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Transepithelial migration of *Toxoplasma gondii* involves an interaction of intercellular adhesion molecule 1 (ICAM-1) with the parasite adhesin MIC2

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Summary

***Toxoplasma gondii* crosses non-permissive biological barriers such as the intestine, the blood–brain barrier and the placenta thereby gaining access to tissues where it most commonly causes severe pathology. Herein we show that in the process of migration *Toxoplasma* initially concentrates around intercellular junctions and probably uses a paracellular pathway to transmigrate across biological barriers. Parasite transmigration required viable and actively motile parasites. Interestingly, the integrity of host cell barriers was not altered during parasite transmigration. As intercellular adhesion molecule 1 (ICAM-1) is upregulated on cellular barriers during *Toxoplasma* infection, we investigated the role of this receptor in parasite transmigration. Soluble human ICAM-1 and ICAM-1 antibodies inhibited transmigration of parasites across cellular barriers implicating this receptor in the process of transmigration. Furthermore, human ICAM-1 immunoprecipitated the mature form of the parasite adhesin MIC2 present on the parasite surface, indicating that this interaction may contribute to cellular migration. These findings reveal that *Toxoplasma* exploits the natural cell trafficking pathways in the host to cross cellular barriers and disseminate to deep tissues.**

Introduction

Biological barriers play crucial roles in homeostasis and represent the first line of defence against microbial infections that would otherwise lead to disseminated disease. Whereas the mechanisms leading to bacterial and viral penetration of biological barriers have been partly elucidated (Kerr, 1999; Hornef *et al*., 2002), very little is known about mechanisms used by parasites. During natural infections, *Toxoplasma* initially crosses the intestinal epithelium, disseminates into the deep tissues and traverses biological barriers in the placenta, the blood–brain barrier and the blood–retina barrier (Barragan and Sibley, 2003). Within these immunologically privileged sites, it causes severe pathology in the developing fetus (Remington *et al*., 1995), immunocompromised individuals (Luft *et al*., 1993), and ocular pathology in immunocompetent individuals (Roberts and McLeod, 1999). We recently demonstrated that *Toxoplasma* actively crosses polarized cell monolayers *in vitro* and that this ability is linked to parasite motility and virulence in the mouse model (Barragan and Sibley, 2002).

Toxoplasma lacks cilia or flagella and uses a unique mode of locomotion, termed gliding motility, to rapidly enter host cells by active penetration (Dobrowolski and Sibley, 1996). Gliding motility is also characteristic of other apicomplexan parasites that are important causative agents of human diseases including Plasmodium, the aetiological agent of malaria (Sibley, 2004). *Toxoplasma* tachyzoites invade a wide variety of cells from different vertebrates, suggesting the presence of ligands for widespread host cell surface molecules. Consistent with this model, interactions with sialic acid (Monteiro *et al*., 1998) and heparan sulphate-like proteoglycans (Ortega-Barria and Boothroyd, 1999; Carruthers *et al*., 2000a; Harper *et al*., 2004) are important in host cell recognition. Whether there are also specific protein–protein interactions that mediate polarized attachment, entry and tissue migration remained uncertain, as these specific interactions have not been extensively studied.

Apicomplexan parasites share an evolutionary conserved family of transmembrane adhesins first described in malaria as TRAP (thrombospondin-related anonymous protein) (Robson *et al*., 1988), and called MIC2 in *Toxoplasma* (Wan *et al*., 1997). The extracellular domains of MIC2 and TRAP contain two adhesive modules, an integrin I/A-domain and one or more thrombospondin type I repeats; these domains bear significant similarities to

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mammalian counterparts involved in cell–cell interactions (Kappe *et al*., 1999; Whittaker and Hynes, 2002). TRAP plays a crucial role in gliding motility and host cell invasion by malaria sporozoites (Sultan *et al*., 1997; Kappe *et al*., 1999), and by analogy MIC2 is thought to participate in these processes in *Toxoplasma*. Consistent with this, genetic disruption of the binding partner of MIC2, a protein called M2AP, results in improper trafficking of MIC2 and greatly reduced invasion of host cells (Huynh *et al*., 2003).

MIC2 is secreted from the micronemes onto the parasite cell surface where it exists in two forms. The full-length form contains a prodomain of approximately 45 amino acids that precedes an integrin A-domain, while the mature form lacks this extension (Carruthers *et al*., 1999; Carruthers *et al*., 2000b). A chymostatin-sensitive parasite protease called MPP2 (microneme-processing protease 2) is responsible for trimming the N-terminal extension and generating the mature form of MIC2 (Carruthers *et al*., 2000b). After translocation to the posterior end of the parasite, a second proteolytic activity termed MPP1 is responsible for shedding the adhesin by cleaving within the transmembrane domain (Zhou *et al*., 2004).

Importantly, only cell surface-associated MIC2 is able to bind to host cells (Carruthers *et al*., 1999), although the receptor involved in this recognition is unknown. Here we demonstrate that the mature form of the parasite adhesin MIC2 is activated for binding to ICAM-1 on host cells and that this interaction probably facilitates transmigration by the parasite.

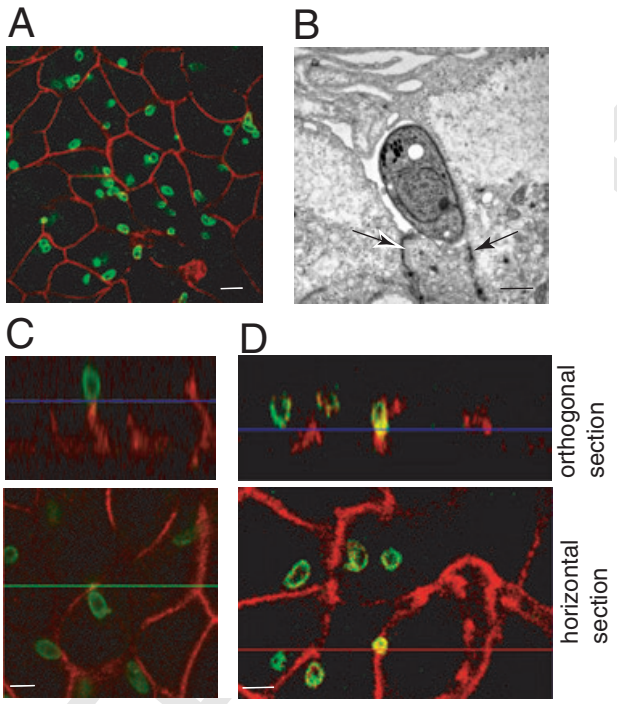

Fig. 1. Interaction of *T. gondii* with polarized epithelial cells suggests a paracellular migration pathway.
A. Parasites (stained for surface antigen SAG1 in green) bind primarily at the cellular junctions (stained for E-cadherin in red) between adjacent MDCK cells. Scale bar = 10 μm.
B. Transmission electron microscopy section showing parasite lying above several tight junctions between adjacent polarized BeWo cells (arrows). Scale bar = 1 μm.
C. Parasites (green) lying directly above the intercellular space of adjacent MDCK cells outlined by E-cadherin (red). Scale bar = 5 μm.
D. The parasite (green) in the centre is migrating across an MDCK monolayer and is surrounded by the intercellular marker E-cadherin (red). Scale bar = 5 μm.

Results

Toxoplasma *transmigrates in close contact with intercellular junctions*

To determine the mechanism used by *Toxoplasma* to cross cellular barriers, we examined parasite migration across polarized Madin Darby Canine Kidney (MDCK) cell monolayers grown on transwells, as described previously (Barragan and Sibley, 2002). During the initial interaction, parasites were primarily concentrated around intercellular junctions, as revealed by co-staining for the basolateral marker E-cadherin and examination by confocal laser scanning microscopy (Fig. 1A). Statistical analysis indicated that a significantly higher proportion of parasites were located within five microns of an intercellular junction than would be expected by chance ($P < 10^{-8}$, χ^2-test; Fig. 1A). When examined by transmission electron microscopy, parasites were observed between the host cells, lying adjacent to tight junctions (Fig. 1B). In transverse Z section analysis of confocal images, parasites were often observed to lie over tight junctions (Fig. 1C) or between adjacent cells (Fig. 1D) as revealed by co-staining for E-cadherin. These data suggest that *Toxoplasma* uses a paracellular pathway to cross polarized cell monolayers.

Parasite transmigration preserves the epithelial barrier integrity

To investigate the effects of parasite transmigration on polarized host cells, the integrity of the monolayer was assessed during *in vitro* transmigration. Parasite transmigration required viable and actively motile parasites (Fig. 2A). During penetration of cells by malaria sporozoites, the host cell membrane is often breached resulting in loss of cell contents and viability (Mota *et al*., 2001; 2002). In contrast, invasion of host cells by *Toxoplasma gondii* rarely leads to disruption of the cell membrane (Suss-Tobey *et al*., 1996). To examine whether cellular damage occurs during the passage of *T. gondii* across polarized monolayers, we used the soluble tracer dextran-FITC to assess membrane integrity and measure

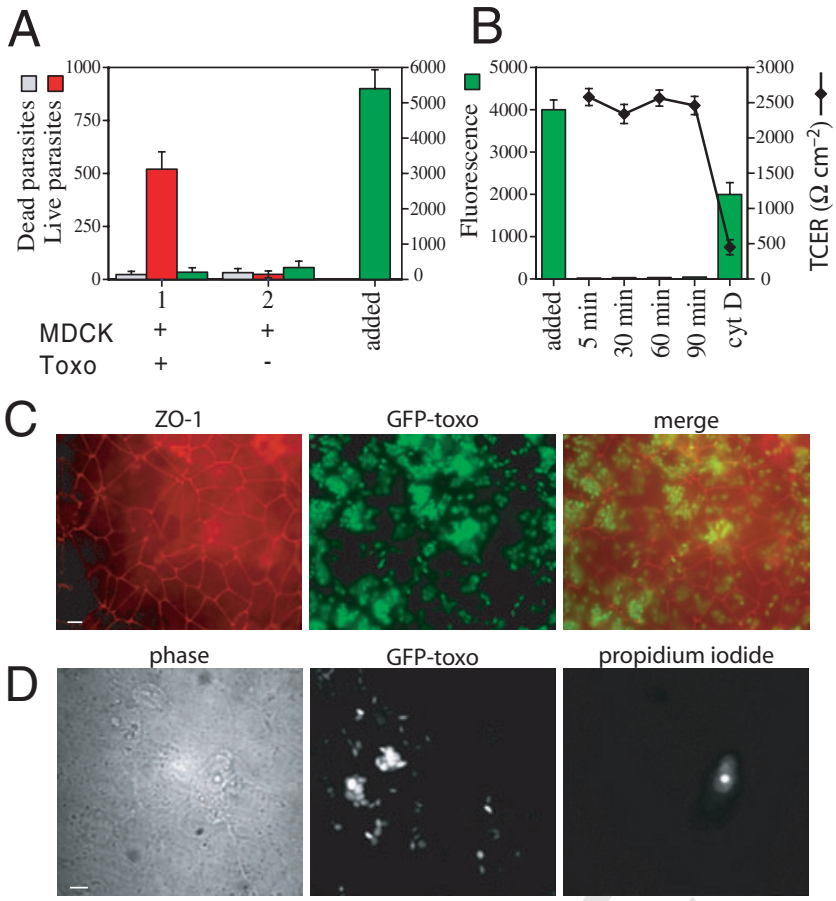

Fig. 2. Monolayer integrity is maintained during active transmigration of *Toxoplasma*.
A. Transmigration of live (red bars) or heat-inactivated (dead, grey bars) parasites (left *y*-axis) across polarized MDCK monolayers grown in transwells. Transmigration of parasites was quantified after 90 min incubation by colorimetric detection of β-galactosidase activity (RH-*lac*Z line). Migration occurred without passage of FITC-dextran to the lower transwell chamber (green bars, arbitrary fluorescence units, right *y*-axis). Bar labelled 'added' reflects the amount of FITC-dextran added to the apical side of the monolayer in a transwell.
B. Transcellular electrical resistance (TCER) was maintained during transmigration (line plot, right *y*-axis). Migration occurred without leakage of the fluid phase marker FITC-dextran (arbitrary fluorescence units, left *y*-axis). The monolayer was depolarized by addition of 1 μm of cytochalasin D (cyt D).
C. Polarized MDCK monolayers grown on transwell filters were heavily challenged with parasites (GFP-expressing intracellular parasites, GFP-toxo) and after 4 h incubation stained for the tight junction marker ZO-1 (red). Conservation of distinct junctional complexes is shown.
D. Live MDCK cell monolayer excludes propidium iodide (except for one floating cell) during heavy infection with GFP-expressing parasites (GFP-toxo).
Scale bars = 10 μm. (A), (B), (C) and (D) were performed three or more times with similar outcome; representative experiments are shown.

electrical resistance to monitor the integrity of the tight junctions. Migration occurred without simultaneous passage of the fluid-phase marker dextran-FITC (Fig. 2A and B) or a decrease in the transcellular electrical resistance (Fig. 2B), indicating there is no disruption in the polarization of the monolayer. When monolayers were exposed to a heavy parasite challenge, leading to a high level of cell invasion and transmigration, no disruption of the junctional complexes was observed based on staining for ZO-1 (Fig. 2C). Staining of host cells with propidium iodide after invasion (Fig. 2D) confirmed the lack of host cell lysis during parasite transmigration. Thus, *T. gondii* is able to transmigrate across polarized monolayers without affecting the integrity of the polarized cell monolayer.

Soluble human ICAM-1 and anti-ICAM-1 antibodies inhibit parasite transmigration

The active migration of *T. gondii* across polarized monolayers suggested that the parasite might engage specific receptors on the apical surface of the host cells. Cell adhesion molecules of the immunoglobulin (Ig) superfamily (ICAMs) present one attractive candidate as this receptor is upregulated after infection and a number of other pathogens are known to interact with ICAMs. To test the participation of ICAM-1 in parasite transmigration, functional assays were performed in the presence of human ICAM-1 (hICAM-1) or blocking ICAM-1 monoclonal antibodies (mAbs). Transmigration was evaluated using polarized monolayers of three independent cell lines including BeWo (human placenta), Caco2 (human intestine) and MDCK (canine kidney) cells. ICAM-1 was abundantly expressed at intercellular junctions and surface of polarized BeWo (data not shown) and Caco2 cell monolayers as detected by specific antibodies (Fig. 3A). Soluble hICAM-1 had an inhibitory effect on transmigration across all cell types (Fig. 3B). MAb 2146Z, which specifically recognizes human ICAM-1, inhibited transmigration in a species-specific manner (Fig. 3B). MAb 2145, which recognizes the D1 domain of ICAM-1, also inhibited parasite transmigration across BeWo cells (86% inhibition) and Caco2 cells (65% inhibition) when added at 5 μg ml^{-1} (data not shown). In contrast, mAbs against ICAM-1 or soluble ICAM-1 did not significantly affect entry of host cells by the parasite, indicating that transmigration requires a specific pathway that is not essential for cell entry (Fig. 3B).

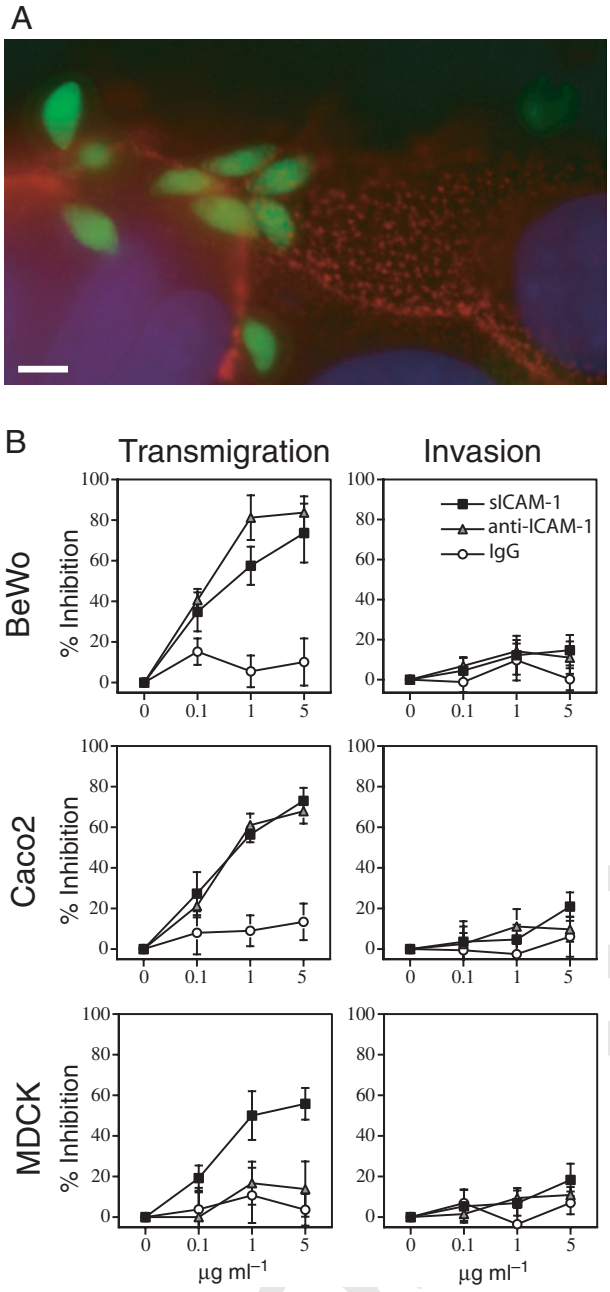

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Fig. 3. Soluble ICAM-1 and anti-ICAM-1 antibodies inhibit transmigration by *Toxoplasma*.
A. Parasites concentrate along the cellular junctions. Polarized Caco2 cells stained for ICAM-1 (mAb 2146Z, red) and challenged with GFP-expressing parasites (green). DAPI was used for nuclear staining (blue). Scale bar = 5 µm.
B. Transmigration was assessed after 4 h incubation using BeWo cells (human placenta), Caco2 cells (human intestine) and MDCK cells (canine kidney). A marked effect of ICAM-1 (sICAM-1) was observed on transmigration across all three cell types while anti-ICAM-1 (mAb 2146Z) was only effective on human cell types. Invasion was not significantly reduced by any of the treatments.
(A) and (B) were performed three or more times with similar outcome; representative experiments performed in triplicate are shown.

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Human ICAM-1 immunoprecipitates the parasite adhesin MIC2

During *in vivo* infections, *Toxoplasma* crosses a number of cellular barriers and consequently is exposed to a variety of cell surface molecules including ICAM-1. As hICAM-1 and ICAM-1 antibodies inhibited parasite transmigration, we examined whether parasite cell surface or secreted proteins would interact with ICAM-1. Immunoprecipitation experiments were performed using human ICAM-1 protein fused to the Fc region of human IgG (ICAM-1/Fc). Biotinylated parasite surface proteins failed to identify any interaction with ICAM-1/Fc (data not shown).

A number of microneme proteins have been shown to bind to host cells and the conservation of adhesive domains in this family of proteins implicates them in cell adhesion (Soldati *et al*., 2001). Consequently, we examined a number of MIC proteins for their potential to bind to ICAM-1 using pull-downs followed by Western blotting. MIC1, MIC4 and MIC6 were not detected by ICAM-1/Fc precipitation and Western blotting using specific antibodies (data not shown). In contrast, the parasite cell surface adhesin MIC2 was specifically precipitated by ICAM-1/Fc but not IgG (Fig. 4A).

Importantly, while a form of MIC2 present in cell lysates was bound to ICAM-1, the fully processed form of MIC2 released into the supernatant was unable to bind ICAM-1 (Fig. 4A). Binding of MIC2 to ICAM-1 required the presence of the C-terminal domain as confirmed by precipitating parasite lystates with ICAM-1/Fc and blotting with antibodies specific to the C-domain (Fig. 4B). The form of MIC2 that bound to ICAM-1/Fc migrated slightly faster than full-length MIC2 present in parasite lysate (Fig. 4A). This migration is reminiscent of the N-terminal processing of MIC2 that removes the propiece of approximately 45 residues (Carruthers *et al*., 2000b). Chymostatin has previously been shown to block the activity of MPP2, which processes the N-terminus of MIC2 (Carruthers *et al*., 2000b). To test the role of MPP2 in the interaction with ICAM-1/Fc, parasites were incubated with chymostatin throughout the preparation of the parasite lysates. Chymostatin prevented the binding of MIC2 to ICAM-1/Fc (Fig. 4C), underlying a critical role of MPP2 in this process. Collectively, these results indicate that full-length MIC2 is inhibited from binding ICAM-1 by the presence of the N-terminal propiece, but that once this segment is removed, the protein in activated for binding (Fig. 4D). Furthermore, release of the protein from the cell surface by MPP1 eliminates ICAM-1 binding (Fig. 4D).

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Discussion

Biological barriers are important for maintaining different solute concentrations, controlling the migration of cells

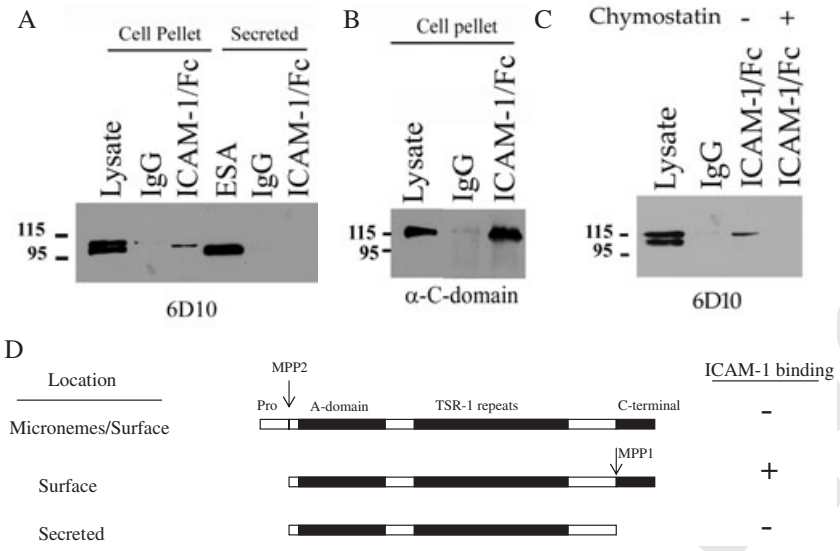

Fig. 4. Interaction of MIC2 with ICAM-1/Fc.
A. A nearly full-length form of MIC2 present in cell lysates binds to ICAM-1/Fc but not IgG. In contrast, MIC2 present in the secreted fraction did not bind to ICAM-1/Fc. Parasite cell lysates or secreted proteins (present in the excretory secretory fraction or ESA) were incubated with IgG- or ICAM-1/Fc-coated beads. Interacting proteins were detected by Western blot using the mAb 6D10 directed against the TSR-1 repeats of MIC2. The upper band in the parasite 'Lysate' lane represents full-length protein, while the lower band results from C-terminal cleavage during the preparation. Lysate and ESA lanes represent input material used in precipitations.
B. The form of MIC2 precipitated from parasite lysates reacts with antibodies to the C-domain. Proteins were incubated with ICAM-1/Fc or IgG and then blotted with rabbit anti-C-domain.
C. The cleavage of MIC2 by MPP2 is required for the interaction with ICAM-1/Fc. Parasites were incubated with or without chymostatin (50 μm) before the preparation of the lysate. The interaction between the MIC2 and ICAM-1/Fc was detected by Western blot using the mAb 6D10.
D. Schematic representation of the processing events that occur during the secretion of MIC2 and their role in the interaction with ICAM-1/Fc. MPP2, microneme-processing protease 2; MPP1, microneme-processing protease 1, Pro, refers to the N-terminal extension.

and preventing entry of microorganisms (Kerr, 1999). Tight attachment between adjacent cells is mediated by cell adhesion proteins including cadherins, integrins, selectins and Ig-domain adhesion proteins (IgCAMs). After oral infection, *Toxoplasma* crosses the intestinal epithelium and disseminates within the host to reach immunologically privileged sites such as the brain, the developing fetus or the retina (Frenkel, 1988). Tissue dissemination is associated with enhanced migration across polarized monolayers and is an important component of virulence (Barragan and Sibley, 2002). Thus, the interaction of *Toxoplasma* with biological barriers is a determinant factor in the development of toxoplasmosis. Here, we present evidence that *Toxoplasma* probably uses a paracellular pathway to efficiently traverse across polarized monolayers by interactions involving ICAM-1 on the host cell surface and the parasite adhesin MIC2.

A number of parasitic, bacterial and viral pathogens have been shown to cross biological barriers using a variety of mechanisms including a trafficking within leukocytes (i.e. Trojan-horse), transcytosis and paracellular migration (Kerr, 1999; Huang and Jong, 2001). Passage of *Toxoplasma* across polarized epithelium *in vitro* occurs by direct penetration but does not result in damage to the monolayer or to individual cells. In contrast, migration of plasmodium sporozoites into the liver occurs by a process that results in damage to some host cells (Mota *et al*., 2001). The process of initial penetration through the first host cells also seems to condition the malarial parasite for productive invasion into subsequent cells (Mota *et al*., 2002). In contrast, our studies demontrate that tachyzoites of *T. gondii* enter cells and pass epithelial junctions without causing cellular damage. After oral challenge with *Toxoplasma* oocysts, rapid penetration of sporozoites beyond the intestinal epithelium and into the lamina propria has also been reported (Dubey *et al*., 1997). Parasites that successfully crossed the epithelium were observed to be extracellular, indicating they travese this barrier actively (Dubey *et al*., 1997).

Neutrophils migrate using both paracellular and transcellular pathways (Feng *et al*., 1998), and it is also possible that parasites are able to cross polarized monolayers using several different mechanisms. While the observations reported here are most consistent with passage via a paracellular route, we cannot rule out the possibility that parasites may also enter the cell apically and then exit from the basolateral side. However, this mechanism seems less likely as *T. gondii* rarely exists a host cell once invasion has occurred (Morisaki *et al*., 1995). Furthermore, the use of a paracellular route for transmigration

bypasses the onset of intracellular replication that occurs upon invasion. Paracellular transmigration may be a useful adaptation to avoid damage to the host cell that would initiate a local inflammatory response.

Intercellular contacts within polarized monolayers are organized into tight junctions to create a restrictive barrier to solute flow and to partition proteins and lipids between the apical and basolateral surfaces. The barrier is maintained by several junctional complex proteins including occludins, claudins and junctional adhesion molecules (JAMs) (Tsukita *et al*., 2001). Despite this rigid organization, leukocytes regularly traffic from the vasculature into the deep tissue by passing through the junctional complexes. Leukocyte trafficking is controlled by a three-step process that involves rolling adhesion (mediated by selectins), tight binding (mediated by ICAMs and integrins) and finally extravasation [mediated by PECAM-1, integrin-associated protein (IAP) and integrins] (Kerr, 1999). *Toxoplasma* may utilize a similar pathway by initially interacting with host cell ICAM-1 through MIC2. By analogy with leukocyte transmigration, it is also likely that additional tightly regulated ligand-receptor interactions take place during transmigration. The elucidation of these processes awaits further investigation.

MIC2 belongs to a family of parasite adhesins referred to as TRAP proteins that contain a well-recognized integrin I/A-domain that is found in a variety of taxa, including prokaryotes, protozoa, plants and metazoan animals (Whittaker and Hynes, 2002). Similar to its role in metazoan animals, this domain is found in extracellular adhesins in apicomplexans (Kappe *et al*., 1999). Notably, TRAP has been shown to be essential for motility and invasion by Plasmodium sporozoites and the I/A-domain is important for productive infection in both vertebrate and invertebrate hosts (Sultan *et al*., 1997; Wengelnik *et al*., 1999; Matuschewski *et al*., 2002). The A-domain of MIC2 is unusual in that it is preceded by an N-terminal extension of approximately 45 residues. We now provide evidence that proteolytic processing to remove this propiece is necessary for binding of MIC2 to ICAM-1. While the molecular identity of MPP2 is unknown, our data suggest a potential role for this protease in activating ligands for receptor engagement. The interaction of mature MIC2 with ICAM-1 is highly reminiscent of the role of integrin A-domains in the β2-integrins LFA-1 and Mac-1 (Hynes, 2002). It is likely this represents an example of recent functional convergence for an otherwise ancient protein domain.

Upregulation of ICAM-1 expression in response to cytokines produced during inflammation is responsible for enhanced leukocyte extravasation in allergic asthma, autoimmune disorders and allogenic transplant rejection (van de Stolpe and van der Saag, 1996; Dietrich, 2002). Several important pathogens also exploit ICAM-1 to gain entry into cells (e.g. rhino and coxsackie viruses) or attach specifically to vascular endothelium (e.g. Plasmodium) (van de Stolpe and van der Saag, 1996; Dietrich, 2002). ICAM-1 is present on the apical surface of a number of biological barriers encountered by *Toxoplasma* and its expression is upregulated during infection of human cells *in vitro* (Nagineni *et al*., 2000) and during human toxoplasmic uveitis *in vivo* (Klok *et al*., 1999). Thus, the enhanced expression of ICAM-1 during infection or underlying inflammatory conditions may potentiate the dissemination of the parasite into deep tissues.

Our studies indicate that *T. gondii* interacts with ICAM-1 and that this facilitates passage across polarized monolayers. Interactions between the parasite adhesin MIC2 and host cell ICAM-1 probably participate in this process. Transmigration may be important for dissemination *in vivo*, in particular by allowing the parasite to advance into tissues that normally restrict access to leukocytes (i.e. CNS, retina, placenta). Defining the precise molecular interactions that lead to cellular migration will be important for understanding virulence and designing improved therapies to prevent infection.

Experimental procedures

Parasites and cell lines

Toxoplasma tachyzoites (RH, RH-*lac*Z, RH-GFPS65T strains) were maintained in human foreskin fibroblast cells as described (Barragan and Sibley, 2002). MDCK cells [American Type Culture Collection (ATCC)], Manassas (VA), colorectal adenocarcinoma-derived Caco2 cells (ATCC) and placenta-derived BeWo cells (Dr Smith, Washington University) were plated onto 24-well transwell filters (3 μm pore, Becton Dickinson Labware, NJ) and grown for at least 7 days to form polarized monolayers as defined by a resistance of $> 1800\ \Omega\ cm^{-2}$ as measured using an Ohmmeter (Millipore, Bedford, MA).

Immunofluorescence microscopy

After infection, monolayers were fixed and stained for immunofluorescence as described previously (Barragan and Sibley, 2002). Host cell markers were detected using antibodies to ZO-1 (mAb 1520, Chemicon Intl., Temecula, CA), ICAM-1 mAbs 2145 (clone RR1/1) and 2146Z (clone P2A4) (Chemicon Intl.) and E-cadherin (mAb rr1, Developmental Studies Hybridoma Bank, Iowa City, IA). Parasites were detected using polyclonal rabbit anti-*Toxoplasma* tachyzoite serum or mAb DG52 to the surface protein SAG1. Goat anti-rabbit or anti-mouse antibodies conjugated to Alexa 488 or to Alexa 594 (Molecular Probes, Eugene, OR) were used as secondary antibodies. Monolayers were examined using a Zeiss LSM 510 laser scanning confocal microscope or a Zeiss Axioscope equipped with phase-contrast and epifluorescence microscopy. Images were digitally captured using an ORCA-ER CCD camera (Hamamatsu, Japan) and processed using OpenLabs v3.0 (Improvision, Lexington, MA) image analysis software. Distances between parasites and tight junctions (ZO-1) were calculated using Zeiss image analysis software (LSM Image Browser R 3.0). Statistical analysis was based on

comparing the number of observed versus expected parasites that were within five microns of a junction using a χ^2 function ($n = 275$ cells).

Transmission electron microscopy

Samples were fixed in 1% glutaraldehyde (Polysciences, Warrington, PA), 1% osmium tetroxide (Electron Microscopy Sciences, Ft. Washington, PA) in 50 mM phosphate buffer, pH 6.3 for 45 min on ice (4°C). Samples were rinsed in dH_2O before *en bloc* staining with 1% aqueous uranyl acetate (Ted Pella, Redding, CA) for 3 h at 4°C. Samples were rinsed in dH_2O, dehydrated in ethanol, and embedded in Eponate 12 resin (Ted Pella). Ultra thin sections were stained with uranyl acetate and lead citrate, and viewed on a JEOL 1200 EX microscope (JEOL USA, Peabody, MA).

Transmigration and invasion assays

Transmigration assay was performed as described (Barragan and Sibley, 2002). Briefly ($0.5–1 \times 10^6$) freshly egressed parasites were added to the upper well of the transwell system containing a polarized monolayer and incubated at 37°C at indicated times. Transmigration of parasites was quantified by colorimetric detection of β-galactosidase activity (RH-*lac*Z) or by visual counting of parasite vacuoles in the underlying human foreskin fibroblast (HFF) monolayer (Barragan and Sibley, 2002). For heat inactivation, parasites were incubated for 30 min at 50°C before use. To assess cell damage, parasites were allowed to transmigrate in the presence of FITC-dextran (MW 3000 Da, 1 mg ml^{-1}, Molecular Probes). Fluorometric quantification was performed with a Chameleon microplate reader (Bioscan, Washington, DC). Alternatively, monolayers were stained with propidium iodide (10 μg ml^{-1}, Sigma) after transmigration.

For invasion assays, green fluorescent protein (GFP)-expressing parasites (RH-GFPS65T) were added to polarized monolayers and allowed to invade for 1 h. The monolayers were extensively washed and mAb DG52 directly conjugated to Alexa 594 fluorophore (Molecular Probes, Eugene, OR) was added to distinguish intracellular parasites (GFP positive but SAG1 negative) from extracellular parasites (double positive). The percentage of intracellular parasites was determined by counting of 20 fields (20×) examined using epifluorescence microscopy.

For inhibition assays, soluble hICAM-1/Fc chimera (Chemicon Intl.), ICAM-1 mAbs, human IgG (Chemicon Intl.) were added at concentrations from 0 to 5 μg ml^{-1} to the monolayers 15 min before the addition of parasites. These additives did not affect the transcellular electric resistance (TCER) or the permeability to FITC-dextran of the polarized cell monolayers. The number of intracellular and transmigrating parasites was measured as described above. Per cent inhibition was determined by comparing the mean of three samples between control (no addition) and treatment groups.

Protein analyses

Parasite cell lysates and secreted microneme proteins were generated as previously described (Carruthers *et al.*, 2000a,b). Sepharose 4B, protein A-coated beads (Zymed, San Francisco, Ca) were incubated in PBS containing $CaCl_2$ (0.1 mM) and BSA (0.1 mg ml^{-1}) with 3 μg of recombinant human ICAM-1/Fc (R&D Systems, Minneapolis, MN) or 3 μg of rabbit IgG overnight at 4°C. Beads were washed three times in PBS and then incubated with fractions containing either parasite lysates or excretory secretory antigens (ESA) for 4 h at 4°C. The beads were then washed four times in PBS and boiled in SDS-PAGE sample buffer. Proteins were detected by Western blot using mAb 6D10 to the TSR-1 repeats or rabbit polyclonal anti-C-domain antibodies to detect MIC2 (Carruthers *et al.*, 2000b), mAb 421-S1 (from Jean-Francois Dubremetz, Montpellier, France) to detect MIC1, mAb 5B1 to detect MIC4 and rabbit polyclonal antibodies to detect MIC6 (obtained from Dominique Soldati, Imperial College, London).

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