

Molecular mechanisms of spontaneous and directed mast cell motility

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ABSTRACT

Migration is a fundamental function of immune cells, and a role for Ca^{2+} in immune cell migration has been an interest of scientific investigations for many decades. Mast cells are the major effector cells in IgE-mediated immune responses, and cross-linking of IgE-Fc ϵ RI complexes at the mast cell surface by antigen activates a signaling cascade that causes mast cell activation, resulting in Ca^{2+} mobilization and granule exocytosis. These cells are known to accumulate at sites of inflammation in response to parasite and bacterial infections. Using real-time imaging, we monitored chemotactic migration of RBL and rat BMMCs in response to a gradient of soluble multivalent antigen. Here, we show that Ca^{2+} influx via Orai1 plays an important role in regulating spontaneous motility and directional migration of mast cells toward antigen via IgER complexes. Inhibition of Ca^{2+} influx or knockdown of the Ca^{2+} entry channel protein Orai1 by shRNA causes inhibition of both of these processes. In addition, a mutant Syk⁻ shows impaired spontaneous motility and chemotaxis toward antigen that is rescued by expression of Syk. Our findings identify a novel Ca^{2+} influx-mediated, Orai1-dependent mechanism for mast cell migration. *J. Leukoc. Biol.* 92: 000–000; 2012.

Introduction

Mast cells are key effector cells in IgE-associated immune responses, including allergic disorders and protective immune responses against certain bacteria and parasites [1]. Mast cells

carry out adaptive immune functions through antigen- and IgE-dependent clustering of the high-affinity IgER, Fc ϵ RI [2]. Cross-linking of IgE-Fc ϵ RI complexes at the mast cell surface activates a signaling cascade that causes mast cell activation, resulting in Ca^{2+} mobilization and consequent release of pre-formed mediators of the allergic response and inflammation [3]. The RBL-2H3 mast cell line has structural and functional characteristics of differentiated mucosal mast cells [4], and it has been used extensively for biochemical and cell biological investigations of mast cell function. Mast cell recruitment into sites of inflammation is associated with helminth and bacterial infections [5, 6] and chronic allergic disorders [7]. In particular, differentiated mucosal mast cells are known to redistribute from the submucosa or crypt area to the lamina propria and intraepithelial regions of jejunal villi during the course of an immune response to certain parasitic infections [8]. This process depends on mast cell motility and is likely to be driven by chemotactic responses, but the mechanisms underlying this process are poorly understood.

The directed migration of leukocytes in response to soluble cues, known as chemotaxis, is induced by various extracellular signals, including chemokines and cytokines, lipid mediators, bacterial factors, and ECM degradation products [9–11]. Chemotactic ligands have been identified for mast cells, including S1P [12], SCF [13], arachidonic acid metabolites leukotriene B4 [14], and PGE₂ [15], as well as several chemokines [16]. In addition, mast cell chemotaxis toward IgE-specific antigen was first reported for RBL cells [17] and later characterized in mouse mast cells [18–20].

A role for Ca^{2+} in directed hematopoietic cell migration has been implicated in some studies [21–23] but not in others [24, 25]. Human neutrophil migration has been reported to slow and/or stop when the extracellular Ca^{2+} is depleted [21, 22, 26], and migration is reduced when intracellular Ca^{2+} is buffered [27, 28]. In contrast, other studies find that neutrophils orient correctly across the chemoattractant gradient without extracellular Ca^{2+} [24, 25] or even migrate faster [25]. A rise in intracellular Ca^{2+} as a result of interaction with APCs

Abbreviations: Δ =change, 2-APB=2-aminoethoxydiphenyl borate, BIM=bisindolylmaleimide I, BSS=buffered saline solution, CRAC= Ca^{2+} release-activated Ca^{2+} , Gd³⁺=gadolinium ion, GdCl₃=gadolinium trichloride, IP₃=inositol-1,4,5-trisphosphate, mRFP=monomeric red fluorescent protein, MSD=mean squared displacement, Orai1=calcium release-activated calcium channel protein 1, RBL=rat basophilic leukemia, RFP=red fluorescent protein, S1P=sphingosine-1-phosphate, SCF=stem cell factor, SH2=Src homology 2, (SH2)₂=tandem Src homology 2, shRNA=small hairpin RNA, SOCE=store-operated calcium entry, STIM=stromal interaction molecule, Syk⁻=RBL cell variant that lacks the tyrosine kinase Syk, TRPC=transient receptor potential channel, yFMI=y forward migration index

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causes T cells to stop crawling [29, 30]. All of these observations suggest that the role of Ca^{2+} in hematopoietic cell polarization and migration may be different for different cell types and circumstances. One of the principal mechanisms for Ca^{2+} influx in eukaryotic cells is via CRAC channels, by which depletion of intracellular Ca^{2+} stores triggers Ca^{2+} influx through the coupling of the ER store Ca^{2+} sensor STIM1 to the plasma membrane channel protein Orai1 [31]. Recently, evidence linking Orai1 and STIM1 to cancer cell migration and metastasis [32], neutrophil recruitment and polarization [33], and vascular smooth muscle cell migration [34, 35] has been described.

In the course of investigating mast cell motility and directional migration, we found that Ca^{2+} influx plays a key role in regulating spontaneous mast cell motility via the SOCE channel protein Orai1. Furthermore, we found that antigen directly elicits a chemotactic response in mucosal mast cells, and this directed migration is dependent on the tyrosine kinase Syk, extracellular Ca^{2+} , and Orai1, as assessed using real-time imaging. These results demonstrate the importance of Ca^{2+} dynamics in mast cell motility and directed migration toward antigen, while revealing roles for Syk and Orai1 in these processes.

MATERIALS AND METHODS

Chemicals, reagents, and constructs

Cytochalasin D, wortmannin, U-73122, 2-APB, and GdCl_3 were purchased from Sigma-Aldrich (St. Louis, MO, USA). Rat rSCF and rIL-3 were from PeproTech (Rocky Hill, NJ, USA). SIP is from Enzo Life Sciences (Farmingdale, NY, USA). Mouse monoclonal anti-DNP IgE was purified, as described previously [36]. Multivalent antigen (DNP-BSA) contained an average 15 DNP groups/protein and was prepared, as described previously [37]. GFP-PLC- γ 1 (SH2)₂ [38] and Syk-CFP (ATCC ID: 10373748) cDNA constructs were gifts from Dr. Tobias Meyer (Stanford University, Stanford, CA, USA). shRNA plasmids targeting Orai1, STIM1, and TRPC1 (OriGene, Rockville, MD, USA) were described previously and characterized in RBL cells [39]. GCaMP3 [40] and R-GECO 1 [41] constructs were obtained from Addgene (Cambridge, MA, USA).

Cell culture

The RBL-2H3, RBL-C1, and Syk⁻ cells were maintained as monolayers in minimal essential medium supplemented with 20% (v/v) FBS and 10 $\mu\text{g}/\text{ml}$ gentamicin. All tissue-culture reagents were obtained from Invitrogen (Carlsbad, CA, USA) unless noted otherwise. For transient transfection, cells were plated in a 35-mm culture dish at 70–80% confluence and transfected with fluorescently tagged Syk or the tandem SH2 domain of PLC γ using FuGENE HD (Roche Diagnostics, Indianapolis, IN, USA), per the manufacturer's instructions, with modifications to enhance transfection efficiency in RBL cells, as described previously [42]. For knock-down studies, cells were transiently transfected by using FuGENE HD or by electroporation of 5×10^6 cells using Gene Pulser X (Bio-Rad, Hercules, CA, USA) with 20 μg each shRNA interfering plasmids specific for Orai1 or STIM1, which also contain a genetically encoded fluorescent protein expression sequence, or with 20 μg shRNA plasmids targeting TRPC1, together with 8 μg of the expression vector that encodes mRFP, as described previously [43]. For controls, cells were transfected with plasmids lacking the shRNA insert. Cells were used 48 h after transfection. Rat BMMCs were differentiated from bone marrow-derived stem cells of Lewis strain rats by culturing for 14–28 days in the presence of rat SCF (50 ng/ml) and rat IL-3 (100 ng/ml), as described previously [44].

Cell motility measurements

RBL-2H3 cells or rat BMMCs were plated at low density ($\sim 1.5 \times 10^5$ cells/dish) overnight in 35-mm dishes with coverslip inserts (MatTek, Ashland, MA, USA). Time-lapse video microscopy of live cells was collected for 2–3 h with images taken every 1–2 min. Images were collected using a 40 $\times$ /0.65 NA or a 10 $\times$ /0.22 NA dry objective with a Leica DMIR microscope with a Photometrics Quantix charge-coupled device camera (Roper Scientific, Tucson, AZ, USA), and a thermally regulated air gun (ASI 400 air stream incubator, Nevtek, Williamsville, VA, USA) was used to maintain the temperature at 37°C throughout the experiment. To quantify cell migration, we developed an automated tracking and analysis algorithm using Matlab, in which the cell bodies were tracked automatically; then, the MSD was calculated based on migration tracks. Roughly speaking, the MSD tabulates the average distance traveled by the cell body in a given time interval (τ). With the use of our tracking algorithms, we measure the positions of the cell body as a function of time [$r(t)$], where the time between measurements (Δt) is 2 min. A single track that persists for T minutes contains $N = T/\Delta t$ individual positions, and the MSD is calculated as follows for time intervals τ between 2 and 20 min

$$MSD(\tau) = \frac{1}{N} \sum_{j=0}^{N-1} (r(j) - r(j + \tau))^2 \quad (1)$$

For random motion in two dimensions, the MSD varies linearly with τ , and the slope is related to the diffusion coefficient (D) as $MSD = 4D\tau$. We find that $MSD(\tau)$ varies approximately linearly with τ for time intervals between 10 and 20 min. We define a mobility coefficient as the slope of this linear segment as follows

$$MSD(10\text{min} < \tau < 20\text{min}) = (\text{Motility coefficient})\tau + \text{constant} \quad (2)$$

Average motility coefficients and SEM were calculated for multiple individual tracks as specified in text and figure legends.

For measurements of cell velocities with transfected RBL cells, positively transfected cells were identified by the expression of GFP or RFP and manually tracked using Manual Tracking plugin for ImageJ (U.S. National Institutes of Health, Bethesda, MD, USA). Cell velocities were determined using the same plugin.

Ca^{2+} imaging and fluorescence spectroscopy

In preparation for Ca^{2+} imaging, RBL cells were transiently transfected with Ca^{2+} sensor GCaMP3 by electroporation and then plated onto 35 mm MatTek dishes. After 24 h, cells were washed with BSS [135 mM NaCl, 5 mM KCl, 1.8 mM CaCl_2 , 1 mM MgCl_2 , 1 mg/ml glucose, 20 mM HEPES (pH 7.2–7.4)] and imaged using a heated (37°C) stage with 25 $\times$ oil immersion objective on a Zeiss 710 confocal microscope. GCaMP3-transfected cells were excited at 488 nm, and fluorescence was monitored at 505–530 nm. Time-lapse images were taken every 2 s for 10–20 min. For measurements without extracellular Ca^{2+} , cells were washed in BSS without Ca^{2+} [135 mM NaCl, 5 mM KCl, 3 mM MgCl_2 , 1 mM EGTA, 1 mg/ml glucose, 20 mM HEPES (pH 7.2–7.4)] and imaged in the same buffer. Pharmacological reagents 2-APB (final concentration of 10 μM) or GdCl_3 (final concentration of 2 μM) were added just prior to initiating data collection.

To evaluate the effect of Orai1 shRNA on stimulated Ca^{2+} mobilization, RBL cells were transfected with this vector or its control shRNA together with R-GECO1 using FuGENE HD as described above. After 48 h, transfected cells were harvested with 1 mM EGTA in PBS, resuspended in BSS without Ca^{2+} , and monitored in an SLM 8000C steady-state fluorimeter using excitation 565 nm and emission 600 nm [37].

Chemotaxis measurements

RBL-2H3 cells or rat BMMCs were plated into the narrow observation channel separating the two reservoirs in a chemotaxis μ -slide chamber (ibidi, Verona, WI, USA) in complete medium. In the cases indicated, cells were sensitized for 1 h with anti-DNP IgE (final concentration of 3 $\mu\text{g}/\text{ml}$) before plating or overnight with anti-DNP IgE (final concentration of 2 $\mu\text{g}/\text{ml}$) simultaneously with plating. Any unbound IgE in the observation

channel was washed away by changing the medium inside of the channel with fresh medium before filling up the reservoirs. RBL mast cells were plated in uncoated ibidi μ -slide chambers, and rat BMMCs were plated into collagen IV-coated ibidi μ -slide chambers. After 24 h of incubation, one of the reservoirs was filled with complete medium with 25 mM HEPES (pH 7.2–7.4). The other reservoir was filled with medium containing a potential chemoattractant, as indicated. Chemoattractant concentrations specify the final concentration used to fill the reservoir. For experiments without extracellular Ca^{2+} , cells cultured overnight were washed into medium containing 4 mM EGTA and 3 mM MgCl_2 and then equilibrated with the same medium for the duration of the experiment. Images were collected using 10 $\times$ /0.22 NA dry objective with the Leica microscope described above and maintained at 37°C throughout the experiment. After 16 h of collecting time-lapse images every 10 min, cells were tracked using the Manual Tracking plugin for ImageJ (U.S. NIH).

The Manual Tracking plugin provides a way to retrieve coordinates (x, y) and velocity of a single cell by manually clicking in the center of that cell body. This click designates the original position of the specified cell (set as 0,0), and the migrating cell is then followed in a coordinate system with positive y in the direction of the lower μ -slide chamber containing the test chemoattractant (or control medium). Multiple individual cells can be visibly tracked in this manner (see Fig. 5). The chemotactic index (yFMI) was determined for the tracked cells with the Chemotaxis and Migration Tool plugin of ImageJ. yFMI quantifies the chemotactic response of cells by dividing the net Δy value of a given track by total accumulated distance traveled to that endpoint. The value for yFMI was calculated as follows

$$yFMI = \text{net change in } y \text{ direction} / \text{Total path length} = \sum \Delta y / \sum (\Delta x^2 + \Delta y^2)^{1/2} \quad (3)$$

The Δx and Δy have positive and negative values, as determined by the coordinate system described above (see Fig. 5). The sums for individual cells in Equation 3 were carried out for coordinates (x, y) determined every 10 min over the observation period of 16 h [45]. Velocities of individual cells (stimulated or unstimulated) were calculated from the Chemotaxis and Migration Tool plugin as the total distance traveled by a cell [$\sum (\Delta x^2 + \Delta y^2)^{1/2}$], divided by the time that the cell was tracked. For experiments with transiently transfected cells, only fluorescent protein-tagged cells were analyzed. Relative velocity was calculated as the velocity of cells transfected with specific shRNA, divided by the velocity of cells transfected with control shRNA.

Statistical analysis

Statistical analysis was performed using unpaired two-tailed Student's t test. Summary data are represented as means $\pm$ SEM. We consider a value of $P < 0.05$, designated by one or more asterisks, to be significant.

RESULTS

Mast cells exhibit spontaneous motility

With the use of RBL-2H3 mast cells as a model, we initially characterized the spontaneous motility of mast cells using real-time video microscopy. RBL-2H3 cells often exhibit distinctive extended membrane protrusions after several hours in culture on glass surfaces (Fig. 1A, left panel). The population of cells migrates spontaneously in all directions, and individual cells often move along tracks that are defined by the elongated protrusions (Fig. 1B, left panel, and Supplemental Movie 1). To evaluate motility characteristics of mast cells, we developed an automated tracking method, which yields a motility coefficient for cells tracked as described in Materials and Methods (Equation 2). The motility coefficient is a measure of the average area that cells survey/unit time, and it is analogous to a two-dimensional diffusion coefficient [46]. In agreement with pre-

vious findings with other hematopoietic cells, inhibition of actin polymerization by 1 μM cytochalasin D completely blocked cell motility in complete medium (Fig. 1C), and inhibition of PI3K by 200 nM wortmannin substantially reduced cell motility (Fig. 1D). Wortmannin is known to be inactivated by components in medium [47], and motility measurements with this inhibitor were carried out in BSS, in which the average motility is less than in complete medium (compare Fig. 1C and D).

To investigate further the molecular bases of spontaneous motility in mast cells, we evaluated the mutant RBL cell line RBL-C1, which is deficient in Fc ϵ RI-mediated activation of Cdc42 and Rac1 and in Cdc42-dependent biosynthetic trafficking [48]. These cells exhibit substantially reduced motility, suggesting significant roles for these Rho family GTPases in this process (Fig. 1C). In addition, we evaluated Syk $^-$ [49] and found that this protein contributes to spontaneous RBL cell motility (Fig. 1C). In contrast, inhibition of PKC with BiM at a concentration that significantly inhibits degranulation [50] does not alter cell motility (Fig. 1D), suggesting differential requirements for intracellular signaling pathways that regulate mast cell motility and granule exocytosis.

Similar to RBL mast cells, primary rat BMMCs have IgERs and the mast cell-specific ganglioside detected with mAb AA4, and they similarly exhibit a mucosal mast cell phenotype [51]. Although BMMCs have heterogeneous morphologies, we observe extended protrusions in a subset of these cells, very reminiscent of those seen with RBL-2H3 mast cells (Fig. 1A, right panel). Rat BMMCs also show spontaneous migration on glass and have motility characteristics similar to RBL-2H3 mast cells (Supplemental Movie 2), with a somewhat lower average motility coefficient value in medium (compare Fig. 1C and E). As for RBL cells, cytochalasin D completely inhibits this motility (Fig. 1E). These results provide evidence that mucosal mast cells migrate spontaneously and that actin polymerization, Rho GTPases, protein tyrosine kinase Syk, and PI3K are involved in regulating this motility.

Ca^{2+} influx regulates spontaneous motility of mast cells

Ca^{2+} mobilization contributes to a diverse range of cell functions, including cell motility and adhesion, and is essential for several mast cell functions [3, 52]. We investigated whether Ca^{2+} dynamics may be involved in the spontaneous migration of resting mast cells using pharmacological inhibitors. RBL-2H3 cells exhibit substantially reduced cell motility in the absence of extracellular Ca^{2+} as compared with control cells (Fig. 2A). We found that chelating intracellular Ca^{2+} with BAPTA-AM did not further reduce their migration (data not shown), and we hypothesized that impaired Ca^{2+} influx is responsible for the reduction in motility. Although 2-APB was first described as an inhibitor of IP $_3$ R-mediated Ca^{2+} release [53], subsequently, it was shown to directly inhibit SOCE in T cells at concentrations between 10 and 50 μM [54]. In RBL-2H3 cells, 2-APB has an inhibitory effect on stimulated Ca^{2+} influx but fails to inhibit IP $_3$ R-mediated Ca^{2+} release from ER stores at concentrations up to 40 μM [43]. As shown in Fig. 2A, 10 μM 2-APB causes a large reduction in RBL cell motility. As RBL cells do not express voltage-gated Ca^{2+} channels [55], Gd^{3+} can be used to specifically block CRAC channels in these

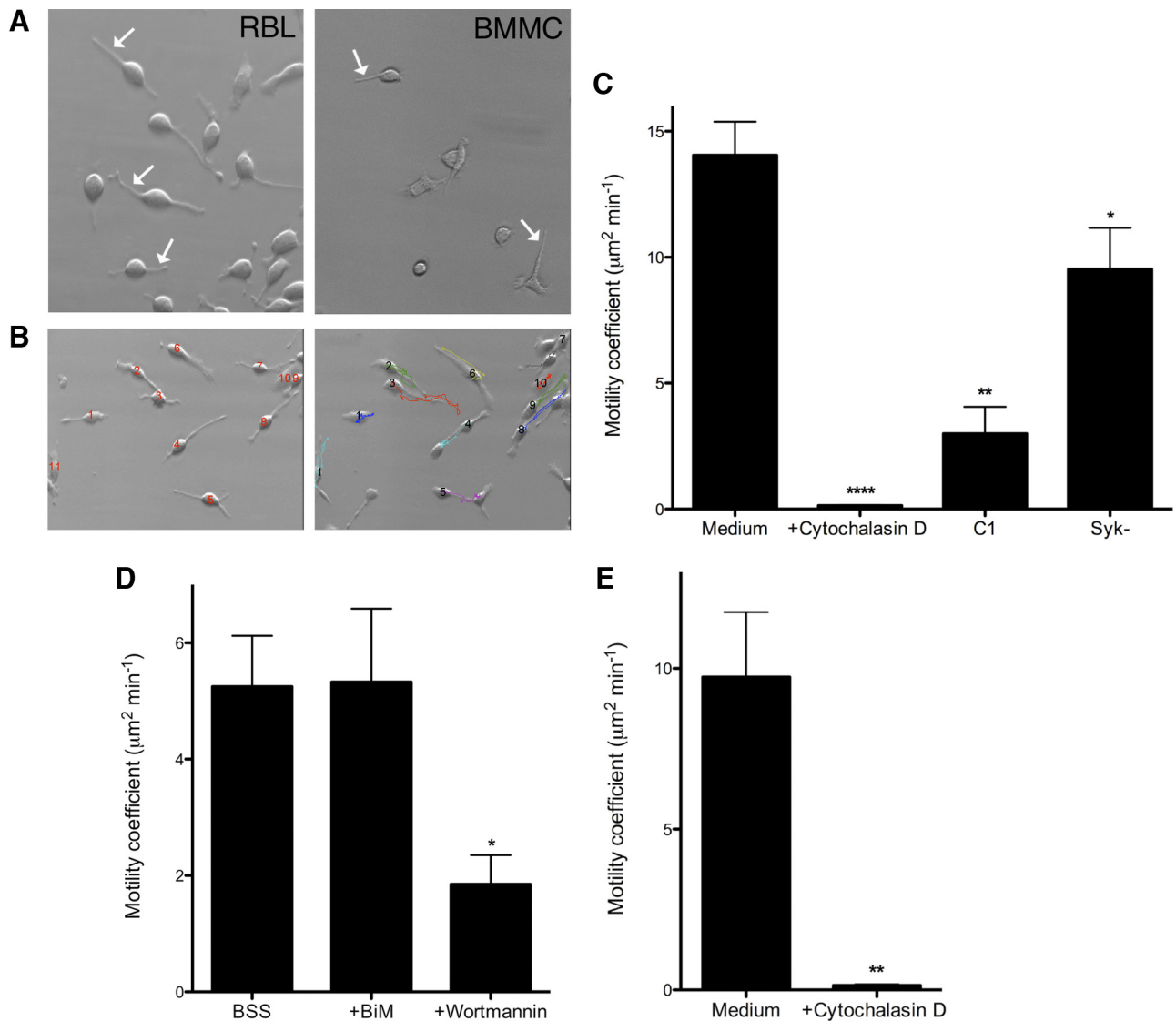


Figure 1. Morphology and motility properties of RBL-2H3 mast cells and rat BMMCs. (A) Phase contrast images of RBL-2H3 cells (left) and rat BMMCs (right) in culture medium. Note polarized morphologies with extended protrusions that are common for these cells after several hours on glass surfaces (arrows). (B) Representative images of first (left) and last (right) snapshots of time-lapse images of RBL-2H3 cells that were automatically tracked for 3 h, as described in Materials and Methods. Numbers identify identical cells in both images, and colored lines in the right panel represent the cell migration tracks. (C and D) Average motility coefficients of RBL-2H3 cells, as determined by Equation 2. Error bars show SEM for $n = 15$ –97 cells for each sample. Cell motility was monitored for 1.5–3 h in culture medium (C) or in BSS with 1 mg/ml BSA (D). Inhibitors (1 μM cytochalasin D, 1 μM BiM hydrochloride, or 200 nM wortmannin) were added just prior to motility measurements. For samples with wortmannin, cell motility was monitored in buffer without BSA. C1 is a mutant RBL cell line, RBL-C1, defective in Rho GTPase activation. (E) Average motility coefficients of rat BMMCs $\pm$ SEM for $n = 30$ –67/each sample. Cytochalasin D (1 μM) was added just prior to motility measurements. * $P < 0.05$; ** $P < 0.01$; **** $P < 0.0001$ compared with untreated control.

cells [56]. When 2 μM Gd^{3+} was added to assess the role of CRAC channels in RBL-2H3 cell motility, we observed a significant reduction in motility, although not as severe as when 2-APB was used. The PLC inhibitor U-73122 also caused a substantial decrease in cell motility (Fig. 2A). This suggests that basal activation of SOCE mediated by IP_3 contributes to spontaneous motility. As U-73122 can react with free sulfhydryl groups present in BSA, these measurements were carried out in BSS without BSA, and the average motility of control RBL

cells is somewhat larger under these conditions than in the presence of BSA (compare Figs. 2A and 1D).

These same trends of reduced motility when Ca^{2+} influx is inhibited are also observed in primary rat BMMCs (Fig. 2B). For these cells, the spontaneous motility coefficient in BSS is only approximately one-third of its value in complete medium (compare Figs. 2B and 1E), suggesting factors in the serum contribute substantially to motility. Collectively, these data demonstrate that absence of extracellular Ca^{2+} or pharmaco-

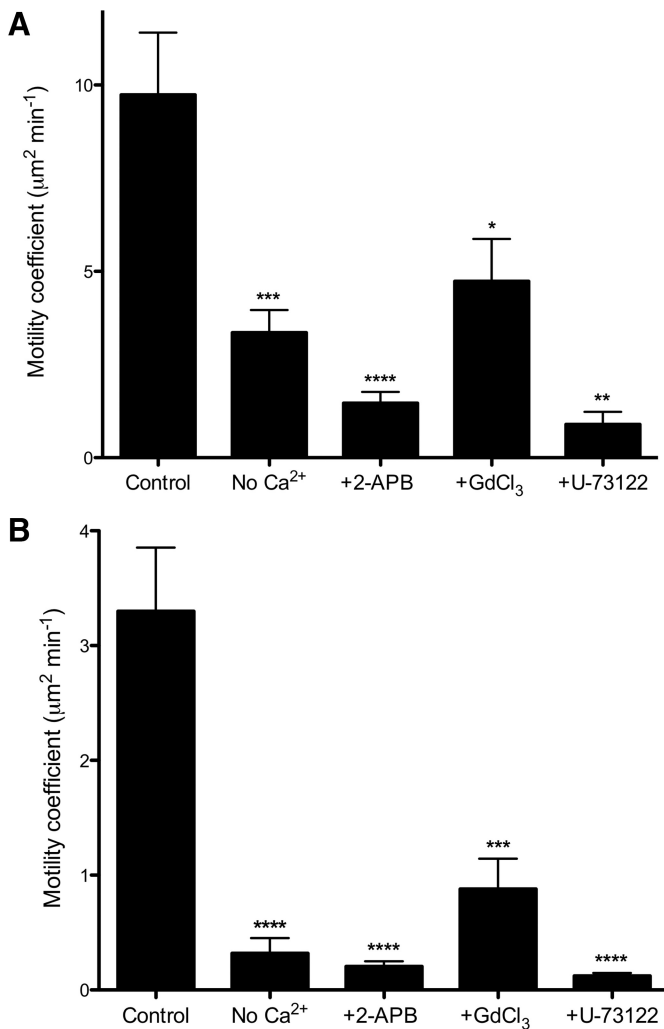


Figure 2. Extracellular Ca^{2+} is important for mast cell motility. Motilities of individual RBL-2H3 cells (A) and rat BMMCs (B) were monitored for 1.5 h in BSS, and average motility coefficients $\pm$ SEM ($n=14-88$ /each sample) calculated from Equation 2 are shown. No Ca^{2+} refers to BSS without CaCl_2 and with 1 mM EGTA and 2 mM MgCl_2 . SOCE inhibitor 2-APB (10 μM), Orai1 channel inhibitor GdCl_3 (2 μM), and PLC inhibitor U-73122 (2 μM) were added prior to motility measurements. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$ compared with control.

logical inhibition of Ca^{2+} influx causes reduction in spontaneous motility of rat mucosal mast cells.

To evaluate further the molecular bases of Ca^{2+} influx in mast cell motility in resting RBL cells, we knocked down Orai1 and STIM1, the major components of CRAC channel activation, using shRNA vectors. A previous study showed that each of these same shRNA vectors causes inhibition of antigen-stimulated SOCE in RBL cells [39]. Consistent with this, we found that Orai1 shRNA coexpression with the genetically encoded Ca^{2+} sensor R-GECO1 results in reduction of SOCE responses to antigen and thapsigargin by 50% and 35%, respectively (Supplemental Fig. 1). Expression of the shRNA for Orai1 significantly reduced spontaneous RBL cell velocity by ~30%

compared with control vector-transfected cells (Fig. 3 and Supplemental Fig. 2). By comparison, STIM1 shRNA did not significantly reduce motility in these cells. A recent study showed that STIM2 is the primary regulator of spontaneous levels of cytoplasmic Ca^{2+} in HeLa and other cells [57], and this other STIM family member may be most important for spontaneous motility in RBL cells. TRPC1 shRNA did not reduce velocity compared with controls but rather caused a small increase in the rate of migration. With the use of this construct, we found previously that TRPC1 contributes to antigen-stimulated Ca^{2+} wave initiation in RBL cells [43]. Our results with shRNA vectors support a positive role for Ca^{2+} influx via Orai1 in spontaneous mast cell motility.

RBL-2H3 mast cells exhibit spontaneous Ca^{2+} transients

Changes in intracellular Ca^{2+} , including Ca^{2+} puffs, waves, and oscillations, follow antigen-stimulated Fc ϵ RI-mediated activation in mast cells as we described previously [43], but spontaneous Ca^{2+} events in unstimulated cells have not been characterized previously. As spontaneous motility in mast cells appears to be regulated by Ca^{2+} influx (Figs. 2 and 3), we investigated Ca^{2+} mobilization events under nonstimulatory conditions using the genetically encoded Ca^{2+} indicator GCaMP3 and real-time confocal microscopy. GCaMP3 is reported to have an increased fluorescence quantum yield, higher affinity for Ca^{2+} , and significantly better signal-to-noise ratio than previously used GCaMP2 [40], making it better-suited for monitoring transient Ca^{2+} mobilization events. As represented in Fig. 4A, we observed short-lived, localized intracellular Ca^{2+} transients that occur frequently in extended pro-

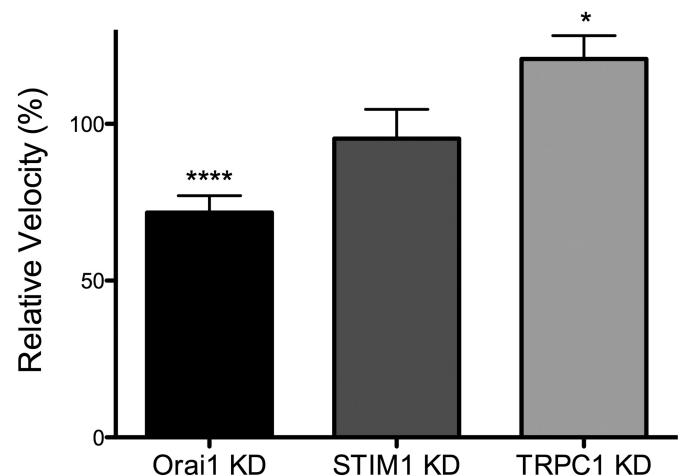


Figure 3. Orai1 is involved in RBL mast cell motility. RBL-2H3 cells were transiently transfected with shRNA specific (KD) for Orai1, STIM1, or parallel control vectors (TRPC1), respectively. TRPC1 shRNA was cotransfected with mRFP, and in control experiments, RBL cells were transiently transfected with mRFP only. Cell tracks were monitored for 1.5 h in media, and relative average velocities are shown $\pm$ SEM ($n=49-76$ cells/each sample). * $P < 0.05$; **** $P < 0.0001$ compared with controls. Averages of absolute velocity values are shown in Supplemental Fig. 1.

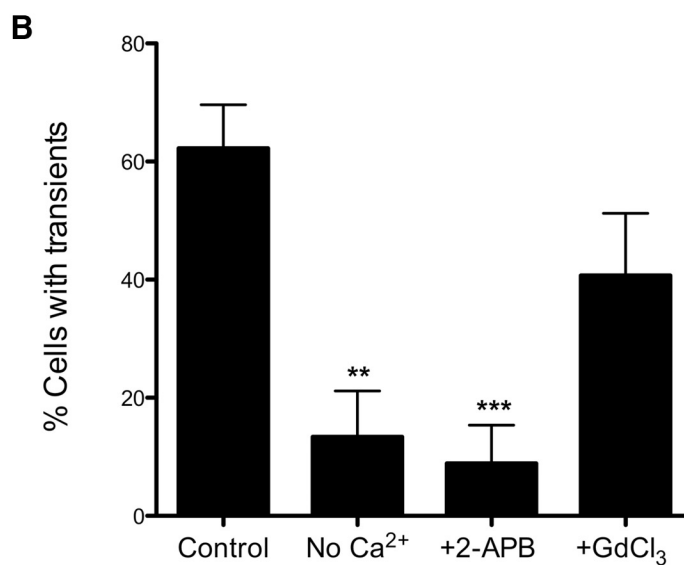
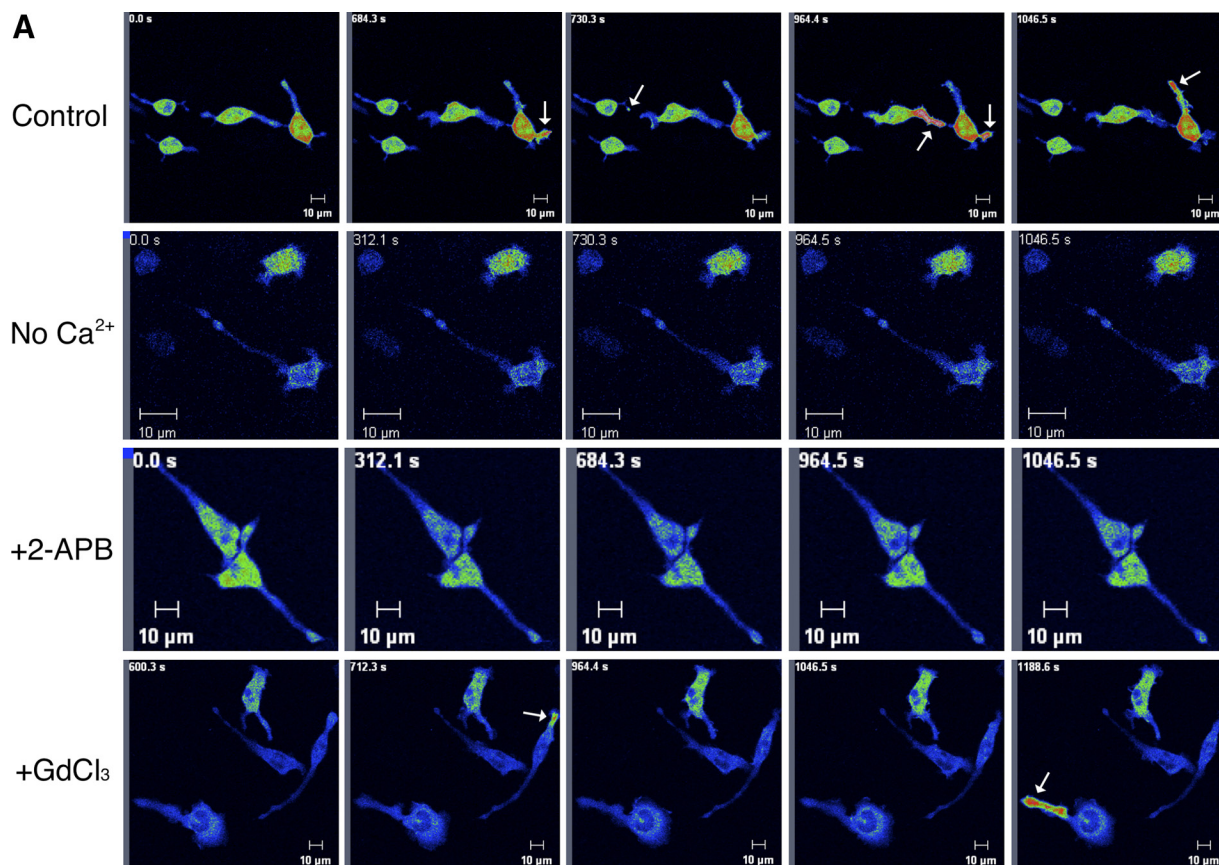


Figure 4. RBL-2H3 mast cells exhibit spontaneous Ca^{2+} transients with influx dependence that correlates with motility. (A) Confocal images of representative RBL-2H3 mast cells expressing GCaMP3. Time-lapse images were taken every 2 s for 20 min. Warmer colors indicate higher Ca^{2+} levels. Note local Ca^{2+} transients (red or white) frequently occurring in protrusions (arrows). No Ca^{2+} refers to BSS without CaCl_2 and with 1 mM EGTA and 2 mM MgCl_2 . Inhibitors 2-APB (10 μM) or GdCl_3 (2 μM) were added just prior to collecting time-lapse images. (B) Summary of average percentages of cells with Ca^{2+} transients compared with total GCaMP3-expressing cells $\pm$ SEM ($n=27\text{--}42$ /each condition; monitored in three experiments). ** $P < 0.01$; *** $P < 0.001$ compared with control.

trusions (Fig. 4A and Supplemental Movie 3). During 20 min of real-time confocal microscopy, an average of $62.3\% \pm 7.4\%$ SEM of GCaMP3-expressing cells exhibited Ca^{2+} transients (Fig. 4B). Strikingly, when the cells were monitored in the absence of extracellular Ca^{2+} , the number of cells that show spontaneous Ca^{2+} transients decreased to $14.3\% \pm 6.4\%$. Adding 2-APB also caused substantial reduction in the number of cells with Ca^{2+} transients ($8.9\% \pm 6.5\%$), and Gd^{3+} caused a smaller reduction to $40.7\% \pm 10.6\%$ (Fig. 4B), similar to the trends observed in RBL-2H3 cell motility under these conditions (Fig. 2A). These results are consistent with the possibility that spontaneous Ca^{2+} transients dependent on SOCE are important for spontaneous motility of these cells.

Mast cells show directed migration toward antigen

To visualize mast cell chemotaxis in real time, we established a chemotaxis assay using μ -slide chambers as described in Materials and Methods. RBL-2H3 cells were plated into a narrow observation channel separating two reservoirs, and the putative chemoattractant was added to one of the reservoirs to establish a spatially defined chemotactic gradient. After imaging cells for 16 h, the cell paths were tracked manually (Fig. 5A and Supplemental Movie 4) and analyzed. As shown in the representative experiments in Fig. 5B and the corresponding Supplemental Movie 5, anti-DNP IgE-sensitized RBL-2H3 cells exhibit net chemotaxis toward antigen in a gradient up to 10 ng/mL DNP-BSA. For analysis within a rectangular coordinate system, the cell migration tracks are plotted after setting the start point for each cell to $x = 0$, and $y = 0$, with the x -axis corresponding to the line separating the reservoirs, and the positive y -axis pointing in the direction toward the reservoir containing the chemoattractant (or control buffer). As represented in Fig. 5B, more cells have a net migration toward the reservoir containing antigen (red cells) than toward the opposite reservoir (black cells), and similar numbers of cells have a net migration in both directions in the absence of antigen (control experiment).

To quantify directed migration, we calculated the chemotactic index, or yFMI, where yFMI was determined by dividing the net Δy value of a given cell track by its accumulated total distance (Equation 3). As summarized in Fig. 6, RBL-2H3 cells migrate toward antigen in a dose-dependent manner, with a maximal response observed with 10 ng/ml DNP-BSA in the reservoir. We observed that these cells do not migrate significantly toward 100 ng/ml antigen, probably as this dose stimulates immobilization and cell spreading (data not shown). As expected, RBL-2H3 cells do not chemotax toward the antigen when the cells are not sensitized with anti-DNP IgE (data not shown). Rat BMMCs showed a small, statistically insignificant chemotactic response toward the antigen over a similar dose range. BMMCs also exhibited substantial directed migration toward the SCF, which is a known chemoattractant for BMMCs (Figs. 5C and 6) [13]. RBL mast cells express constitutively active c-kit [1], a receptor for the SCF, making directed migration of RBL cells toward the SCF unlikely. These results provide strong evidence that rat mucosal mast cells can sense and

directly migrate in response to gradients of antigen and/or the SCF.

Syk participates in RBL mast cell chemotaxis toward antigen

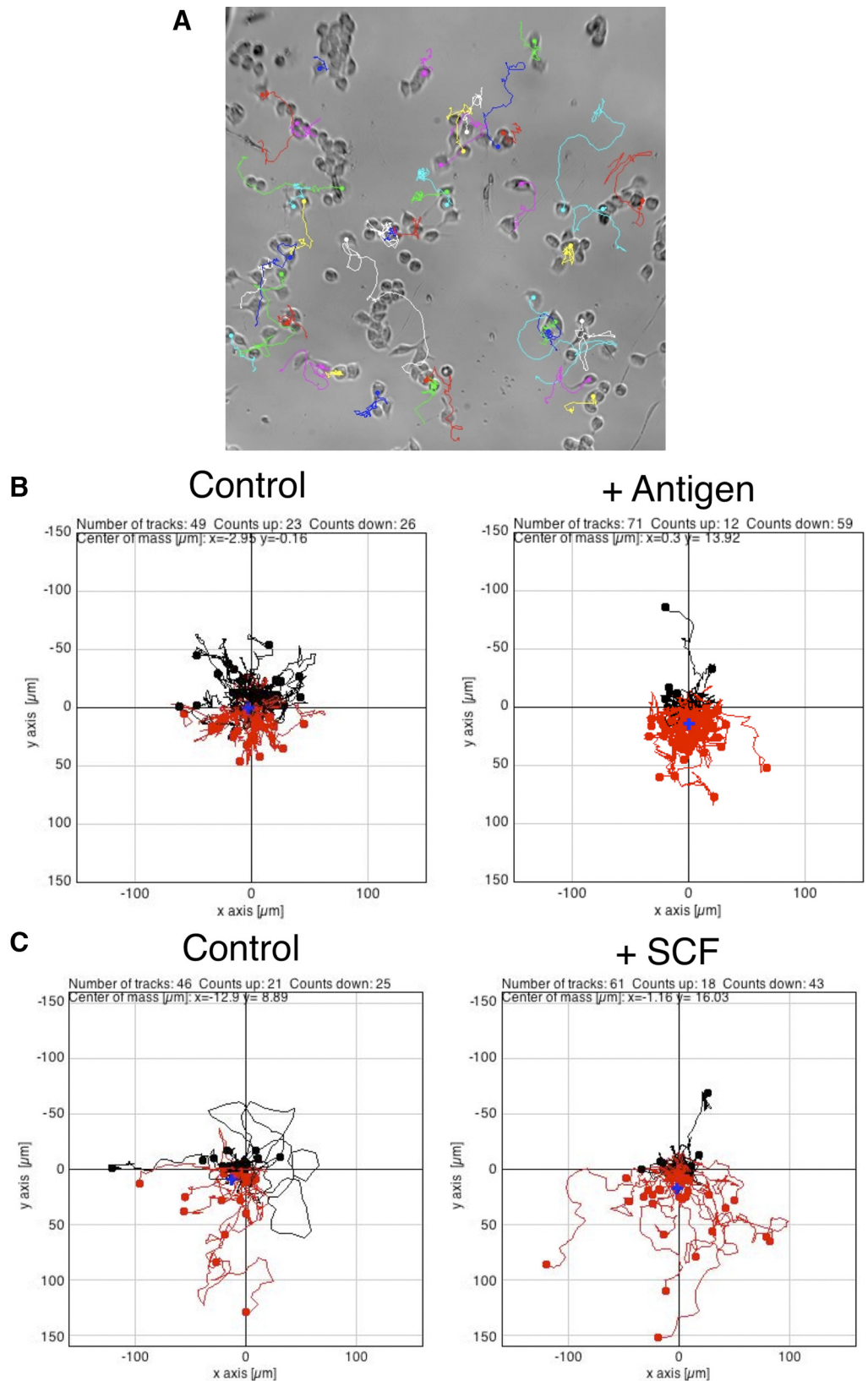
A previous study provided genetic evidence that mouse BMMC chemotaxis toward the antigen depends on tyrosine kinase Syk [19]. To investigate the situation for RBL mast cells, we initially assessed the chemotactic capacity of Syk[−] RBL cells, characterized previously by Zhang et al. [49]. As shown in Fig. 7, Syk[−] cells sensitized with IgE failed to chemotax toward antigen at 10 ng/ml, even though they have similar IgER densities and initial receptor phosphorylation responses as RBL-2H3 cells [49]. In fact, these cells exhibited a greater probability to migrate in the direction opposite the source of the antigen. Individual migrating cells typically reverse directions often during the observation period, and this usually results in a small net migration difference in the absence of chemotaxis. It is unclear at the present time why Syk[−] cells exhibit final yFMI values that are negative on average. Notably, chemotaxis toward the antigen could be restored by transiently expressing Syk-CFP into the Syk[−] cells, consistent with participation of Syk in this chemotactic response (Fig. 7). Syk contains a tandem SH2 domain that might be sufficient to restore the chemotactic response. Therefore, as a control, we transiently expressed in Syk[−] cells the tandem SH2 domain of PLC γ (SH2), which was previously shown to associate with cross-linked IgERs [38]. These cells behaved similarly to untransfected Syk[−] cells, consistent with a requirement for the Syk kinase domain in restoring chemotaxis to antigen.

Ca^{2+} channel protein Orail is important for RBL-2H3 cell migration toward antigen

As we observed decreased spontaneous motility of unstimulated RBL-2H3 mast cells in the absence of extracellular Ca^{2+} and when the Ca^{2+} channel protein Orail was knocked down (Figs. 2 and 3), we asked whether these conditions also impair mast cell chemotaxis toward the antigen. As shown in Fig. 8, when RBL-2H3 cells were monitored in excess EGTA, they show significantly reduced yFMI when compared with cells in normal media. Cells transiently transfected with shRNA specific for Orail also show markedly reduced yFMI when compared with untransfected cells and with cells that were transfected with control shRNA. These data provide strong evidence that Ca^{2+} influx via Orail plays an important role not only in spontaneous mast cell motility (Fig. 3) but also in directed migration to antigen. Previous reports have shown that mast cells generate and secrete SIP upon cross-linking of Fc ϵ RI [12, 20, 58]. SIP can act as a chemoattractant for mast cells [12], and it has been suggested that mast cell chemotaxis toward the antigen may be a result of SIP secreted by Fc ϵ RI activation of cells. When SIP was used as a chemoattractant, RBL-2H3 mast cells show directed migration toward SIP, as demonstrated previously [12] (Fig. 8). However, this chemotaxis does not depend on extracellular Ca^{2+} , suggesting an alternative molecular basis for this process. In contrast, the velocities of RBL cells chemotaxing toward SIP decreased in

Figure 5. Monitoring and analyzing mast cell chemotaxis in real time.

(A) Representative image of RBL-2H3 cells in an ibidi chemotaxis μ -slide after 16 h tracking. Colored lines show migration tracks derived from ImageJ Manual Tracking plugin program. (B) Representative plots from a single experiment showing migration tracks of RBL-2H3 cells with (right) and without (left) 10 ng/mL DNP-BSA in the lower reservoir buffer. (C) Representative plots for rat BMMCs with (right) and without (left) 100 nM SCF in the lower reservoir buffer. The migration tracks were plotted after setting the starting point for each cell at $x = 0$, and $y = 0$, using ImageJ Chemotaxis and Migration Tool plugin. Red tracks indicate individual cells with net migration toward the lower chamber that contained chemoattractant (or control). Blue crosses represent the average endpoints.



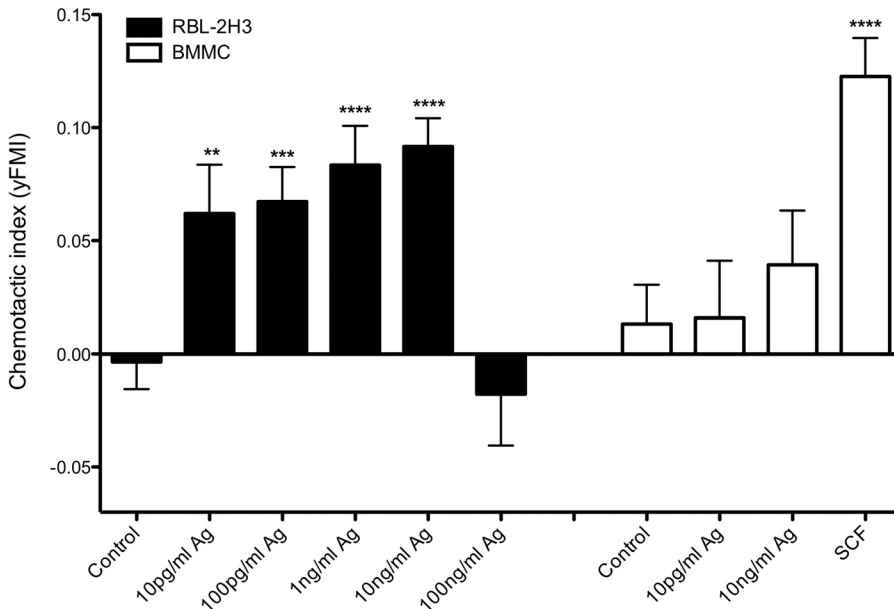


Figure 6. RBL-2H3 mast cells show chemotaxis toward antigen. Mast cell chemotaxis is represented as the average yFMI $\pm$ SEM ($n=36-137$ cells/each condition). yFMI is determined by dividing the net y value of a given track by accumulated distance (Equation 3). RBL-2H3 cells (black bars) or rat BMMCs (open bars) were sensitized with anti-DNP IgE, plated onto ibidi chemotaxis μ -slide chambers overnight, and then monitored for 16 h in the absence (Control) or presence of varying doses of DNP-BSA or SCF, as indicated, in the lower reservoir buffer. ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$ compared with respective control sample.

excess EGTA (Supplemental Fig. 3), indicating that extracellular Ca^{2+} has some involvement in cell migration in the presence of S1P.

DISCUSSION

The role of Ca^{2+} in regulating leukocyte migration has been a question of interest for many decades [21, 24, 25]. Our results using real-time video microscopy provide direct evidence that Ca^{2+} influx is important for mast cell motility

that is spontaneous, as well as for migration that is directed toward an activating antigen. Using pharmacological inhibitors as well as genetic manipulations, we present compelling evidence that SOCE and the CRAC channel protein Orai1 are important for regulating these processes.

PI3Ks have been shown to be key regulators of chemotaxis and cell polarity in T cells and neutrophils [59–63], and we found that spontaneous motility in RBL-2H3 mast cells is reduced substantially by inhibition of PI3K by wortmannin (Fig. 1D). Additionally, by studying the motility properties of the

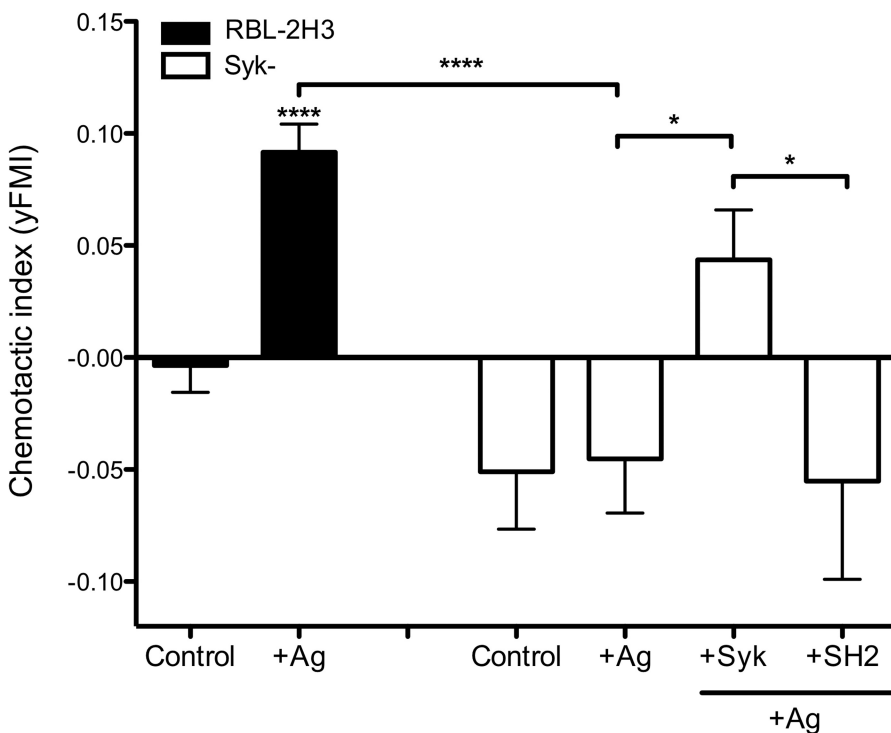
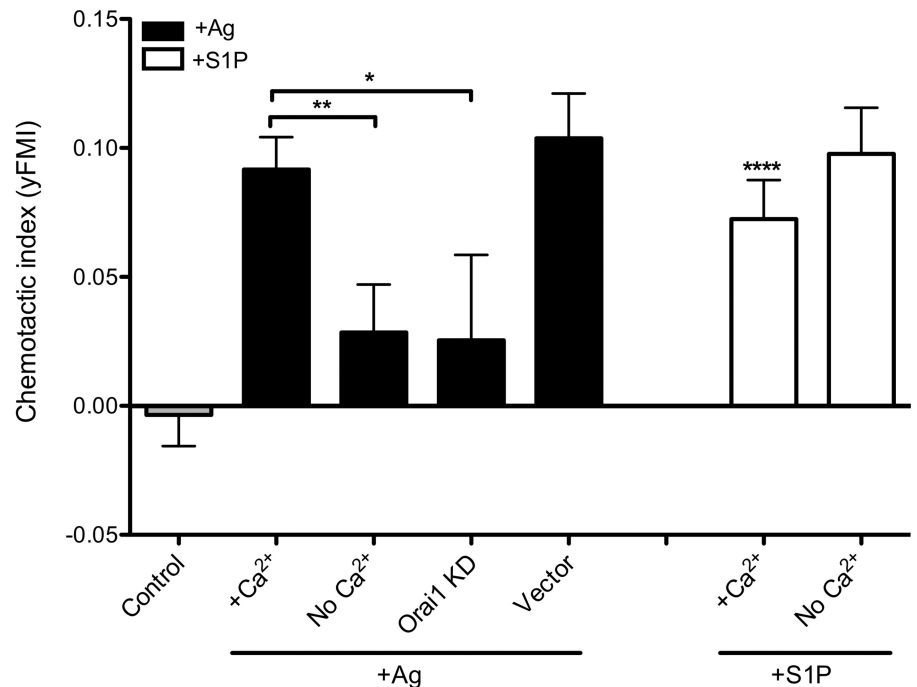


Figure 7. Directed migration of RBL-2H3 cells toward antigen depends on Syk kinase. Syk $^{-}$ cells were sensitized with anti-DNP IgE, plated onto ibidi chemotaxis μ -slide chambers overnight, and then monitored for 16 h in the absence (Control) or presence of 10 ng/mL DNP-BSA (+Ag) in the lower reservoir buffer. +Syk refers to Syk $^{-}$ cells transiently expressing Syk-CFP. +SH2 refers to Syk $^{-}$ cells transiently expressing PLC γ (SH2) $_2$ -GFP. Average yFMI $\pm$ SEM ($n=27-108$ cells/each condition) is shown. * $P < 0.05$; **** $P < 0.0001$ compared with respective control sample.

Figure 8. Orai1 contributes to RBL-2H3 mast cell chemotaxis toward antigen. RBL-2H3 cells were sensitized with anti-DNP IgE, plated onto ibidi chemotaxis μ -slide chambers overnight, and then monitored for 16 h in the absence (Control) or presence of 10 ng/mL DNP-BSA (+Ag). +S1P refers to chemotaxis of RBL cells in the presence of 1 μ M S1P. No Ca^{2+} refers to RBL-2H3 cells in medium with 4 mM EGTA and 3 mM MgCl_2 . RBL-2H3 cells were transiently transfected with shRNA specific for Orai1 (Orai1 KD) or with corresponding control vector (Vector), sensitized with anti-DNP IgE, plated onto ibidi chemotaxis μ -slide chambers overnight, and then monitored for 16 h. Average yFMI $\pm$ SEM ($n=11$ –108 cells/each condition) is shown. * $P < 0.05$; ** $P < 0.01$; **** $P < 0.0001$ compared with respective control sample.



RBL-C1 mutant cell line, which is deficient in Cdc42-dependent biosynthetic trafficking and Fc ϵ RI-mediated activation of Cdc42 and Rac1, we found that Rho family GTPases also appear to participate in mast cell motility (Fig. 1C). Some recent reports indicate that PI3K is dispensable for leukocyte chemotaxis [64–66], and currently, some models are emerging that attempt to bridge these seemingly inconsistent results, including the possibility that the PI3K requirement in chemotaxis depends on the differentiation state or primed status of cells [67]. Mouse BMMCs have been found to depend on PI3K γ for chemotactic responses to antigen [19], and future studies may determine whether a more “primed” condition, e.g., cells undergoing chemotaxis to antigen, causes the PI3K requirement to differ from that of spontaneously migrating mucosal mast cells.

We characterized spontaneous Ca^{2+} transients that have not been described previously in mast cells using a genetically encoded Ca^{2+} indicator GCaMP3 with real-time confocal microscopy (Fig. 4 and Supplemental Movie 3). Interestingly, we observed local Ca^{2+} transients in unstimulated cells, typically more frequently in extended cell protrusions, and these can be inhibited by chelating extracellular Ca^{2+} or by inhibiting SOCE (Fig. 4), correlating with the Ca^{2+} dependence of cell motility (Fig. 2). A recent study by Wei et al. [68] reported that high Ca^{2+} microdomains, “ Ca^{2+} flickers”, are asymmetrically localized to the leading edge of migrating fibroblasts and promote the turning behavior of these cells. In response to the antigen, we have observed more frequent Ca^{2+} transients in the protrusions of RBL cells toward which the cell body is moving (unpublished results), suggesting a correlation between these localized Ca^{2+} transients and RBL cell chemotaxis.

We investigated chemotaxis of mast cells by establishing real-time imaging of this process using μ -slide chambers. This

method allows us to visualize and analyze directed cell migration, and it is especially well suited for long-term studies of these slowly migrating cells [69]. We demonstrated that mast cells exhibit chemotaxis toward antigen (Figs. 5 and 6), and this process depends on tyrosine kinase Syk and Ca^{2+} influx via Orai1 (Figs. 7 and 8). We observed that RBL-2H3 mast cells chemotax toward the antigen in a dose-dependent manner, with a maximal response toward 10 ng/ml antigen and a response that is only a little smaller at 100 ng/ml. We did not observe chemotaxis toward the antigen at 100 ng/ml, which we find to be a level of stimulation leading to cell immobilization and spreading. Mast cells show similar average velocities in the presence or absence of various doses of the antigen as a chemoattractant (Supplemental Fig. 3), implying that the antigen directly elicits a chemotactic response by altering the directionality and sensing of mast cells rather than causing enhancement of chemokinesis in these cells. However, the relationship between velocity and directionality sensing of mast cells under other conditions seems to be more complicated. With 100 ng/ml antigen, mast cells halt their migration and undergo degranulation. When a dose of 1 μ g/ml antigen was added globally to RBL mast cells, they flatten out, ruffle, and stop crawling altogether (data not shown). At 100 ng/ml antigen, mast cells show a maximal degranulation response, whereas at 10 pg/ml antigen, the degranulation response is insignificant [70], suggesting that the mechanism that regulates the chemotactic response of mast cells intersects but differs from the mechanism for mast cell degranulation.

We showed that RBL Syk[−] cells are deficient in their directed migration toward antigen, and this defect can be restored by transient overexpression of Syk-CFP (Fig. 7). Participation of Syk in mouse BMMC chemotaxis toward the antigen has been described previously and was shown to be important

in early FcεRI signaling in this process [19]. Syk contains two tandem SH2 domains and a carboxy-terminal tyrosine kinase domain. We found that overexpression of two tandem SH2 domains from PLCγ in Syk[−] mutant cells fails to reconstitute the deficiency in Syk[−] cell chemotaxis toward antigen. This result suggests that binding of the tandem SH2 domain to FcεRI ITAMs is not sufficient for this process, suggesting a requirement of the kinase activity of Syk. Syk has also been implicated in macrophage chemotaxis [71], lamellipodium formation and chemotaxis of human leukocytes [72], and integrin-mediated signal transduction leading to leukocyte adhesion and migration [73–75]. Syk activity is important in FcγRI-mediated signaling in macrophages and neutrophils [76, 77], and it is essential for FcεRI-mediated signaling in mast cells [78]. Together with our finding that Syk plays a key role in RBL mast cell chemotaxis toward antigen, these studies imply a universal role for Syk in immune cell migration.

As described above, mast cell chemotaxis toward antigen has been demonstrated previously with RBL-2H3 cells and mouse BMMCs [12, 18–20, 79], and involvement of Ca²⁺ in this process was suggested recently [80, 81]. Our principal, new finding is that this process is mediated by Ca²⁺ influx via Orai1 (Fig. 8). Evidence for Orai1 and/or STIM1 participation in cell migration is beginning to emerge in various cell types. Yang and coworkers [32] reported that cell migration and tumor metastasis in breast cancer depend on Orai1 and STIM1, and Orai1 has been shown to regulate integrin-dependent arrest and migration of neutrophils [33]. Roles for Orai1, STIM1, and TRPC1 in vascular smooth muscle cell migration have also been described [34, 35]. We observed less inhibition of spontaneous motility of mast cells by knocking down Orai1 compared with the inhibition of chemotaxis toward antigen (Figs. 3 vs. 8), suggesting that Orai1 plays a larger role in regulating the latter process. Knockdown of TRPC1 by shRNA did not inhibit spontaneous motility of RBL-2H3 mast cells, and STIM1 knockdown caused relatively small, statistically insignificant inhibition. It is possible that Orai1 couples to STIM2 under these conditions, but we cannot rule out insufficient knockdown of STIM1 as an explanation for our results.

Rat BMMCs show smaller, statistically insignificant chemotaxis toward antigen at doses that are optimal for RBL-2H3 cells (Fig. 6). It is possible that variable expression of FcεRI on the surface of BMMCs contributes to these results. We find that a substantial subpopulation of BMMCs does not express detectable surface expression of FcεRI, and this could limit the average chemotactic response (unpublished results).

S1P is known to be a chemoattractant for mast cells [12], and we confirmed directed migration of RBL-2H3 cells toward S1P (Fig. 8). S1P generation and secretion occur after FcεRI aggregation in mast cells and consequent activation of sphingosine phosphate kinases [82]. In DCs, secreted S1P can act in an autocrine and paracrine fashion, binding to its GPCR S1PR1 and then activating one of its downstream targets, Rac1, to enhance migration [83]. It was reported previously that for RBL-2H3 cells, knocking down S1PR1 or sphingosine kinase 1 reduced chemotaxis toward antigen [12], which led to the hypothesis that S1P secreted as a consequence of FcεRI aggregation mediates this chemotaxis response in mast cells. Whether

autocrine-paracrine action of chemotactic factors that are released from the activated mast cells after FcεRI cross-linking are required [12, 19] or not [18, 79] for antigen-stimulated chemotaxis is unclear. Although we cannot rule out the possibility that secreted S1P is mediating chemotaxis toward the antigen, our data clearly demonstrate that in contrast to chemotaxis toward antigen, RBL-2H3 chemotaxis toward S1P is independent of extracellular Ca²⁺, suggesting that different pathway(s) are involved in these processes. This discrepancy may be attributed, at least in part, to the different time scale of these experiments: Jolly et al. [12] monitored chemotactic migration after 3 h, whereas we monitored migration for 16 h. The mechanisms involved are not necessarily mutually exclusive. For example, secreted, S1P-mediated chemotaxis toward the antigen may be more important for the initial, shorter time period, but Ca²⁺ influx may become more important over longer time periods.

In summary, the present study shows that Ca²⁺ influx plays an essential role in mast cell spontaneous motility and directed migration toward antigen, and Orai1 contributes to these processes. Spontaneous motility also depends on Rho GTPases, protein tyrosine kinase Syk, and PI3K. Furthermore, we observed spontaneous Ca²⁺ transients that are inhibited by 2-APB and Gd³⁺ in parallel with their inhibition of cell motility. In addition, antigen stimulates a chemotactic response from IgE-sensitized mast cells that depend on Ca²⁺ influx via Orai1. These results provide new insights into the mechanisms of mucosal mast cell migration.

AUTHORSHIP

J.L. designed and performed experiments, analyzed and interpreted results, and cowrote the manuscript. S.V. developed the method for migration analysis, wrote the program for this analysis, and contributed to data analysis and interpretation. B.B. and D.H. were co-principal investigators who codesigned experiments and cowrote the manuscript.

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KEY WORDS:

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