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# Regulation of Eosinophil Trafficking by SWAP-70 and Its Role in Allergic Airway Inflammation

**Nooshin S. Bahaie,* M. Reza Hosseinkhani,* Xiao Na Ge,* Bit Na Kang,* Sung Gil Ha,* Malcolm S. Blumenthal,† Rolf Jessberger,‡ Savita P. Rao,* and P. Sriramarao*,†**

**Eosinophils are the predominant inflammatory cells recruited to allergic airways. In this article, we show that human and murine eosinophils express SWAP-70, an intracellular RAC-binding signaling protein, and examine its role in mediating eosinophil trafficking and pulmonary recruitment in a murine model of allergic airway inflammation. Compared with wild-type eosinophils, SWAP-70–deficient (*Swap-70*$^{-/-}$) eosinophils revealed altered adhesive interactions within inflamed postcapillary venules under conditions of blood flow by intravital microscopy, exhibiting enhanced slow rolling but decreased firm adhesion. In static adhesion assays, *Swap-70*$^{-/-}$ eosinophils adhered poorly to VCAM-1 and ICAM-1 and exhibited inefficient leading edge and uropod formation. Adherent *Swap-70*$^{-/-}$ eosinophils failed to translocate RAC1 to leading edges and displayed aberrant cell surface localization/distribution of α4 and Mac-1. Chemokine-induced migration of *Swap-70*$^{-/-}$ eosinophils was significantly decreased, correlating with reduced intracellular calcium levels, defective actin polymerization/depolymerization, and altered cytoskeletal rearrangement. In vivo, recruitment of eosinophils to the lungs of allergen-challenged *Swap-70*$^{-/-}$ mice, compared with wild-type mice, was significantly reduced, along with considerable attenuation of airway inflammation, indicated by diminished IL-5, IL-13, and TNF-α levels; reduced mucus secretion; and improved airway function. These findings suggest that regulation of eosinophil trafficking and migration by SWAP-70 is important for the development of eosinophilic inflammation after allergen exposure.** ***The Journal of Immunology*****, 2012, 188: 000–000.**

Eosinophil infiltration to sites of inflammation has long been recognized as one of the hallmarks of allergic inflammation (1). Eosinophils contain highly specific basic granule mediators, such as eosinophil peroxidase, major basic protein (MBP), and eosinophil cationic protein, which, combined with various eosinophil-derived cytokines, chemokines, and growth factors, contribute to the development and maintenance of inflammation and injury at sites of allergic inflammation (2). Chemoattractants such as eotaxins, C5a, C3a, MCP-2, MCP-3, MCP-4, RANTES released by other immune cells such as T cells, macrophages, mast cells (MCs), and in some cases eosinophils themselves play an important role in recruitment and microlocalization of eosinophils (3). In addition, the specific and sequential engagement of cell surface adhesion receptors with vascular counterligands in inflamed blood vessels (4, 5) initiates a cascade of events regulated by various signaling molecules, resulting in F-actin rearrangement and cytoskeletal reorganization that leads to directed cell shape changes as a prelude to cell adhesion and migration into inflamed tissues (6–10).

SWAP-70 is a RAC-binding, F-actin–binding intracellular signaling molecule first identified in the nucleus of activated B cells as part of a complex involved in Ig class switching (11–13). Subsequently, its expression has been demonstrated in MCs (14), primary embryonic fibroblasts (15), dendritic cells (16), trophoblasts (17), and gliomas (18). In B cells, SWAP-70 is involved not only in nuclear events but also in signaling events in the cytoplasm during cell activation (19) and in efficient homing to the lymph nodes (20). SWAP-70–deficient B cells exhibit reduced migration into lymph nodes in vivo due to altered adhesive interactions with the vascular endothelium of lymphoid organs. Studies with immature bone marrow (BM)-derived MCs from SWAP-70–deficient (*Swap-70*$^{-/-}$) mice have demonstrated that SWAP-70 is required for Fcε receptor-mediated reactions as well as for signal transduction from the stem cell factor receptor c-Kit, regulating events such as degranulation, adhesion, migration, differentiation, and survival of MCs (14, 21). Further, IgE-mediated passive cutaneous and systemic anaphylactic responses are strongly reduced in *Swap-70*$^{-/-}$ mice (22). SWAP-70 also regulates surface localization of MHC class II molecules and is therefore important in dendritic cell-dependent immune responses (23). Upon cell stimulation, SWAP-70 has been shown to regulate the F-actin cytoskeleton by binding to nonmuscle F-actin (24, 25) and promoting membrane ruffling (25). Overall, the cytoskeleton-modulating effects of SWAP-70 are probably due to its ability to interact with and regulate levels of activated RAC, a member of the Rho family of GTPases (15, 25), which acts as a central molecular switch in a number of signal transduction pathways relevant to leukocyte recruitment (8, 26). To date, no studies have described the expression of this signaling molecule by eosinophils and the potential role this molecule may play in eosinophil recruitment, particularly in the context of allergic inflammation. In the current study, we have identified an important and novel role for SWAP-70 in mediating eosinophil trafficking.

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Abbreviations used in this article: AHR, airway hyperresponsiveness; BALF, bronchoalveolar lavage fluid; BM, bone marrow; $C_{dyn}$, dynamic compliance; MBP, major basic protein; MC, mast cell; PAS, periodic acid-Schiff; PSGL-1, P-selectin glycoprotein ligand 1; $R_L$, pulmonary resistance; rm, recombinant murine; WT, wild-type.

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## Materials and Methods

### *Mice*

Mice (male and female) on a C57BL/6 background that were rendered deficient for SWAP-70 (*Swap-70*$^{-/-}$) by removing the first exon and part of the 5′ UTR in both alleles, as described previously (13), were bred together, yielding *Swap-70*$^{-/-}$ offspring. Age- and gender-matched C57BL/6 mice were used as WT controls. All studies involving mice were performed following standards and procedures approved by the Institutional Animal Care and Use Committee at the University of Minnesota.

### *Murine and human eosinophils*

Murine eosinophils were cultured from the BM of WT and *Swap-70*$^{-/-}$ mice, as previously described (27), but with minor modifications. Briefly, BM cells collected from the femurs of naive *Swap-70*$^{-/-}$ and WT mice were cultured in RPMI 1640 supplemented with 20% FBS, 2 mM glutamine, 25 mM HEPES, nonessential amino acids, 1 mM sodium pyruvate, and 50 μM 2-ME (all reagents from Invitrogen, Carlsbad, CA). Stem cell factor and FLT3 ligand (PeproTech, Rocky Hill, NJ) were added to the medium at 80 ng/ml during initial culture. On day 4, cells were transferred to medium containing 10 ng/ml recombinant murine (rm) IL-5 (PeproTech) and subcultured every other day up to day 14, maintaining starting cell density at $10^6$/ml each time. Eosinophil differentiation was assessed starting from day 8 by staining cytocentrifuged preparations of BM cultures with the Hema 3 System (Thermo Fisher Scientific, Pittsburgh, PA) and evaluating cells for expression of MBP by confocal microscopy, with rat mAb against murine MBP (2.5 μg/ml), followed by FITC-conjugated goat anti-rat IgG, and of Siglec-F by flow cytometry using PE-conjugated rat anti-mouse Siglec-F (5 μg/ml; BD Biosciences, San Diego, CA). Differentiated cells between days 12 and 14 of culture that were 99% Hema 3 positive and expressed both MBP and Siglec-F were used in studies.

Human eosinophils were isolated from peripheral blood of allergic donors. Granulocytes were first collected from peripheral blood by centrifugation over Ficoll-Paque (GE Healthcare, Piscataway, NJ), and RBCs were removed by lysis with ammonium chloride solution (150 mM ammonium chloride, 10 mM potassium bicarbonate, 0.5 M EDTA, pH 8.0). Eosinophils were then enriched based on negative selection, using EasySep eosinophil enrichment kits according to the manufacturer's protocol (Stemcell Technologies, Vancouver, BC, Canada). Viability and purity were routinely found to be >95%.

### *Expression of SWAP-70 by murine and human eosinophils*

BM-cultured eosinophils were immunostained with affinity-purified rabbit anti-mouse SWAP-70 Abs (10 μg/ml) (11) or with rat mAb against murine MBP (described above), and the bound Abs were detected using FITC-conjugated goat anti-rabbit or anti-rat IgG, as described previously (28). Rabbit IgG (for SWAP-70) and rat IgG (for MBP) were used as isotype controls. Slides were observed at ambient temperature, using a FluoView-FV1000/BX61ConfocalLaserScanningBiologicalMicroscope equipped with a UPlanSApo lens (20 ×/0.85 [oil]) and a PlanApo N lens (60 ×/1.42 [oil]). FV10-ASW 2.0 software was used for image acquisition (Olympus, Melville, NY). Western blot analysis of murine and human eosinophil lysates (40 μg protein/lane/sample on 5–10% Tricine Gels under reduced conditions) was performed using Abs against mouse SWAP-70 (500 ng/ml), followed by HRP-conjugated goat anti-rabbit IgG (1:10000; Cell Signaling Technology, Danvers, MA). Protein bands were detected with ECL Western Blotting Substrate (Pierce Biotechnology, Rockford, IL) and visualized on x-ray films.

Expression of SWAP-70 by human and murine eosinophils at the mRNA level was assessed by RT-PCR. Total RNA (1 μg) extracted with TRIzol Reagent (Invitrogen) was used for RT-PCR with SuperScript III Reverse Transcriptase (Invitrogen) according to the manufacturer's instructions. Primers for murine and human eosinophils specific for the first exon of the SWAP-70 gene (5′-GCTGACCAGGTCTGTTAGGG-3′ and 5′-GACTTGCGACTTGGAGACCT-3′ for murine and 5′-TCCACCATCCATCTGTTGAA-3′ and 5′-GCGTTTCTTTTCCTCGTCTG-3′ for human) were designed using Primer3 (v. 0.4.0) (available at http://frodo.wi.mit.edu/primer3/). Expression of β-actin (for mouse) (29) and expression of GAPDH (for human; primers derived at http://www.rtprimerdb.org) were used as internal controls. PCR amplification was performed using GoTaq Green Master Mix (Promega, Madison, WI) in a DNA Engine PTC-0200 Cycler (Bio-Rad Laboratories, Hercules, CA) under the following conditions: initial denaturation at 95°C for 2 min, followed by 38 cycles of amplification (95°C for 20 s [denature], 60°C for 1 min [anneal], and 72°C for 30 s [extension]), and a final extension at 72°C for 5 min. PCR products were analyzed by 1.5% agarose gel electrophoresis.

### *Migration assay*

Eosinophils from BM of WT and *Swap-70*$^{-/-}$ mice were suspended in DMEM and added to the upper wells ($5 \times 10^4$/well in 50 μl) of 96-well Transwell Chambers. Murine eotaxin-1 (CCL11, 100 nM, PeproTech) or media alone was added to the lower wells and the plates were incubated at 37°C for 3 h. The number of cells that migrated in each case was evaluated under an Olympus CK2 inverted microscope using a 40× objective and cells in three different fields of each well were counted. The assay was performed three times in duplicate. The average number of cells/field/well was determined and results were expressed as fold increase over background (media alone) which was in the range of 12 – 21 cells/field/well for WT and 24 - 31 cells/field/well for *Swap-70*$^{-/-}$ eosinophils.

### *Actin polymerization and cytoskeletal changes*

Actin polymerization was assessed based on phalloidin binding, using rhodamine-phalloidin (Invitrogen) followed by flow cytometry, as previously described (21), but with minor modifications. Eosinophils ($2 \times 10^5$ in 1% BSA in HEPES buffer) were stimulated with 100 nM eotaxin-1 for various time points. Subsequently, cells were fixed and permeabilized with 3.6% formaldehyde and 0.5% saponin in PBS for 15 min. Total cellular F-actin was stained using rhodamine-conjugated phalloidin (2.5 U/ml) and analyzed by flow cytometry. A total of 10,000 events were acquired, and results were expressed as percent increases in cell fluorescence relative to untreated cells.

To assess the effect of eotaxin-1 on cytoskeletal changes in WT and *Swap-70*$^{-/-}$ eosinophils, cells were allowed to attach to chamber slides (Nalgene Nunc International, Rochester, NY) coated with poly-L -lysine (10 μg/ml in PBS, 100 μl/chamber; Sigma-Aldrich, St. Louis, MO) and incubated with 100 nM eotaxin-1 for different durations (10 s to 10 min). Cells were permeabilized, and actin filament rearrangement was evaluated by confocal microscopy, using rhodamine-phalloidin as previously described (30).

### *Calcium ($Ca^{2+}$) flux measurements*

Calcium flux in response to stimulation with eotaxin-1 was measured by flow cytometry, using Indo-1, AM (Invitrogen), a cell-permeant, ratiometric $Ca^{2+}$-binding dye, as described previously (31). WT and *Swap-70*$^{-/-}$ eosinophils ($1 \times 10^6$) in RPMI 1640 containing 10% FBS were loaded with 3 μM Indo-1, AM (concentration identified after initial optimization), for 30 min at 37°C and 5% $CO_2$. Following incubation, cells were washed once and resuspended in medium. Cells were equilibrated at 37°C for 5 min prior to acquisition on a BD LSR II flow cytometer (BD Biosciences). Indo-1, AM, is excited in the UV range and fluoresces at different wavelengths, depending on whether it is bound to $Ca^{2+}$ (~405 nm) or free (~530 nm). The ratio of these two wavelengths, which is indicative of changes in intracellular $Ca^{2+}$ concentration, was measured up to 3 min after the addition of eotaxin-1 (100 nM) or ionomycin (2 μM), which is an ionophore used as a positive control, at the 30-s time point.

### *Assessment of eosinophil trafficking in vivo by intravital microscopy*

Trafficking of WT and *Swap-70*$^{-/-}$ eosinophils in inflamed postcapillary venules of the mouse cremaster muscle microcirculation was evaluated as described previously (32). WT mice were first administered TNF-α (250 ng, intrascrotally), and surgery to expose the cremaster muscle microcirculation was initiated ~4–5 h later. Eosinophils ($1 \times 10^7$) labeled with 5 μM CFSE (Invitrogen) were infused via the carotid artery into the prepared animal (~5–6 h after TNF-α administration), which was placed on the stage of an intravital microscope (Leica Microsystems, Bannockburn, IL). Interaction of fluorescently labeled eosinophils with the vascular endothelium of the cremaster muscle microvessels was made visible by stroboscopic epi-illumination using a xenon lamp, and all images were recorded using StreamPix digital video recording software (NorPix, Montreal, QC, Canada). This step was followed by off-line analysis of recorded digital images to determine the number of rolling and adherent cells, as described in our earlier studies (33). On average, rolling and adhesion were analyzed in four to six microvessels per mouse. In addition, the velocity of rolling eosinophils was analyzed in three to five venules per mouse (34).

### *Expression of cell surface receptors by flow cytometry*

Baseline (nonactivated) expression of α4 integrin (CD49d), LFA-1 (CD11a), Mac-1 (CD11b), L-selectin, P-selectin glycoprotein ligand 1 (PSGL-1), and CCR3 by WT and *Swap-70*$^{-/-}$ eosinophils ($3 \times 10^5$ cells) was evaluated by flow cytometry with a FACScan flow cytometer (BD

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Biosciences) and analyzed with FlowJo software (version 7.1; Tree Star, Ashland, OR). Expression of α4 and LFA-1 was evaluated using rat anti-mouse α4 (clone PS/2) and rat anti-mouse LFA-1 (eBiosciences, San Diego, CA), respectively, followed by FITC-conjugated anti-rat Abs (Jackson ImmunoResearch, West Grove, PA), with rat IgG2a as the isotype-matched control. Expression of L-selectin was assessed with FITC-conjugated rat anti mouse L-selectin (clone Mel-14; BD Biosciences). PSGL-1 and Mac-1 expression was evaluated using PE-conjugated rat anti-mouse PSGL-1 and Mac-1 (BD Biosciences), respectively, whereas expression of CCR3 was assessed using FITC-conjugated rat anti-mouse CCR3 (R&D Systems, Minneapolis, MN). PE- or FITC-conjugated rat IgG2a (BD Biosciences) or unconjugated rat IgG2b (eBiosciences) was used as an isotype-matched control Ab. All Abs were used at a final concentration of 5 μg/ml.

### *Adhesion assay*

Flat-bottomed 96-well plates were precoated with rm ICAM-1 (10 μg/ml; R&D Systems) or VCAM-1 (5 μg/ml) (28) in PBS with $Ca^{2+}$ and $Mg^{2+}$ for 2 h at 37°C. BM-cultured murine eosinophils were labeled with 1 μM Calcein-AM (Invitrogen) (30) and added to the wells ($5 \times 10^4$ in 100 μl PBS/well). After incubation at 37°C for 5 or 30 min, plates were gently washed with PBS four to five times to remove nonadherent cells. Adherent eosinophils were quantitated using a FLUOStar Optima microplate reader (BMG Labtech, Durham, NC) against a generated standard curve of Calcein-AM–labeled eosinophils.

To assess cytoskeletal rearrangement, cell morphology, RAC1, and adhesion molecule expression after attachment, WT and *Swap-70*$^{-/-}$ eosinophils ($5 \times 10^5$) were first allowed to adhere to coverslips coated with rm ICAM-1 or VCAM-1 for 5 or 30 min at 37°C. Nonadherent cells were removed by washing, as described above, and adherent cells were fixed with 4% paraformaldehyde. To evaluate the F-actin cytoskeleton, adherent cells were permeabilized, stained with FITC-phalloidin (2.5 U/ml; Invitrogen), as previously explained (30), and visualized by confocal microscopy. To assess differences in cell morphology, adherent cells were counted after 30 min in five different fields of the coverslips, and the number of polarized cells, defined as cells with a distinct leading edge and uropod compartment, was determined and expressed as a percentage of the total number of adherent cells in the field. To examine RAC1 expression, cells were permeabilized and incubated overnight with polyclonal Abs against RAC1 (20 μg/ml; Santa Cruz Biotechnology, Santa Cruz, CA), or rabbit IgG as the isotype control, followed by rhodamine-conjugated secondary Abs (5 μg/ml; Jackson ImmunoResearch) after washing. Cells were then stained with FITC-phalloidin and visualized by confocal microscopy. To evaluate α4 expression, cells adherent to VCAM-1 were fixed and first incubated with donkey IgG in PBS as blocking buffer for 30 min at room temperature. This step was followed by overnight incubation with goat anti-mouse α4 (5 μg/ml; Santa Cruz Biotechnology) or isotype control and then with rhodamine-conjugated donkey anti-goat IgG (5 μg/ml; Jackson ImmunoResearch). Cells were finally stained with FITC-phalloidin in buffer containing 0.5% saponin and visualized by confocal microscopy. For Mac-1 expression, cells adherent to ICAM-1 were fixed and incubated overnight with rat anti-mouse Mac-1 (10 μg/ml; eBiosciences) or isotype control (rat IgG2b) in 0.1% BSA in PBS and then with rhodamine-conjugated rabbit anti-rat (5 μg/ml, Jackson ImmunoResearch). Cells were stained with FITC-phalloidin, as described above, for α4 before visualization under a confocal microscope.

### *Murine model of acute allergic airway inflammation*

WT and *Swap-70*$^{-/-}$ mice (8–10 wk old) were sensitized with 50 μg OVA (Grade V, Sigma-Aldrich) in 0.5 mg aluminum hydroxide by s.c. injections on days 0, 7, 14, and 21 and then challenged with OVA (20 μg/mouse) intranasally on days 23, 25, and 28, as previously established (35). Control mice were administered saline instead of OVA for sensitization and challenge.

### *Measurement of airway responsiveness*

Airway hyperresponsiveness (AHR) was assessed in control and allergen-challenged WT and *Swap-70*$^{-/-}$ mice, using the FinePointe RC invasive plethysmography system (Buxco, Wilmington, NC) equipped with a nebulizer, ventilator, and heated bed as per the RC system application manual and according to previously published protocols (36, 37) This system is capable of directly measuring pulmonary resistance ($R_L$) and dynamic compliance ($C_{dyn}$). Anesthetized mice were ventilated at a tidal volume of 230 μl and a respiratory frequency of 120 breaths per minute. After monitoring baseline $R_L$ and $C_{dyn}$, changes in airway resistance and compliance were monitored continuously in response to saline followed by increasing concentrations of inhaled methacholine (3–25 mg/ml) nebulized for 18–20 s. To measure the degree of airway responsiveness, mean $R_L$ and $C_{dyn}$ values following each dose of methacholine were expressed as percent change from the baseline value.

### *Sample collection and analysis*

Mice were sacrificed 24 h after the last allergen challenge. Total and differential cell counts were determined in BM, blood, and bronchoalveolar lavage fluid (BALF) based on morphologic and histologic criteria after staining with the Hema 3 System (Thermo Fisher Scientific). BALF supernatants were stored at −70°C until further evaluation. Cells recovered from the BALF of WT mice by cytocentrifugation were dual stained with affinity-purified rabbit Abs against murine SWAP-70 (10 μg/ml) (11) and rat mAb against murine MBP, as described previously in this section, and evaluated for SWAP-70 expression by confocal microscopy. Right lungs were snap-frozen, and left lungs were perfused with 4% paraformaldehyde to preserve pulmonary structure, fixed in 4% paraformadehyde, and paraffin embedded.

### *Lung histology*

Paraffin-embedded tissue sections (4 μm thick) were stained with Harris Modified Hematoxylin and Shandon Instant Eosin (Thermo Fisher Scientific) to determine cellular infiltration. For all immunohistologic analyses, tissue sections were subjected to Ag retrieval followed by quenching of endogenous peroxidase activity prior to staining with specific Abs, and sections were briefly counterstained (5 s) with hematoxylin at the end of the procedure. Appropriate VECTASTAIN ABC kits with biotinylated secondary Abs and avidin-biotin HRP complex (Vector Laboratories, Burlingame, CA) and the Peroxidase AEC (3-amino-9-ethylcarbazole) Substrate Kit (Vector Laboratories) were used for detection. Stained slides were analyzed using a Nikon Microphot EPI-FL microscope (magnification of ×100 or ×400), and images were captured with an Olympus DP71 camera. Expression of SWAP-70 in the lungs of allergen-challenged WT mice was assessed using rabbit anti-mouse SWAP-70 Abs (10 μg/ml). Evaluation and quantitation of eosinophil infiltration were accomplished by immunohistochemical staining for eosinophil-specific MBP with rat mAb against murine MBP, as described earlier (33). Rat IgG (for MBP) and rabbit IgG (for SWAP-70) were used as control Abs. To detect airway mucus production, deparaffinized lung sections were stained with periodic acid-Schiff (PAS) reagent (Sigma-Aldrich), and PAS-positive areas in horizontally sectioned airways were quantitated using ImageJ (38). Results are expressed as square micrometers of PAS-positive area per 100 μm basement membrane length.

### *Measurement of BALF cytokines and chemokines*

Cytokine levels in BALF were measured using Flex Set kits (BD Biosciences) for Th1 (IL-2, IFN-γ)/Th2 (IL-4, IL-5) cytokines as well as IL-13, according to the manufacturer's instructions, with a FACScan flow cytometer and FCAP Array Software (BD Biosciences), using the same volume of BALF for each sample, and results were expressed as picograms per milliliter of BALF. Eotaxin-1 levels were measured using an ELISA kit (R&D Systems), as recommended by the manufacturer.

### *Statistical analysis*

Results are expressed as the mean ± SEM. Statistical significance was determined by an unpaired Student *t* test, using Microsoft Excel. A two-tailed test was used to first establish statistical significance between WT and *Swap-70*$^{-/-}$ eosinophils in cellular trafficking and recruitment in vivo (Figs. 2, 6*C*). A one-tailed test was used for all other analyses. A $p$ value $<0.05$ was considered significant.

## Results

### *Human and murine eosinophils express SWAP-70*

Western blots of lysates of human eosinophils isolated from peripheral blood of allergic donors ($n = 5$) with Abs against murine SWAP-70 demonstrated a band of 70 kDa corresponding to the reported molecular mass of SWAP-70 (Fig. 1*A*, *left panel*, two representative donors shown). In addition, a band corresponding to 70 kDa was observed in lysates of WT BM-cultured murine eosinophils, but not eosinophils from *Swap-70*$^{-/-}$ mice (Fig. 1*A*, *right panel*). Lysates of BM MCs from WT mice known to strongly express SWAP-70 (14) were used as a positive control and demonstrated a band of similar molecular mass, whereas lysates of BM MCs from *Swap-70*$^{-/-}$ mice that served as the negative control did

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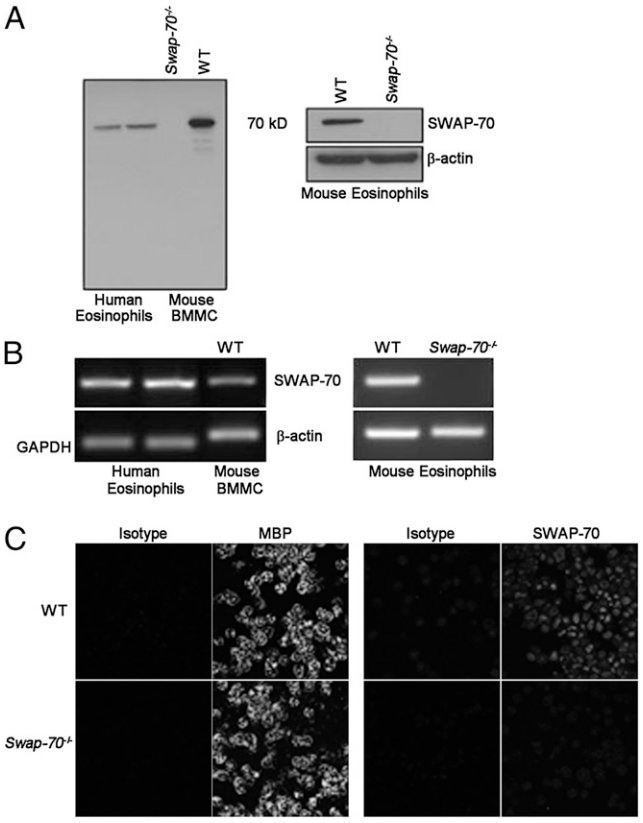


**FIGURE 1.** Expression of SWAP-70 by murine and human eosinophils. *A*, Western blot of human eosinophil lysates (two representative allergic donors) as well as lysates of BM MCs and eosinophils from WT and *Swap-70*$^{-/-}$ mice with affinity-purified polyclonal Abs against murine SWAP-70. Lysates of *Swap-70*$^{-/-}$ and WT BM MCs served as the negative and positive control, respectively. *B*, RT-PCR with total RNA from human eosinophils (two representative allergic donors) as well as eosinophils from WT and *Swap-70*$^{-/-}$ mice, using species-specific primers for the first exon of the SWAP-70 gene. Total RNA from WT BM MCs was used as the positive control. Expression of GAPDH (for human eosinophils) and β-actin (for murine BM MCs and eosinophils) served as internal controls. *C*, Immunofluorescence staining for eosinophil-specific MBP and SWAP-70 expression by WT and *Swap-70*$^{-/-}$ eosinophils, by confocal microscopy with rat anti-mouse MBP Abs and affinity-purified rabbit anti-mouse SWAP-70 Abs, followed by FITC-conjugated goat anti-rat or anti-rabbit IgG, respectively. Rat IgG1 (for MBP) and rabbit IgG (for SWAP-70) were used as isotype controls. Data shown in *C* are representative of three independent experiments, with eosinophils from three different mice for each group.

not demonstrate any bands in this region (Fig. 1*A*, *left panel*). Further, human eosinophils (Fig. 1*B*, *left panel*) and WT, but not *Swap-70*$^{-/-}$, murine eosinophils (Fig. 1*B*, *right panels*) were found to express SWAP-70 mRNA by RT-PCR. Expression of SWAP-70 by murine eosinophils was also confirmed by confocal microscopy (Fig. 1*C*), where WT eosinophils were positive for expression of eosinophil-specific MBP (Fig. 1*C*, *upper left panels*) as well as SWAP-70 (Fig. 1*C*, *upper right panels*), and *Swap-70*$^{-/-}$ eosinophils were positive only for expression of MBP (Fig. 1*C*, *lower left panels*). Together, these studies clearly demonstrate that human and murine eosinophils express SWAP-70.

### *Altered adhesive interactions of Swap-70*$^{-/-}$ *eosinophils under conditions of flow in vivo*

To evaluate whether SWAP-70 is involved in promoting adhesive interactions of eosinophils with the inflamed vascular endothelium, trafficking of fluorescently labeled WT and *Swap-70*$^{-/-}$ eosinophils was examined by intravital microscopy in vivo within TNF-α–treated postcapillary venules under conditions of blood flow. Compared with WT eosinophils, more *Swap-70*$^{-/-}$ eosinophils were found to roll along the vessel wall (Fig. 2*A*). Of interest, however, a large fraction (~70%) of these rolling cells were slow rollers that demonstrated extremely slow rolling, with velocities <10 μm/sec without adhering, whereas the remaining eosinophils (~30%) were intermediate and fast rollers, with velocities in the range of 10–100 μm/sec and >100 μm/sec, respectively (Fig. 2*B*). In contrast, only 35% of WT eosinophils exhibited slow rolling, with nearly equal proportions of intermediate and fast rollers. Further, a significantly larger number of WT eosinophils, compared with *Swap-70*$^{-/-}$ eosinophils, were found to adhere to the vascular endothelium ($p < 0.01$) (Fig. 2*C*). Overall, these findings suggest that SWAP-70 plays a role in regulating adhesive interactions (rolling and adhesion) of eosinophils with the vascular endothelium under conditions of blood flow in vivo.

### *Reduced adhesion of Swap-70*$^{-/-}$ *eosinophils to VCAM-1 and ICAM-1*

Because *Swap-70*$^{-/-}$ eosinophils exhibited considerably decreased adhesion to the vascular endothelium in postcapillary venules,

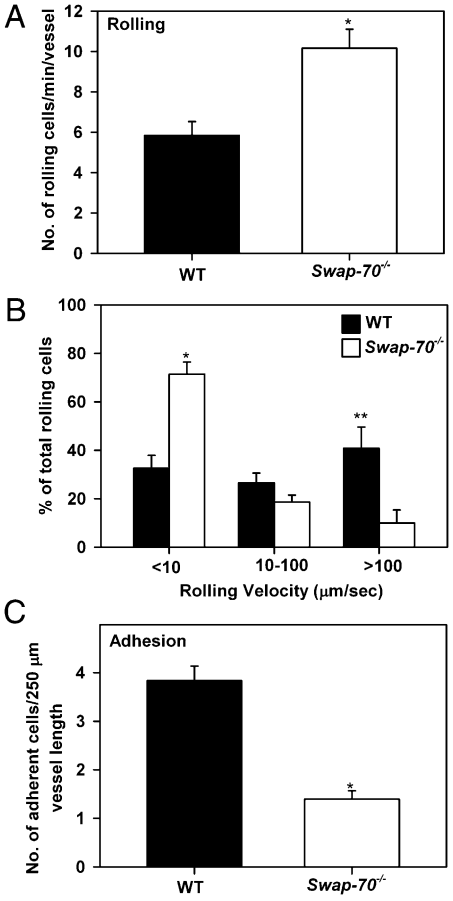


**FIGURE 2.** *Swap-70*$^{-/-}$ eosinophils exhibit altered rolling and decreased adhesion within inflamed blood vessels. *A*, Rolling of infused WT and *Swap-70*$^{-/-}$ eosinophils in inflamed (TNF-α–stimulated) cremaster muscle microvessels of anesthetized mice by intravital microscopy. *B*, Velocities of rolling WT and *Swap-70*$^{-/-}$ eosinophils under conditions of blood flow. Combined data from 55 *Swap-70*$^{-/-}$ and 51 WT rolling eosinophils in three to five vessels per mouse is shown. *C*, Adhesion of infused WT and *Swap-70*$^{-/-}$ eosinophils in cremaster muscle microvessels. Combined data from four to six vessels per mouse is shown for *A* and *C*. Data represent mean ± SEM in *A*–*C*. $n = 3$ mice/group in *A*–*C*. *$p < 0.01$, **$p < 0.05$ for comparison of WT with *Swap-70*$^{-/-}$ eosinophils.

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adhesion to specific vascular adhesion molecules was evaluated by confocal microscopy (Fig. 3*A*). Evaluation of the actin cytoskeleton in ICAM-1– and VCAM-1–adherent eosinophils following phalloidin binding demonstrated subtle differences after 5 min. For example, adherent WT eosinophils displayed phalloidin binding mostly localized to the cell periphery/margin, whereas *Swap-70*$^{-/-}$ eosinophils exhibited more dispersed phalloidin binding, particularly in cells adherent to ICAM-1, although no major difference in cell shape was visible between the two cell types (Fig. 3*A*, *upper panels*). At 30 min, the actin cytoskeleton of eosinophils of both groups was similar, with cells exhibiting dispersed phalloidin binding all over the cell (Fig. 3*A*, *lower panels*). However, at this later time point, many WT eosinophils adherent to VCAM-1 and ICAM-1 exhibited distinct cell polarization, with leading edge formation and development of uropodia and filopodia, whereas *Swap-70*$^{-/-}$ eosinophils exhibited less polarization and more irregular spreading, with no distinct leading edges or filopodia. This finding correlated with the number of adherent cells in static adhesion assays (Fig. 3*B*). At 5 min, *Swap-70*$^{-/-}$ eosinophils, compared with WT eosinophils, showed significantly reduced adhesion to VCAM-1 ($p < 0.01$) or to ICAM-1 ($p < 0.05$). By 30 min, *Swap-70*$^{-/-}$ eosinophils continued to exhibit significantly decreased adhesion to VCAM-1 ($p < 0.01$) than did WT eosinophils, although adhesion to ICAM-1 was similar in both groups. Quantitation of the number of polarized cells, defined as cells with a distinct leading edge and uropod compartment, revealed that a significantly larger percentage of WT eosinophils adherent to VCAM-1 and ICAM-1, compared with *Swap-70*$^{-/-}$ eosinophils, were polarized ($p < 0.01$) (Fig. 3*C*). Immunofluorescence studies were performed to evaluate expression of RAC1, which plays a key role in regulating the actin cytoskeleton and participates in the formation of lamellipodia (39). In *Swap-70*$^{-/-}$ eosinophils adherent to VCAM-1, RAC1 expression was dispersed over the entire cell (Fig. 3*D*, *lower panels*), whereas in polarized WT eosinophils, RAC1 expression translocated to the leading edge of the cells with less expression observed in other areas of the cell (Fig. 3*D*, *upper panels*). The few nonpolarized WT eosinophils exhibited dispersed RAC1 staining similar to that in *Swap-70*$^{-/-}$ eosinophils. However, in both genotypes, no overlapping of RAC1 with F-actin expression was noted, in contrast to previous observations with both MCs (21) and B cells (20).

To determine whether this decreased adhesion of *Swap-70*$^{-/-}$ eosinophils was due to baseline differences in cell surface adhesion molecule expression, LFA-1, Mac-1, L-selectin, PSGL-1, and α4 expression was assessed by flow cytometry and indicated no significant differences between WT and *Swap-70*$^{-/-}$ eosinophils (Fig. 4*A*). We next investigated by confocal microscopy whether the expression and distribution of these receptors on the cell surface during adhesion may be altered in *Swap-70*$^{-/-}$ eosinophils compared with WT cells. Interestingly, distinct differences were

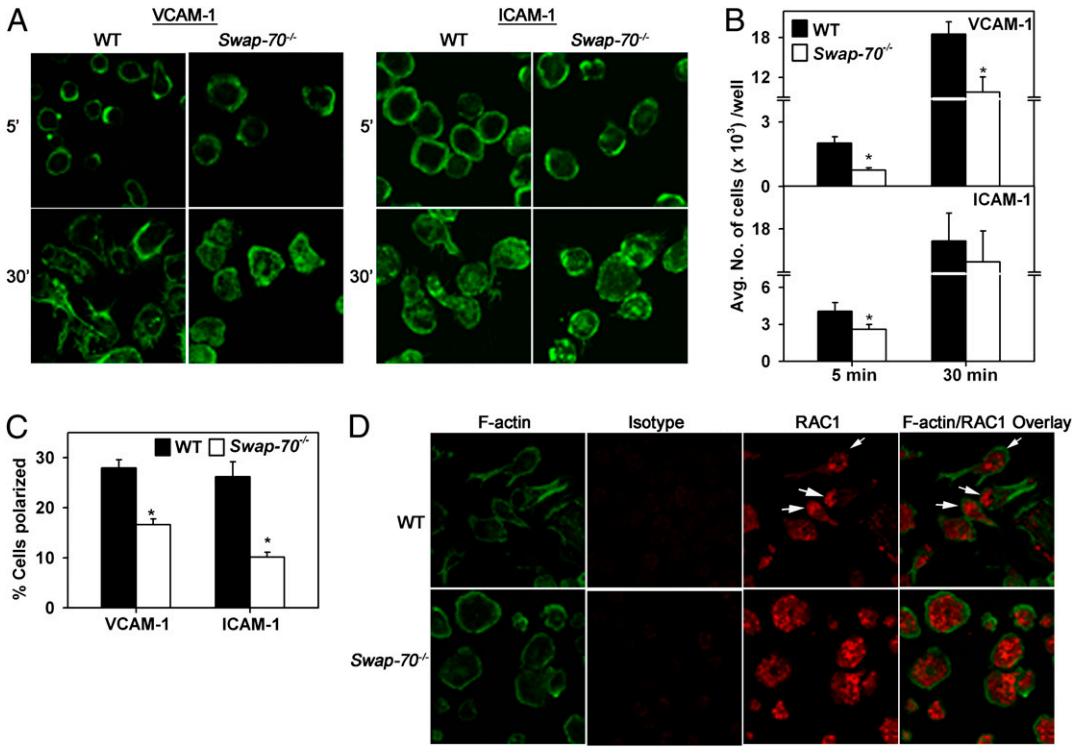


**FIGURE 3.** Decreased adhesion associated with altered cytoskeletal rearrangement and impaired RAC1 translocation in *Swap-70*$^{-/-}$ eosinophils. *A*, Staining of WT and *Swap-70*$^{-/-}$ eosinophils adherent to ICAM-1 or VCAM-1 with FITC-phalloidin, followed by confocal microscopy. Original magnification ×600. B, Adhesion of fluorescently labeled WT and *Swap-70*$^{-/-}$ eosinophils to wells coated with rm VCAM-1 (5 μg/ml) or rm ICAM-1 (10 μg/ml) in static adhesion assays. Adherent cells were quantitated in a microplate reader against a generated standard curve of fluorescently labeled eosinophils. *C*, Polarization of adherent WT and *Swap-70*$^{-/-}$ eosinophils. The number of polarized cells, defined as cells with a distinct leading edge and uropod compartment, after adhesion to VCAM-1 and ICAM-1 for 30 min, was assessed and expressed as a percentage of the total number of cells in a given field. *D*, Immunofluorescence staining for RAC1 expression in WT and *Swap-70*$^{-/-}$ eosinophils adherent to VCAM-1. Adherent cells were stained for F-actin with FITC-phalloidin and for RAC1 using polyclonal Abs against RAC1 (20 μg/ml), followed by rhodamine-conjugated secondary Abs. Rabbit IgG was used as the isotype control. Original magnification ×600. Combined data (mean ± SEM) of two (for VCAM-1) or three (for ICAM-1) independent experiments in triplicate for *B* and of five fields per slide from two independent experiments for *C* are shown. Data in *A* and *D* are representative of two to three independent experiments. For comparison of WT with *Swap-70*$^{-/-}$ eosinophils, *$p < 0.01$ for VCAM-1 and *$p < 0.05$ for ICAM-1 in *A* and *$p < 0.01$ for VCAM-1 and ICAM-1 in *C*.

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observed in the distribution of α4 (Fig. 4*B*). WT eosinophils adherent to VCAM-1 exhibited intense α4 expression at the leading edge of polarized cells, with less expression in the main body and uropod compartment, whereas expression of this receptor by *Swap-70*$^{-/-}$ eosinophils was observed around the main body of the cell. Expression of α4 did not colocalize with F-actin. With Mac-1, not only distribution but even expression was altered in *SWAP-70*$^{-/-}$ eosinophils (Fig. 4*C*). First, WT eosinophils adherent to ICAM-1 demonstrated increased Mac-1 expression, compared with *Swap-70*$^{-/-}$ eosinophils. Although eosinophils express Mac-1 constitutively, studies have shown that it can be increased by exposure to various stimuli (cytokines, chemokines, interaction with endothelial cells) (40). Second, in WT eosinophils, Mac-1 expression was predominantly observed at the cell periphery, with polarized cells exhibiting more intense Mac-1 expression at leading edges. In contrast to Mac-1 expression in WT eosinophils, that in *Swap-70*$^{-/-}$ eosinophils was dispersed all over the cell. F-actin was variably expressed in both cell types; however, F-actin expression colocalized with Mac-1 at the cell periphery in WT eosinophils, but not in *Swap-70*$^{-/-}$ eosinophils. These data suggest that the distribution, but not the level of expression, of α4 on the cell surface of adherent eosinophils is regulated by SWAP-70, whereas for Mac-1, both distribution and level of expression are regulated by this molecule. Expression and spatial distribution of cell surface adhesion receptors are critical for efficient cellular rolling and/or firm adhesion. Therefore, although no significant differences in baseline α4 and Mac-1 expression were observed between WT and *Swap-70*$^{-/-}$ eosinophils by flow cytometry (Fig. 4*A*), aberrations in distribution and level of expression observed with adherent eosinophils may account for the continued slow rolling of *Swap-70*$^{-/-}$ eosinophils with failure to adhere efficiently in vivo under conditions of blood flow.

### *Diminished chemokine-induced migration by Swap-70*$^{-/-}$ *eosinophils*

Previous studies with MCs and B cells have indicated a role for SWAP-70 in cell migration (20, 21, 41). In the current study, migration of WT and *Swap-70*$^{-/-}$ eosinophils in response to eotaxin-1 at concentrations ranging from 0.1 to 100 nM was evaluated in vitro. Relative to WT eosinophils, *Swap-70*$^{-/-}$ eosinophils exhibited significantly decreased migration ($p < 0.01$ at 100 nM) (Fig. 5*A*), although no difference in cell surface expression of the eotaxin-1 receptor CCR3 was noted between *Swap-70*$^{-/-}$ and WT eosinophils by flow cytometry (Fig. 5*B*). Because actin polymerization and cytoskeletal reorganization in response to activation is critical for directional movement and migration, we evaluated F-actin levels and examined the actin cytoskeleton in WT and *Swap-70*$^{-/-}$ eosinophils before and after exposure to eotaxin-1. WT and *Swap-70*$^{-/-}$ eosinophils exhibited a similar pattern of increase and decrease in F-actin levels up to 30 s. However, based on rhodamine-phalloidin binding, the increase in baseline fluorescence (untreated) after treatment with eotaxin-1, which is indicative of total F-actin levels, was higher in *Swap-70*$^{-/-}$ eosinophils than in WT cells at every time point. Although experimental variation was observed in absolute cell fluorescence, the trends were identical and two representative experiments of a total of five are shown (Fig. 5*C*). Difference in actin polymerization was also assessed by confocal microscopy of WT and *Swap-70*$^{-/-}$ eosinophils adherent to poly-L-lysine–coated surfaces (Fig. 5*D*). Untreated WT and *Swap-70*$^{-/-}$ eosinophils (without eotaxin-1) demonstrated a defined pattern of membrane-associated F-actin by confocal microscopy. After exposure to eotaxin-1 for 10 s, both WT and *Swap-70*$^{-/-}$ eosinophils exhibited increased phalloidin binding, mostly localized at one end of the cells. By 2 min, more *Swap-70*$^{-/-}$ eosinophils showed increased phalloidin

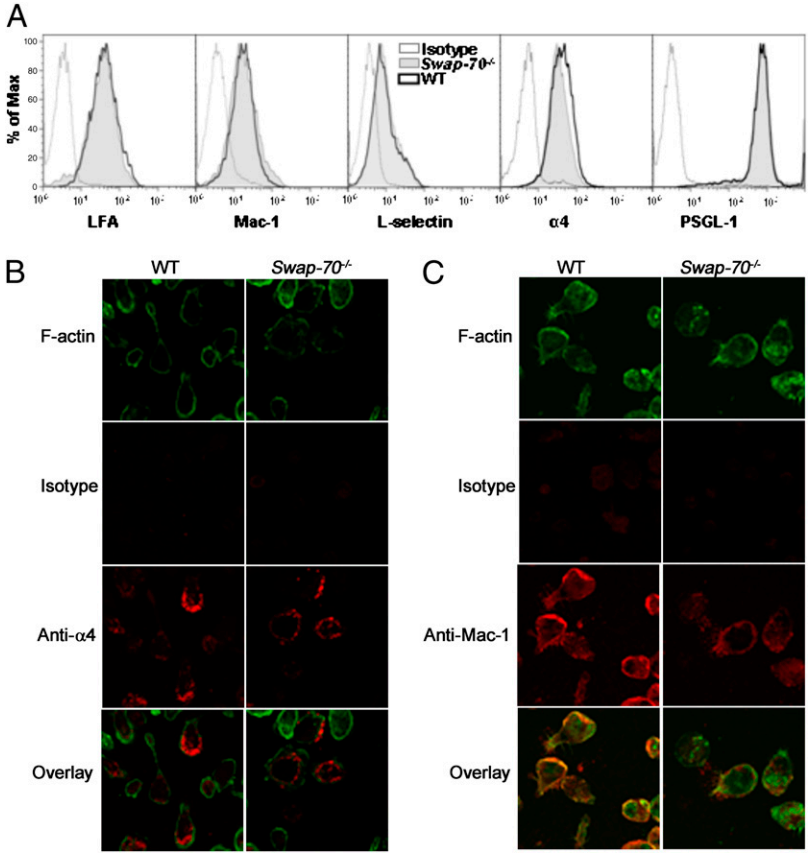


**FIGURE 4.** Adhesion molecule distribution is altered in *Swap-70*$^{-/-}$ eosinophils. *A*, Expression of adhesion molecules by WT and *Swap-70*$^{-/-}$ eosinophils by flow cytometry. Expression of LFA-1 and α4 was evaluated using rat mAbs against LFA-1 and α4, respectively, followed by FITC-conjugated anti-rat Abs, with rat IgG2b as the isotype control. PE-conjugated rat mAbs against Mac-1 and PSGL-1, as well as FITC-conjugated rat mAbs against L-selectin, were used to evaluate expression of Mac-1, PSGL-1, and L-selectin, respectively. PE- or FITC-conjugated rat IgG2a was used as the isotype control. All Abs were used at a concentration of 5 μg/ml. *B* and *C*, Immunofluorescence staining to evaluate distribution of α4 and Mac-1 on the cell surface of WT and *Swap-70*$^{-/-}$ eosinophils adherent to VCAM-1 and ICAM-1, respectively. Adherent cells were first stained with goat anti-mouse α4 (5 μg/ml) or rat anti-mouse Mac-1 (10 μg/ml), followed by rhodamine-conjugated secondary Abs and then with FITC-phalloidin in buffer containing 0.5% saponin. Goat IgG (for anti-α4) or rat IgG2b (for anti–Mac-1) was used as the isotype control. Original magnification ×600. Data shown in *A–C* are representative of three independent experiments, with eosinophils from three different mice for each group.

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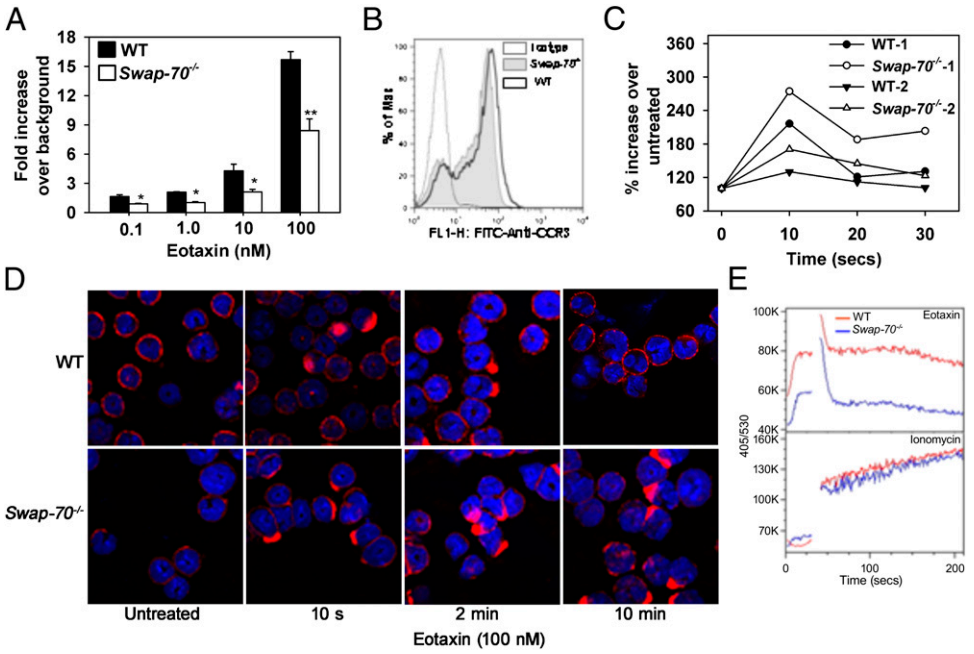


**FIGURE 5.** Eotaxin-1–induced migration is impaired in *Swap-70*$^{-/-}$ eosinophils. *A*, Migration of WT and *Swap-70*$^{-/-}$ eosinophils toward increasing concentrations of murine eotaxin-1 or media alone (background) in Transwell plates. Combined data (mean ± SEM) from three experiments in duplicate are shown. *B*, Expression of CCR3, the receptor for eotaxin-1, by WT and *Swap-70*$^{-/-}$ eosinophils by flow cytometry, using FITC-conjugated anti-mouse CCR3 (5 μg/ml). Rat IgG2a was used as the isotype control. *C*, Total F-actin in WT and *Swap-70*$^{-/-}$ eosinophils by flow cytometry after rhodamine-phalloidin staining of cells before and after treatment with eotaxin-1 (100 nM) for the indicated time points. Data of two representative experiments from a total of five independent experiments are shown. *D*, Staining of WT and *Swap-70*$^{-/-}$ eosinophils adherent to poly-L-Lysine with rhodamine-phalloidin after treatment with PBS (untreated) or eotaxin-1 (100 nM) for the indicated time points. Original magnification ×600. *E*, $Ca^{2+}$ flux in WT and *Swap-70*$^{-/-}$ eosinophils based on Indo-1, AM binding before and after stimulation with 100 nM eotaxin-1 (*upper panel*) or 2 μM ionomycin (*lower panel*) evaluated by flow cytometry. Eotaxin was added at 30 s. Data are shown as wavelengths 405/530, which reflect the ratio of bound to free $Ca^{2+}$. Data shown in *B*, *D*, and *E* are representative of three independent experiments with eosinophils from three different mice. *$p < 0.05$ and **$p < 0.01$ for comparison of WT versus *Swap-70*$^{-/-}$ eosinophils in *A*.

binding, compared with WT eosinophils. Interestingly, by 10 min, the actin cytoskeleton in WT eosinophils reverted back to that observed in untreated cells, whereas *Swap-70*$^{-/-}$ eosinophils continued to demonstrate increased phalloidin binding. Overall, deficiency of SWAP-70 appears to result in irregular F-actin polymerization/depolymerization, causing persistent elevated F-actin levels and altered cytoskeletal changes upon stimulation with eotaxin-1.

Eotaxin-1 is known to induce calcium flux in eosinophils (42). Calcium flux in response to eotaxin-1 (100 nM) in WT and *Swap-70*$^{-/-}$ eosinophils was investigated by flow cytometry (Fig. 5*E*, *upper panel*). Baseline (unstimulated) intracellular $Ca^{2+}$ levels were higher in WT eosinophils than in *Swap-70*$^{-/-}$ cells. Further, although both WT and *Swap-70*$^{-/-}$ eosinophils demonstrated a similar pattern of an initial surge in free intracellular $Ca^{2+}$ in response to eotaxin-1 within a few seconds, followed by a decline, intracellular $Ca^{2+}$ levels remained elevated in WT eosinophils, compared with *Swap-70*$^{-/-}$ eosinophils, up to 3 min. In contrast, both WT and *Swap-70*$^{-/-}$ eosinophils demonstrated a similar response to 2 μM ionomycin (Fig. 5*E*, *lower panel*), indicating that $Ca^{2+}$ release as such is not affected and that the eotaxin-1–induced $Ca^{2+}$ responses are altered in *Swap-70*$^{-/-}$ eosinophils. These data suggest that eotaxin-1–induced migration of eosinophils is dependent upon intracellular SWAP-70 signaling.

### *Allergic airway inflammation results in recruitment of SWAP-70–expressing inflammatory cells, including eosinophils, to the lungs*

The identification of a role for SWAP-70 in regulating trafficking and migration of eosinophils, the predominant cells recruited during allergic inflammation, prompted us to examine whether allergen-exposed lungs demonstrate increased presence of SWAP-70–positive cells in vivo in a murine model of acute allergic airway inflammation. Expression of SWAP-70 in the lungs of WT mice in response to allergen (OVA) challenge was examined immunohistologically using affinity-purified rabbit polyclonal Abs against murine SWAP-70 (Fig. 6*A*). Although no positive staining was detected in sections from control mice sensitized and challenged with saline instead of the allergen, expression of SWAP-70 by inflammatory cells infiltrating the lung tissue after allergen challenge was clearly evident. Further, we investigated specifically whether eosinophils recruited to the airways in response to allergen challenge express SWAP-70. In these studies, cells recovered from the BALF of acute allergen-challenged mice were dual stained with Abs against eosinophil-specific MBP and SWAP-70 and examined by confocal microscopy (Fig. 6*B*). As expected, BALF from allergen-challenged mice was found to contain predominantly cells that expressed eosinophil-specific MBP. All MBP-positive cells were also positive for expression of SWAP-70. Few cells that were positive for SWAP-70 but negative for MBP (Fig. 6*B*, red arrows) or negative for both MBP and SWAP-70 (Fig. 6*B*, white arrows) were observed.

### *Swap-70*$^{-/-}$ *mice exhibit decreased airway inflammation after allergen challenge*

Because SWAP-70 promotes eosinophil adhesion (Fig. 3*B*) as well as migration (Fig. 5*A*) and SWAP-70–expressing eosinophils are recruited to allergen-exposed lungs (Fig. 6*B*), the role of SWAP-70 in the development of allergic airway inflammation was investigated in allergen-challenged *Swap-70*$^{-/-}$ and WT mice. Al-

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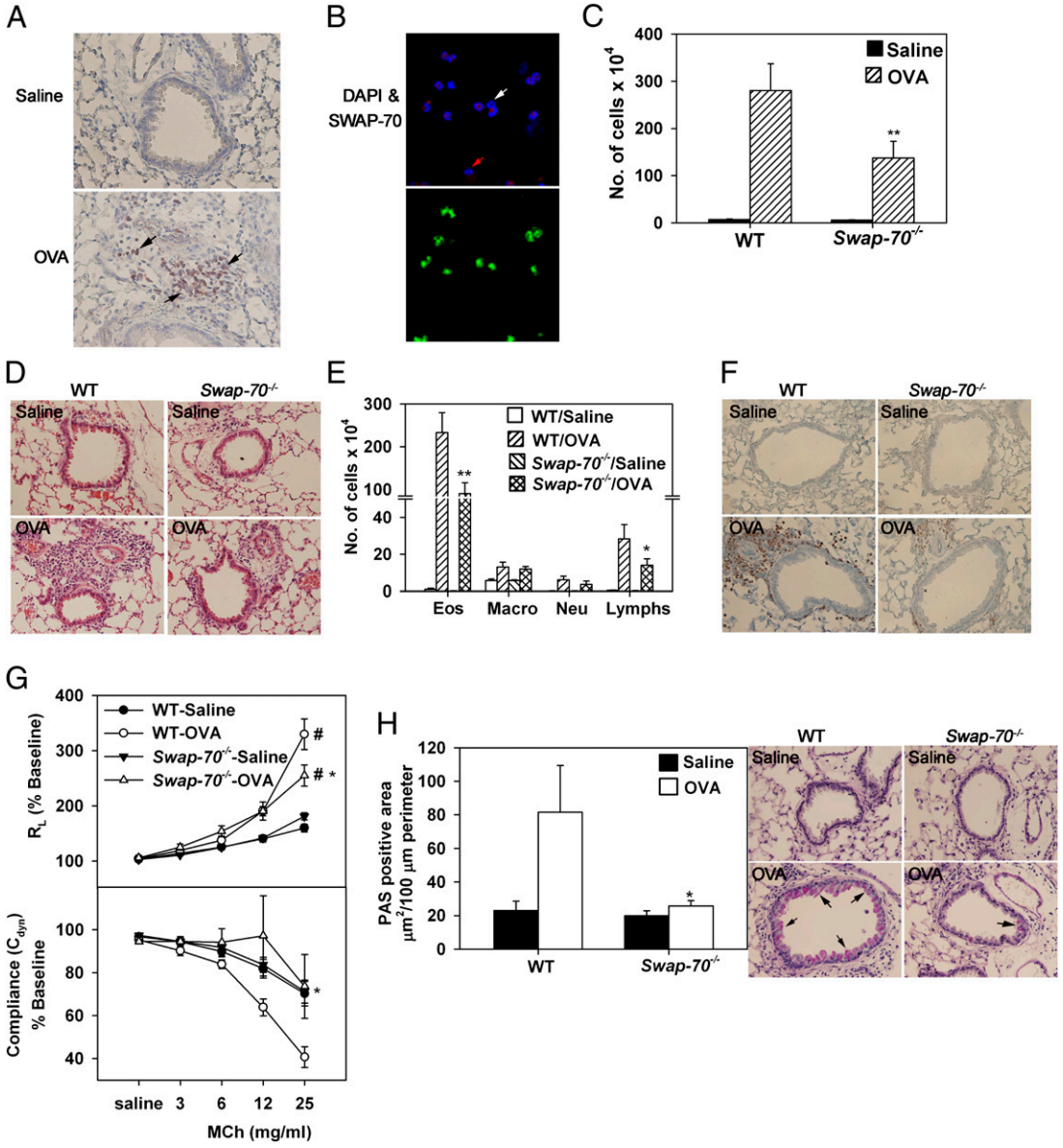


**FIGURE 6.** Allergen-induced airway inflammation is attenuated in *Swap-70*$^{-/-}$ mice. *A*, Immunohistology of paraffin-embedded lung sections from saline- and allergen (OVA)-challenged WT mice with polyclonal Abs against murine SWAP-70. Black arrows in lung sections of allergen-challenged mice indicate cells positive for SWAP-70. Original magnification ×200. *B*, Immunofluorescence staining of BALF cells from allergen-challenged WT mice with polyclonal Abs against SWAP-70 (*upper panel*) and rat mAb against murine MBP (*lower panel*), followed by rhodamine-conjugated goat anti-rabbit IgG and FITC-conjugated goat anti-rat IgG, respectively. Images of the same field with and without DAPI staining are shown. White arrows indicate cells in BALF that are negative for both SWAP-70 and MBP expression, whereas red arrows indicate cells that are positive for SWAP-70, but negative for MBP expression. Original magnification ×600. Representative images are shown for *A* and *B*. *C*, Total cell counts in BALF from saline- and OVA-challenged WT and *Swap-70*$^{-/-}$ mice. *D*, Cellular infiltration of lungs after H&E staining of paraffin-embedded lung sections. Representative images (original magnification ×100) are shown. *E*, Differential cell counts in BALF from saline- and OVA-challenged WT and *Swap-70*$^{-/-}$ mice. Combined data from experiments repeated at least three times are shown for *C* and *E*. $n = 11$ mice for saline groups and 15 mice for OVA-challenged groups. *F*, Infiltration of lung tissue by eosinophils after staining with rat mAb against murine MBP. Representative images (original magnification ×200) are shown for saline- and allergen-challenged groups. *F*, $R_L$ and compliance measured in saline- and OVA-challenged WT and *Swap-70*$^{-/-}$ mice by invasive plethysmography after exposure to increasing concentrations of aerosolized methacholine (MCh). Combined data of three experiments are shown. $n = 7$–9 per group. *G*, Mucus production in airways after PAS staining. PAS-positive area in airways of relatively similar size (3–12 airways per slide) was quantitated by ImageJ analysis of captured images (*left*). Representative images (original magnification ×200) are shown for saline- and allergen-challenged groups (*right*). For *A* and *B*, $n = 5$ mice per group, and for *D*, *F*, and *H*, $n = 9$ mice per group. Data represent mean ± SEM in *C*, *E*, *G*, and *H* (*left panel*). $^{*}p < 0.05$ and $^{**}p < 0.02$ for allergen-challenged *Swap-70*$^{-/-}$ versus WT mice. $^{\#}p < 0.01$ for allergen-challenged group versus respective saline group.

though both groups demonstrated more inflammatory cells in the BALF and lung tissue by H&E staining than did respective control mice, allergen-challenged *Swap-70*$^{-/-}$ mice had significantly fewer cells in the BALF ($p < 0.02$) and lung tissue than did WT counterparts (Fig. 6*C*, 6*D*). Differential cell counts in the BALF indicated a marked reduction in eosinophil recruitment ($p < 0.02$) to the airways of allergen-challenged *Swap-70*$^{-/-}$ mice relative to WT mice (Fig. 6*E*). Further, a significant reduction in recruitment of lymphocytes ($p < 0.05$), but not of macrophages or neutrophils, was noted. Immunohistologic studies to detect lung tissue eosinophils also revealed fewer eosinophil-specific MBP-positive cells in lung sections of allergen-challenged *Swap-70*$^{-/-}$ mice com-

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pared with WT mice (Fig. 6*F*), whereas very few to no MBP-positive cells were detected in control mice of both groups. No difference was observed between WT and *Swap-70*$^{-/-}$ mice with respect to the number of eosinophils in the BM after allergen challenge (16.22 ± 2.76% for WT mice and 12.65 ± 1.56% for *Swap-70*$^{-/-}$ mice, $p = 0.156$), indicating that eosinophil generation is not impaired in SWAP-70–deficient mice. Further, blood eosinophils in naive WT and *Swap-70*$^{-/-}$ mice before sensitization and challenge (2.16 ± 0.32% for WT and 2.05 ± 0.4% for *Swap-70*$^{-/-}$ mice) and their increase following allergen challenge were similar (3.98 ± 0.56% for WT mice and 5.62 ± 0.65% for *Swap-70*$^{-/-}$ mice, $p = 0.065$), showing that the role of SWAP-70 in regulating eosinophil recruitment is more likely at the level of cell adhesion and migration during inflammation.

In association with the increased airway eosinophilia, allergen-challenged WT mice displayed significantly elevated airway reactivity, with increased $R_L$ and decreased compliance ($C_{dyn}$), compared with control mice ($p < 0.01$) in response to aerosolized methacholine by invasive plethysmography (Fig. 6*G*). Allergen-challenged *Swap-70*$^{-/-}$ mice, compared with allergen-challenged WT mice, demonstrated considerable improvement in airway function, with a significant reduction in $R_L$ and improved compliance ($p < 0.05$ at 25 mg/ml methacholine), although airway reactivity was higher than in the respective control group. Next, airway mucus production by goblet cells in lung sections from control and allergen-challenged WT and *Swap-70*$^{-/-}$ mice was assessed after PAS staining (Fig. 6*H*). Negligible PAS-positive staining was observed in lung sections of control mice from both groups. As expected, the bronchial epithelium of allergen-challenged WT mice exhibited more PAS-positive mucus-producing cells and was found to be significantly higher than in allergen-challenged *Swap-70*$^{-/-}$ mice ($p < 0.05$).

### *Decreased Th2 cytokine levels in lungs of allergen-challenged Swap-70*$^{-/-}$ *mice*

Th1 (IL-2, INF-γ)/Th2 (IL-4, IL-5, IL-13) cytokine, TNF-α, and eotaxin-1 levels in the BALF of control and allergen-challenged WT and *Swap-70*$^{-/-}$ mice were quantitated (Table I). In WT mice, levels of IL-5, IL-13, and TNF-α significantly increased relative to control mice in response to allergen challenge, with no change in IL-2 and IFN-γ levels. Although IL-4 and eotaxin-1 levels in allergen-challenged WT mice also tended to be higher than in control mice, the increase was not statistically significant. More importantly, although levels of IL-5, IL-13, and TNF-α were also found to be higher in allergen-challenged *Swap-70*$^{-/-}$ mice compared with corresponding controls, they were significantly lower than in WT counterparts ($p < 0.05$). IL-4 and eotaxin-1 levels in allergen-challenged *Swap-70*$^{-/-}$ mice were similar to those in corresponding control mice.

## Discussion

Eosinophils are central effector cells in an ongoing allergic inflammation and are recruited to the site of inflammation from the peripheral blood. This process is driven by a cascade of events involving the specific and sequential engagement of cell surface adhesion receptors with vascular counterligands in inflamed blood vessels, followed by a series of signaling events that result in actin rearrangement and cytoskeletal reorganization, leading to directed cell shape changes that facilitate cell adhesion and migration into inflamed tissues (5, 8, 9, 43). SWAP-70 is an intracellular signaling protein previously shown to play a role in regulating B cell adhesion and migration (20, 41) and to participate in events such as degranulation, adhesion, migration, differentiation, and survival during MC activation (14, 21).

In the current study, we show for the first time, to our knowledge, that both human and murine eosinophils express SWAP-70 and demonstrate this molecule's role in eosinophil trafficking and migration, as well as its impact on the development of allergic airway inflammation in mice. *Swap-70*$^{-/-}$ eosinophils exhibited altered trafficking in inflamed blood vessels under physiologic conditions of blood flow, with a large fraction of cells demonstrating slow rolling with velocities <10 μm/s, as opposed to the majority of the WT eosinophils, which rolled at higher velocities; this finding is suggestive of aberrant interactions with the vascular endothelium. Of interest, most of the slow-rolling *Swap-70*$^{-/-}$ eosinophils failed to adhere to the vascular endothelium, whereas WT eosinophils exhibited significant adhesion. This finding stands in contrast to previous studies with B cells, in which *Swap-70*$^{-/-}$ cells rolled and adhered as well as WT cells, but accumulated in high endothelial venules of lymph nodes and failed to migrate (20). Consistent with failure to adhere efficiently to the vascular endothelium under conditions of flow, *Swap-70*$^{-/-}$ eosinophils demonstrated reduced attachment to ICAM-1 and even more profoundly to VCAM-1. Based on phalloidin staining, *Swap-70*$^{-/-}$ eosinophils exhibited decreased cell polarization, with poor formation of leading edges, filopodia, and uropods causing more cells to retain an irregular morphology and appear spread out, compared with WT eosinophils. In this respect, *Swap-70*$^{-/-}$ eosinophils appear to behave in a similar fashion to CCL19-stimulated SWAP-70–deficient B cells, which also failed to form uropods or stable lamellipodia after attachment to anti-CD44–coated surfaces and exhibited decreased polarization on ICAM-1 and MadCAM (20, 41). Leukocytes are known to undergo distinct shape change from a round, free-flowing form to a polarized morphology with

Table I. BALF cytokines in allergen-challenged WT and *Swap-70*$^{-/-}$ mice

| | WT Mice, Mean ± SEM pg/ml BALF | | *Swap-70*$^{-/-}$ Mice, Mean ± SEM pg/ml BALF | |
|---|---|---|---|---|
| Cytokine | Saline | OVA | Saline | OVA |
| IL-4 | 3.09 ± 0.43 | 24.77 ± 19.53 | 2.25 ± 0.32 | 4.06 ± 0.93 |
| IL-5 | 7.37 ± 1.37 | 62.22 ± 24.77[a] | 5.8 ± 0.97 | 14.32 ± 4.11[a,b] |
| IL-13 | <0.01 | 16.59 ± 6.03[a] | <0.01 | 1.9 ± 1.0[a,b] |
| IFN-γ | 4.45 ± 0.4 | 3.98 ± 0.9 | 2.59 ± 0.45 | 3.38 0.48 |
| IL-2 | 3.54 ± 0.53 | 3.51 ± 0.69 | 2.64 ± 0.35 | 3.02 ± 0.32 |
| TNF-α | 10.27 ± 1.66 | 24.18 ± 6.0[a] | 5.98 ± 1.15 | 11.15 ± 2.46[a,b] |
| Eotaxin-1 | 13.27 ± 2.41 | 27.05 ± 8.38 | 11.34 ± 2.85 | 13.43 ± 4.61 |

$n = 8$ mice for saline groups and 9 mice for OVA-challenged groups.
[a] $p < 0.05$, saline- versus OVA-challenged groups for WT and *Swap-70*$^{-/-}$ mice.
[b] $p < 0.05$, OVA-challenged *Swap-70*$^{-/-}$ mice versus OVA-challenged WT mice.

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formation of a leading edge and uropod upon activation (44). Therefore, although *Swap-70*$^{-/-}$ eosinophils roll slowly and can potentially become activated by encountering endothelial ligands or immobilized chemokines, lack of formation of a leading edge, which allows directional movement of the cell, may perhaps be responsible for their continued slow rolling with decreased ability to adhere under conditions of flow in vivo.

RAC1 plays a central role in cell motility by regulating the actin cytoskeleton and participating in the formation of lamellipodia and filopodia (39). In the current study, although WT eosinophils attached to VCAM-1 exhibited localization of F-actin to the cell periphery and translocation of RAC1 to the leading edge, in *Swap-70*$^{-/-}$ eosinophils F-actin and RAC1 expression remained dispersed all over the cell and did not localize to the periphery. Similar findings have been reported for B cells and MCs, in which RAC1 and F-actin localize to the cell periphery upon activation (attachment or chemokine stimulation) in a SWAP-70–dependent manner (20, 21), except that in WT and *Swap-70*$^{-/-}$ eosinophils RAC1 and F-actin do not colocalize, as in MCs and B cells. Studies have also shown that SWAP-70 interacts with and regulates RAC1 (15) and also binds to F-actin and promotes membrane ruffling (25). Therefore, absence of SWAP-70 may lead to aberrant RAC1 translocation and F-actin localization, resulting in failure of *Swap-70*$^{-/-}$ eosinophils to efficiently form leading edges and undergo polarization.

α4β1–VCAM-1 and β2 integrin–ICAM-1 interactions together drive eosinophil rolling, tethering, and arrest (5). As observed with B cells (20), absence of SWAP-70 in eosinophils did not appear to affect the basal (nonactivated) level of expression of α4, Mac-1, and LFA-1. However, SWAP-70 was found to be essential for regulating the distribution of α4 and Mac-1 on adherent eosinophils. In addition to the conformational state and receptor and ligand density, the spatial distribution of integrins on the cell surface determines their adhesiveness (45). Accordingly, in the current study, the more adhesive WT eosinophils express α4 localized to the leading edge of polarized cells, whereas in the less adhesive *Swap-70*$^{-/-}$ eosinophils, perhaps owing to the failure to form leading edges, α4 expression does not localize to specific structures and remains dispersed all over the cell. Similarly, Mac-1 expression colocalized with F-actin at the cell periphery in WT eosinophils but remained diffused in *Swap-70*$^{-/-}$ eosinophils without colocalizing with F-actin. α4β1 integrins mediate rolling and initial attachment of eosinophils on VCAM-1 (46) and TNF-α–stimulated HUVEC (47), whereas β2 integrins are thought to act later and mediate eosinophil arrest/firm adhesion (48). Therefore, based on their role in adhesive events promoting cell trafficking, it is conceivable that α4 is presented at the leading edge to initiate attachment with endothelial counterreceptors, and Mac-1 is presented at the periphery of the entire cell to establish firm adhesion along a wide surface area under conditions of blood flow. Using transfected cells, integrins such as α4β1 have previously been shown to localize at surface projections such as membrane ruffles and microvilli, whereas β2 integrins localized to the cell body (49). Along similar lines, absence of SWAP-70 in MCs and B cells was found to alter regulation of LFA-1, resulting in homotypic hyperaggregation of these cells in vitro without affecting its level of expression (20, 21).

Along with the impaired adhesion, *Swap-70*$^{-/-}$ eosinophils, compared with WT cells, demonstrate significantly reduced migration in response to eotaxin-1, associated with decreased intracellular $Ca^{2+}$ levels and altered kinetics of actin polymerization/depolymerization. Eotaxin-1–induced F-actin levels were higher in *Swap-70*$^{-/-}$ eosinophils and persisted for a longer time in adherent (poly-L-lysine) *Swap-70*$^{-/-}$ eosinophils than in WT eosinophils. Further, unlike WT eosinophils, *Swap-70*$^{-/-}$ eosinophils demonstrated increased F-actin localized at one end of the cells without returning to basal (untreated) levels even at 10 min. This finding suggests defective F-actin depolymerization rather than impaired polymerization in the absence of SWAP-70. Remodeling of the actin cytoskeleton, often mediated by the Rho family of GTPases in response to extracellular signals, is critical for cellular activities such as cell motility and active processes, including cell adhesion and migration, and requires the coordinated polymerization and depolymerization of F-actin. The eotaxin-1 receptor CCR3 belongs to the family of G protein-coupled receptors (50), which activate small RAC GTPases upon stimulation (51, 52). Because the level of expression of this receptor is not affected by SWAP-70 deficiency, it is more likely that regulation of signaling through CCR3 is impaired, as indicated by the reduced intracellular $Ca^{2+}$ levels and delayed F-actin depolymerization upon stimulation, resulting in aberrant cytoskeletal rearrangement in *Swap-70*$^{-/-}$ eosinophils that can impact cell polarization toward a chemokine gradient. Absence of SWAP-70 in MCs results in a similar pattern of sustained elevated F-actin positivity and formation of multiple elongated filopodia-like F-actin protrusions in response to stem cell factor, associated with decreased migration toward this chemokine (21).

Because SWAP-70 is required for normal eosinophil trafficking and migration, we investigated its contribution to the development of allergic airway inflammation, which is largely eosinophil associated, in a murine model. In contrast to allergen-challenged WT mice that develop a classical asthmatic response characterized by airway eosinophil infiltration, AHR, elevated eotaxin-1 and Th2 cytokines (IL-5, IL-4, and IL-13), as well as airway mucus secretion, the allergic inflammatory response in *Swap-70*$^{-/-}$ mice was attenuated. Most striking was the significant decrease in airway recruitment of eosinophils. This decrease in airway eosinophil recruitment in *Swap-70*$^{-/-}$ mice is most likely due to impaired trafficking (rolling and adhesion) and chemokine-induced migration of *Swap-70*$^{-/-}$ eosinophils. Although eotaxin-1 levels in the BALF of allergen-challenged WT mice tended to be higher than in *Swap-70*$^{-/-}$ counterparts, the increase was not statistically significant; moreover, in vitro studies indicate that *Swap-70*$^{-/-}$ eosinophils migrate less efficiently than WT eosinophils (despite similar levels of eotaxin-1 and CCR3 expression). In addition to eosinophils, *Swap-70*$^{-/-}$ allergen-challenged mice, compared with WT mice, demonstrated a reduction in the number of total lymphocytes (T and B cells) recruited to the airways. B cell migration (20) and IgE class switching (13) are known to be impaired in *SWAP-70*$^{-/-}$ mice, and because nonactivated T cells do not express SWAP-70 (11), decreased T cell recruitment to the airways may be an indirect effect caused by inhibition of other factors that are known to promote T cell recruitment, including MC-derived mediators (histamine) released during degranulation (53). Indeed, both migration and FcεRI-mediated degranulation of MCs are impaired in the absence of SWAP-70 (14, 21). Alternatively, although not well established, it is conceivable that activated T cells may express SWAP-70 and would thus be affected by its deficiency.

In association with decreased eosinophilia, allergen-challenged *Swap-70*$^{-/-}$ mice displayed significantly reduced AHR, mucus secretion, IL-5, IL-13, and TNF-α, compared with WT mice. Eosinophils, through the release of mediators (leukotrienes) and cytokines (IL-13), are crucial for mucus secretion in the airways and in the development of AHR (54–56). Decreased levels of IL-13 in allergen-challenged *Swap-70*$^{-/-}$ mice may be responsible for the attenuated AHR and mucus secretion. Although reduced IL-13 may also contribute to overall decreased cellular

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recruitment by virtue of its ability to support leukocyte trafficking via induction of VCAM-1 on endothelial cells (57), altered trafficking of *Swap-70*$^{-/-}$ eosinophils within inflamed blood vessels of SWAP-70–competent mice (slow rolling and decreased adhesion), combined with decreased migration, is indicative of an important role for SWAP-70 in eosinophil recruitment during inflammation, irrespective of the potential contribution of the vascular endothelium. Decreased recruitment of lymphocytes to the airways may also in part contribute to decreased BALF cytokines, especially IL-5, which in turn can contribute to reduced eosinophilia. Finally, eosinophils and MCs are a source of TNF-α (58, 59), and although we have not evaluated TNF-α release by *Swap-70*$^{-/-}$ eosinophils, *Swap-70*$^{-/-}$ MCs demonstrate impaired TNF-α release (14). Nonetheless, reduced TNF-α levels in allergen-challenged *Swap-70*$^{-/-}$ mice caused by decreased airway eosinophil recruitment may further attenuate airway inflammation.

In summary, eosinophils deficient in SWAP-70 exhibit altered trafficking (slow rolling with decreased firm adhesion) in inflamed blood vessels, attributable to reduced polarization, failure of RAC1 translocation, and aberrant adhesion molecule localization/distribution, along with impaired calcium flux and defective actin depolymeriztion upon chemokine activation, resulting in reduced ability to migrate in vitro. Further, in a model of allergic airway inflammation, *Swap-70*$^{-/-}$ mice show significantly reduced airway eosinophil recruitment and eosinophil-associated inflammatory responses. Because eosinophils are important contributors to the pathogenesis of allergen-induced airway inflammation, our findings, together with the previously identified role of SWAP-70 in B cell and MC recruitment as well as in Ig class switching, suggest that blockade of SWAP-70 may help in preventing allergic airway inflammation, including asthma.

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## Disclosures

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