Phosphoinositide 3-OH Kinase Regulates Integrin-Dependent Processes in Neutrophils by Signaling through Its Effector ARAP3


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ARAP3, a GTPase activating protein for Rho and Arf family GTPases, is one of many phosphoinositide 3-OH kinase (PI3K) effectors. In this study, we investigate the regulatory input of PI3K upstream of ARAP3 by analyzing neutrophils from an ARAP3 pleckstrin homology (PH) domain point mutation knock-in mouse (R302, 303A), in which ARAP3 is uncoupled from activation by PI3K. ARAP3 PH domain point mutant neutrophils are characterized by disturbed responses linked to stimulation by either integrin ligands or immobilized immune complexes. These cells exhibit increased β2 integrin inside-out signaling (binding affinity and avidity), and our work suggests the disturbed responses to immobilized immune complexes are secondary to this. In vitro, neutrophil chemotaxis is affected in the mutant. In vivo, ARAP3 PH domain point mutant bone marrow chimeras exhibit reduced neutrophil recruitment to the peritoneum on induction of sterile peritonitis and also reduced inflammation in a model for rheumatoid arthritis. The current work suggests a dramatic regulatory input of PI3K into the regulation of β2 integrin activity, and processes dependent on this, by signaling through its effector ARAP3.

Neutrophils are highly specialized cells that form part of the innate immune system (1). These terminally differentiated, short-lived cells are continuously being generated in the bone marrow. Mature neutrophils reside in a quiescent state in the bloodstream. On stimulation, they follow a well-defined activation pathway that includes rolling along the vessel wall, followed by weak and then firm adhesion. Neutrophils finally extravasate and chemotax to sites of damage and/or infection to phagocytose pathogens. Insufficient neutrophil activation leaves individuals susceptible to recurrent infections, as exemplified in leukocyte adhesion deficiency patients, where β2 integrin function is affected. On the flip side, neutrophils contribute to inflammation by producing reactive oxygen species (ROS) and cytoxic enzymes. IgG-coated microorganisms drive the activation of neutrophil FcγRs, triggering neutrophil activation, normally at a site of infection. Immobilized immune complexes formed by autoantibodies reacting with their (host) Ags also trigger FcγR-mediated neutrophil activation targeted against host tissue, leading to exacerbated autoimmune inflammatory responses. Neutrophil activity therefore needs to be tightly controlled to ensure an adequate innate immune response while avoiding exaggerated inflammatory responses. On a molecular level, this control is exerted by many regulators, including agonist-activated (class I) phosphoinositide 3-OH kinases (PI3Ks) and their effectors.

Agonist-activated PI3Ks are heterodimeric proteins that are activated downstream of tyrosine kinase-coupled receptors (class IA) and G protein-coupled receptors (class IB), respectively, as well as Ras. PI3Ks are responsible for the generation of the lipid second messengers phosphatidylinositol-3,4,5-trisphosphate [PtdIns(3,4,5)P3] and phosphatidylinositol-3,4-bisphosphate in the plasma membrane (2). This drives the activation—mediated often at least in part by recruitment to the plasma membrane—of PI3K effectors. Although most attention has been placed on Akt/PKB signaling downstream of PI3K, there are also many other PI3K effectors: it is estimated that most cell types express in excess of 25 different PI3K effectors, which all mediate downstream effects. A large number of PI3K effectors isolated to date are regulators of small GTPases.

ARAP3 is a PI3K effector that was identified from neutrophils in a screen for PtdIns(3,4,5)P3 binding proteins (3). ARAP3 is a GTPase activating protein (GAP) for the small GTPases RhoA and Arf6, and it is regulated by PI3K and Rap-GTP. PtdIns(3,4,5)P3 regulates ARAP3 catalytically as an Arf GAP and causes ARAP3’s recruitment to the plasma membrane, thereby bringing it into the vicinity of its substrates and of Rap-GTP, which activates ARAP3 as a Rho GAP (3, 4). We previously showed that ARAP3 is a regulator of integrin-dependent neutrophil functions (5).

To understand the regulatory input of PI3K into ARAP3 in the neutrophil, we report the analysis of neutrophils from knock-in mice in which ARAP3 carries a double point mutation in the most N-terminal pleckstrin homology (PH) domain (R302, 303A).

Phosphoinositide 3-OH Kinase Regulates Integrin-Dependent Processes in Neutrophils by Signaling through Its Effector ARAP3

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ARAP3, a GTPase activating protein for Rho and Arf family GTPases, is one of many phosphoinositide 3-OH kinase (PI3K) effectors. In this study, we investigate the regulatory input of PI3K upstream of ARAP3 by analyzing neutrophils from an ARAP3 pleckstrin homology (PH) domain point mutation knock-in mouse (R302, 303A), in which ARAP3 is uncoupled from activation by PI3K. ARAP3 PH domain point mutant neutrophils are characterized by disturbed responses linked to stimulation by either integrin ligands or immobilized immune complexes. These cells exhibit increased β2 integrin inside-out signaling (binding affinity and avidity), and our work suggests the disturbed responses to immobilized immune complexes are secondary to this. In vitro, neutrophil chemotaxis is affected in the mutant. In vivo, ARAP3 PH domain point mutant bone marrow chimeras exhibit reduced neutrophil recruitment to the peritoneum on induction of sterile peritonitis and also reduced inflammation in a model for rheumatoid arthritis. The current work suggests a dramatic regulatory input of PI3K into the regulation of β2 integrin activity, and processes dependent on this, by signaling through its effector ARAP3.

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This double point mutation renders ARAP3 unable to interact with and be activated by PtdIns(3,4,5)P$_3$ (3, 6). For ease of reading, in this study we refer to this PH domain point mutation knock-in as PH*. Like ARAP3-deficient neutrophils, Arap3$_{PH*PH*}$ cells exhibited perturbed responses on adhesion-dependent stimulation and showed increased β2 integrin affinity and clustering. Arap3$_{PH*PH*}$ neutrophils were also hyperactive when plated onto immobilized immune complexes, but not on cross-linking FcγR in solution. In vitro chemotaxis assays showed a directional defect of Arap3$_{PH*PH*}$ neutrophils, whereas in vivo, Arap3$_{PH*PH*}$ neutrophils were characterized by reduced recruitment to the peritoneum after induction of sterile peritonitis and by subtly reduced inflammation in an autoantibody-induced arthritis model.

Materials and Methods

Unless otherwise stated, materials were obtained from Sigma (Dorset, U.K.).

Abs

Mouse anti-BSA (clone BSA-33) was from Sigma (Dorset, U.K.); rat anti-FcγR/III (CD16/32; clone 93) was from eBioscience (Hatfield, U.K.); hamster anti-FcγRIV (blocking Ab; clone 9E9) has been described (7) and was a kind gift from Falk Nimmerjahn (Erlangen University, Erlangen, Germany). Sheep anti-ARAP3 has been described (3), and mouse anti-β2-COP was a gift from Nick Kistakis. Mouse anti-RhoA, anti-rap1p, and anti-Arf6 were from Santa Cruz Biotechnology. Alexa Fluor–conjugated, highly cross-absorbed secondary Abs were from BD Invitrogen–Molecular Probes (Eugene, OR, U.S.A.); and goat anti-rabbit and anti-sheep were from Santa Cruz Biotechnology. Alexa Fluor–conjugated, highly cross-absorbed, anti-human Fc Ab, and cells were stained with Cell Signaling Technology/New England Biolabs (Hitchin, Hertfordshire, U.K.). Goat anti-mouse HRP conjugate was obtained from Bio-Rad Laboratories (Hemel Hempstead, U.K.), and goat anti-rabbit and anti-sheep were from Santa Cruz Biotechnology. Alexa Fluor–conjugated, highly cross-activated peroxidase Abs were from BD Biotech International (Cockeysville, MD). FITC-conjugated mouse anti-hamster IgG was from BD Pharmingen (San Diego, CA). Alexa Fluor–conjugated anti-GR1, anti-LFA1, and anti-Mac1 as well as all isotype controls were from eBioscience.

Arap3$_{PH*}$ mouse model

Generation of the Arap3$_{PH*}$ mouse has been previously described. Experiments performed for this work were carried out with mice derived from the BB10 clone, which produces rare homozygous mutants (8). Because the frequency of Arap3$_{PH*PH*}$ mice was very low, we generated cohorts of Arap3$_{PH*PH*}$ and wild-type bone marrow chimeras. In vitro experiments were carried out with matched wild-type and Arap3$_{PH*PH*}$ mice and in parallel with bone marrow chimeras (using chimeras originating from different donors for duplicate experiments). The results obtained in this fashion were pooled. Because ARAP3 is known to be expressed in the murine vasculature, in vivo experiments were only carried out with anti-mouse HRP conjugate was obtained from Bio-Rad (Hemel Hempstead, U.K.), and goat anti-rabbit and anti-sheep were from Santa Cruz Biotechnology. Alexa Fluor–conjugated, highly cross-activated peroxidase Abs were from BD Biotech International (Cockeysville, MD). FITC-conjugated mouse anti-hamster IgG was from BD Pharmingen (San Diego, CA). Fluorescently conjugated anti-GR1, anti-LFA1, and anti-Mac1 as well as all isotype controls were from eBioscience.

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Immobilization of adhesive proteins and immune complexes

Sheep fibrinogen (150 μg/ml) or poly-Arg-Gly-Asp (20 μg/ml) were adsorbed onto tissue culture grade plastics overnight at 4°C (fibrinogen) or for 3 h at room temperature (poly-Arg-Gly-Asp). Surfaces were washed prior to performing any assays. For immune complexes, dishes were coated overnight at 4°C with essentially endotoxin and fatty acid-free BSA in PBS (100 μg/ml), blocked with 1% fat-free milk in PBS for 1 h, and incubated with mouse anti-BSA (1:10,000) for 1 h at room temperature.

FcyR cross-linking

FcyR cross-linking was performed as described elsewhere (11) using rat anti-FcγRII/III and goat anti-rat F(ab’)2.

ROS production assays

ROS production was measured by chemiluminescence using a luminol-based assay in polystyrene 96-well plates (Berthold Technologies, Bad Wildbad, Germany) as described (12). 5 × 10$^5$ cells were incubated with luminol (150 μM) and HRP (18.75 U/ml) for 10 min at 37°C. Where indicated, neutrophils were added manually to wells precoated with fibrinogen or poly-Arg-Gly-Asp or with or without murine TNF-α (20 ng/ml, R&D Systems) or to immobilized immune complexes. For soluble agonist assays with fMLF or poly-Arg-Gly-Asp concentration, cells were primed for 1 h at 37°C in the absence (mock) or presence of mouse TNF-α (1000 U/ml) and GM-CSF (100 ng/ml). For all assays, measurements were started immediately, and light emission was recorded. Data output is relative light unit (RLU) per second or total RLU integrated over indicated measured periods of time.

Chemotaxis assays

For micropipette assays, neutrophils were resuspended in HBSS, 15 mM HEPES pH 7.4, 0.05% BSA, and allowed to attach to a glass coverslip. Cells were stimulated at 37°C with a point source of fMLF delivered from a microinjection pipette (femtosecond; Eppendorf, Cambridge, U.K.) using 50 mbar pressure as described (5) and monitored by time-lapse imaging for 30 min using an inverted Zeiss axiowert microscope (Welwyn, Hertfordshire, U.K.) and Axiovision software. Dunn chamber chemotaxis assays were carried out as described (10, 13) using fMLF or MP2 as chemoattractant. Cells were tracked using “manual tracking,” and tracks were analyzed using the Chemotaxis Tool plug-ins (Ibidi, Planegg, Germany) in ImageJ.

MAPK, ERK, and PKB activation assays

These assays were carried out with cells plated onto poly-Arg-Gly-Asp, BSA–anti-BSA immune complex–coated, or hiFCS-blocked dishes essentially as described (14) or cells were lysed at indicated time points after FcγRII/III cross-linking as described (11).

Degranulation assay

Release of gelatinase granules after plating onto a fibrinogen-coated or immune complex–coated surface or as induced by stimulation with fMLF and cytochalasin B was detected by zymography as described (15).

Analysis of β2 integrins

Surface integrins were visualized by FACs analysis of stained cell populations. Bone marrow cells were stained with anti-GR1 FITC and anti-integrin Cy5. GR1$^{Hi}$ cells were gated and analyzed for level of integrin expression. Cell surface labeling was analyzed by confocal microscopy of unstained, stained cells that were kept in solution at the time of assaying and that were allowed to settle onto electrostatic slides (superfrost +; VWR, Lutterworth, U.K.) after fixation. To measure integrin binding affinity, unstained, stimulated cells were allowed to bind to ICAM1–Fc chimera (R&D Systems, Abingdon, U.K.) in solution; after fixation, bound ICAM1–Fc was stained using a highly cross-absorbed Alexa Fluor–conjugated anti-human Fc Ab, and cells were allowed to settle onto electrostatic slides. Bound ICAM1 was measured using quantitative immunofluorescence as described (5).

K/BxN serum transfer arthritis model

The K/BxN serum transfer model was performed as described (16) using lethally irradiated recipients reconstituted with Arap3$_{PH*}$ or matched control bone marrows. Mice were injected with 150 μl of arthritogenic or control serum, and ankle thickness and clinical scores were measured for the following 14 d. Sample preparation for histology was as described (17).
Small GTPase activity assays

RhôA-GTP was determined by G-LISA assay (Cytoskeleton, Denver, CO); Arf6-GTP and Rap1-GTP were identified by pull-down assay as described (5).

Results

ARAP3 is a PI3K- and Rap-regulated dual Rho and Arf GAP that was isolated in a screen for PtdIns(3,4,5)P3 binding proteins from neutrophils. PtdIns(3,4,5)P3 binds ARAP3 in a PH domain–dependent fashion and regulates ARAP3 catalytically as well as causing its plasma membrane recruitment. ARAP3 can be uncoupled from activation by PI3K by insertion of a double point mutation in its most N-terminal PH domain (3, 6). We previously showed that introducing this PH domain mutation (R302, 303A; in this study termed PH*) into ARAP3 led to embryonic lethality (8). A small number of homozygous mutant pups from Arap3PH*PH* intercrosses from mice derived from one of two independently targeted embryonic stem cell clones were viable and fertile (8) (Table I). Arap3PH*PH* knock-ins which survived to adulthood were healthy and fertile. We analyzed their neutrophils and those isolated from lethally irradiated recipient mice that had been reconstituted with bone marrow cells from Arap3PH*PH* or matched wild-type control mice to elucidate the regulatory input of PI3K into ARAP3 in the neutrophil.

Expression of ARAP3 protein was not affected by the presence of the PH domain point mutation in heterozygous or homozygous mutants (Fig. 1A). We detected no differences in expression of the ARAP family members ARAP1 and ARAP2 (data not shown). Peripheral blood cell counts from chimeric mice reconstituted with control and Arap3PH*PH* bone marrow cells were very similar (Fig. 1B).

Adhesion-dependent events are upregulated in Arap3PH*/PH* neutrophils

Neutrophils produce ROS in a well-characterized manner in response to a variety of stimuli. This allows the analysis of the machinery required for ROS production and also of signaling pathways required for ROS production after a particular stimulus. ROS production of purified wild-type control and Arap3PH*/PH* neutrophils on stimulation with the soluble agonist fMLF (Fig. 2A, 2D) or with the nonphysiological soluble stimulus PMA (data not shown) was very similar, indicating ARAP3 is not required downstream of PI3K for ROS generation per se. However, when we assayed ROS production after plating neutrophils onto the physiological Mac1 ligand fibrinogen or onto the synthetic multivalent integrin ligand polyRGD, ROS production was significantly increased in Arap3PH*/PH* compared with the control neutrophils (Fig. 2B, 2C, 2E, 2F). For stimulation by fibrinogen, neutrophils require a costimulatory event, for example by a proinflammatory cytokine like TNF-α (18). Notably, plating Arap3PH*/PH* onto fibrinogen caused significantly increased ROS production even in the absence of any costimulation (Fig. 2B, 2E), suggesting that the knock-in cells were present in a preactivated state.

We investigated whether signaling events known to lie downstream of β2 integrin ligation (outside-in signaling) were affected by uncoupling ARAP3 from PI3K. We assessed adhesion-dependent activation of PKB (also known as Akt) and p38 MAPK using phospho-specific Abs and observed significantly enhanced PKB and p38 phosphorylation in Arap3PH*/PH* neutrophils on stimulation with the soluble agonist fMLF (Fig. 2G–I).

To address why integrin ligation caused these enhanced responses we assayed ROS production after plating neutrophils onto the physiological Mac1 ligand fibrinogen or onto the synthetic multivalent integrin ligand polyRGD. ROS production was significantly increased in Arap3PH*/PH* compared with the control neutrophils (Fig. 2B, 2C, 2E, 2F). For stimulation by fibrinogen, neutrophils require a costimulatory event, for example by a proinflammatory cytokine like TNF-α (18). Notably, plating Arap3PH*/PH* onto fibrinogen caused significantly increased ROS production even in the absence of any costimulation (Fig. 2B, 2E), suggesting that the knock-in cells were present in a preactivated state.

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We next analyzed the effect of uncoupling ARAP3 from PI3K on neutrophil degranulation by assaying gelatinase activity in supernatants of neutrophils that had or had not been stimulated. Like ROS production, this can be triggered by soluble stimuli or by ligation of integrins. In line with the results obtained with the ROS assays, Arap3PH*/PH* and control neutrophils released very similar amounts of gelatinase granules on stimulation with a soluble agonist (fMLF in the presence of cytochalasin B), whereas granule release in the PH domain point mutants was enhanced on being plated onto the integrin ligand fibrinogen in the presence of TNF-α (Fig. 2J, 2K).

We finally compared the ability of control and Arap3PH*/PH* neutrophils to adhere and spread. Arap3PH*/PH* neutrophils plated onto polyRGD adhered and spread significantly more than controls (Fig. 2L–N); we observed the same pattern with fibrinogen in the presence of TNF-α (data not shown).

In summary, our results indicated that uncoupling ARAP3 from PI3K perturbed neutrophil responses on β2 integrin ligation, giving results typical for increased outside-in signaling.

β2 integrin inside-out signaling is increased in Arap3PH*/PH* neutrophils

To address why integrin ligation caused these enhanced responses with Arap3PH*/PH* neutrophils, we compared the major neutrophil β2 integrins, LFA1 and Mac1, of knock-in and control
FIGURE 2. Integrin-dependent events are upregulated in Arap3PH*/PH* neutrophils. (A–F) Bone marrow–derived Arap3PH*/PH* (PH*) and matched wild-type control (WT) neutrophils were prepared, primed with TNF-α and GM-CSF, or mock primed (A, D), and (all) preincubated with luminol as described in Materials and Methods. Cells (5 × 10⁶) were plated into 96-well plates containing fMLF as a soluble stimulus (A, D) or that had been coated with fibrinogen (B, E) or polyRGD (C, F). Light emission was measured in a Berthold Microtiter Plus luminometer. Data were recorded in duplicate. Data (means ± range) from a representative experiment are shown in (A)–(C), and (D)–(F) represent accumulated light emissions (means ± SEM) from four separate, pooled experiments expressed as a percentage of the responses obtained with control neutrophils. (G–I) Neutrophils were allowed to adhere to heat-inactivated serum-blocked (hiFCS) or polyRGD-coated plastic. Lysates were prepared and immunoblotted with Abs specific for phospho-PKB (Ser 473) or phospho-p38 (T180, Y182) or β-COP as a loading control. A representative example is shown (G). Blots were quantitated using ImageJ software; integrated data obtained from five independent experiments are shown (H, I). (J and K) Gelatinase granule release was measured by zymography of supernatants of neutrophils that were allowed to adhere to hiFCS-blocked or fibrinogen-coated dishes. As a control, neutrophils were stimulated with IMLF in the presence of cytochalasin B (CB). A representative experiment is shown. For (J), the samples were run on two separate gels and are pasted next to one another for ease of viewing; this is indicated by a dotted line. Integrated, quantitated data obtained from four independent experiments are plotted (K). (L–N) Neutrophils were allowed to adhere to polyRGD-coated tissue culture dishes, washed, and fixed. Numbers of attached cells (phase dark) per field of view and the percentage of spread cells (phase light) were counted. Integrated data obtained from three separate experiments (L, M), and representative examples (N) are shown. All raw data were analyzed by t test (Mann–Whitney). *p < 0.05; **p < 0.01, ***p < 0.001.
neutrophils. Surface expression of Mac1 and LFA1 was not affected by the presence of the PH domain point mutation (Fig. 3A).

Integrins are regulated by inside-out signaling (affinity and avidity modulation) (19). Because we are not aware of any activation state–specific Abs for murine β2 integrins, we analyzed ligand binding affinity experimentally. We tested the ability of neutrophils to bind to ICAM1, a ligand for LFA1, in solution in the absence of any stimulation. We also microscopically analyzed integrin lateral mobility (clustering), a readout for integrin avidity, in unstimulated cells that were kept in suspension. In confocal images of unstimulated neutrophils, distribution of LFA1 and Mac1 resembled that of wild-type cells. LFA1 on wild-type cells.

We also microscopically analyzed integrin lateral mobility (clustering), a readout for integrin avidity, in unstimulated cells that were kept in suspension. In confocal images of unstimulated control cells, distribution of LFA1 and Mac1 resembled that of uniform beads on a string (as did the neutrophil surface marker GR1; data not shown). In contrast, Arap3PH*/PH* neutrophils had a distinctively more clustered (patchy) LFA1 and Mac1 distribution (Fig. 3C). This suggested that the avidity of β2 integrins in Arap3PH*/PH* neutrophils was increased compared with controls. In summary, whereas the surface expression of Mac1 and LFA1 was unaltered by the PH domain point mutation in ARAP3, these integrins were characterized by increased inside-out signaling in Arap3PH*/PH* neutrophils.

Immobilized immune complex–dependent events are upregulated in Arap3PH*/PH* neutrophils

Immune complex binding–induced cross-linking of FcγRs activates neutrophils, a function important for autoimmune responses. We assayed ROS production after plating neutrophils onto immobilized immune complexes and found this to be significantly increased in Arap3PH*/PH* cells (Fig. 4A, 4B). In line with this observation, we also observed significantly enhanced PKB and p38 phosphorylation, gelatinase granule release, and cell spreading with Arap3PH*/PH* neutrophils that had been plated onto immobilized immune complexes (Fig. 4C–J). Therefore, responses induced by immobilized immune complexes were perturbed in Arap3PH*/PH* neutrophils.

FcγR signaling is not directly affected in Arap3PH*/PH* neutrophils

To address why Arap3PH*/PH* neutrophils displayed these perturbed responses on being plated onto immobilized immune complexes, we analyzed FcγR signaling further. We did not detect significantly different surface expression of FcγRII/III or of FcγRIV between control and Arap3PH*/PH* neutrophils (Fig. 5A). Using specific blocking Abs in ROS assays with neutrophils plated onto immobilized immune complexes, we found blocking of FcγRII/III caused a significant reduction in both wild-type and Arap3PH*/PH* neutrophils, whereas blocking of FcγRIV alone had very little effect. Using both blocking Abs together virtually

FIGURE 3. β2 integrins on Arap3PH*/PH* neutrophils have higher affinity and avidity. (A) Wild-type control and Arap3PH*/PH* bone marrow cells were or were not preincubated with 20 ng/ml TNF-α at 37°C before being labeled with PE-conjugated anti-GR1, allophycocyanin-conjugated anti-Mac1, and FITC-conjugated anti-LFA1. For FACS analysis, GR1+ cells were gated, and Mac1 and LFA1 staining was measured. Results were analyzed with FlowJo V6.4.7 software. Cells from 15 knock-in and 11 control chimeras obtained from three separate reconstitutions were analyzed in three experiments. Representative traces are shown. Black lines, wild-type; gray lines, Arap3PH*/PH*; broken lines, no TNF-α; solid lines, with TNF-α. (B) Unstimulated, bone marrow–derived control (WT) and Arap3PH*/PH* (PH*) neutrophils were allowed to bind to ICAM1–Fc in solution in the absence of any stimulation. Cells were washed and fixed, and bound ICAM1 was stained with a fluorescently conjugated secondary Ab. Washed, stained cells were allowed to settle on electrostatically coated slides, and signal strength was measured by quantitative immunofluorescence. Mean fluorescence intensity obtained from 64 knock-in and 50 control cells in three independent experiments is plotted (mean ± SEM), and representative examples are shown. (C) Integrin clustering was analyzed in unstimulated control and Arap3PH*/PH* neutrophils in solution. Bone marrow–derived neutrophils were prepared and incubated at 37°C in the presence of anti-LFA1 or anti-Mac1, washed, fixed, stained with a fluorescently conjugated secondary Ab, and allowed to settle on electrostatically labeled slides. Distribution of fluorescence was assessed by confocal microscopy. Representative examples (stained for LFA1) are shown together with their corresponding heat maps (obtained by analysis with ImageJ). Pooled results stem from four independent experiments, in each of which 25 cells per genotype were analyzed and plotted (mean ± SEM). (B and C) Results obtained were analyzed by paired t tests. *p < 0.05, **p < 0.01.
FIGURE 4. Immune complex–induced events are up-regulated in Arap3PH*/PH* neutrophils. (A and B) Bone marrow–derived Arap3PH*/PH* (PH*) and matched wild-type control (WT) neutrophils were prepared and preincubated with luminol as described in Materials and Methods. Cells (5 × 10^6) were plated into 96-well plates that had been coated with an immune complex (BSA–anti-BSA). Light emission was measured in a Berthold Lumilux Plus luminometer. Data were recorded in duplicate. Data (means ± range) from a representative experiment are shown in (A), and (B) represents accumulated light emissions (means ± SEM) from four independent experiments expressed as a percentage of the responses obtained with wild-type control neutrophils. (C–E) Neutrophils were allowed to adhere to heat-inactivated BSA-blocked (BSA) or immune complex–coated (BSA–αBSA) plastic. Lysates were prepared and subjected to immunoblotting with Abs specific for phospho-PKB (Ser 473) or phospho-p38 (T180, Y182) or β-COP as a loading control. A representative example is shown (C). Blots were quantitated using ImageJ software; integrated data obtained from five independent experiments are shown (D and E). (F and G) Gelatinase granule release was measured by zymography of supernatants of neutrophils that were allowed to adhere to BSA-blocked or immune complex–coated dishes. A representative experiment is shown. For (F), the samples were not in this order on the original gel and have been pasted next to one another for ease of viewing; this is indicated by a dotted line. Integrated, quantified data obtained from four independent experiments are plotted (G). (H–J) Neutrophils were allowed to adhere to immune complex–coated tissue culture dishes, washed, and fixed. Numbers of attached cells (phase dark) per field of view and the percentage of spread cells (phase light) were counted. Integrated data obtained from three separate experiments are plotted (I and J), and representative examples are shown (H). All raw data were analyzed by t test (Mann–Whitney). *p < 0.05, **p < 0.01, ***p < 0.001.

abolished the ROS production in both control and Arap3PH*/PH* neutrophils (Fig. 5B), in agreement with published work (15), and confirmed that immune complex–induced ROS production was indeed FcyR dependent.

To test whether ARAP3 affected FcyR signaling directly, we stimulated cells in solution by Ab-mediated FcyR cross-linking and measured ROS production. We observed very similar ROS production by primed or unprimed Arap3PH*/PH* neutrophils compared with controls on cross-linking FcyRII/III (Fig. 5C; we were unable to obtain meaningful results on cross-linking FcyRIV in a similar manner). We also measured PKB and p38 phosphorylation on FcyRII/III cross-linking in solution. Again, we observed very similar activations in control and Arap3PH*/PH* neutrophils (Fig. 5D). This work suggested that the presence of the PH domain mutation in ARAP3 did not affect signaling through FcyR per se, but rather that we had observed an indirect effect when assaying Arap3PH*/PH* neutrophils that had been plated onto immobilized immune complexes.

Adhesion-dependent RhoA activation is increased in Arap3PH*/PH* neutrophils

ARAP3 is a functional GAP protein for RhoA and Arf6 that is activated by PI3K and Rap. To analyze the mechanism underlying our observations, we carried out activity assays. Because Rap is known to regulate integrins, we assayed Rap1-GTP in Arap3PH*/PH* neutrophils that were kept in suspension or plated onto polyRGD. Rap1-GTP was significantly increased on plating neutrophils of either genotype onto polyRGD, but we detected no significant differences between genotypes in suspension cells or those plated onto polyRGD (Fig. 6A). We next analyzed Arf6-GTP in neutrophils that were kept in suspension or plated onto polyRGD. Arf6 was strongly activated by plating control and Arap3PH*/PH* neutrophils onto polyRGD. No difference was observed between the two genotypes (Fig. 6B). RhoA was also significantly activated on plating control and Arap3PH*/PH* neutrophils onto polyRGD. Our measurements indicated that RhoA fold activation was significantly higher in Arap3PH*/PH* than in control neutrophils (Fig. 6C), suggesting that the incorporation of the PH* mutation into ARAP3 affected RhoA signaling in neutrophils. Because RhoA and Rac are known to cross talk, we also analyzed Rac. In agreement with previously published data from human neutrophils (20), neither Rac1 nor Rac2 were activated in neutrophils plated onto polyRGD. We did not observe any differences in Rac1 and Rac2 activities between wild-type control and Arap3PH*/PH* neutrophils (data not shown). To summarize, RhoA signaling was affected in Arap3PH*/PH* neutrophils plated onto integrin ligands, which is suggestive of an involvement of RhoA signaling in the adhesion-dependent Arap3PH*/PH* phenotype we observed.

We monitored phospho-myosin L chain (pMLC), a RhoA effector, as an indirect readout for the localization of RhoA activation in the neutrophils. In wild-type control cells, F-actin and pMLC staining were mutually exclusive. In contrast, pMLC localization was perturbed in Arap3PH*/PH* neutrophils that had or had not
Arap3<sup>PH*/PH*</sup> neutrophils have a chemotaxis defect in vitro

To test whether uncoupling ARAP3 from PI3K affects the ability of neutrophils to chemotax, we performed Dunn chamber chemotaxis assays. We assayed cells migrating toward fMLF, a commonly used chemoattractant in this context, which has been shown to support PI3K-mediated and p38 MAPK–mediated chemotaxis. We also analyzed cells migrating toward MIP2, the murine equivalent of human IL-8 that has been shown to promote chemotaxis independently of p38, in a PI3K-dependent fashion (21, 22). Tracking of cell trajectories (Fig. 7A) followed by computational analysis of the tracks showed that Arap3<sup>PH*/PH*</sup> neutrophils traveled longer total but shorter Euclidean distances exhibiting reduced directionality compared with controls (Fig. 7B, 7C) when migrating toward fMLF. When the cells moved toward MIP2 (Fig. 7D–F), the defect observed was more severe, with Arap3<sup>PH*/PH*</sup> neutrophils migrating shorter accumulated and Euclidean distances than their control counterparts while the directionality defect remained. These measurements confirmed that uncoupling ARAP3 from PI3K caused an in vitro neutrophil chemotaxis defect, the nature of which is dependent on the chemoattractant used and the signaling intermediates it relies on.

Arap3<sup>PH*/PH*</sup> cells have a recruitment defect in vivo

To analyze in vivo chemotaxis, we assessed neutrophil recruitment to the peritoneum of control and Arap3<sup>PH*/PH*</sup> bone marrow chimeras in which sterile peritonitis had been induced by thioglycollate. In this assay, significantly reduced numbers of Arap3<sup>PH*/PH*</sup> neutrophils were recruited to the peritoneum (Fig. 7G). In the lights of the elevated responses of Arap3<sup>PH*/PH*</sup> neutrophils we had observed on plating neutrophils onto immobilized immune complexes in vitro, we next tested the effect of uncoupling ARAP3 from PI3K in the context of a model of an autoimmune disease. For this we made use of the well-characterized K/BxN serum transfer model for the effector phase of rheumatoid arthritis (23, 24). In this model, arthritogenic K/BxN serum is transferred to healthy mice, resulting in neutrophil-dependent inflammation of their joints. Sera were injected into lethally irradiated recipient mice reconstituted with control bone marrows, and ankle thickness and clinical score were measured over 14 d (Fig. 7H). Unexpectedly, arthritogenic serum-injected Arap3<sup>PH*/PH*</sup> bone marrow chimeras were less affected than control chimeras. Hence, Arap3<sup>PH*/PH*</sup> neutrophils conferred mild protection from immunarthritosis. The inflammatory response in the K/BxN model depends on efficient neutrophil recruitment to the joints. We analyzed this in ankle sections from chimeras that had been injected with control or arthritogenic serum and observed reduced tissue infiltration in joints from Arap3<sup>PH*/PH*</sup> chimeras (Fig. 7I). This suggests the protection from arthritis in these animals was due to a leukocyte recruitment defect conferred by uncoupling ARAP3 from activation by PI3K.

Discussion

We recently showed that ARAP3 is an important regulator of neutrophils that is involved in the modulation of integrin inside-out signaling and regulation of responses downstream of integrin ligation. ARAP3 is regulated by the PI3K lipid product PtdIns(3,4,5)P<sub>3</sub> that has been stimulated with fMLF (Fig. 6D and not shown), suggestive of mislocalized RhoA-GTP in these cells.

FIGURE 5. FcyR are not affected in Arap3<sup>PH*/PH*</sup> neutrophils. (A) Surface distribution of FcyRII/III and FcyRIV was analyzed by FACS analysis of bone marrow cell populations. Seven wild-type and seven knock-in chimeras obtained from three different pairs of donors were analyzed in two independent experiments. A representative example is shown. Black lines represent control cells and gray lines represent Arap3<sup>PH*/PH*</sup>; FcyRII/III FACS: broken lines represent unstimulated and solid lines represent TNF-α-stimulated samples. FcyRIV FACS: dotted lines represent isotype control. (B) Immune complex–induced ROS formation depends on signaling through FcγRs. Neutrophils were or were not preincubating with FcγR blocking Abs and/or isotype controls as indicated before being plated onto immobilized immune complexes for ROS assays. Pooled results from three independent experiments are plotted (mean ± SEM). Differences between wild-type and Arap3<sup>PH*/PH*</sup> were highly significant (p < 0.001) for all conditions except anti-FcγRII/III plus anti-FcγRIV (p > 0.05); two-way ANOVA with Bonferroni posttest. (C) Cross-linking FcγRII/III does not cause increased ROS production in Arap3<sup>PH*/PH*</sup> neutrophils. Bone marrow–derived neutrophils were prepared from control and knock-in mice and incubated with anti FcγRII/III with or without priming. After washing, ROS production was measured on cross-linking the FcγR with an F(ab′)<sub>2</sub> fragment. A representative example is shown (left), and integrated results from five independent experiments are plotted (mean ± SEM). Differences did not reach statistical significance (p > 0.1; Mann–Whitney test). (D) Control and Arap3<sup>PH*/PH*</sup> neutrophils were incubated with FcγRII/III Ab, washed, and stimulated by F(ab′)<sub>2</sub> fragment–mediated cross-linking for the indicated amounts of time. Time 0, No cross-linking. Lysates were prepared and protein subjected to immunoblotting. PKB and p38 phosphorylation was analyzed using phosphospecific Abs; β-COP served as loading control. A representative experiment from four independent experiments is shown.
P3 and by Rap-GTP. To analyze the regulatory contribution of PI3K on ARAP3 in the neutrophil, we analyzed neutrophils in which ARAP3 harbored a PH domain point mutation that uncoupled it from activation by PI3K. The current work argues that ARAP3 is an important PI3K effector in neutrophils.

We demonstrated before that ARAP3 is regulated by PI3K catalytically and it is also recruited to the plasma membrane in a PI3K-dependent and PH domain–dependent fashion. Therefore, the regulatory role of PI3K upstream of ARAP3 could contain direct and/or indirect components. PI3K has been shown to regulate integrins in several cell types (25–27), and our current work argues that it does this in the neutrophil at least in part by signaling through ARAP3. However, lack of PtdIns(3,4,5)P3-mediated recruitment of the ARAP3 PH domain point mutant to the plasma membrane could also have impinged on ARAP3’s ability to be regulated by Rap, an established regulator of integrin inside-out signaling (28–30). We are currently devising an ARAP3 Ras binding domain point mutation knock-in to address this possibility.

We noted that PKB phosphorylation, a commonly used readout of PI3K activity, was increased in Arap3PH*/PH* neutrophils that had been plated onto polyRGD or immobilized immune complexes (Figs. 2, 4). In contrast, Rap-GTP was not affected by genotypes (Fig. 6), suggesting that Rap is not involved in a feedback loop involving ARAP3 and integrin signaling, whereas PI3K might be.
**FIGURE 7.** *Arap3PH*/PH* neutrophils have a chemotaxis defect. (A–F) In vitro chemotaxis assays. Bone marrow–derived *Arap3PH*/PH* and control neutrophils were allowed to chemotax toward 300 nM fMLF (A–C) or toward 10 nM MIP2 (D–F) in Dunn chambers. Movements were recorded by time-lapse imaging. (A and D) Pooled tracks of individual cells from experiments carried out with three separate cell preparations were plotted using the Ibidi chemotaxis tool plug-in in ImageJ. The source of chemoattractant is at the bottom center of the diagram. The tracks were analyzed using the Ibidi chemotaxis tool’s statistics features. Accumulated and Euclidean distances and directionality are plotted [mean ± SEM (B, C, E, and F)]. (G) Neutrophil recruitment to the peritoneum in a model for sterile peritonitis. Bone marrow chimeras (generated with four bone marrow donors per genotype) were i.p. injected with 0.25 ml thioglycollate-containing broth. Mice were sacrificed 4.5 h after injection, their peritonea were flushed, and Mac1high GR1+ neutrophils were counted. Pooled results obtained from two separate experiments are plotted. (B–G) Data were analyzed by *t* tests (Mann–Whitney). *p < 0.05, ***p < 0.001. (H and I) Serum transfer arthritis. Twelve wild-type and 13 *Arap3PH*/PH* bone marrow chimeras were injected with 150 ml arthritogenic serum, and six wild-type and six *Arap3PH*/PH* bone marrow chimeras were injected with 150 ml control serum in two separate experiments. Joints were scored daily for 2 wk. Ankle thickness and clinical score are plotted. Circles and dotted lines, wild-type bone marrow chimeras; triangles and solid lines, *Arap3PH*/PH* bone marrow chimeras. Blue symbols, control serum; red symbols, arthritogenic serum. The area under the graph was compared by *t* test (Mann–Whitney): ankle thickness, *p = 0.053; clinical score, *p = 0.023. (I) Wax sections of decalcified joints from chimeras reconstituted with control (WT) or *Arap3PH*/PH* (PH*) bone marrows induced as indicated were H&E stained to visualize leukocyte infiltration on day 4 after serum injection using a 4× objective. Representative examples from sections obtained with six arthritogenic and two control serum–injected mice in two independent experiments are shown.
We carried out activity assays to elucidate the mechanism downstream of ARAP3 in this complex phenotype. Plating neutrophils onto polyRGD caused activation of RhoA and Arf6, but only RhoA fold-activation was increased in the knock-in cells, suggesting a regulatory role for RhoA downstream of ARAP3. Indeed, RhoA has been shown in a variety of cell types to regulate integrins (26, 31–34). Although RhoA and Rac have been shown to have an antagonistic relationship in many experimental systems (35), cross talk between Rho and Rac does not appear to play a major role in this context. We noticed that the distribution of pMLC was disturbed in *Arap*<sup>3PlypH</sup> neutrophils, suggesting indirectly that their RhoA-GTP localization may be affected. This will be investigated further in future work. Potentially, perturbed distribution of RhoA-GTP could have contributed to the chemotaxis defect we observed.

In our in vitro assays, immune complex–induced responses were found to be increased in *Arap*<sup>3PlypH</sup> neutrophils in addition to integrin-dependent ones. Because activating cells by cross-linking their FcγRIII/III did not cause similarly increased responses, uncoupling ARAP3 from activation through PI3K does not directly affect signaling through FcγRIII/III. We cannot exclude a potential direct effect on the activating FcγRIV (7), which acts together with FcγRIII to regulate immobilized immune complex–dependent activation of neutrophils (15), as we were unable to test this receptor experimentally in similar cross-linking experiments. An alternative explanation for our data could be that cross talk between β2 integrins and FcγRs takes place when FcγR-dependent signaling is induced by immobilized immune complexes. The preactivated β2 integrins of *Arap*<sup>3PlypH</sup> neutrophils might be able to modulate FcγR-dependent signaling. Cross talk between integrin and FcγR signaling has previously been described both in vitro (36–38) and with in vivo models (39–41). Although the molecular mechanisms of such cross talk have yet to be elucidated, it has been clearly demonstrated that both classes of receptors share common downstream signaling adapters and further components of their signaling cascades (42).

We observed a chemotaxis defect with *Arap*<sup>3PlypH</sup> neutrophils in vitro, which was more pronounced when MIP2 rather than IMLF was used as a chemotactic agent, in line with the distinct dependency on PI3K by these two chemoattractants (21, 22). We also noted that preincubation with MIP2 rather than fMLF inhibited PI3K activity for such recruitment to occur in an efficient manner. Notably, our findings for such recruitment to occur in an efficient manner strongly support the idea that PI3K activity is required for the stimulation of Rac activity in response to chemotactic gradients.


