Miro-1 links mitochondria and microtubule dynein motors to control lymphocyte migration and polarity

Running Title: Miro-1 controls lymphocyte migration and polarity

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Abstract

The recruitment of leukocytes to sites of inflammation is crucial for a functional immune response. In the present work, we explore the role of mitochondria in lymphocyte adhesion, polarity and migration. We show that during adhesion to the activated endothelium under physiological flow conditions, lymphocyte mitochondria redistribute to the adhesion zone together with the microtubule-organizing center (MTOC) in an integrin-dependent manner. Mitochondrial redistribution and efficient lymphocyte adhesion to the endothelium requires Miro-1 function, an adapter molecule that couples mitochondria to microtubules. Our data demonstrate that Miro-1 associates with the dynein complex. Moreover, mitochondria accumulate around the MTOC in response to the chemokine CXCL12/SDF-1α; this redistribution is regulated by Miro-1. CXCL12-dependent cell polarization and migration is reduced in Miro-1-interfered cells, due to impaired myosin II activation at the cell uropod, and diminished actin polymerization. These data point to a key role of Miro-1 in the control of lymphocyte adhesion and migration through the regulation of mitochondrial redistribution.
Introduction

The recruitment of blood leukocytes to the site of inflammation involves a sequential, multistep adhesion cascade between the leukocyte and endothelial-cell adhesion molecules that mediates leukocyte rolling, firm adhesion, and transmigration across the endothelium (1). Firm arrest of leukocytes on endothelial cells is mediated by the interaction of leukocyte integrins, mainly very late antigen-4 (VLA-4; $\alpha_4\beta_1$) and lymphocyte function-associated antigen-1 (LFA-1; $\alpha_L\beta_2$), with their respective endothelial counter-receptors vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1) (2). During this process the leukocyte and endothelium cytoskeletons undergo an extensive reorganization that ensures sufficient duration of the contact and enables leukocyte extravasation. Leukocyte navigation through tissues is governed by extracellular signals, such as chemoattractant gradients (chemotaxis) and adhesion signals. Chemokines, by activating specific receptors of the G-protein-coupled receptor (GPCR) family, generate leukocyte polarity by inducing the formation of a leading edge (through actin polymerization) and a uropod, a slender posterior appendage characterized by actin–myosin-driven contraction (3, 4).

Mitochondria are highly dynamic organelles continuously remodelling their shape and size through fission and fusion events. They are actively relocalized within the cell through a cytoskeleton-based transportation system (5). These organelles move along microtubules in both anterograde and retrograde directions, using kinesin and dynein motor proteins, respectively (6). Specific subcellular locations with high energy and Ca$^{2+}$-buffering requirements, such as cellular synapses and other polarized structures, require the establishment of a high local density of mitochondria (7, 8). In leukocytes, mitochondria relocalize to the uropod during chemotaxis (9) and to the immune synapse (IS) to enable modulation of its architecture and downstream

3
signalling (10-12). The molecular mechanisms driving this mitochondrial positioning to specific subcellular locations in lymphocytes are however not well understood.

It has been reported that the atypical Rho GTPase Miro-1 plays an essential role in the regulation of mitochondrial morphogenesis and trafficking along microtubules (13, 14), and might serve as a calcium-dependent sensor for the control of mitochondrial motility (15-17). Miro-1 binds the cytoplasmic adaptor protein milton and kinesin heavy chain through its cytoplasmic domains, thereby connecting mitochondria to microtubules (18). Miro-1 contains a transmembrane domain that anchors it to the outer mitochondrial membrane, two GTPase domains and two Ca\(^{2+}\)-sensing EF-hand domains that protrude into the cytoplasm (14). Upon binding of Ca\(^{2+}\) to its EF-hand domains, Miro-1 dissociates from microtubules (19). The expression of a Miro-1 form with a double mutation in its EF-hands prevents the arrest of mitochondria in response to cytoplasmic Ca\(^{2+}\) elevation (15-17) and decreases the level of Ca\(^{2+}\) entering mitochondria (20).

Here we show that the lymphocyte mitochondria specifically redistribute to the adhesion zone in close contact with the endothelium. Our results indicate that Miro-1, through the regulation of mitochondria movement along microtubules and its association with dynein/dynactin motors, influences mitochondria positioning. Deficiency in Miro-1 prevents correct interaction with inflamed endothelium, lymphocyte polarization and chemotactic migration.
Materials and Methods

Cells, plasmids and cell transfection.

HUVEC were obtained and cultured as previously described (21). To activate HUVEC, TNF-α (20 ng/ml; R&D Systems) was added to the culture medium 20 h before adhesion/migration assay. Human PBLs and T lymphoblasts were obtained and cultured as described elsewhere (22). CEM T cells, CH7C17 T cells, and the human erythroleukemic cell line K562 stably overexpressing α4 or αL integrin were grown in RPMI 1640 medium (GIBCO BRL) supplemented with 10% fetal calf serum (FCS) (Invitrogen), 50 IU/ml penicillin, 50 μg/ml streptomycin. Medium for CH7C17 cells was further supplemented with 400 μg/ml hygromycin B and 4 μg/ml puromycin, and medium for K562 with 1 mg/ml G418. The HEK293T cell line was cultured in DMEM (Sigma) supplemented with 10% FCS.

Plasmids encoding fluorescent-tagged ICAM-1 proteins (GFP, YFP and CFP) were described previously (23). Plasmids encoding mtRFP or mtYFP (24) were generously provided by Dr L. Scorrano (University of Padua, Italy). The EB1-GFP construct was a kind gift from Dr. Anna Akhmanova (Utrecht University, The Netherlands). The GFP-Miro construct was a gift from Dr J. T. Kittler (University College London); the double K208, K328 Miro-1 mutation (Miro-1 KK) was generated by site-directed mutagenesis with the QuickChange protocol (Stratagene). SiRNAs against human DHC were previously described (25); those against Miro-1 were a SMARTpool of 4 distinct siRNAs (5’-GCUUAUCUGACUCGCAAA-3’; 5’-CCAGAGGGAGAGACAGGAA-3’; 5’-GCAAUUGCGAGGCUGUA-3’; 5’-UGUGCAGUGUCAGCGA-3’) obtained, together with the negative control siRNA (5’-AACUCCGAGCUAGCAGCUAC-3’), from Thermo Scientific Dharmacon. T-cell lines, human T lymphoblasts and HUVEC were transfected with DNA plasmids or with specific doublestranded siRNAs by electroporation using
the Gene Pulser II electroporation system (Bio-Rad Laboratories) or the Nucleofector system (Amaxa Biosystems). HEK293T cells were transfected using Lipofectamine (Invitrogen). CEM T cells were electroporated twice (at 0 and 72 h) with specific siRNA at a final concentration of 2μM. The efficiency of gene silencing was checked by western blot, and cells were used for experiments on day 6 after the first electroporation. Transiently transfected HUVEC were used 24–48 h after transfection.

Reagents and antibodies.

Recombinant human fibronectin (FN), poly-L-lysine (PLL), human γ-globulin, oligomycin, FITC-conjugated or unconjugated anti-α-tubulin and anti–DHC (clone 440) were from Sigma-Aldrich. The mitochondrial calcium uptake inhibitor Ru360 and antibiotic G418 were from Calbiochem. The fluorescent cell tracker BCECF-AM, Alexa 488 and 647 phalloidin conjugates, JC-1, Fluo4-AM, Mitotracker Orange, Mitotracker Green, DiO, DiD and Dil Vybrant Cell-Labeling Solutions and all fluorochrome-conjugated secondary antibodies were from Invitrogen. Puromycin was from InvivoGen, and hygromycin B from Roche. PureCol (Advanced BioMatrix) 3 mg ml⁻¹ and Collagen I Bovine (GIBCO) were employed respectively for standard collagen assays and for collagen titration experiments. The antibodies D3/9 (anti-CD45), HP 2/6 (anti-CD4), the HP2/1 (anti-α4 integrin) and TS1/11 (anti-αL integrin) were produced in our laboratory (23). TP 1/25 (anti ICAM-3) and TS2/16 antibody (anti-β1 integrin) were described previously (21, 26). Hu5/3 antibody (anti-ICAM-1) was kindly provided by F.W Luscinskas (Brigham and Women Hospital, Boston). Polyclonal antibody against ezrin/moesin (90/3) was provided by Dr. Heinz Furthmayr (Stanford University, Stanford, CA). The rabbit polyclonal anti-Mn-SOD antibody was from Rockland. Anti Erk1/2 and anti p-74 were from Millipore;
phospho-Erk1/2 and anti pSer19 MLC from Cell Signaling Technology; monoclonal p50-
dynamitin, anti p150-glued and anti CXCR-4 from BD Transduction Laboratories; and anti-GFP
from Living Colors (Clontech Laboratories, Inc.). Anti-Miro-1 for western blot was from Abanova
and for IP from Abcam. Anti Dynein Heavy Chain antibody was from Santa Cruz. Anti-Giantin
was from Abcam. HRP-conjugated secondary antibodies were from Pierce. Recombinant human
CXCL12 was from ImmunoTools. Integrin inhibitors BIO5192 and BIRT377 were provided by
Biogen Idec and Boehringer Ingelheim, respectively (27).

Fluorescence confocal microscopy.
For immunofluorescence assays, lymphocytes were plated on slides coated with PLL or FN or on
confluent HUVEC for 20 min at 37°C, fixed with 4% paraformaldehyde (PFA) (Electron
Microscopy Sciences), stained with the indicated antibodies and mounted in Prolong (Invitrogen).
Where indicated, the PLL or FN matrix included 10 nM CXCL12. To detect intracellular proteins,
cells were permeabilized for 3 min with 0.2% Triton X-100 in PBS before staining. Confocal
images were obtained with a Leica TCS-SP5 confocal scanning laser unit with an inverted
epifluorescence microscope (DMI6000B). Microscopes were maintained at 37°C, 5% CO2 and
covered by a full acrylic box, allowing live-cell imaging. For time-lapse fluorescence imaging,
cells were plated onto coated 35 mm dishes (MatTek).
Redistribution of lymphocyte mitochondria towards the endothelium was calculated as
percentage of the fluorescence intensity (FI) of lymphocyte mitochondria located in the third of
the cell near the HUVEC contact area. FI was measured using ImageJ or Metamorph. Cellular
polarity was assessed by the elliptical form factor, calculated from images with membrane protein
staining using Metamorph software. pMLC concentration at the uropod with respect to the
leading edge and the three-dimensional distance between the mitochondrial network and the MTOC were respectively quantified using the Fluorescence Ratio and Distance Measure plugins for ImageJ.

**Calcium measurement.**

For measurement of intracellular Ca\(^{2+}\)-flux, control and Miro-1-interfered CEM cells were respectively pre-incubated with DiD and Dil to distinguish them. The pooled cells were loaded with Fluo-4-AM (12) and settled onto MatTek glass bottom dishes covered by a TNF-\(\alpha\)-activated HUVEC monolayer. Images were acquired every 10s, using a TCS SP5 confocal microscope. The mean Fluo-4 fluorescence intensity of each migrating cell in each frame was calculated by Wimasis GmbH. Calcium peak intensity was calculated by subtracting the basal fluorescence intensity from the maximum fluorescence registered in each peak. Peak duration was calculated by multiplying the number of frames in which it was registered for frame duration in seconds.

**Fluorescence Image analysis.**

Images were analysed with Leica LASAF (Leica Microsystems), Metamorph (Universal Imaging Corporation, Downingtown, PA) or ImageJ (NIH) softwares. Mitochondria and MTOC 3-D reconstructions were obtained using Imaris 7.6 software (Bitplane).

**Fluorescence ratios.**

To measure the relative fluorescence between two regions we computed the ratio between the average fluorescence in the two areas corrected for background fluorescence according to the following model. The model assumes that the observed fluorescence in the different areas can be
modeled as the contribution of background fluorescence (Bg), fluorescence in region of interest A and fluorescence in region of interest B:

\[
\begin{pmatrix}
B_{\text{obs}} \\
A_{\text{obs}} \\
B_{\text{obs}}
\end{pmatrix} = \begin{pmatrix}
1 & 0 & 0 \\
1 & 1 & 1 \\
1 & 0 & 1
\end{pmatrix}
\begin{pmatrix}
B_g \\
A \\
B
\end{pmatrix}.
\]

In the model, fluorescence observed in region A is the sum of the background fluorescence and the fluorescences of regions A and B, while the fluorescence observed in region B is the contribution of the background fluorescence and the fluorescence of region B.

Background-corrected contributions can be estimated by inverting the model:

\[
\begin{pmatrix}
B_g \\
A \\
B
\end{pmatrix} = \begin{pmatrix}
1 & 0 & 0 \\
0 & 1 & -1 \\
-1 & 0 & 1
\end{pmatrix}
\begin{pmatrix}
B_{\text{obs}} \\
A_{\text{obs}} \\
B_{\text{obs}}
\end{pmatrix}.
\]

Note that the estimated A and B have already been corrected for the background fluorescence, and that A no longer has a contribution from region B (it is the pure contribution of region A).

Finally, we compute the relative fluorescence ratio as:

\[r = \frac{A + B}{B}.
\]

Distance measurements.

To measure the distance between two three-dimensional fluorescence distributions in independent channels, we binarized both grey-scale stacks (the user can select two thresholds, so that the region of interest is isolated in both channels). We then computed the Euclidean distance between every possible pair of voxels (one in distribution A, and the other in distribution B). This gives us the exact distribution of pairwise distances. Note that the calculation is fully computed in 3D and therefore that the actual distance between the two regions of interest is calculated. Finally, we
computed a truncated mean by computing the distance mean after discarding the 5% lower and upper values. This mean is known to be a robust estimate of the distribution mean.

**Total internal reflection fluorescence microscopy (TIRFM).**

For TIRFM, control and Miro-1-interfered CEM cells, transfected with EB1-mGFP, were sorted to warrant transfection and cultured for 24h. Cells were allowed to settle onto FN coated glass bottomed microwell dishes, No 1.5 (Mattek; Ashland, MA, US). Recording was initiated 3 min after cell plating and cells were visualized with a Leica AM TIRF MC M mounted on a Leica DMI 6000B microscope coupled to an Andor-DU8285_VP-4094 camera. Images were acquired with a HCX PL APO 100.0x1.46 OIL objective and processed with the accompanying confocal software (LCS; Leica) as described (28). For EB1-GFP microtubule plus tip tracking, penetrance was 150 nm for 488 nm laser channel with same objective angle and 0.26 s exposure time. Synchronization was performed through the Leica software. Images were analyzed and converted into movies using Imaris software (Bitplane, Switzerland). Quantification of the number of microtubule nucleation events, tracking of microtubule movement trajectories, and computation of microtubule growth were performed with the ‘connected components’ algorithm for particle tracker. EB1-GFP comets were detected by the surface algorithm detection based on channel intensity and using a local background. Analysis was conducted on movie lengths of 300 s that were captured at a rate of 0.5 frame/s. To exclude microtubules that move ins and out of z-plane, events shorter than 2 \(\mu\)m were not incorporated into the final analysis.

**Ligand-coated microspheres.**

Styrene divinilbenzene latex beads (6\(\mu\)m diameter, Sigma), were coated with VCAM-1-Fc or
ICAM-1-Fc, produced in our laboratory. (29) CH7C17 cells preloaded with mitotracker orange (2 \(10^5\)) were mixed with pre-coated latex beads (2 \(10^5\)), incubated for 30 min at 37°C in HBSS, 2% BSA, and plated on FN-coated slides. Slides were fixed and immunostained for tubulin. Redistribution of mitochondria and MTOC was considered to have occurred if they were more abundant in the third of the cell centred on the contact with the latex bead.

**Immunoblotting and co-immunoprecipitation.**

After the indicated treatment, CEM and CH7C17 T cells were lysed as described (12). For co-immunoprecipitation assays transfected HEK293T cells or primary T lymphoblasts were lysed in ice-cold lysis buffer as described (14) containing 0.5% Triton X-100. Lysates were centrifugated at 16,000g for 10 min at 4°C, and the resulting supernatants were precleared for 1 h at 4°C with Protein G Dynabeads (Life Technologies) and incubated for 3 h with anti-GFP or with anti-Miro-1. Immunocomplexes were collected by incubation with protein G Dynabeads for 1 h. The samples were thereafter washed six times with ice-cold lysis buffer and processed for western blotting. Proteins were separated by SDS-PAGE and visualized with FUJIFILM LAS-3000 after membrane incubation with specific primary antibodies and peroxidase-conjugated secondary antibodies. Band intensities were quantified using Image Gauge software (Fujifilm) and results normalized to \(\alpha\)-tubulin or, for phosphoproteins, to total protein expression.

**Parallel plate flow chamber analysis of endothelial-leukocyte interactions and detachment.**

PBLs, T lymphoblasts, PBMCs and CEM cells (1\(x\)10\(^6\)/mL) were drawn across activated confluent HUVEC monolayers at an estimated shear stress of 1.8 dyn/cm\(^2\) (assumed as
physiological for human post-capillary venules where transmigration takes place) for the required time. The shear stress was applied as described (23).

Control and Miro-1-interfered CEM cells were first labeled with DiO, Dil or Did and then with Mitotracker Orange or Green. Cells (10^6/ml from each type) were drawn across on activated confluent HUVEC monolayers with a shear stress of 1.8 dynes/cm². Images were acquired every 30 sec. Cells labeled with different cell trackers or Mitotrackers displayed similar results, confirming that there were no interference with the cellular processes analysed.

For detachment experiments, control and Miro-1-interfered CEM cells were pre-incubated with Mitotracker Orange or Green to distinguish them and allowed to adhere for 5 min at 37°C to activated HUVEC monolayers. Shear stress was started at 0.5 dyn/cm² and increased up to 15 dyn/cm². Cell detachment was calculated by normalizing the number of adhered cells to the number observed at the flow rate of 0.5 dyn/cm².

Adhesion assays.

HUVEC were grown to confluence in 96-microwell plates (Costar) and activated with TNF-α. CEM or CH7C17 cells were labeled with 1μM BCECF-AM, and adhered to the activated HUVEC (21). In other experiments, CEM cells (1 x 10^6/mL) were left untreated or pretreated with 4μM olygomicin or 10μM Ru360 (30min) and washed and resuspended in HBSS, 2% FCS.

The cells were then adhered to activated HUVEC in 6-well plates for 30 min at 37°C, under static conditions or with continuous rotation (60 rpm). After incubation the plates were washed three times with HBSS, adhered cells were fixed with 4% PFA. The effects of inhibitors on lymphocyte adhesion were assessed by counting the number of adhered cells using the CellCounter ImageJ plugin. Results were normalized to untreated cells adhered to activated
HUVEC. Cells adhered to non-activated HUVEC were used as a negative control.

Migration and transmigration assays.

Migration assays were performed in 5 μm pore Transwell cell culture chambers (Costar) (30). Trasmigration through a confluent monolayer of activated HUVEC was assayed in 5μm pore Transwell plates (23). The number of migrated cells was measured by flow cytometry.

3-D migration assay.

Migration of CEM T cells through a 3D-collagen matrix was assayed according to manufacturer’s instructions (ibidi). Control and Miro-1-interfered CEM cells were pre-incubated with DiD or Dil, and images were acquired every 5 min for standard assays (Fig. 4C) and every 3 min for titration experiments (Fig. 4D). Images were analysed using the Manualtracking and Chemotaxis Tool ImageJ plugins.

F-actin determination.

Levels of polymerized actin were measured as described (31). Briefly, cells (1x10^5 per well of a 96-well plate) were incubated with 10 nM CXCL12 at 37°C for the indicated times. Cells were then fixed, permeabilized and stained with Phalloidin-Alexa647 (Invitrogen). Fluorescence intensity was determined in a FACSCanto flow cytometer (BD Biosciences) and analysed with FlowJo Software. Results are expressed as the fold increase in mean fluorescence intensity relative to the cells not exposed to CXCL12.

Flow cytometry calcium measurement.
To track intracellular calcium levels in cells in suspension, control and Miro-1-interfered CEM cells were incubated with DiD or Dil and Fluo4-AM and, after 1 min baseline recording, CXCL12 was added (10 nM). Calcium flux was measured over time using a FACSCanto flow cytometer. Ionomycin (0.5 mg/ml) was used as a positive control. Data were analysed with Flow-Jo Software.

**ATP measurement.**

For intracellular ATP quantifications, frozen cells were homogenized with a Lysis Buffer (25 mM Tris-HCl pH= 7.5; 0.1% Triton X-100) and ATP was rapidly measured by a coupled luciferin/luciferase reaction (32) with ATP determination kit (Invitrogen, Paisley, United Kingdom). For protein quantification, a colorimetric method (Pierce BCA Protein Assay Kit) was used following the manufacturer’s instructions.

**Mitochondrial membrane potential.**

The mitochondria-directed fluorescent sensitive probe JC-1 was used to determine variations in mitochondrial membrane potential ($\Delta\psi_m$) (12). Briefly, CEM T cells ($1 \times 10^5$ per well of a 96-well plate) were preloaded with JC-1 (2 mg/ml) and stimulated with 10 nM CXCL12 at 37°C for the indicated times. FL1 (520 nm) and FL2 (590 nm) emissions were recorded in a FACSCanto flow cytometer and analysed with Flow-Jo Software.

**Statistical analysis.**

Statistical analysis and comparisons were made with GraphPad Prism5. Data were tested for normality using the D’Agostino-Pearson omnibus normality test, or the Kolmogorov–Smirnov
test when the sample was small due to experimental conditions. Differences between means were tested by Student’s t-test for normal data. Data was analyzed using one-way ANOVA with Bonferroni post-test (Fig. 2D; Fig. 5D and Fig. S4A) and with Tukey post-test (Fig. 7D), two-way ANOVA with Bonferroni post-test (Fig. 3D).
Results

Mitochondria positioning to the adhesion zone during leukocyte–endothelial cell interaction.

We first assessed the specific redistribution of leukocyte mitochondria during the interaction between leukocyte and endothelial cells. Using time-lapse confocal microscopy, we tracked lymphocyte mitochondria using mitochondrial-targeted fluorescent proteins or with Mitotracker Orange, during their interaction with TNF-α-activated primary human umbilical vein endothelial cells (HUVEC). During static adhesion of human T-lymphoblasts to activated HUVEC, most lymphocyte mitochondria localized to the leukocyte-HUVEC contact zone. During subsequent polarization of the migrating lymphoblast we observed a further relocalization of mitochondria towards the uropod (Fig. 1A; see Movie S1 in the supplemental material). The initial redistribution of mitochondria to the contact zone during endothelial adhesion was also observed in the T-cell line CEM both in static and flow conditions (Fig. 1B). This biphasic mitochondria repositioning, with initial concentration at the endothelial contact zone followed by polarization to the uropod in migrating cells, was also seen under physiological flow conditions, and with freshly isolated leukocyte populations (see Fig. S1A and B in the supplemental material). These data indicate that redistribution of mitochondria to the adhesion zone and later to the uropod constitutes a cellular signature of the attachment of leukocytes to endothelium and their subsequent migration.

Mitochondria bind microtubule-associated proteins (MAPs) and move along the microtubule network (6). To monitor the redistribution of the microtubule-organizing centre (MTOC) and mitochondria in lymphocytes adhering to activated endothelium (Fig. 1C), we transfected human T lymphoblasts with Paxillin-GFP (33) and mt-RFP to simultaneously track MTOC and
mitochondria (Fig. 1D). During lymphocyte adhesion, most lymphocyte mitochondria and the MTOC moved in a concerted fashion to the contact zone with the endothelium (Fig. 1D). In the reverse experiment, in which endothelial mitochondria were tracked by labelling with mt-YFP, we did not detect a significant shift of position upon lymphocyte adhesion (Fig. 1E). Confocal microscopy revealed that endothelial mitochondria co-localized with tubulin but not with the actin cytoskeleton (see Fig. S1C in the supplemental material). In addition, endothelial mitochondria were not present at the docking structure (23) formed after leukocyte arrest on the endothelium (see Fig. S1D in the supplemental material). These results indicate that mitochondrial redistribution is a specific process in leukocytes during interaction with endothelium.

Mitochondria redistribution depends on lymphocyte integrin engagement.

We next assessed whether mitochondria relocalization to the contact zone with endothelium is dependent on specific interactions between lymphocyte integrins and adhesion molecules present on activated endothelium. For this, we added lymphoblasts to resting or TNF-α-activated HUVEC and quantified mitochondrial density as the percentage of the total mitochondrial fluorescence intensity located in the contact area (the third of the cell adjacent to the contact site; Fig. 2A). Upon adhesion to activated endothelial cells, about 60% of mitochondrial fluorescence was present at the contact area, contrasting with 30% upon adhesion to non-activated endothelium (Fig. 2A). To dissect the role of lymphocyte integrins, we incubated T lymphocytes with beads coated with ICAM-1 or VCAM-1, the ligands of integrins LFA-1 and VLA-4, respectively, or with BSA as a negative control (Fig. 2B). Engagement of VLA-4 or LFA-1 by their specific ligands significantly increased translocation of both
mitochondria and MTOC. The translocation rates of both MTOC and mitochondria in response
to VLA-4 engagement (with a fold induction in comparison to BSA of 2.1 for MTOC and 1.65
for mitochondria) were higher than those observed with LFA-1 (respectively of 1.5 and 1.25)
(Fig. 2B and C). Furthermore, T lymphoblasts, treated with specific LFA-1 or VLA-4 inhibitors
and added to TNF-α-activated HUVEC, showed a clear reduction in mitochondria translocation
in comparison to the untreated cells (Fig. 2D), assed by mitochondrial fluorescence intensity
localized at the contact area with endothelium. These data demonstrate that specific engagement
of both VLA-4 and LFA-1 is sufficient to promote MTOC and mitochondria translocation
towards the cell-to-cell contact during lymphocyte adhesion to endothelium.

Retrograde movement of mitochondria along microtubules (towards the minus end where MTOC
is allocated) is mediated by dynein (6). Cytoplasmic dynein is a multisubunit microtubule motor
complex that, together with its activator dynactin, drives vesicular cargo towards the minus ends
of microtubules (34). Specific silencing of dynein heavy chain (DHC) in T lymphocytes (see Fig.
S2A in the supplemental material) uncoupled mitochondria from the MTOC and impaired the
recruitment of both organelles to the contact zone (see Fig. S2B in the supplemental material).
These cells showed an impaired the ability to adhere to activated HUVEC (see Fig.S2C in the
supplemental material).

Miro-1 regulates mitochondria redistribution and lymphocyte firm adhesion
to activated endothelium.

Miro-1 is a calcium-dependent sensor that controls mitochondrial motility along
microtubules, and therefore mitochondrial trafficking (14-17). We examined the possible
involvement of Miro-1 in mitochondrial translocation during lymphocyte adhesion. Depletion of
Miro-1 with a specific siRNA (Fig. 3A), that did not affect Miro-2 expression (see Fig. 3SA in supplemental material), decreased mitochondrial translocation in T cells adhered to activated HUVEC (Fig. 3B), suggesting that Miro-1 controls mitochondria positioning in response to integrin engagement in T cells. In contrast to DHC-interfered cells, Miro-1-depleted ones showed a slight but non-significant decrease of cell adhesion under static conditions (Fig. 3C). A critical step during lymphocyte interaction with the endothelium is firm adhesion. Therefore, we analyzed the role of Miro-1 in firm adhesion by measuring the resistance of T lymphocytes to be detached from endothelium under increasing shear stress. Importantly, Miro-1-silenced T cells detached more readily than controls from HUVEC at a flow rate of 5 dyn/cm² or above (Fig. 3D), suggesting a specific role of Miro-1 in regulating lymphocyte adhesiveness strength. To explore the role of Miro-1 in lymphocyte polarization, we analyzed the shape of lymphocytes during adhesion to HUVEC under flow conditions by measuring their elliptical form factor. Interestingly, under these conditions, hyperpolarized cells were present in control but not Miro-1-interfered cells (see Fig. 3S in the supplemental material). We next investigated whether the defects observed under physiological flow conditions both in firm adhesion and polarization were accompanied with impaired mitochondrial dynamics. Miro-1-depleted cells presented an evident impaired ability to firmly adhere, polarize and migrate (see Movie S2 in the supplemental material). Moreover, mitochondrial redistribution to the uropod during polarization and the subsequent migration was greatly affected (Fig. 3E; see Movie S3 in the supplemental material).

**Miro-1 knockdown impairs lymphocyte chemotaxis.**

To assess whether Miro-1 regulates lymphocyte migration capability, we examined CXCL12-induced migration of control and Miro-1-interfered cells in modified Boyden
chemotaxis chambers coated with a TNF-α activated HUVEC monolayer or uncoated. In both cases, Miro-1-silenced cells migrated less efficiently along the chemotactic gradient (Fig. 4A and B). Miro-1 knockdown also disrupted the directionality and reduced the speed of T-cell migration towards CXCL12 in 3D collagen gels (Fig. 4C). These data indicate that expression of Miro-1 is essential for proper directional lymphocyte migration in response to a chemotactic stimulus not only in conditions greatly dependent on integrin function, but also in an integrin-independent migration mode (35). We further analyzed the contribution of Miro-1 during lymphocyte migration within 3D gels containing different collagen concentrations. Remarkably, Miro-1 interfered cells migrate slower than control cells through different collagen densities (Fig. 4D). It is noteworthy that both, control and Miro-1-depleted cells, showed the same trend in the decrease of mean velocity at increasing collagen densities (35).

Miro-1 controls CXCL12-mediated lymphocyte polarization and mitochondria-MTOC association.

Chemokines can increase lymphocyte adhesion to endothelium, and also induce the acquisition of a polarized, pro-migratory cell shape (36). CXCL12-induced polarization was impaired in Miro-1-interfered cells (Fig. 5A). Upon Ca²⁺ binding to its EF-hands, Miro-1 undergoes a conformational change resulting in the dissociation of mitochondria from microtubule track (19). Therefore, we studied polarization after CXCL12 stimulation in lymphocytes overexpressing GFP (as a negative control), GFP-Miro or GFP-MiroKK, a Miro-1 form with a double mutation E208K/E328K unable of Ca²⁺ binding in its EF-motifs(14). The overexpression of Miro-1KK with EF-motif mutations was able to decrease CXCL12-induced polarization (Fig. 5B; see Movie S4 in the supplemental material). Moreover,
we assessed the effect on CXCL12-mediated polarization of Miro-1 \(^{KK}\) overexpression in cells previously silenced for Miro-1. As shown in Fig. S4A of the supplemental material, the overexpression of GFP-Miro \(^{KK}\) did not restore cell polarity whereas GFP-Miro rescued the phenotype observed in control cells, underlining the importance of Miro-1 calcium binding domains for its correct function in chemokine-induced lymphocyte polarity.

We next assessed whether the defect in adhesion strength of Miro-1-silenced cells as well as in polarization in both Miro-1-interfered and Miro \(^{KK}\) -expressing cells might be related to impaired association of mitochondria with the MTOC. For this, we plated T cells on a fibronectin (FN) or poly-L-lysine (PLL) matrix containing CXCL12 and measured the distance between the centre-of-mass of mitochondrial fluorescence and the MTOC. In control cells, CXCL12 stimulation reduced the distance between mitochondria and the MTOC at the uropod (Fig. 5C and D; see Movie S5 in the supplemental material). This effect of CXCL12 was not observed in Miro-1-silenced cells (Fig. 5C and D; see Fig. S4B and Movie S5 in the supplemental material), supporting the importance of Miro-1 in the relocalization of MTOC and mitochondria to the uropod. Moreover, T cells overexpressing GFP-Miro \(^{KK}\) showed a reduced proximity of MTOC and mitochondria induced by CXCL12 in comparison with T cells overexpressing GFP-Miro (Fig. 5E and F; see Fig. S4C and Movie S6 in the supplemental material). Upon chemokine stimulation, mitochondria first redistribute towards the uropod (9) together with the MTOC. Our results indicate that Ca\(^{2+}\) binding to Miro-1 EF-hands might lead to a conformational change in Miro-1, resulting in the halt of mitochondria in this specific subcellular location.

The effect of targeting Miro-1 by either silencing or GFP-Miro \(^{KK}\) was exerted specifically at the mitochondria/MTOC polarized association, as other cell polarity parameters as membrane receptors or cytoskeletal proteins (ICAM-3 or ERM) remained unaltered (see Fig. S4B and C in the supplemental material).
To rule out possible pleiotropic effects produced by Miro-1 knock-down on the microtubular skeleton, we have assessed parameters as the general conformation and dynamics of the tubulin network and the localization of the Golgi Apparatus (GA) (37). Neither tubulin network nor GA were apparently altered. Hence, GA showed its usual localization around the MTOC in both control and silenced cells (see Fig. S5A in the supplemental material). Moreover, the analysis of microtubule dynamics in control and Miro-1-silenced cells showed similar microtubule growing rates (siCtrl=14.60 ± 3.11 μm.min⁻¹ and siMiro=15.01 ± 2.82 μm.min⁻¹) (see Fig. S5B and Movie S7 in the supplemental material). Furthermore, membrane expression of several cell markers, including integrins and the CXCL12-receptor CXCR4, as well as the CXCL12-mediated endocytosis of CXCR4 were largely unaffected (see Fig. S5C and D in the supplemental material).

These results indicate that Miro-1 mediates mitochondria positioning and MTOC association, a phenomenon specifically implicated in the chemokine-induced acquisition of a polarized morphology by lymphocytes.

**Miro-1 associates with dynein complex and contributes to regulate CXCL12-induced lymphocyte activation.**

To investigate how Miro-1 controls mitochondrial positioning we transfected HEK293T cells with GFP-Miro-1 or GFP alone and examined GFP immunoprecipitates from these cells. The dynein p74 intermediate chain and the dynactin p50 subunit were specifically detected in GFP-Miro-1 immunoprecipitates (Fig. 6A). Therefore, we analyzed this interaction in endogenous complexes from human T lymphoblasts lysates. Dynein heavy chain and p150-glued component of dynactin were co-immunoprecipitated with Miro-1 in these cells (Fig. 6A right
panel). Hence, Miro-1 appears to be a specific mediator of the interaction between mitochondria and the dynein/dynactin motor complex.

To explore the molecular basis of inhibited polarization in Miro-1-silenced T-cells, we studied molecular hallmarks of chemokine-induced lymphocyte polarization. One of these hallmarks is rapid actin polymerization (38), and we detected decreased actin polymerization in response to CXCL12 in Miro-1-interfered cells (Fig. 6B and C). Another polarity hallmark in T cells is myosin light chain (MLC) phosphorylation, required for efficient retraction of the uropod by Myosin II (30). Interestingly, CXCL12-induced MLC phosphorylation was inhibited in the uropod of Miro-1-silenced cells (Fig. 6D), suggesting a specific role of Miro-1 in cell polarization.

We next examined the intracellular calcium dynamics in response to CXCL12 stimulation and during lymphocyte migration on activated endothelium. Interestingly, when cells were stimulated with CXCL12 in suspension, we observed no significant differences between control and Miro-1 interfered-cells (Fig. 7A). In contrast, when T cells adhered and migrated on activated endothelium, Miro-1 depletion had a negative impact on the intensity and duration of calcium peaks (Fig. 7B and C). These data indicate that Miro-1 regulates intracellular calcium not in a general scale but in a subcellular context.

Mitochondrial activity is reflected in the electrical potential (Δψm) across the inner membrane, and has a direct influence on their axonal transport (39). It has been shown that an increased Δψm enhances the rate of mitochondrial transport, most obviously retrograde movement (40). We found that CXCL12 increased Δψm, but no significant differences were observed between the responses of control and Miro-1-silenced T cells (see Fig. S6 in the supplemental material). Miro-1 depletion thus appears to affect CXCL12-induced movement of mitochondria without
significantly affecting their intrinsic activation capacity. Moreover, to rule out possible alterations in mitochondrial functioning due to the down-modulation of Miro-1 expression, we assessed ATP cellular content in both control and Miro-1-silenced cells. Control and Miro-1-interfered cells presented similar ATP content and CXCL12 stimulation led to a significant reduction in ATP levels in both cells (Fig. 7D). Chemokine receptors activate multiple signaling pathways, leading to varied functional outcomes, including adhesion, polarization, and chemotaxis (41). Among these, the ERK1/2 phosphorylation is one of most characterized cell signalling signature (42). Therefore, we assessed the role of Miro-1 in the modulation of signaling downstream of CXCL12 receptor through the study of the phosphorylation kinetics of ERK1/2. Maximal ERK1/2 phosphorylation was detected within 2 min of chemokine stimulation in control T cells, and this effect was clearly inhibited in Miro-1-silenced cells (Fig. 7E).
Discussion

In this study, we demonstrate that mitochondria relocalize to the contact area during lymphocyte adhesion to endothelial monolayers, and that this is directed by the GTPase Miro-1. Redistribution of mitochondria is specific to the leukocyte, and is not observed in endothelial cells. The movement of mitochondria involves the LFA-1/ICAM-1 and VLA-4/VCAM-1 receptor-countereceptor pairs.

It has been reported that MTOC and mitochondria can be recruited by chemokine-activated LFA-1 at the immunological synapse (43). Here, we provide evidence that the signal generated by VLA-4 is even more efficient in the redistribution of both MTOC and mitochondria. In our model, mitochondrial arrest in the cellular zone of integrin engagement might be related to local actin polymerization and increased intracellular Ca\(^{2+}\). The cytoplasmic tail of the \(\alpha_4\) subunit of VLA-4 interacts with paxillin (44), which is a docking protein for other cytoskeletal proteins such as F-actin, for tyrosine kinases and GTPase activating proteins (45). Moreover, VLA-4 engagement can promote the tyrosine phosphorylation of a broad range of proteins, including proline-rich tyrosine kinase-2 (Pyk2), focal adhesion kinase (FAK) and c-Src (46), resulting in the activation of several pathways. Accordingly, increased intracellular Ca\(^{2+}\) and LFA-1 engagement trigger activation and redistribution of Pyk2 to the MTOC in T-lymphoblasts (47).

All these signalling cascades might be involved in the confinement of mitochondria and MTOC at the contact zone.

The regulation of the interaction of mitochondria with the cytoskeleton is crucial for proper mitochondrial function at specific sites of action (6). In our search for a specific adaptor between mitochondria and microtubules, we focused on Miro-1, a member of the mitochondrial Rho GTPase family of proteins together with Miro-2. Our observations on Miro-2 expression level
suggest us that Miro-1 plays unique functions in mitochondrial localization that cannot be
replaced by Miro-2. Miro-1 localizes at the outer mitochondrial membrane and has two EF-hand
Ca²⁺-binding domains (13). Our data show, for the first time, that Miro-1 interacts with the
dynein/dynactin complex, whereas interaction of Miro-1 with kinesin was previously described to
promote anterograde mitochondrial movement (14, 15, 17, 18). This interaction may account for
previously described effects on retrograde mitochondria movement (48). In this regard, kinesin
and dynein are interdependent in axonal transport (49), interacting directly through kinesin light
chains (50) and indirectly through the same region of p150Glued dynactin (51). These actions
coordinate bi-directional movement of organelles along axon microtubules. Recent evidence
suggests that kinesin and dynein can interact with the same mitochondrion, determining its
complex mobility patterns in axons (52). Mitochondria can buffer the Ca²⁺ that enters T-
lymphocytes via store-operated Ca²⁺ channels (53). A recent study identified Miro-1 as a Ca²⁺
sensor involved in the regulation of mitochondrial mobility in neurons and heart cell lines (16).
Upon Ca²⁺ binding to the EF-hand domains, a conformational change in Miro-1 provokes the
release of mitochondria from microtubules and their arrest at the subcellular site of calcium
influx, where they can contribute to the cell’s response to this second messenger (15-17). Miro-1
function has been studied primarily in neuronal cells where soma and synaptic structures can be
distant from each other. It is therefore conceivable that mitochondrial positioning influences
synaptic transmission by local ATP production and local Ca²⁺ buffering. Although changes of
mitochondrial positioning within a spherical small cell like lymphocytes may be more modest
(54), it is emerging the evidence on how mitochondrial morphology and positioning are crucial
for cellular signal threshold in immune cells (9-12, 43, 55). Our data demonstrate how changes of
only few micrometres in lymphocyte mitochondria positioning to the adhesion zone or to the
uropod are able to significantly affect both adhesion and migration. Unlike lymphocytes,
migrating epithelial cells require an anterior mitochondrial localization for correct migration. In these cells Miro-1 knockdown also perturbs mitochondrial localization and reduces their migratory capability (56), in agreement with our data. The mitochondrial localization correlates with MTOC localization in leukocytes and epithelial cells (57, 58) and calcium levels distribution (59).

In our model, the accumulation of mitochondria at the cell-to-cell contact suggests involvement of calcium in the polymerization and contraction of the actin cytoskeleton. This is in agreement with the defect in actin polymerization and in myosin light chain phosphorylation found in Miro-1-silenced cells, correlating with the deficiency in mitochondria polarization. These data suggest a possible function for the accumulation of mitochondria at the contact zone of the lymphocyte with the endothelium. Mitochondria may indeed supply the ATP necessary for the activity of the various kinases involved in these processes, as well as for actin polymerization and MLC phosphorylation required to establish and maintain persistent cell polarization. During firm adhesion, the combination of integrin signaling and exposure to immobilized chemokines on the apical surface of endothelial cells induces a marked change in the leukocyte morphology (60). Miro-1-interfered cells were unable to polarize properly under shear stress conditions, and these cells as well as MiroKK-expressing cells showed impaired polarization in response to CXCL12 stimulation, highlighting the importance of Miro-1 in mitochondria redistribution during cell polarization (61). These results indicate that Ca$^{2+}$-binding might dissociate Miro-1 from dynein/dinactin complex, as previously described for kinesin interaction (15, 17). However, additional studies are required to demonstrate this issue.

The loss of mitochondrial accumulation around the MTOC in Miro-1-silenced cells upon CXCL12 stimulation was accompanied by impaired migration, transmigration, as well as loss of directionality, reduced Ca$^{2+}$ oscillation during migration and inhibition of ERK1/2
phosphorylation. It is therefore conceivable that Miro-1 regulates, through Ca\(^{2+}\) binding to its EF-motifs, the redistribution and relocation of mitochondria around the MTOC at the uropod, where they not only provide ATP to sustain actomyosin contraction (9), but also contribute to Ca\(^{2+}\)-influx at the rear of the migrating cell. The localized Ca\(^{2+}\)-influx may contribute, through calmodulin-based machinery, to the activation of the MLC kinase required for uropod retraction during migration (62, 63). At the same time, mitochondrial Ca\(^{2+}\)-influx can increase ATP production by activating the tricarboxylic acid cycle and enhancing the activities of the electron transport-chain enzymes and the ATP synthase complex (64). Whether the impaired mitochondria redistribution to the uropod caused by Miro-1 down-regulation is able to reduce Ca\(^{2+}\) entry at this subcellular location remains to be elucidated. Nevertheless, we cannot rule out that Miro-1 might play additional functions unrelated to mitochondrial positioning that might contribute to its effects on actin polymerization, calcium oscillations and cell migration.

In summary, we demonstrate that Miro-1 can contribute to the regulation of mitochondrial translocation towards the MTOC during lymphocyte adhesion to the inflamed endothelium and the subsequent directed migration towards the site of inflammation, demonstrating the importance of mitochondrial subcellular localization in these processes. Considering the importance of an appropriate and rapid recruitment of leukocyte for an efficient immune response, our results shed new light about the regulation of adhesion and migration processes that could help the design of new more specific strategies for the treatment of a large variety of immunological diseases in which there is deregulated leukocyte recruitment.

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References


**Figure Legends**

Fig. 1. Mitochondria dynamics during leukocyte-endothelium interaction. (A) Representative images from a time-lapse video microscopy analysis of human T lymphoblasts loaded with Mitotracker Orange (red) and adhered to a TNF-\(\alpha\)-activated HUVEC monolayer transfected with ICAM1-GFP (green). Maximal projections of stacks near to endothelial monolayer are shown; discontinuous line delineates cell contour of adhering lymphocyte; arrows mark sites of leukocyte mitochondria accumulation; Ad (adhered lymphocyte), LE (lymphocyte leading edge), U (lymphocyte uropod). (B) Time-lapse sequence of orthogonal maximal projections of CEM T cells transfected with mt-RFP (fluorescence intensity colour gradient) adhering to an activated HUVEC monolayer transfected with ICAM1-YFP (scale of greys). Graph shows the percentage of T-cell mitochondrial fluorescence intensity at the contact area (illustrated in boxed region at time = 0, representing a third of the cell close to the HUVEC monolayer) during the adhesion, under flow conditions (1.8 dyn/cm\(^2\)), of CEM cells. (C) Left panel. Representative horizontal and orthogonal sections of a lymphoblast adhered to an activated HUVEC monolayer. Cells were stained with anti-MnSOD (red), anti-ICAM-1 (blue), and anti-tubulin-FITC (green). Right panel. Histograms show quantification of cells showing translocation of mitochondria and MTOC towards the contact zone in human lymphoblasts adhered to activated HUVEC monolayers. Data are means ± s.d. of two independent experiments (n=205 cells). NS: non-significant difference. (D) Representative orthogonal maximal projections from a time-lapse of a human T lymphoblast transfected with paxillin-GFP (green) and mt-RFP (red) adhering to an activated HUVEC monolayer (not shown); dashed line delineates cell contour of adhering lymphocyte. (E) Time-lapse of a human PBL (asterisk) migrating towards an activated HUVEC monolayer transfected.
with mt-YFP (yellow). Arrows mark the endothelial area in contact with the PBL. Scale bars, 10 μm.

Fig. 2. Mitochondria redistribution depends on integrin engagement. (A) Quantification of mitochondria redistribution in lymphoblasts adhered to resting or activated HUVEC monolayers. Mitochondria redistribution is calculated as the percentage of mitochondrial fluorescence intensity (maximal Z-stack projections of Mitotracker Orange staining) located in the contact area with endothelial cells (defined as the third of the cell close to the HUVEC monolayer; see scheme). Data are shown in a scatter plot; horizontal lines depict medians of 23 and 25 not polarized cell adhered respectively on either TNF-α-activated or non-activated HUVEC. Three independent experiments were performed (**P<0.001). The scheme illustrates the method used to quantify mitochondrial redistribution. (B) Representative images of conjugates formed between CH7C17 cells and latex beads coated with BSA, ICAM-1 or VCAM-1, stained for α-tubulin (green) and mitochondria (red). Scale bars, 10 μm. (C) Fold inductions in comparison to BSA negative control of MTOC and mitochondria relocalization towards the coated beads. A minimum of 30 (BSA), 95 (VCAM-1), or 140 (ICAM-1) cells were analysed for each experiment. (D) Graph shows quantification of mitochondria redistribution in lymphoblasts untreated (n=43) and pretreated (15 min at 37 ºC) with BIRT377 (n=41) or BIO5192 (n=31) compounds to block LFA-1 or VLA-4 integrins and incubated with activated TNF-α-HUVECs for 20 min. Data are shown in a scatter plot; horizontal lines depict medians from two independent experiments of non-polarized cells adhered activated HUVEC (**P<0.001, **P<0.01, one-way Anova).
Fig. 3. Miro-1 regulates mitochondria redistribution during adhesion and migration on endothelium. (A) Western blot analysis of siRNA Miro-1 silencing in CEM T cells. (B) Box and whiskers plot (min to max) of mitochondria redistribution in control and Miro-1-interfered cells adhered to TNF-α-activated HUVEC monolayers. Mitochondria redistribution is calculated as the percentage of mitochondrial fluorescence intensity located at the contact area (defined as the third of the cell close to the HUVEC monolayer). Data are median ± interquartile range of 247 (siCtrl) and 291 (siMiro-1) cells from three independent experiments (*** P<0.001). (C) Quantification of lymphocyte adhesion on TNF-α-activated HUVEC monolayer in control and Miro-1 silenced cells. Data are means ± s.e.m out of three independent experiments. (D) Representative images of control CEM T cells (siCtrl; Mitotracker Green) and Miro-1-interfered cells (siMiro; Mitotracker Orange) adhered to activated HUVEC under increasing shear stress (flow rate from 0.5 to 15 dyn/cm²). At the right panel, the graph presents adhered cells at different flow rates as a proportion of adherence at 0.5 dyn/cm². Data are from a representative experiment and are the means ± s.e.m of nine fields; ***P<0.001 (two-way ANOVA). (E) Representative images from a time-lapse video microscopy of control and Miro-1 silenced cells pre-stained with Mitotracker Orange and with DiO (siCtrl; green) and DiD (siMiro; blue) adhering and migrating on a TNF-α-activated HUVEC pre-treated with CXCL12 (1μM), under physiological laminar flow conditions (1.8 dyn/cm²). At the right part of each image is shown the fluorescence intensity distribution (grey values) across the cells (depicted by white arrows). Scale bars, 10 μm.

Fig. 4. Miro-1 controls lymphocyte migration and chemotaxis. (A) Quantification of the transmigration of control and Miro-1-interfered CEM T cells across transwell filters (5μm pore) coated by a TNF-α activated HUVEC monolayer (see Materials and methods). Data are
expressed as a percentage of transmigrated control cells. (B) Migration of control and Miro-1-interfered CEM T cells across uncoated transwell filters. Migration is calculated as a percentage of input cells. Data in A and B are means ± s.e.m of two independent experiments run by duplicate (*** P<0.001; ** P<0.01). (C) Cell trajectory plots of control and Miro-1-silenced cells migrating toward CXCL12 in a collagen matrix (1.6 mg ml⁻¹). The upper panels show a representative experiment out of three. Lower panels show quantification of movement toward the chemokine (Y forward migration index) and cell speed (μm min⁻¹) of 98 control and 102 Miro-1-interfered cells from three independent experiments (mean ± s.e.m) (*** P<0.001). (D) Velocities of single migrating control and Miro-1-interfered CEM cells (dots) in collagen gels with varying densities. Data are shown in a scatter plot; horizontal lines depict means out of three independent experiments (*** P<0.001, ** P<0.01).

**Fig. 5. Miro-1 regulates cell polarity and mitochondria relocalization to MTOC.** (A) Quantification of elliptical form factor in control (1213 cells) and Miro-1-silenced (1245 cells) CEM T cells plated on matrix containing CXCL12 (left panel). Data are shown in a scatter plot; horizontal lines depict medians from two independent experiments; ***P<0.001. Right panels show representative cells from each treatment group. Scale bars, 10μm. (B) Quantification of elliptical form factor in CEM T cells overexpressing GFP (345 cells), GFP-Miro-1 (512 cells) and GFP-Miro KK (431 cells) and plated on matrix containing CXCL12. Data are shown in a scatter plot; horizontal lines depict medians from two independent experiments; * P<0.05. (C) 3-D reconstruction of mitochondria (red) and MTOC (α-tubulin, green) in control and Miro-1-silenced CEM T cells plated on matrix containing or lacking CXCL12. Scale bars, 2 μm. (D) Graph showing the distance between mitochondria and MTOC in cells as in (C). Data are means ± s.e.m from two independent experiments; ***P<0.001 (one-way Anova). The numbers of cells are 38.
analysed for each condition were 73 (siCrtl), 60 (CXCL12 siCrtl), 66 (siMiro), and 64 (CXCL12 siMiro). (E) 3-D reconstruction of GFP proteins (green), mitochondria (red) and MTOC (α-tubulin, cyan) in CEM T cells overexpressing GFP, GFP-Miro-1 or GFP-MiroKK and plated on matrix containing CXCL12. Scale bars, 2 μm. (F) Graph showing the distance between mitochondria and MTOC in cells as in (D). The numbers of cells analysed for each condition were 115 (GFP), 103 (GFP-Miro-1) and 113 (GFP-MiroKK). Data are means ± s.e.m from two independent experiments; ***P<0.001, **P<0.01.

Fig. 6. Miro-1 connects mitochondria to the microtubule retrograde transport machinery and regulates CXCL12-induced cytoskeleton activation. (A) Co-immunoprecipitation analysis showing association of Miro-1 with the dynein motor complex. Complexes (IP) were precipitated from cell lysates of transfected HEK293 cells (Input) with anti-GFP and probed with anti-p74, anti-p50 and anti-GFP (left panel) or from human primary T lymphoblasts with anti-Miro-1 antibody and probed with anti-Dynein Heavy Chain (DHC), anti-p150 and anti-Miro-1 (right panel). (B) Actin polymerization dynamics in control or Miro-1 silenced CEM T cells exposed to CXCL12. F-actin content was measured by flow cytometry and is expressed relative to t=0. Data are presented as means ± s.e.m of three independent experiments; ***P<0.001 (Multifactorial Anova). (C) Representative images of F-actin (phalloidin, green), mitochondria (mitotracker orange, red) and ICAM-3 to mark the uropod (cyan) in maximum projections of a confocal Z-stack of CEM T cells plated on matrix containing CXCL12. (D) Representative images of pMLC distribution in control and Miro-1-silenced CEM T cells plated on CXCL12 matrix. Cells were stained for pMLC (green), Mitotracker Orange (red) and ERMs (cyan). Graph of pMLC fluorescence-intensity ratios of the uropod and the leading edge. Data are shown in a scatter plot; horizontal lines depict medians as medians from two independent experiments; *P<0.05.
Fig. 7. Miro-1 regulates mitochondria dependent calcium influx during adhesion and migration.

(A) Flow cytometry analysis of intracellular calcium dynamics (Fluo4-AM staining) in response to the addition of CXCL12 (10 nM) to a suspension of control and Miro-1-silenced CEM T cells (prestained with DiD and DiL, respectively). A representative experiment is shown out of three performed. (B) Representative intracellular calcium oscillation in control and Miro-1-interfered CEM T cells migrating on TNF-α activated HUVEC. (C) Distribution of relative calcium peak intensity (left) and peak duration in seconds (right). Data are shown in a scatter plot; horizontal lines depict medians out of two independent experiments (67 control and 62 Miro-1-silenced cells) (*** P<0.001). (D) Graph showing ATP amount in unstimulated and CXCL12-stimulated (10 min) control and Miro-1-interfered cells. Data are means ± s.e.m from two independent experiments; ***P<0.001; *P<0.05 (one-way Anova). (E) Time course of the phosphorylation status of ERK 1/2 in control and Miro-1-silenced CEM T cells activated with CXCL12. Total protein expression is shown as loading control. The right panel shows pERK/total ERK band intensity ratios relative to the ratio in control cells at t=0. Data are presented as the mean ± s.d. out of two independent experiments.
Figure 1
Figure 2
Figure 3

**Figure 3**

A) Western blot analysis of Miro-1 and Tubulin expression levels in siCtrl and siMiro conditions.

B) Box plot showing the % of fluorescence at contact area for siCtrl and siMiro conditions.

C) Bar graph illustrating the % of adhesion for siCtrl and siMiro conditions with p-value = 0.06.

D) Detachment assay images showing the ratio of cell number over flow rate (dyn/cm²) for siCtrl and siMiro conditions.

E) Representative images of Lipophilic Tracer (DIO) Mitochondria for siCtrl and siMiro conditions at different time points (0 sec, 5 sec, 5/10 sec, 2490 sec) along with their corresponding distance plots.
Figure 4
Figure 5
Figure 6
Figure 7