Supplemental Methods, Figures and Movie legends

Supplemental Methods and associated references

Generation of Myo1g Conditional Knockout Mice.

Mouse genomic DNA harboring the myo1g locus was isolated from the BAC clone RP23-69P8 (http://bacpac.chori.org/). Using recombineering (Liu et al., 2003), LoxP recombination sites and a neomycin selection cassette were introduced flanking the second and third coding exons of the mouse Myo1g genomic locus. The resulting targeting vector was linearized by NotI digestion, and ES cells were electroporated. After positive and negative selection with Geneticin and ganciclovir, respectively, genomic DNA of surviving ES cell colonies were screened for homologous recombination by Southern hybridization (**Fig. S1a-b**). Correctly targeted ES cell clones were identified and used for the generation of the Myo1g conditional knockout or full knockout mouse strain.

To generate the Myo1g FLOX allele, the neomycin cassette was removed by crossing Myo1g conditional knockout mice with β-actin–Flpe transgenic mice (The Jackson Laboratory). The Myo1g NULL (Myo1g-/-) allele was generated by crossing Myo1g FLOX mice with β-actin–Cre transgenic mice (The Jackson Laboratory).

Mice

Myo1g-/- mice were crossed with Ovalbumin (OVA)-specific TCR–transgenic OTI CD45.1+/+ in order to get OTI CD45.1+ Myo1g-/- mice. These mice, C57/Bl6 (The Jackson Laboratory and Simonsen), Actin-CFP, “mTomato” (mTmG mice (Muzumdar et al., 2007) in the absence of Cre expression, The Jackson Laboratory) and OTI CD45.1+/+ mice were housed and bred under specific pathogen-free conditions at the University of California Animal Barrier Facility. All experiments involving mice were approved by the Institutional Animal Care and Use Committee of the University of California.

Generation of plasmid encoding for YFP-Myo1g constructs

Mouse Myo1g cDNA (FL-Myo1g) (accession number NP_848534.2) was amplified using the primers $^{5'}$GGA CTG AAG CTT TAG GAG GCG GAT CAG GTG GCG GTG GAA GTG GAG GTG GTG GAT CTC TTG CAG TGG GCA GGA$^{3'}$ and $^{5'}$ACT GCG GGT ACC TCA GTG GCT TGG CCA GAG G$^{3'}$. Mouse Myo1g IQ-Tail cDNA (2247 to 3307bp) was amplified with the primers $^{5'}$CGC AGT AAG CTT TAG GAG GCG GAT CAG GTG GCG GTG GAA GTG GAG GTG GTG GAT CTC TGA TTC CCA TCA TTG TGT TAT TGC TGC$^{3'}$ and $^{5'}$ACT GCG GGT ACC TCA GTG GCT TGG CCA GAG G$^{3'}$. Constructs were cloned into pEYFP-C1 (Clontech) using the cloning sites HindIII and KpnI. The resulting YFP-Myo1g construct chimeras were then sub-cloned into MCSV using the restriction sites AgeI, SspI and ScaI.

Mouse Myo1g IQ-Tail (2247 to 3307bp) was amplified with the primers $^{5'}$CGC AGT AAG CTT TAG GAG GCG GAT CAG GTG GCG GTG GAA GTG GAG GTG GTG GAT CTC TGA TTC CCA TCA TTG TGT TAT TGC TGC$^{3'}$ and $^{5'}$ACT GCG GGT ACC TCA GTG GCT TGG CCA GAG G$^{3'}$ and cloned into pEYFP-C1 (Clontech) using the cloning sites HindIII and KpnI. The resulting YFP-Myo1g chimera was then sub-cloned into MCSV using the restriction sites AgeI, SspI and ScaI.

Cell isolation, activation and retroviral infection

T cells were isolated from lymph nodes and spleen of 6 to 12 weeks old mice. Selection was carried out using a negative CD8 isolation kit (STEMCELL Technologies Inc.). Naïve T cells were activated with 2μg/ml plate-bound CD3 Ab and 2μg/ml CD28 Ab. Plasmid encoding for YFP-Myo1g was transfected in the phoenix packaging cell line using standard calcium phosphate methodology. Virus-containing supernatant from these cells was used on two consecutive days (days 2 and 3 after activation) to spin-infect activated T cells. Transduced cells were used between 4 and 8 days after activation.

Membrane tension and membrane reservoir measurements

Single tether force assays (Dai and Sheetz, 1995, 1999; Hochmuth et al., 1996) were performed as follows: 1.0μL of 2.0μm diameter, streptavidin-coated microspheres (Spherotech, Inc.; 0.05% w/v, 0.46nmol biotin binding capacity per mg) were incubated with 50μL biotinylated-concanavalin A (Sigma; 4-8mol of biotin per mole of protein) at a concentration of 0.001μg/μL for half an hour. The microsphere coating density was selected so that the probability of multiple tether formation was minimal. The

microspheres were then centrifuged at 1600xg and re-suspended in 100μL of Hank's Balanced Salt Solution (HBSS -$MgCl_2$, -$CaCl_2$) supplemented with 0.1% BSA.

Prior to an experiment, cells were centrifuged at 200xg and re-suspended in 1mL of RPMI with IL-2. Several volumes (~50μL) of this solution were then introduced into a chamber constructed using a glass slide and a coverslip coated with a 1% solution of poly-l-lysine, separated by double-sided tape. Cells were allowed to adhere to the coverslip at 37°C in 5% CO_2 for 15 minutes. Several volumes of HBSS/microspheres were then washed through the chamber, which was then sealed and incubated at 37°C in 5% CO_2 for 10 minutes.

For optical Trapping, The sample chamber was mounted on a Nikon TE2000U microscope equipped with an optical trap (Ashkin, 1997) (MMI Cell Tools) and a piezoelectric stage (Mad City Labs). Videos of experiments were acquired at 30 frames/second using a CCD camera controlled by LabVIEW-based software modules. The microsphere position was obtained with a template matching software, also written in LabVIEW (Carter et al., 2005). The trap was calibrated using viscous drag forces as previously described (Nambiar et al., 2009).

For Tether Force Assays, a microsphere was captured with a trap of stiffness, k_{Trap} = 0.5pN/nm. The equilibrium position of the microsphere was recorded for about a second. Subsequently, the microsphere was briefly brought into contact with the membranes of leukocytes, which were not migrating. The stage was then translated at a constant speed, v_{Stage} = 3155nm/s for 5.0μm causing the cell to move away from the microsphere resulting in a membrane tether ~5.0μm in length. The steady-state tether force for a 5μm tether was recorded for several hundred frames.

Hypotonic buffer treatment

Activated T cells were plated on ICAM-1-coated coverslips and incubated at 37°C for 1-2 hours. Cells were then gently washed with complete media (RPMI 10%FCS) to remove non-adherent cells, and complete media + 0.1% low melting agarose was added on the cells. Cells were imaged for 20 min at 30-second intervals with a modified microscope (Axiovert 200M; Carl Zeiss, Inc.) with Plan-Neofluar 20x objective (Carl Zeiss, Inc.). During imaging, we added an equal volume of hypotonic buffer (ddH2O + 1 mM MgCl2 + 1.2 mM CaCl2) to reduce osmolarity by approximately 170 mOsm.

Microchannel fabrication and imaging

Microchannel fluidic devices were fabricated by a soft lithography technique with PDMS (polydimethylsiloxane; Sylgard Elastomer 184 kit; Dow Corning) and bonded to a glass-bottomed imaging dish as described (Faure-Andre et al., 2008; Jacobelli et al., 2010). Feature sizes (heights of channels) were 7μm, whereas width size was variable and indicated in the legend. ICAM-1 solution (5μg/ml) was injected into the entry area of the microchannels and after 1h of incubation at 37°C, microchannels were washed out with PBS. Activated T cells were added into a loading port and cells were allowed to spontaneously enter at 37°C for 2–4h.

Migration in collagen lattices

WT and Myo1g-/- T cells were labeled with 1μm CFSE or 10μm CMTMR, respectively, mixed at ratio 1:1 and resuspended in RPMI 10%FCS. PureCol (INAMED, Fremont, CA) in 1× minimum essential medium eagle (Sigma-Aldrich) and 0.4% sodium bicarbonate (Sigma-Aldrich) was mixed with cells in RPMI (Invitrogen), 10% fetal calf serum (FCS; Invitrogen) at a 2:1 ratio, resulting in gels with a collagen concentration of 1.6 mg/mL. Final cell concentration was 3-4 × 10^6 cells/ml. After 1 h incubation at 37°C to let the collagen fully polymerize, cells were imaged as described above. Imaris (Bitplane) was used for calculation of cell speed and directionality.

Chemotaxis assays.

Chemotaxis in Boyden chambers: The inner and outer face of Transwells (Costar; 5-μm pore size) were coated with 0.5% BSA for 2 h at RT. Purified WT and Myo1g-/- T cells were labeled with 1um CFSE and 10 um CMTMR respectively, for 30 minutes in PBS, then washed extensively in RPMI 10% FCS. Cells were mixed at a ratio 1:1 and resuspended at 2.5 x 10^5 in 150 μl RPMI 0.1% BSA. Cells were loaded in a BSA-coated transwell, which was placed into a 24-well plate containing 250ul RPMI supplemented with 0.1% BSA and various concentrations of SDF1α. After 2 hours at 37°C, the cells that migrated into the lower chamber were collected and counted by flow cytometry.

3D chemotaxis in collagen was essentially performed as in (Lammermann et al., 2009). Briefly, Cells were embedded in collagen as described above. Collagen-cell mixtures were cast in μ-slide Chemotaxis 3D (Ibidi). After 1 hour at 37C, or when collagen lattices were polymerized, SDF1α (300ng/ml in complete media) was added to one corner of the

chamber, whereas the other corners were filled with complete media only. Cells were imaged for 3 hours at 30 to 60-second intervals with a modified microscope (Axiovert 200M; Carl Zeiss, Inc.) with Plan-Neofluar 20x objective (Carl Zeiss, Inc.). Data was analyzed using the Chemotaxis and Migration tool on Imagej.

Measurement of T cell activation and proliferation in vitro.

WT or Myo1g-/- OTI cells were labeled with CFSE when indicated and activated with LPS-activated BMDCs (APCs) loaded with the indicated concentration of OVA peptide.

Twenty-four hours after activation, cells were stained for CD25 and CD69 (eBiosciences) in FACS Buffer for 20 min at 4°C. Cells were washed and resuspended in FACS buffer containing 1% PFA and analyzed by flow cytometry. CFSE dilution profiles were analyzed by flow cytometry after 3 days.

Adhesion assay.

The 96-well flat-bottomed plates were precoated with 5 µg/mL of recombinant mouse ICAM-1/Fc or VCAM-1/Fc (R&D Systems) or 2% BSA as a control. Cultured T cells from WT or Myo1g-/- animals were washed 3 times with PBS, resuspended in PBS at 1×10^6 cell/mL, and labeled with 5 µg/mL BCECF for 30 minutes at 37°C and washed 3 times with HBSS media containing 1mM HEPES, 0.1% BSA, 1 mM $CaCl_2$, 1mM $MgCl_2$ (HBSS buffer), with or without 1mM $MnCl_2$. Labeled cells (0.5×10^5) were plated into each well, and incubated at 37°C for 25 minutes. The wells were washed vigorously 3 times with HBSS buffer and the remaining adherent cell number assessed using a fluorescence microplate reader (Bio-Tek) using excitation and emission wavelengths of 485 and 528 nm.

TCR Microcluster analysis and TIRF microscopy.

Experiment was done as in (Beemiller et al., 2012). Small unilamellar vesicles consisting of 96.5% POPC, 2% DGS-NTA-Ni, 1% Biotinyl-CAP-PE, and 0.5% PEG5,000-PE were prepared by rehydrating dried phospholipid cakes into PBS and extruding through 100-nm pore-size filters using a LiposoFast extruder (Avestin). Lipid bilayers on glass were blocked with 1% BSA, loaded with 5 µg/ml streptavidin, and then loaded with 12×his-ICAM and 25ng/ml biotinylated pMHC (SIINFEKL-loaded H2-k^b). The surface TCRs of WT and Myo1g-/- naïve T cells was labeled by resuspending 2×10^6 cells in ~0.1 ml complete RPMI and 1µg Alexa Fluor 568-labeled H57-597 anti-TCRβ (H57-597 was

from BioXCell and was conjugated to Alexa Fluor 568 at the hybridoma facility of the University of California, San Francisco). After 30 minutes on ice, cells were washed with complete RPMI (10%FCS, no phenol red) and maintained at 4C until imaging. Cells (10^5) were deposited onto the bilayer and imaged by TIRF microscopy.

The TIRF microscope was a Zeiss Axiovert 200M, with a Laser TIRF I system and a 1.45 NA, 100× Plan-Fluar objective (Jacobelli et al., 2009). TIRF images were acquired at 1 or 2-second intervals using 33– 100 ms exposure lengths for 3– 5 minutes. Cells were typically imaged for 10– 15 min and were analyzed with Methamorph and Imaris as described in Beemiller et al. 2012.

Immunization, measurement of T cell activation and expansion in vivo

CD45.1+ WT or CD45.1+ Myo1g-/- OTI cells ($5x10^4$ or $5x10^3$) were transferred in WT recipient mice. Sixteen hours after transfer, mice were immunized subcutaneously in the flank with the indicated number of Bone Marrow-Derived Dendritic Cells (BMDCs). BMDCs generated by culturing bone marrow cells from C57Bl/6 mice for 8–11 days with GM-CSF. IL4 was added for the last 2 days of culture. LPS (1ug/ml) and OVA peptide SL8 (AnaSpec 100ng/ml) was added to cultures 4-6 hours before immunization.

For assessment of T cell activation, draining LNs were excised 24h after immunization. For assessment of T cell expansion, draining LNs were excised 6 days after immunization. Cell suspension was washed in PBS, and blocked in FACS buffer (PBS, 2% FCS, 2mM EDTA, 0.1% sodium azide) containing CD16/32 Ab. Staining for surface proteins with conjugated-antibodies against CD45.1, CD45.2, CD69 (for T cell activation only) and CD8 (eBiosciences) was performed in FACS Buffer for 20 min at 4°C. Cells were washed and resuspended in FACS buffer containing 1% PFA. The percentage of CD69+ cells among CD45.1+ cells (T cell activation) or the percentage of CD45.1+ cells among CD8 cells was analyzed by flow cytometry (T cell expansion).

Listeria monocytogenes infection.

Mice were transferred with 5×10^3 WT or Myo1g-/- OTI cells and challenged with a sub-lethal dose of LM expressing Ovalbumin (LM-OVA (Pope et al., 2001)) ($0.25\times LD_{50}$). Survival was monitored for 15 days.

References

Ashkin, A. (1997). Optical trapping and manipulation of neutral particles using lasers. Proceedings of the National Academy of Sciences of the United States of America *94*, 4853-4860.

Beemiller, P., Jacobelli, J., and Krummel, M.F. (2012). Integration of the movement of signaling microclusters with cellular motility in immunological synapses. Nature immunology *13*, 787-795.

Carter, B.C., Shubeita, G.T., and Gross, S.P. (2005). Tracking single particles: a user-friendly quantitative evaluation. Physical biology *2*, 60-72.

Dai, J., and Sheetz, M.P. (1995). Mechanical properties of neuronal growth cone membranes studied by tether formation with laser optical tweezers. Biophysical journal *68*, 988-996.

Dai, J., and Sheetz, M.P. (1999). Membrane tether formation from blebbing cells. Biophysical journal *77*, 3363-3370.

Faure-Andre, G., Vargas, P., Yuseff, M.I., Heuze, M., Diaz, J., Lankar, D., Steri, V., Manry, J., Hugues, S., Vascotto, F.*, et al.* (2008). Regulation of dendritic cell migration by CD74, the MHC class II-associated invariant chain. Science *322*, 1705-1710.

Hochmuth, F.M., Shao, J.Y., Dai, J., and Sheetz, M.P. (1996). Deformation and flow of membrane into tethers extracted from neuronal growth cones. Biophysical journal *70*, 358-369.

Jacobelli, J., Bennett, F.C., Pandurangi, P., Tooley, A.J., and Krummel, M.F. (2009). Myosin-IIA and ICAM-1 regulate the interchange between two distinct modes of T cell migration. J Immunol *182*, 2041-2050.

Jacobelli, J., Friedman, R.S., Conti, M.A., Lennon-Dumenil, A.M., Piel, M., Sorensen, C.M., Adelstein, R.S., and Krummel, M.F. (2010). Confinement-optimized three-dimensional T cell amoeboid motility is modulated via myosin IIA-regulated adhesions. Nature immunology *11*, 953-961.

Lammermann, T., Renkawitz, J., Wu, X., Hirsch, K., Brakebusch, C., and Sixt, M. (2009). Cdc42-dependent leading edge coordination is essential for interstitial dendritic cell migration. Blood *113*, 5703-5710.

Liu, P., Jenkins, N.A., and Copeland, N.G. (2003). A highly efficient recombineering-based method for generating conditional knockout mutations. Genome research *13*, 476-484.

Muzumdar, M.D., Tasic, B., Miyamichi, K., Li, L., and Luo, L. (2007). A global double-fluorescent Cre reporter mouse. Genesis *45*, 593-605.

Nambiar, R., McConnell, R.E., and Tyska, M.J. (2009). Control of cell membrane tension by myosin-I. Proceedings of the National Academy of Sciences of the United States of America *106*, 11972-11977.

Pope, C., Kim, S.K., Marzo, A., Masopust, D., Williams, K., Jiang, J., Shen, H., and Lefrancois, L. (2001). Organ-specific regulation of the CD8 T cell response to Listeria monocytogenes infection. J Immunol *166*, 3402-3409.

Supplemental Figure Legends

Figure S1: Generation of conditional allele of Myo1g and Myo1g localization by TIRF microscopy. Related to Figure 1.

a- Schematic representation of the targeting strategy; the targeting vector is depicted at the top. The 5' arm is 3.7 kilobase (kb) in length. The middle fragment, which contains a 2.0 kb-genomic fragment bearing exon 2 and 3 of Myo1g and a pGKneobpa cassette bordered by frt sites, is flanked by loxP sites. The 3' arm is 5.9 kb in length. In the middle is the WT allele showing the position of the probes. The targeted allele is depicted underneath. After expression of Flpe recombinase, the neo-cassette was removed to generate the floxed allele. Subsequent expression of the Cre recombinase caused removal of exons 2 and 3 to ablate Myo1g protein expression. The locations of the XbaI and NsiI sites, as well as the 5', 3' probes used to screen the clones by Southern blotting, are represented. **b-** Genomic DNA from representative embryonic stem (ES) cell clones was digested with XbaI (left panel) and NsiI (right panel) and probed by Southern blotting with the probes shown in (a). **c-** Expression of Myo1g protein in spleen cells from the indicated mice (WT +/+, Myo1g Het +/-, Myo1gKO -/-) was assessed by immunoblotting of total cell lysates with Myo1g antibodies (Rabbit Ab, Rockland). ß-Actin expression was evaluated as control. **d-** Flow cytometry analysis of Thymic CD4 and CD8 profiles of two independent mice. **e-** YFP-Myo1g expressing T cells were allowed to migrate on ICAM-1-coated coverslip and labeled with the membrane marker DiD. Snapshot shows representative localization of Myo1g according to the plasma membrane analyzed by TIRF microscopy (n=10). Left panel: TIRF image of YFP-Myo1g; middle panel: TIRF image of DID; Right panel: Merge image. Scale bar = 3μm.

Figure S2: Myo1g-/- cell morphology according to migration environment. Related to Figure 2.

Activated WT (black) and Myo1g-/- (red) T cells were allowed to migrate in multi-sized (8-20um) microchannels (**a** and **d**), on ICAM-1 coated coverslip (**b**) or in collagen lattices (**c**). **a-** Graph shows the percentage of cells migrating in microchannels exhibiting a rounded morphology, accessed by the absence of clear uropod and leading edge. Data are from 2 independent experiments (n>50). Each dot corresponds to a field. *p<0.05. **b-** Cells were fixed and stained for CD44 and Actin to analyze cell polarization. Graph shows the percentage of polarized cells (n>200). Data are from 2 independent

experiments. **c-** Graph shows the percentage of cells migrating in collagen lattices exhibiting a rounded morphology. Data are from 2 independent experiments (n=30). Each dot corresponds to a field. ***p<0.001. **d-** Graph shows instantaneous turning angle during a turn relative to instantaneous speed displayed by the cell the last 1-2 minutes before making a turn. Each dot represents a cell. Data are from 3 independent experiments. **p<0.01.

Figure S3: Rescue speed and turning angle altered by loss of Myo1g. Related to Figure 3.

a- WT (left panel) and Myo1g-/- (right panel) T cells were fluorescently labeled, mixed at a ratio 1:1 and allowed to migrate for 1h on ICAM-1 coated coverslip. Cells were then incubated in hypotonic buffer to increase membrane tension. Cell migration was recorded throughout the experiment. Graphs show cell speed before (pre-) and after (post-) hypotonic treatment. Each dot corresponds to a given field. Gray lines connect the same field before and after treatment. ***p<0.001. n>100. **(b-c)** Activated WT T cells were transduced with YFP-FL Myo1g or YFP-IQ-Tail Myo1g and harvested 4 to 5 days after activation. Cells were allowed to migrate into ICAM-1 coated microchannels for 2h before imaging. Cells were imaged for 5 minutes at 30-second intervals, using a 40X objective. **b-** Snapshot showing representative YFP localization in cells migrating into 3D environment. Scale bar = 5μm. **c-** Quantification of transient YFP accumulation in cell migrating in 3D environment. Data correspond to 3 independent experiments. ***p<0.001. (**d-e**) Activated Myo1g-/- T cells were transduced with YFP, YFP-FL Myo1g or YFP-IQ-Tail Myo1g and harvested 4 to 5 days after activation. Cells were allowed to migrate into ICAM-1 coated microchannels for 2h before imaging. Cells were imaged for 90 minutes at 1-minute intervals, using a 20X objective. Graphs show speed (**d**) and weaving angle (**e**) of YFP positive T cells. Data correspond to 3 independent experiments. Each dot corresponds to a cell. ***p<0.001.

Figure S4: Myo1g-/- T cells are not defective in chemotaxis and regulates T cell scanning of DCs. Related to Figure 4 and 5.

WT and Myo1g-/- T cells were fluorescently labeled, mixed at a ratio 1:1 and allowed to migrate side-by-side in collagen gels. SDF1α (300ng/ml) was added in a position corresponding to the lower left quadrant of the displayed graph and cells were imaged for 3 hours at 1-minute intervals. **a-** Graphs show tracks of WT (left panel) and Myo1g-/-

(right panel) T cells. **b-** Graph shows average displacement of center of mass of WT (black) and Myo1g-/- (red) T cells, which represents the averaged point of all cell endpoints. Data are from 2 independent experiments. n>30. (**c-f**) 12-hour long tracks of WT (black) and Myo1g-/- (red) T cells have been extrapolated from 2-Photon experiments. Path length (**c**), mean displacement (**d**) and the ratio between path length and mean displacement (**e**) of simulated WT and Myo1g-/- OTI cells tracks over square root of time. **f-** Scan efficiency (probability a track touches a target, or *Search*) of simulated WT (black) and Myo1g-/- (red) 12-hour tracks relative to the diameter of the sphere where target are randomly placed. **g-** Mice bearing WT (black) or Myo1g-/- (red) OTI cells were transferred with activated BMDCs. Explanted draining LN was subjected to 2Photon microscopy. Graph shows the fraction of cells still in contact with a DC over time. ***p<0.001. n=70

Figure S5: Myo1g-/- T cells are not defective in TcR activation. Related to Figure 6.

a- WT (black) or Myo1g-/- (red) T cells were activated with 2□g plate-bound anti-CD3 and 2□g anti-CD28. Sixteen hours after activation, T cell activation was analyzed by CD69 up-regulation using flow cytometry. **b-** CFSE-labeled WT (black) or Myo1g-/- (red) T cells were activated with 2□g plate-bound anti-CD3 and 2□g anti-CD28. Three days after activation, CFSE profiles were analysed by flow cytometry. **(c-d)** WT and Myo1g-/- naïve OT-I T cells were stained for their TCR and deposited on bilayers. Microcluster movement and cSMAC formation was characterized by TIRF imaging. Graphs show microcluster track speed (**c**) and track straightness (**d**) of microclusters in WT cells (black, *n* = 12) and Myo1g-/- cells (red, *n* = 10). **e-** Mice were immunized s.c with OVA peptide-pulsed CD45.1^{+} BMDCs. Draining, inguinal LNs from immunized mice were collected when indicated. Cell suspension was stained for CD45, Cd11c and CD45.1 and analyzed by flow cytometry.

Supplemental Movie Legends

Movie S1: YFP-Myo1g localization after local application of pressure, related to Figure 1.

Representative video of YFP-Myo1g recruitment during application of pressure relative to plasma membrane. mTomato T cells were transduced with YFP-Myo1g and allowed to migrate onto ICAM-1 coated coverslips. Cells were gently pushed with a needle tip. Imaged were taken at 4" intervals.

Movie S2: Example of WT and Myo1g-/- T cells migrating in 8umx7um microchannel, related to Figure 2.

Representative video of WT (top) and Myo1g-/- (bottom) T cells migrating in 8umx7um microchannel. T cells were introduced into ICAM-1 coated microchannels. 16 hours after, cells were imaged sat 1' intervals. Elapsed time is displayed in minutes:seconds.

Movie S3: Example of a stopped cell re-initiating migration after being "pushed" along by other cells, related to Figure 2.

Representative video of T cells starting to migrate after encountering other migrating cells. WT T cells were introduced into ICAM-1 coated 12umx7um microchannels. 2 hours after, cells were imaged at 1' intervals. Elapsed time is displayed in minutes:seconds.

Movie S4: Re-initiation of T cell polarization after blebbstatin treatment, related to Figure 2.

Representative video of WT and Myo1g-/- T cell behavior during re-polarization. WT or Myo1g-/- T cells were allowed to migrate onto ICAM-1 coated coverslips and treated with 100 mM blebbistatin for 10'. Cells were washed with media and imaged at 1' intervals. Elapsed time is displayed in minutes:seconds.

Movie S5: Example of WT and Myo1g-/- T cells migrating in wide microchannels, related to Figure 3.

Representative video of WT (top) and Myo1g-/- (bottom) T cells migrating in a wide microchannel. Activated T cells were introduced into ICAM-1 coated 20umx7um

microchannels. 2 hours after, cells were imaged at 1' intervals. Elapsed time is displayed in minutes:seconds.

Movie S6: Example of WT and Myo1g-/- T cells migrating in LNs, related to Figure 4.

Representative zoom in video of WT (green) and Myo1g-/- (red) T cell behaviour migrating in LN. Naive WT T cells and Myo1g-/- T cells were labeled with CFSE and CMTMR, respectively. Cells were then introduced into naive recipient mice. 16 hours after, LNs were explanted and subjected to 2-Photon microscopy. The video shows a Maximum Intensity Projection rendering of an imaging volume of 280 (x) × 280 (y) × 99 µm (z). Elapsed time is displayed in hours:minutes:seconds. Note that the time lapse repeats three times after the zoom. First repeat shows WT (green) and Myo1g-/- (red) migrating cells. Second and Third repeats respectively show WT and Myo1g-/- cells migrating together with cell tracks color-coded by cell speed.

Movie S7: Example of WT and Myo1g-/- T cells forming a cSMAC on lipid bilayer, related to Figure S7.

Representative video of time lapse TIRF imaging showing naïve WT (left) and Myo1g-/- (right) T cell generating microcluster flow and forming a centralized immune synapse. Cells were stained for their TCR, and deposited on a bilayer containing ICAM-1 and cognate OVA peptide coupled to MHC class I. TCR microclusters and cSMAC formation was characterized by TIRF microscopy. The image sequence consists of 0.1 s exposures acquired at 1 s intervals. Elapsed time is displayed in seconds.

Movie S8: Example of WT and Myo1g-/- T cells stopping in LNs following Ag encounter, related to Figure 6.

Representative video of WT (red) and Myo1g-/- (green) T cell stopping behavior in LN following TCR triggering. Naive WT T cells and Myo1g-/- T cells were labeled with CMTMR and CFSE, respectively. Cells were then introduced into naive recipient mice. 16 hours after, LNs were explanted and subjected to 2-Photon microscopy. OVA peptide was added to the flow media 10 minutes after the beginning of imaging. The video shows a Maximum Intensity Projection rendering of an imaging volume of 200 (x) × 200 (y) × 80 µm (z). Elapsed time is displayed in seconds.