation of β1 integrins (36). Mock-infected cells formed clusters of activated β1 integrins, resembling focal adhesion structures, and spread over the surface of the fibronectin (Fig. 1A). Strikingly, there was a dramatic reduction in the clustering of activated β1 integrins in T. gondii–infected cells (Fig. 1, A and C). An analysis of total αβ1 integrin (VLA-4) or activated β1 integrin on the surface of monocytes by flow cytometry (Fig. 1E) revealed no differences in infected or uninfected cells, indicating that T. gondii caused a reduction in activated integrin clustering to ligand without affecting integrin cell surface expression.

To determine whether the disruption in β1 integrin clustering was specific to T. gondii infection or simply because of monocyte activation, we also examined β1 integrin clustering in THP-1 cells treated with lipopolysaccharide or exposed to *Escherichia coli* and settled onto fibronectin (Fig. 1B). In the T. gondii and *E. coli* conditions, the cells were imaged both at the cell base and in the z-plane at the cell center to permit visualization of the intracellular pathogen. In contrast to T. gondii infection, neither LPS nor *E. coli* impaired β1 integrin clustering, as cells in these conditions exhibited similar or higher numbers of β1 integrin clusters compared with mock-treated cells (Fig. 1C). Although differences in the surface area of β1 integrin clusters in each condition did meet statistical detection (Fig. 1D), the magnitude of these changes was small, suggesting that T. gondii infection predominantly affected the number of β1 integrin clusters in adherent cells, rather than the size of the clusters. The T. gondii–induced impairment in activated β1 integrin clustering was also observed when we examined total αβ1 integrin (Fig. 1F). Taken together, these data indicate that T. gondii dysregulation of β1 integrin clustering in infected monocytes is not a general feature of microbial stimulation.

**Focal adhesion signaling is disrupted downstream of β1 integrins**

To investigate the downstream effects of a decrease in β1 integrin clustering on the adhesome complex, we examined a key regulator of integrin signaling and focal adhesion formation, focal adhesion kinase. Freshly elutriated human peripheral blood monocytes were mock infected or infected with T. gondii for 4 h and either left unsettled or settled on fibronectin. Lysates from the cells were prepared after 15, 30, or 60 min post settling or from control unsettled cells, and Western blotting was performed for total FAK and p-FAK Tyr-397, the initial autophosphorylation event induced by β1 integrin clustering (Fig. 2A). In mock–treated cells settled on fibronectin, an increase in p-FAK Tyr-397 was observed as early as 15 min after settling and persisted at 30 min. In contrast, there was no comparable induction in the levels of p-FAK Tyr-397 in cells infected with T. gondii (Fig. 2B).

In addition to FAK, monocytes highly express the related kinase proline-rich tyrosine kinase 2 (Pyk2). By qPCR analysis,
transcripts for both PTK2 and PTK2B (the gene names of FAK and Pyk2, respectively) were detected in freshly elutriated monocytes (Fig. 2D). PTK2B transcripts were more abundantly expressed than PTK2 relative to GAPDH, consistent with previous reports that monocytes predominately express Pyk2 (20). Given the potential role for Pyk2 in focal adhesion formation in monocytes, we also investigated total Pyk2 and the phosphorylation of tyrosine residue 402 (p-Pyk2 Tyr-402) in infected pri-

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**Figure 1. Activated β1 integrin clustering in human monocytic cells.**

A. THP-1 cells were mock infected or infected with GFP-expressing *T. gondii* for 4 h, settled onto fibronectin-coated coverslips for 30 min, fixed, and stained with a mAb specific for the active (open and extended) conformation of β1 integrins and DAPI. Micrographs of mock-infected cells and cells harboring *T. gondii* were acquired at the cell base in contact with fibronectin. Representative images from five independent experiments are shown. B, THP-1 monocytic cells were mock treated or cultured with LPS, GFP-expressing *T. gondii* or CFSE-labeled *E. coli* for 4 h and settled onto fibronectin-coated coverslips. The cells were fixed and stained for the active conformation of β1 integrins and DAPI. Micrographs were acquired at the cell base and at the cell center in the *T. gondii* and *E. coli* conditions to permit visualization of the intracellular microbes (in green). Representative images from four independent experiments are shown. C and D, for all conditions in (B), the number (C) and area (D) of activated β1 integrin clusters per cell were calculated using the method published by Horzum et al. (58). n = 2000 randomly selected β1 integrin clusters from 50–84 cells in each condition. In all the box-and-whisker plots, the whiskers represent the 5th and 95th percentiles (i.e. not the standard deviation). **, p < 0.01; ***, p < 0.001; one-way ANOVA with a Bonferroni post hoc test. E, THP-1 cells were mock infected (black histograms) or infected with *T. gondii* (green histograms) for 4 h, stained with a control Ig (clg), anti-α4β1 (VLA-4) integrin mAb, or anti-activated β1 integrin mAb, and analyzed by flow cytometry. Representative histograms from three independent experiments are shown.

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Transcripts for both PTK2 and PTK2B (the gene names of FAK and Pyk2, respectively) were detected in freshly elutriated monocytes (Fig. 2D). PTK2B transcripts were more abundantly expressed than PTK2 relative to GAPDH, consistent with previous reports that monocytes predominately express Pyk2 (20). Given the potential role for Pyk2 in focal adhesion formation in monocytes, we also investigated total Pyk2 and the phosphorylation of tyrosine residue 402 (p-Pyk2 Tyr-402) in infected pri-
Western blotting using antibodies for total FAK, p-FAK Tyr-397, and (the gene names for FAK and Pyk2, respectively) relative to PTK2B (*, p display the mean bars and adhesion targeting (G) domain. FAT association with Src and signals for adhesome formation. The association results in Src phosphorylation of FAK at Tyr-925, located within the C-terminal focal simplified model of FAK phosphorylation and subsequent FAK-mediated signaling for cell adhesion is illustrated. Autophosphorylation at Tyr-397 induces FAK (T. gondii Prugniaud) LPS (Fig. 2), but this effect was not observed in monocytes treated with phosphorylation of Tyr-925 of FAK (38) (Fig. 2). Interest-T. gondii–infected cells. Impaired in T. gondii–infected monocytes downstream of β1 integrin activation.

FAK, paxillin, and vinculin localization to focal adhesions is impaired in T. gondii–infected monocytes

During the formation of nascent focal adhesions, FAK is recruited to the cytoplasmic tails of β1 integrins (39). To study the dynamics of FAK localization during the adhesion of T. gondii–infected cells, we used retroviral transduction to generate a line of THP-1 monocytic cells expressing an eGFP-FAK fusion protein (called eGFP-FAK THP-1 cells) (Fig. 3). Among the transduced cells, there was heterogeneity in eGFP-FAK expression. Single-cell clones were isolated using limiting dilution to create a homogeneous population of eGFP-FAK expressing cells (Fig. 3B). Lysates from uninfected or T. gondii–infected
Utilizing the eGFP-FAK THP-1 cells, we examined the recruitment of FAK to clusters of activated β1 integrins. The cells were mock infected or infected with Type II tdTomato-expressing *T. gondii* for 4 h, and settled on fibronectin for 15, 30, or 60 min. The cells were then fixed and stained for activated β1 integrins (Fig. 4A). The z-plane at the center of infected cells was also imaged to permit visualization of the intracellular parasites in the cytosol, as indicated by the arrows. The surface area of the infected cells was significantly reduced compared with uninfected cells at each time point examined, indicating a reduction in cell spreading over fibronectin because of infection (Fig. 4B and Table S1). By quantifying the area and number of eGFP-FAK adhesions per cell, we observed a significant decrease in the number of adhesions per cell in infected compared with the uninfected cells, suggesting that infection reduced the density of adhesions (Fig. 4C). Furthermore, the colocalization of eGFP-FAK and activated β1 integrins in infected and uninfected cells was calculated using Manders’ coefficient, in which a value closer to 1 indicates a greater degree of spatial overlap in signals (40). A reduced colocalization of eGFP-FAK and activated β1 integrins was observed in the *T. gondii*-infected cells compared with uninfected cells (Fig. 4C and Table S1). Finally, we also examined the localization of the focal adhesion components paxillin and vinculin, indicators of nascent and mature focal adhesions, respectively, in mock and *T. gondii*-infected THP-1 cells during settling (Fig. 5, A and B) and found that infection resulted in a significant reduction in the number of adhesions marked by either paxillin or vinculin and in the colocalization of both of these proteins with β1 integrin (Fig. 5C and Table S1). There was also a statistically detectable reduction in the area of adhesions marked by either paxillin or vinculin (Fig. 5C), although the magnitude of these changes was not large. Collectively, these data suggest that the decrease in p-FAK Tyr-397 and p-Pyk2 Tyr-402 in infected cells resulted from fewer β1 integrin clusters and was associated with an impairment of paxillin, FAK, and vinculin localization to focal adhesions.

**Live microscopy confirms impaired focal adhesion formation in *T. gondii*-infected cells**

To observe the organization of FAK during focal adhesion formation, the clonal line of eGFP-FAK THP-1 cells (Fig. 3B) was live imaged by time-lapse confocal microscopy during a settling period of 40 min on fibronectin (Fig. 6A and Videos S1 and S2). Uninfected monocytes spread over the fibronectin surface and formed structures resembling focal adhesions, which were visible as small regions of high eGFP-FAK density (Fig. 6A). Notably, these structures increased in mock-infected cells over time, particularly during the first 15 min of settling, after which point, they appeared to stabilize (Fig. 6B). In contrast, *T. gondii*-infected cells formed fewer aggregations of eGFP-FAK, with no net increase over time (Fig. 6B). Most notably, the *T. gondii*-infected cells changed shape rapidly as they roamed over the ECM (Fig. 6, A and C and Video S2) and moved at significantly higher speeds (Fig. 6D) than uninfected cells, consistent with the increased motility of *T. gondii*-infected monocytes (28) and the microscopy analyses of cells fixed at specific time points after settling on fibronectin (Fig. 4A).
Discussion

Integrins function as critical regulators of cell adhesion and motility in immune cells and are characterized by bidirectional signaling. They assume an open, extended “activated” conformation through inside-out signaling, and also transduce signals from extracellular ligands to intracellular pathways via outside-in signaling. Several studies have demonstrated that immune cells infected with *T. gondii* become hypermotile (27–34, 41), an effect that has been proposed to facilitate the dissemination of the intracellular parasite in the infected host. In the present study, we have demonstrated that the hypermotility of *T. gondii*–infected human monocytes is linked to a dysregulation in integrin-dependent cell adhesion through defects in FAK-regulated focal adhesions. Notably, *T. gondii* infection did not affect the expression of integrins on the cell surface (Fig. 1E) or the ability of integrins to become activated through inside-out signaling (27). Rather, *T. gondii* impeded outside-in signaling and integrin clustering upon ligand engagement. These findings are consistent with the hypothesis that the level of activated integrins expressed on the cell surface does not necessarily predict the adhesive properties of the cell (42). Interestingly, although integrin clustering is impaired in *T. gondii*–infected monocytes, these cells, nonetheless, require functional integrins for crawling on cellular substrates, as blocking integrins or their ligands with neutralizing antibodies prevents *T. gondii*–induced hypermotility (28). Taken together, these findings indicate that the parasite modulates the activity of integrins on infected monocytes, rather than blocking integrin function completely.

The *T. gondii*–induced disruption in integrin-mediated adhesion is linked to a dysregulation of components of the focal adhesion complex. In response to extracellular ligand binding, focal adhesion proteins are recruited to the complex in a hierarchical manner. FAK is a key regulator of the assembly and disassembly of focal adhesions and functions as both a signaling kinase and a scaffolding protein in the focal adhesome. In the autoinhibited state, the FERM domain of FAK structurally inhibits the kinase domain, and it is the displacement of the FERM domain that allows for the initiation of the kinase activity of FAK and autophosphorylation of Tyr-397 (43). FAK then

![Figure 4. Localization of eGFP-FAK and activated β1 integrins during cell adhesion.](image-url)

A

Mock

T. gondii

B

C

Toxoplasma gondii dysregulates β1 integrin signaling


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recruits talin (13), which may function as the architectural framework for the adhesome machinery (44, 45). The knockout of FAK in mice results in embryonic lethality because of a defect in mesoderm development, and interestingly, cells from these embryos have reduced motility, indicating a role for FAK in focal adhesion disassembly (19). In FAK-null cells, key adhesome proteins such as paxillin, talin, and vinculin localize to the cell periphery more slowly, resulting in a delay in actin restructuring. In addition, focal adhesion turnover is severely reduced (46). Based on the reduced mobility of FAK knock-out mouse embryonic fibroblasts, it is perhaps surprising that *T. gondii*–induced hypermotility in monocytes is associated with a reduction in FAK phosphorylation. However, it has also been shown that FAK knockdown leads to increased motility of cancer cells and fibroblasts (47), suggesting that FAK regulation of motility may also be dependent on the expression and function of other components of the focal adhesome. Indeed, Pyk2 expression increases in FAK knock-out mouse embryonic fibroblasts (48), endothelial cells (49), and mammary tumor cells (50), and Pyk2 can functionally substitute for FAK in these cells, supporting a compensatory function of Pyk2 in the motility of FAK-deficient cells. In our studies, we have observed a reduction in 1) p-FAK Tyr-397 and p-Pyk2 Tyr-402; 2) FAK, paxillin, and vinculin recruitment to focal adhesions; and 3) the total number of focal adhesions formed in *T. gondii*–infected monocytes during settling. Although additional mechanisms aside from FAK and Pyk2 activation may contribute to parasite-induced hypermotility, our data suggest that *T. gondii* impairs focal adhesion formation. We, therefore, favor the hypothesis that the parasite-induced dysregulation occurs after integrin binding/activation and before FAK phosphorylation in the signaling pathway connecting fibronectin to the actin cytoskeleton.

The conformational activation of integrins has been heavily studied, as it is a primary mechanism of regulating integrin function. However, the regulation of outside-in signaling through integrin clustering remains less well understood. Kindlins, although structurally similar to talin, occupy nonredundant roles in integrin-mediated signaling and recycling, and bind to β integrins at a separate NPXY motif (42). Kindlin-2, although not required for α5β1-mediated adhesion of endothelial cells (42), facilitates integrin αIIbβ3 clustering in Chinese hamster ovary cells during binding to fibrinogen (51). Additionally, the interactions between kindlin-2 and integrin-linked kinase (ILK) are necessary for outside-in αIIbβ3 integrin signaling and cell adhesion, although mutations preventing this binding do not affect integrin activation (52). It is possible that *T. gondii* disrupts kindlin function in infected monocytes, dysregulating β1 integrin outside-in signaling, but not inside-out signaling. As a result, monocyte-sustained adhesion is reduced, yet the cells maintain the ability to migrate and cross endothelial barriers. Of course, further studies examining the localization, activation, and binding interactions of kindlins are necessary to elucidate their role in *T. gondii* infection–induced hypermotility.

*T. gondii* is known to manipulate host cells via proteins secreted into the cell during invasion or released when the parasite is harbored within the specialized parasitophorous vacuole (53). Recently, it was found that *T. gondii* secretes a 14-3-3 protein (Tg14-3-3) into the parasitophorous vacuole and sequesters host 14-3-3 (54), a family of adapter proteins.
involved in numerous signaling pathways including adhesion. The introduction of purified Tg14-3-3 into DCs or the expression of Tg14-3-3 in DCs is sufficient to induce hypermotility (54). In addition, a peptide from the *Toxoplasma gondii* dense granule protein GRA5 increases the CCR7-mediated chemotaxis of DCs (55). There is also evidence that soluble *Toxoplasma gondii* proteins can affect immune cell chemotaxis, as cyclophilin-18 (C-18), a soluble protein secreted by *Toxoplasma gondii*, recruits DCs (56) in a CCR5-dependent manner.

The current report demonstrates a disruption to FAK and Pyk2 activation in *T. gondii*–infected monocytes, which may underlie monocyte hypermotility. These findings provide another example of *T. gondii* manipulation of cellular adhesion, interestingly, by impairing integrin-mediated clustering without affecting integrin expression or conformational activation. Because FAK, through its various binding partners and protein substrates, lies at the junction of many intracellular signaling pathways, these findings also suggest that *T. gondii* dysregulation of FAK activation may have broader implications for parasite manipulation of host cell signaling platforms.

**Experimental procedures**

**Mammalian and parasite cell culture and infections**

THP-1 monocytic cells were grown in RPMI 1640 medium (GE Healthcare) supplemented with 10% heat-inactivated fetal
Toxoplasma gondii dysregulates β1 integrin signaling

bovine serum (FBS) (Omega Scientific, Tarzana, CA), 2 mm l-glutamine, 100 units ml⁻¹ penicillin, and 100 μg ml⁻¹ streptomycin (R-10%). Primary monocytes were isolated from peripheral blood mononuclear cells (PBMC) using counterflow elutriation, as described previously (28). Isolated primary monocytes were resuspended in R-10% and used immediately. Blood was collected by the University of California, Irvine Institute for Clinical and Translational Science in accordance with guidelines and approval of the University of California, Irvine Institutional Review Board.

Type II Prugniaud parasites expressing tdTomato or GFP and Type I RH parasites expressing GFP were grown in human foreskin fibroblasts (HFFs) maintained in Dulbecco’s modified Eagle medium (DMEM) (GE Healthcare) with 10% heat-inactivated FBS, 2 mm l-glutamine, 100 units ml⁻¹ penicillin, and 100 μg ml⁻¹ streptomycin (D-10%), as previously described (57). For infection experiments of THP-1 or primary monocytes, infected human foreskin fibroblasts were syringe lysed and washed once with D-10%. The lysate was filtered through a 5-μm low protein binding Durapore membrane (EMD Millipore), washed with D-10%, and resuspended in R-10%. Parasites were added to the monocytes at a multiplicity of infection (m.o.i.) of 2 to 3 and incubated at 37 °C for 3 to 4 h before the cells were used in settling assays. Mock-infected cells are those in which the same volume of R-10% media that was used for the parasite infections was added to the monocytes in place of parasites. All parasite and mammalian cell cultures were tested monthly for Mycoplasma contamination and confirmed to be negative.

Retroviral transduction
eGFP-FAK-expressing THP-1 cells were generated using retroviral transduction. The pMXs-puro-eGFP-FAK plasmid, deposited by Noboru Mizushima (University of Tokyo, Japan), was purchased from Addgene (Cambridge, MA). 293T Phoenix cells were transfected with the plasmid using Lipofectamine 2000 (Thermo Fisher Scientific) to produce replication-incompetent retrovirus. The pMXs-puro-eGFP-FAK plasmid, deposited by Noboru Mizushima (University of Tokyo, Japan), was purchased from Addgene (Cambridge, MA). 293T Phoenix cells were transfected with the plasmid using Lipofectamine 2000 (Thermo Fisher Scientific) to produce replication-incompetent retrovirus. THP-1 cells were infected with retrovirus by centrifugation at 2500 rpm for 3 h at 25 °C. Three days post transduction, the cells were selected in R-10% with 2 μg ml⁻¹ puromycin. Single-cell cloning by serial dilution produced a line of THP-1 with uniform levels of eGFP-FAK expression. eGFP-FAK expression of clonal isolates was confirmed by flow cytometry using a BD FACSCalibur analyzer (BD Biosciences), and the data were analyzed by FlowJo software (FlowJo, Ashland, OR).

E. coli and LPS treatment
E. coli were resuspended in the cytoplasmic dye carboxyfluorescein succinimidyl ester (CFSE) (Thermo Fisher Scientific) and washed with PBS before addition to THP-1 cells. Where indicated, THP-1 cells were treated with 100 μg ml⁻¹ of LPS in R-10%.

Immunofluorescence microscopy and adhesion analysis
To investigate cell settling, 3 × 10⁵ uninfected or T. gondii–infected monocytes were settled onto fibronectin-coated glass coverslips for 15, 30, or 60 min at 37 °C. The samples were fixed with 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA), blocked with PBS and 5% normal goat serum (SouthernBiotech, Birmingham, AL), permeabilized with 0.01% saponin (Sigma-Aldrich), and probed with antibodies against activated β1 integrins (12G10, EMD Millipore) (36), VLA-4 (9F10, BioLegend, San Diego, CA), paxillin (mAb ab32084, Abcam, Cambridge, MA), or vinculin (pAb ab73412, Abcam). Alexa Fluor 488 conjugated goat anti-rabbit IgG, Alexa Fluor 594 conjugated goat anti-mouse IgG, or Alexa Fluor 647 conjugated goat anti-mouse IgG (Thermo Fisher Scientific) were used as secondary antibodies. The coverslips were mounted to slides using VectaShield with DAPI (Vector Laboratories, Burlingame, CA).

The cells were imaged using either a Nikon Eclipse Ti inverted microscope with a CFI Plan Apo VC 60× oil immersion objective with a 1.4 numerical aperture (Nikon Instruments Inc., Melville, NY) or a Leica TCS SP8 confocal microscope with a HC PL Apochromat 63× oil immersion objective with a 1.4 numerical aperture (Leica Microsystems GmbH, Menarini, Germany). Surface area and Manders’ coefficient were computed by a self-designed MATLAB (The MathWorks, Inc., Natick, MA) program: github.com/LodoenLab/FAK-Paper. The Manders’ coefficient was determined using the algorithm described by Manders et al. (40). The identification and measurement of adhesions in microscopy images was conducted using ImageJ (National Institutes of Health, Bethesda, MD) following the algorithm published by Horzum et al. (58).

The algorithm was modified to optimize adhesion identification in our microscopy images: the Laplacian of Gaussian filter was not used, the rolling ball radius for background subtraction was set at 10 pixels, and the minimum size for particle detection was set at 0.15 μm. The algorithm can be found here: github.com/LodoenLab/FAK-Paper. Graphs were generated using Prism (GraphPad Software, Inc., La Jolla, CA).

Flow cytometry
Mock and T. gondii–infected THP-1 cells were resuspended in fluorescence-activated cell sorting (FACS) buffer (PBS with 2% FBS) containing the Fc receptor blocking solution, Human TrueStain FcX (BioLegend, San Diego, CA). The cells were incubated on ice for 10 min then resuspended in a control IgG-phycocerythrin (PE) (MOPC-21, BioLegend), anti-α4β1 integrin-PE antibody (9F10, BioLegend), unconjugated control IgG (MOPC-21), or anti-activated β1 integrin antibody (12G10, EMD Millipore). The cells were incubated for 30 min on ice, and excess antibody was removed by washing with FACS buffer. The cells stained with the unconjugated control IgG or the anti-activated β1 integrin antibodies were incubated with PE goat anti-mouse IgG antibodies for 15 min on ice. The cells were acquired by flow cytometry (BD FACSCalibur, BD Biosciences), and the data were analyzed using FlowJo software (FlowJo).

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Statistics

One-way or two-way analysis of variance (ANOVA) or Student’s t-test were used for statistical analysis, as indicated in the figure legends. For the one-way ANOVA, a Bonferroni post hoc test was used. For all the box-and-whisker plots, the boxes represent the first and third quartiles, the band represents the median, and the whiskers represent the 5th and 95th percentiles.

Western blotting and densitometry

Cell lysates were prepared with 2× Laemmli buffer and 10% β-mercaptoethanol, and 2 × 10⁵ cell equivalents for each sample were loaded and separated on 10% SDS-PAGE. Gels were transferred to polyvinylidene difluoride (PVDF) (Bio-Rad, Hercules, CA) and probed using antibodies for FAK (D2R2E), phospho-FAK Tyr-397 (D20B1), or polyclonal antibodies against phospho-FAK Tyr-925, Pyk2 (H364), phospho-Pyk2 Tyr-402 from Cell Signaling Technology (Danvers, MA); vinculin (7F9) or talin (TA205) from EMD Millipore; or β-actin (AC-15, Sigma Aldrich). The membranes were then probed with HRP-conjugated anti-mouse or anti-rabbit IgG (Jackson Immuno-Research, West Grove, PA) and visualized using enhanced chemiluminescence (GE Healthcare Life Sciences). Images were captured by a Nikon camera as described previously (27). Band intensity was quantified using ImageJ Gel tools (National Institutes of Health, Bethesda, MD).

Time-lapse videomicroscopy

Live imaging was performed on a Zeiss Laser Scanning Microscope 780 (Zeiss, Oberkochen, Germany) using an incubation chamber maintained at 37 °C and 5% CO₂. Eight-chambered Lab-Tek no. 1.0 borosilicate cover glasses (Thermo Fisher Scientific). Relative gene expression was quantified using iQ SYBR Green Supermix (Bio-Rad, Hercules, CA), an iCycler PCR system (Bio-Rad), and the Strand Synthesis kit (Thermo Fisher Scientific). Relative gene expression was calculated using the 2ΔΔCt method (59), where the levels of expression have been normalized to levels of the housekeeping gene GAPDH. The primers used for GAPDH have been previously described (60). The PTK2 and PTK2B (the gene names of FAK and Pyk2, respectively) primers were as follows: PTK2, 5′-GCT-GCAATCCCAACATCTT-3′ (sense) and 5′-TCCGCAATG-GTTAGGGATG-3′ (antisense); PTK2B, 5′-TGTGAAGCTG-GGGGGATTTG-3′ (sense) and 5′-AGGATCTCCACATGCACAC-3′ (antisense). All primers were designed such that the amplicon spanned an intron/exon boundary. All PCR was performed in triplicate and with the following negative controls: samples without SuperScript III reverse transcriptase or with water in the place of a DNA template, and no signal was observed in these samples.

Author contributions—M. B. L. conceived and coordinated the research and supervised the project. N. U. and J. H. C. designed, performed, and analyzed the experiments. J. H. C. and M. B. L. wrote the manuscript. All authors reviewed the results and approved the final version of the manuscript.

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