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Immobilized IL-8 Triggers Phagocytosis and Dynamic Changes in Membrane Microtopology in Human Neutrophils

Michael T. Beste[†], Elena B. Lomakina[‡], Daniel A. Hammer[†], and Richard E. Waugh[§]

[†]Departments Chemical and Biomolecular Engineering, University of Pennsylvania, Philadelphia, Pennsylvania

[‡]Department of Pharmacology and Physiology, University of Rochester, Medical Center, Rochester, New York

[§]Department of Biomedical Engineering, University of Rochester, Medical Center, Rochester, New York

Abstract

The interaction of leukocytes with surface bound ligands can be limited by the location of the molecules relative to the surface topology of the cell. In this report, we examine the dynamic response of neutrophils to IL-8 – fractalkine chimera immobilized on bead surfaces taking into account changes in receptor occupancy resulting from changes in surface topography. As a readout for receptor signaling, we observe the dynamics of calcium release in neutrophils following contact with the IL-8 coated surface. After a delay that depended on the initial area of contact and the surface density of IL-8, the cell began to phagocytose the IL-8 coated bead. This appeared to be a pre-requisite for release of calcium, which typically followed shortly after the initiation of phagocytosis. In separate experiments, effective kinetic coefficients for the formation of bonds between immobilized IL-8 and receptors on the cell surface were determined. Using these coefficients, we were able to estimate the number of bound receptors in the nascent contact zone. Kinetic modeling of the signaling response predicted that cell spreading and a concomitant increase in the density of occupied receptors would be required for the experimentally observed calcium dynamics. Postulating that there is an increase in receptor occupancy resulting from smoothing of the cell surface as it is stretched over the bead enabled us to obtain model predictions consistent with experimental observations. This study reveals the likely importance of membrane microtopology as a rate-limiting property and potential means of regulation of cell responses stimulated by two-dimensional surface interactions.

INTRODUCTION

A wide variety of biological phenomena in leukocyte biology are regulated by receptor-ligand interactions that occur at a two-dimensional cell-cell or cell-substratum interface. Notable examples include cell adhesion (1), signal activation within molecular synapses (2),

To whom correspondence should be addressed: Richard Waugh, University of Rochester, Box 270168, Rochester, NY 14627, Phone: 585-275-3768, waugh@bme.rochester.edu, Daniel A. Hammer, University of Pennsylvania, 240 Skirkanich Hall, 210 S. 33rd St., Philadelphia, PA 19104, Phone: 215-573-6761, hammer@seas.upenn.edu.

pathogen phagocytosis (3), and haptotaxis (4). Significant progress has been made towards characterizing these processes in terms of the intrinsic kinetic properties that govern molecular recognition, particularly through the use of two-dimensional binding assays (5–7). However, there is growing appreciation that the observed receptor-ligand kinetics are strongly influenced by how effectively receptors and/or ligands are presented at the interface. Importantly, a highly irregular microtopology between opposing cell surfaces due to the presence of microvilli and surface folds may significantly restrict receptor and/or ligand presentation, resulting in limited bond formation (8, 9). In such geometries, remodeling of the cell surface morphology – in response to mechanical deformation or active cell processes – may significantly enhance ligand presentation and the rate of adhesion and/or cell activation.

We previously reported that microbeads coated with the chemokine interleukin 8 (IL-8) specifically bind receptors on the surface of resting human neutrophils (10). Unexpectedly, adherent beads were phagocytosed within several minutes of initial cell contact. Engulfment occurred more frequently at high IL-8 densities, suggesting that passive engagement of a threshold number of IL-8 receptors was sufficient to trigger pseudopod formation and extension over the bead surface. Active engulfment was predicted to further enhance IL-8 presentation, but the dynamics and magnitude of this enhancement remained uncertain as receptor occupancy could not be probed beyond the passive binding phase.

In this report, we use a combination of micromanipulation, fluorescence imaging, and mathematical modeling to further explore the dynamics of receptor engagement by neutrophils at a two-dimensional interface. In particular, we rely on intracellular calcium flux as an auxiliary reporter for CXCR1/2 ligation to characterize how active membrane processes leads to enhanced ligand binding. By comparing the dynamics of bead engulfment and calcium flux, we demonstrate that the timing and progression of receptor-mediated signals leading to calcium release are tightly coupled to the onset of phagocytosis. Together with simulated estimates of receptor occupancy, our results point to a critical role for surface remodeling as a mechanism that enhances intimate contact and receptor presentation between neutrophils and their substrate.

ANALYSIS

Binding Kinetics Between Resting Neutrophils and Immobilized IL-8

We first consider receptor binding in the initial moments following contact between immobilized IL-8 and resting neutrophils during a micromanipulation experiment (Fig. 1). During this initial phase the macroscopic contact area A_c is approximately constant, and we assume that the interfacial topology between the cell and bead surface remains unperturbed. For a bead radius R_b , A_c is related to the projected contact length, L_c , by

$$A_c = 2\pi R_b \left(R_b - \sqrt{R_b^2 - \left(\frac{L_c}{2}\right)^2} \right). \quad \text{Eq. 1}$$

Provided that bond formation follows first-order kinetics and does not significantly deplete available IL-8 from the contact area, the time-dependent increase in expected bond number, $\langle n \rangle(t)$, may be related to the two-dimensional equilibrium constant, K_a , and the intrinsic dissociation rate constant, k_r (5, 7),

$$\langle n \rangle(t) = \phi_0 A_c \rho_r \rho_l K_a (1 - e^{-k_r t}), \quad \text{Eq. 2}$$

where ϕ_0 is the initial fraction of the macroscopic contact area available for binding, and ρ_r and ρ_l are the density of receptors and ligands on the cell and bead surfaces, respectively. The parameter ϕ_0 encapsulates the effects of topological features (i.e. microvilli and surface folds) which limit the effective contact area to a value less than A_c (8). In practice, ϕ_0 is indeterminate and lumped into an apparent equilibrium constant $K_a' = \phi_0 K_a$.

Cell-Activated Receptor Engagement

The onset of a second, active phase of IL-8 presentation is marked by the development of a phagocytic pseudopod at the perimeter of the contact area (Fig. 1 B). Pseudopod extension proceeds until the entire bead is encapsulated into a phagosome, which subsequently moves to the cell interior as the neutrophil attempts to reestablish a spherical morphology (10, 11). During this process, the neutrophil must redistribute plasma membrane at its outer surface to accommodate the complete internalization of the bead. While various modes of membrane expansion have been postulated, our present analysis is concerned with active cell processes (e.g. cytoskeletal polymerization) that increase membrane cortical tension and unfurl surface wrinkles and villi (12). Mechanical ‘unzipping’ of these surface features is predicted to significantly augment the extent of microscopic contacts between the bead and cell, giving rise to an effective increase in the apparent binding affinity, K_a' .

To estimate when microscopic surface features disappear during the course of engulfment, we consider the evolution of the visible cell surface area and its relation to the membrane cortical tension. Prior to stimulation, the macroscopic surface area of the aspirated cell, A_a , is given by the relation (derived from geometrical considerations):

$$A_a = 4\pi R_a^2 + 2\pi R_p \left[L_p - (R_0 - \sqrt{R_0^2 - R_p^2}) \right] \quad \text{Eq. 3}$$

where R_0 is the resting cell radius (4.5 μm), $R_a = R$ is the aspirated cell radius (4.36 μm), R_p , is the pipette bore radius (1.6 μm), and L_p is the cell protrusion length into the pipette (4.4 μm) (Fig. 1 A). For simplicity, we assume that the total area of expansion during engulfment is directed to the formation of a spherical phagocytic cup. This allows the total visible surface area of the cell, $A(t)$, to be approximated as

$$A(t) = A_a + 2 [A_c(t) - A_c(0)] \quad \text{Eq. 4}$$

where $A_c(t)$ is now the observed time-dependent increase in macroscopic contact area. While this geometry idealizes the physical situation, we believe it provides a plausible upper limit on $A(t)$ during the duration of engulfment.

Following the analyses of Herant and colleagues, increases in visible surface area $A(t)$ may be related to changes in the membrane cortical tension, $\gamma(t)$, during the process of area expansion (11, 13). The total cortical tension arises from the sum of elastic and viscous contributions:

$$\gamma(t) = \gamma_0 + k \frac{A(t) - A_0}{A_0} + v \frac{dA/dt}{A(t)} \quad \text{Eq. 5}$$

where γ_0 is the resting surface tension, k is the elastic expansion modulus, $A_0 = 4\pi R_0^2$ is the resting cell area, and v is the surface viscosity. For fractional expansion up to an elastic threshold of 26%, ($A/A_0 \leq 1.26$) Herant et al. report $\gamma_0 = 0.010$ mN/m, $k = 0.16$ mN/m and $v = 0$ mN/m; above 26%, $\gamma_0 = -0.50$ mN/m, $k = 2.14$ mN/m, and $v = 75$ mN/m (13). Combining Eqs. 4 and 5 thus yields an approximate model for the membrane cortical tension as a function of observable changes in visible cell surface area. In our subsequent analysis, we consider how the dynamics of cortical tension correlate with the observed cell response to IL-8 presentation.

Kinetic Model for Receptor-Mediated Calcium Release

During the passive phase of receptor binding, CXCR1/2 occupancy may be assessed directly by repeatedly measuring the probability for specific adhesion upon removing the bead from the cell surface. This method becomes inefficient and eventually impossible during the engulfment phase, as the strength of the adhesive contact becomes too great to separate cell and bead. To circumvent this difficulty, we turned to measurements of intracellular Ca^{2+} flux as an indirect reporter for IL-8 recognition (14, 15).

To define the quantitative relationship between IL-8 receptor occupancy and Ca^{2+} release, we developed a kinetic mass-action model of signaling events based on previous models for G-protein coupled receptor (GPCR) signal transduction (16–18). Mechanistically, the reaction network describes GPCR ligation (19), collision-coupled G-protein activation (20), catalytic activation of PLC (18, 21), and IP_3 regulated Ca^{2+} flux between the endoplasmic reticulum and cytosol (22). A single activated receptor can activate many G-proteins. Listed in Table I, the composite reaction network consists of nine association/catalysis reactions and four transport rates characterizing Ca^{2+} flux. This represents a minimal reaction network required to generate agonist-stimulated Ca^{2+} oscillations and does not encompass other well-characterized features of Ca^{2+} regulation such as store-operated influx or IP_3 channel inhibition (17, 18). Our principal goal in formulating a simplified model is to provide a means for inferring the dynamics of IL-8 binding at the cell-bead interface; incorporating more complex mechanistic descriptions will be the focus of future work. Requisite parameter values for the reaction and transport mechanism were selected by calibrating model predictions against published dose-response and time series data for IL-8 signaling responses in human neutrophils (23–26). A detailed description of the parameter calibration procedure used to obtain the optimized values given in Table I is discussed in Supporting Materials.

Within the model, the aspirated neutrophil is abstracted as a spherical membrane enclosing a cytosolic compartment of constant volume. An impermeable spherical core comprising 37%

of the total cell volume represents the intracellular space excluded by the nucleus, granules, and mitochondria (27, 28). Rather than simulating the complex evolution of membrane morphology during phagocytosis, bead engulfment is idealized by defining an equivalent circular contact area (corresponding to A_c) that enlarges over time towards a maximum, $A_c(t \rightarrow \infty) = 4\pi R_b^2$. IL-8 molecules are presented exclusively within this contact area while the model cell retains its spherical geometry. Accordingly, the IL-8 coating density and observed contact area dynamics represent experimental input to the calculation. To simulate a dynamic increase in the microscopic contact area - the principal unknown in the experimental setup - the fractional contact area, $\phi(t)$, is varied according to the desired temporal profile.

Because the cytosolic components of the signaling pathway are highly diffusive ($D = 10^2 \mu\text{m}^2/\text{s}$), the model neglects intracellular gradients and treats the cytoplasm as a single compartment. In contrast, membrane-associated signaling components are expected to be significantly less mobile ($D = 10^0 \mu\text{m}^2/\text{s}$) and may remain spatially segregated following a heterogeneous surface stimulus (29). To resolve such effects, the cell membrane is discretized into coaxial surface elements along the polar dimension defined by the micropipette axis. The level of discretization is chosen such that incremental changes in A_c fall below 5% of the maximal contact area. After populating the cytosol and each surface compartment with a number of molecules corresponding to the equilibrium concentration (Table II), the signaling network is evolved according to the reaction-diffusion master equation (30). We generate solutions to the master equation using the Next Subvolume Method, which executes reaction and diffusion events within each surface element in order of their relative propensity (31).

MATERIALS AND METHODS

Bead Preparation

Procedures for chemokine immobilization have been published previously (10). Briefly, two sizes (2.8 μm and 4.5 μm diameter) of protein G coated beads (Dynal, Lake Success, NY) were coated with recombinant chimera of IL-8 with the mucin-like stalk of human fractalkine terminated with a His-Tag sequence. Beads were first treated with BlockAid solution (Molecular Probes, Eugene, OR) to reduce non-specific binding, then coated with antibody against His-Tag, using dimethyl pimelimidate (Sigma, St. Louis, MO) to cross-link the Fc portion of antibody to the protein G. Ten micrograms of IL-8/chimera was added to enable binding of the His-Tag sequence on the chimera to the anti-His-Tag antibody on the beads. Beads were stored at 4°C in the buffer containing 0.1% sodium azide and the chimera. The density of protein binding sites on the surface of the beads was determined using flow cytometry. Beads were incubated at 4°C overnight with FITC conjugated antibody against human IL-8 (Clone #6217, R&D Systems) or with FITC-conjugated isotype control. To correlate fluorescence intensity with the number of bound antibodies on the beads, the fluorescence signal was calibrated using Quantum Simply Cellular Beads (Flow Cytometry Standards Corp., Fishers, IN). To correct for non-specific binding, the number of non-specific “sites” detected using isotype control antibody was subtracted from the total number of sites detected using the specific antibody. In the dynamic response experiments, coating

densities were determined to be 28,000 sites/bead (990 sites/ μm^2) for small, 2.8 μm diameter beads and 6,500 sites/bead (90 sites/ μm^2) for large, 4.5 μm diameter beads.

Two-Dimensional IL-8 Binding Kinetics

Binding kinetics between immobilized IL-8 and surface CXCR1/2 receptors were measured using a protocol previously adapted by our laboratory to quantify adhesion kinetics between immobilized ICAM-1 and LFA-1 (7). Neutrophils were obtained from healthy donors by diluting a drop of peripheral blood in 4% FBS in BSS (balanced saline solution: 5 mM KCl, 146 mM NaCl, 5.5 mM Glucose, 1 mM Ca^{2+} , 1 mM Mg^{2+} made with low endotoxin water obtained from Invitrogen Corp.) containing 10 mM N-[2-Hydroxyethyl] piperazine-N'-[2-ethanesulfonic acid] (HEPES, Sigma, Saint Louis, MO), pH 7.4, 290 mOsm. 2.8 μm diameter beads were prepared at low IL-8 density by mixing IL-8 chimera with the identical construct but with IL-8 replaced with monocyte chemotactic protein-1 (MCP-1, R&D Systems). The final IL-8 concentration was 80 sites/ μm^2 . MCP-1 coated beads were used as controls for non-specific binding. Cells and beads were placed together in a chamber on the microscope stage and two micropipettes were used to manipulate a single cell into repeated contact with a ligand-coated bead for controlled durations. The adhesion probability, $P_{adhesion}$ (corrected for non-specific binding) was determined as the fraction of contacts that resulted in cell deformation upon separation. The expected bond number in the contact zone, $\langle n \rangle$ is directly related to the adhesion probability (5),

$$\langle n \rangle = -\ln(1 - P_{adhesion}). \quad \text{Eq. 6}$$

To ensure that the number of IL-8 molecules presented to the cell was approximately constant between test cycles, the macroscopic contact area was maintained at $\sim 5 \mu\text{m}^2$. The apparent two-dimensional binding affinity, K_a' , and intrinsic dissociation rate, k_r , were determined by fitting the dynamic increase in expected bond number to Eq. 2.

Calcium Mobilization

Changes in intracellular free calcium concentration were measured using the fluorescent probe fluo-4 as described previously (32). Neutrophils in 3 μl whole blood from finger sticks were incubated in HBSS containing 10 mM HEPES, 0.1% BSA and 0.05 μM fluo-4 for 30 min at 37°C, washed, and resuspended in HBSS. For inhibition experiments, cells were co-loaded with 20 μM 1,2-Bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (BAPTA) for 30 min at 37°C prior to Ca^{2+} mobilization experiments. Cells were diluted in 4% FBS in BSS plus 10 mM HEPES and placed in a chamber on the microscope stage with two micropipettes. As a cell and IL-8 coated bead are brought into contact, the progression of phagocytosis is observed and recorded while fluorescence emission at 488 nm was measured using a spectrofluorometer (Photon Technology International, Birmingham, NJ) attached to the microscope.

RESULTS

Binding Kinetics Between Immobilized IL-8 and CXCR1/2

Binding kinetics between immobilized IL-8 and surface CXCR1/2 receptors on resting neutrophils were determined by monitoring the time-dependent adhesion between IL-8 coated beads and neutrophils as described in Materials and Methods. Although CXCR1 and CXCR2 are known to bind IL-8 in solution with slightly distinct affinities (33), the 2-D forward and reverse binding rates are most likely limited by factors other than intrinsic molecular affinities, and can be assumed to be identical. The transient increase in expected bond number illustrated in Fig. 1 exhibited first order kinetics that were fit well by Eq. 2 ($R^2 = 0.72$). The apparent two-dimensional affinity and reverse binding rate were estimated to be $K_a' = 6.0 \pm 0.7 \times 10^{-6} \mu\text{m}^2$ and $k_r = 0.49 \pm 0.19 \text{ s}^{-1}$, respectively. Maximum adhesion was achieved within 10 s of contact.

Active CXCR1/2 Engagement During Phagocytosis

To examine receptor engagement during phagocytosis, bead engulfment and calcium flux in fluo-4 loaded neutrophils were measured simultaneously following contact with IL-8 coated beads. Illustrated in Figure 2, beads were briefly held to the neutrophil surface (< 5 s) and released. After a variable latent period, cells formed a phagocytic pseudopod that quickly spread over the bead surface. Phagocytosis was quantified by recording the transient increase in macroscopic contact area, $A_c(t)$, at 5 s intervals until beads were fully engulfed (Fig. 3B). The observed increase in contact area could be described by a phenomenological, dynamic function of the form

$$A_c(t) = \frac{4\pi R_b^2 - A_c(0)}{1 + e^{-\alpha(t-\tau)}} + A_c(0) \quad \text{Eq. 7}$$

where α is the characteristic rate for phagocytosis and τ is the time for half-maximal engulfment. Both large diameter beads (4.5 μm) coated at 90 IL-8 molecules/ μm^2 and small diameter beads (2.8 μm) coated at 990 IL-8 molecules/ μm^2 were completely engulfed. Control beads coated at comparable densities of E-selectin remained adherent to the neutrophil surface but were not engulfed, indicating that the phagocytic response was specific for IL-8 (data not shown).

After a similar latent period, fluo-4 fluorescence intensity throughout the cytosol exhibited a sharp rise and subsequent decline to resting levels, indicative of a diffuse, transient calcium wave (Fig. 2C). Stimulating neutrophils in calcium-depleted media resulted in a marginal reduction in the calcium response, implicating intracellular stores as the dominant source of calcium entry (data not shown). In all experiments, intracellular calcium remained at resting levels immediately following contact with IL-8 coated beads and throughout the period of passive IL-8 engagement. The initial calcium ‘burst’ was consistently rapid, but exhibited slight variations in magnitude and duration between experiments.

Prior studies have established that neutrophil phagocytosis in response to immunoglobulin (IgG) and complement (C3bi) presentation proceeds via calcium-dependent and independent mechanisms, respectively (34, 35). To rule out the possibility that phagocytosis resulting

from IL-8 stimulation was dependent upon an increase in intracellular calcium, cells were pretreated with the calcium chelator BAPTA. Calcium levels in buffered cells remained at baseline levels following contact; however, the phagocytic response was unchanged (data not shown). These observations were consistent with previous data linking actin polymerization and pseudopod formation to a distinct calcium-independent pathway downstream of CXCR1/2 ligation (36).

Repeated observations of IL-8 stimulation revealed significant variability in the latent period, t_{engulf} , between bead contact and visible detection of engulfment. Engulfment occurred within 15–100s following contact with small beads coated at 990 sites/ μm^2 and within 20–200s for large beads coated at 90 sites/ μm^2 . We hypothesized that such substantial variation in response timing reflected an underlying variability in the small number of CXCR1/2 receptors (< 15) engaged at the cell-bead interface. To explore this possibility, beads were mechanically impinged upon the neutrophil, thereby increasing the macroscopic contact area and total number of IL-8 molecules available to the neutrophil. Shown in Figure 3, both mean response time and population heterogeneity decreased significantly with increasing $A_c(0)$. Using the apparent binding affinity ($K_a' = 6.0 \times 10^{-6} \mu\text{m}^2$) and a total of 9.5×10^4 IL-8 receptors per cell (10) to convert the imposed contact area to an estimated number of bound IL-8 receptors, response data for small and large beads collapsed onto a single curve describing an inverse relationship between the response time and number of receptors bound during passive ligand presentation (Fig. 3C). These data suggested that the neutrophil engulfment machinery is exquisitely sensitive to IL-8 presentation and can initiate a robust phagocytic response in response to as few as 10 bound IL-8 receptors.

The observed cell-to-cell variation in t_{engulf} was closely mirrored in the timing of calcium release, t_{release} , (Fig. 4). Careful inspection of all micromanipulation experiments revealed that t_{release} consistently lagged but never preceded the onset of engulfment, regardless of the initial contact area. The delay between engulfment and calcium release $t_{\text{release}} - t_{\text{engulf}}$, was likewise variable (2–49 s), but statistically independent of IL-8 density (Fig. 4B, inset). In all but a few experiments, calcium release commenced prior to complete bead engulfment. This strong temporal coupling indicated that active cell spreading over the surface of large and small diameter beads was a necessary prerequisite to transmitting robust activation signals via CXCR1/2 receptors.

Cellular Remodeling at the Binding Interface

Surprisingly, calcium transients in neutrophils presented with small and large microbeads were similar both in magnitude and duration, despite a ten-fold difference in IL-8 coating density (Fig. 5). Although we first hypothesized that this similarity might reflect a saturated signaling response, a simple stoichiometric comparison revealed that both small and large beads contained only enough IL-8 molecules to ligate a fraction of the available CXCR1/2 receptors. Specifically, small beads could maximally bind 29% (28,000 IL-8/95,000 CXCR1/2) and large beads 7% (6,500 IL-8/95,000 CXCR1/2) of the total number of surface receptors. Based on previous studies of receptor occupancy and calcium release in response to soluble IL-8 (23, 26), we concluded that neither group of microbeads were capable of

stimulating the full dynamic range of the calcium response and that the observed transients fell below the saturation limit.

We instead reasoned that the response similarity between small and large bead stimuli could reflect underlying differences in the extent of microscopic contacts within the visible contact interface. To assess this possibility, we considered the evolution of $A_c(t)$ and its relation to both visible and imperceptible contributions to IL-8 presentation. Figs. 6 A–C illustrate this comparison for the corresponding calcium dynamics in Fig. 5 A. Phagocytosis of large diameter beads required a fractional area expansion exceeding 50% of the resting cell surface area compared 23% for small diameter beads (Fig. 5 B). Accounting for the respective IL-8 and CXCR1/2 receptor densities, bond formation with small, high density beads was limited by the number of available CXCR1/2 receptors, whereas the number of IL-8 molecules limited bond formation for large, low density bead stimuli (Fig. 5 C).

Applying Eq. 5 indicated that large beads exceeded the elastic threshold of 26% area expansion early during engulfment, consistent with previous findings that internalization of microbeads larger than $\sim 3 \mu\text{m}$ in diameter required viscous membrane expansion (13). This transition to viscous expansion is appropriately reflected in the sudden, discontinuous increase in the estimated membrane cortical tension (Fig. 5 D). Although the discontinuity reflects an artifact of discontinuous parameters for Eq. 5, the overall forty-fold increase in cortical tension strongly suggested that engulfment of large beads required significant, if not complete, unfurling of topological membrane features during the internalization process. In contrast, a modest two-fold increase in cortical tension indicated that an equivalent level of membrane remodeling was unlikely for small diameter beads, whose internalization remained within the elastic expansion regime. Because the resolution of topological features will enhance the fractional cell-bead contact area, the predicted twenty-fold disparity in cortical tension is indicative of a substantial difference in the extent to which neutrophils engaged IL-8 molecules while engulfing large versus small diameter beads. This discrepancy may in fact offset the relative difference in IL-8 density and contribute to the similar level of calcium release observed between bead sizes.

Simulations of Receptor Engagement During Phagocytosis

To further characterize how the morphology of the contact interface changes dynamically during bead engulfment, we performed simulations of CXCR1/2 receptor binding using the kinetic model for receptor-mediated calcium release. Because the magnitude and dynamics of fractional contact area remained indeterminate in the micromanipulation assay, we supplied model calculations with candidate temporal profiles for $\phi(t)$, and inferred which dynamical profile best reproduced the features of observed calcium transients (Fig. 6). In all simulations, we considered the engulfment of small diameter beads coated at 990 IL-8 sites/ μm^2 using the measured kinetic parameters $K_a' = \phi_0 K_a = 6.0 \times 10^{-6} \mu\text{m}^2$ and $k_r = 0.49 \text{ s}^{-1}$. The evolution of the macroscopic contact area was specified by Eq. 9 using characteristic values $R_b = 1.4 \mu\text{m}$, $A_c(0) = 5 \mu\text{m}^2$, $\alpha = 0.1 \text{ s}^{-1}$, and $\tau = 45 \text{ s}$.

In the simplest (albeit unrealistic) scenario, we assumed that the fractional contact area increased by a constant factor over the initial value, $\phi(t)/\phi_0 = 10^0\text{--}10^3$, immediately following cell-bead contact (Fig. 6A). Importantly, a minimum ten-fold increase over ϕ_0

was necessary to elicit any detectable calcium response; the increase in macroscopic contact area due to engulfment alone was insufficient to engage the minimum number of IL-8 receptors required to stimulate downstream effectors. During the latent binding phase that preceded phagocytosis, the model predicted that an instantaneous rise in fractional contact area ten-fold or greater would stimulate significant calcium mobilization. Such an early response was conspicuously absent in all experiments. Furthermore, the second phase of calcium release during the ensuing engulfment period was predicted to be gradual and prolonged, which contrasted with the sharp rise in fluorescent intensity observed experimentally. We therefore concluded that significant changes in membrane topology do not occur during the passive binding phase and begin only after engulfment has commenced.

We next considered the limiting scenario in which the fractional contact area increased by a constant factor only at the conclusion of engulfment, $\phi(t > 70 \text{ s})/\phi_0 = 10^0\text{--}10^3$ (Fig. 6B). In each case, the model predicted very limited receptor engagement during the passive binding phase and phagocytosis, and calcium release was accordingly inhibited. The predicted increase in receptor binding at the moment of contact area enhancement was rapid, and reflected in a sharp rise in calcium concentration. This more closely mimicked experimental observations, with the exception that in the experiments, calcium release nearly always began before engulfment was complete.

Finally, we simulated an intermediate scenario in which the microscopic contact area increased 10^3 -fold at variable times during bead engulfment. On average, neutrophils phagocytosed small diameter beads within 30 s of pseudopod formation. We therefore modeled increases in fractional contact area 15 s prior to, at, and 15 s after half-maximal engulfment, $\phi(t > 30 \text{ s}, 45 \text{ s}, 60 \text{ s})/\phi_0 = 10^3$ (Fig. 6C). The predicted calcium response was rapid at each interval, but both the magnitude and duration of the response varied with timing. Specifically, a sudden increase in ϕ early during phagocytosis resulted in a calcium wave smaller in absolute magnitude and longer in duration. This dynamic reflected an underlying asynchrony in receptor engagement and downstream signaling: effectors activated by receptors bound early during engulfment became attenuated as additional receptors entered the contact area, resulting in a sub-maximal and prolonged calcium response. Delaying the increase in ϕ to later times within the engulfment phase minimized asynchronous IL-8 binding and resulted in calcium waves of greater magnitude and shorter duration. This systematic variation between calcium wave magnitude and duration was similarly observed in micromanipulation experiments (cf. Fig 4A), confirming that topological changes at the cell-bead interface likely occur at variable times during bead engulfment.

DISCUSSION

A recurring dilemma in assaying two-dimensional binding kinetics between leukocyte receptors and their ligands has been determining the relative importance of topological effects at the binding interface. Molecules must be placed in sufficient proximity for lateral and rotational diffusion to facilitate molecular collisions, but the presence of surface features often restrict such intimate contact. Because fine membrane foldings cannot be resolved by light microscopy, the fraction of the visible contact area that is truly available for binding

during kinetic experiments remains unknown and is generally lumped into an apparent binding affinity (Eq. 2) (5). This stopgap approach has in fact yielded excellent insights into the kinetic properties of a number of complexes including CD16/IgG, LFA-1/ICAM-1, and P-selectin/PSGL-1 (9, 37, 38). Here, we extend these measurements by providing the first report of two-dimensional binding parameters between an immobilized chemokine, IL-8 and its two counter receptors, CXCR1 and CXCR2. Both the chemokine-receptor dissociation rate ($k_r = 0.49 \text{ s}^{-1}$) and apparent binding affinity ($A_c K_a' = 3.0 \times 10^{-5} \mu\text{m}^4$) were strikingly similar in magnitude to other high-affinity leukocyte receptor-ligand interactions (8, 38, 39).

Despite the relative ease in assessing apparent rates of molecular association, the desire remains to identify the precise dynamics by which cells conform to their surroundings, particularly in regard to engaging effector molecules that stimulate cell activity. The methods presented herein constitute a novel approach to address this challenge. Specifically, we take advantage of well-characterized signaling events that occur downstream of CXCR1/2 binding to infer the dynamics of the otherwise invisible surface morphology at the two-dimensional contact interface. For human neutrophils interacting with immobilized IL-8, the morphological response appears to hinge upon the activation of cell spreading and pseudopod extension, which in the present case culminates in microsphere phagocytosis.

In the moments immediately following contact, the absence of a detectable calcium response suggests that the resting membrane microtopology remains largely intact. This conclusion is supported by a number of earlier observations of leukocyte-substratum adhesion. In rat basophilic leukemia cells, membrane remodeling relies entirely upon passive redistribution of surface receptors (40). Electron micrographs revealed that long-range diffusion of IgE receptors into a two-dimensional contact interface alone was sufficient to resolve membrane ruffles following contact with fluid vesicles. However, this passive contact maturation took place over time scales much longer (5–10 min.) than those observed in the present study. Similarly long time scales have been described for the development of mature immunological synapses between T lymphocytes and antigen presenting surfaces (2). In this latter case, diffusive segregation of peptide-MHC/TCR complexes and LFA-1/ICAM-1 is thought to limit the rate at which lymphocytes achieve close apposition with their underlying substrate. Thus, the contribution of passive mechanisms to contact maturation (namely diffusion) is likely minor for neutrophils encountering immobilized IL-8 in our micromanipulation experiments.

Remarkably, neutrophils proceeded to spread over the surface of microbeads despite minimal levels of CXCR1/2 engagement (~ 25 bound receptors). While our data do not exclude the possibility of a purely mechanical response mechanism, they do imply that low levels of IL-8 signaling are sufficient to trigger the reorganization of cytoskeletal components near the contact interface. The time required to mount this response was highly variable, but markedly accelerated by enlarging the size of the initial contact region. In a previous report, we demonstrated that contact area enlargement is expected to be significant in the physiological context of neutrophil rolling as the result of mechanical indentation (41). Therefore, we expect that the activation of cell spreading may likewise be sensitized in high shear environments, where adherent neutrophils will be subjected to significant deformation.

A key variable that will remain subject to further refinement is the initial fraction of visible membrane area that is oriented in sufficient proximity to facilitate receptor/ligand binding. Chesla and co-workers estimated that CHO cells – which share a similar surface morphology with human neutrophils – only engage 2.5% of a smooth contact interface (5). Our simulations of receptor engagement predict that detectable calcium release requires a minimum ten-fold increase in fractional contact area, which would similarly put the initial fractional contact area at or below 10%. These changes in membrane area are driven largely by the cortical tension. We envision that other complementary experimental approaches may help shed light on this elusive property, as well as the overall dynamics of cell surface remodeling. Such methods including total internal reflection fluorescent microscopy (TIRF) and reflection interference contrast microscopy (RICM) (42, 43). Both techniques are capable of resolving membrane-substrate separation distances on molecular length scales, which would facilitate a direct quantitation of focal contacts. These methodologies should prove particularly useful for receptor-ligand interactions that occur in the absence of active membrane remodeling.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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FIGURE 1. Micromanipulation Set-up for Neutrophil Stimulation

(A) Video micrograph of IL-8 coated microbead contact with aspirated neutrophil. The projected contact length, L_c was determined using on-screen calipers. Bead radius, R_b , aspirated cell radius, R_a , pipette bore radius, R_p ($2\ \mu\text{m}$) and cell protrusion length, L_p , are indicated. (B) Pseudopod extension at the onset of phagocytosis. (C) The apparent affinity ($K_a' = 6 \times 10^{-6}\ \mu\text{m}^2$) and dissociation rate ($k_r = 0.49\ \text{s}^{-1}$) for IL-8 binding were determined by fitting the first order increase in expected bond number, $\langle n \rangle$, with Eq. 2.

FIGURE 2. Phagocytosis and Calcium Release in Stimulated Neutrophils

(A) Characteristic video and fluorescent micrographs of bead engulfment at indicated time points. (B) Phagocytosis was quantified as the change in macroscopic contact area, $A_c(t)$ at 5 s intervals following stimulation. The dynamics of $A_c(t)$ were parameterized according to Eq. 7. The onset of phagocytosis, t_{engulf} , was defined by the first visible signs of pseudopod extension. (C) Calcium dynamics corresponding to the cell response in (B). Changes in intracellular calcium concentration were determined by fluo-4 fluorescence intensity. The

beginning of calcium release, t_{release} , was defined at the onset of fluorescence rise over baseline levels.

FIGURE 3. Influence of Initial Contact Area on Bead Phagocytosis

(A) Individual cell responses following contact with large, 4.8 μm diameter beads coated at 90 IL-8 sites/ μm^2 (○) and small, 3.0 μm diameter beads coated at 990 IL-8 sites/ μm^2 (●). (B) Data from (A) grouped according to initial contact area, $A_c(0)$. Error bars represent the standard deviations for the distributions of contact area (horizontal) and onset of engulfment (vertical). (C) Eq. 2 was used to convert the mean $\pm$ s.d. values of $A_c(0)$ to the equivalent number of bound IL-8 molecules.

FIGURE 4. Timing Analysis of Phagocytosis and Calcium Release

(A) Characteristic variations in t_{engulf} and t_{release} for small diameter beads coated at 990 IL-8 sites/ μm^2 . Response profiles were synchronized to the time of initial bead-cell contact ($t = 0$). (B) Correlation between t_{release} and t_{engulf} for small and large beads. For all observed cells, calcium release lagged the onset of phagocytosis indicated by points lying above $t_{\text{release}} = t_{\text{engulf}}$. Variation in the delay between engulfment and calcium response was statistically insignificant between low and high IL-8 density stimuli.

FIGURE 5. Calcium and Membrane Dynamics During Phagocytosis of Small and Large Diameter Beads

(A) Characteristic calcium dynamics observed upon engulfing small, high IL-8 density beads (left) and large, low IL-8 density beads (right). (B) Experimental $A_c(t)$ dynamics were used to determine the fractional area expansion according to Eq. 4. (C) Number of CXCR1/2 and IL-8 molecules within the contact zone. (D) Predicted dynamics in membrane cortical tension were estimated from Eq. 5.5.

FIGURE 6. Simulated Dynamics of Surface Remodeling, Receptor Engagement, and Calcium Response

The number of occupied CXCR1/2 receptors, $\langle n \rangle$, and calcium response were predicted for the specified fractional contact area dynamics within the contact zone, $\phi/\phi_0(t)$ (left panels, A–C). The maximum number of CXCR1/2 receptors available for binding is indicated (dashed line, center panels). In (A), $\phi/\phi_0(t)$ was increased by a constant factor immediately following cell-bead contact ($t = 0$ s). $\phi/\phi_0(t)$ was increased at the conclusion or at intermediate times of engulfment in (B) and (C), respectively.

Table I

Kinetic Model for Receptor-Mediated Calcium Release

Reaction	Rate Law	Rate Parameters
$L + R \leftrightarrow L.R$	$k_f[L] \cdot [R] - k_r \cdot [L.R]$	$k_f = 169 \mu M^{-1} s^{-1}$
$L + R_p \leftrightarrow L.R_p$	$k_f[L] \cdot [R_p] - k_r \cdot [L.R_p]$	$k_r = 0.49 s^{-1}$
$R \rightarrow R_p$	$k \cdot [G_{\beta\gamma}.GRK] \cdot [R]$	$k = 1 \mu M^{-1} s^{-1}$
$L.R \rightarrow L.R_p$	$k \cdot [G_{\beta\gamma}.GRK] \cdot [L.R]$	
$G_{\alpha\beta\gamma} \rightarrow G_{\alpha}^{GTP} + G_{\beta\gamma}$	$k \cdot [L.R] \cdot [G_{\alpha\beta\gamma}]$	$k = 2.3 \mu M^{-1} s^{-1}$
$G_{\alpha}^{GTP} \rightarrow G_{\alpha}^{GDP}$	$k \cdot [G_{\alpha}^{GTP}]$	$k = 1 s^{-1}$
$G_{\alpha}^{GDP} + G_{\beta\gamma} \rightarrow G_{\alpha\beta\gamma}$	$k \cdot [G_{\alpha}^{GDP}] \cdot [G_{\beta\gamma}]$	$k = 71 \mu M^{-1} s^{-1}$
$G_{\beta\gamma} + GRK \leftrightarrow G_{\beta\gamma}.GRK$	$k_f[G_{\beta\gamma}] \cdot [GRK] - k_r \cdot [G_{\beta\gamma}.GRK]$	$k_f = 10 \mu M^{-1} s^{-1}$
		$k_r = 1 s^{-1}$
$PIP2 \rightarrow IP_3$	$k_1 \cdot [PIP2_0] + k_2 \cdot [PLC\beta_0] \cdot [Ca]_i / (K_{M,I} + [Ca]_i) \cdot [G_{\beta\gamma}] / (K_{M,2} + [G_{\beta\gamma}]) \cdot [PIP2_0]$	$k_1 = 0.05 s^{-1}$
		$k_2 \cdot [PLC\beta_0] = 0.26 s^{-1}$
		$K_{M,I} = 0.1 \mu M$
		$K_{M,2} = 1.4 \mu M$
$IP_3 \rightarrow \emptyset$	$k \cdot [IP_3]$	$k = 0.1 s^{-1}$
$Ca_{ER} \leftrightarrow Ca_i$	$J_{IP3} + J_{leak} - J_{SERCA}$	$v_1 = 1.2 s^{-1}$
		$v_2 = 10^{-4} s^{-1}$
	$J_{IP3} = v_1 P^3 \cdot ([Ca]_{ER} - [Ca]_i)$	$K_I = 20 \mu M$
	$J_{leak} = v_2 \cdot ([Ca]_{ER} - [Ca]_i)$	$K_2 = 0.01 \mu M$
	$J_{SERCA} = v_3 \cdot [Ca_i]^2 / (K_3^2 + [Ca_i]^2)$	$v_3 = 8.9 \mu M s^{-1}$
		$K_3 = 0.22 \mu M$
	$P = [IP_3] / (K_I + [IP_3]) \cdot [Ca_i] / (K_2 + [Ca_i]) \cdot h$	$\phi = .156$
		$[Ca]_{cell} = 30 \mu M$
	$[Ca]_{ER} = ([Ca]_{cell} - (1 - \phi) \cdot [Ca]_i) / \phi$	$h = 0.99$

Table II**Concentration Estimates for Human Neutrophils**

Species	Range	Resting Concentration [†]	Unit	Reference
IL-8	[L]	0	μM	(26)
CXCR1/2	[R]	9.5×10^4	#/cell	this study
G-protein	[G _{q/12}]	5×10^4	#/cell	(19)
G-protein coupled receptor kinase	[GRK]	3.0×10^{-3}	μM	(18)
phosphatidylinositol biphosphate	[PIP ₂]	$4.4\text{--}6.6 \times 10^6$	#/cell	(18)
Inositol-1,4,5-triphosphate	[IP ₃]	3	μM	(25)
Calcium (intracellular)	[Ca] _i	0.11	μM	(26)
Calcium (endoplasmic reticulum)	[Ca] _{ER}	200	μM	(18)

[†] Assuming membrane area = $254 \mu\text{m}^2$ and cytosolic volume = $125 \mu\text{m}^3$ (27).