Complement C5a-Induced Changes in Neutrophil Morphology During Inflammation


Abstract

The complement and neutrophil defence systems, as major components of innate immunity, are activated during inflammation and infection. For neutrophil migration to the inflamed region, we hypothesized that the complement activation product C5a induces significant changes in cellular morphology before chemotaxis. Exposure of human neutrophils to C5a dose- and time-dependently resulted in a rapid C5a receptor-1 (C5aR1)-dependent shape change, indicated by enhanced flow cytometric forward-scatter area values. Similar changes were observed after incubation with zymosan-activated serum and in blood neutrophils during murine sepsis, but not in mice lacking the C5aR1. In human neutrophils, Amnis high-resolution digital imaging revealed a C5a-induced decrease in circularity and increase in the cellular length/width ratio. Biomechanically, microfluidic optical stretching experiments indicated significantly increased neutrophil deformability early after C5a stimulation. The C5a-induced shape changes were inhibited by pharmacological blockade of either the Cl-/HCO3-exchanger or the Cl- channel. Furthermore, actin polymerization assays revealed that C5a exposure resulted in a significant polarization of the neutrophils. The functional polarization process triggered by ATP-P2X7/purinoceptor interaction was also involved in the C5a-induced shape changes, because pretreatment with suramin blocked not only the shape changes but also the subsequent C5a-dependent chemotactic activity. In conclusion, the data suggest that the anaphylatoxin C5a regulates basic neutrophil cell processes by increasing the membrane elasticity and cell size as a consequence of actin-cytoskeleton polymerization and reorganization, transforming the neutrophil into a migratory cell able to invade the inflammatory site and subsequently clear pathogens and molecular debris.

Introduction

The complement system plays a key role in the recognition, marking and clearance of pathogen- and damage-associated molecular patterns (PAMPs and DAMPs, respectively) during inflammation and infection [1–3]. Upon complement activation, the generated anaphylatoxin C5a exerts manifold pro-inflammatory effects, including neutrophil recruitment, activation and an increase in intracellular pH in concert with extracellular acidification [4, 5]. Evidence suggests that to accomplish migration and tissue penetration for bacterial defence, neutrophils may undergo some volume and extreme morphological changes, for example, from a spherical to a flattened shape, which is accompanied by rapid ‘expansion’ of the plasma membrane surface [6]. More than two decades earlier, it was reported that exposure of neutrophils to chemoattractants, including N-formylmethionine-leucyl-phenylalanine (fMLP), interleukin 8 and C5a, induced calcium-independent actin polymerization and ruffling and polarization of the cells [7]. Mechanistically, the polymerization of actin filaments and the reorganization of the cytoskeletal network are major contributors to adjusting the mechanical properties of neutrophils during interstitial migration [8]. In this regard, C5a in its active and desarginated forms was shown to alter cellular morphology and surface adhesion in several cell types, including neutrophils [9]. Moreover, an autocrine purinergic signalling system, especially via the ATP-activated P2X7 receptor, is involved in shape polarization of these cells [10, 11].
To ensure the functionality of such cellular processes, a balanced intracellular-volume regulation appears to be of fundamental importance. In this regard, studies have shown that pathophysiologic conditions, including hypoxia and ischaemia [12, 13], and inflammatory processes [14] are associated with changes in neutrophil cellular volume. Furthermore, an actively regulated and directed influx of fluid via different ion channels and transporter systems is regarded as a prerequisite for neutrophil chemotactic activity, because cell migration is abolished in a hyperosmolar environment [15–18]. In this context, activation of the electro-neutral sodium-potassium chloride cotransporter (NKCC) induces a synchronous influx of Na\(^{+}\), K\(^{+}\) and Cl\(^{-}\) into the cell, leading to an osmotically triggered water influx. Additionally, the coupled activation of the ubiquitous sodium-proton exchanger 1 (NHE1) and the chloride-bicarbonate exchanger results in the net uptake of sodium chloride and fluid. Although the sodium bicarbonate cotransporter (NBC) is mainly known for its role in HCO\(_{3}^{-}\) reabsorption in the kidney, there is experimental evidence indicating that NBC activity is also involved in volume regulation [19] and was found to be expressed by neutrophils [20]. To date, several chemoattractants, hormones and growth factors have been shown to alter the activity of these transport systems. However, the respective activation mechanisms and the subsequent cellular effects are not fully understood.

Here, we provide evidence that interaction between the complement activation product C5a and its cellular receptor C5aR1 leads to increased deformability and a marked alteration of the neutrophil cell shape that is dependent on cellular influx of osmotically active chloride ions, actin polymerization and autocrine P2X/Y signalling. In a translational animal model, we found similar alterations in neutrophil morphology during experimental sepsis.

Materials and methods

Reagents. Unless otherwise indicated, all reagents were purchased from Sigma-Aldrich (Steinheim, Germany). For Amnis high-resolution digital imaging, neutrophils were isolated as described by Barnes et al. [21].

Preparation of zymosan-activated serum. To prepare zymosan-activated serum, 0.04 g zymosan was diluted in 1 ml distilled water. The solution was centrifuged at 300 \(\times\) g for 5 min at room temperature (RT), and the pellet was washed with DPBS. After a further centrifugation step, the supernatant was removed, and the zymosan pellet was resuspended in 20 ml DPBS. A total of 1 ml of the zymosan-DPBS stock solution was centrifuged (3500 \(\times\) g, 5 min, RT), and the supernatant was removed. For serum activation, the zymosan pellet was resuspended in 200 \(\mu\)l serum to a final concentration of 10 mg zymosan/ml serum and incubated at 37\(^\circ\)C for 30 min to activate the complement cascade.

Neutrophil stimulation. Isolated neutrophils (2 \(\times\) 10\(^6\) cells/ml) were incubated with human recombinant C5a (0.1–20 nm), C3a (1000 ng/ml) (Calbiochem, Darmstadt, Germany), zymosan-activated serum (5, 10, 20 \(\mu\)l) or non-stimulated serum for up to 30 min at 37\(^\circ\)C.

For inhibition of the C5aR1, neutrophils were pretreated with the selective small peptide C5aR1 antagonist (C5aR1A) AcF[OPdChaWR] [22, 23] (10 \(\mu\)g/ml) for 10 min at 37\(^\circ\)C before C5a stimulation. For the blockade of central volume-associated ion-transporter systems, neutrophils were pretreated with the respective inhibitor for 20 min at 37\(^\circ\)C before C5a incubation. Herein, (4-cyano-benzo[bl]thiophene-2-carbonyl)guanidine methanesulfonate (NHE1 inhibitor, 5–100 \(\mu\)M) was used as a Na\(^{+}\)/H\(^{+}\)-exchanger inhibitor, amiloride (200 \(\mu\)M) as a Na\(^{+}\)-channel inhibitor, bumetanide (100 \(\mu\)M) as an NKCC inhibitor, omeprazole (10 \(\mu\)M) as a K\(^{+}\)/H\(^{+}\)-ATPase inhibitor, ouabain (100 \(\mu\)M) as a Na\(^{+}\)/K\(^{+}\)-ATPase inhibitor, UK5099 (400 \(\mu\)M) as a Cl\(^{-}\)/HCO\(_{3}^{-}\)-exchanger inhibitor, S0859 (30 \(\mu\)M) as a Na\(^{+}\)/HCO\(_{3}^{-}\)-cotransporter inhibitor and 5-nitro-2-(3-phenylpropylamino)benzoic acid (NPPB, 100 \(\mu\)M) as a Cl\(^{-}\)-channel inhibitor. Furthermore, suramin (200 \(\mu\)M) was used to block P2X/Y-purinoceptors. For inhibition experiments, cells were incubated in HBSS medium ± inhibitors. Experiments using the Na\(^{+}\)/HCO\(_{3}^{-}\} sodium bicarbonate cotransporter inhibitor S0859 were performed in RPMI medium, containing 24 mM sodium bicarbonate.

Chemotaxis assay. Isolated neutrophils (5 \(\times\) 10\(^6\) cells/ml in DPBS) were labelled with 2',7'-bis-(2-carboxyethyl)-5-(and -6)-carboxyfluorescein, Molecular Probes, Eugene, OR, USA). Following a washing step with DPBS, cells were pre-incubated with suramin (200 \(\mu\)M) for 15 min at 37\(^\circ\)C. The neutrophils were loaded into the upper chambers of a 96-well chemotaxis mini-chamber (Neuroprobe, Cabin John, MD, USA). The lower chamber was loaded with C5a (10 nm) and separated from the upper chamber by a polycarbonate membrane of 3-\(\mu\)m porosity (Neuroprobe). The cells were incubated in the mini-chamber for 30 min at 37\(^\circ\)C. To determine the neutrophil

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chemotactic activity, the number of cells that had migrated through the polycarbonate filters to the lower surface was determined by cytofluorometry (Cytofluor II; PerSeptive Biosystems, Framingham, MA, USA). Samples were measured at least in quadruplicates.

**Cell sizing by flow cytometry (FACS Canto II).** For cell size analysis, human and murine neutrophils were analysed for forward-scattered (FSC) light with a FACS Canto II (BD Biosciences, Heidelberg, Germany) using a 488-nm laser. The extent of scattering is dependent not only on particle size, but also on cell shape and surface topology [24, 25]. Herein, the integrals (‘areas’) of the FSC pulses (FSC-A) generated by neutrophils were determined. To calculate neutrophil cell size, the FSC-A values generated by polystyrene beads of different diameters were used to prepare a standard curve (see Fig. 1A, B), allowing a direct association between FSC-A values and cell diameters. A minimum of 10,000 neutrophils were analysed per sample.

**High-resolution digital imaging.** The Amnis ImageStream system (Merck, Darmstadt, Germany) was used to obtain morphological information of cells by capturing high-resolution multispectral images. After stimulation of isolated neutrophils with C5a (20 nm) or control (DPBS) for 5–10 min at 37 °C, neutrophils were identified in the scatter plot, and data from 10,000 cells per sample were collected. Changes in cell shape were detected by predefined features using the IDEAS image analysis software (Merck). This included determination of the mean neutrophil circularity, which is the mean distance of the cell boundary from its centre divided by the variation of this distance. The shape ratio, the minimal thickness of the cell divided by the length of the cell, was also determined.

**Confocal microscopy.** For confocal microscopy analysis, isolated neutrophils (6 × 10⁶ cells/ml) were stimulated using C5a (20 nm) for up to 17 min at 37 °C. A 50-μl cell suspension per condition was allowed to settle on the glass bottom of the microwell dishes and was then examined (without a cover slip) using a Zeiss 7000 scanning confocal microscope (Zeiss, Oberkochen, Germany).

**Microfluidic optical stretching.** C5a was dissolved in water and added to the neutrophils 1 h before experiments. Mechanical properties of the neutrophils were determined by optical stretching as described previously [26–29]. Optical stretching allows the assessment of cellular deformability without mechanical contact, which may otherwise lead to cellular stimulation. Deformation (stretching) of cells suspended in a microfluidic channel is induced by forces generated by the momentum transfer from two counterpropagating laser beams to the cellular surface (see Fig. 5B inset).

**Actin-cytoskeleton polymerization assay.** Isolated human neutrophils were incubated for 10 min at 37 °C in medium (RPMI with 5% foetal calf serum) with C5a after pre-incubation for 15 min with either C5aR1A or the Cl−channel blocker and intracellular ATP blocker NPPB. For control, cells were left untreated or treated with C5aRA or NPPB alone. After incubation, cells were fixed and then F-actin was stained using phalloidin-fluorescein isothiocyanate (FITC) (green). Images of the cells were recorded using an iMic digital microscope (Till Photonics, Gräfelfing, Germany).

**Caecal ligation and puncture-induced murine sepsis.** All animal studies were approved by the University of Ulm Committee on the Use and Care of Animals (approval number 988). Mice had unrestricted access to water and food. For experimental murine sepsis, male C57BL/6 mice (weighing 25–30 g, purchased from Jackson Laboratory, Bar Harbor, USA) were used. Sepsis was induced by caecal ligation and puncture (CLP) as previously described [30]. Mice were anaesthetized using a 2.5% sevoflurane (Sevoflurane; Abbott, Wiesbaden, Germany) and 97.5% oxygen mixture under a continuous flow via an inhalation mask. For analgesia, mice received buprenorphine 0.03 mg/kg body weight subcutaneously every 6–8 h after surgery. Following abdominal midline incision, 50 % of the caecum was ligated below the ileocaecal valve, punctured using a 21-gauge needle and a small portion of faeces was extruded to induce high-grade sepsis. The abdominal incision was closed in layers. Whole blood was collected 24 h after surgery into ethylenediaminetetraacetic acid-containing syringes and FSC-A values of a minimum of 10,000 neutrophils were determined by flow cytometry.

In vitro stimulation of neutrophils from knockout mice. For the in vitro stimulation experiments, blood was drawn from homozygous C5aR1 (C5aR1−/−) or C5aR2 (C5aR2−/−) gene knockout mice with a C57BL/6 background and from strain-matched wild-type (WT) animals (5–6 months old, weighing 25–35 g). The knockout mice were generated by Dr. Craig Gerard (Boston Children’s Hospital, Boston, MA). After hypotonic lysis of red blood cells, leucocytes were stimulated using murine recombinant C5a (10 nm) for up to 10 min at 37 °C and the FSC-A values of a minimum of 10,000 neutrophils were determined by flow cytometry.

**Statistical analyses.** Results are presented as the mean ± standard error of the mean (SEM). A one-way analysis of variance followed by the Student–Newman–Keuls post hoc test for multiple comparisons was performed to determine significant differences between experimental means when comparing several groups. For comparisons between two groups, statistical significance was calculated using Mann–Whitney U-test. A P value of less than 0.05 was considered statistically significant.

**Results**

**C5a-induced increase in neutrophil size as determined by FSC-A measurements**

Flow cytometric analysis of isolated human neutrophils revealed an increase in the FSC-A values as early as 1 min
Figure 1 Neutrophil shape changes following C5a stimulus. (A) Representative dot plots (upper images) and histograms (lower images) of neutrophil light-scatter properties after control (Ctrl, left panel) or C5a (right panel) stimulation in comparison with beads of defined sizes in flow cytometric measurements using a BD FACSCanto II. Neutrophils are shown in red; beads are depicted in green (10 μm, P2), blue (15 μm, P3) and purple (20 μm, P4). (B) Correlation between FSC-A signal and bead diameter in flow cytometric assessment (BD FACSCanto II); n = 22. (C) Time-dependent effect of C5a on cell size as assessed by FSC-A; n = 3–4; *, P < 0.05 compared to control. (D) Dose-dependent neutrophil size increase as assessed by FSC-A; n = 4; *, P < 0.05 compared to 0 nM.
and was significantly different 5 min after C5a stimulation in comparison with control incubation and as referenced to polystyrene beads of different predefined sizes (10, 15, 20 μm in diameter) (Fig. 1A). The linear correlation between FSC-A values and particle diameters within the range 10–20 μm (Fig. 1B) provides an estimation of the neutrophil size by flow cytometry. Based on this relationship, we found that incubation of neutrophils with C5a led to an early increase in neutrophil length, reaching a maximum after 10 min (Fig. 1C), whereas C3a failed to alter neutrophil size (data not shown). Furthermore, increasing concentrations of C5a for 10 min led to a concentration-dependent increase in neutrophil length (Fig. 1D).

The C5a-induced increase in the FSC-A values could be completely abolished by pretreatment of neutrophils using the C5aR1 antagonist C5aR1A (Fig. 2A). In a more complex system, exposure of neutrophils to zymosan-activated autologous serum (with generated C5a) also resulted in a concentration-dependent increase in cell length, whereas non-stimulated serum did not alter the cell dimensions. Noteworthy, this zymosan-induced size effect could also be completely abolished by C5aR1 antagonism (Fig. 2B). Moreover, neutrophils from WT mice and C5aR2 knockout (C5aR2<sup>−/−</sup>) mice exhibited a significant increase in cell length 5 min after stimulation with murine C5a. In striking contrast, under similar conditions, neutrophils from C5aR1 knockout mice (C5aR1<sup>−/−</sup>) failed to increase in cell size (Fig. 2C). Interestingly, unstimulated C5aR2<sup>−/−</sup> animals displayed visibly increased FSC-A values compared to WT and C5aR1<sup>−/−</sup> mice. These flow cytometric results indicate that the C5a-induced FSC-A effects, reflecting to some extent the cell dimensions, were found with both human and mouse neutrophils and were dependent on C5aR1 signalling.

**Sepsis-induced changes in neutrophil size**

Because increased C5a plasma levels have been found during experimental and human sepsis, we determined neutrophil cell size in murine CLP-induced sepsis. The flow cytometry-based analysis of neutrophils from mice with sepsis showed a significant increase in the FSC-A values 24 h after CLP induction when compared to neutrophils of control mice (Fig. 2D).

**Potential mechanisms of the C5a-induced shape change**

To investigate possible mechanisms of the C5a-induced effects in neutrophils, we blocked central ion channels/transporters that may be associated with intracellular-volume regulation (Supplementary Table 1). Inhibition of the NHE1 by amiloride or a specific NHE1 inhibitor (4-cyano-benzo[b]thiophene-2-carbonylguanidine, methanesulfonate) failed to reduce the C5a-mediated increase in cell size, neither alone nor in combination with the H<sup>+</sup>/K<sup>+</sup>-ATPase inhibitor omeprazole. Likewise, blockade of the Na<sup>+</sup>/HCO<sub>3</sub>−-cotransporter by S0859 or the Na<sup>+</sup>/K<sup>+</sup>/2Cl<sup>−</sup>-cotransporter by bumetanide revealed no significant inhibitory effects. Additionally, the K<sup>+</sup>-channel blocker tetraethylammonium chloride, which can also inhibit aquaporins to some extent [31], did not alter the C5a-induced size increase (Supplementary Table 1). In contrast, the Cl<sup>−</sup>/HCO<sub>3</sub>−-exchanger inhibitor UK5099, which blocks the exchange of intracellular bicarbonate with extracellular chloride, partially reduced the C5a effect on neutrophils (Fig. 3A). To determine whether the C5a-induced increase in neutrophil length was mediated by the influx of chloride ions, we examined the action of the Cl<sup>−</sup>-channel blocker NPPB. Pre-incubation of neutrophils with NPPB also significantly reduced the C5a-induced increase in their length (Fig. 3B), indicating that C5a stimulation resulted in an influx of osmotically active chloride ions into neutrophils.

**Specific morphological changes of neutrophils by anaphylatoxin C5a**

To investigate the C5a-mediated neutrophil shape alteration in more morphological detail, we used the Amnis ImageStream system, which combines the features of a flow cytometer and a fluorescence microscope. Surprisingly, the analysis of the mean cell area by IDEAS software revealed only marginal increases in the overall cell size after C5a treatment in terms of cellular swelling (data not shown). However, we also determined the mean circularity of the neutrophils by analysis of the mean distance of the cell boundary from its centre divided by the variation of this distance. The more the shape deviates from a circle, the greater the variation and therefore the lower the circularity value. We found that incubation of neutrophils with C5a (in this set of experiments with 200 ng/ml which revealed maximal effects in a pilot dose-response study, data not shown) resulted in a significant reduction of cell circularity after 5 min based on a cut-off value of 10 when compared to control samples (Fig. 4A,B). Furthermore, we analysed the shape ratio of the neutrophils, which was defined by the minimal cell thickness divided by the cell length. When neutrophils were stimulated with C5a, the number of cells that displayed a shape ratio of less than 0.66 was increased (to 54%) in comparison with neutrophils after control incubation (39%). Moreover, the combined analysis of the shape ratio (<0.66) together with low circularity values (<10) of neutrophils revealed that the percentage of these cells was significantly increased after 5 min of C5a stimulation (Fig. 4C–E).

For further morphological evaluation, neutrophils were analysed by confocal microscopy. After control incubation, the cells appeared uniformly spherical, whereas upon C5a exposure, they lost their circularity, adopting a rather polarized cell shape (Fig. 5A).

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Biomechanical changes of neutrophils upon exposure to C5a

Whether such a polarized cell shape was also associated with altered cellular mechanics was the objective of the next set of experiments. Optical stretching was applied to compare the deformability of neutrophils exposed to 10 nM C5a with that of untreated cells (Fig. 5B). Pooled data for neutrophils from five healthy individuals demonstrated...
Addition of C5a induces neutrophil shape changes, as analysed by high-resolution spectral imaging in a flow cytometry environment. Neutrophils were maintained at 37 °C for 5 or 10 min (controls) or reacted with 20 nm C5a for the same time periods. The samples were kept on ice for 20 min and brought to room temperature just before analysis. Changes in both the circularity (A, B) and shape ratio (C) induced by C5a are evident. Low circularity values (<10) represent a high deviation from circular shape. Dot plots that evaluate both the shape ratio and circularity (D,E) display the same trends. All points were run in duplicate. There was a significant difference between the samples examined after 5 min for C5a treatment versus the untreated samples for low circularity and for the shape ratio and low circularity combined (P = 0.04 and P = 0.03, respectively). When taking the mean of the results for both time points, the differences between C5a-treated and untreated samples were significant for both criteria (P = 0.004 and P = 0.002, respectively). Representative of two separate experiments.
that the majority of neutrophils exhibited significantly increased deformability in response to C5a (Fig. 5B).

**Figure 5** Morphological changes induced by C5a as assessed by confocal microscopy and optical stretching. (A) Neutrophils were stimulated using C5a (20 nM) for up to 10 min at 37 °C. Cells were allowed to settle on the glass bottom of microwell dishes and were then examined using a Zeiss 7000 scanning confocal microscope. (B) Deformation of neutrophils by optical stretching. Insert: neutrophils (green) suspended in a microfluidic channel (light blue) were deformed by forces (red) produced by the momentum transfer from counter-propagating laser beams (light red) to the cellular surface. The scatter plot depicts the relative peak deformation of individual neutrophils as determined by the changes of the cells’ diameter (insert, yellow arrow). Neutrophils were exposed to optical forces for 2 s. Peak deformability of neutrophils was significantly increased by 10 nM C5a. The horizontal lines represent the mean relative peak deformation ± SEM; ****, P < 0.0001.

C5a-induced change in cell morphology involves actin–cytoskeleton reorganization and is dependent on ATP signalling

A major driver for cellular mechanics and shape changes is the actin cytoskeleton. Early after C5a exposure, human blood neutrophils stained with phalloidin-FITC (green) displayed clear evidence of F-actin polymerization and polarization (Fig. 6A). The signs of C5a-induced actin reorganization were abolished in the presence of either C5aRA or NPPB (Fig. 6A) but not in the presence of UK5099 (data not shown).

To determine whether the C5a-mediated changes of the neutrophil features were associated with functional cell polarization, we used suramin to block the P2X/Y-
purinoceptors that induce a polarization process after ATP stimulus [32]. To screen for cell shape changes, we again determined the FSC-A values by flow cytometry. The C5a-induced increase of the FSC-A could be significantly reduced by inhibition of the purinoceptors (Fig. 6B), suggesting that the C5a-induced changes in the neutrophil shape were dependent on P2X/Y signalling. Because changes in cell shape are regarded as a prerequisite for migrational function, the effect of P2X/Y inhibition on C5a-stimulated chemotaxis was investigated. After pre-incubation of neutrophils with suramin, the chemotactic activity towards C5a was significantly reduced (Fig. 6C), indicating that the C5a-mediated activation of the P2X/Y signalling and the associated changes in cell shape were required for neutrophil chemotaxis.

Discussion
Manifold effects of the complement activation product C5a on key functions of neutrophils have been identified [33]. Because the functional activity of neutrophils is dependent on intracellular-volume regulation, we investigated the effect of C5a on neutrophil cell volume and cell shape and the regulatory signalling mechanisms (Fig. 7).

Neutrophil stimulation using C5a resulted in a concentration- and time-dependent increase of FSC-A values during flow cytometric analysis as an indication of increased cell length or size (Fig. 1). In this context, O’Flaherty et al. have shown that neutrophil stimulation using chemotactic agents leads to their aggregation [34]. However, in the present study, a C5a-induced aggregation...
of neutrophils could be excluded by a discriminative analysis of FSC-A and FSC-height signals (data not shown). The observed effect could be effectively blocked by a C5aR1 antagonist. Furthermore, there was no shape change in neutrophils from C5aR1 knockout mice after C5a stimulation in contrast to neutrophils from C5aR2 knockout and WT animals (Fig. 2). These data suggest that the C5a stimulus induces a C5aR1-dependent intracellular signalling cascade with subsequent osmotic swelling and cellular shape change. In previous studies, similar shape changes by various chemoattractants, including C5a in excessive amounts, have been described [35] and linked to functional alterations such as induction of the oxidative burst [36].

On a technical level, it should be indicated that determination of cell size by flow cytometric means is by far more complex than so far appreciated [37]. Although various optical scatter and fluorescence parameters (FSC-A, FSC width, side-scatter area, and 450/50 area) may estimate the cellular volume (and were all increased after C5a exposure in the present study, data not shown), an investigation of electronic cell-volume distributions by Coulter counting showed only a trend of an enhanced neutrophil diameter after C5a exposure (resulting in an approximately 5% total increase, data not shown). This indicates that enhanced FSC-A values alone failed to precisely determine cell volume, but may reflect an increased cell length with marginal if any volume alterations.

However, several previous studies addressing cell-volume regulation revealed that neutrophil stimulation with chemoattractants resulted in cell swelling and that neutrophil size was increased after migration into inflamed tissue [17, 38, 39]. Remarkably, in tumour cells, stimulation with zymosan-activated serum — known to generate substantial amounts of C5a — resulted in a 20% increase in cell diameter, which was associated with an enhanced chemotactic migration behaviour [40]. Similarly, in neutrophils, an fMLP- or osmosis-induced increase in cellular volume was accompanied by enhanced chemotactic activity [15, 17], which was associated with some reorganization of the actin cytoskeleton [41]. Here, we demonstrated that C5a induces rapid actin polymerization and polarization of the cells (Fig. 6A). Of note, in the actin-cytoskeleton reorganization experiments, C5a was applied by micro-injection from a distinct direction; however, uniform alignment of the cells in direction of the gradient was not apparent in the present staining. This might indicate a general activation and priming of the cells upon sensing a C5a gradient or the gradient was no longer present after the incubation period. As a limitation of the study, no combined exposure to PAMPs and C5a was performed.
which may result in a clearer response to the molecular danger signal. In regard to actin disassembly, cofilin-dependent (de)polymerization is known to be a pH-sensitive process [42]. However, inhibition of the NHE1, the $H^+$/K$^+$-ATPase or the sodium bicarbonate cotransporter did not reduce the C5a effect in the present investigation (Supplementary Table 1), whereas fMLP-induced volume changes could be blocked by combined inhibition of the NHE1 and $H^+$/K$^+$-ATPase in earlier studies [16]. Furthermore, activation of aquaporins as a driving mechanism shown in neutrophils after stimulation with fMLP [31] could be excluded.

It is somewhat surprising that amiloride was incapable of inhibiting C5a-induced cellular shape change, although it was most potent to inhibit an increase in intracellular pH in our recent report [5]. However, in the context of the C5a-induced shape change/cellular volume, we could not find this effect using amiloride. In a previous study, Shimizu Y et al. studied the Cl$^-$ efflux from neutrophils, which was triggered by factors elevating intracellular calcium such as C5a. This C5a effect was not inhibited by amiloride [43], supporting our data. Furthermore, replacing extracellular Na$^+$, but not the presence of amiloride, was shown to inhibit fMLP-caused neutrophil polarization [44].

In contrast, the blockade of the Cl$^-$/HCO$_3^-$-exchanger significantly reduced the C5a-induced shape change, indicating an underlying Cl$^-$-mediated mechanism as suggested by Giambelluca et al. [45]. This is supported by the inhibitory effects of global chloride-channel blockade (Fig. 3) and might be even more pronounced by combined inhibition of chloride channels and exchangers. Of note, the inhibitory effect of NPPB on the C5a response might not be restricted to its blocking function on chloride channels. NPPB also acts as an effective protonophore, potentially disturbing cytosolic pH and subsequent intracellular ion and fluid load [46]. Furthermore, NPPB treatment leads to depletion of intracellular ATP [46], which, together with the P2X/Y-blocking results in the present study, suggests an involvement of ATP signalling in the C5a-induced alteration of the neutrophil cell shape as discussed below.

Alteration in Cl$^-$-homeostasis by various inflammatory mediators seems to play an important functional role in neutrophils. Chloride is considered crucial for the host defence of neutrophils by generating potent chlorine bleach, contributing to antimicrobial micromilieu in phagosomes and thereby killing bacteria [55]. Exposure of neutrophils to fMLP, IL-8 or C5a resulted in an enhanced Cl$^-$ efflux which was proposed not to be causal for the shape change [43]. Another report described that fMLP activates the anion transporters CIC-3 and ICI$_{swell}$ [47]. Niflumic acid (NFA) and phloretin as non-selective inhibitors of CIC-3 and ICI$_{swell}$ function, both reduced chemotactic activity and velocity, whereas NFA in a mM range and tamoxifen in a $\mu$m range (as specific ICI$_{swell}$ inhibitor) were capable to inhibit cellular shape change in response to a fMLP-gradient [47]. Future studies should address the effect of C5a on these anion transporters.

Further analysis of neutrophils by Amnis ImageStream found only marginal increases in the mean cell area (data not shown). Instead, a C5a-induced change in the cell shape could be detected consisting of a reduction of cell circularity and an increase of the cell length in comparison with the cell width (Fig. 4). Therefore, the increase of FSC-A values after C5a stimulation might rather represent a change in the cellular shape and presumably formation of pseudopodia over the entire cellular surface than an increase in the overall cell volume. This shape change was associated with a C5a-induced increase in neutrophil deformability as assessed by optical stretching [29] (Fig. 5). Altered neutrophil mechanics with substantial cellular deformation determined by this technique have been described as a requirement for migration processes through very small pores [29], suggesting that C5a influences neutrophil motility in confined spaces. Formation of pseudopodia was apparently induced by local alkalinization via the NHE1 and influx of fluid following directed/targeted activation of fMLP receptors on neutrophils [41, 48]. However, in the present study, the NHE1 appears to play only a minor role, if at all, in the C5a-associated cellular shape changes.

In the case of the chemoattractant fMLP, a local release of ATP has been observed, which lead to reorganization of the actin cytoskeleton and polarization of neutrophils [11, 32, 49]. This pathway can also be activated by membrane deformation due to mechanical or osmotic stress [50, 51]. To investigate whether the C5a-induced polarization in our study was dependent on ATP interactions, we blocked the P2Y receptor using suramin, which significantly reduced the C5a-mediated increase of the FSC-A values (Fig. 6). Furthermore, the C5a-induced chemotaxis was significantly reduced by blocking the P2Y signalling, which may be due to the inhibition of the P2Y-mediated polarization process in neutrophils. In contrast to the in vivo chemotaxis situation where neutrophils are exposed to a C5a gradient, the global excessive generation of C5a during systemic inflammation in vivo [33, 52] may be responsible for the shape changes and might not necessarily require a specific chemoattractant gradient.

As a limitation of the study, we cannot exclude other mechanisms to be also involved in cell shape regulation by C5a as we did not perform broad-range dose-response experiments for most inhibitors that were used and might have missed other specific ion-transporter/exchange systems relevant for the cellular volume and ion balance.

In our animal experiments, neutrophils from C5aR1$^{-/-}$, but not from C5aR2$^{-/-}$ mice, were resistant to the C5a-induced cell shape changes. In a translational approach, it is tempting to speculate that specific blockade of the C5aR1 might inhibit excessive and ubiquitous neutrophil migration into host tissue. Of note, there was a visible alteration in
basal cell shape in C5aR2−/− mice (Fig. 2), which might indicate that absence of C5aR2 as a postulated modulator of the pro-inflammatory response [53, 54] leads to a permanently enhanced pro-inflammatory signalling via C5aR1 with subsequent shape change. Future studies need to clarify to what extent C5aR1 blockade during the inflammatory response will modulate cellular shape change and subsequent functions.

In conclusion, our data suggest that the complement activation product C5a is involved in basic cell regulatory processes (Fig. 7) by modulation of chloride channels and transporters and changing the cell shape (increased length and decreased width) as well as membrane formability. This occurs via actin-cytoskeleton reorganization and polarization, all of which transform the neutrophil into a migratory cell able to invade inflammatory sites and target and clear pathogens and debris.

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Author contributions
SD, RW, EMC, MAL, KP, TM and DM performed the research; RPT, SP, HB, FG and MSHL designed the research study; JDL contributed the C5aR1A; SD, RW, EMC, TE, MW, EB, MK and TM analysed the data; SD, RPT, RW, SP, HB and MSHL wrote the manuscript. All authors revised and approved the final version of the manuscript.

Conflicts of interest
The authors declare no conflict of interest.

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Supporting Information

Additional Supporting Information may be found online in the supporting information tab for this article:

Table S1 Effect of different ion-channel/transporter inhibitors on the C5a-induced increase in neutrophil length as determined by forward-scatter area.