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*J Immunol* 2013; 190:381-391; Prepublished online 23 November 2012;
doi: 10.4049/jimmunol.1201330
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Phosphoinositide 3-OH Kinase Regulates Integrin-Dependent Processes in Neutrophils by Signaling through Its Effector ARAP3

Laure Gambardella,* Karen E. Anderson,* Zoltán Jakus,‡ Miklós Kovács,† Susann Voigt,*,1 Phillip T. Hawkins,* Len Stephens,* Attila Mócsai, † and Sonja Vermeren*,‡

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. The Journal of Immunology, 2013, 190: 381–391.

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 cytotoxic 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-triphosphate [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).
This double point mutation renders ARAP3 unable to interact with and be activated by PtdIns(3,4,5)P3 (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, Arap3PH*/PH* cells exhibited perturbed responses on adhesion-dependent stimulation and showed increased β2 integrin affinity and clustering. Arap3PH*/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 Arap3PH*/PH* neutrophils, whereas in vivo, Arap3PH*/PH* neutrophils were characterized by reduced recruitment to the peritoneum after induction of sterile peritonitis and by subtly reduced inflammation in an autoimmune-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-FcγRI/III (CD16/32, clone 93) was from eBioscience (Hatfield, U.K.); hamster anti-FcγRIV (blocking Ab; clone 9E9) that 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-β1-COP was a gift from Nick Ktistakis. Mouse anti-RhoA, anti-Rap1, and anti-Arf6 were from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit anti-Arf1, anti-Arf3, anti-Arf6, anti-Arp2, and anti-α-tubulin were obtained from Sigma (Dorset, U.K.). Mouse anti-BSA (clone BSA-33) was from Sigma (Dorset, U.K.); rat anti-FcγRI/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). Goat anti-ARAP3 was a kind gift from Falk Nimmerjahn (Erlangen University, Erlangen, Germany). Goat anti-rat F(ab')2 was a kind gift from Falk Nimmerjahn (Erlangen University, Erlangen, Germany). Goat anti-mouse HRP conjugate was obtained from Bio-Rad (Paisley, U.K.). Alexa Fluor–conjugated, highly cross-absorbed Alexa Fluor–conjugated anti-human Fc Ab, and cells were counted using “manual tracking,” and tracks were analyzed using the Chemotaxis Tool plug-ins (Ibid, Planegg, Germany) in ImageJ.

MAPK, ERK, and PKB activation assays

These assays were carried out with cells plated onto polyRGD, BSA–anti-BSA immune complex–coated, or hiFCS-blocked dishes essentially as described (14) or cells were lysed at indicated time points after FcγRI/III PH*/PH* mouse model

Generation of the Arap3PH* 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 Arap3PH*/PH* mice was very low, we generated cohorts of Arap3PH*/PH* and wild-type bone marrow chimeras. In vitro experiments were carried out with matched wild-type and Arap3PH*/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 bone marrow chimeras, ensuring only neutrophil-dependent defects were assessed. Animal work carried out at Babraham Institute was approved by United Kingdom Home Office Project licenses PPL08/175 and PPL80/2335. Experiments carried out at Semmelweis University were approved by the Semmelweis University Animal Experimentation Review Board.

Reconstitutions

Cohorts of C57BL/6 mice were lethally irradiated and subsequently reconstituted by tail vein injection with 4 × 10^6 bone marrow cells from Arap3PH*/PH* mice or from sex- and age-matched wild-type controls. Complete reconstitution of the hematopoietic system was confirmed by flow cytometry.

Neutrophil purification

Neutrophils were isolated from bone marrows of 12- to 14-wk-old sex- and age-matched C57BL/6 mice using discontinuous Percoll gradient (GE Healthcare Amersham, Uppsala, Sweden) as described (9, 10) using endotoxin-free reagents throughout. Chimeric mice were used 6–8 wk after reconstitution.

Analysis of peripheral blood

Tail vein blood was collected in EDTA-coated microcentres (Sarstedt, Nümbrecht, Germany) for analysis using a Vetabc animal blood cell counter.

Im mobilization of adhesive proteins and immune complexes

Sheep fibrinogen (150 µg/ml) or polyRGD (20 µg/ml) were adsorbed onto tissue culture grade plastics overnight at 4°C (fibrinogen) or for 3 h at room temperature (polyRGD). 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.

FcγR cross-linking

FcγR 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 polyRGD with or without murine TNF-α (20 ng/ml, R&D Systems) or to immobilized immune complexes. For soluble agonist assays with fMLF (10 µM final 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 (femtomist; Eppendorf, Cambridge, U.K.) using 50 mbar pressure as described (5) and monitored by time-lapse imaging for 30 min using an inverted Zeiss axiovert microscope (Welwyn Garden City, U.K.) and Axiovision software. Dunn chamber chemotaxis assays were carried out as described (10, 13) using iMLF or MP2 as chemotactic attractant. Cells were tracked using “manual tracking,” and tracks were analyzed using the Chemotaxis Tool plug-ins (Ibid, Planegg, Germany) in ImageJ.

MAPK, ERK, and PKB activation assays

These assays were carried out with cells plated onto polyRGD, BSA–anti-BSA immune complex–coated, or hiFCS-blocked dishes essentially as described (14) or cells were lysed at indicated time points after FcγRI/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 iMLF 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 cotained with anti-GR1 FITC and anti-integrin Cy5. GR1PH* cells were gated and analyzed for level of integrin expression. Staining by flow cytometry was analyzed by confocal microscopy of unstained, stained cells that were kept in solution at the time of assays and that were allowed to settle onto electrostatic slides (superfrost +; VWR, Lutterworth, U.K.) after fixation. To measure integrin binding affinity, unstained 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 Arap3PH* 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

RhoA-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 PI3K by inactivation 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). Peripheral blood cell counts from chimeric mice reconstituted with control and Arap3PH*/PH* bone marrow cells were very similar (Fig. 1B). 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). 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* knock-in cells as compared with the control neutrophils (Fig. 2G–J). 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

Table I. Litters from Arap3R302,3A intercrosses

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<td>—</td>
<td>80</td>
<td>10</td>
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FIGURE 1. Incorporating a PH domain point mutation does not affect ARAP3 expression. (A) Protein from 1 × 10⁶ bone marrow–derived neutrophils from control (Arap3+/+), heterozygous (Arap3+/PH*), or homozygous mutant (Arap3PH*/PH*) mice was immunoblotted for ARAP3 (top panel) or β-COP (bottom panel) as a loading control. Representative experiment shown from four independent experiments. (B) Neutrophils were purified by Ficoll-Paque and reconstituted with Arap3PH*/PH* or matched control (WT) hematopoietic stem cells. Data shown (means ± SEM) were obtained with 14 wild-type and 12 knock-in chimeras generated with three individual bone marrow donors per genotype.
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 Microluminat 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 fMLF 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 pRGD-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. 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 immuno-fluorescence. 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 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. As a 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 immuno-fluorescence. 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. (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 immuno-fluorescence. 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.

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 FcγRI alone had very little effect. Using both blocking Abs together virtually
either genotype onto polyRGD, but we detected no significant differences between genotypes in 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 that were kept in suspension or 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

**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 Luminopect 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 FcγR dependent.

To test whether ARAP3 affected FcγR signaling directly, we stimulated cells in solution by Ab-mediated FcγR 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 FcγRII/III (Fig. 5C; we were unable to obtain meaningful results on cross-linking FcγRIV in a similar manner). We also measured PKB and p38 phosphorylation on FcγRII/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 FcγR 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
been stimulated with fMLF (Fig. 6D and not shown), suggestive of mislocalized RhoA-GTP in these cells.

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 immunarthrosis. 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> by guest on July 26, 2017 http://www.jimmunol.org/ Downloaded from
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 6. RhoA activation is affected in Arap3PH*/PH* neutrophils. (A–C) Wild-type control (WT) and Arap3PH*/PH* (PH*) neutrophils were kept in suspension or plated onto polyRGD-coated tissue culture plastic. Cells were lysed with ice-cold lysis buffer. (A and B) Clarified lysates were subjected to “pull-down” assays using GST-Ral GDS as bait to determine GTP-loaded fractions of Rap (A) and using GST-MT2 bait to determine GTP-loaded Arf6 (B). Results obtained from a minimum of five independent experiments were pooled and plotted (mean ± SEM, left); representative examples are shown (right). Blots were probed with an Ab specific for Rap1 (A) and Arf6 (B). (C) Clarified lysates were used in RhoA G-LISA assays to determine GTP-loaded RhoA. Results from five pooled, independent experiments are presented (mean ± SEM). (A–C) Raw data were analyzed by paired t tests. *p < 0.05, ** p < 0.001. (D) Indirect assessment of localization of RhoA activation. Neutrophils were or were not stimulated for the indicated time with 1 μM fMLF in solution, allowed to settle on a glass coverslip for 3 min, fixed, and stained using phalloidin to visualize F-actin and anti-pMLC (60× objective and 4× zoom). Representative cells from three independent experiments are shown. L, Lysates; PD, pull-downs.
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 μl arthritogenic serum, and six wild-type and six Arap3PH*/PH* bone marrow chimeras were injected with 150 μl 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. Phating 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 Arap3PHPH-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 Arap3PHPH-neutrophils in addition to integrin-dependent ones. Because activating cells by cross-linking their FcγRIII did not cause similarly increased responses, uncoupling ARAP3 from activation through PI3K does not directly affect signaling through FcγRIII. We cannot exclude a potential direct effect on the activating FcγR IV (7), which acts together with FcγRII 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 Arap3PHPH-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 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 Arap3PHPH-neutrophils in vitro, which was more pronounced when MIP2 rather than IL-8 was used as a chemotactic agent, in line with the distinct dependency on PI3K by these two chemoattractants (21, 22). We also noted evidence for recruitment defects to the peritoneum and joints, respectively, on inducing sterile inflammation in these locations. Neutrophil recruitment to the periphery is known to depend on β2 integrins (43), and our data suggest that PI3K-dependent, ARAP3-mediated fine tuning of β2 integrin inside-out signaling is required for such recruitment to occur in an efficient manner. Notably, our result are in line with findings from several other laboratories who reported that constitutively activating β2 integrins (Mac1 or LFA1) either genetically by introducing activating mutations or pharmacologically by using specific small-molecule agonists results in reduced leukocyte extravasation, reduced neutrophil recruitment in sterile peritonitis, and reduced inflammation in a number of animal model systems (44, 45).

Acknowledgments
We thank Simon Walker for help with image analysis, Anne Segond-Pichon for help with statistical analysis, Falk Nimnjarham (University of Erlangen) and Nick Krisakis for Abs, Anthony Green (Cambridge Institute for Medical Research) for the use of a blood cell counter, and Su Kulkarni, Tamara Chessa, George Damoulakis, and Alison Condliffe (Cambridge University Medical School) for helpful discussions.

Disclosures
The authors have no financial conflicts of interest.


