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Quantifying the Sensitivity of Human Immune Cells to Chemoattractant

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ABSTRACT The efficient recruitment of immune cells is a vital cornerstone of our defense against infections and a key challenge of immunotherapeutic applications. It relies on the ability of chemotaxing cells to prioritize their responses to different stimuli. For example, immune cells are known to abandon gradients of host-cell-produced cytokines in favor of complement-derived anaphylatoxins, which then guide the cells toward nearby pathogen surfaces. The aptitude to triage stimuli depends on the cells' specific sensitivities to different chemoattractants. We here use human neutrophils as uniquely capable biodetectors to map out the anaphylatoxic cloud that surrounds microbes in the presence of host serum. We quantify the neutrophil sensitivity in terms of the ratio between the chemoattractant concentration c and the production rate j_0 of the chemoattractant at the source surface. An integrative experimental/theoretical approach allows us to estimate the c/j_0-threshold at which human neutrophils first detect nearby β-glucan surfaces as $c/j_0 \approx 0.0044$ s/μm.

How does an immune cell cope with situations in which it faces multiple chemotactic stimuli? How does the cell decide on a particular response? Such questions touch on the core of our mechanistic understanding of immune-cell behavior, and have inspired the paradigm that immunotaxis comprises an intricate spatiotemporal hierarchy of distinct chemotactic processes (1–6). The systematic dissection of this hierarchy is an enormous interdisciplinary challenge that requires, among others, quantitative analyses of the stimulus-specific sensitivity of the responding cells.

Complement-mediated chemotaxis has emerged as a universal, short-range homing mechanism by which chemotaxing immune cells can implement a last-minute course correction toward pathogenic bacteria and fungi. Recent single-cell experiments have validated human neutrophils as uniquely capable biodetectors of minuscule amounts of complement-derived anaphylatoxins in the proximity of microbial and model pathogens (Fig. 1) (6–8). But the question just how sensitive these immune cells are was not addressed by earlier studies.

We here use an integrative theoretical/experimental strategy to tackle this difficult question. A recently found closed-form solution of the appropriate reaction-diffusion problem (V.H., E.A.F., and W.D. Simpson, unpublished data) predicts the spatiotemporal distribution of anaphylatoxins as a function of the time t and the radial distance from the source, $\Delta r = r - R$ (Fig. 1 *B*; Supporting Material). In the considered scenario, the source of chemoattractant is a sphere of radius R that, at time $t = 0$, starts releasing anaphylatoxins at a constant rate given by the boundary flux j_0. The chemoattractant is redistributed in the surrounding infinite space by diffusion. A realistic estimate gives a diffusion coefficient of $D \approx 130\ \mu m^2/s$ for the dominant anaphylatoxin C5a (V.H., E.A.F., and W.D. Simpson, unpublished data). We further model the deactivation of chemoattractant by carboxypeptidases as an irreversible removal reaction with a typical kinetic off-rate constant of $k \approx 0.011\ s^{-1}$ (V.H., E.A.F., and W.D. Simpson, unpublished data). This removal process is vital because it prevents potentially dangerous overstimulation of the host organism by an unchecked buildup of chemoattractant.

We apply this model to single-cell/single-target experiments in which individual human neutrophils are exposed to anaphylatoxins produced at the surface of fungal model particles in the presence of autologous serum (added to the experiment buffer at 20%; Fig. 1 *B*). The most suitable targets for our purpose turned out to be β-glucan particles. These particles tended to cluster, which allowed us to use a variety of targets with different sizes but the same chemical surface composition (Fig. 2). The ability to vary R, a key control parameter in our analysis, considerably increased the robustness of our results.

This integrative strategy suggests two possible approaches to characterize the sensitivity of human neutrophils in their role as biosensors. First, one could measure

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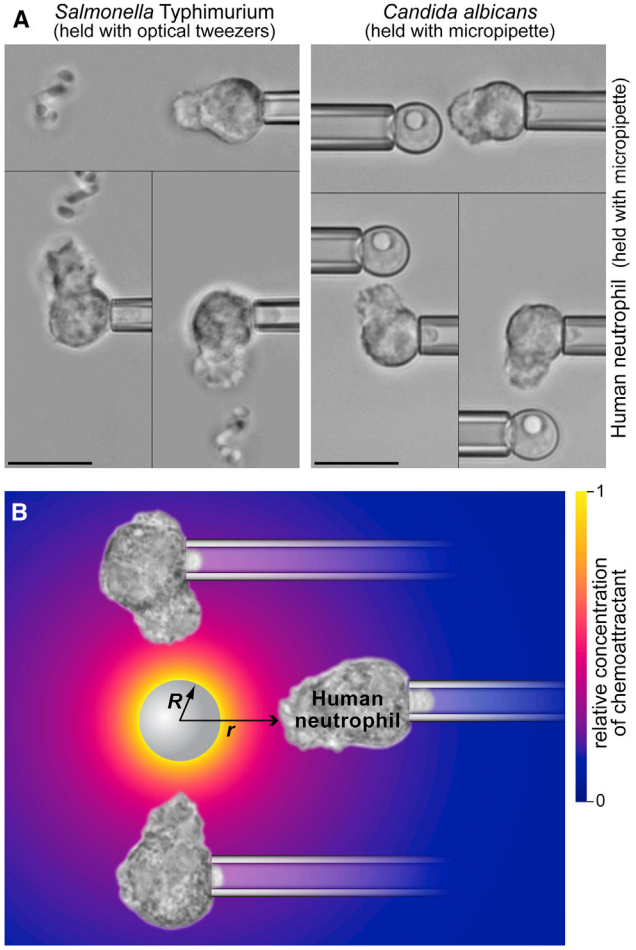

FIGURE 1 Human neutrophils as biodetectors. (*A*) Neutrophils report the presence of nearby bacterial and fungal pathogens by extending directed pseudopods. Repositioning the target allows us to triple-check the specificity of the response. Scale bars denote 10 μm. (*B*) Chemoattractant anaphylatoxins like C5a are produced by the host's complement system on the surface of foreign particles and released. Neutrophils detect these anaphylatoxins through G-protein-coupled receptors (e.g., the C5a receptor CD88).

the time lag from placing a target particle in the cell's proximity to the onset of the formation of a pseudopod directed toward the target. However, this lag includes not only the sought time required to reach the concentration threshold triggering the cell response, but also the time it takes a quiescent neutrophil to subsequently start up its internal actin-remodeling machinery. The latter time is not accurately known, nor is it negligibly small. For example, the typical time lag from placing a zymosan particle near a neutrophil to the first sign of a newly forming chemotactic pseudopod was found to be on the order of ~60 s (9). Similar results were obtained for bacterial and fungal pathogens (6,8). On the other hand, for the typical cell-target distances ($\Delta r \approx 5$ μm) and target sizes ($R \approx 2.5$ μm) used in those experiments, Eq. S4 in the Supporting Material predicts that the concentration of chemoattractant at the front of the cell rises to near-steady-state values in <5 s. Thus, it is the time required for cell activation rather than for the formation of the anaphylatoxic cloud that dominates the measured time lag in this case. Consequently, this type of analysis is not suited to characterize the cells' sensitivity.

The second, more promising type of analysis is based on the determination of the maximum cell-target distance that triggers a cell response. In experiments specifically designed for this purpose, we stepwise reduced the cell-target distance, giving the cell enough time (~2 min) at each distance to sample the local near-steady-state concentration of chemoattractant. We performed >50 recognition experiments with β-glucan particles of different sizes (Fig. 2; Movie S1) and recorded the yes/no answers to the question whether or not a response was triggered at a particular distance. We then superimposed these data onto a concentration map that predicted the profile of the anaphylatoxic cloud as a function of Δr and R at the 2-min time point (Fig. 3 *A*).

Two lines of constant concentration included in Fig. 3 *A* subdivide this map into three regions: one where recognition always occurred; one where recognition never occurred; and an intermediate region where some, but not all, neutrophils detected the chemoattractant. For each cell-target pair, the source-size-dependent recognition distance was smaller than the respective value of the right boundary of the intermediate region (*blue line*), and in almost all cases it was larger than the value of the left boundary (*orange line*). Accordingly, the concentration threshold that triggered the formation of a chemotactic pseudopod can be assumed to lie within the range of concentrations bounded by the two lines. We choose the average ($c/j_0 \approx 0.0044$ s/μm) of the relative concentration values of these contour lines as a representative estimate of this threshold. This estimate is specific to β-glucan surfaces exposed to 20% serum, because j_0, and hence c/j_0, depends on these features. Yet as long as the targets had similar sizes, we did not observe noticeable differences between the chemotactic responses of neutrophils to β-glucan particles, *Candida albicans* cells, clusters of *Salmonella* Typhimurium, and other targets. Therefore, we believe that the critical concentration of $c/j_0 \approx 0.0044$ s/μm is a representative estimate for the surfaces of all of these types of target.

Based on this estimate, we can characterize the effective spatial extent and dynamics of the anaphylatoxic cloud surrounding these target surfaces under near-physiological conditions. Fig. 3 *B* shows that for $c/j_0 \approx 0.0044$ s/μm, the effective spatial reach of the anaphylatoxic cloud spans ~1–3.5 source diameters for the depicted target-size range. The smaller spatial reach of ~1 source diameter is typical for target sizes up to $R \approx 2.5$ μm, whereas a reach of ~3.5 source diameters is characteristic for target sizes in the R-range from ~7.5 to 10 μm. Finally, Fig. 3 *C* confirms

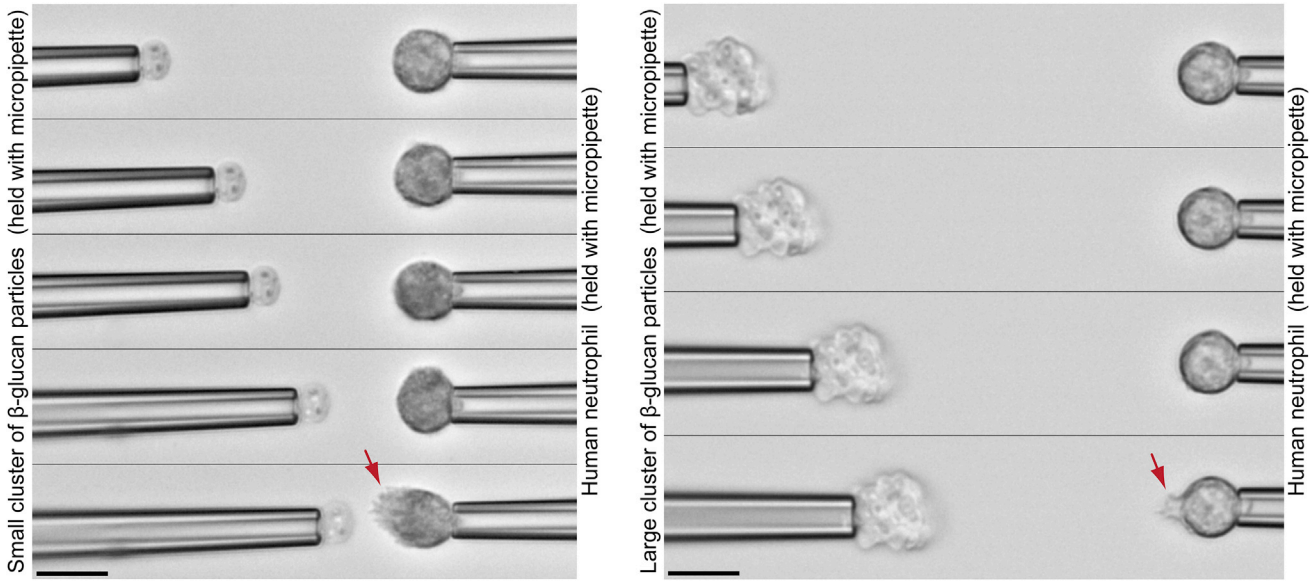

FIGURE 2 Measurement of the response distance of human neutrophils to clusters of β-glucan particles. Two sequences of video images demonstrate how the cell-target distance is stepwise reduced every 2 min (see also Movie S1). The distance at which the cell first forms a pseudopod directed toward the cluster of β-glucan particles (*video snapshot at the bottom*) is the sought response distance. The two panels confirm that the response distance depends strongly on the target size. Scale bars denote 10 μm. To see this figure in color, go online.

that the anaphylatoxic cloud forms rapidly compared to typically observed cell-response times. For example, at a cell-target distance of one source diameter, the critical concentration of chemoattractant is reached in <~3 s for all considered source sizes.

We caution that, due to natural cell-to-cell variability of live human neutrophils, the measured c/j_0-threshold should be viewed as a very rough estimate. The c/j_0-values of the two lines in Fig. 3 *A* could be taken as best-case error margins of this estimate. But because of other uncertainties (Supporting Material; V.H., E.A.F., and W.D. Simpson, unpublished data), the error margins could be larger. We conservatively view the value of $c/j_0 \approx 0.0044$ s/μm as an order-of-magnitude estimate.

The ultimate completion of the calibration of immune cells as detectors of chemoattractant requires knowledge of the value of the source flux j_0. We hope to be able to estimate this value for β-glucan surfaces in future work, which will then allow us to pinpoint the absolute concentration threshold of anaphylatoxins required to trigger a chemotactic response by human neutrophils. In conclusion, this

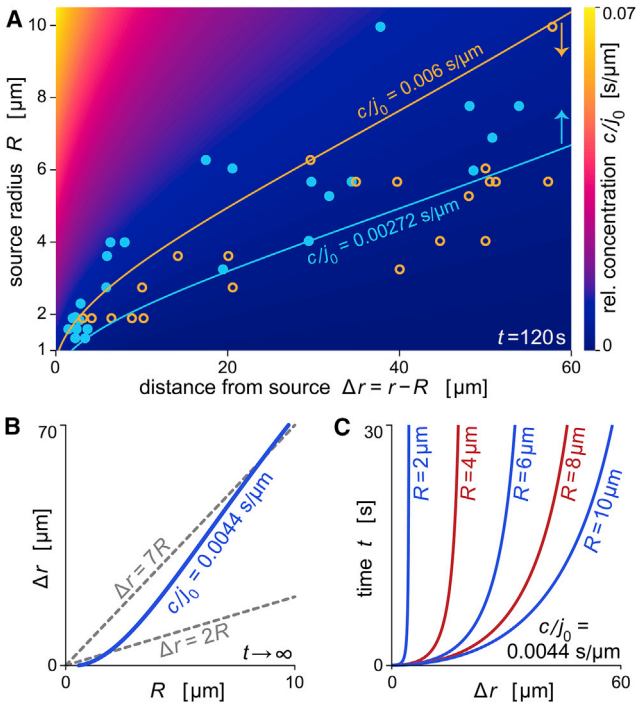

FIGURE 3 Calibration of the sensitivity of human neutrophils to C5a. (*A*) Density map of the relative concentration c/j_0 as a function of Δr and R calculated at $t = 120$ s. Overlaid open circles (*orange*) show the locations of experiments where no neutrophil response was observed. The contour line of constant concentration at $c/j_0 = 0.006$ s/μm (*orange line*) marks the upper concentration limit of the no-response data. Solid circles (*light blue*) show the locations of experiments where neutrophils did detect anaphylatoxins. The contour line at $c/j_0 = 0.00272$ s/μm (*light-blue line*) marks the lower concentration limit of the data corresponding to positive responses. (*B*) Response distance Δr as a function of the source size R for the concentration threshold $c/j_0 = 0.0044$ s/μm. Here, this relationship is shown for the steady state. Dashed lines with slopes of 2 and 7 are included for comparison. (*C*) Time required to reach the critical concentration $c/j_0 = 0.0044$ s/μm as a function of Δr for five different source sizes.

study demonstrates how superb experimental control over one-on-one encounters between immune cells and pathogenic targets (10), in conjunction with realistic mathematical modeling, provides key insights into mechanisms of vital cellular behavior that are inaccessible to traditional biological methods.

SUPPORTING MATERIAL

Supporting Materials and Methods and one movie are available at http://www.biophysj.org/biophysj/supplemental/S0006-3495(17)30104-2.

AUTHOR CONTRIBUTIONS

E.A.F. and V.H. designed the experiments and analyzed the data; E.A.F. performed the experiments; and V.H. developed the theory, prepared the figures, the movie, and the Letter.

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Supplemental Information

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Materials and Methods

Our dual-micropipette manipulation setup and procedure were described in detail in previous publications (1, 2).

Human neutrophil isolation

Written informed consent was obtained from all subjects. The Institutional Review Board of the University of California Davis approved the protocol covering this study.

Neutrophils were isolated from whole blood of healthy donors by immunomagnetic negative selection using the EasySep Direct Human Neutrophil Isolation Kit (STEMCELL Technologies, Vancouver, BC). First, 25 μL each of the isolation cocktail and the magnetic bead solution were added to a small volume of blood (~0.5 mL) to facilitate cross-linking of cells other than neutrophils to magnetic beads via tetrameric antibody complexes. After 5 minutes, the sample was diluted with phosphate-buffered saline (PBS) containing 2% fetal bovine serum (FBS) and 1mM EDTA (EasySep Buffer, STEMCELL Technologies) at a minimum 2:1 ratio, and placed adjacent to a magnet. After 10 minutes, 0.5-1.0 mL of the enriched, lighter-colored neutrophil fraction was carefully transferred into a new test tube, and another 25 μL of the magnetic bead solution was added. After two more 5-minute magnetic separation cycles, the cells were re-suspended in calcium and magnesium-free Hank's balanced salt solution (HBSS; Thermo Fisher Scientific, Waltham, MA) and gently rotated until use.

All experiments using human neutrophils were performed at room temperature.

Preparation of β-glucan particles

Whole 1,3/1,6-β-glucan particles (WGP Dispersible; InvivoGen, San Diego, CA) were suspended in PBS (IBI Scientific, Peosta, IA) at 2-10 mg/mL. After three washes in PBS, the solution was sonicated for 30-60 minutes. The solution was stored at 4°C. Sonication was repeated on each day of experimentation.

Data collection for the determination of the cell-target distance triggering chemotaxis

For each selected pair of cell and target, we stepwise decreased the cell-target distance $\Delta r = r - R$ (see Fig. 1B for notation) until the formation of a chemotactic pseudopod signaled that the cell had detected the target. Depending on the outcome of this procedure, each tested cell contributed either one or two data points to the map of Fig. 3A. In cases where the cell responded to the target at the first tried cell-target distance, this result was registered as a single, "positive-response" event in Fig. 3A. On the other hand, if the response switched from "negative" to "positive" upon reduction of Δr, we included both events as data points in Fig. 3A.

The time at which we evaluated the concentration map of Fig. 3A (2 min) was chosen to coincide with the duration of each test interval at a given cell-target distance. The concentration profile predicted by this map corresponds to the experimental situation at the end of the first tried 2-minute test interval for each cell-target pair. Because we were unable to wash away chemoattractant after reducing Δr, the subsequent test intervals did not strictly start from a clean slate of zero-concentration that would allow us to reset the clock to $t = 0$. Consequently, the local concentrations predicted by Fig. 3A were reached in less than 2 min in these tests, and the actual concentrations at the end of the respective intervals were somewhat higher than predicted. However, this discrepancy is mitigated by the fact that for the considered range of cell-target distances and target sizes, the concentration profile at 2 min does not appreciably differ from the steady-state profile. In other words, the local concentration reached at $t = 2$ min remains essentially unchanged at later times. Moreover, a "no-response" event observed at a given concentration is also a "no-response" event at any smaller concentration, and thus may safely be registered as a respective data point in Fig. 3A. Finally, it is highly unlikely—but cannot be excluded—that the imperceptibly small difference between the actual local concentration at the end of a test interval and the (smaller) concentration predicted by Fig. 3A could have caused "false-positive" events to be registered in Fig. 3A.

Concentration of chemoattractant as a function of the distance from the source and time

The reaction-diffusion scenario described in the main text translates into the partial differential equation

$$\frac{\partial \tilde{c}}{\partial t} = D\left(\frac{2}{r}\frac{\partial \tilde{c}}{\partial r} + \frac{\partial^2 \tilde{c}}{\partial r^2}\right) - k\tilde{c} \tag{S1}$$

with initial condition

$$\tilde{c}(r,0) = 0 \qquad \text{for} \qquad R \le r < \infty \tag{S2}$$

and boundary conditions

$$\begin{aligned} \tilde{c}(r,t)\big|_{r\to\infty} &= 0 \qquad \text{for} \qquad 0<t<\infty \\ \left.\frac{\partial\tilde{c}(r,t)}{\partial r}\right|_{r=R} &= -\frac{j_0}{D} \qquad \text{for} \qquad 0<t<\infty \end{aligned} \tag{S3}$$

Here, the following notation was used:

$\tilde{c}$ … concentration of chemoattractant in units of *number of molecules* per *volume*, where the tilde indicates the functional dependence of the concentration on the distance r from the source center (cf. Fig. 1B) and on time t

j_0 … constant outward source flux that only exists at the particle surface and has units of *number of molecules* per *area* per *time*

R … radius of the spherical particle that is the source of chemoattractant

D … diffusion coefficient of the chemoattractant in the given medium

k … kinetic off-rate constant of the removal reaction of chemoattractant

The second boundary condition of Eq. (S3) is Fick's first law applied locally to the source surface.

This problem can be solved analytically (3). For $c(\Delta r,t)=\tilde{c}(\Delta r+R,t)$ the solution reads

$$c(\Delta r,t)=\frac{j_0R^2}{\Delta r+R}\left\{\begin{aligned} &\frac{1}{2\left(D+R\sqrt{Dk}\right)}\exp\left(-\frac{\Delta r}{\sqrt{D/k}}\right)\operatorname{erfc}\left(\frac{\Delta r}{2\sqrt{Dt}}-\sqrt{kt}\right) \\ &+\frac{1}{2\left(D-R\sqrt{Dk}\right)}\exp\left(\frac{\Delta r}{\sqrt{D/k}}\right)\operatorname{erfc}\left(\frac{\Delta r}{2\sqrt{Dt}}+\sqrt{kt}\right) \\ &-\frac{1}{D-kR^2}\exp\left[\frac{\Delta r}{R}+\left(\frac{D}{R^2}-k\right)t\right]\operatorname{erfc}\left(\frac{\Delta r}{2\sqrt{Dt}}+\frac{\sqrt{Dt}}{R}\right) \end{aligned}\right\} \tag{S4}$$

where Δr denotes the distance from the surface of the source, and erfc is the complementary error function. The applicability of this model to our experiments is contingent on the validity of the following assumptions (for details see (3)):

- <u>*Convection (or drift) is assumed to be negligible.*</u> Some fluid drift almost always occurs in open chambers used in micropipette experiments, mainly because evaporation cools the air-water interface, which in turn causes convection loops. In chambers that are open on two sides, we minimized this effect by carrying out the experiments at a location that is roughly equidistant from the two air-water interfaces.

  Even without this precaution, we generally do not observe drift velocities v_{drift} exceeding ~1 µm/s in micropipette experiments (estimated based on the motion of free small particles that occasionally can be seen and tracked in the background). The Péclet number in this case is

$$\mathrm{Pe} = \frac{L}{D} v_{\mathrm{drift}} \lesssim \left(0.0077\ \mu\mathrm{m}^{-1}\right) L \tag{S5}$$

for the diffusion coefficient of C5a, $D = 130\,\mu\mathrm{m}^2/\mathrm{s}$ (see main text), and a typical length scale L. This upper limit is smaller than 0.5 for the largest distance $L \sim \Delta r = 60\,\mu\mathrm{m}$ included in Fig. 3A, and it decreases linearly with Δr. Therefore, diffusion is the dominant form of transport of chemoattractant in the experiments of this study. In particular at small distances from the source, drift effects can be neglected. Indeed, in "triple-check" experiments such as shown in Fig. 1A, pseudopod formation did not appear to depend on the location of the target particle. On the other hand, if convection is sufficiently strong, it will deform the cloud of chemoattractant into a plume that is not radially symmetric anymore. Especially at large distances, this distortion increases the relative impact of drift. Thus we cannot exclude that convection may occasionally have a small effect on our measurements, in which case it contributes to the uncertainty of our results that the main text cautions about.

- *The production rate of chemoattractant at the source surface is assumed to remain constant during experiments.* It is important to bear in mind that pathogenic microbes or surrogate particles are not the actual chemical sources of anaphylatoxins. Instead, special host enzymes called convertases produce the chemoattractant peptides. These convertases are assembled on pathogen surfaces by the host's complement system. The surface-bound convertases continually cleave anaphylatoxins like C5a from serum-based precursor proteins such as complement component C5.

  In our experiments, we always expose target particles to autologous serum for at least one hour before using them in single-cell chemotaxis tests. Therefore, it is reasonable to assume that the complement machinery is fully assembled on the particle surface at the beginning of each test. As long as precursor proteins like C5 are available in excess in the surrounding medium—which we assume to be the case for the experiment times used here—the convertases produce fresh anaphylatoxins at a constant rate. As a result, a constant number of chemoattractant molecules per unit target-surface area per unit time are released into the surroundings. Our assumption of a constant source flux j_0 accurately reflects this physiological-like reality (at the current level of simplification). This assumption is also supported by our observation that the vigor of the chemotactic response of a given neutrophil does not appear to diminish over the time course of an experiment.

  As seen from Eq. (S4), the concentration of chemoattractant $c(\Delta r, t)$ is scaled by the source flux j_0. The prediction of absolute concentration values using Eq. (S4) would thus require knowledge of the value of j_0, which in turn depends on the chemical composition of the particle surface. As mentioned in the main text, we hope to be able to estimate the value of j_0 in future work.

- *The formation of the anaphylatoxic cloud is assumed to be unaffected by nearby walls.* The problem setup in Eqs. (S1)-(S3) assumes that the radial symmetry of the considered scenario extends to infinity. In practice this means that the space available for unobstructed diffusion of chemoattractant should be large compared to the source size. We generally try to ensure that this is the case by conducting experiments at a height of at least ten source radii above the

chamber bottom. Any effects on the spatiotemporal distribution of anaphylatoxins due to the presence of micropipettes and the pipette-held immune cell are neglected.

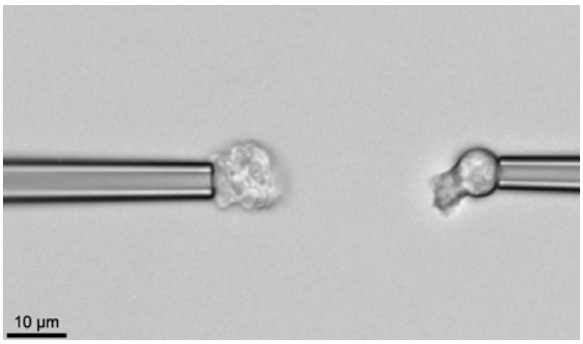
- *The used parameter values are assumed to be valid estimates.* Our prediction of the concentration profile of anaphylatoxins relies on estimates of the diffusion coefficient D and the kinetic rate constant k of the removal reaction (for details see (3)). Our estimate of D is based on the Stokes-Einstein equation. The size of the diffusing molecules was obtained from the molecular weight of C5a using the empirical formula provided in (4). The viscosity of the used buffer was measured using a custom-designed microliter viscometer.

  Our estimate of k is based on a linear approximation of quasi-steady-state Michaelis-Menten kinetics, assuming that the concentration of anaphylatoxins is small compared to the Michaelis constant of the carboxypeptidase that deactivates C5a. Our value of k is a representative average based on a fairly large spread of kinetic parameters of carboxypeptidase N found in the literature.

The above discussion shows that our model (Eq. (S4)) is physically realistic, biologically plausible, and based on reasonable assumptions. It captures the biophysical and biochemical processes known to underlie the formation of the anaphylatoxic cloud. We have not encountered discrepancies between the predictions of this model and our experimental observations.

For example, the behavior of human neutrophils in hundreds of previous single-cell chemotaxis experiments was consistent with the existence of a rapidly forming steady-state distribution of chemoattractant. At cell-target distances greater than the recognition distance, neutrophils did not respond to the presence of a target, no matter how long we waited. If, after a positive neutrophil response, the target was removed beyond the recognition distance, the cell started retracting the pseudopod in a matter of seconds. Similarly, if the target was moved to a different side of the cell in a wide arc, the cell responded by starting to retract the former pseudopod within seconds and forming a new pseudopod toward the repositioned target. On the other hand, if the target was kept within the recognition distance as it was slowly repositioned, the pseudopod appeared to follow the target motion. These observations support that, as long as the source of chemoattractant moves sufficiently slow, one may picture the 3D distribution of chemoattractant as a stationary cloud that persistently surrounds the target. This evidence of the existence of a steady state underlines the importance of the inclusion of a removal term in realistic mathematical models. As mentioned in the main text, the removal of potent chemoattractants like anaphylatoxins is also of vital physiological importance. Our model includes the simplest possible type of removal reaction that ensures the existence of a steady state.

Movie Legends



Movie S1. Human neutrophil as a sensitive biodetector of chemoattractant. The video illustrates how a human neutrophil and a cluster of β-glucan particles (both held at the tip of micropipettes) are initially placed at a large distance from each other. The cell-target distance is then stepwise reduced every ~2 min. The distance at which the neutrophil first forms a pseudopod directed toward the cluster of β-glucan particles marks the threshold at which the cell detects the presence of anaphylatoxins produced at the target surface. The real-time duration of this experiment was ~14 min.

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