Integrin-dependent migratory switches regulate the translocation of *Toxoplasma*-infected dendritic cells across brain endothelial monolayers

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10

Abstract

12 Multiple cellular processes, such as immune responses and cancer cell metastasis,
13 crucially depend on interconvertible migration modes. However, knowledge is scarce on
14 how infectious agents impact the processes of cell adhesion and migration at restrictive
15 biological barriers. In extracellular matrix, dendritic cells (DCs) infected by the obligate
16 intracellular protozoan *Toxoplasma gondii* undergo mesenchymal-to-amoeboid transition
17 (MAT) for rapid integrin-independent migration. Here, in a cellular model of the blood-
18 brain barrier, we report that parasitised DCs adhere to polarised endothelium and shift to
19 integrin-dependent motility, accompanied by elevated transendothelial migration (TEM).
20 Upon contact with endothelium, parasitised DCs dramatically reduced velocities and
21 adhered under both static and shear stress conditions, thereby obliterating the infection-
22 induced amoeboid motility displayed in collagen matrix. The motility of adherent
23 parasitised DCs on endothelial monolayers was restored by blockade of $\beta 1$ and $\beta 2$
24 integrins or ICAM-1, which conversely reduced motility on collagen-coated surfaces.
25 Moreover, parasitised DCs exhibited enhanced translocation across highly polarised
26 primary murine brain endothelial cell monolayers. Blockade of $\beta 1$, $\beta 2$ integrins, ICAM-1
27 and PECAM-1 reduced TEM frequencies. Finally, gene silencing of the pan-integrin-
28 cytoskeleton linker talin (*Tln1*) or of $\beta 1$ integrin (*Itgb1*) in primary DCs resulted in
29 increased motility on endothelium and decreased TEM. Adding to the paradigms of
30 leukocyte diapedesis, the findings provide novel insights in how an intracellular pathogen
31 impacts the migratory plasticity of leukocytes in response to the cellular environment, to
32 promote infection-related dissemination.

33

34 **Keywords:** leukocyte; blood-brain barrier; Apicomplexa; immune cell; cell migration;
35 cell adhesion molecule

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Introduction

Dendritic cells (DCs) are central to mount immune responses and to combat infections [1]. Their effector functions are crucially linked to a remarkable migratory plasticity, for example the ability to transition from mesenchymal to amoeboid (MAT) migration modes [2]. This requires major changes in the actin cytoskeleton, and enables high-speed locomotion through extracellular matrix (ECM), when travelling from the periphery to secondary lymphoid organs, or to reach biological barriers, on their way from the blood into tissues [3,4]. Amoeboid migration is especially suited for rapid locomotion of leukocytes in cellular networks and tissues [5]. In contrast to integrin-dependent mesenchymal migration on 2-dimensional (D) surfaces, amoeboid migration in 3D confinements occurs independently of adhesion to specific substrates or ECM degradation and relies primarily on the protrusive flow of actin filaments for efficient migration through interstitial tissues [6-8]. However, upon reaching biological barriers, DCs in the circulation also need to adhere to the vascular endothelium.

The blood-brain barrier (BBB) regulates traffic of leukocytes and protects the brain from pathogens, toxins, cell damage and inflammation [9]. Specialised endothelial cells, held together by tight junctions (TJs), form a highly restrictive barrier that tightly regulates homeostasis in the central nervous system (CNS) [10]. The cerebral endothelium is also in constant crosstalk with astrocytes, microglia, neurons, pericytes and circulating immune cells [11], that all contribute to protect the CNS from external insults.

Upon inflammation, recruitment of leukocytes, such as DCs, and their interaction with the vascular endothelium is a critical step in the immune response [12]. Endothelial cells attract and direct leukocytes to enter underlying tissue, by exposing a variety of cell adhesion molecules (CAMs), such as ICAM-1 and PECAM-1, at their surface that capture travelling immune cells in the bloodstream [13]. Leukocytes on the other hand, express various integrins on their surface, which include $\beta 1$ and $\beta 2$ integrins that bind to CAMs on the endothelial cell surface [14]. Talin-1 connects integrins to actin and contributes to the regulation of integrin adhesion complexes [15]. Jointly, these

69 interactions mediate firm adhesion and spreading of leukocytes on the endothelium,
70 which leads to leukocyte crawling and eventually transmigration through the endothelial
71 barrier to reach sites of inflammation [16].

72 Understanding the biology of leukocyte migration can be aided through the study
73 of host-pathogen interactions, owing to the microbial ability to hijack host cell functions.
74 *Toxoplasma gondii* is a model parasite [17] and obligate intracellular pathogen that
75 infects warm-blooded vertebrates. It is estimated that one third of the global human
76 population encounters *T. gondii* during a life-time [18]. *T. gondii* has a remarkable ability
77 to cross non-permissive cellular barriers to reach immunoprivileged sites, such as the
78 CNS [19]. Primary infection is usually subclinical, and once the parasite reaches the
79 CNS, it can chronically persist inside cysts. In immunocompromised individuals or in the
80 developing foetus, reactivation of latent infection or acute disseminated infection,
81 respectively, can be life-threatening and cause encephalitis [20].

82 Mononuclear phagocytes, such as DCs, can act as 'Trojan horses' for *T. gondii*
83 dissemination in mice [21-23]. When actively invaded by *T. gondii*, DCs undergo
84 cytoskeletal changes with redistribution of integrins, reminiscent of MAT in ECM [24-
85 26]. This migratory activation -termed hypermotility- [27] requires live intracellular
86 parasites [25], does not rely on chemotaxis [28] and is instead mediated by non-canonical
87 GABAergic signalling and MAP kinase activation [23,29-32]. However, it has remained
88 unknown how highly-migratory parasitised DCs interact with polarised endothelium.

89 In the present study, we addressed the interaction of parasitised DCs with
90 polarised primary brain endothelial cell monolayers, and identified a migratory shift
91 associated to transendothelial migration (TEM), with a central role for integrins and
92 CAMs.

93

94 **Materials and Methods**

95 **Parasite culture and cell lines.** The GFP or RFP-expressing PTG/ME49 *T. gondii*
96 strains (type II) [33,34] were maintained by serial 48 h passaging in human foreskin
97 fibroblasts (HFFs; CRL-2088, American Type Culture Collection). HFFs were cultured
98 in Dulbecco's modified Eagle's medium (DMEM, Thermofisher scientific) with 10%
99 heat inactivated fetal bovine serum (FBS, Sigma), gentamicin (20 µg/ml, Gibco), L-
100 glutamine (2 mM, Gibco) and HEPES (10 mM, Gibco), referred to as D10. bEnd.3 cells

(CRL-2299, American Type Culture Collection) were cultured in D10. All cell cultures and parasites were grown in a humidified atmosphere containing 5% CO₂ at 37°C.

103

Primary dendritic cells (DCs) and macrophages. Murine bone marrow-derived DCs were generated as previously described [28]. Briefly, cells from bone marrow of 6 to 10-week old male or female C57BL/6NCr mice (Charles River) were cultivated in RPMI 1640 with 10% fetal bovine serum (FBS), gentamicin (20 µg/ml), glutamine (2mM) and HEPES (0.01 M), referred to as complete medium (CM), and supplemented with recombinant mouse GM-CSF (20 ng/ml, Peprotech). Loosely adherent cells (DCs) were harvested after 6 or 8 days. To generate macrophages, cells from bone marrow were cultivated in CM supplemented with M-CSF (20 ng/ml, ImmunoTools). Loosely adherent cells were discarded and adherent cells were harvested after 6 or 8 days.

113

Primary murine brain endothelial cells (MBECs). Six to 8-week old C57BL/6NCr mice, housed under specific pathogen-free conditions at Stockholm University, were euthanised and brains were extracted. The brain tissue was freed from the cerebellum, hypothalamus, olfactory bulb and kept in ice-cold PBS. Preparation of cells was performed as described [35], with modifications [36]. The tissue was digested in collagenase IV (1 mg/ml, Gibco) for 1 h at 37° C, homogenised and washed with wash buffer (PBS, 0.5% FBS and 2mM EDTA). The tissue suspension was mixed with density gradient medium. Percoll (Easycoll, Biochrom) in PBS was added to the tissue suspension at a final concentration of 30% and centrifuged for 30 min at 500 g. After removal of the myelin on the top layer, the cell pellet was washed with wash buffer. A second digestion was carried out by incubating the cell pellet with collagenase IV for 30 min at 37° C. Positive selection of CD31-expressing cells was performed by magnetic activated cell sorting (MACS, Myltenyi Biotec) and CD31 MicroBeads (Myltenyi Biotec), according to the manufacturer's instructions. CD31⁺-enriched cell suspensions were finally plated onto transwell filters (8 µm pore size; BD Biosciences) at cell yield from 1 brain per 4 transwell inserts pre-coated with 0.1% gelatin (Gibco), or in 12-well culture plates and monitored for confluence and polarisation. Cells were cultured in EBM-2/EGM-2 medium (Lonza) with 12% FBS, glutamine (200 mM, Gibco) and growth supplements (Bulletkit™, Lonza) minus the vascular endothelial growth factor (mVEGF) supplement. EBM-2/EGM-2 medium was supplemented with puromycin (1 mg/ml, Gibco) for 3 d, after which medium was changed every 2 d in absence of puromycin. The

cellular monolayers had high and stable expression of PECAM1/CD31 (>99%), TJ markers ZO-1, occludin and claudin-5, with low expression of astrocyte marker GFAP (<1%), as previously characterised [36].

Polarisation parameters: permeability assay and transendothelial electrical resistance (TEER). bEnd.3 cells were cultured to 80% confluence then seeded onto transwells (8 µm pore size; BD Biosciences) and grown for 5 d until they reached polarisation, as defined below. MBECs were seeded directly after isolation and reached a polarisation plateau after ~ 11-12 d, which was maintained beyond 14 d, as defined below. Experiments were performed on d 13-14. For evaluation of cell monolayer permeability following treatments or transmigration, FITC-dextran (3 kDa; Life tech) was added to the upper compartment of the transwell at a concentration of 12.5 µg/ml for 90 min. Medium was collected from the lower compartment, and fluorescence was measured in a fluorometer (EnSpire Multimode Plate Reader, Perkin Elmer) at 485 nm excitation 520 nm emission.

MBECs and bEnd.3 cells were cultured to form polarised monolayers defined by a TEER $\geq 250 \Omega \cdot \text{cm}^2$, as measured using an Ohmmeter (Millipore, Bedford, MA) and correcting measurements with the formula: Unit Area Resistance (TEER) = Resistance (Ω) • Effective Membrane Area (cm^2). TEER was measured before and after transmigration or treatments. Values are shown as percentage (%) of TEER related to TEER prior to treatment or transmigration.

Reagents. Blocking antibodies, isotype controls and inhibitor of VLA-4 were used at 1 µg/ml: LEAF™ purified anti-CD29 (clone HMβ1-1, 102209, BioLegend), LEAF™ purified anti-CD31 (clone MEC13.3, 102511, BioLegend), LEAF™ purified anti-CD18 (clone M18/2, 101409, BioLegend), LEAF™ Purified Rat IgG2a, κ Isotype Ctrl (clone RTK2758, 400515, BioLegend), Ultra-LEAF™ Purified Armenian Hamster IgG Isotype Ctrl (clone HTK888, 400969, BioLegend), BIO 5192 (R&D systems), anti-CD54 (ICAM-1; clone YN1/1.7.4; eBioscience).

Immunostainings. *T. gondii*-challenged DCs were plated on coverslips coated with bovine collagen I (1 mg/ml, Life Technologies). After fixation (4% PFA, Sigma-Aldrich), cells were permeabilised (0.5% Triton X-100, Sigma-Aldrich) and stained with phalloidin

168 Alexa Fluor 595 (Invitrogen). Micrographs were generated using a 63x objective (DMi8,
169 Leica Microsystems). Primary MBECs were seeded on coverslips pre-coated with 0.1 %
170 gelatin (BioRad). Fixation and permeabilisation steps were carried out as for DCs,
171 followed by blocking (5% FBS in PBS for 2 h). Cells were then incubated with primary
172 antibodies to ZO-1 (Thermofisher) and Occludin (Thermofisher) ON at 4° C at 1:500.
173 Cells were then stained with Alexa Fluor 594-conjugated secondary antibodies
174 (Invitrogen) and DAPI for 2 h, mounted and imaged by confocal microscopy (LSM 800,
175 Zeiss).

176

177 **Transmigration assays.** Day 6-8, DCs or macrophages were challenged with freshly
178 egressed tachyzoites (ME49/PTG-GFP or ME49-RFP, MOI 2, 4 h), resulting in 60-70%
179 infection frequency and ~1,2 tachyzoites / infected cell [22]. Cells were then transferred
180 to transwell filters (8 µm pore size; BD Biosciences), with pre-cultured polarised
181 monolayers of bEnd.3 cells in CM or MBECs in EBM-2/EGM-2. After 16 h,
182 transmigrated DCs were put on ice for 1 h and macrophages trypsinised (TrypLE™
183 Express; Gibco) for 10 min to disassociate adherent cells. Cells were then resuspended
184 and counted in a Bürker chamber.

185

186 **Motility assays.** Motility assays were performed as previously described [25]. Briefly,
187 DCs were cultured with CM ± freshly egressed *T. gondii* tachyzoites (ME49/PTG-GFP or
188 ME49-RFP, MOI 3, 4 h, resulting in 70-80% infection frequency) and with soluble
189 reagents as indicated. DCs were then added to 96-well plates pre-cultured with bEnd.3
190 cells or embedded in bovine collagen I (1 mg/ml, Life Technologies). Live cell imaging
191 was performed for 1 h, 1 frame/ min, at 10x magnification (Z1 Observer with Zen 2 Blue
192 v. 4.0.3, Zeiss). Time-lapse images were consolidated into stacks and motility data was
193 obtained from 30 cells/condition (Manual Tracking, ImageJ) yielding mean velocities
194 (Chemotaxis and migration tool, v. 2.0). Infected cells were defined by GFP⁺ or RFP⁺
195 cells, as indicated.

196

197 **Flow condition assays.** DCs were cultured as stated in motility assay and then added to
198 fluidic channels (µ-Slide VI^{0.4}; Ibidi) with confluent bEnd.3 cell monolayers or pre-
199 coated with collagen I (1 mg/ml, Life Technologies), and allowed to adhere for 10 min.
200 Phase-contrast and fluorescence images were first captured in static condition. Fluidic
201 shear stress was then applied by flowing CM at 0.2 dyn/cm² through the channels. Live

cell imaging was immediately initiated and images acquired every 5 seconds for up to 10 min, at 10x magnification. Fluidic shear stress was then increased to 1 dyn/cm² and live cell imaging immediately initiated. Time-lapse images were consolidated into stacks and motility and path-length data obtained from 20 cells (Manual Tracking, ImageJ) yielding mean velocities and pathlengths (Chemotaxis and migration tool, v .2.0). The percentage of remaining adherent cells was calculated by dividing the number of cells per frame following shear stress, by the number of cells in the same frame in static condition. Representative track plots were created in Python 3.6.9 (<https://www.python.org/>) with pandas 1.1.5 and matplotlib 3.1.3.

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Flow cytometry. Bone marrow-derived DCs were cultured in CM ± freshly egressed *T. gondii* tachyzoites (ME49/PTG-GFP, MOI 1) or LPS (100 ng/ml, serotype 011:B4, Sigma-Aldrich) for 4 or 24 h. Cells were stained on ice in FACS buffer (1% FBS and 1 mM EDTA in PBS) with Live/Dead Violet (L34955, Life technologies), anti-CD11c (clone N418, 25-0144-82, eBioscience) and anti-CD18 (clone M18/2, 101407, BioLegend), -CD29 (clone HMβ1-1, 102213, BioLegend) -CD54 (clone YN1/1.7.4, 116119, BioLegend) or isotype control antibodies (clone R35-95, 553930, BD Pharmingen; clone HTK888, 400924, BioLegend; clone RTK4530, 400611, BioLegend), fixed with 2% PFA and analysed on a BD LSRFortessa flow cytometer (BD Biosciences) with FlowJo software (v. 10, FlowJo LLC). Prior to staining, cells were blocked in FACS buffer supplemented with anti-CD16/CD32 antibody (Fc Block, BD Pharmingen).

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Lentiviral vector production and *in vitro* transduction. Self-complementary hairpin DNA oligos targeting *Itgb1* (shITGB1, TRCN0000066645, Genscript) or *Tln1* (shTln1, TRCN0000294832, Genscript) mRNA were on self-inactivating lentiviral vectors (pLL3.7) with eGFP reporter expression (**Table S1**). Transfer plasmid (shRNA targeting ITGB1, Tln1 or Luc) was co-transfected with psPAX2 (12260, Addgene) packaging vector and pCMV-VSVg (8454, Addgene) envelope vector into Lenti-X 293T cells (Clontech) using Lipofectamine 2000 (Invitrogen). The resulting supernatant was harvested 24 h and 48 h post-transfection. Supernatants were centrifuged to eliminate cell debris and filtered through 0.45-mm cellulose acetate filters. DCs (3 days post-bone marrow extraction) were transduced by adding lentiviral supernatants in the presence of DEAE dextran (8 µg/ml; Sigma-Aldrich) to cells for 4 h. After 5-7 days, transduction efficiency was examined for eGFP expression by epifluorescence microscopy (Z1

Observer with Zen 2 Blue v. 4.0.3, Zeiss) followed by expression analysis by qPCR for knockdown of targeted mRNA.

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Polymerase chain reaction (PCR). Total RNA was extracted using TRIzol reagent (Sigma-Aldrich). First-strand cDNA was synthesised with Superscript IV Reverse Transcriptase (Invitrogen). Real-time quantitative polymerase chain reaction (qPCR) was performed using SYBR green PCR master mix (Kapa biosystems), forward and reverse primers (200 nM), and cDNA (100 ng) with a QuantStudio™ 5 real-time PCR system (Thermofisher). Glyceraldehyde 3-phosphatase dehydrogenase (GAPDH) was used as a house-keeping gene to generate ΔC_t values; $2^{-\Delta C_t}$ values were used to calculate relative knock-down efficiency. All primers (Invitrogen) were designed using the Get-prime or Primer-BLAST software (**Table S2**).

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Statistical analyses. All statistics were performed with Prism software (v. 8, GraphPad). In all statistical tests, values of $P \geq 0.05$ were defined as non-significant and $P < 0.05$ were defined as significant.

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253

254 **Results**

***T. gondii*-infected DCs transmigrate across polarised monolayers of primary mouse brain endothelial cells (MBECs) and bEnd.3 cells.**

To assess if *T. gondii*-infected DCs can cross polarising and TJ forming brain endothelial cell monolayers, primary MBECs from murine brains or bEnd.3 cells were grown to confluency and polarisation in a transwell system (**Fig. 1a, Supplementary Fig. S1**). Upon exposure to *T. gondii*-challenged DCs or unchallenged DCs, MBEC monolayers maintained low permeability to the low molecular weight tracer FITC-dextran (3kDa) (**Fig. 1b**) and high transendothelial electrical resistance (TEER) (**Fig. 1c**), consistent with characteristics of cerebral endothelium [36]. Importantly, *T. gondii*-challenged DCs transmigrated across polarised MBEC and bEnd.3 monolayers, at significantly higher frequencies compared with unchallenged DCs (**Fig. 1d, g**). In contrast, *T. gondii*-challenged macrophages transmigrated at significantly lower frequencies, with non-significant differences compared with unchallenged macrophages (**Fig. 1e, f, g**). We

conclude that *T. gondii*-challenged DCs transmigrate across polarised endothelial monolayers, with undetectable perturbation of cellular barrier integrity.

Absence of hypermotility by *T. gondii*-infected DCs on endothelial cell monolayers

T. gondii-infected DCs are hypermotile in 2D and 3D confinements with collagen [23,30,26]. To determine their migratory behavior on endothelium, we compared the motility of DCs on bEnd.3 endothelial cell monolayers and on collagen-coated surfaces (Fig. 2a). In line with previous studies, *T. gondii*-infected DCs exhibited hypermotility on collagen, with elevations in migrated distances and velocity (Fig. 2b, c). In stark contrast, we found that *T. gondii*-infected DCs exhibited non-significant differences in velocity and migrated distances on bEnd.3 cell monolayers, compared with unchallenged DCs (Fig. 2b, c). The unexpected absence of hypermotility by *T. gondii*-infected DCs on endothelial cell monolayers motivated a further exploration of the cell-cell interactions in play.

Integrins and CAMs are implicated in the motility of *T. gondii*-challenged DCs on endothelial cell monolayers

CAMs and integrins play central roles in adhesion, crawling and extravasation of leukocytes across endothelium [16]. We previously reported that integrin-blocking monoclonal antibodies (mAbs) non-significantly impacted the migration of parasitised DCs in a 3D collagen matrix [26]. To address the putative implication of integrins and CAMs on the motility of parasitised DCs on endothelium, we first assessed the expression of $\beta 1/\beta 2$ integrins and ICAM-1 upon *T. gondii* challenge by flow cytometry (Supplementary Fig. S2a, b). In parasitised DCs, the expression of $\beta 1/\beta 2$ integrins was maintained, with small elevations or non-significant differences compared to unchallenged DCs (Fig. 3a, b) or bystander DCs (Supplementary Fig. S2c, d) and with, overall, a relatively inferior response compared to stimulation with LPS (Fig. 3a, b; Supplementary Fig. S2c, d). Interestingly, the expression of ICAM-1 was significantly elevated by 24 h in *T. gondii*-infected DCs (Fig. 3c) but not in by-stander DCs (Supplementary Fig. S2d) Next, analyses of cell velocity were performed in presence of blocking mAbs. As expected, blockade of $\beta 1$ and $\beta 2$ integrins reduced the motility of parasitised DCs on collagen-coated surfaces (2D), with a non-significant impact by CAM blockade and antibody isotype controls (Fig. 3d). In sharp contrast, on bEnd.3 endothelial monolayers, blockade of ICAM-1 and $\beta 1$ and $\beta 2$ integrins conversely elevated the

motility of *T. gondii*-challenged DCs (**Fig. 3e**). Blockade of PECAM-1, $\alpha 4\beta 1$ integrin and isotype controls non-significantly impacted DC motility (**Fig. 3e**). Thus, ICAM-1- and $\beta 1$ - and $\beta 2$ - integrin blockades had opposite effects on DC velocities on collagen and endothelial cell monolayers (**Fig. 3f**). These dramatic and seemingly contraposed motility effects motivated further analyses of parasitised DCs under shear stress.

Adhesion and motility of *T. gondii*-challenged DCs under flow conditions

To assess DC motility under more physiological conditions, unchallenged or *T. gondii*-challenged DCs were tracked under shear stress in flow chambers coated with collagen or bEnd.3 cells (**Fig. 4a, b**). On collagen, the numbers of adhered DCs were reduced upon *T. gondii* challenge (**Fig. 4c**). The mean pathlength of *T. gondii*-challenged DCs (related to unchallenged) was increased, and their mean velocities were elevated following 0.2 dyn/cm² shear stress conditions (**Fig. 4d, e**). However, non-significant differences were observed between unchallenged and *T. gondii*-challenged DCs at 1 dyn/cm², indicating detachment at higher shear stress (**Fig. 4d, e**). In contrast, on bEnd.3 cell monolayers, adhesion of *T. gondii*-challenged DCs was elevated compared with unchallenged DCs following 0.2 dyn/cm² flow, with non-significant differences after 1 dyn/cm² (**Fig. 4f**). While pathlengths and velocities remained high compared to collagen condition, non-significant differences in pathlength or velocity were observed between unchallenged and *T. gondii*-challenged DCs (**Fig. 4g, h**), in line with observations under static conditions (**Fig 2c**). Thus, under shear stress, collagen and endothelium had opposite effects on the adhesion of parasitised DCs by reducing and elevating numbers of adhered cells, respectively, related to unchallenged DCs (**Fig. 4i**). Moreover, the pathlengths of adherent parasitised DCs were elevated in collagen but not on endothelium at low shear stress, related to unchallenged DCs (**Fig. 4j**). Altogether, this extends and corroborates results under static conditions (Figs. 2 and 3). Jointly, the data indicate that the *T. gondii*-induced hypermotility of DCs is maintained on collagen under low shear stress but is abolished upon binding of parasitised DCs to the endothelium. Nonetheless, adherent parasitised DCs were motile on endothelial monolayers.

Implication of CAMs and integrins in the transmigration of *T. gondii*-challenged DCs across endothelium

Because CAMs and integrins play a central role in the extravasation of leukocytes across endothelium [13], we assessed the effects of CAM and integrin blockade on the TEM of

unchallenged and *T. gondii*-challenged DCs across polarised bEnd.3 cell monolayers. First, we performed a transcriptional profiling for relevant integrins and CAMs (Supplementary Fig. S3) and controlled that the treatments non-significantly impacted permeability (Fig. 5a, b) and TEER (Fig. 5c, d). Interestingly, blockade of ICAM-1, PECAM-1, $\alpha 4\beta 1$, $\beta 1$ and $\beta 2$ integrins significantly reduced the TEM frequency of *T. gondii*-challenged DCs (Fig. 5e), but non-significantly impacted the TEM frequency of unchallenged DCs (Fig. 5f). Jointly with motility assays (Fig. 3), this indicates that parasitised DCs utilise integrin and CAM interactions to adhere and perform TEM, which is potentiated by *T. gondii* infection.

Gene silencing of talin-1 (*Tln1*) or of $\beta 1$ integrin (*Itgb1*) impacts motility and TEM of *T. gondii*-infected DCs on endothelium under static and flow conditions

Consistent with paradigms of DC migration [5], we previously showed that amoeboid migration of parasitised DCs in collagen matrix is an integrin-independent process [26]. However, extravasation of DCs across endothelium is integrin-dependent [16] and integrins contribute to the migration of parasitised DCs [30]. To further assess the role of integrins in motility and TEM of *T. gondii*-infected primary DCs, we gene silenced the integrin-linking protein talin (*Tln1*) and $\beta 1$ integrin (*ITGB1*). In transduced DCs, significant reductions in *Tln1* and *Itgb1* mRNA expression, respectively, were measured, related to control shLuc-transduced DCs (Fig. 6a, b). First, to confirm the impact of gene silencing on integrin-mediated motility, transduced cells were challenged with *T. gondii* and allowed to migrate on collagen-coated surfaces (Supplementary Fig. S4). Motility and mean velocities of shITGB1 and *Tln1*-transduced *T. gondii*-infected DCs were significantly reduced on collagen (Fig. 6c, d). In contrast, on bEnd.3 cell monolayers, shITGB1- and *Tln1*-transduced *T. gondii*-infected DCs exhibited increased velocities compared to mock and shLuc-transduced cells (Fig. 6e, f). A similar effect was confirmed under flow, with reduced adhesion (Fig. 6g) and elevations in pathlengths and velocities of transduced infected DCs (Fig. 6h, i). Importantly, the TEM frequency of *T. gondii*-challenged DCs across polarised endothelial monolayers (Fig. 6 j, k) was significantly decreased in shITGB1 and sh*Tln1*-transduced cells compared to shLuc- or mock-transduced cells (Fig. 6l). We conclude that gene silencing of talin-1 (*Tln1*) or of $\beta 1$ integrin (*Itgb1*) inhibits hypermotility and TEM but elevates the motility of *T. gondii*-infected DCs on endothelium.

370 Discussion

371 The interconversion between migratory states of leukocytes and the crosstalk between
372 CAMs and integrins are crucial for diapedesis [16]. Here, we explored the interplay
373 between endothelium and *T. gondii*-challenged DCs and found that intracellular
374 parasitisation impacts migration in a cell environment-related fashion, with a pivotal role
375 for integrins.

376 We report that infection with *T. gondii* induces dramatic migratory changes in
377 primary DCs, which impact on locomotion, adhesion and enhanced transmigration across
378 polarised endothelial cell monolayers. Upon *T. gondii* infection, DCs undergo MAT in
379 extracellular matrix and enhanced amoeboid migration -termed hypermotility-
380 [26,24,30,31], which promotes parasite dissemination *in vivo* [22,28]. Surprisingly, we
381 found that the hypermotility of parasitised DCs in 3D and 2D collagen confinements (**Fig**
382 **7a, b**) is lost upon direct contact with endothelium (**Fig. 7c**), and that the mean velocities
383 and migrated distances became comparable between unchallenged and *T. gondii*-infected
384 DCs. The reduction of motility of DCs on endothelium suggests instead, arrest and
385 adhesion, which is regulated by integrin-CAM crosstalk [37,16], and precedes leukocyte
386 TEM (**Fig 7d**).

387 Under shear stress conditions, parasitised DCs elevated or maintained adhesion to
388 endothelium, while adhesion was conversely reduced on collagen. The elevated adhesion
389 on endothelium is in contrast with the MAT-related morphological changes that
390 parasitised DCs present on collagen and in collagen matrix, including round-cell
391 morphology, decreased adhesion and redistribution of integrins [25,26,24]. Moreover,
392 under flow conditions, the pathlengths of parasitised DCs were maintained high with
393 reduced velocities, indicating crawling of DCs [16]. *In vivo*, leukocyte diapedesis mainly
394 occurs at the postcapillary venules of inflamed tissue, at low shear stress levels [13].
395 Further, exposure to shear stress can increase transcription levels of ICAM-1, PECAM-1
396 and other endothelial CAMs [12], which interact with leukocyte integrins for diapedesis.
397 This motivated a further investigation of the role of integrins.

398 We demonstrate that integrin signalling plays a pivotal role in the infection-
399 related arrested motility of DCs on endothelium. Interestingly, disruption of the integrin-
400 CAM interaction by blockade of $\beta 1$ and $\beta 2$ integrins, along with ICAM-1, partially
401 restored the hypermotility of infected DCs on endothelium. In contrast, on collagen-
402 coated surfaces, the hypermotility phenotype in *T. gondii*-infected DCs was reduced upon

403 $\beta 1$ and $\beta 2$ integrin blockade, and gene silencing of $\beta 1$ integrin or talin. We recently
 404 reported a role for $\beta 1$ integrin in a motogenic TIMP1-CD63-ITGB1-FAK signalling axis
 405 associated with MAT [30]. Here, we add that $\beta 1$ integrin is equally important for,
 406 presumably, amoeboid-to-mesenchymal migratory conversion and adhesion to
 407 endothelium. Not in contraposition with the above, amoeboid migration of DCs is
 408 integrin-independent in ECM [5], and integrin-blocking antibodies non-significantly
 409 impacted the migration of *T. gondii*-infected DCs in a 3D collagen matrix [26]. More
 410 recent studies have also shown that talin-deficient T cells, lacking integrin-based force
 411 transmission, are unable to adhere to or migrate on 2D surfaces [38]. Similarly, the switch
 412 of cancer cells between amoeboid and mesenchymal migration modes appears to be
 413 advantageous in tumour invasion, depending on their extracellular environment [39].
 414 Migrating leukocytes have the ability to slow down or speed up migration in seconds,
 415 switching from high-speed actin-flow driven motility, to slow adhesion-driven motility
 416 mediated by integrins [4]. The difference in motility observed between parasitised DCs
 417 on collagen versus endothelium, implies a pivotal role for integrins in each scenario. It
 418 also reflects the ability of DCs to switch between different modes of migration depending
 419 on the extracellular environment (**Fig. 7**). On the one hand, infection-induced
 420 hypermotility in matrix is linked to increased dissemination [28,29,23], but upon reaching
 421 barriers such as the BBB, adhesion is necessary for TEM and thus facilitates parasite
 422 dissemination across the vasculature.

423 Mesenchymal cell migration is characterized by cell polarisation, adhesive
 424 interactions with the substratum and proteolytic extracellular matrix remodelling,
 425 followed by retraction of the cell rear to achieve cellular movement [6]. In fact, the
 426 motility of parasitised DCs on 2D collagen or endothelial cell surfaces fulfils few criteria
 427 to be classified as strictly mesenchymal. Our data show that this motility relies on
 428 adhesive interactions, in contrast to motility in 3D matrix which is integrin-independent
 429 [26]. However, a number of observations also argue against strict mesenchymal motility.
 430 Specifically, parasitised DCs abrogate pericellular matrix proteolysis by secretion of
 431 TIMP-1 [24] and undergo gross morphological changes, encompassing rounding-up and
 432 irreversible dissolution of podosomes with redistribution of integrins that are, in fact,
 433 more consistent with amoeboid motility [25]. The intracellular parasite achieves
 434 migratory activation of DCs by interfering with MAP kinase signalling [30,31] and
 435 calcium signalling [29] via activation of GABAergic signalling in the infected cell
 436 [28,23]. Here, we add that the hypermigration of parasitised DCs [40] is modulated by

external cues in the cellular environment. In the blood circulation, it is unlikely that the sequestration of parasitised DCs to endothelium solely depends on the migratory mode *per se* but rather on the ability to adhere. Thus, that hypermotile parasitised DCs adhere to brain endothelial monolayers in an integrin-dependent fashion bears relevance for concepts of parasite dissemination.

We demonstrate that integrins contribute to the enhanced TEM of *T. gondii*-infected DCs across polarised primary brain endothelial cell monolayers (**Fig. 7d**). Interestingly, transmigration occurred without measurable perturbation of polarisation or disruption of the cell barrier integrity, in line with previous reports [41] and indicative of regulated cell-cell interactions. Indeed, the TEM of infected DCs was reduced upon blockade of adhesion molecules, ICAM-1 and PECAM-1, and $\beta 1$ and $\beta 2$ integrins. Moreover, gene silencing of the integrin-cytoskeleton linker talin in DCs demonstrated a crucial role for integrins. Taken together with the motogenic role of $\beta 1$ integrin [30], here we add a role in adhesion to endothelium and TEM. Thus, while $\beta 2$ integrins contribute, we identify a pivotal role for $\beta 1$ integrins. Jointly, this shows that, potentiated by *T. gondii* infection, integrin-CAM interactions mediated TEM. It remains largely unknown how intracellular pathogens modulate the migration modes of shuttle-leukocytes to enter the CNS, especially initially during infection in the absence of CNS inflammation [42]. However, integrin-CAM interactions are likely to be in play, as shown here for *T. gondii*-infected DCs. A recent report using a fungal infection model in mice showed that $\alpha 4 \beta 1$ integrin (VLA-4) mediated recruitment of infected monocytes to the BBB, which subsequently migrated to the brain parenchyma [43]. In HIV infection, disrupting the interaction between $\alpha L \beta 2$ (LFA-1) on CD4⁺ T cells and ICAM-1 on DCs by mAbs inhibited viral transmission between T cells and DCs [44,45], and thus similar interactions may be implicated at the BBB. Beyond infection, the pathogenesis of multiple sclerosis implicates a recruitment of DCs and T cells to the CNS [46] and has opened up for mAb-based therapies targeting integrins, for example $\alpha 4 \beta 1$ [47]. Of note, parasitised DCs, but not by-stander DCs, elevated their expression of ICAM-1, indicating effects related to intracellular parasitisation. Further, this also indicates that blockade of ICAM-1 in our study may inhibit adhesion mediated by both DC and endothelial cell ICAM-1 [48,49]. Reciprocally, blockade of both DC and endothelial integrins may also take place [50-52]. Additionally, future studies need to address if the infection-induced cytoskeletal changes with redistribution of integrins alter adhesion [25] and if the activation state of integrins is altered in parasitised DCs [53]. Also, given the profound

471 impact of talin on the migratory responses described here, its role in integrin activation
472 and mechanotransduction in parasitised DCs awaits further investigation [15].

473 Our data shows that the TEM frequency of parasitised leukocytes not only
474 depends on the cellular environment but also is related to cell type. The TEM frequency
475 of *T. gondii*-infected macrophages, derived from the same donor mice, was 10-20-fold
476 inferior to that of infected DCs. For human monocytes, comparable transmigration
477 frequencies have been reported for unchallenged and *T. gondii*-challenged monocytes
478 across filters and non-polarizing endothelial monolayers [54,53]. *T. gondii*-challenged
479 monocytes have also been reported to exhibit hypermotility but reduced transmigration
480 [55], contrasting with the elevated transmigration of parasitised DCs and microglia
481 [22,56]. Further, elevated TEM was observed in infected rat peripheral blood
482 mononuclear cells (PBMCs) [57] and, under shear stress conditions on HUVEC
483 monolayers, the motility and TEM frequencies of infected monocytes increased
484 compared to static conditions [53,58]. Altogether, this highlights that the cellular
485 environment likely impacts migratory responses in a cell type-related fashion [59] and
486 that DCs may provide dissemination advantages over other mononuclear phagocytes.

487 In experimental mouse toxoplasmosis, the transportation of *T. gondii* by DCs and
488 other monocytic cells in the circulation has been associated with higher parasitic loads in
489 the CNS [21,22]. DCs migrate into the brain parenchyma in an integrin-dependent
490 fashion during toxoplasmosis [60]. However, it remains unclear if DCs can transport *T.*
491 *gondii* into the CNS, as extracellular parasites can also invade and transmigrate across
492 endothelium [61,36]. Here, we provide *in vitro* proof-of-concept that transmigration of
493 DCs across highly-polarised primary endothelium is potentiated by infection. In a broader
494 context, leukocytes can be activated to perform diapedesis by chemotactic signalling [62].
495 However, activation does not automatically imply enhanced TEM. For example, upon
496 LPS-stimulation, both reduced and elevated TEM by leukocytes have been reported [63-
497 65]. The role of chemokines in homing of leukocytes to the CNS remains partly
498 unelucidated [66]. Also, natural primary *T. gondii* infection of humans and vertebrates is
499 asymptomatic or accompanied by mild symptomatology. Thus, the initial penetration of
500 *T. gondii* to the CNS likely happens in absence of generalized inflammation or reactive
501 leukocyte infiltration to the parenchyma [67]. Nevertheless, it remains to be investigated
502 if inflammatory mediators, such as IFN- γ and IL-12, impact the interaction of
503 endothelium with parasitised leukocytes. *T. gondii* induces amoeboid migration of DCs

and other mononuclear phagocytes by activating alternative signalling pathways, including GABAergic signalling and MAP kinase activation [23,31], which can synergise with chemotaxis *in vitro* [28,25]. Thus, we postulate that the infection-related enhanced TEM shown here *in vitro* may facilitate the diapedesis of parasitised DCs to the brain parenchyma, synergistically with chemotactic and inflammatory cues. The elucidation of alternative activation pathways that can drive leukocyte TEM will further our knowledge on how leukocytes and other migratory cells regulate their migratory states for vital biological processes.

Declarations

Ethics approval. The Regional Animal Research Ethical Board, Stockholm, Sweden, approved protocols involving extraction of cells from mice, following proceedings described in EU legislation (Council Directive 2010/63/EU).

Data availability. The datasets used and/or analysed in this study are available from the corresponding author on reasonable request.

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Conflict of interest. The authors declare that there is no duality of interest associated with this manuscript.

Author's contributions. E.C.R and ALH performed experiments and analysed the data. All authors contributed to the generation of figures and writing of this manuscript.

Code availability: not applicable

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Figure legends

Figure 1. Transmigration of *T. gondii*-challenged DCs across polarised MBEC and bEnd.3 monolayers

(a) Top cartoon shows experimental setup in a 2-chamber transwell system and assessment of endothelial cell polarisation and permeability, as indicated in Materials and Methods. (1) Measurement of transcellular electrical resistance (TEER), (2) *T. gondii*-challenged or unchallenged cells were added to upper chamber, (3) The permeability tracer, FITC-dextran (3 kDa), added to upper chamber and measured in lower chamber, (4) Transmigrated cells were collected and counted. Bottom micrograph represents a DC infected with GFP-expressing *T. gondii* (green) and stained for F-actin (red) and nuclei (blue). Scale bar = 10 μ m.

(b) Permeability of MBEC monolayers to FITC-dextran (3 kDa) following DC transmigration. Data is shown as arbitrary fluorescence units (a.u).

(c) TEER under same conditions as in (b). For each condition, TEER values ($\Omega \cdot \text{cm}^2$) relative to TEER values at the initiation of the assay (100%) are indicated, as described in Materials and Methods.

(d) Relative transendothelial migration (TEM) frequency of DCs across MBEC monolayers shown as percentage (%) of DCs added in the upper well, normalised to unchallenged DCs (set to 1.0).

(e) Permeability of bEnd.3 cell monolayers to FITC-dextran (3 kDa) following transmigration of unchallenged or *T. gondii*-challenged dendritic cells (DCs) or macrophages (M Φ).

(f) TEER values relative to TEER values at initiation of the assay (100%).

(g) Relative transendothelial migration (TEM) frequency of DCs or M Φ across bEnd.3 monolayers shown as percentage (%) of DCs or M Φ added in the upper well, normalised to unchallenged DCs (set to 1.0).

Bar graphs represent the mean $\pm$ s.e.m of 3-4 independent experiments (n = 3-4). **P* < 0.05, ***P* < 0.01, ns: non-significant, by one-way ANOVA with Dunnett's post-hoc test (b, c, e, f), Students' *t*-test (d, g),

Figure 2. Motility analyses of *T. gondii*-infected DCs on collagen and endothelium

749 **(a)** Representative micrographs of unchallenged DCs and GFP-expressing *T. gondii*-
750 challenged DCs on collagen (top row) and bEnd.3 cell monolayers (bottom row). Scale
751 bar = 100 μ m, inset = 10 μ m.
752 **(b)** Representative motility plots of DCs as described and represented in (a).
753 **(c)** Mean velocity of unchallenged and *T. gondii*-infected DCs. Data represent the mean $\pm$
754 s.e.m from 3 independent experiments (n = 3). **P* < 0.05, ns; non-significant by one-way
755 ANOVA, Sidak's post-hoc test.

756

757 **Figure 3. Motility of *T. gondii*-infected DCs upon blockade of integrins and CAMs**

758 **(a, b, c)** Bar graphs show the relative expression of β 1 (CD29), β 2 (CD18) integrins and
759 ICAM-1 (CD54), respectively, at 4 and 24 h post-challenge of CD11c⁺ cells with *T.*
760 *gondii* tachyzoites (GFP-expressing ME49, MOI 1) or LPS (100 ng/ml), assessed by flow
761 cytometry (see Supplementary Fig. S2 for gating strategy). For each time point, the mean
762 fluorescence intensity (MFI) was related to that of unchallenged CD11c⁺ cells in
763 complete medium (CM, normalised to 1). Histograms show fluorescence intensity
764 distributions for indicated cell populations/conditions and for corresponding isotype
765 controls (unfilled curve with grey line).

766 **(d, e)** Mean velocity of unchallenged and *T. gondii*-challenged DCs treated with
767 inhibitors and antibodies as stated, on (a) collagen or (b) bEnd.3 monolayers,
768 respectively. CM indicates complete medium.

769 **(f)** Percentage (%) change in velocity of *T. gondii*-challenged DCs compared to
770 unchallenged DCs for each condition.

771 All data are presented as mean $\pm$ s.e.m from 3 independent experiments (n = 3) **P* < 0.05,
772 ***P* < 0.01, ****P* < 0.001, *****P* < 0.0001, ns; non-significant by one-way ANOVA,
773 Dunnett's post-hoc test.

774

775 **Figure 4. Adhesion and motility of *T. gondii*-challenged DCs under flow conditions**

776 **(a, b)** Representative cell traces of unchallenged or *T. gondii*-challenged DCs on (a)
777 collagen or (b) bEnd.3 cell monolayers, at indicated shear stress (dyn/cm²). Each color-
778 coded trace shows the euclidian displacement distance of a single cell. White dots
779 indicate the start point of randomly selected track lengths, showing that cells migrated in
780 the direction of flow.

781 **(c)** Percentage (%) of DCs adhered to collagen, following fluidic shear stress (dyn/cm²),
782 related to total number of DCs in the same frame in static condition as indicated under
783 Materials and Methods.

784 **(d, e)** Mean pathlength and velocity, respectively, of tracked cells on collagen.

785 **(f)** Percentage (%) of DCs adhered to bEnd.3 cell monolayers, following fluidic shear
786 stress (dyn/cm²), related to total number of DCs in the same frame in static condition.

787 **(g, h)** Mean pathlength and velocity, respectively of tracked cells on bEnd.3 cell
788 monolayers.

789 **(i, j)** Percentage change in adherence and velocity, respectively of *T. gondii*-challenged
790 DCs related to unchallenged DCs, for each condition.

791 Bar graphs show mean $\pm$ s.e.m. from 4 independent experiments ($n = 4$). * $P < 0.05$, ** P
792 < 0.01 ; ns, non-significant by one-way ANOVA, Sidaks post-hoc test (c, d, e, f, g, h) or
793 Students' t -test (i, j).

794

795 **Figure 5. TEM of *T. gondii*-challenged DC upon integrin CAM blockade**

796 **(a, b)** Permeability of bEnd.3 monolayers to FITC-dextran (3 kDa) after 16 h exposure to
797 *T. gondii*-challenged DCs or unchallenged DCs $\pm$ antibodies or inhibitors, respectively,
798 and as indicated. CM indicates complete medium.

799 **(c, d)** TEER (%) of bEnd.3 monolayers as in (a) and (b), respectively.

800 **(e, f)** Relative TEM (%) of *T. gondii*-challenged DCs or unchallenged DCs, respectively.
801 Relative TEM frequency of DCs across bEnd.3 monolayers are shown as percentage (%)
802 of DCs added, normalised to unchallenged DC condition for each experimental condition
803 (set to 1.0, light blue bar 'CM').

804 Data are presented as mean $\pm$ s.e.m. from 4 independent experiments ($n = 4$). * $P < 0.05$,
805 ** $P < 0.01$, ns; non-significant by one-way ANOVA, Dunnett's post-hoc test (a-f).

806

807 **Figure 6. Impact of gene silencing of talin (*Tln1*) and $\beta 1$ integrin (*Itgb1*) on the**
808 **motility and transmigration of *T. gondii*-challenged DCs**

809 **(a)** *Tln1* mRNA expression ($2^{-\Delta Ct}$) in DCs transduced with shTln1 or control shLuc
810 lentivirus relative to mock-treated cells (mock set to 1.0).

811 **(b)** *Itgb1* mRNA expression ($2^{-\Delta Ct}$) in DCs transduced with shITGB1 or control shLuc
812 lentivirus relative to mock-treated cells.

813 **(c)** Representative motility plots of mock-treated DCs and DCs transduced with lentiviral
814 vectors targeting *Itgb1* mRNA (shITGB1), *Tln1* mRNA (shTln1) or a non-expressed
815 target (shLuc) challenged with *T. gondii*, on collagen.

816 **(d)** Box-and-whisker and scattered dot plots represent median velocities ($\mu\text{m}/\text{min}$) of DCs
817 on collagen, as in (c). Grey circles represent velocities from individual cells.

818 **(e)** Representative motility plots of mock-treated DCs and DCs transduced with lentiviral
819 vectors targeting *Itgb1* mRNA (shITGB1), *Tln1* mRNA (shTln1) or a non-expressed
820 target (shLuc) challenged with *T. gondii*, on bEnd.3 monolayers.

821 **(f)** Box-and-whisker and scattered dot plots represent median velocities ($\mu\text{m}/\text{min}$) of DCs
822 on bEnd.3, as in (e). Grey circles represent velocities from individual cells.

823 **(g)** Bar graphs show percentage (%) of transduced DCs adhered to bEnd.3 monolayers
824 related to DCs in the same frame in static condition, following fluidic shear stress (0.2
825 dyn/cm^2).

826 **(h, i)** Box-and-whisker and scattered dot plots represent median pathlengths (μm) and
827 velocities ($\mu\text{m}/\text{s}$), respectively, of tracked cells on bEnd.3 monolayers. Grey circles
828 represent pathlengths or velocities, respectively, from individual cells.

829 **(j, k)** Permeability to FITC-dextran (3 kDa) and TEER (%), respectively, of bEnd.3 cell
830 monolayers following DC transmigration.

831 **(l)** TEM frequency shown as percentage (%) of DCs added in the upper well.

832 Bar graphs show mean $\pm$ s.e.m and box plots show median from 3-4 independent
833 experiments ($n = 3-4$). In box-and-whisker plots, boxes mark 25th to 75th percentile and
834 whiskers mark 10th and 90th percentiles of the datasets. * $P < 0.05$, ** $P < 0.01$, *** $P <$

0.001, **** $P < 0.0001$; ns, non-significant by Students' *t*-test (a, b), One-way ANOVA, Dunnett's post-hoc test (d, f), Kruskal-Wallis, Dunn's post-hoc test (h, i), repeated measures one-way ANOVA, Sidak's post-hoc test (j, k, l).

838

Figure 7. Schematic representation of the integrin-dependent motility switches in *T. gondii*-infected DCs leading to transendothelial migration

(a) *T. gondii* (Tg) actively invades DCs and induces rapid integrin-independent DC migration, termed hypermotility, in 3-dimensional (3D) extracellular matrix (ECM) confinements, such as collagen. *T. gondii*-induced hypermotility has features of amoeboid motility [26].

(b) The hypermotility of *T. gondii*-infected DCs on 2D collagen-coated surfaces has the adhesion/integrin-dependency feature of mesenchymal migration, however with abrogated proteolysis [24,30]. Blockade of $\beta 1$ or $\beta 2$ integrin, or gene silencing of talin (shTln1) or $\beta 1$ integrin (shITGB1) abolishes hypermotility of *T. gondii*-infected DCs on collagen.

(c) On endothelium, *T. gondii*-infected DCs adhere to cell monolayers under static and flow conditions. In relation to hypermotility on collagen and in ECM (a, b), the reduced motility by parasitised DCs on endothelium is integrin- and CAM-dependent, with features of mesenchymal migration. Specifically, Ab blockade of $\beta 1$ or $\beta 2$ integrins, or gene silencing of talin (shTln1) or $\beta 1$ integrin (shITGB1) elevates *T. gondii*-infected DC motility on endothelium, while the same treatments reduce motility on collagen-coated surfaces (b).

(d) *T. gondii*-infected DCs exhibit elevated transmigration across polarised endothelial monolayers, related to non-infected DCs or infected monocytes/macrophages. Ab blockade of $\beta 1$ or $\beta 2$ integrins, ICAM-1, PECAM-1 or gene silencing of talin (shTln1) or $\beta 1$ integrin (shITGB1) decreases the transmigration frequency of *T. gondii*-infected DCs across endothelium.

862

Supplementary material

Supplementary Table S1. Sequences for shRNAs.

Supplementary Table S2. Sequences for qPCR primers.

Supplementary Figure S1. Immunostainings of endothelial tight junctions

(a, b) Representative micrographs of primary mouse brain endothelial cells (MBECs) stained for (a) ZO-1 (red), (b) occludin (red), with DAPI-stained nuclei (blue). Scale bars = 50 μ m.

Supplementary Figure S2. Gating strategy for flow cytometry and characterizations of CD11c⁺ cells and by-stander cells.

(a) Bivariate dot plots show gating strategy for the experiments displayed in Figure 3, exemplified with bone marrow-derived cells challenged with GFP-expressing *T. gondii*. Histogram shows fluorescence intensity distributions and gates used to define CD11c⁺ cells. Numbers indicate percentage (%) of cells in corresponding gate.

(b) Bivariate contour plots of live unchallenged DCs stained for CD11c vs. β 1, β 2 integrins or ICAM-1, respectively.

(c, d) Bar graphs show the relative expression of β 1 (CD29), β 2 (CD18) integrins and ICAM-1 (CD54), respectively, at 4 h (c) and 24 h (d) post-challenge of cells (CD11c⁺ and CD11c⁻) with *T. gondii* tachyzoites (GFP-expressing ME49, MOI 1) or LPS (100 ng/ml), assessed by flow cytometry. For each time point and condition, the mean fluorescence intensity (MFI) was related to unchallenged cells in complete medium (CM, normalised to 1). 'By-stander' indicates GFP⁻ cells (non-infected) in cell suspensions challenged with *T. gondii*.

Data are presented as mean $\pm$ s.e.m from 3 independent experiments (n = 3) ***P* < 0.01, *****P* < 0.0001, ns; non-significant by two-way ANOVA, Sidak's post-hoc test.

Supplementary Figure S3. Transcriptional analyses of bEnd.3 cells and DCs

(a) mRNA expression of indicated genes in bEnd.3 cells challenged with *T. gondii*, unchallenged DCs, *T. gondii*-challenged DCs or LPS, relative to CM condition, after 6 h.

(b) mRNA expression of unchallenged bEnd.3 cells and DCs.

Bar graphs show mean $\pm$ s.e.m. from 2-3 independent experiments. * *P* < 0.05, ***P* < 0.01, ****P* < 0.001, *****P* < 0.0001; ns, non-significant by two-way ANOVA, Dunnett's post-hoc test.

Supplementary Figure S4. Transductions of primary DCs with shRNAs

Transductions were performed as indicated under Materials and Methods. (a-d) For each condition, representative micrographs show unchallenged DCs (left) and *T. gondii*-challenged DCs (right), mock-treated or treated with control shRNA (shLuc) or shRNA to indicated target gene. Transduced cells were distinguished by expression of the reporter eGFP (green) and *T. gondii* by expression of RFP (red). Infected cells were defined as green + red cells (indicated by white arrows). Scale bar = 100 μ m.

Figure 1

Figure 2

Figure 4

Figure 7