Toxoplasma gondii disrupts β1 integrin signaling and focal adhesion formation during monocyte hypermotility

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ABSTRACT

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 have investigated the molecular basis for the hypermotility of primary human peripheral blood monocytes and THP-1 cells infected with T. gondii. Compared to 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 LPS or E. coli. Furthermore, T. gondii infection disrupted the phosphorylation of focal adhesion kinase (FAK) at tyrosine 397 (Y397) and Y925 and of the related protein proline-rich tyrosine kinase (Pyk2) at Y402. 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 eGFP-FAK during settling on fibronectin, we found that T. gondii-induced monocyte hypermotility was characterized by a reduced number of eGFP-FAK-containing clusters over time compared to 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.

INTRODUCTION

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). 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 non-covalently 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 (11–13), 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 Y397) 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 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), 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 Y397 and Y925 and Pyk2 Y402 than uninfected monocytes during adhesion to fibronectin. Additionally, eGFP-FAK-
expressing monocytes infected with *T. gondii* exhibited decreased colocalization of eGFP-FAK and β1 integrins compared to uninfected cells. Furthermore, real-time imaging of human monocytes during cell settling revealed increased motility and fewer eGFP-FAK clusters in infected cells compared to 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 monocytic cells were mock-infected with media alone or infected with green fluorescent protein (GFP)-expressing Type II *T. gondii* for 4 hours (hr) and settled on fibronectin for 30 minutes (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. 1A and C). An analysis of total α4β1 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 without affecting integrin cell surface expression.

To determine if the disruption in β1 integrin clustering was specific to *T. gondii* infection or simply due to monocyte activation, we also examined β1 integrin clustering in THP-1 cells treated with lipopolysaccharide (LPS) or exposed to *E. 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 to 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 α4β1 integrin (VLA-4, Fig. S1). 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 (FAK). Freshly elutriated human peripheral blood monocytes were mock-infected or infected with *T. gondii* for 4 hr 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 Y397, the initial autophosphorylation event downstream of β1 integrin signaling (Fig. 2A). In mock-treated cells settled on fibronectin, an increase in p-FAK Y397 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 Y397 in cells infected with *T. gondii* (Fig. 2B).

In addition to FAK, monocytes highly express the related tyrosine 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 Y402) in infected primary monocytes settled on fibronectin, in parallel with tracking total FAK and p-FAK Y397 (Fig. 2A and C). Y402 of Pyk2 is functionally analogous to Y397 of FAK (37). Notably, the reduced detection of p-Pyk Y402 recapitulated the reduced levels of p-FAK Y397 in *T. gondii*-infected cells. Interestingly, in two out of three blood donors, we detected three bands with the antibody against p-Pyk2 Y402, which may reflect detection of the previously reported splice variants of Pyk2 (20).

Infection of monocytes with either Type I (RH) or Type II (Prugniaud) *T. gondii* resulted in reduced levels of p-FAK Y397, but this effect was not observed in monocytes treated with LPS (Fig 2E), indicating a specific effect of *T. gondii* infection.

The formation of focal adhesion structures and signaling for actin cytoskeletal rearrangements involves interactions between Src kinase and FAK, culminating in the Src-dependent phosphorylation of Y925 of FAK (38) (Fig. 2F). *T. gondii* infection also resulted in a significant reduction in the phosphorylation of Y925 of FAK (Fig. 2G and H), suggesting a defect in the formation of the FAK-Src complex in infected cells. Collectively, these data on the phosphorylation of Pyk2 and FAK demonstrate a disruption of focal adhesion signaling 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 monocyteic cells expressing an enhanced green fluorescent protein (eGFP)-FAK fusion protein (called eGFP-FAK THP-1 cells) (Fig. 3A). 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 eGFP-FAK THP-1 cells that were unsettled or settled on fibronectin were probed by Western blot for eGFP-FAK and for phosphorylated eGFP-FAK at Y397 (hereafter referred to as p-FAK Y397 in these cells), along with additional components of the adhesome (Fig. 3C). Although less dramatic than in cells expressing only endogenous FAK, *T. gondii* infection of the eGFP-FAK cells also resulted in lower levels of p-FAK Y397 compared to mock infection (Fig. 3C and D). The reduced magnitude of the effect of infection on p-FAK Y397 in the eGFP-FAK THP-1 cells may indicate that the parasite is less effective at inhibiting the phosphorylation of FAK when FAK is highly overexpressed, as in the eGFP-FAK cells (Fig. S2). The levels of the adhesome components talin and vinculin were unchanged in the mock and *T. gondii*-infected eGFP-FAK THP-1 cells (Fig. 3C).

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 hr, settled on fibronectin for 15, 30, or 60 min, 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 to uninfected cells at each time point examined, indicating a reduction in cell spreading over fibronectin due to 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 to the uninfected cells, suggesting reduced density of adhesions due to infection (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 one 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 to 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. 5A and B) and found that infection resulted in a significant reduction in the number of adhesions.
intracellular pathways via outside-in signaling. transduce signals from extracellular ligands to cells infected with Trendy. Several studies have demonstrated that immune conformation through inside-out signaling, and also assume an open, extended "activated" characterised by bidirectional signaling: they cell adhesion and motility in immune cells and are Integrins function as critical regulators of DISCUSSION facilitate the dissemination of the intracellular parasite in the infected host. In the present study, we have demonstrated that the hypermotility of Toxoplasma 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 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 auto-inhibited 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 Y397 (43). FAK then recruits talin (13) which may function as the architectural framework for the adhesome machinery (44, 45). The knock-out of FAK in mice results in embryonic lethality due to 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 MEFs,
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 knock-down leads to increased motility of cancer cells and fibroblasts (47), suggesting that FAK regulation of motility may be dependent on the expression and function of other components of the focal adhesome. Indeed, Pyk2 expression increases in FAK knock-out MEFs (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 Y397 and p-Pyk2 Y402, 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 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 activity. However, the regulation of outside-in signaling through integrin clustering remains less well understood. Kindlins, while structurally similar to talin, occupy non-redundant roles in integrin-mediated signaling and recycling, and bind to β integrins at a separate NPXY motif (42). Kindlin-2, though 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 are necessary for outside-in αIIbβ3 integrin signaling and cell adhesion, though 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 *T. gondii* dense granule protein GRA5 increases the CCR5-mediated chemotaxis of DCs (55). There is also evidence that soluble *T. gondii* proteins can affect immune cell chemotaxis, as cyclophilin-18 (C-18), a soluble protein secreted by *T. 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. Since 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 (GE Healthcare, Logan, UT) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Omega Scientific, Tarzana, CA), 2 mM L-glutamine, 100 U 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 previously described (28). Isolated primary monocytes were resuspended in R-10% and used immediately. Blood was collected by the University
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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, Logan, UT) with 10% heat-inactivated FBS, 2 mM L-glutamine, 100 U 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 HFFs were syringe lysed and washed once with D-10%. The lysate was filtered through a 5 µm Low Protein Binding Durapore® Membrane (EMD Millipore, Billerica, MA), washed with D-10%, and resuspended in R-10%. Parasites were added to the monocytes at a multiplicity of infection (MOI) of 2-3 and incubated at 37ºC for 3-4 hr 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 A cells were transfected with the plasmid using Lipofectamine LTX (Thermo Fisher Scientific, Carlsbad, CA) to produce replication-incompetent retrovirus. THP-1 cells were infected with retrovirus by centrifugation at 2500 rpm for 3 hr at 25ºC. 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, San Jose, CA), 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, Carlsbad, CA) 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 x 10⁵ uninfected or T. gondii-infected monocytes were settled onto fibronectin-coated glass coverslips for 15, 30, or 60 min at 37ºC. 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, St. Louis, MO), and probed with antibodies against activated β1 integrins (12G10, EMD Millipore, Temecula, CA) (36), VLA-4 (9F10, BioLegend, San Diego, CA), paxillin (mAb ab32084, Abcam, Cambridge, MA), or vinculin (pAb ab73412, Abcam, Cambridge, MA). 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, Carlsbad, CA) 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 60X 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 63X 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, which is available at the following URL: https://webfiles.uci.edu/xythoswfs/webview/_xy-5945316_1. 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 (NIH, Bethesda, MD) following the algorithm published by Horzum et al. (58). The algorithm was modified to optimize adhesion identification in our microscopy images:
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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. This modified ImageJ script is available here: https://webfiles.uci.edu/xythoswfs/webview/xy-5987011_1. 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% fetal bovine serum) containing the Fc receptor blocking solution, Human TruStain FcX (Biolegend, San Diego, CA). The cells were incubated on ice for 10 min then resuspended in a control IgG-PE (MOPC-21, Biolegend, San Diego, CA), anti-α4β1 integrin-PE antibody (9F10, Biolegend, San Diego, CA), unconjugated control IgG (MOPC-21), or anti-activated β1 integrin antibody (12G10, EMD Millipore, Temecula, CA). 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, San Jose, CA), and the data were analyzed using FlowJo software (FlowJo, Ashland, OR).

Statistics
One-way or two-way 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-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 2x Laemmli buffer and 10% β-mercaptoethanol, and 2 x 10^5 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 Y397 (D20B1) or polyclonal antibodies against phospho-FAK Y925, Pyk2 (H364), phospho-Pyk2 Y402 from Cell Signaling Technology (Danvers, MA), vinculin (7F9) or talin (TA205) from EMD Millipore (Billeric, MA), or β-actin (AC-15, Sigma Aldrich, St. Louis, MO). 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, Pittsburgh, PA). Images were captured by a Nikon camera as previously described (27). Band intensity was quantified using ImageJ Gel tools (NIH, 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 #1.0 Borosilicate Coverglasses (Thermo Fisher Scientific, Waltham, MA) were coated with fibronectin for 1 hour prior to use. Images were captured as z-stacks 0.5 μm apart starting from just below the cell base to the top of the cell at a frequency of 1 min⁻¹.

qPCR
RNA was extracted using the RNasy Minikit (Qiagen, Germantown, MD) and treated with DNase I (Thermo Fisher Scientific, Carlsbad, CA). cDNA was synthesized using SuperScript III First-Strand synthesis kit (Thermo Fisher Scientific, Carlsbad, CA). Relative gene expression was quantified using iQ SYBR Green Supermix (Bio-Rad, Hercules, CA), an iCycler PCR system (Bio-Rad, Hercules, CA), and the cycle threshold (CT) method (59), where the levels of expression have been normalized to levels of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (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’-GCTGCAATCCCACACATCTT-3’ (sense) and 5’-TCCGCAATGGTTAGGGATGG-3’ (antisense), PTK2B: 5’-TGTGAAGCTGGGGGACTTTG-3’ (sense) and 5’-AGGATCTCCCACATGCACAC-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
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SuperScript III reverse transcriptase or with water in the place of a DNA template, and no signal was observed in these samples.

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CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest with the contents of this article.

AUTHOR CONTRIBUTIONS

MBL conceived and coordinated the research and supervised the project. NU and JHC designed, performed, and analyzed the experiments. JHC and MBL wrote the manuscript. All authors reviewed the results and approved the final version of the manuscript.
REFERENCES

1. Imhof, B. A., and Aurrand-Lions, M. (2004) Adhesion mechanisms regulating the migration of monocytes. *Nat. Rev. Immunol.* 4, 432–444

2. Ley, K., Laudanna, C., Cybulsky, M. I., and Nourshargh, S. (2007) Getting to the site of inflammation: the leukocyte adhesion cascade updated. *Nat. Rev. Immunol.* 7, 678–89

3. Ginsberg, M. H., Partridge, A., and Shattil, S. J. (2005) Integrin regulation. *Curr. Opin. Cell Biol.* 17, 509–516

4. Luo, B.-H., Carman, C. V, and Springer, T. a (2007) Structural basis of integrin regulation and signaling. *Annu. Rev. Immunol.* 25, 619–47

5. Shattil, S. J., Kim, C., and Ginsberg, M. H. (2010) The final steps of integrin activation: the end game. *Nat. Rev. Mol. Cell Biol.* 11, 288–300

6. Laudanna, C., Kim, J., Constantin, G., and Butcher, E. (2002) Rapid leukocyte integrin activation by chemokines. *Immunol. Rev.* 186, 37–46

7. Mitra, S. K., Hanson, D. A., and Schlaepfer, D. D. (2005) Focal adhesion kinase: in command and control of cell motility. *Nat. Rev. Mol. Cell Biol.* 6, 509–516

8. Schaller, M. D., Borgman, C. A., Cobb, B. S., Vines, R. R., Reynolds, A. B., and Parsons, J. T. (1992) pp125FAK a structurally distinctive protein-tyrosine kinase associated with focal adhesions. *Proc. Natl. Acad. Sci. U. S. A.* 89, 5192–6

9. Hanks, S. K., Calalb, M. B., Harper, M. C., and Patel, S. K. (1992) Focal adhesion protein-tyrosine kinase phosphorylated in response to cell attachment to fibronectin. *Proc. Natl. Acad. Sci. U. S. A.* 89, 8487–8491

10. Guan, J. L., and Shalloway, D. (1992) Regulation of focal adhesion-associated protein tyrosine kinase by both cellular adhesion and oncogenic transformation. *Nature.* 358, 690–692

11. Schlaepfer, D. D., Mitra, S. K., and Ilic, D. (2004) Control of motile and invasive cell phenotypes by focal adhesion kinase. *Biochim. Biophys. Acta - Mol. Cell Res.* 1692, 77–102

12. Sulzmaier, F. J., Jean, C., and Schlaepfer, D. D. (2014) FAK in cancer: mechanistic findings and clinical applications. *Nat. Rev. Cancer.* 14, 598–610

13. Lawson, C., Lim, S. T., Uryu, S., Chen, X. L., Calderwood, D. A., and Schlaepfer, D. D. (2012) FAK promotes recruitment of talin to nascent adhesions to control cell motility. *J. Cell Biol.* 196, 223–232

14. Turner, C. E. (2000) Paxillin interactions. *J Cell Sci.* 113 Pt 23, 4139–4140

15. Serrels, B., Serrels, A., Brunton, V. G., Holt, M., McLean, G. W., Gray, C. H., Jones, G. E., and Frame, M. C. (2007) Focal adhesion kinase controls actin assembly via a FERM-mediated interaction with the Arp2/3 complex. *Nat. Cell Biol.* 9, 1046–1056

16. Serrels, B., Sandilands, E., Serrels, A., Baillie, G., Houslay, M. D., Brunton, V. G., Canel, M., MacHesky, L. M., Anderson, K. I., and Frame, M. C. (2010) A complex between FAK, RACK1, and PDE4D5 controls spreading initiation and cancer cell polarity. *Curr. Biol.* 20, 1086–1092

17. Thomas, J. W., Ellis, B., Boerner, R. J., Knight, W. B., White, G. C., and Schaller, M. D. (1998) SH2- and SH3-mediated interactions between focal adhesion kinase and Src. *J. Biol. Chem.* 273, 577–83

18. Ruest, P. J., Roy, S., Shi, E., Mernaugh, R. L., and Hanks, S. K. (2000) Phosphospecific antibodies reveal focal adhesion kinase activation loop phosphorylation in nascent and mature focal adhesions and requirement for the autophosphorylation site. *Cell Growth Differ.* 11, 41–8

19. Ilić, D., Furuta, Y., Kanazawa, S., Takeda, N., Sobue, K., Nakatsuji, N., Nomura, S., Fujimoto, J., Okada, M., and Yamamoto, T. (1995) Reduced cell motility and enhanced focal adhesion contact formation in cells from FAK-deficient mice. *Nature.* 377, 539–544

20. Li, X., Hunter, D., Morris, J., Haskill, J. S., and Earp, H. S. (1998) A calcium-dependent tyrosine kinase splice variant in human monocytes. Activation by a two-stage process involving adherence and a subsequent intracellular signal. *J. Biol. Chem.* 273, 9361–4

21. Lev, S., Moreno, H., Martinez, R., Canoll, P., Peles, E., Musacchio, J. M., Plowman, G. D., Rudy,
Toxoplasma gondii dysregulates β1 integrin signaling

B., and Schlessinger, J. (1995) Protein tyrosine kinase PYK2 involved in Ca2+-induced regulation of ion channel and MAP kinase functions. *Nature*. **376**, 737–745

22. Watson, J. M., Harding, T. W., Golubovskaya, V., Morris, J. S., Hunter, D., Li, X., Haskell, J. S., and Earp, H. S. (2001) Inhibition of the Calcium-dependent Tyrosine Kinase (CADTK) Blocks Monocyte Spreading and Motility. *J. Biol. Chem.* **276**, 3536–3542

23. Klingbeil, C. K., Hauck, C. R., Hsia, D. A., Jones, K. C., Reider, S. R., and Schlaepfer, D. D. (2001) Targeting Pyk2 to beta 1-integrin-containing focal contacts rescues fibronectin-stimulated signaling and haptotactic motility defects of focal adhesion kinase-null cells. *J. Cell Biol.* **152**, 97–110

24. Montoya, J. G., and Remington, J. S. (2008) Clinical Practice: Management of Toxoplasma gondii Infection during Pregnancy. *Clin. Infect. Dis.* **47**, 554–566

25. Dunay, I. R., and Sibley, L. D. (2010) Monocytes mediate mucosal immunity to Toxoplasma gondii. *Curr. Opin. Immunol.* **22**, 461–466

26. Courret, N., Darche, S., Sonigo, P., Milon, G., Buzoni-Gâtel, D., and Tardieux, I. (2006) CD11c- and CD11b-expressing mouse leukocytes transport single Toxoplasma gondii tachyzoites to the brain. *Blood*. **107**, 309–316

27. Harker, K. S., Ueno, N., Wang, T., Bonhomme, C., Liu, W., and Lodoen, M. B. (2013) Toxoplasma gondii modulates the dynamics of human monocyte adhesion to vascular endothelium under fluidic shear stress. *J. Leukoc. Biol.* **93**, 789–800

28. Ueno, N., Harker, K. S., Clarke, E. V., McWhorter, F. Y., Liu, W. F., Tenner, A. J., and Lodoen, M. B. (2014) Real-time imaging of Toxoplasma-infected human monocytes under fluidic shear stress reveals rapid translocation of intracellular parasites across endothelial barriers. *Cell. Microbiol.* **16**, 580–595

29. Coombes, J. L., Charsar, B. A., Han, S. J., Halkias, J., Chan, S. W., Koshi, A. A., Striepen, B., and Robey, E. A. (2013) Motile invaded neutrophils in the small intestine of Toxoplasma gondii-infected mice reveal a potential mechanism for parasite spread. *Proc Natl Acad Sci U S A*. **110**, E1913-22

30. Ueno, N., Lodoen, M. B., Hickey, G. L., Robey, E. a, and Coombes, J. L. (2014) Toxoplasma gondii-infected natural killer cells display a hypermotility phenotype in vivo. *Immunol. Cell Biol.* **93**, 508–13

31. Lambert, H., Hitziger, N., Dellacasa, I., Svensson, M., and Barragan, A. (2006) Induction of dendritic cell migration upon Toxoplasma gondii infection potentiates parasite dissemination. *Cell. Microbiol.* **8**, 1611–1623

32. Weidner, J. M., Kanatani, S., Hernández-Castañeda, M. A., Fuks, J. M., Rethi, B., Wallin, R. P. A., and Barragan, A. (2013) Rapid cytoskeleton remodelling in dendritic cells following invasion by Toxoplasma gondii coincides with the onset of a hypermigratory phenotype. *Cell. Microbiol.* **15**, 1735–1752

33. Fuks, J. M., Arrighi, R. B. G., Weidner, J. M., Kumar Mendu, S., Jin, Z., Wallin, R. P. A., Rethi, B., Birnir, B., and Barragan, A. (2012) GABAergic Signaling Is Linked to a Hypermigratory Phenotype in Dendritic Cells Infected by Toxoplasma gondii. *PLoS Pathog.* 10.1371/journal.ppat.1003051

34. Kanatani, S., Uhlén, P., and Barragan, A. (2015) Infection by toxoplasma gondii induces amoeboid-like migration of dendritic cells in a three-dimensional collagen matrix. *PLoS One*. **10**, 1–16

35. Iwamoto, D. V, and Calderwood, D. A. (2015) Regulation of integrin-mediated adhesions. *Curr. Opin. Cell Biol.* **36**, 41–47

36. Su, Y., Xia, W., Li, J., Walz, T., Humphries, M. J., Vestweber, D., Cabañas, C., Lu, C., and Springer, T. A. (2016) Relating conformation to function in integrin α5β1. *Proc. Natl. Acad. Sci. U. S. A*. **113**, E3872-81

37. Schlaepfer, D. D., Hauck, C. R., and Sieg, D. J. (1999) Signaling through focal adhesion kinase. *Prog Biophys Mol Biol*. **71**, 435–478
Toxoplasma gondii dysregulates β1 integrin signaling

38. Schlaepfer, D. D., and Hunter, T. (1996) Evidence for in vivo phosphorylation of the Grb2 SH2-domain binding site on focal adhesion kinase by Src-family protein-tyrosine kinases. Mol. Cell. Biol. 16, 5623–33

39. Miyamoto, S., Teramoto, H., Coso, O. A., Gutkind, J. S., Burbelo, P. D., Akiyama, S. K., and Yamada, K. M. (1995) Integrin function: Molecular hierarchies of cytoskeletal and signaling molecules. J. Cell Biol. 131, 791–805

40. Manders, E. M. M., Verbeek, F. J., and Ate, J. A. (1993) Measurement of co-localisation of objects in dual-colour confocal images. J. Microsc. 169, 375–382

41. Lambert, H., and Barragan, A. (2010) Modelling parasite dissemination: Host cell subversion and immune evasion by Toxoplasma gondii. Cell. Microbiol. 12, 292–300

42. Margadant, C., Kreft, M., de Groot, D.-J., Norman, J. C., and Sonnenberg, A. (2012) Distinct roles of talin and kindlin in regulating integrin α5β1 function and trafficking. Curr. Biol. 22, 1554–63

43. Lietha, D., Cai, X., Ceccarelli, D. F. J., Li, Y., Schaller, M. D., and Eck, M. J. (2007) Structural Basis for the Autoinhibition of Focal Adhesion Kinase. Cell. 129, 1177–1187

44. Yano, H., Mazaki, Y., Kurokawa, K., Hanks, S. K., Matsuda, M., and Sabe, H. (2004) Roles played by a subset of integrin signaling molecules in cadherin-based cell-cell adhesion. J. Cell Biol. 166, 283–295

45. Fan, H., and Guan, J.-L. (2011) Compensatory function of Pyk2 protein in the promotion of focal adhesion kinase (FAK)-null mammary cancer stem cell tumorigenicity and metastatic activity. J. Biol. Chem. 286, 18573–82

46. Fukuda, K., Bledzka, K., Yang, J., Perera, H. D., Plow, E. F., and Qin, J. (2014) Molecular basis of kindlin-2 binding to integrin-linked kinase pseudokinase for regulating cell adhesion. J. Biol. Chem. 289, 28363–28375

47. Boothroyd, J. C., and Dubremetz, J.-F. (2008) Kiss and spit: the dual roles of Toxoplasma rhoptries. Nat. Rev. Microbiol. 6, 79–88

48. Weidner, J. M., Kanatani, S., Uchtenhagen, H., Varas-Godoy, M., Schulte, T., Engelberg, K., Gubbels, M. J., Sun, H. S., Harrison, R. E., Achour, A., and Barragan, A. (2016) Migratory activation of parasitized dendritic cells by the protozoan Toxoplasma gondii 14-3-3 protein. Cell. Microbiol. 18, 1537–1550
**Toxoplasma gondii dysregulates β1 integrin signaling**

Toxoplasma gondii triggers human dendritic cells’ migration. *J. Leukoc. Biol.* **92**, 1241–50

56. Aliberti, J., Valenzuela, J. G., Carruthers, V. B., Hieny, S., Andersen, J., Charest, H., Reis e Sousa, C., Fairlamb, A., Ribeiro, J. M., and Sher, A. (2003) Molecular mimicry of a CCR5 binding-domain in the microbial activation of dendritic cells. *Nat. Immunol.* **4**, 485–90

57. Morgado, P., Sudarshana, D. M., Gov, L., Harker, K. S., Lam, T., Casali, P., Boyle, J. P., and Lodoen, M. B. (2014) Type II Toxoplasma gondii induction of CD40 on infected macrophages enhances interleukin-12 responses. *Infect. Immun.* **82**, 4047–4055

58. Horzum, U., Ozdil, B., and Pesen-Okvur, D. (2014) Step-by-step quantitative analysis of focal adhesions. *MethodsX*. **1**, 56–9

59. Livak, K. J., and Schmittgen, T. D. (2001) Analysis of relative gene expression data using real-time quantitative PCR and. *Methods*. **25**, 402–408

60. Wong, B. C. K., Chan, K. C. A., Chan, A. T. C., Leung, S. F., Chan, L. Y. S., Chow, K. C. K., and Lo, Y. M. D. (2006) Reduced plasma RNA integrity in nasopharyngeal carcinoma patients. *Clin. Cancer Res.* **12**, 2512–2516
Footnote

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**Figure Legends**

**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 hr, 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 5 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 hr 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 4 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 hr, stained with a control Ig (cIg), anti-α4β1 (VLA-4) integrin mAb, or anti-activated β1 integrin mAb, and analyzed by flow cytometry. Representative histograms from 3 independent experiments are shown.

**Figure 2. Phosphorylation of FAK and Pyk2 in monocytes during cell adhesion.** A) Primary human monocytes were mock-infected or infected with *T. gondii* for 4 hr and either lysed in suspension or settled on fibronectin for 15, 30, or 60 min prior to lysis. Total lysates were separated by SDS-PAGE and examined by Western blotting for total FAK, p-FAK Y397, total Pyk2, p-Pyk2 Y402, and β-actin. Molecular weight (MW) markers are shown to the right of each blot. Representative blots from 3
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Independent experiments are shown. Densitometry was performed on Western blots of B) FAK and C) Pyk2 by normalizing band intensities to β-actin. p-FAK Y397 and p-Pyk2 Y402 levels were normalized to total FAK or total Pyk2 band intensities, respectively. Bars display the mean ± SD from 3 independent experiments (*p < 0.05, one-way ANOVA with a Bonferroni post-hoc test). D) Transcript levels of PTK2 and PTK2B (the gene names for FAK and Pyk2, respectively) relative to GAPDH were quantified by qPCR. RNA was isolated from freshly elutriated primary human monocytes (0 hr) or monocytes cultured for 4 hr in mock conditions or with T. gondii. Bars indicate the mean ± SD from 3 independent experiments. (*p < 0.05, **p < 0.01, one-way ANOVA with a Bonferroni post-hoc test). E) Primary human monocytes were mock treated or cultured with T. gondii of Type I (RH strain) or Type II (Prugniaud strain) lineage for 4 hr or treated with LPS. Monocytes were then settled onto immobilized fibronectin, lysed, and analyzed by Western blotting using antibodies for total FAK, p-FAK Y397, and β-actin. Representative blots from 3 independent experiments are shown. F) A simplified model of FAK phosphorylation and subsequent FAK-mediated signaling for cell adhesion is illustrated. Autophosphorylation at Y397 induces FAK association with Src and signals for adhesome formation. The association results in Src phosphorylation of FAK at Y925, located within the C-terminal focal adhesion targeting (FAT) domain. G) Primary human monocytes were mock infected or cultured with T. gondii for 4 hr, settled onto fibronectin for 30 min, lysed, and analyzed by Western blotting using antibodies for total FAK, p-FAK Y397, p-FAK Y925, and β-actin. Representative blots from 4 independent experiments are shown. H) Quantification of p-FAK Y925 levels relative to total FAK at 30 min post settling was performed by using densitometry of Western blots. Bars display the mean ± SD from 4 independent experiments. (*p < 0.05, Student’s t-test).

Figure 3. Generation of eGFP-FAK THP-1 cells. A) eGFP-FAK was expressed in THP-1 monocytic cells by retroviral transduction, and the cells were imaged by live cell fluorescence microscopy. B) The transduced cells were single-cell cloned, and the clonal population was compared to the parental THP-1
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cells by flow cytometry. Debris and dead cells were gated out based on a propidium iodide (PI) viability
stain, and eGFP expression of the THP-1 and eGFP-FAK THP-1 cells was represented as histograms. C)
eGFP-FAK THP-1 cells were mock infected or infected with T. gondii for 4 hr and left unsettled or
settled onto fibronectin for 30 min. Cells were lysed and analyzed by Western blotting using antibodies
for total eGFP-FAK, phosphorylated eGFP-FAK at Y397 (p-FAK Y397), talin, vinculin, and β-actin.
Representative blots from 4 independent experiments are shown. D) Densitometry was performed on
Western blots of p-FAK Y397 by normalizing band intensities to total FAK. Bars display the mean ± SD
from 4 independent experiments (*p < 0.05, Student’s t-test).

**Figure 4. Localization of eGFP-FAK and activated β1 integrins during cell adhesion.** eGFP-FAK
THP-1 cells were mock infected or infected with T. gondii for 4 hr and settled onto fibronectin-coated
coverslips. A) At the designated times, cells were fixed and stained with DAPI and for β1 integrins using
an active conformation-specific mAb. Confocal micrographs of mock-infected and T. gondii-infected
cells were acquired at the cell base, and micrographs of cells harboring T. gondii were acquired at the cell
base and cell center to visualize intracellular parasites (arrows). Representative images from 3
independent experiments are shown. B) The surface area of the cell base of mock and infected monocytes
was quantified. C) The area and number of adhesions containing eGFP-FAK per cell were measured
using previously described methods (58). The colocalization of eGFP-FAK with activated β1 integrin at
the cell base was measured using Manders coefficient. For (B) and (C), n = 600 randomly selected β1
integrin clusters from 61-112 cells in each condition. (**p < 0.001, one-way ANOVA with a Bonferroni
post-hoc test).

**Figure 5. Localization of paxillin and vinculin to activated β1 integrins during cell adhesion.** THP-1
cells were mock-infected or infected with T. gondii for 4 hr and settled onto fibronectin-coated coverslips.
At the designated times, cells were fixed and stained with DAPI and with antibodies for activated β1
Toxoplasma gondii dysregulates β1 integrin signaling integrin and either (A) paxillin or (B) vinculin. The cells were imaged by confocal microscopy at the plane of contact with fibronectin to permit visualization of the adhesions and the infected cells were also imaged at the middle of the cell to capture the intracellular parasites (arrows). Representative images from 4 independent experiments are shown. C) The area and number of adhesions containing paxillin or vinculin per cell were measured using previously described methods (58). The colocalization of paxillin and vinculin with activated β1 integrin at the cell base was measured using Manders coefficient. n = 600 randomly selected β1 integrin clusters from 55-84 cells in each condition (*p < 0.05, **p < 0.01, ***p < 0.001, one-way ANOVA with a Bonferroni post-hoc test).

Figure 6. Real-time microscopy of FAK recruitment to the contact interface during cell adhesion and motility. Uninfected or T. gondii-infected eGFP-FAK THP-1 cells were settled onto fibronectin-coated chamber slides and imaged on a confocal microscope in an environmental chamber maintained at 37ºC and 5% CO2. The cells were imaged as z-stacks for 40 min at a frequency of 1 stack per min. A) Images shown are five sequential frames summed together (t-projection) with the time stamp indicating the time-point of the middle frame. The arrow indicates an intracellular T. gondii. These filmstrips are representative of videos from 14 independent experiments. B) The number of adhesions formed by uninfected or T. gondii-infected eGFP-FAK THP-1 cells was measured over time using previously established methods (58). n = 13 cells in each condition (p < 0.001 for infection and p = 0.7950 for time, using a two-way ANOVA). C) Flower plots depict the path lengths of individual uninfected or T. gondii-infected eGFP-FAK THP-1 cells during imaging. Each track represents a single cell. D) The average speed of the cells tracked in panels (B) and (C) during imaging is shown. Each dot represents a single cell, and the colors of the dots correspond with the colors of the tracks in panel (B) (**p < 0.01, Student’s t-test).
Figure 3
Figure 4
Figure 5

A) Paxillin
B) Vinculin
C) Graphs showing area of actin (µm²), number of actin filaments, and co-localization with vinculin (Mander's overlap coefficient) over time (min).
Figure 6

A

Uninfected

FAK

T. gondii

B

Number of adhesions (cell^-1)

Time (min)

Uninfected

T. gondii

C

Uninfected

T. gondii

D

Uninfected

T. gondii
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