ARTICLE

Arhgef6 (alpha-PIX) cytoskeletal regulator signals to GTPases and Cofilin to couple T cell migration speed and persistence

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Abstract
Immunity is governed by successful T cell migration, optimized to enable a T cell to fully scan its environment without wasted movement by balancing speed and turning. Here we report that the Arhgef6 RhoGEF (aka alpha-PIX; 𝛼PIX; Cool-2), an activator of small GTPases, is required to restrain cell migration speed and cell turning during spontaneous migration on 2D surfaces. In Arhgef6−/− T cells, expression of Arhgef7 (beta-PIX; 𝛽PIX; Cool-1), a homolog of Arhgef6, was increased and correlated with defective activation and localization of Rac1 and CDC42 GTPases, respectively. Downstream of Arhgef6, PAK2 (p21-activated kinase 2) and LIMK1 phosphorylation was reduced, leading to increased activation of Cofilin, the actin-severing factor. Consistent with defects in these signaling pathways, Arhgef6−/− T cells displayed abnormal bilobed lamellipodia and migrated faster, turned more, and arrested less than wild-type (WT) T cells. Using pharmacologic inhibition of LIMK1 (LIM domain kinase 1) to induce Cofilin activation in WT T cells, we observed increased migration speed but not increased cell turning. In contrast, inhibition of Cdc42 increased cell turning but not speed. These results suggested that the increased speed of the Arhgef6−/− T cells is due to hyperactive Cofilin while the increased turning may be due to abnormal GTPase activation and recruitment. Together, these findings reveal that Arhgef6 acts as a repressor of T cell speed and turning by limiting actin polymerization and lamellipodia formation.

KEYWORDS
Cofilin, GTPases, lamellipodia, migration, RhoGEF

1 | INTRODUCTION

T cell migration is a complex, multistep process that enables the cell to scan its environment for antigenic signals. On 2D surfaces, these steps begin with a broad and thin protrusion called a lamellipodium, consisting of polymerized, branched actin. Actin polymerization at the cell membrane forms a lamellipodium that forces filaments back toward the cell center, a process known as retrograde actin flow. As the lamellipodium grows and attaches to the substrate using integrin-based adhesions, retrograde actin flow is transformed into a force that propels the cell forward. Eventually the lamellipodium retracts and becomes reabsorbed while another protrusion forms. The new protrusion dictates the migration direction. Therefore, the angle between two successive lamellipodia determines the straightness, or the persistence, of the cell migration path. The frequency of cell turning is also linked to cell speed, as the disassembly of an old lamellipodium inhibits retrograde actin flow, forcing the cell to pause and slowing overall migration speed. Assembly of a new lamellipodium enables the cell to resume forward motion in a new direction. The cell migration path straightness and speed are optimized to ensure that exploratory

Abbreviations: 2D, two-dimensional; Pak, p21-activated kinase; PIX, Pak-interacting exchange factor; GEF, guanine nucleotide exchange factor; WT, wildtype; KO, knockout; OD, optical density; TIRF, total internal reflection fluorescence; CH, calponin-homology.

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T cells can maximally scan their local environment while randomly migrating.4

Cell turning during migration is controlled by proteins that regulate protrusion or lamellipodia formation.2 The GTPase Cdc42 has long been known as a polarity factor in yeast, where it is required for placement of new buds after recruiting its activating GEF (Guanine nucleotide exchange factor).3,6 Knockout of Cdc42 in dendritic cells results in multiple protrusions around the periphery of cells and highly twisting trajectories; thus Cdc42 suppresses protrusions in order to promote a single, dominant protrusion.7 The GTPase Rac1 promotes lamellipodial extension by activating actin polymerization at the tips of lamellipodia.8 Knockdown of Rac1 in fibroblasts inhibits peripheral protrusions resulting in an elongated cell that migrates persistently, whereas overexpression of Rac1 induces multiple lateral protrusions and twisting migration paths.2,9 Rac1 activation of the WAVE (WASP-family verprolin homologous protein 1) regulatory complex induces the Arp2/3 actin-branching complex required for stable lamellipodia, and inhibitors of Arp2/3 such as Arpin or Coronin affect protrusion formation, migration speed, and cell turning.10–12 Rac1 and Cdc42 also promote polymerized actin in cell protrusions by inducing LIMK to inhibit Cofilin, which otherwise severs actin.13 Thus, Cdc42 and Rac1 regulate cell protrusions, turning, and cell speed.

Although many actin-related proteins play roles in T cell migration, relatively few are negative regulators of T cell speed. One such protein is Arhgef6 (alpha-PIX), a RhoGEF exchange factor for Rho GTPases Rac1 and Cdc42. Thymocytes and marginal zone B cells lacking Arhgef6 migrate faster than their wild-type (WT) counterparts.14,15 And Arhgef7 has been shown to increase cell speed by turning over nascent focal adhesions in fibroblasts.16 Arhgef6 and its homolog Arhgef7 were first isolated as p21-activated kinase (PAK)-interacting proteins that activate Rac1 and Cdc42 by catalyzing a GDP-GTP nucleotide exchange. Arhgef6 and Arhgef7 bind constitutively to the GTPase-activating proteins GIT1 and GIT2, members of the Arf-GAP family of proteins that inactivate Arf (ADP-ribosylation factor) GTPases.17 This complex is referred to as the PIX-GIT (p21-activated protein kinase exchange factor - GRK-interacting protein) complex and it is notable for connecting two major classes of GTPases: Rho GTPases, activators of actin dynamics, and the Arf GTPases, regulators of the WAVE complex, Arp2/3 and lamellipodia.17 Together, the PIX-GIT complex relays signals from receptors to actin cytoskeletal rearrangements required for signaling.

Arhgef6 is an X-linked protein and has been identified as a mutation in patients with X-linked intellectual disabilities.18 In neurons, Arhgef6 and Arhgef7 are required for formation of actin structures including neurites, spines, and growth cones,18–24 and signal to Cofilin in these processes.25–27 In Arhgef6−/− mice, pyramidal neurons show increased dendritic length and spine density, while the mice display impairments in learning and behavior.21 However, despite its importance to neuronal functions, the roles of Arhgef6 in T cells are less clear. Arhgef6 protein is highly expressed in immune cells compared to Arhgef717,28 and is required for formation of the immune synapse downstream of TCR signaling.29 Additionally, Arhgef6−/− mice have developmental defects at an early stage of T cell development in the thymus due to increased migration speed and reduced contact with antigen-presenting cells.14 In order to investigate the mechanisms behind the increased speed of Arhgef6−/− T cells, we employed immunocytochemistry and 2D integrin-based migration assays to assess lamellipodia, their regulators, and cell migration trajectories. We found that T cells lacking Arhgef6 have increased active Rac1, mislocalized Cdc42, and increased Cofilin activity, as well as larger and more frequent lamellipodia. The Arhgef6−/− T cells migrated both faster and turned more than WT cells, revealing a role for Arhgef6 in coupling T cell speed and persistence in cell migration.

2 METHODS

2.1 Mice, T cells, and culture

Mice were housed in specific pathogen-free conditions according to institutional guidelines. All procedures were performed in accordance with the institutional guidelines for health and care of experimental animals and were approved by the Landesverwaltungsamt Halle (representing the state of Saxony-Anhalt), Germany. Lymph node T cells were obtained from 8- to 12-wk old C57BL/6 mice and Arhgef6−/− mice of either sex using a CD4+ T cell isolation kit (Miltenyi, Bergisch Gladbach, Germany) to deplete non-CD4+ T cells (>95% purity). T cells were cultured in RPMI 1640 with stable glutamine supplemented with 10% FCS, penicillin/streptomycin, beta-mercaptoethanol, nonessential amino acids, sodium pyruvate, recombinant murine IL-2, and recombinant murine IL-7 in 96-well plates on wells coated with 5 μg/ml anti-CD3 (clone 2C11) and 1 μg/ml anti-CD28 (clone 37.51). Highly motile T cell blasts were obtained after 7–9 d of culture. In some experiments the cells were additionally treated by adding LIMK1 inhibitor LIMKi330–32 (10 μM) (4745, Tocris, Wiesbaden, Germany) for 4 h before harvesting or with ethanol alone as a vehicle control at 0.05%. To inhibit Cdc42, T cells were treated with Casin35 (17694, Cayman Chemical, Ann Arbor, MI) at 10 μM or 0.1% DMSO as a vehicle control for 24 h before harvesting.

2.2 Immunoblotting and immunocytochemistry

For Western blot analysis, CD4+ T cells were resuspended in lysis buffer (300 mM NaCl, 20 mM HEPES, 20 mM NaF, 5 mM EDTA, 2 mM Na3VO4, 1 mM DTT, 0.5 mM PMSF, 1% Triton X-100, 1x Complete [Roche], 1x PhosSTOP [Roche]) (Sigma, Darmstadt, Germany)) and centrifuged at 14,000 × g for 5 min to clear lysates. Protein concentration in the supernatant was determined using the BCA protein assay (Pierce, Thermo Fisher Scientific, Waltham, MA). Supernatants were mixed with 4x SDS loading buffer and denatured at 95°C for 5 min. Proteins were then immunoblotted using standard procedures and detected with ECL (Amersham, Thermo Fisher Scientific, Waltham, MA) and an Agfa Curix 60 or Syngene GeneGnome XRQ developer or with fluorescently labelled secondary antibodies and the Odyssey Infrared Imaging system (LI-COR, Bad Homburg, Germany). Quantifications of band intensities were performed either by
using ImageJ (for ECL films) or Odyssey software v2.1 (for Li-COR scans). Following background subtraction, all optical density (OD) values were normalized to β-actin or GAPDH as a loading control; when using phospho-specific antibodies, signals from the corresponding nonphospho antibody recognizing total protein were used as the internal loading control. For normalization, the loading control sample with the highest or mean OD was identified, after which all loading control values were divided by the highest or mean OD value to get values between 0 and 1. The target protein values were then divided by the corresponding relative OD value of the loading control. The average OD of the signal obtained from WT cells was taken as 100% and the signal of Arhgef6−/− cells (obtained after normalization to corresponding loading control) was expressed as a percentage of WT.

For immunocytochemistry, slides (15 well μ-slides, 81506 Ibidi, Gräfelfing, Germany) were coated overnight with 10 μg/ml ICAM-1 (796-1C, R&D, Minneapolis, MN) or 5 μg/ml ICAM-1 for membrane dye experiments, washed with HBSS (L-2035 Biochrom, Berlin, Germany) and blocked with HBSS + 2% fatty acid-free BSA (Roth, Karlsruhe, Germany). Cultured CD4+ T cells were harvested at days 7–9, seeded on coated slides (20,000 cells/well), and incubated at 37°C for 45 min. Cells were fixed and permeabilized using Cytofix/Cytoperm solutions (554714, Becton-Dickinson, Heidelberg, Germany). Cells were incubated overnight with primary antibodies at 4°C and with secondary antibodies for 1 h, both diluted in Cytoperm, at room temperature and kept in PBS + 0.2% BSA for imaging. Antibodies used for immunoblotting and immunocytochemistry were: Arhgef6 (C23D2, Cell Signaling, Frankfurt am Main, Germany), Arhgef7 (4515, Cell Signaling), β-actin (A5316, Sigma, Darmstadt, Germany), Cdc42 (sc-87, Santa Cruz, Dallas, TX), Cdc42-GTP (26905, New East Biosciences Biomol, Hamburg, Germany), Rac1 (610651, Becton-Dickinson, Heidelberg, Germany), Rac1-GTP (26903, New East Biosciences Biomol, Hamburg, Germany), Pak2 (60651, BD, phospho Pak1 (Thr 423)/phospho-Pak2 (Thr 402) (2601, Cell Signaling, Frankfurt am Main, Germany), GAPDH (mAB374, Merck Millipore, Darmstadt, Germany), LIMK1 (611748, Becton-Dickinson, Heidelberg, Germany), phospho-LIMK1 pThr508 (SAB4300103, Sigma, Darmstadt, Germany), Cofilin (612144, Becton-Dickinson, Heidelberg, Germany), and phospho Cofilin Ser3 (clone 77G2, 3313, Cell Signaling, Frankfurt am Main, Germany).

2.3 Image acquisition and analysis

Confocal images were acquired using Leica TCS SP5 confocal microscope (LAS AF software, version 2.0.2; 1024 × 1024 pixel display resolution; 12 bit dynamic range; 63x objective; 4x optical zoom, Leica Microsystems, Wetzlar, Germany). Brightfield and total internal reflection fluorescence (TIRF) images were acquired using the Leica DMi6000 microscope (LAS AF software, version 2.0.2; 10x objective for brightfield cell tracking; 100x objective for additional cell tracking, lamellipodia morphology and TIRF imaging).

For quantifications of fluorescence intensities (FIs) and morphologic parameters, all microscope settings were kept constant within one experiment. For each condition, a minimum of 60 cells were acquired and analyzed using ImageJ (NIH). TIRF imaging was used to detect FI at the contact region of a cell with the substrate using a penetration depth of 200 nm. Confocal LSM (laser-scanning microscopy) images were quantified to detect total immune-fluorescence signal intensity of cells. To quantify mean fluorescent intensity (MFI) of cells acquired by confocal microscopy, z-projections of image stacks (20 steps at 500 nm each) were made using the sum slices algorithm in ImageJ. For both confocal and TIRF images, thresholding was used to define cell outlines for quantification of area and of total immunofluorescence within the cell. ImageJ values for MFI were noted for each complete outline cell in the image along with two noncell areas for background subtraction. Resulting MFI values for WT cells from all images in one experiment with cells derived from one mouse per genotype were averaged, then individual values for WT and for knockout cells were divided by the WT average of the experiment and multiplied by 100 to get MFI values as percentages relative to the WT average in the experiment.

To quantify MFI in lamellipodia, T cells were first stained for Cdc42, Cdc42-GTP, or ARHGEF7, then were labeled with the CellBrite555 membrane dye (Biotium, Fremont, CA) at 1:1000 in PBS for 15 min at RT and analyzed by TIRF microscopy. Cells were outlined in ImageJ using thresholding and FIs within the resulting outlines were measured as mean gray values and referred as total cell FIs. To check for changes in peripheral distributions of FI, a second ROI was created for each cell that was 2 μm smaller than the first cell outline and set to approximately half of the cell to encompass the FI at the leading edge. The ratios of FI from the peripheral ROI to the total cell FI were then normalized to the WT average set as 100% for both WT and knockout cells. To measure lamellipodial size, lamellipodia were defined by manual free-hand bordering of thin sheet-like processes at the leading edge of cells in brightfield images. Each lamellipodium was characterized by area, length, and width. The angle between old and newly formed lamellipodia was calculated by drawing a line through the tips of consecutive dominant lamellipodial filaments at the leading edge and a vertex located in the visual center of the nucleus.

2.4 Migration assays

CD4+ T cells were resuspended in migration buffer (1x HBSS, 2% fatty acid-free BSA, 1 mM HEPES) and seeded at a density of 12,000 cells/well on slides coated with ICAM-1 as described earlier at 37°C. Loose and dead cells were carefully washed off with prewarmed migration buffer. Cells were imaged for 20 min at 20 s intervals using a 10x objective. Cells were manually tracked using the ImageJ manual tracking plugin. Tracks were analyzed using the Ibidi Chemotaxis tool v.2.0 for velocity, displacement, and straightness (displacement over path length). Arrest coefficient is the percentage of time that a cell is not moving (speed between frames at <2 μm/min) over the total time of the cell track. Autocorrelation, idling plots, cell tracks with idling marked, and plots of idling/active paths vs. angle changes were calculated using Excel VBA programs from Gorelik and Gautreau and plotted using GraphPad Prism v.7.
2.5 | Statistical analysis

All the data are reported as mean ± SD or SEM, as indicated, and means of different groups were compared using unpaired Student’s t-test or 2-way ANOVA in GraphPad Prism v. 7. The difference between two data sets was considered significant for P-values <0.05.

3 | RESULTS

3.1 | Arhgef6 controls the recruitment and activation of GTPases and the Cofilin pathway

In previous work, we found that the loss of Arhgef6 alters the expression of its homolog and binding partner, Arhgef7, in thymocytes. To test the expression of Arhgef7 in T cells, we checked lysates from WT and Arhgef6−/− CD4+ T cells by Western blot and found increased expression of Arhgef7 in the absence of Arhgef6 (Fig. 1A). Similarly, immunocytochemistry of WT and Arhgef6−/− CD4+ T cells on ICAM1-coated slides revealed an increase in Arhgef7 levels in Arhgef6−/− cells by confocal microscopy (Fig. 1B). In our earlier work, the increased Arhgef7 levels in thymocytes were accompanied by increased activation of Rac1 GTPase. First, total levels of Rac1 and Cdc42 in WT and Arhgef6−/− cells were compared by Western blot and found normal levels of Arhgef7 in the absence of Arhgef6 (Fig. 1A). Similarly, immunocytochemistry of WT and Arhgef6−/− CD4+ T cells on ICAM1-coated slides revealed an increase in Arhgef7 levels in Arhgef6−/− cells by confocal microscopy (Fig. 1B). In our earlier work, the increased Arhgef7 levels in thymocytes were accompanied by increased activation of Rac1 GTPase. First, total levels of Rac1 and Cdc42 in WT and Arhgef6−/− cells were compared by Western blot and were normal (Fig. 1C, D). Therefore, we next tested activation of both GTPases known to bind to both Arhgef6 and Arhgef7, Rac1 and Cdc42. To this end, we used immunocytochemistry, as the antibodies specific for the GTP-bound form of Rac1 and Cdc42 do not work in direct Western blotting of T cell extracts. These results showed that in the Arhgef6−/− T cells, despite normal total levels of Rac1, Rac1-GTP was notably increased (Fig. 1E). We then tested levels of active Cdc42 by staining for total Cdc42 and Cdc42-GTP, the active form. The total Cdc42 was essentially normal, with only a modest reduction in total Cdc42 (Arhgef6−/− levels at 94% of WT) whereas Cdc42-GTP was normal in Arhgef6−/− T cells (Fig. 1F).

The lack of marked differences in Cdc42 activation despite increased levels of Arhgef7 in Arhgef6−/− T cells prompted us to investigate potential defects in Arhgef7 and Cdc42 localization using TIRF microscopy to collect fluorescent signals at a depth of 200 nm from the cell-coverslip contact zone. TIRF imaging revealed a strong increase in localized Arhgef7 and of Cdc42 (Fig. 2A, B). Cdc42-GTP was also increased in the TIRF contact plane but proportionally to Cdc42; thus Cdc42 activity was normal and only recruitment was increased (Fig. 2B). We also assessed Arhgef7 and Cdc42 recruitment to the periphery of T cells where the leading edge of the lamellipodia would be found and observed slightly increased levels of recruitment of Arhgef7, Cdc42, and Cdc42-GTP to the periphery with respect to the total cell (Fig. 2A, B). Furthermore, when we repeated the same analysis on beta-actin, the levels of actin at the TIRF contact zone were also increased (Fig. 2C), despite normal levels of beta-actin by Western blot in the Arhgef6−/− T cells (Fig. 2D). Together, these data indicate that although overall levels of Cdc42 and beta-actin are normal, there is increased recruitment of both and of Arhgef7 to the integrin-ligand contact plane of the cell.

Arhgef6 and Arhgef7 bind to a Rac1 and Cdc42 effector, PAK2 kinase, which is the dominant PAK isoform in T cells. Western blot analysis of total PAK2 and phospho-PAK2 in WT and Arhgef6−/− T cells showed that expression of total PAK2 was higher in Arhgef6 knockout T cells but the level of phosphorylated PAK2 was lower (Fig. 3A). One target of active PAK2 kinase is the kinase LIMK1. LIMK1 is phosphorylated to become active and can then phosphorylate Cofilin on serine 3 to inactivate it. We therefore examined phosphorylation of LIMK1 and Cofilin in Arhgef6−/− T cells by Western blot and found that the phosphorylated forms of both LIMK1 and Cofilin were greatly reduced in Arhgef6−/− T cells (Fig. 3B, C). As a loading control for pLIMK1, we used GAPDH (Fig. 3B) because the LIMK1 antibody did not work in Western blot, although it did work in immunocytochemistry. Thus, Arhgef6 is required for normal activation of a signaling pathway that includes PAK2, LIMK1, and Cofilin.

3.2 | Arhgef6 restrains lamellipodia spread and inhibits local actin polymerization in T cells

The increased Cofilin activity suggested a possible defect in actin polymerization in Arhgef6−/− T cells. To investigate, we imaged WT and Arhgef6−/− T cells at 100x magnification and quantified lamellipodia size and numbers. One early observation was that Arhgef6−/− T cells frequently displayed multilobed protrusions that appeared to consist of newly forming lamellipodia that coexisted with previous lamellipodia. In contrast, WT T cells typically displayed only a single lamellipodium (Fig. 4A). Moreover, the lamellipodia on Arhgef6−/− T cells were wider and larger overall than those on WT cells. To quantify, we measured lamellipodial length, width, overall area, and the angles between two subsequent lamellipodia (Fig. 4B). A histogram of small (<70 μm2) and large (>70 μm2) lamellipodia illustrated the observation that WT cells displayed a majority of small ones, whereas in contrast Arhgef6−/− T cells had mainly large ones (Fig. 4C). The width was normal in Arhgef6−/− T cells (Fig. 4D) but the length and the corresponding angle from the centroid to the tips of two subsequent lamellipodia were significantly increased in the Arhgef6−/− T cells (Fig. 4E and F). Additionally, the overall area of the lamellipodia was higher in the mutant T cells (Fig. 4G). In order to show these defects in Arhgef6−/− T cells, we stained membranes and imaged cells randomly migrating on ICAM1-coated slides using TIRF microscopy. The Arhgef6−/− T cells displayed larger lamellipodia that rapidly shifted orientation around the cell surface, leading the cell to migrate in unexpected directions (Fig. 4H and Supporting Information Videos S1 [WT T cells] and S2 [Arhgef6−/− T cells]). Together, these observations show that Arhgef6 restricts lamellipodial size and placement.

3.3 | Arhgef6 restrains speed and turning in T cells

Because lamellipodia are involved in the directionality of migrating cells, we compared the migration trajectories of WT and Arhgef6−/− T cells. Cells were settled on ICAM-1-coated slides and imaged for 20 min while spontaneously migrating. A representative trajectory plot showed that Arhgef6−/− T cell tracks appeared longer and less straight than WT (Fig. 5A and Supporting Information Video S3 [WT T cells]).
FIGURE 1  Increased expression of Arhgef7 and activity of Rac1 in Arhgef6−/− T cells. (A) Western blots of Arhgef7 in wild-type (WT) and Arhgef6−/− (KO) CD4+ T cells with densitometric quantification (n = 3 each). (B) Immunofluorescent images of CD4+ T cells stained for Arhgef7 and quantification of total fluorescence intensity (n = 61 WT and n = 66 KO cells pooled from 3 experiments, one mouse of each genotype per experiment). (C, D) Western blots of Rac1 (C) or Cdc42 (D) in WT and Arhgef6−/− CD4+ T cells with quantification (n = 3 each). (E) Immunofluorescent images of CD4+ T cells stained for Rac1 or Rac1-GTP show normal levels of Rac1 but a significant increase in Rac1-GTP to Rac1 ratios in Arhgef6−/− cells (n = 103 WT and n = 83 KO cells pooled from 4 experiments, one mouse of each genotype per experiment). (F) Immunofluorescent images of CD4+ T cells stained for Cdc42 and Cdc42-GTP show normal levels of activation (n = 315 WT and n = 308 KO cells pooled from 3 experiments, one mouse of each genotype per experiment). All quantifications show results from Arhgef6−/− cells normalized to WT results. ns = not significant, *P < 0.05, **P < 0.01, ***P < 0.001, by Student’s t-test. Values are mean ± SEM. Scale bars, 20 μm.
T cell migration at 10x] and Supporting Information Video S4 [KO T cell migration at 10x]). Quantification of the averages of multiple experiments revealed a statistically significant increase in velocity and decrease in straightness for Arhgef6-/- T cells (Fig. 5B). To visualize these differences, we plotted straightness against velocity for each cell tracked in all experiments and observed a clear relationship between straightness and velocity for WT cells: high speed (>10 μm/min) corresponds to a high level of straightness. However, this relationship did not hold for the Arhgef6-/- T cells, as the faster cells displayed a wide range of straightness values (Fig. 5C). To confirm this, we quantified the displacement, and straightness values for the faster cells (velocity > 10 μm/min). The displacement was slightly reduced for Arhgef6-/- T cells (P = 0.11, t-test), and the straightness was significantly reduced (Fig. 5D). To apply an additional test of straightness, we calculated the direction autocorrelation values, a statistical measure of correlation between turning angles in a cell’s trajectory. The higher position of the line for WT cells indicates greater correlation between turning angles, consistent with straighter trajectories than for Arhgef6-/- T cells (Fig. 5E). The increased turning of Arhgef6-/- T cells suggested that Cdc42 was involved. To test the role of Cdc42 in T cell turning, we treated T cells with a Cdc42 inhibitor, Casin. Casin had no effect on T cell speed for either genotype and also no effect on overall track straightness (Fig. 5F). As expected, Arhgef6-/- T cells were faster and twistier than WT cells (Fig. 5F). However, Casin significantly reduced straightness of fast-moving WT cells, showing that Cdc42 is required for maintaining a straighter cell migration path for fast-moving cells (Fig. 5F). Together, these data showed that Cdc42 may be involved in T cell turning and that Arhgef6-/- T cells migrated faster than WT cells but turned more.

Increased cell migration speed is normally associated with reduced cell turning, and proteins that induce cell turning, such as Arpin, reduce cell speed. In contrast, the Arhgef6-/- T cells migrate faster and turn more than WT cells. To further investigate the increased turning, we used several programs from the Gautreau group to qua-
tify correlations between turning and cell arrest in Arhgef6−/− T cells. A visual depiction of the velocities of 50 individual cells in a typical movie used blue squares to indicate velocity over 2 μm/min and white squares to show cell arrest between frames, defined as velocity under 2 μm/min. The white squares were reduced in tracks from Arhgef6−/− T cells (Fig. 6A). Confirming this visualization, the average arrest coefficients from each 20 min movie of WT or Arhgef6−/− migrating T cells showed a significant reduction in percentage of time spent in arrest for Arhgef6−/− T cells (Fig. 6B). A visual depiction of the tracks with pauses under 2 μm/min marked with red dots showed that for WT T cells, pausing is often associated with cell turning, as has been reported for other cell types (Fig. 6C). However, these illustrations also revealed reduced pausing in Arhgef6−/− T cells (Fig. 6C). To quantify the association between pausing and turning in Arhgef6−/− T cells, we assessed turn angles in cells that either paused or migrated faster than 2 μm/min for 1, 2, or 3 frames (Fig. 6D). Turning angles are expected to be lower for actively moving cells than for idling cells as idling is associated with turning, and this was the case for both WT and Arhgef6−/− T cells (Fig. 6D). In addition, there was no difference between WT and Arhgef6−/− T cells in the turning angles of idling cells (Fig. 6D, “idling” cells, left panel). However, the actively moving Arhgef6−/− T cells (“active” cells, right panel) displayed consistently higher turning angles than those of WT (Fig. 6D). Together, these data show that in Arhgef6−/− T cells, cell turning is increased and is uncoupled from cell pausing.

### 3.4 LIMK inhibition increases T cell size and speed

To determine if the defective Cofilin pathway in Arhgef6−/− T cells was responsible for one or both of the velocity and turning defects, we treated WT and mutant T cells with the LIMK1 inhibitor, LIMK3. Arhgef6−/− T cells already displayed the expected extremes in lamellipodia in untreated conditions, and did not change upon LIMK3 treatment; however, WT cells underwent a shape change upon inhibitor treatment, which we observed in T cells stained with a membrane dye and imaged with TIRF microscopy as in Figure 4 (Fig. 7A and Supporting Information Videos S5–S8). WT and Arhgef6−/− T cells treated with a EtOH vehicle as controls were not affected by the EtOH (Supporting Information Videos S5 and S6; WT T cells with EtOH; KO T cells with EtOH), as they showed no differences from untreated migrating cells analyzed in Figure 4H (Supporting Information Videos S1 and S2) although Arhgef6−/− T cells displayed the expected increased speed and morphologic changes. Upon LIMK3 treatment, quantification of lamellipodia sizes revealed a significant increase in lamellipodial length and area in WT cells, consistent with an increase in Cofilin activation (Fig. 7B). We next tested the effects of LIMK3 on T cell migration. Similar to the lamellipodia, migration of Arhgef6−/− T cells was unaffected by LIMK3 treatment, because velocity and straightness were already significantly abnormal (Fig. 7C and D, and Supporting Information Videos S7 and S8; WT T cells with LIMK3; KO T cells with LIMK3). However, LIMK1 inhibition strongly affected the velocity of WT T cells, which increased from approximately 10 to 14 μm/min, the average velocity of Arhgef6−/− T cells (Fig. 7B). Surprisingly, LIMK1 inhibition did not reduce the straightness of WT T cells to the levels of Arhgef6−/− T cells, indicating that Cofilin does not regulate the migration persistence of Arhgef6−/− T cells (Fig. 7B and Supporting Information Videos S7 and S8). Together, these data suggest that Arhgef6 controls lamellipodial size and cell migration speed via Cofilin and path straightness via a Cofilin-independent pathway.

### 4 Discussion

Random T cell migration enables the cell to thoroughly scan an environment for antigens without unnecessary energy expenditures. To achieve an efficient search pattern, T cells must tightly control both migration speeds and cell turn angles. In this study, we have shown
FIGURE 4  Defective lamellipodia on Arhgef6−/− T cells. (A) Representative brightfield images of bilobed lamellipodia found in Arhgef6−/− CD4+ T cells compared to typical lamellipodia on wild-type (WT) CD4+ T cells, on which a new lamellipodia protrudes as an old one retracts. P = protrusion, R = retraction. Right: quantification of incidence of bilobed protrusions on cells normalized to WT (n = 3 experiments with 15 cells each, one mouse of each genotype per experiment). Mean ± SEM. Scale bar, 5 μm. (B) Diagram of lamellipodial parameters including width, length, area, and angle between old and new lamellipodia. (C) Frequency distribution of small and large size lamellipodia discriminated by area threshold of 70 μm². Numbers on bars show numbers of cells per group (n = 30 cells total pooled from 3 experiments, one mouse of each genotype per experiment). (D–G) Quantification of width (D), length (E), angle (F), and area (G) on WT and Arhgef6−/− CD4+ T cells (n = 30 for length, width, area; n = 20 for angles, from 3 experiments with one mouse per genotype each). ns = not significant, *P < 0.05, ****P < 0.0001, by Student’s t-test. Values are mean ± SD. (H) Images from Supporting Information Videos S1 and S2 (t = 10 s intervals) of WT and Arhgef6−/− CD4+ T cells stained with CellBrite live cell membrane dye migrating on ICAM-1. Scale bar, 5 μm
**FIGURE 5** *Arhgef6*−/− T cells migrate faster and less persistently than wild-type (WT). (A) Representative track plots of WT and *Arhgef6*−/− CD4+ T cells migrating on ICAM-1. (B) Velocity, displacement, and straightness ratio of WT and *Arhgef6*−/− cell migration tracks. Data points represent averages of 50 individual cell tracks from one experiment (n = 9 experiments with 50 tracks each, one mouse of each genotype per experiment). (C) Straightness ratio vs. velocity of individual cell tracks. Vertical line at velocity 10 μm/min indicates threshold used for track quantification in (D). (D) Displacement and straightness ratio of cells with velocities over 10 μm/min (pooled from n = 9 experiments in A, B). (E) Direction autocorrelation of WT and *Arhgef6*−/− CD4+ T cell turning angles. Higher autocorrelation between turning angles indicates smaller variation between angles on a straighter migration path. Values are mean ± SD. (F) Effects of Casin (Cdc42 inhibitor) pretreatment (10 μm for 24 h) on velocity of all cells, straightness of all cells, and straightness of fast cells (velocities over 10 μm/min). Data points represent averages of individual cell tracks per experiment (n = 12 (WT) and 10 (KO) experiments with 50 tracks each, one mouse of each genotype per experiment). ns = not significant, *P < 0.05, **P < 0.01, ***P < 0.001, by Student’s t-test. Values are mean ± SD.
FIGURE 6  Arhgef6 couples T cell idling and turning. (A) Idling plots of wild-type (WT) and Arhgef6−/− CD4+ T cell tracks, depicted as rows of squares, from a representative movie (50 tracks, each with 61 images acquired over 20 min). Blue squares: migration between frames (20 s apart) with velocity over 2 μm/min, white squares: migration between frames with velocity under 2 μm/min. (B) Arrest coefficients of WT and Arhgef6−/− CD4+ T cells (values are mean ± SD; n = 8 experiments). (C) Representative cell track plots with pausing (velocity under 2 μm/min) indicated with red dots. (D) Angle changes in idling and actively migrating WT and Arhgef6−/− CD4+ T cells. Cells are classified as active or idling if frame-to-frame velocity is over 2 μm/min (active) or under 2 μm/min (idling) for n = 1, 2, or 3 frames between a start and end frame. Angle changes for each idling or active event are averaged (±SEM) and plotted against number of idling or active frames (multiple angles per track, 50 tracks per movie, 9 movies per genotype). *P < 0.05 by Student’s t-test

that Arhgef6 is essential for reducing lamellipodia formation, migration speed, and cell turning angles. In Arhgef6−/− T cells, several proximal signaling proteins dependent on Arhgef6 were dysregulated: expression of Arhgef7 and Rac1 activity was increased, whereas Cdc42 was mislocalized. In addition, PAK2 and LIMK1 phosphorylations are reduced, resulting in decreased phosphorylation and inhibition of Cofilin. Active Cofilin, the nonphosphorylated form, supplies g-actin for de novo actin polymerization by severing and depolymerizing old filaments. The increased Cofilin activity in Arhgef6−/− T cells leads to enlarged lamellipodia that are not limited to one pole of the cell but project randomly around the cell periphery. We found that the inhibition of LIMK in WT T cells, and by extension the Cofilin pathway, only partially mimicked the Arhgef6−/− T cell phenotype: T cell speed was increased but cell twistiness was not increased. In contrast, inhibition
FIGURE 7 LIMK1 inhibition increases wild-type (WT) T cell migration speed. (A) Total internal reflection fluorescence (TIRF) images from Supporting Information Videos S5 to S8 (t = 10 s each) of WT and Arhgef6−/− CD4+ T cells treated with control ethanol vehicle or LIMK1 inhibitor, LIMKi3 (10 μM for 4 h), stained with CellBrite live cell membrane dye and migrating on ICAM-1 display increased lamellipodia size for WT cells. Scale bar, 5 μm. (B) LIMK1 inhibition in WT T cells causes increased lamellipodial width and area but has no effect on Arhgef6−/− T cells. Quantification of lamellipodial length (left), width (center), and area (right) for WT and Arhgef6−/− T cells either untreated vehicle control (ctrl) or treated with LIMKi3 as shown in (A). Bars = mean ± SEM, (n = 3 experiments with 30 cells each). ns = not significant, ****P < 0.0001, by 2-way ANOVA followed by Bonferroni’s post hoc test. (C) Representative track plots for WT and Arhgef6−/− T cells migrating on ICAM-1 either untreated (ctrl) or treated with LIMKi3. (D) Quantification of velocity, displacement and straightness for the samples shown in (c). Mean ± SD. *P < 0.05, ***P < 0.001, ****P < 0.0001, by Student’s t-test.
FIGURE 8 Schematic representation of Arhgef6-controlled signaling pathways. In wild-type (WT) cells, Arhgef6 and Arhgef7, RhoGEFs for Rac1 and Cdc42, repress signaling to actin reorganization and restrict lamellipodial formation to limit cell speed and maintain relative straightness. In T cells lacking Arhgef6, cells migrate faster and turn more. Cdc42 is mislocalized to the ICAM1-coated migration surface and Rac1 is overactivated. Moreover, Pak2, LIMK1, and Cofilin are all hypophosphorylated meaning that Cofilin, which promotes actin severing and polymerization, is overactivated. The mechanisms for Rac1 activation of lamellipodia extension are not characterized here but may include hyperactivation of WAVE and Arp2/3, both required for lamellipodia extension. Arhgef7 expression is increased, likely due to its taking the place of Arhgef6 in the PIX-GIT complex, but it cannot compensate fully for the absence of Arhgef6 as the immune cell-specific Arhgef6 may be required for targeting the complex to T cell specific receptors.

of Cdc42 resulted in increased twistiness of fast cells but cell speed was unchanged. These data suggest that Arhgef6 controls two pathways critical to T cell migration: cell speed via Cofilin-mediated actin polymerization and cell turning, possibly via localization of Cdc42.

These observations highlight a role for Arhgef6 as a repressor of lamellipodia formation and of cell migration speed in T cells (Fig. 8). We hypothesize that Arhgef6 signals through Pak2 and LIMK1 to inhibit Cofilin activation of actin severing and also restricts a newly formed lamellipodia to a site close to the old lamellipodia. Arhgef6 and Arhgef7 (aka PIX proteins) are normally found in a constitutive complex with GIT1 and GIT2 proteins. In the absence of Arhgef6, Arhgef7 may be protected from degradation by taking the place of Arhgef6 in the complex, thus increasing Arhgef7 expression overall. However, Arhgef7 may not be able to compensate properly for Arhgef6 in the complex, possibly because Arhgef6 possesses an actin-binding domain, called a CH domain, that is absent in Arhgef7 and which may target the PIX-GIT complex to actin at receptors in T cells. The increased Arhgef7 expression is accompanied by increased Rac1-GTP activity, which may activate WAVE and Arp2/3 complexes in the formation of lamellipodia. And although activated Rac1 is increased, it fails to direct the Pak-LIMK signaling pathway to inhibit Cofilin, likely because the role of Arhgef6 is to localize the GTPases to a single, dominant lamellipodia. Thus, increased Arhgef7 at the periphery of T cells could mislocalize the complex of PIX-GIT proteins and Cdc42 and fail to repress Rac1.

Further support for the idea that Arhgef6 and Arhgef7 control placement of cellular projections by regulating expression and localization of downstream signaling proteins is found in other cell systems. In fibroblasts, Arhgef7 is initially recruited by Cdc42 to a nascent lamellipodium, where it then subsequently recruits Rac1 to polymerize actin and induce protrusion formation. In neurons, GIT1 is enriched in synapses and acts with Arhgef7 to stabilize synaptic spines. However, overexpression of Arhgef7 or of a truncated form of GIT1 mislocalizes these proteins, causing multiple dendritic protrusions. Aberrant expression—either higher or lower, of Arhgef6-controlled proteins is often associated with multipolar protrusions. For example, overexpression of Arhgef7 in fibroblasts or its deletion in anterior visceral endoderm cells both cause multiple protrusions. Moreover, knockdown of Cofilin can either induce or repress multiple protrusion formation in different cells lines, possibly in response to metastatic cues. Similarly, Cdc42 knockdown or deletion causes multiple protrusions in both dendritic cells and neutrophils. Further, overexpression of active Rac1V12 in neurons and metastatic cell lines is sufficient to induce multiple protrusions. Together, these observations
illustrate a model of Arhgef6 and Arhgef7 controlling Rac1, Cdc42, and Cofilin in restricting protrusions to specific subcellular locations.

Cell migration speed and turning are linked by localized actin polymerization. Migrating cells move in the direction of their lamellipodia. A stable, actin-rich lamellipodium correlates with comparatively fast retrograde actin flow and migration along a straight path. And in the converse case, unstable lamellipodia correlate with arrested actin flow, formation of new lamellipodia, and a turning migration path. The unrestricted lamellipodia formation around the perimeter of Arhgef6−/− T cells caused twistier migration paths. However, Arhgef6−/− T cells were also 20–25% faster than WT cells. Our results reveal a role for Arhgef6 in coupling T cell speeds with T cells turning. The Arp2/3 inhibitor, Arpin, is also involved in cell speed and turning but differs from Arhgef6 in that Arpin reduces cell speed because it increases cell turning. In contrast, Arhgef6 represses both cell speed and cell turning, likely by signaling upstream of Rac1, Cofilin, WAVE complex activation and Arp2/3, and thus represents a nexus between the placement of a new lamellipodium and the extension of that lamellipodium. It has been demonstrated that actin flows mediate coupling between cell speed and turning, and we show here that Arhgef6 is integral to this mechanism.

Immune cells in general, and T cells in particular, are the most motile cells in the body and can migrate at remarkable speeds through vessels as well as tissues and organs. It is possible that T cells use Arhgef6 and Arhgef7 to act as the brakes on a migration motor that is always running, and then release the immune-specific Arhgef6 as needed at specific integrins or TCR to unleash actin reorganization. We reported earlier that Arhgef6−/− thymocytes displayed increased migration speeds and reduced arrest coefficients, leading to inefficient scanning of antigen-presenting cells and impaired T cell development. We also observed decreased arrest of Arhgef6−/− T cells here. Defects in scanning of antigen-presenting cells are also linked to cell turning in the case of T cells with a mutation in Myo1g. Therefore, the loss of Arhgef6 control of lamellipodia formation is a molecular mechanism that would likely lead to defective T cell-based immunity in Arhgef6 knockout mice. In conclusion, Arhgef6 and Arhgef7 control Rac1, Cdc42, and Cofilin to restrain lamellipodia formation, cell turning, and cell migration speed.

AUTHORSHIP

D.M., M.K., J.H., and A.G. designed and performed the experiments. D.M., M.K., J.H., A.G., and K.T. participated in the data analysis. K.T. and K-D.F. prepared the manuscript.

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ACKNOWLEDGMENTS

The authors thank Christoph Schwarzer for excellent technical assistance and Dr. Kristina Langnäse for many helpful discussions. This work was supported by the ABINEP graduate school funded by the Ministry for Economics, Science, and Digitization of the State Saxony-Anhalt, and by the European Funds for Social and Regional Development (EFRE, ESF) to K-D.F. and by a GRK 1167 fellowship from Deutsche Forschungsgemeinschaft to D.M.

Open access funding enabled and organized by Projekt DEAL.

DISCLOSURES

The authors declare no conflicts of interest.

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SUPPORTING INFORMATION

Additional information may be found online in the Supporting Information section at the end of the article.

How to cite this article: Mamula D, Korthals M, Hradsky J, Gottfried A, Fischer K-D, Tedford K. Arhgef6 (alpha-PIX) cytoskeletal regulator signals to GTPases and Cofilin to couple T cell migration speed and persistence. J Leukoc Biol. 2021;110:839-852. https://doi.org/10.1002/JLB.1A1219-719R