Regulation of immature dendritic cell migration by RhoA guanine nucleotide exchange factor ARHGEF5
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Running Title: ARHGEF5 regulates RhoA and DC migration

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There are a large number of Rho guanine nucleotide exchange factors, most of which have no known functions. Here, we carried out shRNA-based functional screen of Rho GEFs for their roles in leukocyte chemotaxis and identified ARHGEF5 as an important factor in chemotaxis of a macrophage phage-like RAW264.7 cell line. ARHGEF5 can strongly activate RhoA and RhoB and weakly RhoC and RhoG, but not Rac1, RhoQ, RhoD, or RhoV, in transfected HEK293 cells. In addition, Gβγ interacts with ARHGEF5 and can stimulate ARHGEF5-mediated activation of RhoA in an in vitro assay. In vivo roles of ARHGEF5 were investigated using an ARHGEF-5-null mouse line. ARHGEF5-deficiency did not affect chemotaxis of mouse macrophages, T and B lymphocytes, and bone marrow derived mature dendritic cells (DCs), but it abrogated MIP1α-induced chemotaxis of immature DCs and impaired migration of DCs from the skin to lymph node. In addition, ARHGEF5-deficiency attenuated allergic airway inflammation. Therefore, this study provides new insights into signaling mechanisms for DC migration regulation.

Leukocyte chemotaxis underlies leukocyte migration, infiltration, trafficking, and homing that are not only important for normal leukocyte functions, but also have an important role in inflammation-related diseases. Leukocyte chemotaxis is regulated by leukocyte chemoattractants that include bacterial by-products such as fMLP, complement proteolytic fragments such as C5a, and the superfamily of chemotactic cytokines, chemokines. These chemoattractants bind to their specific cell G protein-coupled receptors and are primarily coupled to the Gi family of G proteins to regulate leukocyte chemotaxis. Previous studies have established that the Rho family of small GTPases regulates leukocyte migration1,2. Rac, Cdc42 and RhoA are the three best studied Rho small GTPases. In myeloid cells, Cdc42 regulates directionality by directing where F actin and lamellipodia is formed, and Rac regulates F actin formation in the lamellipodia, which provides a driving force for cell motility3-6. On the other hand, Rho A regulates the formation and contractility of the actomyosin structure at the back that provides a pushing force7,8. Rho GEFs are key regulators for the activity of these small GTPases. GEFs activate small GTPases by promoting the loading of GTP to the small GTPases, a rate limiting step in GTPase regulation9-11. Previous biochemical and genetic studies have revealed how Cdc42 and Rac may be regulated by chemokine receptors in leukocytes. Chemokine receptors can regulate Cdc42 via a Rho GEF PIα, which
is regulated by Gβγ from the Gi proteins via the interactions between Gβγ and Pak1 and between Pak1 and PI(x)α in myeloid cells 12. On the other hand, in neutrophils chemokine receptors regulate Rac2 via another Rho GEF P-Rex1, which is directly regulated by Gβγ 13-15. Two RhoGEFs have been implicated in regulation of RhoA in neutrophils. GEF115 was found in the leading edges of polarized mouse neutrophils, whereas PDZRhoGEF was found in the uropods of differentiated HL-60 cells. Both RhoGEFs were believed to mediate Pertussis toxin-resistant activation of RhoA in these cells. However, there is a significant portion of RhoA activity in leukocytes that is Pt-x-sensitive, which is presumably regulated by the α and/or βγ subunits from the Gi proteins. The signaling mechanism for this Pt-x-sensitive RhoA regulation by chemokine receptors remains largely elusive.

Molecular cloning and genomic sequencing have identified more than 70 Rho guanine nucleotide exchange factors (GEFs) in mammals 16-20. Many of these Rho GEFs have been shown to activate RhoA in in vitro and overexpression assays 16-20. However, it is not known if any of them may regulate RhoA in vivo, because we have found that although P1Xα is a specific GEF for Cdc42 in neutrophils 12 despite its potent activity on Rac in in vitro and overexpression assays 21,22. Therefore, we used a siRNA-based loss of function screen in an attempt to identify the GEFs that regulate myeloid cell migration and RhoA activity. One of the candidates, ARHGEF5, was found to be directly activated by Gβγ to regulate RhoA and has an important role in immature DC migration. In addition, GEF5-deficiency attenuated allergic airway inflammation in a mouse model.

MATERIALS AND METHODS

Plasmids, protein preparation, cell culture and transfection

The full length ARHGEF5 cDNA and its truncated mutants were amplified and subcloned into a mammalian expression vector carrying a Flag tag. The exchange activity-deficient ARHGEF5 mutant ARHGEF5DH was generated by substituting Ala residues for Leu245 and Leu246. For the in vitro binding assay, the ARHGEF5 cDNA was subcloned into the pET21-His vector. His-tagged ARHGEF5 was expressed in BL21 (DE3) competent cells and subsequently purified with Ni-NTA (Qiagen). Recombinant RhoA, Rhoteckin-RBD and PAK-PBD and Elmo were prepared from bacteria as GST fusion proteins. The Gβγ2 protein was prepared as previously described 12. The cDNAs of RhoB, RhoC, RhoD, RhoG, RhoQ as well as the dominant negative forms of RhoC, RhoD, and RhoF were acquired from UMR cDNA Resource Center carrying an HA-tag at their N-termini. DNA sequences of all expression constructs were verified by sequencing. Antibodies specific to HA, His, and Flag were acquired from Covance.

HEK293T cells and Raw264.7 cells were maintained in Dulbecco’s modified Eagle's medium (Cellgro) supplemented with penicillin/streptomycin and 10% fetal bovine serum (Hyclone). The B lymphoid cells were cultured in RPMI-1640 medium (Cellgro) supplemented with penicillin/streptomycin and 10% heat-inactivated fetal bovine serum (Hyclone). Transfection was carried out using Lipofectamine Plus (Invitrogen) by following manufacture’s instructions.

The method to culture immature and mature dendritic cells from mouse bone marrows was described in detail 44. Briefly, mouse bone marrow cells were collected by flushing the femurs. After lysis of the red blood cells, the cells were cultured in the Dulbecco’s modified Eagle’s medium supplemented with 20 ng/ml rmGM-CSF (Pepro Tech). After 5 days, cultured cells were collected and used for immature DC migration assays. Alternatively, the immature DCs were treated with 1 μg/ml LPS (Sigma) for 24 hours and used for mature DC migration assays.
ShRNAs screening and chemotaxis assays.
The shRNA vector, which was named as pAS, was modified based on pSuper by incorporating a GFP-luciferase fusion protein expression unit. The sequences for these shRNAs are shown in Supplementary Table I. For the screening, the shRNAs and control vector were transfected into Raw264.7 cells using Lipofectamine Plus. The cells were collected 48 hours later by trypsinization and resuspended in DMEM containing 1% FBS. They were then loaded into the upper chambers of 24-well transwell plates (Costar, 5μM pore size). The lower chambers were filled with the same medium, but supplemented with 10 nM C5a (Sigma). The plates were incubated at 37°C for 4 hours. Migrated cells were detached from the lower surface of the transwell inserts by trypsin and EDTA and lysed for luciferase assays. The chemotactic indices were calculated by dividing the luciferase activity of migrated cells in the presence of C5a by that of its absence.

For DC migration assays, BM derived immature DCs or mature DCs were collected and resuspended in DMEM containing 1% FBS and loaded onto the upper chambers of transwell plates. Immature DCs were stimulated with 300 ng/ml MIP-1α (Pepro Tech) whereas mature DCs with 60 ng/ml CCL-19 (Pepro Tech) for 4 hours at 37°C. Migrated cells were then counted and stained with CD11c for flowcytometric analysis. The chemotaxis indices were calculated by dividing the number of migrated immature (CD11c_{mid}) or mature (CD11c_{high}) DCs in the presence of a chemotactic ligand by that in its absence.

To evaluate the role of ARHGEF5 and 15 in mature DC chemotaxis, bone marrow-derived immature DCs were collected and transfected with synthetic siRNA duplex oligos of ARHGEF5 and ARHGEF15 using the Amaxa Nucleofector system (Amaxa Inc). Twenty-four hours after transfection, LPS was added into the culture to induce DC maturation. Mature DCs were collected for the transwell migration assay as described above 24 hours after the induction. The target sequences for the ARHGEF5 and ARHGEF15 siRNAs are CAGGAGGAATTTAATAATACA and AAGTATTAAATTAATAATA, respectively.

For evaluating lymphocyte migration, splenocytes were used in the transwell assay in response to 10 nM SDF-1. After 3 hours incubation at 37°C, migrated cells were counted and stained with anti-CD3-FITC and anti-B220-RPE. The numbers of migrated T cells and B cells were determined based on the cell number and their relative percentages, and chemotactic indices were computed as described above.

GTPase pull-down assays
The pull down assays for determining the activity of small GTPases were carried essentially as previously described. In brief, HEK293T cells were cotransfected with ARHGEF5 or its inactive mutant ARHGEF5DH with one of the HA-tagged small GTPases. After 24h transfection, cells were lysed with the lysis buffer [50mM Tris–HCl (pH 7.3), 10mM MgCl2 and 0.2M NaCl, 2% NP40, 10% glycerol, 2mM orthovanadate] containing recombinant GST-Rho/ Arctin-RBD for RhoA, RhoB, and RhoC pull-down, GST-PAK-CRIB for Rac, RhoD, and RhoQ pull-down, or GST-Elmo for RhoG pull-down. Bound GTPases were detected by Western analysis with an anti-HA antibody.

RBD binding assay
Cells were stimulated with or without 30 ng/ml SDF-1 for 15s before they were fixed with 4% para-formaldehyde. The cells were then washed and permeabilized by 0.1% Triton X-100 RT for 5min. After washing, cells were blocked by 1% BSA and incubated with purified GST-RBD at RT for one hour followed by Alexa-633-conjugated
anti-GST antibody (Molecular Probes, Inc). After washing, cells were analyzed by a flow-cytometer, and the mean fluorescence intensity (MFI) of GFP-positive populations that represent cells carrying the pAS vector was determined.

Guanine nucleotide loading assays
The exchange activity of ARHGEF5 was determine for its ability to promote the loading of N-methylanthraniloyl–GTP (Mant-GTP, from JENA Bioscience) to recombinant RhoA as previously described.

45. In brief, purified RhoA (0.12 uM) was incubated in an assay buffer containing 20 mM Tris, pH 7.5, 150 mM NaCl, 5mM MgCl2, and 1 mM dithiothreitol with 10uM MANT-GTP and recombinant proteins of 0.85 uM ARHGEF5 and/or 1 uM Gβγ. Immediately after mixing, fluorescence intensity was determined by a fluorometer (Wallac Vector, 1420 multilabel counter) with the excitation wavelength of 360nm and emission wavelength of 440nm.

Luciferase reporter assays
HEK293T cells were seeded in 24-well culture plates and transfected with the luciferase reporter construct SRE-luc, normalization plasmid GFP, and other plasmids shown in the figures by using Lipofectamine Plus. After transfection, cells were cultured in serum free medium for 24 hours before the GFP intensity was measured by a fluorometer. The cells were then lysed, and their luciferase activities were determined by a luminometer. Data are presented after the luciferase activity was normalized against the GFP intensity.

Immunoprecipitation
HEK293T cells were cotransfected with Gβ1γ2 and Flag-tagged ARHGEF5 or its mutants in for 24 hours. The cells were lysed with a lysis buffer(1% NP-40, 1% Sodium deoxycholate, 0.1% SDS, 0.15M NaCl, 0.01M sodium phosphate, 50mM sodium fluoride, 2mM EDTA, PH 7.2). Immunoprecipitation was then carried out with an anti-Flag antibody and Protein A/G beads at 4 °C for 1 hour. The immunocomplexes were subjected to Western analysis with an anti-Gβ1 antibody.

RT–PCR
Total RNAs were extracted from BM derived immature and mature DCs using the Trizol reagent (Invitrogen) according to manufacturer’s instructions. RNA was reverse-transcribed using the iScript cDNA synthesis kit (Bio-Rad). RT-PCR was performed. The sense oligo for Ephexin is GAAGCTGATCGACAGTGTGA, and the antisense oligo is ATCTTCGCAGACACCTCTCATT. The sense oligo for ARHGEF15 is ATCAGCTCAGCCAAGAGTGG, and the antisense oligo is AGATGGTGCTGGGGAACAG. The sense oligo for ARHARHGEF5 is TATGCACTAGTGAAGTTCCT. The antisense oligo is ACCTGACTGATAGGTTCCT.

In vivo DC migration assay
The migration of DCs from the skin to lymph node was determined by FITC skin painting as previously described.

46. In brief, FITC (Sigma-Aldrich) was dissolved in a 50:50 (vol/vol) acetone-dibutylphthalate mixture at the concentration of 5 mg/ml. Mice were anesthetized and their abdominal furs were shaved. The FITC solution (0.25ml/ animal) was applied onto the shaved skins. Twenty four hours later, inguinal lymph nodes were harvested and treated with collagenase D (1mg/ml, Roche) for 20 minutes at 37°C. The lymph nodes were then smashed onto 70 uM cell strainers to produce cell suspensions. The cells were collected and stained with PE-CD11c and analyzed by a flowcytometer.

Generation of ARHGEF5-null mice
A BAC clone that contains the ARHGEF5 gene was acquired from the BACPAC Resources Center at Children's Hospital Oakland Research Institute in Oakland, California. Exons 5 to 9, which encode residues Ala1153-Lys1355 in the DH domain of ARHGEF5, were floxed with the LoxP sequences in the gene-targeting
construct. The ARHGEF5 mutant mouse line was generated at the Gene Targeting and Transgenic Facility of the University of Connecticut Health Center using the ES cell line 129S6 derived from 129SvEvTac/C57BL/6J F1 blastocysts. The chimeric mice were crossed with 129S1-Hprt1-Cre from JAX to produce germline excision of the sequences between the two LoxP sites. Finally, mice heterozygous for the disrupted ARHGEF5 gene were interbred to produce mice homozygous of ARHGEF5 KO mice. Animals from F1 to F3 were used in this study.

Ovalbumin-induced asthma model
Mice were sensitized by IP injection of with 200ul OVA-alum suspension [0.5mg/ml Ovalbumin (OVA, Sigma) in PBS mixed with equal volume of 20mg/ml Aluminum Hydroxide]. Eight days after immunization, mice were challenged with an aerosol of 1% FITC-OVA in PBS, delivered by an ultrasonic nebulizer (OMRON, Compair) for 20 min. FITC-OVA was prepared by mixing 2mg/ml FITC solution in carbonate buffer (220mM, PH 9.6) with OVA at 10mg/ml. The mixture was gently rotated at 4°C for overnight in the dark. Unbounded FITC was removed by ultrafiltration using a 10 Kd molecular weight cut-off membrane in a 15ml filtration cell (Amicon). One day after challenge, the mice were anesthetized. Their trachea was cannulated, and their lungs were lavaged five times with 1ml pre-chilled PBS. The bronchial lymph nodes were then collected. Cells in the bronchoalveolar lavage (BAL) fluid were collected by centrifugation at 4000 rpm for 5 min. Cytospin preparations of cells were stained with Diff-Quik (Dade Behring and differential were performed on 200 cells based on morphology and staining characteristics. The supernatant of BAL fluids were analyzed for IL-4 levels using a mouse IL-4 ELISA kit (Endogen). Lymph nodes were passed through a cell strainer (BD Falcon), and cells were counted and stained with RPE-CD11c (BD Pharmingen) and analyzed by a flow cytometry (Caliber, BD).

Intravaginal infection of HSV-2.
The thymidine kinase mutant HSV-2 viruses were prepared and inoculated intravaginally (ivag) into GEF5 and wildtype littermates (1 × 10⁶ plaque-forming units of HSV-2) as previously described ⁴⁷. Vaginal washes were collected daily, and the levels of IFN-γ and IL-12 were determined by ELISA. On Day 6, mice were euthanized, and CD4⁺ or CD8⁺ T cells were isolated from iliac and inguinal lymph nodes. The T cells were cocultured with naïve WT splenocytes and heat-inactivated HSV2 of varying pfu (for CD4⁺ T cells) or 1µg/ml gB peptide (for CD8⁺ T cells) for 3 days. The levels of IFN-γ in the conditioned media were determined by ELISA.

Statistical analysis
Statistical comparisons between different groups or treatments were performed by unpaired two-tailed Student’s T test and p< 0.05 was considered statistically significant.

RESULTS
To investigate the roles of Rho-GEFs in leukocyte chemotaxis, we carried out a siRNA-based functional screen for Rho GEFs that may play a role in migration of macrophage-like Raw264.7 cells. We generated a mini vector-based shRNA library targeting 38 Rho GEFs, whose expression could be detected by RT-PCR in RAW264.7 cells (data not shown). The shRNA vector was modified based on pSuper ²³ by incorporating a GFP-luciferase fusion protein expression unit; thus, cells producing shRNA can be monitored by the expression of GFP and/or luciferase. This vector was named as pAS. All of the GEF shRNAs were validated based their ability to knock down endogenous GEF expression detected by quantitative RT-PCR and/or coexpressed cDNAs if available (data not shown). To test the effects of these shRNAs on RAW264.7 cell migration, cells were transfected with one of the shRNAs, and the empty pAS was used as a negative
control. Two days after transfection, transwell migration assays were carried out, and the migratory ability of the cells expressing a GEF shRNA was compared with that of the control cells. Out of 38 GEF shRNAs we screened, 6 shRNAs showed more than 50% inhibition (Fig. 1A and Supplementary Table 1). Because siRNAs are known to have off-target effects, we tried to validate the effects by constructing a second shRNA expression plasmid that has a different targeting sequence for these 6 putative hits. We successfully generated the second shRNAs for 5 out of these 6 putative hits. Among these 5 shRNAs, only ARHGEF5 shRNA showed more than 50% inhibition of RAW264.7 cell migration (Supplementary Table 1, Fig. 1A). To further validate the siRNA specificity, we coexpressed an ARHGEF5 expression plasmid together with its shRNA plasmid to determine if siRNA’s effects can be rescued. To prevent the silencing effect of the shRNA on the expression of exogenous ARHGEF5, we introduced silent mutations at the shRNA targeting sequence of the ARHGEF5 cDNA. As shown in Fig 1B, expression of ARHGEF5, but not an ARHGEF5 mutant with its DH domain mutated, led to a strong increase in the levels of active RhoA and RhoB, while resulting in weak activation of RhoC and RhoG. Expression of ARHGEF5 had no effects on the level of active RhoD, RhoV, RhoQ, or Rac1 (Fig. 2A). Previous studies also showed that GEFs generally exhibit high affinities for the GTP-free mutant forms of small GTPases they regulate. Consistent with the pull-down assay results, ARHGEF5 co-immunoprecipitated with RhoA-N19, but not RhoC-N19 or RhoD-N31 (Fig. 2B). In addition, RhoF-N33 did not show detectable interaction with ARHGEF5 (Fig. 2B), suggesting that ARHGEF5 may not regulate RhoF. These results indicate that ARHGEF5, Ephexin1, and ARHGEF15, which are more homologous in amino acid sequences than SGEF, belong to a subgroup that potently activates RhoA rather than RhoG.

Next, we wanted to assess the significance of ARHGEF5 in chemoattractant-induced small GTPase activation. Because of the relative low transfection efficiency for leukocytes and the large number of cells required for the pull-down assays particularly for detection of endogenous proteins, we developed a flowcytometry-based approach to assess the GTPase activities. J774 cells expressing ARHGEF5 shRNA (G5pAS) or the control vector (pAS) were stimulated with SDF-1. They were then fixed, permeabilized, and incubated with purified GST-RBD and Alexa633-conjugated anti-GST antibody. The mean fluorescence
intensity (MFI) of GFP positive populations that represent cells carrying the pAS vector was determined by a flow cytometer. As shown in Fig. 2C, SDF-1 treatment resulted in marked right-shift in MFI in cells expressing the control vector, suggesting that SDF-1 stimulates the activation of small GTPase that can bind to RBD in these cells. However, in cells expressing ARHGEF5 shRNA, SDF-1-induced MFI increase was blunted (Fig. 2C). This result suggests that ARHGEF5 has a significant role in regulation of endogenous Rho GTPases.

Because RhoA and its close homolog RhoB can be potently activated by ARHGEF5, we interpret the inhibitory effect of the ARHGEF5 shRNA on RBD binding to suggest that ARHGEF5 may be involved in chemoattractant-mediated activation of RhoA and/or RhoB. In leukocytes, Gβγ subunits have been shown to mediate many of the chemoattractant signaling events. Thus, we examined if Gβγ regulates ARHGEF5. We first performed the SRE.L-luciferase reporter gene assay, which was previously shown to be specifically activated by Rho GTPases. As shown in Fig. 2D, coexpression of Gβ1γ2 and ARHGEF5 led to synergistic activation of the reporter gene activity, suggesting that Gβγ and ARHGEF5 may function in the same signaling pathway. This conclusion is supported by the observation that coexpression of ARHGEF5, Gβ1γ2 and RhoA led to the highest levels of active RhoA (Fig. 2E).

To further investigate the relationship between Gβγ and ARHGEF5, we examined if Gβγ can interact with ARHGEF5 in an immunoprecipitation assay. We found that Gβγ and ARHGEF5 coimmunoprecipitated in HEK293 cells expressing both proteins (Fig. 3A & B). We went on to delineate the sequences on ARHGEF5 that are required for its interaction with Gβγ. A series of ARHGEF5 deletion mutants were generated as depicted in Fig. 3A. These ARHGEF5 mutants were tested for their ability to coimmunoprecipitate with Gβ1γ2 in HEK293 cells. We found that the sequence encompassing Residues Val261-Thr395 is minimal for retaining the full ability to interact with Gβγ (Fig. 3B). The fact that the sequences encompassing Residues Val261, Arg313 and Trp312-Glu424 are also able to bind to Gβγ (Fig. 3B) support the idea that the Gβγ-binding site is located in and near the PH domain. To determine if the interaction between Gβγ and ARHGEF5 is direct, we carry out an in vitro pull-down assay using recombinant ARHGEF5 protein prepared from a bacterial expression system and Gβ1γ2 protein prepared from a baculoviral expression system. The pull-down assay shows that these two proteins can interact directly (Fig. 3C).

The aforementioned results together suggest that Gβγ binds to and may activate ARHGEF5, which in turn activates RhoA. To demonstrate that Gβγ can activate RhoA via ARHGEF5 in a biochemically defined system, we carried out an in vitro GTP loading assay. In this assay, we tested if recombinant Gβ1γ2 and ARHGEF5 proteins were able to stimulate the loading of a fluorogenic GTP analog, Mant-GTP, to recombinant RhoA, which represents the activation of the small GTPase. We found that, while ARHGEF5 alone stimulated GTP loading to RhoA as expected, addition of Gβ1γ2 resulted in a further increase in GTP loading (Fig. 3D) and that Gβ1γ2 regulated this RhoA activity in a dose-dependent manner (Fig. 3E). These results demonstrate that Gβγ is able to directly activate ARHGEF5, which in turn activates RhoA.

Next we investigated the roles that ARHGEF5 plays in vivo by generating a mouse line in which the ARHGEF5 gene is disrupted (Fig. S1). Because siRNA-mediated knocking down of ARHGEF5 expression led to impaired chemotaxis of a macrophage, we first examined whether ARHGEF5-deficiency affected primary mouse macrophage chemotaxis. The migration of neither peritoneal macrophages...
in response to C5a was, however, not affected (Fig. 4A). We also examined chemotaxis of spleen T and B lymphocytes in response to SDF-1 and of bone marrow neutrophils to fMLP. GEF5-deficiency did not affect the chemotactic responses of these cells (Fig. 4B; data not shown).

Dendritic cells (DCs) play important roles in modulating adapted immune system, and some of them share the same ontogeny as macrophages. Therefore, we examined chemotaxis of bone marrow-derived immature DCs in vitro and found that ARHGEF-5 deficiency markedly impaired immature DC chemotaxis in response to MIP-1α (Fig. 4C). We also examined the RhoA activation in response to MIP-1α and found that MIP-1α-induced RhoA activation is significantly reduced in the immature DCs from ARHGEF5-null mice compared to those from the wildtype littermates (Fig. 4D). These results indicate that chemokines may regulate immature DC migration via ARHGEF5 and RhoA. However, when we examined the effect of ARHGEF5-deficiency on migration of mature DCs, we found that the lack of ARHGEF5 had little effect (Fig. 4D). We also examined DC migration in vivo using the skin painting assay. There was a partial (35%), but statistically significant reduction in DC migration from the skin to lymph nodes in GEF5-null mice compared to the wildtype controls (Fig. 4F). Because DCs start to mature upon antigen engagement, the differential effects of ARHGEF5-deficiency on immature and mature DCs may explain this less robust effect of GEF5-deficiency on DC migration in vivo.

We postulated that the lack of effect of ARHGEF5-deficiency on mature DC migration may be due to the molecular redundancy as there are several ARHGEF5 close homologues, whose expression may be upregulated in mature DCs to functionally compensate the lack of ARHGEF5. Therefore, we examined the expression of ARHGEF5 and its two homologs Ephexin-1 and ARHGEF15 in immature and mature DCs. While ARHGEF5 could be detected in both mature and immature DCs, Ephexin-1 and ARHGEF15 could only be detected in mature DCs by RT-PCR (Fig. 4G), suggesting that the relative contribution of ARHGEF5 may diminish along the increases in Ephexin-1 and ARHGEF15 expression during DC maturation. To test whether these ARHGEF5 homologs are indeed functional redundancy in regulation of mature DC migration, we attempted knocking down ARHGEF5, ARHGEF15, and both in cultured mature DCs with their specific siRNAs. As shown in Fig. 4H, transfection of mouse bone marrow derived mature DCs with both siRNAs led a significant reduction in mature DC chemotaxis in response to CCL-19, while transfection of either siRNAs alone showed insignificant effects.

We also investigated the in vivo roles of GEF5 using two mouse model systems, in which DCs are known to be involved: ovalbumin-induced allergic airway inflammation and vaginal infection of herpes simplex virus type 2 (HSV-2). After OVA immunization and OVA challenge, we found that GEF5-deficiency reduced the number of eosinophils infiltrating into the respiratory tract, CD11c+ DC cells migrating to the bronchial lymph node, and the levels of IL-4 in the bronchoalveolar lavage (BAL) (Fig. 5A-C). However, GEF5-deficiency did not cause significantly changes in the levels of IL-12 or IFN-γ in the vaginal washes. Furthermore, induction and differentiation of CD4 T cells as measured by IFN-γ production from CD4+ T cells stimulated by heat-inactivated HSV-2 or CD8+ T cells by the gB peptide in the HSV-2 infection model were not significantly affected in GEF5-deficient mice (Fig. 5D-F). These results suggest that GEF5-deficiency may affect the Th2 responses in an OVA-induced asthma model, but not Th1 responses induced following a viral infection with HSV-2. In addition, the results shown in Fig. 5F indicate that intrinsic T cell responses are not affected by GEF5-deficiency.
DISCUSSION

We screened a mini-Rho GEF shRNA library and identified ARHGEF5 as an important GEF in the regulation of cultured myeloid cells. By studying of ARHGEF5-null mice, we demonstrated that ARHGEF5 is a RhoA regulator in vivo and has an important role in the regulation of immature mouse DC chemotaxis. Together with the finding that ARHGEF5 is directly activated by Gβγ, we have characterized a novel signaling pathway from chemokine receptors to RhoA activation for regulation of leukocyte migration. In this signaling pathway, chemokine receptors presumably act through the Gβγ subunits of the G protein to activate ARHGEF5, which in turn activates RhoA. RhoA are known to regulate the formation and contractility of the actomyosin structure that provide a pushing force for leukocyte migration.

In this report, we provide strong evidence showing that ARHGEF5 is directly regulated by Gβγ. Gβγ binds to the linker region between the DH and PH domains of ARHGEF5 as well as part of the PH domain. ARHGEF5 is the first RhoA GEF that has been found to be directly regulated by Gβγ. Gβγ may regulate ARHGEF5 through a mechanism similar to that by which Gαq regulates p63RhoGEF revealed by a recent structural study. It is also possible that Gβγ may induce ARHGEF5 membrane translocation as it does to a Rac-specific GEF, P-Rex1. Additional studies, particularly structural ones, are needed to further determine the molecular mechanisms by which Gβγ regulates ARHGEF5 activity.

The study of ARHGEF5-null mice revealed that ARHGEF5 is a significant regulator of RhoA in immature DCs. This observation is consistent with the effect of ARHGEF5-deficiency on immature DC migration. Because of upregulation of ARHGEF5 homologs in mature DCs as shown in Fig. 7, ARHGEF5 is no longer the primary GEF for RhoA regulation, and hence its deficiency no longer has a significant impact on mature DC migration. Immature DCs, once activated by antigens, start to migrate towards lymph nodes accompanied by their maturation process. It is reasonable to speculate that upregulation of GEF5 homolog expression upon DC maturation would mask some of the in vivo effects of GEF5 deficiency. This molecular redundancy may help to explain the differential effects of GEF5-deficiency on the two model studies shown in Fig. 5. DCs are known to play important roles in both of the model systems, even though these two models are primarily mediated by T11 and T12 cells, respectively. The lack of significant effects of GEF5-deficiency on the responses in the HSV-2 infection model may be due to early maturation of DCs or an involvement of different types of DCs in which GEF5 has no primary role. In fact, DCs are rapidly activated through innate recognition of HSV-2 virions through TLR9, and inflammatory monocytes are recruited to the site of infection and there differentiate into DCs. Although significant reduction in DC infiltration into the bronchial lymph nodes observed in GEF5-null mice should contribute to the reduction in eosinophil infiltration and IL-4 production in the OVA-induced asthma model, our results do not exclude other possible contributions by GEF5-deficiency to the attenuation of allergic airway inflammatory responses. Further studies are needed to clarify these questions. In addition, the presence of Ephexin-1 and ARHGEF15 in neutrophils and macrophages (data not shown) may explain why GEF5-deficiency failed to affect migration of these leukocytes in response to chemoattractants. Inactivation of additional GEFs in this class may be needed to reveal if these GEFs are actually involved in chemotaxis of these leukocytes and to more comprehensively investigate the significance of this signaling pathway in immune responses in vivo.
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REFERENCES:

1. Ridley AJ. Rho GTPases and cell migration. J Cell Sci. 2001;114:2713-2722.

2. Etienne-Manneville S, Hall A. Rho GTPases in cell biology. Nature. 2002;420:629-635.

3. Allen WE, Zicha D, Ridley AJ, Jones GE. A role for Cdc42 in macrophage chemotaxis. J Cell Biol. 1998;141:1147-1157.

4. Srinivasan S, Wang F, Glavas S, et al. Rac and Cdc42 play distinct roles in regulating PI(3,4,5)P3 and polarity during neutrophil chemotaxis. J Cell Biol. 2003;160:375-385.

5. Meili R, Firtel RA. Two poles and a compass. Cell. 2003;114:153-156.

6. Gu Y, Filippi MD, Cancelas JA, et al. Hematopoietic cell regulation by Rac1 and Rac2 guanosine triphosphatases. Science. 2003;302:445-449.

7. Xu J, Wang F, Van Keymeulen A, et al. Divergent signals and cytoskeletal assemblies regulate self-organizing polarity in neutrophils. Cell. 2003;114:201-214.

8. Ridley AJ. Rho family proteins: coordinating cell responses. Trends Cell Biol. 2001;11:471-477.

9. Burridge K, Wennerberg K. Rho and Rac take center stage. Cell. 2004;116:167-179.

10. Wennerberg K, Der CJ. Rho-family GTPases: it's not only Rac and Rho (and I like it). J Cell Sci. 2004;117:1301-1312.

11. Schwartz M. Rho signalling at a glance. J Cell Sci. 2004;117:5457-5458.

12. Li Z, Hannigan M, Mo Z, et al. Directional sensing requires G beta gamma-mediated PAK1 and PIX alpha-dependent activation of Cdc42. Cell. 2003;114:215-227.

13. Welch HC, Coadwell WJ, Ellson CD, et al. P-Rex1, a PtdIns(3,4,5)P3- and Gbetagamma-regulated guanine-nucleotide exchange factor for Rac. Cell. 2002;108:809-821.

14. Welch HC, Condliffe AM, Milne LJ, et al. P-Rex1 regulates neutrophil function. Curr Biol. 2005;15:1867-1873.

15. Dong X, Mo Z, Bokoch G, Guo C, Li Z, Wu D. P-Rex1 is a primary Rac2 guanine nucleotide exchange factor in mouse neutrophils. Curr Biol. 2005;15:1874-1879.

16. Schmidt A, Hall A. Guanine nucleotide exchange factors for Rho GTPases: turning on the switch. Genes Dev. 2002;16:1587-1609.

17. Garcia-Mata R, Burridge K. Catching a GEF by its tail. Trends Cell Biol. 2007;17:36-43.

18. Rossman KL, Der CJ, Sondek J. GEF means go: turning on RHO GTPases with guanine nucleotide-exchange factors. Nat Rev Mol Cell Biol. 2005;6:167-180.

19. Buchsbaum RJ. Rho activation at a glance. J Cell Sci. 2007;120:1149-1152.
20. Erickson JW, Cerione RA. Structural elements, mechanism, and evolutionary convergence of Rho protein-guanine nucleotide exchange factor complexes. Biochemistry. 2004;43:837-842.

21. Obermeier A, Ahmed S, Manser E, Yen SC, Hall C, Lim L. PAK promotes morphological changes by acting upstream of Rac. Embo J. 1998;17:4328-4339.

22. Bagrodia S, Bailey D, Lenard Z, et al. A tyrosine-phosphorylated protein that binds to an important regulatory region on the cool family of p21-activated kinase-binding proteins. Journal of Biological Chemistry. 1999;274:22393-22400.

23. Brummelkamp TR, Bernards R, Agami R. A system for stable expression of short interfering RNAs in mammalian cells. Science. 2002;296:550-553.

24. Ogita H, Kunimoto S, Kamioka Y, Sawa H, Masuda M, Mochizuki N. EphA4-mediated Rho activation via Vsm-RhoGEF expressed specifically in vascular smooth muscle cells. Circ Res. 2003;93:23-31.

25. Shamah SM, Lin MZ, Goldberg JL, et al. EphA receptors regulate growth cone dynamics through the novel guanine nucleotide exchange factor ephexin. Cell. 2001;105:233-244.

26. Xie X, Chang SW, Tatsumoto T, Chan AM, Miki T. TIM, a Dbl-related protein, regulates cell shape and cytoskeletal organization in a Rho-dependent manner. Cell Signal. 2005;17:461-471.

27. Ellerbroek SM, Wennerberg K, Arthur WT, et al. SGEF, a RhoG guanine nucleotide exchange factor that stimulates macropinocytosis. Mol Biol Cell. 2004;15:3309-3319.

28. Ren XD, Kiosses WB, Schwartz MA. Regulation of the small GTP-binding protein Rho by cell adhesion and the cytoskeleton. EMBO Journal. 1999;18:578-585.

29. Aspenstrom P, Fransson A, Saras J. Rho GTPases have diverse effects on the organization of the actin filament system. Biochem J. 2004;377:327-337.

30. Benard V, Bohl BP, Bokoch GM. Characterization of rac and cdc42 activation in chemoattractant-stimulated human neutrophils using a novel assay for active GTPases. Journal of Biological Chemistry. 1999;274:13198-13204.

31. Gumienny TL, Brugnera E, Tosello-Trampont AC, et al. CED-12/ELMO, a novel member of the CrkII/Dock180/Rac pathway, is required for phagocytosis and cell migration. Cell. 2001;107:27-41.

32. Feng Q, Baird D, Cerione RA. Novel regulatory mechanisms for the Dbl family guanine nucleotide exchange factor Cool-2/alpha-Pix. Embo J. 2004;23:3492-3504.

33. Meller N, Irani-Tehrani M, Kiosses WB, Del Pozo MA, Schwartz MA. Zizimin1, a novel Cdc42 activator, reveals a new GEF domain for Rho proteins. Nat Cell Biol. 2002;4:639-647.

34. Hill CS, Wynne J, Treisman R. The Rho family GTPase RhoA, Rac1 and CDC42Hs regulate transcriptional activation of SRF. Cell. 1995;81:1159-1170.
35. Mao J, Yuan H, Xie W, Wu D. Specific involvement of G proteins in regulation of SRF by receptors. J Biol Chem. 1998;273:27118-27123.

36. Li Z, Paik JH, Wang Z, Hla T, Wu D. Role of guanine nucleotide exchange factor P-Rex-2b in sphingosine 1-phosphate-induced Rac1 activation and cell migration in endothelial cells. Prostaglandins Other Lipid Mediat. 2005;76:95-104.

37. Remmers AE, Posner R, Neubig RR. Fluorescent guanine nucleotide analogs and G protein activation. J Biol Chem. 1994;269:13771-13778.

38. Mellman I, Steinman RM. Dendritic cells: specialized and regulated antigen processing machines. Cell. 2001;106:255-258.

39. Burns S, Thrasher AJ. Dendritic cells: the bare bones of immunity. Curr Biol. 2004;14:R965-967.

40. Lutz S, Shankaranarayanan A, Coco C, et al. Structure of Galphaq-p63RhoGEF-RhoA complex reveals a pathway for the activation of RhoA by GPCRs. Science. 2007;318:1923-1927.

41. Barber MA, Donald S, Thelen S, Anderson KE, Thelen M, Welch HC. Membrane translocation of P-Rex1 is mediated by G protein beta gamma subunits and phosphoinositide 3-kinase. J Biol Chem. 2007.

42. Lund J, Sato A, Akira S, Medzhitov R, Iwasaki A. Toll-like receptor 9-mediated recognition of Herpes simplex virus-2 by plasmacytoid dendritic cells. J Exp Med. 2003;198:513-520.

43. Iijima N, Linehan MM, Saeland S, Iwasaki A. Vaginal epithelial dendritic cells renew from bone marrow precursors. Proc Natl Acad Sci U S A. 2007;104:19061-19066.

44. Lutz MB, Kukutsch N, Ogilvie AL, et al. An advanced culture method for generating large quantities of highly pure dendritic cells from mouse bone marrow. J Immunol Methods. 1999;223:77-92.

45. Rojas RJ, Kimple RJ, Rossman KL, Siderovski DP, Sondek J. Established and emerging fluorescence-based assays for G-protein function: Ras-superfamily GTPases. Comb Chem High Throughput Screen. 2003;6:409-418.

46. Macatonia SE, Knight SC, Edwards AJ, Griffiths S, Fryer P. Localization of antigen on lymph node dendritic cells after exposure to the contact sensitizer fluorescein isothiocyanate. Functional and morphological studies. J Exp Med. 1987;166:1654-1667.

47. Sato A, Iwasaki A. Induction of antiviral immunity requires Toll-like receptor signaling in both stromal and dendritic cell compartments. Proc Natl Acad Sci U S A. 2004;101:16274-16279.
FIGURE LEGENDS

Figure 1. Effects of GEF shRNAs on chemotaxis. A) Effects of the GEF shRNAs on RAW264.7 cell chemotaxis in response to C5a. The chemotactic index of cells expressing pAS is taken as 1, and the relative chemotactic activity of cells expressing each GEF shRNA was calculated. Data shown are log of the relative chemotactic activities. The raw data are shown in Supplementary Table I. The open circles denote the second shRNAs used for validation of the initial hits. B & C) Rescuing the effect of ARHGEF5 shRNA by expressing an ARHGEF5 mutant containing a silent mutation. Raw 264.7 cells (B) and J774 cells (C) were cotransfected with pAS, ARHGEF5 shRNA (pAS-G5), and/or silently mutated ARHGEF5 (G5*) for 48 hours. Chemotactic assays were carried out using the transwell plate in the presence of C5a for RAW264.7 cells and SDF-1 for J774 cells. CI stands for chemotactic index. D) GEF5 shRNA validation. HEK293T cells were cotransfected with the plasmids expressing GEF5 and GFP and pAS or pAS-G5. Western analyses were carried out two days after transfection.

Figure 2. Regulation of Rho small GTPases by ARHGEF5. A) Pull-down assays. HEK 293T cells were cotransfected with one of the small GTPases and LacZ (Z), ARHGEF5 (G5) or the loss-of-function mutant of ARHGEF5 (DH). The RBD pull-down assays were carried out for cells expressing RhoA, RhoB, and RhoC, whereas the PBD pull-down assay was done for Rac, RhoQ, RhoD, and RhoV. The activity of RhoG was determined by a pull-down using GST-Elmo. Both precipitated and total GTPases were detected by Western analysis using an antibody specific for the HA-tag carried by these small GTPases. B) Interaction of dominant negative GTPases with ARHGEF5. HA-tagged RhoA-N19 (A), RhoC-N19 (C), RhoD-N31 (D), RhoF-N33 (F), and LacZ were cotransfected with Flag-tagged ARHGEF5 in HEK293T cells. Immunoprecipitation was carried out with an anti-HA antibody and detected with an anti-Flag antibody. C) Knocking down of ARHGEF5 reduced SDF-1-induced RBD binding. J774 cells were transfected with pAS or ARHGEF5 shRNA for 48 hours and stimulated with or without 30 ng/ml SDF-1 for 15 seconds. Cells were fixed, permeabilized, stained with GST-RBD and Alexa633-labeled secondary antibody, and analyzed by a flow cytometer. Mean fluorescence intensity (MFI) of Alexa-633 in cells gated for GFP is shown. Three experiments were performed. A representative one is shown. D) SRE-Luciferase assay. HEK293T cells were cotransfected with the SRE-Luciferase reporter gene, GFP, LacZ, Gβγ, and/or ARHGEF5. Cells were lysed and the GFP levels and luciferase activity were determined. The luciferase activity was normalized against the GFP level.

Figure 3. Gβγ interacts with and directly activates ARHGEF5. A) Schematic representation of ARHGEF5 and its mutants. The diagrams are not drawn in scale. The numbers at the side correspond to those in Panel B. B) Interaction of Gβγ with ARHGEF5 and its deletion mutants. Flag-tagged ARHGEF5 and its mutant were cotransfected with Gβ1γ2 in HEK293T cells. Immunoprecipitation was carried out using the anti-Flag antibody and detected using an anti-Gβ1 antibody. N, no ARHGEF5 transfected. C) Direct interaction between ARHGEF5 and Gβ1γ2. His-tagged ARHGEF5 protein purified from a bacterial expression system was incubated with Gβγ from a baculoviral expression system. Pull-down was carried out by an anti-His antibody and detected by an anti-Gβ1 antibody. D) Direct regulation of ARHGEF5 by Gβ1γ2. Purified recombinant proteins of Gβ1γ2 (1 μM),
ARHGEF5 (0.5 μM), and/or RhoA (0.12 μM) were incubated as indicated in the figure in the presence of Mant-GTP.  

**E)** Dose-dependent activation of RhoA by Gβγ. A different doses of Gβ1γ2 were incubated with RhoA (0.12 μM) and ARHGEF5 (0.5 μM).

**Figure 4.** ARHARHGEF5 has an important role in immature DC migration.  

**A-C)** Transwell migration assays. Chemotactic activity of peritoneal macrophages (A), spleen T and B lymphocytes (B), and bone marrow-derived immature DCs (C) from wildtype (G5+/+) or GEF5-deficient (G5-/-) mice was determined using the transwell assay. C5a (10 ng/ml, A), SDF-1 (100ng/ml, B), and MIF1α (300 ng/ml, C) were used.  

**D)** RhoA activity in immature DCs. MIP-1α-induced Rho activation was determined by using an ELISA kit that determines the levels of active RhoA.  

**P** =0.012.  

**E)** Transwell migration assay of bone marrow-derived mature DCs in response to CCL19 (60 ng/ml).  

**F)** In vivo migration of DCs. Mice were painted with FITC and cells from inguinal lymph nodes were isolated and stained with CD11c. The percentage of DCs migrated from the skins (FITC/CD11c double positive) were determined by flowcytometry. n=14,  

**G)** Expression of ARHGEF5 and its close homologs ARHGEF15 and Ephexin1 detected by RT-PCR in immature DCs (imDC) and mature DCs (mDC).  

**H)** Knocking down of both ARHGEF5 and 15 reduces migration of bone marrow-derived mature DCs in response to CCL-19. Bone marrow-derived mature DCs were transfected with no oligo (C) or synthetic siRNA oligos targeting ARHGEF5 (G5), ARHGEF15 (G15) or both (G5/15).  

**P**<0.05 Bar 4 vs. others.

**Figure 5.** Effects of ARHGEF5-deficiency on two in vivo disease models.  

**A-C)** Effects of ARHGEF5-deficiency on an OVA-induced allergic airway inflammation model. The numbers of eosinophils in BAL (A) and FITC+/CD11C+ cells in the bronchial lymph nodes (B) and the levels of IL-4 in BAL (C) were determined. n=7.  

**D-F)** Effects of ARHGEF5-deficiency on a HSV-2 infection model. The levels of IFN-γ (D) and IL-12 (E) in vaginal washes were collected daily and determined. CD4+ or CD8+ T cells were isolated from iliac and inguinal lymph nodes. They were co-cultured with naïve WT splenocytes and heat-inactivated HSV-2 of indicated pfu (for CD4+ T cells) or 1μg/ml gB peptide (for CD8+ T cells) for 3 days. The levels of IFN-γ in the conditioned media were determined by ELISA (F). n= 3 (wt) or 4 (GEF5-/−).
Figure 1

A

Effect of siRNA (log)

| GEF shRNA | pAS-G5 | pAS-G5+G5* |
|-----------|--------|------------|
| GEF5      |        |            |
| GEF5-2    |        |            |

B

Cl (Fold)

| pAS | pAS-G5 | pAS-G5+G5* |
|-----|--------|------------|
|     |        |            |

C

p<0.05

D

GEF5-GFP-

GFP-
Figure 2

A

Pull-down

Input

GTPase  RhoA   RhoC   RhoB   Rac

Pull-down

Input

RhoQ   RhoG   RhoD   RhoV

B

IP

Input

C

Cell Number

MFI

D

Luciferase activity (fold)

E

GEF5: - + - - + -
DH: - - + - - +
Gβγ: - - - + + +
RhoA: + + + + +

Pull-down

Gβ1-

GEF5

GFP-RhoA-

Total
Figure 3

A

B

C

D

E
Figure 5

A. Eosinophils (10^6)

B. FITC+/CD11c+ (10^6)

C. BAL IL-4 (pg/ml)

D. IFNγ [ng/ml]

E. IL-12 [ng/ml]

F. IFNγ [ng/ml]

WT and G5-/- mice were challenged with OVA or HSV-2. The graphs show the expression levels of various cytokines and cell populations. The p-values indicate statistical significance between WT and G5-/- groups. The graphs depict data from days 0 to 7 after infection.