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Aquaporin 9 phosphorylation mediates membrane localization and neutrophil polarization.

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Summary: Aquaporin 9 localizes to the leading edge and enhances polarization and migration in primary mouse neutrophils through a phosphorylation-dependent mechanism

Running title: AQP9 phosphorylation in polarizing neutrophils

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ABBREVIATIONS

AQP - Aquaporin
CCM - Calcium-containing medium
fMLF - formyl-Methionyl-Leucyl-Phenylalanine
GFP - Green fluorescent protein
KRG - Krebs Ringer Glucose buffer
Mock - Empty vector
PIPKI - phosphatidylinositol phosphate kinase type I
PI3K - Phosphoinositide 3-kinase
PKC - Protein kinase C
PLC - Phospholipase C
PMNL - Polymorphonuclear leukocytes
S11A - Serine11 substituted for an alanine
S11D - Serine11 substituted for an aspartate
WGA - Wheat germ agglutinin
Wt - Wild type
ABSTRACT

Neutrophils are of prime importance in the host innate defence against invading microorganisms by employing two primary mechanisms, locomotion towards and phagocytosis of the prey. Recent research points to pivotal roles for water channels known as aquaporins in cell motility. Here, we focused on the role of aquaporin 9 (AQP9) in chemoattractant-induced polarization and migration of primary mouse neutrophils and neutrophil-like HL60 cells. We found that AQP9 is phosphorylated downstream of the fMLF receptor or PMA stimulation in primary human neutrophils. The dynamics of AQP9 was assessed using GFP-tagged AQP9 constructs and other fluorescent markers through various live-cell imaging techniques. Expression of wild-type or the phosphomimic S11D AQP9 changed cell volume regulation as a response to hyperosmotic changes and enhanced neutrophil polarization and chemotaxis. Wt AQP9 and S11D AQP9 displayed a very dynamic distribution at the cell membrane, whereas the phosphorylation-deficient S11A AQP9 failed to localize to the plasma membrane. Furthermore, we found that Rac1 regulated the translocation of AQP9 to the plasma membrane. Our results show that AQP9 plays an active role in neutrophil volume regulation and migration. The display of AQP9 at the plasma membrane depends on AQP9 phosphorylation, which appeared to be regulated through a Rac1-dependent pathway.
INTRODUCTION

Polymorphonuclear leukocytes (PMNL), or neutrophils, provide an ensemble of defence mechanisms against potentially harmful microorganisms. They sense bacteria and move towards them by chemotactic, directional motility [1-3], then ingest and inactivate them via intraphagosomal release of proteases and reactive oxygen species [4-6]. During these events, the neutrophils change shape and adhesive behaviour rapidly and variably, depending on environmental signals provided by the tissue and site of action. Shape change, e.g. polarization, is evident using in vitro chemotaxis assays that are analyzed by live-cell microscopy. In vivo the neutrophils also have to squeeze through the endothelium and extracellular matrices, which requires them to profoundly modify their shape, volume and adhesiveness. Filamentous actin (F-actin) plays a crucial role in this process by responding to and promoting the molecular signals involved in the structure-function dynamics of the neutrophil cytoskeleton.

In principle, the membrane lipid bilayer is impermeable to water, ions and other polar molecules; it constitutes a diffusion barrier which has to be overcome by an array of various transmembrane transporters. Here, water-selective AQPs fine-tune volume and internal pressure, and thereby help determine the shape of cells. The roles of AQPs in renal, lung and plant cells are well established [7-9]. Although major structural diversity is present between AQPs, they all share the capacity to transport water. Their function is also influenced by other factors such as pH, Ca$^{2+}$, phosphorylations and truncations affecting their localization and water permeability [10]. The expression of the aquaglyceroporin AQP9, is known to increase water transport over the plasma membrane 7-fold [11]. We have previously provided evidence that human AQP9 is a key component in promoting directional motility in neutrophils [12]. Indeed, several AQPs have been shown to have
major impact on cell motility [13], and to facilitate shape changes as a result of increased hydrostatic pressure and more rapid diffusion of actin monomers due to localized influx of water [13, 14]. Their role in filopodia formation and endocytosis has, however, only recently emerged, even though the modulation of neutrophil migration by osmolarity changes has been known for decades [12, 15-17]. To further address how AQP9 affects the migratory behaviour and morphology of cells, that do not express AQP9 endogenously, wt and mutant human AQP9 fused with GFP were expressed in cells void of this aquaglyceroporin [18]. Thus, in both fibroblasts and epithelial cells, this yielded a markedly filopodial phenotype, with abundant, slender membrane protrusions all around the cells, including the dorsal surface. This depended on Cdc42 and atypical PKC interactions with AQP9 [18]. Yet, the temporal-spatial expression of AQP9 and the effect of its phosphorylation in the context of neutrophil directional migration had yet to be elucidated. We describe here experiments that address, in real time, the localization and possible functions of AQP9 during neutrophil polarization.

Based on extensive experimental evidence [19-22] several comprehensive models have been articulated, describing how distinct molecular events in the cytosol allow for the protrusion and retraction of the plasma membrane-cytoskeleton complex. Some membrane protrusions are broad and flat (lamellipodia), while others are long and thin (filopodia) [23]. Variation in shape is apparently regulated by differences among the small GTPases or G-proteins [24-27]. Cdc42 appears to play a critical role in the formation of filopodia by binding and activating the WASP proteins. In turn, the latter activate the actin-nucleating complex Arp2/3 [28, 29], IRSp53 and the Ena/ VASP family protein Mena, which localizes near the filopodial tip to protect the elongating filaments from capping [30, 31]. Another small GTPase, Rif [32], has been shown to promote filopodial protrusions from the barbed end of actin filaments, yielding
unbranched, rather parallel, linear structures [33]. Interplay between several small GTPases and AQPs has also been reported [34-36].

The small GTPases belonging to the Rac subfamily influence fundamental neutrophil functions, including directional orientation (Rac1-specific), actin polymerization and reactive oxygen species formation (predominantly Rac2) [2, 37-39]. Moreover, it was recently shown that Rac1 regulates actin uncapping and that Rac2 is responsible for ~70% of actin assembly by promoting cofillin activation and Arp2/3 de novo nucleation [39, 40]. Using a new transfection procedure and real-time imaging of primary neutrophils, Magalhaes et al [41, 42] showed that Rac1 and Rac2 are selectively recruited to the plasma membrane and phagosome membrane, respectively, during cell locomotion and phagocytosis.

The aim of the present investigation was to further delineate the role and the mechanisms that regulate AQP9 activity in neutrophil polarization and motility. With the novel transfection technique of mouse neutrophils and live-cell, confocal imaging [41, 42], we found that the expression of AQP9 increased neutrophil polarization in response to fMLF. Furthermore, employing two mutants, S11D and S11A AQP9, mimicking a constitutively phosphorylated and an unphosphorylated AQP9, respectively, showed that this effect was dependent on phosphorylation of AQP9 on serine11. Increasing levels of endogenous AQP9 phosphorylation upon stimulation with fMLF and PMA were also confirmed in primary human neutrophils. As a human correlate to mouse neutrophils we also used stably transfected HL60 cells differentiated into neutrophil-like cells, where wt AQP9 increased migration and CD11b expression. We conclude that the phosphorylation of AQP9 is essential for its proper plasma membrane localization, which is accomplished through a Rac1-dependent pathway.
MATERIALS AND METHODS

Mouse neutrophil isolation

All procedures described were performed in accordance with the Guide for the Humane Use and Care of Laboratory Animals and were approved by the University of Toronto Animal Care Committee. Rac1-conditional null mice were generated according to a protocol described in Glogauer et al. (2003) [43]. This approach generated Rac1 deletion in neutrophils at birth. Rac1 conditionals were cross-bred with Rac2 null mice [38] and the resulting offspring enabled the generation of mice with neutrophils deficient in either Rac1, Rac2, or both. Neutrophil isolation was performed as described in Sun et al (2007) [40]. More than 85% of cells isolated were neutrophils as assessed by Wright-Giemsa staining. Viability as determined by trypan blue exclusion was >90%.

Human neutrophil isolation

Primary human neutrophils were isolated from blood obtained from healthy blood donors using a slightly modified protocol from [44]. In brief, heparinized whole blood was separated on Polymorphprep™ (Fresenius Kabi, Oslo, Norway) by centrifugation at 480g for 30 min at room temperature (RT). The band containing the granulocytes was transferred to a sterile test tube and washed in PBS. Erythrocytes were then lysed by hypotonic treatment for 30 sec with H₂O. The granulocytes were washed once more and the pellet was subsequently dissolved in KRG. The cells were kept on ice until used to avoid activation of the neutrophils.

Preparation of AQP9 plasmids

AQP9 expression plasmids were prepared according to Loitto et al [18] and included GFP-tagged wt, S11A and S11D AQP9 mutants. The ds-Red wt AQP9 construct was also used in the colocalization studies. The S11A carries an alanine substitution that prevents its
phosphorylation by atypical PKC whereas the S11D construct mimics a constant phosphorylation state. As membrane probes, fluorescent Cholera Toxin B- Alexa 647 (Molecular Probes-Invitrogen, ON, Canada), rhodaminated Wheat Germ Agglutinin (E-Y lab Inc, San Mateo, CA, USA) and RFP-Hras was used. Rac1WT-GFP and Rac1Q61L-GFP (Rac1CA) constructs have been described previously [45]. The EK-GFP probe has been described in [46].

Transfections

For imaging experiments, primary bone marrow mouse neutrophils were isolated and cotransfected with vectors expressing green and red fluorescent constructs of AQP9 [42]. In brief, mouse neutrophils were suspended in 100 μl of Nucleofector Solution V (Amaxa Biosystems, now Lonza, Allendale, NJ, USA) and supplemented with 4-6 μg of vector DNA or GFP control vector (pmaxGFP DNA construct, Amaxa Biosystems). The cells were transfected using the program Y-001 (Amaxa Biosystems) and carefully recovered with 1 ml of 37 °C Iscove’s Modified Dulbecco’s medium (Gibco BRL/Invitrogen, Carlsbad, CA, USA) and transferred to 1.5 ml of Iscove’s Modified Dulbecco’s medium with 10% FBS, followed by a 2-h recovery time in non-tissue culture 12-well plates in a humidified 37°C, 5% CO₂ incubator. The cells were further analysed by fluorescence microscopy and EPICS ALTRA flow cytometer (Beckman Coulter, Mississauga, Canada) to monitor the transfection efficiency. Further details are described in the following reports [40, 42, 47-49]. For HL60 cells, a retroviral transfection with the plasmid pRetroQAcGFP1C1 (Clontech Laboratories Inc., Mountain View, CA) as backbone was used to generate stable AQP9 transfections. Retroviral particles containing GFP-tagged wt AQP9, S11A AQP9, S11D AQP9 and only GFP (Mock) were generated according to the manufactures protocol (Clontech).
**Immunofluorescence**

For immunostaining, cells were fixed in 4% paraformaldehyde (PFA; Sigma-Aldrich, St Louis, MO, USA) in CCM (KRG with 1 mM Ca²⁺) for 15 min. To label the membrane, cells were incubated with rhodamine-conjugated WGA (1:500, E-Y lab Inc.) for 10 min at RT and subsequently washed three times in PBS. Cells were then permeabilized in 0.1% Triton X-100 (Sigma) for 5 min at RT and subsequently washed in PBS. The cells were incubated for 60 min at RT in a blocking solution composed of PBS with 1% BSA, 3% goat serum (Chemicon, St. Charles, MI, USA) and washed three times in PBS. The primary antibody directed against AQP9 (ab85910, Abcam, Cambridge, UK; 1:500) was added in blocking solution and incubated overnight at 4 °C. Cells were washed three times in PBS and incubated with secondary antibody, Alexa Fluor 568-conjugated IgG goat anti-rabbit (2 µg/ml; Molecular Probes-Invitrogen) in the blocking solution for 60 min at RT. The antibodies used for labeling of CD11b were primary antibody mouse anti-CD11b (1:250 MERCK, Nottingham, England) and secondary antibody Alexa Fluor 568-conjugated goat anti-mouse (2 µg/ml; Molecular Probes-Invitrogen). The nuclei were stained with DAPI (5 µM; Molecular Probes-Invitrogen) in permeabilized cells for 30 min at RT and subsequently washed three times in PBS. For measurements of CD11b, a threshold was set for individual cells, captured with the same exposure time, and the fluorescence intensity was measured. The microscope used for imaging of HL60 cells and primary human neutrophils was a Zeiss Axiovert 200 equipped with a spinning disc, structured illumination aperture correlation unit (Vivatome, Zeiss, Jena, Germany) and a 100x (NA 1.4; Zeiss) and 63x (NA 1.25; Zeiss) objective. For live cell imaging the objectives were preheated to 37 °C with a objective heater (Pecon, Erbach-Bach, Germany).
**Cell culture**

HL60 cells (ATCC, Teddington, UK) were grown at 37 °C in humidified environment with 5% CO₂ in Roswell Park Memorial Institute (RPMI, Gibco BRL-Invitrogen, Carlsbad, CA, USA) 1640 medium supplemented with 10% FBS, 100 µg/ml streptomycin, 100 units/ml penicillin, and 2 mmol/l L-glutamine (all obtained from GIBCO BRL-Invitrogen). The medium was changed every 2-3 days, and the cells were split at around 1:5. HL60 cells infected with retrovirus were cultured in the same medium with addition of 1-2 µg/ml puromycin (Sigma). They were stimulated to differentiate into neutrophil-like cells by adding 1.3% dimethyl sulfoxide (DMSO; Sigma) to the medium for 5-6 days [50, 51].

**Quantification of directed cell migration**

The lower wells of a Transwell dish (Corning, NY, USA) were filled with 600 µl of CCM, containing 10 nM fMLF (Sigma) or an identical control solution without chemoattractant. The dish was pre-heated to 37 °C. Neutrophil-like HL60 cells were pelleted, resuspended in KRG, counted in a Bürker chamber (Sigma), and diluted to a concentration of 10⁶ cells/ml in CCM. Then 200 µl was added to the 6.5 mm Transwell insert, containing a tissue culture-treated polycarbonate membrane with 3.0-µm pore size. The cells were allowed to migrate through the membrane for 3 h at 37 °C in 5% CO₂ and the insert was subsequently removed. For each well, 20 random images were captured with a ProgRes C10 Plus camera (Jenoptik, Jena, Germany) at 10X magnification (NA 0.3; air; Olympus, Tokyo, Japan), and the number of cells visible in each image was counted.

**Quantification of morphologically polarized cells**

Cells were treated as for bright-field and epi-fluorescence imaging. Ten random bright-field images were captured prior to, and 5, 10, 15, and 20 min after stimulation with 10 nM of...
fMLF using a 40X objective (NA 1.3) on a Zeiss Axiovert 200 with a 37 °C preheated objective (Pecon). The images were obtained with a cooled CCD Axiocam MRm camera (Zeiss) at each time point, and the number of non-polarized and polarized cells in each image was counted.

**Real-time imaging of protein expression and localization of fluorescent probes during chemotaxis**

Before microscopy, the cells were pelleted for 30 s at 12000g (Eppendorf 5415, Missisagua, Canada), remixed by pipetting in 500 µl PBS with Ca²⁺ and Mg²⁺, and placed on a 0.05% BSA-coated 20 mm round coverslip, mounted in an Attofluor chamber (Invitrogen). The neutrophils were then exposed to uniform stimulation with 100 nM fMLF added directly to the chamber. Digital images were recorded using a Leica DMIRE2 (Leica Microsystems, Wetzlar, Germany) inverted fluorescence microscope equipped with high numerical aperture objectives (1.4 NA, x63 or x100 with a 1.5X magnification ocular lens - Spectral Applied Research, Richmond Hill, Canada), and a Hamamatsu Back-Thinned EM-CCD camera and spinning disk (SD) confocal scanning head. Experiments were done on a heated stage (37 °C) and by time-lapse sampling (0.3 s Red-Green, 6 s between frames). Images were acquired and analysed using the Volocity 4.2 software (Improvision, Coventry, UK) and Image J for further analyses. TIRF imaging was done using a Zeiss Axiovert 200 inverted microscope with a TIRF slider coupled to 2 separate diode-pumped solid-state laser lines (488 nm, 561 nm, Spectral Applied Research) and a Hamamatsu Back-Thinned EM-CD camera (Hammamatsu Photonics, K-K, Japan). The mean fluorescence intensity of the areas of interest was measured and compared with the average cytoplasmic area. Where indicated, the HRas-RFP probe was used to highlight the plasma membrane. HRas-Tail-RFP inserts into the plasma membrane by three palmitate moieties and has been consistently used as a marker for the plasma membrane [52].
Immunoprecipitation

Isolated primary human neutrophils (1x10^7 cells) were seeded into 10 cm tissue-cultured treated petri-dishes in 10 ml CCM. The cells were either un-stimulated, stimulated with 10 nM fMLF or 100 nM phorbol-esters (PMA, Sigma) for 20 min at 37 °C in humidified environment with 5% CO₂. After this, the cells were washed in ice-cold PBS and lysed with radioimmunoprecipitation assay (RIPA) buffer (50 mM TrisCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40 (NP-40), 0.5% sodium deoxycholat, 1% sodium dodecyl sulfate (SDS)) (all obtained from Sigma) supplemented with 1.5x protease inhibitor cocktail (Proteoblock; Fermentas, Vilnius, Lithuania) and 5 µM EDTA (Fermentas). The lysates were scraped of the dishes, and transferred into a 1.5 ml Eppendorf tube and subsequently allowed to rotate for 30 min at 4 °C. Protein concentration was determined using BioRad DC protein assay kit (BioRad, Hercules, CA, USA). The samples were equilibrated to 0.66 mg/ml and monoclonal anti-phosphoserine-conjugated agarose beads were added to the samples (10 µl/sample, A0876, Sigma) that were allowed to rotate at 4 °C overnight. As a negative control, lysis buffer and the monoclonal anti-phosphoserine-conjugated agarose beads were used. The immunoprecipitates were then spun down at 6000g for 2 min at 4 °C. The supernatant was removed and the beads were resuspended in ice-cold RIPA buffer. This was repeated three times before the precipitates were released from the beads by incubation with 25 ul of 100 mM glycine pH 2.8 (Sigma) for 3 min. Then 5 µl of 4x LDS buffer (Fermentas) and 2.2 ul of DTT (500 mM, Fermentas) were added and the samples were heated to 70 °C for 10 min before loaded on a 4-12% Bis-Tris gel (Nupage, Invitrogen). The gel was allowed to run for 60 min at 200 V and the samples were subsequently transferred to a PVDF-membrane (Millipore, Billerica, MA, USA) for 90 min at 30 V. The membrane was
blocked with 5% sterile filtered BSA in TBS-T (20 mM Trizma base, 150 mM NaCl, 0.1% Tween in destilled H$_2$O) for 60 min at RT. The primary polyclonal rabbit anti-human AQP9 antibody (1:1000, ab85910, Abcam) was added in blocking buffer and the membrane was incubated over night at 4 °C under gentle agitation. The day after, the membrane was washed 5x6 min in TBS-T and the secondary antibody HRP-conjugated goat anti-rabbit (1:2000, Dako, Glostrup, Denmark) was added and incubated for 60 min at RT. The membrane was once again washed 5x6 min in TBS-T and subsequently developed with Super signal West Pico Chemiluminescent substrate (Thermo Scientific, Waltham, MA) according to the manufacturer protocol. The same protocol was used to analyze AQP9 in whole cell lysates. After cell lysis, approximately 20 µg of the total protein concentration was loaded onto the gel.

Membrane/cytoplasm fractionation

Subcellular fractionation to obtain the membrane and the cytosol fraction was carried out with Proteojet™ membrane protein extraction kit (K0321, Fermentas) according to manufacturer’s description. In brief, $10^7$ cells were seeded onto a 10 cm tissue-cultured petri-dish in 10 ml CCM. The cells were allowed to adhere at 37 °C for 10 min and subsequently rinsed with 3 ml ice cold cell wash solution and further kept on ice from this point. The medium was carefully removed and 1.5 ml permeabilization buffer (with protease inhibitors and EGTA (Proteoblock, Fermentas)) was added to the cells, which were incubated at 4 °C under gentle agitation for 10 min. The supernatant was carefully transferred to a new tube and subjected to centrifugation at 16000g for 15 min at 4 °C. The resulting supernatant containing the cytoplasmic fraction was aliquoted and stored at -70 °C. The previously permeabilized cells were covered with 1 ml ice cold membrane extraction buffer and subsequently incubated 15 min at 4 °C under agitation. The
supernatant was then transferred to Eppendorf tubes and centrifuged at 16000g for 15 min at 4 °C. 440 µl of the membrane fraction was added to a Microcon10 spin column (Millipore) and centrifuged at 14000g for 50 min at 4 °C. The spin column filter was flipped and the concentrated sample was spun down in a new Eppendorf tube, yielding approx 50 µl/spin column. The membrane protein fraction was also aliquoted and stored at -70 °C. Samples were thawed on ice, and the protein concentration determined with the BioRad DC protein assay kit (BioRad) were equilibrated to 1 mg/ml and subjected to gel-electrophoresis and Western blot using the same protocol as in the immunoprecipitation experiments.

Western blot of differentiated HL60 cells

Differentiated HL60 cells were seeded into a 10 cm fibronectin-coated petri-dish and stimulated with 10^{-8} M fMLF for 30 min. The cells were then place on ice, washed once in ice-cold PBS, and treated with 500 µl of lysis buffer (50 mM Tris, 150 mM NaCl, 1% Triton X-100, 0.5 mM MgCl₂, all obtained from Sigma) with protease inhibitors (Complete protease inhibitor, Roche, Basel, Switzerland). The cell lysates were scraped off the dishes and transferred into 1.5 ml Eppendorf tubes that were allowed to rotate for 30 min at 4 °C whereafter undissolved debris was spun down at 16000g for 4 min at 4 °C. The protein concentrations were equilibrated using the BioRad DC protein assay kit (BioRad) and subjected to gel-electrophoresis and Western blot, using the same protocol as in the immunoprecipitation experiments. The primary antibody for these experiments was polyclonal goat anti-human AQP9 (1:200, Sc-14989, Santa Cruz Biotechnology, Santa Cruz, CA, USA)
Supplemental Fig. 1. (A) Apical localization of wt AQP9, S11A AQP9 and S11D AQP9, in murine neutrophils. (B) Transfection efficiency of the wt, and AQP9 mutants. This was calculated by counting the relative number of GFP positive cells in multiple fields. No significant difference was found among the constructs. (C) Imaging of a primary mouse neutrophils transfected with S11D AQP9 in a basal and an apical z-plane revealed a filopodial phenotype localized to the basal part of the cell. (D) Nuclear membrane localization of AQP9 in human PMN. Human PMN were allowed to adhere and stained for AQP9 (green) and DAPI (blue). A clear perinuclear accumulation of AQP9 was observed. The presence of AQP9 in these regions was further confirmed by subcellular fractionation.

Supplemental Fig. 2. Effects of AQP9 on neutrophil volume regulation in response to osmotic changes

We analyzed the relative cell volume response of HL60 cells by using solutions with increased osmolarity through to the addition of Duphalac®. First, all cells reacted by decreasing their cell volume; the most dramatic change was observed at 325 mOsm. When the cells were exposed to (A) 313 mOsm the relative volume decrease after 10s was 12% for wt and S11D transfectants, and 4 and 7% in S11A- and Mock-transfected cells, respectively, suggesting that osmolarity-dependent volume changes are affected by the phosphorylation status of serine11. Second, the rate of the response differed between the different cell types where the most dramatic effect was seen in cells over-expressing the S11A construct when subjected to (B) the 325mOsm solution.
Supplemental Movie 1. Time-lapse movie of a mouse neutrophil expressing S11D AQP9. Note the presence of a clear filopodial phenotype containing AQP9. Images were acquired with a spinning disk confocal microscope (see Material and Methods section). 1 frame = 6 seconds. 7fps

Supplemental Movie 2. Time series showing the localization of wt AQP9 (green) to membrane protrusions after fMLF stimulation. The HRAS tail-RFP construct (red) was used as a membrane marker. 1 frame = 6.5 seconds. 7 fps

Supplemental Movie 3. Time series showing the localization of S11A AQP9 after fMLF stimulation. Note that S11A AQP9 (green) does not accumulate at membrane ruffles/protrusions after fMLF stimulation. The Alexa-CTB-647 marker (blue) was used as a membrane marker. 1 frame = 6.5 seconds. 7 fps

EXTENDED METHODS

Gradient centrifugation fractionation

Isolated human neutrophils (6.4x10^7) were suspended in 10 ml 0.25 M sucrose with 1 mM EDTA and a mixture of protease inhibitors, disintegrated (> 90%) using a chilled (4C) LOX-press [53], followed by 5 strokes in loose-fitting Teflon-glass homogenizer. The homogenate was then directly subjected to centrifugation at 400g for 10 min to retrieve the nuclear fraction using a Beckman Coulter, Avanti J-26 XPI centrifuge and a JA 25.50 fixed angle rotor (Beckman Coulter, Fullerton, CA, USA). The samples were subjected to gel-electrophoresis and Western blot, using the same protocol as in the immunoprecipitation experiments.

Cell volume analysis
Cells were pelleted and resuspended in KRG. During the experiment the temperature of all solutions was kept constantly at 37 °C. After resuspension of the pelleted cells in PBS, the relative volume was measured with a CoulterCounter (Coulter Electronics, Luton, England). The experiment was repeated with the addition of Duphalac® (Lactulose; Ferrosan, Sweden) to change the conditions from isoosmotic (300 mOsm) to hyperosmotic osmolarity of either 313 or 325 mOsm respectively. We used the median volume of the population at each time point as a measure of the relative volume of the cells during a 30 sec-period after the addition of Duphalac®. The experiments were repeated 3 times with each osmolarity and transfectant, and the relative volume-change curve plotted versus time.
RESULTS

AQP9 localization in primary neutrophils and neutrophil like HL60 cells

Here, we used three GFP-tagged AQP9 constructs, wt AQP9, S11A and S11D AQP9 mimicking non-phosphorylated and constantly phosphorylated variants, respectively. These mainly localized to the plasma membrane but were also observed in the cytoplasm similar to the distribution of endogenous AQP9 (Figs. 1A and 1D). The localization of AQP9 in these regions was further confirmed by detection of the protein in both the membrane and cytoplasmic fractions of primary neutrophils (Fig 1B and 1C). AQP9 was also detected at the nuclear membrane in cells with different expression levels and in immunostained human PMN (Supplemental Figs. 1 A and 1D). Furthermore, expression of AQP9 induced a filopodial phenotype in the basal part of the cell in both mouse neutrophils and HL60 cells (Figs. 1D, Supplemental Fig. 1C and Supplemental Movie 1). The general transfection efficiency of the mouse neutrophils was ~ 50-60% and did not vary among the constructs tested (Supplemental Fig. 1B). Transfection of HL60 cells resulted in a filopodial phenotype and an AQP9 distribution resembling that of the transfected mouse neutrophils and human PMNs stained for AQP9 (Fig. 1D).

Endogenous expression of AQP9 in HL60 cells was detected as shown in Fig. 1E.

Endogenous phosphorylation of AQP9 increases upon stimulation of migration in primary human neutrophils

We hypothesized that AQP phosphorylation and membrane translocation is a critical step in neutrophil activation. To test that, we have analysed the expression and phosphorylation status of AQP9 in neutrophils. We have detected AQP9 as two bands, at 29 kDa and weak band around 33 kDa (Fig. 2A, left panel). To investigate whether
AQP9 was endogenously phosphorylated, we performed immunoprecipitation against phosphorylated serine residues and immunoblotted for AQP9 in non-stimulated cells and cells stimulated with fMLF or PMA. Phosphorylated AQP9 was detected in resting cells at 33 kDa (Fig. 2A right panel). We show here that stimulation with either fMLF or PMA significantly increased the relative amount of phosphorylated AQP9 (1.7-fold and 2.2-fold respectively, Fig. 2B). The low molecular-weight band at 25 kDa most likely reflected the light chain of the precipitation antibody since it was also visualized in the negative control where lysate was not present.

Neutrophils transfected with S11A AQP9 fail to polarize upon fMLF stimulation

Neutrophils are highly motile cells and their long-range locomotion depends on both their adhesiveness to the substratum and the presence of chemoattractants, such as bacteria-derived formylated peptides [54-56], where fMLF represents the most commonly used experimental chemotactic agent. The polarization and motile phenotypes include the formation of filopodia, lamellipodia and elongated cells with a distinct leading edge and trailing uropod. We thus analyzed the polarization and motility of both primary mouse neutrophils and HL60 cells, using a previously described Zigmond chamber chemotaxis assay [57] and Transwell assays. Both mouse neutrophils and HL60 cells showed an increased polarization towards fMLF when wt AQP9 was expressed (Figs. 3A and 3C). Similarly, the expression of wt AQP9 significantly increased the chemotaxis of HL60 cells (Fig. 3B). In order to determine whether AQP9 phosphorylation is involved in this response, primary mouse neutrophils were transfected with the S11A or S11D constructs, and analysed for cell polarization. The expression of wt AQP9 increased cell polarization compared to GFP control (Fig. 3C). The expression of S11D AQP9 alone induced polarization of neutrophils in the absence of stimulus while S11A AQP9 expressing cells failed to polarize towards fMLF (Fig. 3C)
AQP9 induced a highly filopodial phenotype (Figs. 1D, Supplemental Fig. 1C and Supplemental Movie 1), that also yields a cell with an increased cell surface area. This enables more sites for insertion of adhesion molecules, such as integrins, that could contribute to the pronounced morphological phenotype. Hence, we measured the expression of β2-integrins (CD11b) in these cells by capturing the fluorescence intensity with the same exposure time of cells labeled for CD11b. We found a clear up-regulation of the β2-integrin expression (Fig. 3E); AQP9 wt transfected cells displayed a significantly higher fluorescence intensity (33060 ± 3120; mean ± SEM) compared with Mock-transfected cells (25150 ± 761, P<0.01, Student’s T-test).

**S11A AQP9 fails to translocate to the leading edge membrane of migrating neutrophils**

To further analyze the mechanisms by which the AQP9 phosphorylation may regulate neutrophil motility, GFP-wt AQP9, S11A AQP9 and S11D AQP9 were transfected into primary mouse neutrophils, followed by a micropipette-delivered chemoattractant assay. Consistent with previous results, all constructs localized at the nuclear membrane as well as the plasma membrane (Fig. 4A), but there was a clear difference in the relative accumulation at the plasma membrane, especially when the cells were stimulated with fMLF. Both wt AQP9 and S11D AQP9 accumulated at the leading edge membrane (Supplemental Movie 2), while S11A AQP9 failed to do so (Fig. 4B, Supplemental Movie 3). This finding suggests that serine11 phosphorylation is crucial for its translocation to the plasma membrane after receptor stimulation and thereby alters the local water-transporting ability. The relative fluorescence intensity of all the AQP9 constructs was similar at the nuclear membrane compared with the background cytoplasmic levels (Fig. 4C). In SD confocal images, the wt AQP9 fluorescence was punctate, but time-lapse image acquisition clearly showed that the structures were highly dynamic, seemingly forming, reforming, and/or moving along the
membrane in a linear fashion. They also moved intracellularly, seemingly in vesicles along cytoplasmic filaments (Supplemental Movie 2).

Rac1 regulates AQP9 recruitment to the plasma membrane in neutrophils

Since the Rac subfamily of small GTPases is known to serve crucial functions in neutrophil migration, we hypothesized that these proteins might regulate AQP9 localization. We thus, analysed the contribution of Rac1 and Rac2 activity in the context of AQP9 membrane targeting. We observed in mouse neutrophils co-transfected with DsRed-wt AQP9 and Rac1-Q61L-GFP a strong co-localization of the markers (Pearson Correlation 0.8264 +/- 0.03), (Fig. 5A). To elucidate the role of Rac1 in AQP9 translocation and function, neutrophils lacking either Rac1 or Rac2 were transfected with wt AQP9 and analyzed by TIRF microscopy under resting and fMLF- stimulated conditions. Rac1 null neutrophils still had a distinct nuclear membrane distribution but displayed much less AQP9 in the cell periphery, and at the very cell edge (Figs. 5B and 5D). The EK-GFP probe [46] and Hras-RFP were used as controls as they display a uniform plasma membrane localization. Despite the differences in overall morphology, Rac2 null neutrophils had a similar distribution of AQP9 as observed in control (wt) neutrophils (Figs. 5B and 5C). These results indicate that Rac1 is specifically involved in regulating the localization and/or activity of AQP9 and the targeting of this protein to the plasma membrane after receptor activation.
DISCUSSION

In this investigation, we analyzed the distribution and membrane recruitment of AQP9 in primary neutrophils undergoing dynamic shape changes during polarization and chemotaxis [9]. We achieved direct visualization of AQP9 dynamics in primary mouse neutrophils and the myelocytic cell line HL60 using transient and stable transfection techniques, respectively. We also describe that AQP9 is phosphorylated downstream of fMLF and PMA activation. In our experiments, the localization of endogenous AQP9 in primary human PMN was similar to that in transfected cells with over-expressed protein. Also, we were able to localize AQP9 to membrane and cytoplasmic fractions, supporting a notion that the localization is not an artifact of overexpression. Although highly expressed in primary human neutrophils, endogenous AQP9 in mouse neutrophils was not detectable (data not shown), making them a suitable expression system for this protein. The various forms of AQP9 used in this study were all shown to be functional in volume regulation, as the cells responded to hyperosmotic conditions (Supplemental Fig. 2). The decreased response seen in S11A AQP9-transfected cells, which was smaller and slower than for cells only expressing GFP was likely due to a dominant negative effect of the S11A mutant since HL60 cells are neutrophil-like cells with endogenous AQP9 expression (Fig. 1E). This is consistent with previous results from Loitto et al., [18] who showed that cells transfected with wt AQP9 but not S11A AQP9 were more susceptible to hypo-osmotic changes. Our results indicate that AQP9 localizes at both the nuclear membrane and leading edge of migrating neutrophils. The recruitment of AQP9 to the leading edge depended on the phosphorylation of residue S11 and was regulated downstream of Rac1. Notably, the expression of either wt AQP9 or the constitutively phosphorylated S11D AQP9 significantly enhanced the polarization and motility of neutrophils. However, in contrast to S11D AQP9-transfected cells, wt AQP9 expression
required stimulation with fMLF to mediate this effect (Fig. 3C). Phosphorylation of endogenous AQP9 was indeed shown to increase upon stimulation with fMLF, (Fig. 2), suggesting that activation of the motility machinery also includes phosphorylation of AQP9. Evidently, neutrophils have a fine-tuned water regulation mechanism that controls not only cell volume but also functions in polarization and chemotaxis.

It has previously been found that immunoprecipitated wt EGFP-AQP9 and S11A AQP9 incubated with active, recombinant PKCζ and γ[32P]ATP, leads to wt-PKCζ-mediated phosphorylation of wt AQP9 in vitro. In the experiments, substitution of Ser11 to Ala markedly inhibited phosphorylation [18]. Furthermore, the site-directed substitutions of serine had distinct effects on the filopodial phenotype in C3H10T1/2 fibroblasts. Except for the S11D-mutant, the morphology of these cells was strikingly different from cells expressing wt AQP9. Consistent with our data (Figs. 1A and 1D), the S11A mutation caused a smoother cell periphery with fewer AQP9-induced filopodia; the S11D mutation yielded a slightly more polarized phenotype but with seemingly shorter filopodial protrusions [18]. Here, we also showed that endogenous AQP9 phosphorylation was increased upon stimulation of diacylglycerol-dependent PKC suggesting that other PKCs than the atypical PKCζ likely also affect AQP9 phosphorylation status, either directly or indirectly. Recent studies have shown that aquaporins, such as AQP4 are localized to membrane microdomains in neurons and in glial cells adhering to a laminin-coated substratum, allowing for integrin-mediated cell activation [58-60]. These findings suggest that water channels could be key components of the signaling pathways in many cell types [61, 62]. Despite substantial information available about AQP9 functions in other tissues, its role in highly motile cells and how water channels regulate cell chemotaxis are presently unclear.
In our experiments, AQP9 expression increased the polarization response after fMLF stimulation, which highlights a close correlation between water dynamics and directional cell migration (Figs. 3A-C). The changes in shape and volume depend on the remodeling of the filamentous actin cytoskeleton (F-actin), which is crucially regulated by compartmentalized activity of the Rho family of GTPases. In neutrophils, Rac and Cdc42 are primarily found in the leading edge lamellipodium and RhoA in the tail/uropod [27, 63, 64].

In a broader perspective, an ensemble of molecules and cellular structures like phosphoinositides, and phosphoinositide kinases (PIPKI and PI3K), Rho family of small GTPases, microtubules, vesicle transport, membrane microdomains (lipid rafts), and integrin-mediated adhesion all contribute to the regulation of directional motility, polarization and phagocytosis in neutrophils [3, 21, 65, 66]. Our finding that Rac1, but not Rac2, is required for the localization of AQP9 in our mouse model, suggest a very specific regulatory mechanisms that may be invoked to explain the differential localization of Rac1 and Rac2. Magalhães and Glogauer [46] have previously shown differential intracellular targeting of Rac1 (inner leaflet if the plasma membrane) and Rac2 (internal membranes) in neutrophils based on membrane charges. This differential localization, recapitulated in the colocalization of AQP9 and Rac1, suggests the specific regulation of AQP9 localization and function by Rac1. Based on our data, only a fraction of AQP9 ~25% is affected by Rac1KO (Fig. 5C). There is a resident population of AQP9 at the cell membranes in the resting state. After stimulation, a population of phosphorylated AQP translocate to the membrane. We speculate that this population is recruited through a Rac1-dependent pathway.

We propose that AQP9 has an important role in regulating water influx and cell shape changes during neutrophil migration. Our results show that AQP9 localization at the leading edge membrane depends on its phosphorylation (Fig. 4B), possibly through a Rac1-dependent pathway. The finding that Rac1 is necessary for the membrane localization of
AQP9 highlights the importance of AQPs in membrane protrusion. However, the mechanism behind this interaction remains to be elucidated. In fibroblasts, AQP9 phosphorylation was recently shown to be mediated by atypical PKCζ [18], which is a kinase known to act downstream of Rac signaling [67, 68]. Activation of the G-coupled fMLF receptor might therefore result in AQP9 phosphorylation through a Rac1 and PKCζ-mediated pathway. Furthermore, the increased phosphorylation of AQP9 observed upon stimulation with PMA (Fig. 2) suggests involvement of classical or novel PKCs. In this context, we propose a model in conjunction with well-established chemoattractant-associated phospholipase C (PLC) and PI3K pathways (reviewed in [69]) that results in AQP9 phosphorylation and membrane targeting. Here, G-protein-coupled activation of PLCβ results in activation of classical and novel PKCs by generating calcium release and diacylglycerol (DAG) from PtdIns(4, 5)P₂. Activation of these PKCs is dependent on DAG and will results in a direct or indirect phosphorylation of AQP9. Simultaneously, PI3Kγ is activated, and generates PtdIns(3, 4, 5)P₃ that is known to stimulate Rho GTPases such as Rac1 and Cdc42 through activation of RhoGEFs. Rac1 and Cdc42 are then able to further stimulate PLCβ [70] and separately activate atypical PKCζ that phosphorylates AQP9 [18]. This phosphorylation will subsequently localize AQP9 to the leading edge membrane (Fig. 6B). An interplay between accumulated ion- and water channels at these sites may create a localized osmotic gradient that would drive the influx of water close to the site of the growing protrusion [71-74]. This would generate an increased localized hydrostatic pressure, located between the cortical actin filaments and the plasma membrane at the site of water influx, which could dissociate the membrane from the cytoskeleton in a similar fashion as seen in acto-myosin dependent membrane constriction [14, 75-77]. Such an effect would help to expose barbed ends of previously membrane-anchored actin filaments and thereby create space for de novo actin assembly, which could
help form the growing protrusion (Fig 6C). Accordingly, inward flux of water may promote a
gel-to-sol transformation of the cytoplasm, and perhaps 10-100-fold more rapid diffusion of
reactants regulating actin polymerization. On the contrary, at the trailing end of the cell,
outward pumping of water may create a de facto contraction of the cytoplasm and a forward
pressure on the nucleus and cytoplasm in the front of the cell. In the nuclear membrane,
opening of AQPs could be involved in promoting diffusion of transcription regulators to
allow for gene activation or silencing. Since the distribution of AQPs was uneven along the
nuclear membrane (Fig. 1A), it is tempting to speculate that they could be associated with
nuclear pores and their regulation.

In conclusion, we present a novel model for mechanisms involved in the
regulation of cell shape and migration of leukocytes. This concept complements the
established actin-mediated model and may represent a key feature of the activation of highly
motile cells that form leading edge lamellar protrusions rapidly in response to
chemoattractants. The next challenge is to clarify further, the specific mechanisms that
regulate the recruitment and activation of aquaporins in motile cells, especially the possible
direct role of small GTPases in the co-regulation of compartmentalized actin dynamics and
water influx.
AUTHORSHIP

T. K. performed experiments, analyzed data and contributed to writing the manuscript. M. G. collaborated and consulted. R. P. E. consulted, provided the facility and contributed to writing the manuscript, V. M. L. consulted, contributed with designing the research, experimental design and vector construction. K. E. M. and M. A. O. M are the Principal Investigators and designed the research performed experiments, analyzed data, and contributed to writing and finalizing the manuscript.
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DISCLOSURES

The authors declare no direct financial interests.
REFERENCES


FIGURE LEGENDS

Fig. 1. AQP9 expression and localization in neutrophils

(A) AQP9 localization in mouse neutrophils. Mouse neutrophils were transfected with the wt and AQP9 mutants and plated on a glass-bottom imaging chamber. Images were acquired with a spinning disk confocal microscope. They are representatives of the distribution of AQP9 in three independent experiments. Arrows indicate AQP9 accumulation in the plasma membrane. (B) Immunostaining of AQP9 in primary human neutrophils. The cells were fixed, and stained for AQP9 together with WGA-rhodamine as a membrane marker. AQP9 clearly localized primarily to the membrane but also in the cytoplasm, as indicated by the membrane/cytoplasm ratio. (C) Subcellular fractionation of the membrane and cytoplasm in primary human neutrophils, where AQP9 appears at 29kDa in both the membrane and the cytoplasmic fractions. The appearance of AQP9 as two bands has previously been shown to be dependent on glycosylation of the protein [53]. (D, upper panel) AQP9 induced a filopodial phenotype in HL60 cells. Representative epi-florescent images of HL60 cells overexpressing wt, S11D, S11A AQP9 or Mock-constructs. (D, lower panel) Representative images of a intensity profile plot of AQP9 (red line). Distinct membrane localization in cells transfected with S11D or wt AQP9 was observed in comparison to cells with S11A AQP9 or empty vector. (E) Representative western blot confirming the endogenous expression of AQP9 in non-transfected HL60 cells.

Fig. 2. AQP9 is endogenously phosphorylated in primary human neutrophils

(A) Representative western blot analysis of AQP9 expression and phosphorylation in human PMN. AQP9 was detected as two bands, one at 29 kDa and a weak band around 33 kDa. The phosphoserine immunoprecipitation revealed that the upper band (33 kDa) contained phospho AQP9. (B) Quantification of the relative amount of phospho
AQP9. The relative density of the bands was analysed and normalized to the loading control.

Fig. 3. Neutrophils expressing S11A AQP9 fail to maintain polarity

(A) HL60 cells were seeded onto glass-bottom culture dishes stimulated with the chemotactic peptide fMLF. Polarization was assessed as described in the material and methods section. Results are based on evaluation >70 cells per condition/timepoint. (B) Expression of wt AQP9 increased the chemotaxis of HL60 cells. The data are presented as mean number of cells/image in the 20 random images captured in each well for three independent experiments. Significant differences were found between wt AQP9 (18.3±2.3; mean ± SEM) and Mock-transfected cells (12.2±1.0; P<0.01). No significant differences were found when comparing wt AQP9 and Mock-transfected cells without chemotactic stimulation (Control; P>0.05, Student’s T-test). (C) Expression of wt and S11D AQP9 but not S11A AQP9 increased neutrophil polarization towards a gradient of fMLF in a Zigmont cell motility chamber. Images were analyzed using time-lapse DIC microscopy and were acquired every 20 s for 10 min. Only cells expressing the GFP constructs were considered for the analysis. An average of 250 cells was analyzed per group. (D) Representative merged DIC and confocal images of mouse neutrophils mounted on a Zigmont chamber polarizing towards the fMLF gradient. (E) Wt AQP9-transfected cells showed increased CD11b (β2-integrin) expression compared to Mock-transfected cells. The diagram illustrates the mean cell fluorescence of wt AQP9 and Mock-transfected cells of labeled β2–integrin (CD11b). The β2–integrin expression was significantly different in these two cases (Error bars equal SEM, P<0.01, Student’s T-test)

Fig. 4. S11A AQP9 fails to translocate to the leading edge membrane
(A) Neutrophils were transfected with the AQP9 constructs and subjected to chemotaxis experiments with micropipette-delivered fMLF. Images were acquired every 6 s for 5 min by spinning disk confocal microscopy. Results are based on the analysis of 12 cells per group. The Alexa CTB 647 was used as membrane marker. (B) Quantification of the relative accumulation of AQP9 at the leading edge membrane after fMLF stimulation (N=3, P<0.05 between wt-S11A and S11A-S11D). (C) Quantification of the relative accumulation of AQP9 at the nuclear membrane.

Fig. 5. Rac1 regulates the membrane translocation of AQP9

(A) Neutrophils were transfected with the DsRed-wt AQP9 and Rac1Q61L-GFP and plated on a glass coverslip. The image represents the analyses of over 30 cells, and it shows the colocalization of wt AQP9 and Rac1 at the plasma membrane. (B) Neutrophils were transfected with the AQP9 constructs and uniformly stimulated with fMLF. Images were acquired using TIRF microscopy. Results are based on the analysis of 15 cells per group. EK-GFP was used as a control since it uniformly associates with the plasma membrane of neutrophils. White arrows indicate the accumulation of AQP9 at the cell edges. (C) Quantification of the relative accumulation of wt AQP9 at the periphery of neutrophils. Rac1 KO neutrophils failed to translocate AQP9 to the cell periphery, whereas images of Rac2 KO and control neutrophils were alike (P<0.01 between WT and Rac1KO, P>0.05 between WT and Rac2KO). (D) A 3-dimensional image of a Rac1 KO neutrophil expressing Hras tail-RFP as a membrane marker and wt AQP9-GFP was constructed. AQP9 clearly failed to localize to the plasma membrane in these cells.

Fig. 6. Working model for AQP9 accumulation and function at the leading edge
(A) In the resting non-polarized neutrophil the cortical actin cytoskeleton is at a steady state where few growing filaments are seen at the membrane due to inhibition by capping proteins and sterical hindrance of the membrane. (B) Stimulation with fMLF activates the G-protein coupled fMLF-receptor that subsequently activates PI3Kγ and PLCβ. The latter converts PI(4, 5)P₂ into DAG and I(1, 4, 5)P₃ that results in intracellular calcium release and activation of classical and novel PKCs. These kinases will then directly or indirectly phosphorylate AQP9. The PI3Kγ will through generation of PI(3, 4, 5)P₃, activate RhoGEFs that catalyze the substitution of GDP to GTP in GTPases such as Rac1 and Cdc42. These will in turn (i) further stimulate PLCβ and (ii) activate atypical PKCζ that directly phosphorylates AQP9. (C) In the activated polarized neutrophil, AQP9 is phosphorylated and thereby localizes to the membrane where interplay between accumulated AQP9 and ion-transporters at the leading edge build up an osmotic gradient that enhances inward flux of water that can increase the localized pressure between the actin cytoskeleton and the membrane. The pressure will tear the actin cytoskeleton from the membrane and push the membrane outwards. Furthermore, the space created by the influx of water will lead to a low concentration of submembraneous actin monomers which will create a concentration gradient, directing forward flux of actin monomers to the site of the membrane protrusion. Together these events create space and availability for actin polymerization at the leading edge.
Figure 1

A Mouse neutrophils

GFP-Wt AQP9  GFP-S11A AQP9  GFP-S11D AQP9

B Human PMN

AQP9 immunostaining  WGA staining  Merge

C

<table>
<thead>
<tr>
<th>Membrane</th>
<th>Cytoplasm</th>
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<tbody>
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<td>30kDa AQP9</td>
<td>25kDa</td>
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D HL60

GFP-Wt AQP9  Mock  GFP-S11A AQP9  GFP-S11D AQP9

E

<table>
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<tbody>
<tr>
<td>30 kDa</td>
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<td>25 kDa</td>
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</table>
Figure 2

A

Whole cell lysate

Antigen

35kDa

30kDa

25kDa

Neg Ctrl

PMA

fMLF

Ctrl

Anti-AQP9

IP phospho-ser

phospho-AQP9

35kDa

30kDa

28kDa

25kDa

B

Relative density

Ctrl

fMLF

PMA
Figure 3

A. fMLF-induced HL60 polarization

B. fMLF-induced HL60 cell chemotaxis

C. fMLF-induced primary neutrophil polarization

D. AQP9 - S11A

D. AQP9 - S11D

E. CD11b presentation in HL60 cells
Figure 4

AQP9-WT

AQP9-S11A

AQP9-S11D

**AQP9 translocation to leading edge membrane**

**AQP9 translocation to nuclear membrane**

**Figure 4**

AQP9-WT

AQP9-S11A

AQP9-S11D

**ALEXA-CTB 647**
**Figure 5**

**A**

Rac1Q61L-GFP

AQP9-WT-DSRED

MERGED

**B**

WT EK-GFP

WT AQP9-WT

Rac1 null AQP9-WT

Rac2 null AQP9-WT

Scale bar: 5 μm

**C**

TIRF field

Nucleus

Cell periphery

**D**

Rac1 null AQP9-WT

Rac1 null Hras-RFP

Rac1 null MERGED

**Graph**

AQP9 accumulation at cell periphery

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>Rac1KO</th>
<th>Rac2KO</th>
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<td>Normalized fluorescence</td>
<td>125</td>
<td>100</td>
<td>*</td>
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Note: * indicates a significant difference.
Figure 6

A

B

C

[Diagram showing various biological processes involving molecules such as Actin, AQP9, fMLF receptor, H2O, GTP, PKCζ, fMLF, PI(3,4,5)P3, Rac1/Cdc42, PI3Kγ, Ca2+, DAG, PKC, PLCβ, I(1,4,5)P3, and RhoGEF.]