Modeling neutrophil migration in dynamic chemoattractant gradients: assessing the role of exosomes during signal relay

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ABSTRACT Migrating cells often exhibit signal relay, a process in which cells migrating in response to a chemotactic gradient release a secondary chemoattractant to enhance directional migration. In neutrophils, signal relay toward the primary chemoattractant N-formylmethionyl-leucyl-phenylalanine (fMLP) is mediated by leukotriene B\textsubscript{4} (LTB\textsubscript{4}). Recent evidence suggests that the release of LTB\textsubscript{4} from cells occurs through packaging in exosomes. Here we present a mathematical model of neutrophil signal relay that focuses on LTB\textsubscript{4} and its exosome-mediated secretion. We describe neutrophil chemotaxis in response to a combination of a defined gradient of fMLP and an evolving gradient of LTB\textsubscript{4}, generated by cells in response to fMLP. Our model enables us to determine the gradient of LTB\textsubscript{4} arising either through directed secretion from cells or through time-varying release from exosomes. We predict that the secondary release of LTB\textsubscript{4} increases recruitment range and show that the exosomes provide a time delay mechanism that regulates the development of LTB\textsubscript{4} gradients. Additionally, we show that under decaying primary gradients, secondary gradients are more stable when secreted through exosomes as compared with direct secretion. Our chemotactic model, calibrated from observed responses of cells to gradients, thereby provides insight into chemotactic signal relay in neutrophils during inflammation.

INTRODUCTION

Many biological processes such as wound healing, angiogenesis, and immune responses require cells to migrate directionally when subjected to external chemical gradients (Jin et al., 2008). Many of these chemotactic events feature signal relay, a process by which cells, on exposure to a primary end-point chemoattractant, release a secondary chemoattractant to increase the robustness of the initial chemotactic response by mediating intercellular communication (Majumdar et al., 2014). Signal relay has been well studied in the social amoeba Dictyostelium discoideum, where cells chemotaxing toward cAMP regulate collective motility by further releasing cAMP (Garcia and Parent, 2008). In addition, CCL3 and CXCL18 have been shown to be released by monocytes and dendritic cells as secondary chemoattractants in response to the primary chemoattractant serum amyloid A (Gouwy et al., 2015); T-cells secrete the XCR1 ligand XCL1 (Kelner et al., 1994), which has been shown to attract dendritic cells and regulate T-cell effector function in vitro (Dorner et al., 2009).

Neutrophils use signal relay to coordinate their motion through the release of the lipid eicosanoid leukotriene B\textsubscript{4} (LTB\textsubscript{4}) (Afonso et al., 2012). Small molecules such as complement factors, released during tissue injury, or formyl peptides such as N-formylmethionyl-leucyl-phenylalanine (fMLP), released during bacterial infection, constitute primary chemotactic mediators of neutrophil chemotaxis. Ligand binding to cell surface receptors initiates leukotriene

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biosynthesis, which results in the release of arachidonic acid (AA) from membrane phospholipids and its subsequent conversion to LTB₄ (Peters-Golden and Henderson, 2007). LTB₄, released as a secondary chemoattractant, forms a gradient to coordinate neutrophil motility through its interaction with its cognate receptor BLT1. Failure to form or detect the secondary chemoattractant has been shown to cause impaired chemotactic response both in vitro (Afonso et al., 2012) and in vivo (Lämmermann et al., 2013). Although prior work on LTB₄-mediated signal relay in neutrophils showed that paracrine signal relay enhances directed cell migration, this process is not well understood; that is, it is not known whether LTB₄ gradients extend the spatial range over which cells can be guided, amplify noisy signals, prolong the duration for which cells can be guided beyond what a physiological primary gradient would allow, or influence chemotaxis by some other mode of action. This subject is difficult to study as it is not currently possible to image the time-varying gradients of primary chemoattractant and LTB₄. LTB₄ gradient dynamics are further complicated by the mechanism of its release. It was recently shown that LTB₄ and its synthesizing enzymes are packaged in multivesicular body-derived extracellular vesicles, termed exosomes, which are then secreted (Majumdar et al., 2016). Although exosomes and similar vesicles have been shown to be involved in generating various gradients (Yoon and Gho, 2014), the ways by which exosomal secretion (as compared with direct secretion) enhances signal relay have not yet been identified. Thus, we have developed a mathematical model to determine how LTB₄ signal relay enhances collective migration and how exosome-mediated LTB₄ secretion modulates this process. Although limited by the absence of precise quantitative data on certain features, the model provides significant insight on how signal relay can regulate neutrophil chemotaxis.

MODEL Overview
Our model, illustrated in Figure 1, describes the behavior of cells that can sense a combination of chemoattractant gradients. In this model, cell movement proceeds during a series of discrete timesteps of Δt = 1 min, which is based on an estimate of the persistence time for neutrophils (Vicker et al., 1986). Moreover, our own experience with neutrophil-like HL-60 cells suggest an approximate persistence time of less than 1 min. As shown in Supplemental Movie S1 and Figure 2A, a neutrophil-like HL-60 cell takes ∼50 s to reorient when an fMLP-filled micropipette is moved opposite to the cell’s initial direction of motion. At the start of each timestep, every...
neutrophil samples the fMLP and LTB$_4$ concentrations at a given position in the gradient. After sampling, the neutrophil is oriented with the gradient based on the differential receptor occupancy (DFRO), which is the difference in the fraction of ligand-bound receptors across the length of the moving cell. The probability that a cell is oriented toward or away from the gradient is a function of DFRO; the higher the DFRO, the more likely the cell is to be oriented with the gradient. It is assumed that, over the course of the time step, neutrophils move at a constant speed in new directions. The fMLP concentration also controls the rate at which each neutrophil secretes LTB$_4$- and LTB$_4$-containing exosomes (Figure 1). LTB$_4$ secretion (directly and through exosomes) causes LTB$_4$ levels to increase, offset by diffusion and dissipation. This cycle repeats, with neutrophils responding to the fMLP and LTB$_4$ gradients they experience at their new positions.

Parameters
The baseline parameters we used are shown in Table 1. Many of these values are well known, namely the length, migration speed, and persistence time of neutrophils. Rather than directly specifying values for the LTB$_4$ secretion rates ($\sigma_{CLD}, \sigma_{CED},$ and $\sigma_{ELD}$) or the cross-sectional area of the simulation domain, $A$, we set these values in terms of an overall secretion rate, $r_L$, and the fraction of LTB$_4$ that is secreted via exosomes, $\phi_E$. We report results for $r_L$ varying over several orders of magnitude and $\phi_E$ having values between 0 and 1. Concentrations of fMLP and LTB$_4$ are normalized by their respective values of $K_d$.

Distribution of fMLP
Unless otherwise mentioned, we consider the distributions of fMLP to be exponential,

$$F = \exp\left( -\frac{X-X_0}{\ell_F} \right)$$

(1)

where $F$ is the concentration of fMLP and $X_0$ is the position in the simulation domain at which the fMLP concentration is 1 (in units of $K_d$). We focus on exponential distributions because, compared with linear gradients, not only are they more representative of gradients that are likely to form in vivo (Oates et al., 2009; Wartlick et al., 2009) but also, as will be discussed later, they are necessary for signal relay to be observed (also see Figure 3). The characteristic length, $\ell_F$, represents how shallow or steep an exponential curve is; specifically, it is the distance over which the concentration decreases by a factor of 1/e. The characteristic length of gradients formed by formyl peptides in vivo has not been measured; the value we use corresponds to the length scale of gradients that form in the under-agarose assay (Lauffenburger and Zigmond, 1981; Uden et al., 1986).
Exosome activity decay rate

Cross-sectional area for diffusion

fMLP concentration leading to half-maximal exosome secretion rate

Maximum exosome secretion rate per cell

Length of volume in which cells migrate

Sensitivity of neutrophils to fMLP

Maximum LTB

fMLP-induced desensitization to LTB

Neutrophil length

Neutrophil persistence time

LTB

Parameter

Value

0.27/min

4

200

0.27/min

0.01/min

2.4 × 10^4 μm^2/min

1 min

200

10 mm

10 µm/min

100 µm

100 µm/min

500

Varies

Varies

1 K_d/min per exosome

10 K_d

Varies

TABLE 1: Model parameters.

fMLP-induced LTB_4 and exosome secretion rates

A neutrophil secretes LTB_4 (directly) and exosomes (that contain LTB_4) at rates \( \sigma_{CL} \) and \( \sigma_{CE} \), respectively. These rates are assumed to vary with \( F \) as

\[
\sigma_{CL} = \frac{\sigma_{CL0} F}{F + F_L} \tag{2}
\]

and

\[
\sigma_{CE} = \frac{\sigma_{CE0} F}{F + F_E} \tag{3}
\]

Here \( \sigma_{CL0} \) is the maximum LTB_4 secretion rate and \( \sigma_{CE0} \) is the maximum rate of secretion of exosomes by a neutrophil. We treat these secretion rates as functions only of the fMLP concentration (per Eq. 1) and not otherwise varying in time. Although neutrophils secrete exosomes and LTB_4 at time-varying rates even at fixed fMLP concentrations, we neglect this for the sake of simplicity.

Exosome activity distribution

In the following section, we provide an equation for the rate of change of local LTB_4 concentration, in which exosome distribution is represented as a function of position and time. Two considerations have been taken into account in modeling exosome distribution and activity. First, a large number of exosomes would be secreted by a population of neutrophils over the simulation period, so it is not feasible to represent each exosome individually. Second, given that exosomes can only release a finite amount of LTB_4 content. The exosome activity distribution will be a stable event as trails of CD6-positive vesicles are still visible 2 h after the initiation of migration (Figure 2C and Supplemental Movie S2).

The discrete Dirac delta, \( \delta_h \), which represents how the exosomes secreted by a neutrophil add to the activity in the bin that the neutrophil currently occupies, is approximated as

\[
\delta_h(r) = \begin{cases} 1/h, & |r| \leq h \\ 0, & \text{otherwise} \end{cases} \tag{6}
\]

Modeling rate variation of LTB_4 from cells and exosomes

The local rate of change of LTB_4 concentration is given by a reaction–diffusion equation,

\[
\frac{dL}{dt} = D_L \frac{d^2L}{dx^2} - \gamma_L L + \sigma_{EL0} E + \frac{1}{A} \sum_{k=1}^{N} \delta_h(x - X_k) \sigma_{CL,k} \tag{7}
\]
where $L$ is the concentration of LTB$_4$ at point $x$ and $D_L$ is the diffusion coefficient for LTB$_4$. Similarly to Eq. 5, the $k$th neutrophil has an fMLP-dependent free LTB$_4$ secretion rate of $\sigma_{CE,k}$. Exosomes secrete LTB$_4$ at a rate of $\sigma_{CE0}$, and the LTB$_4$ concentration in the medium is assumed to decrease intrinsically (other than by diffusion) at a rate of $\gamma_L$ due to various mechanisms, including perhaps aggregation or adsorption to the extracellular matrix. Under Materials and Methods, we describe how we solve Eqs. 5 and 7, linking results to secretion rates in terms of an overall LTB$_4$ secretion rate, $r_L$, and the fraction of LTB$_4$ that is secreted via exosomes, $\phi_E$; these were the two main parameters that we varied directly. The concentrations of LTB$_4$ that cells sense are determined by the total rate at which cells secrete LTB$_4$ (directly and via exosomes) and by how much space LTB$_4$ can be diluted into. If each cell were secreting at the maximum rate possible, and the loss of LTB$_4$ were negligible ($\gamma_L = 0$), then the LTB$_4$ concentration would tend to increase at the rate

$$r_L = \frac{(\sigma_{CEL} + \sigma_{CE0}\sigma_{EL0}/\gamma_E)N}{A\ell}$$

In this expression, the contribution from exosomes, $\sigma_{CE0}\sigma_{EL0}/\gamma_E$, implicitly assumes that each exosome secretes a finite amount of LTB$_4$, at a rate that decays over time. The fraction of LTB$_4$ secreted via exosomes is given by

$$\phi_E = \frac{\sigma_{CE0}\sigma_{EL0}/\gamma_E}{\sigma_{CEL} + \sigma_{CE0}\sigma_{EL0}/\gamma_E}$$

and can vary from 0 (all LTB$_4$ is secreted directly) to 1 (all LTB$_4$ is secreted via exosomes).

LTB$_4$ gradients have not been measured directly but arachidonic acid (AA), the precursor to LTB$_4$, was observed to move a shorter distance than fMLP (Uden et al., 1986). However, if LTB$_4$ moved as a freely diffusing monomer, it should diffuse farther than fMLP, due to its lower molecular weight. Observed AA distributions

FIGURE 3: Cell motility in linear or exponential gradients governed by differential receptor occupancy. Cells were subjected to linear (A) or exponential (B) gradients of fMLP. For exponential gradients, the concentration of fMLP increased by a factor of $e$ every $400 \mu$m (see Eq. 1). Colored tracks show motion of individual neutrophils, simulated for 1 h in a linear gradient (C) and in an exponential gradient (D); final positions are shown as circles. Results are shown for a 3-mm segment in the middle of a 10-mm simulation domain. (E, F) DFRO (see Eq. 11). A cell would have a higher DFRO where the gradient is steep but the concentration is not saturating. Higher DFRO causes the orientation distribution of cells to be biased in the direction of increasing chemoattractant concentration.
(Uden et al., 1986) resemble predictions for hindered gradients—
gradients that evolve by diffusion but with molecules adsorbed
onto surfaces (Dahlgren et al., 1984). Because LTβ4 is a lipid-
derived hydrophobic molecule, it could bind to surfaces or form
micelles or aggregates; it could also bind to diffusible carrier pro-
teins. For the sake of simplicity, we account for these effects by
Treating LTβ4 concentration as decreasing with first-order kinetics
at a rate γ. Based on its molecular weight, we assume that LTβ4
has a diffusion coefficient $D_L = 2.4 \times 10^{-5}$ mm$^2$/min. This yields a
characteristic length for LTβ4 gradients of $\ell_L = \sqrt{D_L / \gamma}$: in a simple
exponential gradient generated by a single source secreting LTβ4,
$\ell_L$ is the distance over which the LTβ4 concentration increases by a
factor of $e$. To approximately match LTβ4 distributions measured
previously (Uden et al., 1986; Foxman et al., 1997), we set $\gamma_L = 0.27$/min so $\ell_L = 300$ μm.

MODELING DIRECTED CELL MOTION GUIDED BY EVOLVING
CHEMOTACTANT GRADIENTS

Directional sensing biases the movement of neutrophils toward the
direction of increasing chemotactant concentration. Based on
current evidence, the change in receptor occupancy across the
length of the cell is the best predictor of cell bias. The fractional re-
ceptor occupancy (FRO) at a point on the cell surface is

$$\text{FRO} = \frac{c}{c + K_d}$$

(10)

where $c$ is the chemotactant concentration at the surface and $K_d$
is the dissociation coefficient for the chemotactant-receptor-interac-
tion. The DFRO across the length of the cell is obtained by taking
the derivative of FRO with respect to $x$ (the direction in which con-
centration varies), and scaling by the length, $\ell_C$, of the cell,

$$\text{DFRO} = \frac{\ell_C}{K_d} \frac{d}{dx} \left( \frac{1}{(c/K_d + 1)^2} \right)$$

(11)

This is approximately equal to the difference in fractional recep-
tor occupancy between the points on the cell located farthest up
and farthest down the gradient. DFRO has been shown to be roughly propor-
tional to the chemotactic index or mean cell velocity for a variety of cell types, including neutrophils (Tranquillo et al.,
1988; Herzmark et al., 2007), dendritic cells (Haessler et al., 2011;
Wang and Irvine, 2013), T-cells (Wang and Irvine, 2013), and breast
cancer cells (Kim et al., 2013). There are a variety of sources of noise
that interfere with chemotaxis, including stochastic binding of
chemoattractants to the receptors (Berg and Purcell, 1977) amplifi-
cation of gradient signals and conversion of those signals into cell
motion. Rather than accounting for each of these complex pro-
ceses separately, we use DFRO to determine a realistic overall level
of noise.

In our model, both fMLP and LTβ4 can direct neutrophils, but when a cell senses fMLP its sensitivity to LTβ4 decreases. We repre-
sent the combined gradient signal, $\kappa$, as a weighted sum of the
DFRO for each gradient,

$$\kappa = S_F \cdot \text{DFRO}_F + S_L \cdot \exp(-F/F_{c,\text{LTβ4}}) \cdot \text{DFRO}_L$$

(12)

where $S_F$ and $S_L$ are the sensitivities of neutrophils to gradients
of fMLP and LTβ4, respectively. The exponential term in this expression
accounts for neutrophils being less sensitive to LTβ4 when they can
sense fMLP (Heit et al., 2002), with $F_{c,\text{LTβ4}}$ being the fMLP concentration
leading to an e-fold decrease in LTβ4 sensitivity.

Under Materials and Methods, we describe how we estimated the sensitivities $S_F$ and $S_L$. Neutrophils favor formyl peptides over
intermediate chemoattractants such as LTβ4 (Heit et al., 2002); therefore, we adjusted $F_{c,\text{LTβ4}}$ to be just low enough that we did not
observe cells migrating up an LTβ4 gradient when opposed by an
fMLP gradient under normal conditions. At each time step, the di-
rection of neutrophil locomotion was determined by a biased ran-
dom process, such that higher $\kappa$ values make it more likely that the
neutrophil is aligned with the gradient. The neutrophil then moves
in this direction at a speed $v$ for a period $\Delta t$. After that point, the
steps shown in Figure 1 repeat.

RESULTS

EXPERIMENTAL CHEMOTACTANT GRADIENTS DIRECT CELL MIGRATION
BETTER THAN LINEAR GRADIENTS

We first investigated cell response to two fMLP gradient shapes: a
linear gradient (Figure 3A) and an exponential gradient (Figure 3B)
with a characteristic length $\ell_F = 400$ μm (see Eq. 1). As seen in Figure
3C, in a linear gradient the cells were most strongly directed in areas
with low concentrations (<1 mm). In contrast, for exponential gradi-
ents, the cells were most directed in areas where the concentration
was near the $K_L$ in Figure 3D, roughly 1–2 mm, with concentration
ranging from 0.3 to 3.5 $K_L$. This difference in directed cell motion is
due to differences in where cells have high DFRO (Figure 3, E and
F). In a linear gradient, DFRO is highest where the concentration is
lowest (Figure 3E). In contrast, DFRO is highest in an exponential
gradient where the concentration is approximately the $K_L$ (Figure
3F). These findings agree with the observations by Herzmark et al.
(2007) showing that chemotactic index is highest at the low concen-
tration end of a linear gradient or in the part of an exponential gradi-
ent where the concentration is close to $K_L$. We find that, although
the maximum DFRO is higher in the linear gradient, an effective
DFRO (>0.005) is sustained over a greater spatial range in the expo-
ential gradient. As signal relay extends the spatial range over
which cells can be directed, it is necessary that we model neutro-
phils in conditions where the gradient signal is weak far from a
chemoattractant source. We focus on neutrophil response to expo-
ential gradients because, in exponential gradients, signal relay
could potentially attract cells in areas where the slope of the gradi-
ent is shallow. In linear gradients, the slope is uniform; variation in
directionality arises from high concentrations leading to saturation
of chemoattractant receptors. Therefore, as stated under Model, we
used an exponentially decaying gradient of fMLP for all other simula-
tions.

LTβ4 MEDIATES SIGNAL RELAY IN CHEMOTACTIC NEUTROPHILS

We previously established a role for LTβ4 signal relay during neutro-
phil chemotaxis by mixing wild-type (WT) neutrophils with neutro-
phils that cannot sense fMLP (Afonso et al., 2012; Majumdar et al.,
2016). We showed that the migration defects of neutrophils that do
not have functional formyl peptide receptors (FPR) can be rescued by
mixing them with WT neutrophils that are capable of producing
LTβ4. This fundamental behavior, central to the concept of signal
relay, is recapitulated by our model in Figure 4. We show that WT
neutrophils migrating in an fMLP gradient (Figure 4A) exhibit robust
chemotaxis (Figure 4D) and secrete LTβ4 (Figure 4G), while cells lack-
ing FPR show neither chemotaxis (Figure 4E) nor LTβ4 production
under similar conditions (Figure 4H). When combined with WT neu-
trophils, neutrophils lacking FPR regain the ability to chemotax
(Figure 4F) by detecting the LTβ4 gradient created by the WT neutro-
phils (Figure 4I). The shape of the resultant LTβ4 gradient remains
similar to that of the original gradient (Figure 4, A and G).

We studied the effect of signal relay on populations of cells
migrating in response to fMLP by comparing the migration of cells

incapable of detecting LTB₄, and hence defective in signal relay (BLT-, Figure 5, left), with the migration of LTB₄-sensitive cells capable of signal relay (BLT+, Figure 5, right). As shown in Figure 5, C and D, while both BLT- and BLT+ cells responded in the steep parts of gradients, only the BLT+ cells were capable of directed cell migration in regions where the primary fMLP gradient is too shallow for effective chemotaxis (<0.002 Kₘ/µm), shown here as a shaded region. This indicates that signal relay increases the spatial range over which cells can be recruited. Together, these findings show that our model faithfully recapitulates the biological process of LTB₄ signal relay during neutrophil chemotaxis.

**Exosomes regulate the evolution of LTB₄ gradients and control the time required to reach equilibrium concentrations**

We next sought to explain how packaging of LTB₄ in exosomes and its subsequent release affects the evolution of LTB₄ gradients and, in turn, affects directed cell motion. For purposes of comparison, we set LTB₄ secretion rates such that, on fMLP stimulation, cells secrete LTB₄ at the same rate regardless of whether the LTB₄ is secreted directly or is packaged in exosomes. Figure 6 shows how cell response differs if LTB₄ is secreted via exosomes (right panel) rather than directly (left panel). When LTB₄ is gradually released from exosomes, the time taken for the LTB₄ profile to reach a steady state is subject to the decay rate of the exosomal LTB₄ (Figure 6E), and high LTB₄ secretion rates are not reached until 1 h into the simulations (Figure 6G). In contrast, an equilibrium profile is reached within 6 min if LTB₄ is secreted directly (Figure 6F). We assume that when cells secrete LTB₄ directly, it is immediately available to affect cell motion, whereas when cells secrete LTB₄-containing exosomes, the exosomes then gradually secrete the LTB₄. To characterize the effect of the profiles on the behavior of the cells, we calculated the directionality of the cells. We define “directionality” as the mean of the cosine of the orientation angles that cells would have at a given time and position, as determined by the biased distribution of cell orientations in response to the chemoattractant gradient (given by Eq. 12). This is comparable to the chemotactic index, but, while the chemotactic index is used to measure the directed motion of cells over time, we use directionality to capture the average motion of cells at a particular time and position. As shown in Figure 6, H and I, directionality reaches its maximum value more rapidly for direct rather than exosomal secretion. Therefore, secretion of LTB₄ via exosomes can mediate signal relay similarly to direct secretion of LTB₄, but relay begins more gradually. Thus, exosomes may play a critical role in pathophysiological conditions such as sepsis, where large quantities of LTB₄ are expected to be released into tissues and saturate cell surface receptors. By gradually releasing LTB₄, exosomes may prevent LTB₄ profiles from rapidly reaching such saturating concentrations.

**Maximum range of cell recruitment occurs at intermediate LTB₄ secretion rates and is dependent on exosomal secretion**

We next quantified the effect of signal relay on the distance over which cells can be recruited. For this purpose, we define the recruitment range as the length of the zone over which directionality is at least 0.5. Data were obtained from simulations conducted with a wide range of LTB₄ secretion rates, rₑ (see Eq. 8). We also varied the fraction of LTB₄ that is secreted by being packaged into exosomes, φₑ, on secretion, the exosomes gradually release LTB₄ (per Eq. 4). As seen in Figure 7A, the recruitment range was calculated to be...
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...tion rates, the directionality is high in two different regions (Figure 7B, ix), one dominated by the LTB\textsubscript{4} gradient and another due to the fMLP; signal relay would not be useful under these circumstances. To understand how the secretion of LTB\textsubscript{4} by exosomes affects recruitment range, we varied $\phi_E$, the fraction of LTB\textsubscript{4} secreted from exosomes (Eq. 9). LTB\textsubscript{4} can be secreted directly ($\phi_E = 0$), entirely via exosomes ($\phi_E = 1$), or by a combination of the two modes of secretion (0 < $\phi_E$ < 1). The maximum recruitment range is not very different when LTB\textsubscript{4} is either released directly ($\phi_E = 0$), or released exclusively from exosomes ($\phi_E = 1$), or by a combination of the two modes of secretion (0 < $\phi_E$ < 1). The main overall difference in recruitment range due to direct or exosomal secretion of LTB\textsubscript{4} is that for a higher release rate ($r_L$), an optimal recruitment range is obtained if a greater fraction of LTB\textsubscript{4} is released via exosomes (higher $\phi_E$). This effect is due to the time delay involved in exosomal secretion, as mentioned in the previous section. For a given $r_L$, the amount of LTB\textsubscript{4} in solution at 1 h is higher for direct secretion. At that point, with exosome-mediated LTB\textsubscript{4} secretion, much of the LTB\textsubscript{4} is still contained in exosomes and...
Exosomes stabilize LTB₄ gradients under conditions of time-decaying primary chemoattractant gradients

For rapid inflammatory pathophysiological conditions, such as injury, ischemia, or during the early onset of infection, the concentration cannot be sensed by cells. Therefore, exosome-mediated secretion keeps LTB₄ concentrations from rising too high in response to primary chemoattractants during the initiation of an inflammatory phase.
of primary chemoattractants, such as formylated peptides belonging to damage-associated molecular patterns (DAMPs), is expected to rise rapidly in the tissue followed by gradual decay in concentration (Land, 2015). We modeled this by subjecting neutrophils to an fMLP gradient for 1 h and then causing the fMLP concentration to decay exponentially at a rate of 0.2/min. Figure 8, C and D, shows results as a function of time for the first 1 h after the fMLP gradient begins to decay. When LTB₄ is secreted directly, the LTB₄ profile
FIGURE 8: Exosomes prolong directionality of cell migration in decaying fMLP gradient. Results shown for neutrophils secreting LTB₄ directly (left panel) or via exosomes (right panel). (A, B) Tracks showing motion of neutrophils; final positions are shown as circles. (C, D) Concentration of the primary chemoattractant, fMLP; concentration decreases exponentially at a rate of 0.2 min⁻¹. Curves from later time points are shown in progressively darker shades. (E) Exosome activity, that is, the rate of LTB₄ secretion via exosomes. (There is no exosome activity in the case shown in the left column.) (F, G) Concentration of LTB₄. (H, I) Directionality of migrating cells. Curves displayed in C–I show levels at 6 min increments over a total simulation time of 1 h. A value of kₗ = 4 Kᵢₗ/min was used. Darker colors indicate later time points.

mimics the time-varying fMLP profile (Figure 8F), which falls rapidly, causing a decrease in directionality and recruitment of other neutrophils (Figure 8H). In contrast, when LTB₄ is secreted via exosomes, directed migration is maintained. This is because the early fMLP profile causes exosomes to be predominantly secreted in areas with high fMLP concentration (Figure 8E). As the fMLP concentrations fall, the rate at which cells secrete exosomes decreases; even so, in comparison with direct LTB₄ secretion, the rate at which LTB₄ enters
solution is more stable. Hence, even after the fMLP concentration falls, the LTB₄ profile is maintained for at least 1 h (Figure 8G). Consequently, with exosomal LTB₄ secretion, the directionality of cell migration is maintained even in extremely shallow gradients of fMLP (Figure 8).

To quantify the stabilization of LTB₄ gradients via exosomal secretion and its effect on directionality, we repeated these simulations for various values of γₑ, the rate at which exosomal secretion of LTB₄ decays (Figure 9). Our model predicts that intermediate exosomal LTB₄ secretion rates are best for sustaining directed migration. At a high rate of LTB₄ secretion (e.g., 1.0/s) directionality drops in the first 30 min because the exosomes rapidly run out of LTB₄ and, although low levels of LTB₄ secretion (e.g., 0.001/s) enable LTB₄ signaling to occur for a relatively long duration, the signal is weak (<0.2 after 20 min). In contrast, an intermediate rate (0.01/s) maintains directionality above 0.4 for more than 1 h.

**DISCUSSION**

In our model, a cell migrates by detecting differences in chemoattractant concentrations, implicitly transducing the differences in receptor occupancy into an intracellular gradient, and, finally, migrating in the direction of more ligand-bound receptors. A similar approach has been used previously to model neutrophil motion, employing a “chemotaxis coefficient” to account for DFRO and receptor down-regulation (Tranquillo et al., 1988). In this study, we used DFRO not only to determine the spatial distribution of directions in which cells move but also to account for response to multiple chemoattractants. In agreement with previous experimental findings, our model shows that migrating neutrophils can generate gradients of LTB₄ that guide neutrophils that cannot sense fMLP. The model can also replicate the finding that impairing LTB₄-mediated signal relay decreases the directed motion of neutrophils toward fMLP. In addition, our model shows that exponential gradients are better at directing cell migration than are linear gradients, and, in accordance with existing literature (Afonso et al., 2012; Lämmermann et al., 2013; Majumdar et al., 2016), that cells with LTB₄ receptors are more directed in the shallow parts of fMLP gradients than are cells lacking such receptors. Furthermore, our model predicts that LTB₄-mediated signal relay acts by extending the range over which cells can be directed (the recruitment range) by a factor of two to three, again reproducing experimental data (Lämmermann et al., 2013). Results show that the recruitment range is maximized for γₑ in the range of 1–100 K/min, which corresponds to rates that are attainable for neutrophils. Indeed, the LTB₄ secretion rate per neutrophil is related to the secretion rate γₑ divided by the number of neutrophils per unit volume. Assuming that neutrophils are tightly packed, with one neutrophil per 10-µm cube, the secretion rate that optimizes signal relay is between 1 × 10⁻¹² and 1 × 10⁻¹⁰ moles of LTB₄/min/neutrophil. The maximum rate at which neutrophils secrete LTB₄ has been measured to be in the range of 3 × 10⁻¹⁰ to 3 × 10⁻¹² mol LTB₄/min (Afonso et al., 2012). Therefore, neutrophil LTB₄ secretion rates are adequate to increase the recruitment range. Concentration levels and gradient slopes are linked not just to the secretion rates of individual neutrophils but also by cell density.

Our model shows that, although releasing LTB₄ through exosomes does not necessarily translate into higher recruitment ranges or higher directionality in steady fMLP gradients, it plays a pivotal role in decaying fMLP gradients. We show that by their time-delayed release of LTB₄, exosomes can better preserve LTB₄ after fMLP stimulus decreases. The model predicts that under such conditions exosomes maintain persistence of cell migration by conserving gradients over time periods that are determined by the rate at which exosomes are depleted of LTB₄. This could explain why the recruitment of neutrophils to sites of infection occurs in multiple phases, even after the initial recruitment signal dissipates (Ng et al., 2011). We also show that under high tissue concentrations of primary chemotactant, for example, bolus production of LTB₄ in response to *Mycobacterium* infection (El-Ahmady et al., 1997), packaging of LTB₄ in exosomes could prevent receptor saturation and maintain cell motion. Conversely, exosomes may help sequester LTB₄ in situations where it is rapidly removed from the tissue space, for example, near a draining lymphatic vessel. The use of vesicles as a secretion mechanism is not unique to LTB₄ and is important for the formation of morphogen gradients during *Drosophila* embryogenesis (Entchev and González-Gaitán, 2002) and the diffusion of lipid-adducted molecules such as Wnt (The and Perrimon, 2000).

Neutrophil gradient sensing is best predicted by differences in chemoattractant receptor occupancy or DFRO (Tranquillo et al., 1988). DFRO has commonly been treated as proportional to cell flux (Tranquillo et al., 1988) or chemotactic index (Herzmark et al., 2007). The problem with this assumed proportionality is that, for a high enough DFRO, a chemotactic index of greater than 1 would be predicted, which is not possible. To overcome this problem, we previously developed a model of cell migration in which DFRO determined the probability distributions of cell orientations (Szatmary and Nossal, 2017). From these probability distributions, we calculated fluxes of cells in chemotaxis assays. To study signal relay, rather than calculating fluxes of ensembles of cells, we assigned orientations to cells based on their individual probability distributions. Notably, our model also differs from early models of group migration by accounting for individual cells rather than cell densities (e.g., Keller and Segel, 1971; Tranquillo et al., 1988). Models of the mechanisms underlying transduction of gradient signals have clarified how gradient sensing works in individual cells (Irinia et al., 2009; Van Haastert, 2010; Xiong et al., 2010). Nevertheless, at this point, determining cell orientations from DFRO is the most effective way to realistically model gradient sensing in the context of migration of a large number of cells.

**FIGURE 9:** Directed migration is best preserved if exosomes release LTB₄ at an intermediate rate. Mean cell directionality in a 1 mm × 1 mm simulation area is shown for cells when the fMLP gradient decays (see Figure 8), as a function of time for various exosomal LTB₄ activity rates.
In our model, neutrophil sensitivity to formyl peptides, $S_{\theta}$, is calibrated to a systematically collected data set (Zigmond, 1977). Neutrophil sensitivity to LTB$_4$, $S_{\lambda}$, has not been measured as reliably, so we assume $S_{\theta} = S_{\lambda}$. Neutrophils preferentially respond to formyl peptides relative to LTB$_4$ (Heit et al., 2002). We expressed this in our model by using Eq. 12 and selecting a value for $P_{\theta}$ that allowed the model to recapitulate this observation. Despite uncertainty about some properties of neutrophil response, our model recapitulates the in vivo observations of Lämmermann et al. (2013) that LTB$_4$-mediated signal relay increases recruitment range and that LTB$_4$ is involved in prolonging recruitment.

Our model accounts for LTB$_4$ diffusion in one dimension (1D). This is appropriate because, in the problems we consider here, the iMLP concentration varies only in 1D, and LTB$_4$ secretion is driven by the iMLP concentration. Also, LTB$_4$ is secreted by a large number of evenly distributed cells. Therefore, we expect the LTB$_4$ concentration to vary primarily in one direction. At the level of a single cell, the secreted LTB$_4$ spreads out in 2D, so the resulting gradient would differ from what a 1D model would predict. However, in the present situation, 2D gradients arising from many secreting cells coalesce into a single gradient that is effectively 1D for the cells that are guided by it. Therefore, the effect of this approximation is negligible. Thus, accounting for only 1D diffusion is sufficient for the particular problems analyzed in this work. Of course, accounting for diffusion in 1D is not adequate for modeling every signal relay process. For example, accounting for diffusion in at least two dimensions is important for modeling the streaming of Dictyostelium cells (Guven et al., 2013).

In the absence of detailed data, we assumed that neutrophil secretion rates depend only on the current concentration of iMLP and that exosomes secrete LTB$_4$ at exponentially decaying rates. Because LTB$_4$ is a sparingly soluble lipid, we also assumed that an LTB$_4$ molecule undergoes pure diffusion, followed by irreversible removal from solution with first-order kinetics. However, the secretion, motion, or removal of LTB$_4$ may be more complex than this, and many of the parameters required to build this model are not well known. Subsequent modeling efforts that explore the effects of varying these parameters can indicate how signal relay depends on changes in these parameters, such as may occur in disease states. While accounting for these features is not necessary to model gross aspects of signal relay, including them in future modeling efforts may reveal important aspects of this phenomenon.

Measuring the effects of LTB$_4$ on recruitment range is difficult with most existing assays. We suggest that relay works by neutrophils generating an LTB$_4$ profile that extends beyond where the primary iMLP gradient is steep enough to be sensed. Study of this aspect of relay in vitro requires assays in which slopes are shallower farther from the primary gradient source. The bridge (Zigmond, 1977), Dunn (Zicha et al., 1991), Taxiscan (Kanegasaki et al., 2003), and filter (Boyden, 1962) assays are not suitable for measuring recruitment range because they generate gradients of uniform slope. Finally, although microfluidic mixers can be used to generate and sustain nonlinear gradients (e.g., in Wang et al., 2004), they do so by continuously flowing the medium through the chamber, which would disrupt secondary gradients. It is, therefore, difficult to precisely design an in vitro measurement of neutrophil signal relay and chemotaxis assays such as the under-agarose assay are, at best, approximations. While in vivo methods are currently the best way to set up conditions in which changes in recruitment range can be observed, in vitro methods are better suited for making measurements in well-defined environments. Mathematical models can unite these approaches.

The present work primarily models signal relay in neutrophils but can be easily applied to other systems where a secondary chemoattractant plays an important role in group migration. Given the widespread use of exosomes as means to distribute morphogens and other gradient forming agents, we envision that exosomes may be important in shaping gradients in other systems as well.

**MATERIALS AND METHODS**

**Cell orientation**

We have previously described our methods for modeling gradient sensing and chemoattractant transport (Szatmary and Nossal, 2017) and offer a brief overview here. We treat the cells as having orientations that fall on a von Mises–Fisher distribution, which is a kind of bell curve. This is used to represent the observation that stronger gradient signals (i.e., higher DFRO) cause cell orientations to be more biased toward the gradient direction. The von Mises–Fisher distribution is given by

$$f(\theta; \kappa) = \frac{\exp(\kappa \cos(\theta))}{2\pi I_0(\kappa)}$$

where $\theta$ is the angle defined with respect to the direction of the chemoattractant gradient and $I_0(\kappa)$ is the modified Bessel function of order 0; $f(\theta; \kappa)$ is used here for representing 2D cell migration. The $\kappa$ parameter represents the “bias” in the cell orientation distribution; a more-biased distribution has a greater number of cells oriented more directly up the gradient. We assume that bias is proportional to the difference in fractional receptor occupancy, that is,

$$\kappa = S \cdot DFRO$$

where $S$ is the “sensitivity.” This $S$ parameter depends on the cell type and identity of the chemoattractant. We previously estimated the sensitivity of neutrophils to formyl peptides (Szatmary and Nossal, 2017) by comparing Zigmond’s observations of cell orientation distributions with gradient conditions (Zigmond, 1977). Similar measurements have not been made for neutrophil response to LTB$_4$ so, for simplicity, we assume that a neutrophil would be equally responsive in an LTB$_4$ gradient as in an equivalent iMLP gradient.

For each cell at each timestep, we used Eq. 12 to calculate $\kappa$: The Scipy vonmises function then generated a random angle drawn from the von Mises distribution with this particular $\kappa$, and the cell then traveled at this angle during the next time step.

**Determination of chemoattractant gradients**

To determine the distributions of LTB$_4$ and exosomes, we solved Eqs. 5 and 7 using numerical methods. Chemoattractant concentration profiles were calculated by solving the diffusion equation with the finite-difference method using Adams predictor-corrector methods for time stepping; this was implemented with the odeint function from SciPy (Oliphant, 2007), which is an interface for the LSODE solver from ODEPACK (Hindmarsh, 1983). We previously validated our numerical methods (Szatmary et al., 2014) by comparison with the findings of Lauffenburger and Zigmond (1981).

**Cell lines and constructs**

PLB985 cells expressing mCherry-5LO and CD63-GFP as well as coexpressing both CD63-GFP and mCherry-5LO were created using a retroviral approach as described previously (Majumdar et al., 2016).

**Chemotaxis assay and image acquisition**

HL-60 cells were differentiated at a density of $4.5 \times 10^5$ cells/ml for 6 d in culture medium containing 1.3% dimethyl sulfoxide (DMSO), and the status of differentiation was monitored by CD11b staining. Differentiated cells were plated on chambered cover slides coated...
with fibronectin (10 µg/mL), and a chemotactic gradient was generated using an Eppendorf microinjector with Femtotips (Eppendorf, Germany) loaded with 1 µM fMLP. For steady-state cell migration, the under-agarose assay, described elsewhere, was performed 2 h post addition of fMLP (Majumdar et al., 2016). Images of exosome release by migrating cells were acquired using Instant Structured Illumination Microscope (iSIM) super-resolution microscopy as described previously (Curd et al., 2015).

ACKNOWLEDGMENTS

We thank the members of the Nossal and Parent laboratories for helpful discussions. We also thank Hari Shroff, National Institute of Biomedical Imaging and Bioengineering, National Institutes of Health, for his help in iSIM microscopy. This study was supported by the Intramural Research Programs of the Eunice Kennedy Shriver National Institute of Child Health and Human Development and the Center for Cancer Research, National Cancer Institute, National Institutes of Health.

REFERENCES

Afonso PV, Janka-Junttila M, Lee YJ, McCann CP, Oliver CM, Aamer KA, Losert W, Cicerone MT, Parent CA (2012). LTBA is a signal-relay molecule during neutrophil chemotaxis. Dev Cell 22, 1079–1091.

Boydren S (1962). The chemotactic effect of mixtures of antibody and antigen on polymorphonuclear leucocytes. J Exp Med 115, 453–466.

Curd A, Cleasby A, Makowska K, York A, Shroff H, Feckham M (2015). Construction of an instant structured illumination microscope. Methods 88, 37–47.

Dahlgren C, Magnusson KE, Sundqvist T (1984). Concentration gradients in the under-agarose chemotaxis assay system with attractant surface adsorption. J Immunol Methods 75, 23–29.

Dorner BG, Dorner MB, Zhou X, Opitz C, Mora AK, Gutierrez S, Hutloff A, Entchev EV, González-Gaitán MA (2002). Morphogen gradient formation. J Leukoc Biol 39, 27–35.

El-Ahmady O, Mansour M, Zoeir H, Mansour O (1997). Elevated concentrations of interleukins and leukotriene in response to mycobacterium tuberculosis infection. Ann Clin Biochem 34, 160–164.

Entchev EV, Gonzalez-Gaitán MA (2002). Morphogen gradient formation and vesicular trafficking. Traffic 3, 98–109.

Foxman EF, Campbell JJ, Butcher EC (1997). Multistep navigation and maintenance of gradients during directed cell migration. Curr Opin Cell Biol 9, 193–199.

Garcia GL, Parent CA (2008). Signal relay during chemotaxis. J Microsc 231, 529–534.

Gouwy M, De Buck M, Portner N, Opdenakker G, Proost P, Struyf S, Van Damme J (2015). Serum amyloid A chemoattracts immature dendritic cells and indirectly provokes monocyte chemotaxis by induction of cooperating CC and CXC chemokines. Eur J Immunol 45, 101–112.

Guven C, Rericha E, Ott E, Losert W (2013). Modeling and measuring signal relay in noisy directed migration of cell groups. PLoS Comput Biol 9, e1003041.

Haidar U, Pesano M, Wu M, Swartz MA (2011). Dendritic cell chemotaxis in 3D under defined chemokine gradients reveals differential response to ligands CCL21 and CCL19. Proc Natl Acad Sci USA 108, 5614–5619.

Hett B, Tavener S, Raharo E, Kubek P (2002). An intracellular signalling hierarchy determines direction of migration in opposing chemotactic gradients. J Cell Biol 159, 91–102.

Herzmark P, Campbell K, Wang F, Wong K, El-Samad H, Groisman A, Bourne HR (2007). Bound attractant at the leading vs. the trailing edge determines chemotactic prowess. Proc Natl Acad Sci USA 104, 13349–13354.

Hindmarsh AC (1983). ODEPACK, A Systematized Collection of ODE Solvers, IMACS Transactions on Scientific Computation, Vol. 1, ed. RS Stepleman et al., Amsterdam: North-Holland, 55–64.

Irimia D, Balazs G, Agrawal N, Toner M (2009). Adaptive-control model for neutrophil orientation in the direction of chemical gradients. Biophys J 96, 3897–3916.

Jin T, Xu X, Herold D (2008). Chemotaxis, chemokine receptors and human disease. Cytokine 44, 1–8.

Kim BJ, Hanaanta-Anan P, Chau M, Kim YS, Swartz MA, Wu MM (2013). Cooperative roles of SDF-1 alpha and EGF gradients on tumor cell migration revealed by a robust 3D microfluidic model. PLoS One 8, e69422.

Lämmermann T, Afnos PV, Angermann BR, Wang JM, Kastenmuller W, Parent CA, Germain RN (2013). Neutrophil swarms require LTBP4 and integrins at sites of cell death in vivo. Nature 498, 371–375.

Land WG (2015). The role of damage-associated molecular patterns in human diseases: Part I. Promoting inflammation and immunity. Sultan Qaboos Univ Med J 15, e9–e21.

Lauffenburger DA, Zigmond SH (1981). Chemotactic factor concentration gradients in chemotaxis assay systems. J Immunol Methods 40, 45–60.

Majumdar R, Sixt M, Parent CA (2014). New paradigms in the establishment and maintenance of gradients during directed cell migration. Curr Opin Cell Biol 30, 33–40.

Majumdar R, Takawali Tameh A, Parent CA (2016). Esosomes mediate LTBA release during neutrophil chemotaxis. PLoS Biol 14, e1002336.

Ng LG, Qin JS, Roediger B, Wang Y, Jain R, Cavanagh LL, Smith AL, Jones CA, de Veer M, Grimaldeston MA, et al. (2011). Visualizing the neutrophil response to sterile tissue injury in mouse dermis reveals a three-phase cascade of events. J Invest Dermatol 131, 2058–2068.

Oates AC, Gorflinkel N, Gonzalez-Gaitan M, Hesenberg C-P (2009). Quantitative approaches in development biology. Nat Rev Genet 10, 517–530.

Oliphant TE (2007). Python for scientific computing. Comput Sci Eng 9, 10–20.

Peters-Golden M, Henderson WRJ (2007). Leukotrienes. N Engl J Med 357, 1841–1854.

Szatmary AC, Nossal R (2017). Determining whether observed eukaryotic cell migration indicates chemotactic responsiveness or random chemokinetic motion. J Theor Biol 425, 103–112.

Szatmary AC, Stuelten CH, Nossal R (2014). Improving the design of the agarose spot assay for eukaryotic cell chemotaxis. RSC Adv 4, 57343–57349.

The I, Perrimon N (2000). Morphogen diffusion: the case of the Wingless protein. Nat Cell Biol 2, E79–E82.

Tranquillo RT, Zigmond SH, Lauffenburger DA (1988). Measurement of the chemotaxis coefficient for human neutrophils in the under-agarose migration assay. Cell Motil Cytoskeleton 11, 1–15.

Uden AM, Hafstrom I, Palmblad J (1986). Relation to chemotactic factor concentrations of chemotactic factor activities. J Cell Sci 99(Pt 4), 769–775.

Van Haastert PJ (2010). A stochastic model for chemotaxis based on the ordered extension of pseudopods. Biophys J 99, 3345–3354.

Vicker MG, Lackie JM, Schill W (1986). Neutrophil leucocyte chemotaxis is not inhibited by a spatial gradient of chemotactant. J Cell Sci 84, 263–280.

Wang SJ, Saadi W, Lin F, Minh-Canh Nguyen C, Li Jeon N (2004). Differential effects of EGF gradient profiles on MDA-MB-231 breast cancer cell chemotaxis. Exp Cell Res 301, 180–189.

Wang Y, Irvine DJ (2013). Convolution of chemotactant secretion rate, source density, and receptor desensitization direct diverse migration patterns in leukocytes. Integr Biol (Camb) 5, 481–494.

Wartlick O, Kicheva A, Gonzalez-Gaitan M (2009). Morphogen gradient formation. Cold Spring Harb Perspect Biol 1, a001255.

Xiong Y, Huang CH, Iglesias PA, Devreotes PN (2010). Cells navigate with a local-excitation, global-inhibition-biased excitable network. Proc Natl Acad Sci USA 107, 17079–17086.

Yoon YJ, Gho YS (2014). Extracellular vesicles as emerging intercellular communicomnes. BMB Rep 47, 531–539.

Zicha D, Dunn GA, Brown AF (1991). A new direct-viewing chemotaxis chamber. J Cell Sci 99(Pt 4), 769–775.

Zigmond SH (1977). Ability of polymorphonuclear leukocytes to orient in gradients of chemotactic factors. J Cell Biol 75, 606–616.