Positive feedback amplification in swarming immune cell populations (#)

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Abstract

Several immune cell types (neutrophils, eosinophils, T cells, and innate-like lymphocytes) display coordinated migration patterns when a population, formed of individually responding cells, moves through inflamed or infected tissues. “Swarming” refers to the process in which a population of migrating leukocytes switches from random motility to highly directed chemotaxis to form local cell clusters. Positive feedback amplification underlies this behavior and results from intercellular communication in the immune cell population. We here highlight recent findings on neutrophil swarming from mouse models, zebrafish larvae and in vitro platforms for human cells, which together advanced our understanding of the principles and molecular mechanisms that shape immune cell swarming.
Introduction

With the advent of intravital microscopy the direct observation of different immune cell subsets has revealed movement patterns indicative of coordinated population dynamics in inflamed and infected mouse tissues. One of these phenomena is termed “swarming” and refers to a population of individually migrating immune cells that switch from random motility to highly directed and coordinated chemotaxis, ultimately leading to their accumulation and clustering. Swarming behavior was first observed for neutrophils by imaging with two-photon intravital microscopy in infected mouse tissues [1,2]. Neutrophil swarming has since been observed in several inflammatory and infectious conditions, ranging from sterile inflammation to infections with bacteria, parasites, viruses and fungi [3,4]. Moreover, swarming dynamics have also been reported for other immune cell types, including eosinophils [5], cytotoxic T cells [6] and innate-like lymphocytes [7]. For many of these examples, intercellular communication among immune cells amplifies cell aggregation and acts as a major underlying mechanism for coordinated swarming responses [5,6,8]. In this regard, immune cell swarming is reminiscent of the accumulation and aggregation behavior of swarming insects [9,10] or streaming Dictyostelium [11], which rely on self-amplifying positive feedback control. Neutrophil swarms, the best studied example of immune cell swarms, have gained much attention over the last years and we will summarize here recent findings from mice, zebrafish and in vitro platforms for the study of human neutrophil swarms.

Positive feedback amplification drives swarming

Intravital imaging studies in inflamed interstitial tissues of mice have characterized distinct sequential phases when a population of neutrophils responds to tissue injury or locally occurring cell death [3,4]. On tissue or cell damage, a few “pioneer” neutrophils, commonly the ones close to the damage site, respond by switching from exploratory patrolling to chemotactic movement toward the tissue lesion within a few minutes. Pioneer neutrophils then release signals to attract a second wave of neutrophils that, with a time delay of some minutes, also begin to migrate in a directed fashion toward the damage site. This amplified recruitment from more distant sites can be maintained for several minutes before neutrophils accumulate and form stable multicellular clusters to isolate the local site of injury or infection from healthy tissue (Figure 1). The phases from initiation to stabilization of a persistent
neutrophil swarm in mice take around 30-60 minutes, depending on the number of neutrophils involved [8].

Central to neutrophil swarming is a positive feedback amplification mechanism that is mediated by the lipid leukotriene B4 (LTB4). LTB4 is an eicosanoid that is synthesized from arachidonic acid in two enzymatic steps (driven by the enzymes 5-lipoxygenase and leukotriene A4 hydrolase) and released by neutrophils within a few minutes after their activation. Imaging experiments in mice revealed that neutrophils lacking LTB4R1 (alternative name: BLT1), the high-affinity receptor for LTB4, were severely impaired in their amplified recruitment and clustering during swarm formation [8]. LTB4 secreted from neutrophils, but not other myeloid cells, was crucial for efficient swarming in mouse tissues, highlighting the paracrine role of LTB4 to attract distant neutrophils to a developing swarm [8]. In hindsight, the critical role of intercellular communication among neutrophils justifies the initially chosen “swarming” term.

Recent developments in microfabricated devices now allow for the replication of neutrophil swarming dynamics in a culture dish, thereby providing a system that is also amenable for studying human neutrophils. In these assay systems, human neutrophils undergo distinct sequential phases of swarming [12], comparable to the swarm dynamics of mouse neutrophils in vivo. It has been shown that swarm formation of human neutrophils in microscale arrays of patterned zymosan particles also depends on LTB4 [12]. Moreover, further developments of this methodology and other lab-on-a-chip devices revealed an involvement of neutrophil-derived LTB4 in containing the growth of the pathogenic fungus Candida albicans or Aspergillus fumigatus [13•,14].

Small swarms of 10-30 neutrophils can also be studied in zebrafish larvae. Wound studies in this model system probably compare best to injuries of wet epithelial tissues of land-living mammals (e.g. airway epithelium, upper digestive tracts, and potentially also lungs) [15]. As a consequence, the neutrophil response to tissue injury in zebrafish embryos also depends on redox gradients generated by the fin epithelium and the osmolarity of the surrounding water [16,17]. These signaling pathways still need to be explored for neutrophil swarming in nonepithelial mammalian tissues. However, neutrophil swarms manifest clearly under conditions of local injection of living bacteria into zebrafish larvae [18-21]. This swarming response is characterized by the same sequential phases as observed for neutrophils in mice [20]. Refreshing conceptual and mechanistic insights on neutrophil swarming have been
derived from the zebrafish model [19]. Recent studies also confirmed a crucial role of neutrophil-derived LTB4 for controlling the swarming response to sterile and bacteria-infected wounds in zebrafish [20,21]. Thus, the secretion of LTB4 works the same way in different species, allowing neutrophils to communicate among each other and promoting the self-organization of neutrophil swarming and aggregation.

**Triggering positive feedback amplification**

But what triggers the initial release of LTB4 in early-recruited pioneer neutrophils? Many stimulants, including N-formyl peptides (e.g. fMLF), complement factors, immune complexes, lipids, fungal cell wall components and cathelicidins, have long been known to activate LTB4 secretion by neutrophils *in vitro* [3]. On binding to the relevant cell surface receptors, most of these factors lead to elevated intracellular calcium levels, causing the translocation of the enzyme 5-lipoxygenase to nuclear and perinuclear membranes, a crucial step to initiate LTB4 synthesis from arachidonic acid [22]. Which LTB4-stimulating factors are sensed by pioneer neutrophils and induce the amplified swarming response in mouse tissues remained largely unknown. However, it is evident that neutrophil-derived LTB4 acts on top of early-released chemoattractants from tissue lesions, as even in the complete absence of leukotrienes neutrophils close to an injury site could still form very small clusters [8]. The same unidentified early-released attractants were speculated to also induce LTB4 synthesis. Discussed as the most interesting candidates were N-formyl peptides that can be released from damaged mitochondria of necrotic cells and are prominent inducers of chemotaxis. Elegant *in vitro* work showed that the fMLF-induced secretion of LTB4 acts in an autocrine and paracrine fashion to amplify neutrophil chemotaxis in shallow fMLF gradients, causing signal relay to distant neutrophils that follow the pioneer cells [23]. Another trigger factor was identified for neutrophil swarming *in vivo* in the context of lung infection with the fungus *C. albicans* in mice. In this example, the capture of living fungi by neutrophils depended on complement-induced chemotaxis. Complement activation also induced LTB4 synthesis, which led to the subsequent recruitment of larger neutrophil numbers and the formation of intravascular cell aggregates [24]. Interestingly, eosinophils, another granulocyte type, also use paracrine LTB4 signaling for their swarming and clustering response around parasitic worms [5]. However, the worm-derived trigger factors for LTB4 secretion have not yet been investigated.

Recent work by the team of Sarris used the power of zebrafish genetics in combination with
live imaging in translucent larvae to fill an important gap in our understanding of the initiating events of neutrophil swarming [21••]. By visualizing calcium dynamics and the perinuclear translocation of 5-lipoxygenase as a readout for LTB4 biosynthesis, her team provided unprecedented novel insights into the molecular events of nascent neutrophil swarms. Pioneer neutrophils that contacted a necrotic wound area underwent sustained calcium fluxes that rapidly propagated across clustering neutrophils in a developing swarm. This “calcium alarm signal” triggered LTB4 biosynthesis in clustering neutrophils at the wound core rather than in individual migrating cells outside the wound [21••]. The propagation of the calcium signals in a growing neutrophil cluster depended on connexin-43 (Cx43) hemichannels, which allowed active ATP release from neutrophils, promoting an autocrine and juxtacrine amplification of the initial damage signaling. ATP-gated calcium channels, such as P2X1, and extracellular calcium entry were required to promote the calcium signal in neighboring neutrophils. Taken together, this study provides an explanation of how intercellular signal amplification within a nascent neutrophil cluster promotes the local biosynthesis of LTB4 to form a powerful chemoattractant gradient source [21••]. Thus, cell–cell contact–dependent signal amplification in a small group of clustering neutrophils precedes the feed-forward amplification through secreted LTB4 to communicate with more distant cells and attract them for further swarm growth (Figure 2). In the zebrafish model another signal relay mechanism that originates from the injured tissue appears to act in addition to neutrophil-derived signals. Wounding in zebrafish larvae causes hydrogen peroxide gradients in the injured fin epithelium. Recent work has now shown that this peroxide promoted chemotaxis without being chemotactic but forms together with wound-released arachidonic acid long-range lipid peroxidation gradients that attract distant neutrophils [25•].

The direct access of pioneer neutrophils to the necrotic site is important to initiate sustained calcium fluxes in the first arriving cells, a process that also depended on the sensing of ATP [21••]. While the detailed mechanisms for this very early event remain to be fully explored, it was speculated that pioneer neutrophils might experience death-specific signals (such as fMLF) that could activate the opening of Cx43 hemichannels [21••]. These studies are in agreement with the general view that the initiation of neutrophil swarming is determined by the size of the initial tissue injury or the extent of locally occurring cell death, the latter directly depending on the presence of death-inducing factors, for example pathogenic microbes or toxins [3]. Neutrophils fail to swarm to small necrotic lesions in mouse tissues
where resident tissue macrophages quickly respond to the cell damage by forming pseudopod extensions that ensheathe the necrotic area [26,27*]. Similar to neutrophils, this macrophage “cloaking” response depends on the sensing of nucleotides but also involves the secondary detection of alarmins [27*]. By covering the dead cell with macrophage membrane, resident macrophages deny pioneer neutrophils the direct access to the necrotic area and prevent the formation of any attractant gradients directly from the injured tissue or from nascent neutrophil swarms (Figure 2).

While the lipid LTB4 has gained the most attention over the last years, other chemotactic factors also contribute to the positive feedback amplification in neutrophil swarms. This includes the chemokine CXCL2, which can be secreted by mouse neutrophils [28,29]. Mouse neutrophils lacking the corresponding receptor CXCR2 showed normal recruitment to developing swarms but were substantially impaired in neutrophil clustering. In addition, CXCL2 expression could be detected in neutrophil swarm clusters [8]. This suggests that neutrophil-secreted CXCL2 may exert a local effect within the neutrophil cluster, probably because of its binding capacity to glycosaminoglycans that may prevent diffusion in the tissue [30] (Figure 2). While homologs of CXCR2 and its related receptor CXCR1 also exist in other species, direct comparison of the relevant receptor-ligand pairs between model systems is difficult [18]. However, the swarming of human neutrophils in vitro also depends on CXCR2 signaling and complete blockade of swarming is only achieved on interference with both LTB4R1 and CXCR2 function [12]. This highlights the synergistic role of both pathways in the swarming of mammalian neutrophils.

**Maintaining and terminating the amplification signal**

How long does the self-generated LTB4 gradient persist in inflamed tissues? The exact distribution of neutrophil-released LTB4 in swarms remains unknown because of limitations in directly imaging this lipid. Characterized as a sparingly soluble lipid, LTB4 likely undergoes pure diffusion once it is secreted from activated neutrophils [31,32]. Because of its small size (MW = 336 Da), LTB4 released from growing clusters of swarming neutrophils would likely diffuse quickly and create rather transient gradients. In addition, activated neutrophils were shown to package the whole set of LTB4-synthesizing enzymes into multivesicular bodies, which could then be released in the form of exosomes, small extracellular vesicles (EVs) with sizes of 30-100 nm [33]. Packaging of LTB4 and exosome
deposition was suggested as a mechanism to better preserve LTB4 in the extracellular environment and release it with a time delay to prolong its gradient effects. Under conditions of a time-decaying primary chemoattractant gradient (e.g. by attractants directly from a wound site) exosomes might stabilize LTB4 gradients and contribute to the relay of initial signal to distant neutrophils [32,33]. EVs, including exosomes, were also recently detected in \textit{in vitro} platforms of human neutrophil swarms. Here, the release of EVs was suggested to impact neutrophil activation and contain other pro-inflammatory mediators [34]. The direct visualization of small-sized exosomes in zebrafish and mammalian tissues has not yet been achieved because of the resolution limits of intravital microscopy techniques. However, a recent intravital imaging study suggested a functional role of exosomal LTB4 during the process of extravasation when circulating neutrophils traverse the blood endothelium to reach into the inflamed extravascular space. Chemical inhibitors that interfered with exosome release impaired the arrest of neutrophils along the activated endothelium, suggesting that LTB4-synthesizing EVs were shed and deposited by neutrophils in the vascular lumen [35].

Taken together, it is currently assumed that the neutrophil self-generated LTB4 gradient feeds from nonexosomal and exosomal LTB4 sources.

How is LTB4-driven amplification stopped and neutrophil recruitment terminated? As one scenario, the amplification signal may just wane or become degraded. Newly synthesized LTB4 has a short half-life inside neutrophils where it can be rapidly turned into LTB4 metabolites. When externally applied onto neutrophils, these LTB4 metabolites can act as inhibitors and impair chemotaxis [36]. However, it is currently unclear if such metabolic inactivation of LTB4 can also occur in the extracellular tissue milieu [37]. Assuming the hypothetical deposition of LTB4-synthesizing exosomes in the extracellular tissue space, tissue-resident macrophages would be likely candidates to quickly phagocytose EVs, fulfilling their role as professional phagocytes of cellular debris. Other myeloid cells may contribute to breaking down the LTB4 gradient. We have previously observed that the cessation of neutrophil swarming in mice correlates with the late recruitment of monocytes/macrophages, which form a surrounding cover around a neutrophil cluster [8]. \textit{In vitro} swarming experiments with human leukocytes could show that neutrophils increase monocyte migration during the late stages of the swarm response [38].
finding of cell collectives. These studies provided evidence that groups of cells can self-generate gradients by consuming attractants of a homogenous attractant field \([39,40]\). It appears plausible that attractant consumption in an already established gradient may also contribute to its ultimate disappearance. Whether such a mechanism exists for swarming neutrophils remains to be tested. Finally, swarming human neutrophils were shown to produce lipids involved in the resolution of inflammation, including lipoxin A4 (LXA4), and they were suggested as swarming stop signals \([12]\). As these *in vitro* experiments used an excess amount of external LXA4\([12,36]\), several orders of magnitude higher than the amount released from neutrophils \([12]\), the functional relevance of this mechanism for swarming in a physiological tissue context remains to be explored.

Once neutrophils have clustered and the amplification signal is waning, neutrophils may disperse and leave the site again. Localized degradation of a chemoattractant could support cell dispersal from aggregates \([41]\), but other mechanisms may also underlie this process. A recent zebrafish study provided an example of how a two-chemokine receptor system can fine-tune these antagonistic cell behaviors at sites of tissue damage \([42]\). By investigating the zebrafish homologs of the human CXCL8 receptor CXCR1 and CXCR2, this study showed that the differential trafficking of these two receptors in neutrophils allows the coordination of clustering and dispersal, promoting a self-resolving migratory response. CXCR1 promoted neutrophil clustering but was rapidly desensitized and internalized at wounds. This was critical to allow the transition to signaling through CXCR2, which, unlike CXCR1, was sustained at the plasma membrane and promoted bidirectional migration and neutrophil dispersal \([42]\). Similar mechanisms may also operate in mammalian neutrophils, which commonly express \(>30\) receptors on their surface to respond to various chemokines and chemoattractants \([43]\). Neutrophil migration out of cell clusters is commonly observed in infected mouse tissue in situations of transient swarming when neutrophils move out of one swarm center to detect a novel site of cell death and start a new swarm in the tissue. Although not directly proven, it is currently believed that such rerouted neutrophils respond to “end-target” chemoattractants (e.g. N-formyl peptides, C5a) whose signaling dominates over “intermediate-target” attractants (e.g. CXCL2, LTB4) \([44,45]\).

Very recently, two-photon imaging of injured skin and infected lymph nodes of mice showed that the desensitization of LTB4R1 and CXCR2 play critical roles during neutrophil swarming \([46]\). At sites where swarming neutrophils accumulate and self-generate local
fields of high swarm attractant concentration, GPCR desensitization was crucial to stop the migration of neutrophils [46••]. The GPCR kinase GRK2 was identified as key regulator to control the desensitization of the swarm-mediating GPCRs LTB4R1 and CXCR2, whereas it had only a minor impact on other neutrophil-expressed GPCRs [46••]. Unexpectedly, mice with GRK2-deficient neutrophils, which moved faster and explored larger areas of bacteria-infected tissues, showed impaired rather than enhanced bacterial clearance. This study showed that GPCR desensitization acts as a cell-intrinsic negative feedback control mechanism to self-limit neutrophil swarming. It highlighted that impaired neutrophil arrest during swarming comes at the cost of suboptimal phagocytosis and containment of bacteria [46••,47].

Conclusion

Neutrophil swarming is one of the most fascinating phenomena discovered by imaging immune cells in living tissues. Positive amplification signals provide neutrophil swarms a level of self-organization for robust navigation toward sites of tissue damage in a complex inflammatory tissue setting (Figure 2). The exact neutrophil swarm phenotype is determined by several factors: the size of the initial tissue injury or extent of local cell death, the occurrence of only one or several damage sites in the tissue, the presence of pathogens and the number of recruited neutrophils. This combinatorial variability of different control layers explains the heterogeneous appearance of neutrophil swarms in tissues. Studies with human, mouse and zebrafish neutrophils have started to peel the different layers of the swarming response and to identify some of the underlying molecular mechanisms. Some of these basic principles may also prove relevant for other swarming immune cells [6•]. Using the full power of all model organisms, future work will uncover the mechanisms that fine-tune and counter-regulate positive amplification signals and dissect to which extent they differ between species.

Conflict of Interest

The authors declare no conflict of interest.

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This study showed that when cytotoxic T cells (CTLs) identify tumor cells in tumoroids in vitro, they release the chemokines CCL3 and CCL4, which attract other CTLs and recruit them to the target site. Experiments and computer simulations revealed a correlation between the number of attracted CTLs and the amplification through the secreted chemokine signal.


This study builds on previous work in ref. 12 and expands a technical in vitro platform to study human neutrophil swarming toward patterns of living pathogens. By studying the swarming response to several pathogenic fungal species, this paper finds that neutrophil swarms delay the growth of fungal clusters and identifies some of the molecules, including amplifying chemoattractants, that control this process.


19. Huang C, Niethammer P: **Tissue Damage Signaling Is a Prerequisite for Protective Neutrophil Recruitment to Microbial Infection in Zebrafish.** *Immunity* 2018, **48**:1006-1013 e1006.


A delightful piece of work, which used novel biosensors and live imaging in zebrafish to visualize the intracellular events leading to LTB4 synthesis in individual neutrophils. The study identifies connexin channels as a key determinant of neutrophil swarming and highlights a novel mechanism how cell-cell contact dependent communication amplifies calcium fluxes in cell clusters to locally promote the production of a self-generated attractant gradient.


23. Afonso PV, Janka-Juntila M, Lee YJ, McCann CP, Oliver CM, Aamer KA, Losert W, Cicerone MT, Parent CA: **LTB4 is a signal-relay molecule during neutrophil chemotaxis.** *Dev Cell* 2012, **22**:1079-1091.


This zebrafish study describes the functional interplay of hydrogen peroxide and arachidonic acid, two wound-released signals, and brings new light into the controversial topic of H2O2 as a chemoattractant.


Using intravital microscopy of the peritoneal serosa of mice, this study characterizes how tissue-resident macrophages respond to small laser-induced lesions by sending out spreading pseudopods, as previously described in ref. 26 for microglia in the brain. The coverage of small lesions with macrophage membrane prevented the induction of neutrophil swelling.


Using intravital microscopy of extravasating neutrophils in mouse skin, this study observes extracellular vesicles in the vascular lumen and suggests an involvement of LTB4-synthesizing exosomes in the control of neutrophil arrest along activated endothelium.


Although this paper and ref. 39 do not include experiments with neutrophils, both studies highlight a rarely considered concept that likely has implications for many areas of biology. Experimental work in microfluidic mazes and computational modeling reveal the importance of self-generated gradients by local attractant consumption for the navigation of cell collectives.


This study used biosensors and imaging in translucent zebrafish larvae to visualize chemokine receptor internalization and plasma membrane localization in individual neutrophils at tissue wounds. Rigorous mutagenesis of the receptors CXCR1 and CXCR2 revealed that the differential trafficking of these two chemokine receptors coordinates the two antagonistic processes of cell clustering and dispersal.


This study in mice shows that GPCR desensitization acts as cell-intrinsic stop mechanism for the self-organization of neutrophil swarms in infected tissues, which is based on sensing the local accumulation of the same cell-secreted attractants that amplify swarming during early stages. This mechanism allows neutrophils to self-limit swarming responses and ensures optimal elimination of bacteria.

Figure 1: The multistep process of neutrophil swarming. A population of neutrophils (blue) that has infiltrated inflamed or infected tissue responds to damaged tissue or locally occurring cell death in sequential phases. Neutrophils closest to the injury site sense signals from the tissue lesion (yellow), causing a switch from random motility during patrolling behavior to chemotactic movement toward the injury site (1). These pioneer neutrophils are followed by nearby neutrophils and they together form small nascent cell clusters at the damage site (2). Amplified recruitment of more distant neutrophils often occurs as a second wave of neutrophil migration from surrounding tissue areas (3). Neutrophils aggregate and form large cell clusters at sites of initial tissue damage (4). At one point, neutrophil recruitment and cluster growth ceases, a process that correlates in some tissues with a time-delayed accumulation of monocytes/macrophages (purple) around the neutrophil cluster (5).
Figure 2: Discussed concepts and mechanisms underlying the initiation and positive feedback amplification of neutrophil swarming. (Left) Pioneer neutrophils (blue) respond to primary signals released from a necrotic tissue site. The exact nature of primary signals is not entirely clear and may also depend on the studied model system. In mice, resident tissue macrophages (purple) can sense ATP and alarmins to form a membrane shield around small necrotic lesion, which prevents nascent clustering and swarm formation. (Right) Several layers of self-generated signal amplification promote neutrophil swarming and clustering in a feed-forward manner. Zebrafish studies revealed sustained calcium fluxes that propagated across nascent neutrophil clusters in a connexin43- and ATP-dependent mechanism. This “calcium alarm signal” triggered the release of LTB4 in early-arriving neutrophils to increase the radius of neutrophil attraction and also recruit more distant cells. In vitro experiments suggest an involvement of LTB4-synthesizing exosomes in this signal relay model. Once neutrophils have formed prominent clusters, they increase the local concentration of self-produced CXCL2 and LTB4, which further promotes aggregation.