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Neutrophil migration: moving from zebrafish models to human autoimmunity

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Summary: There has been a resurgence of interest in the neutrophil's role in autoimmune disease. Classically considered an early responder that dies at the site of inflammation, new findings using live imaging of embryonic zebrafish and other modalities suggest that neutrophils can reverse migrate away from sites of inflammation. These 'inflammation-sensitized' neutrophils, as well as the neutrophil extracellular traps and other products made by neutrophils in general, may have many implications for autoimmunity. Here, we review what is known about the role of neutrophils in three different autoimmune diseases: rheumatoid arthritis, systemic lupus erythematosus, and small vessel vasculitis. We then highlight recent findings related to several cytoskeletal regulators that guide neutrophil recruitment including Lyn, Rac2, and SHIP. Finally, we discuss how our improved understanding of the molecules that control neutrophil chemotaxis may impact our knowledge of autoimmunity.

Keywords: *neutrophil, migration, autoimmune, Lyn, Rac2, SHIP*

Introduction

Neutrophils are classically known as critical first responders in an immune response. They are generated in the bone marrow and circulate in the bloodstream until they are attracted into the tissue by numerous different types of molecules (1). In the tissue, activated neutrophils produce reactive oxygen species and release cytoplasmic granules filled with molecules like alarmins and antimicrobial peptides (2), which result in the killing of pathogens as well as significant host cell death and tissue damage. Neutrophils also can die in a process called NETosis in which chromatin is extruded as part of a nuclear extracellular trap (NET) (2). These NETs contain antimicrobial proteins and are important for the innate immune response against pathogens, but they have also been implicated in autoimmunity, as discussed below.

Neutrophils have many immediate effects mediated by their antimicrobial products and reactive oxygen species, but they also have more long-lasting effects related to the cytokines that they secrete and their influence on other immune cells. They can recruit macrophages to sites of inflammation,

where the macrophages have the potential to perform many pro- and anti-inflammatory activities, in part, dependent on signals from neutrophils (3). Neutrophils also have been shown to carry antigens from sites of inflammation to lymph nodes in mice (4) and have been reported to have an average lifespan greater than 5 days, much longer than the previously generally accepted lifespan of less than 1 day (5). What these longer-lived and/or 'inflammation-sensitized' neutrophils might be doing is not known. However, there is evidence that neutrophils can alter the long-lasting adaptive immune response through interactions with B and T cells. For example, neutrophils colonize an area near the splenic marginal zone where they can expand the production of antibodies made by B-lineage cells (6). Furthermore, they can present antigens to T cells (7) and appear, in different studies, to be able to activate (8) and/or repress (9) T-cell activity. Thus, neutrophils may be playing a complex role in regulating adaptive immunity.

All of these neutrophil effects depend on neutrophils arriving at sites of inflammation, and they have a very specific mechanism for doing so (10). First, they are captured by selectins on the vessel wall leading to intraluminal crawling, then rolling, and then firm adhesion via β_2 integrins (CD11/CD18) and chemokines. The next step is neutrophil extravasation through the vessel wall, either between endothelial cells or through an individual endothelial cell, in a process called diapedesis. Finally, they migrate through the basement membrane, perivascular region, and into the tissue. There is a large literature about cytoskeletal control of neutrophil motility that is derived largely from *in vitro* studies (11–14). We are interested in inflammatory disease, and have therefore developed and studied the zebrafish system, which allows us to look at cytoskeletal regulatory pathways in the context of *in vivo* neutrophil movements.

Zebrafish are an excellent model system for imaging cell movement, as embryonic zebrafish are transparent, allowing an observer to track cells *in vivo* using time-lapse imaging. One of our early observations in zebrafish is neutrophil reverse migration. Using a transgenic zebrafish whose neutrophils express green fluorescence protein, time-lapse imaging showed that neutrophils not only migrate to a wound (a type of sterile inflammation) but also away from the wound back to the vasculature to resolve the local inflammatory response (15). This reverse migration may be how neutrophils carry antigens to the lymph nodes in mice. Using transgenic zebrafish whose neutrophils express a photoconvertible fluorescent reporter, Dendra2 (16), individual migrating neutrophils can be photolabeled and then tracked as they migrate elsewhere in the living fish. Using this model, we found that neutrophils migrate to and from the wound repeatedly and then disperse throughout the body as the wound heals (16). Thus, in addition to wound resolution, this process leads to the dissemination of 'wound-sensitized' neutrophils. Similar events may be occurring in mammals during inflammation, although the role of the inflammation-sensitized neutrophils is unknown. However, it is interesting to hypothesize how these experienced neutrophils might influence other immune cells. In this review, we focus on how live imaging of neutrophil movements in zebrafish has shed light on the roles of several important cytoskeletal regulatory molecules and what the implications may be for neutrophil migration and human autoimmunity.

Neutrophils in autoimmune diseases

Rheumatoid arthritis

Rheumatoid arthritis is an inflammatory, destructive arthritis that affects about 1% of people. It is an autoimmune disease characterized by antibodies against citrullinated proteins. However, the innate immune system and inflammatory cytokines like tumor necrosis factor-α (TNFα) and interleukin-6 (IL-6) play a large role. A hallmark of rheumatoid arthritis is the transformation of the normal synovial lining, which surrounds the joint and synovial fluid, into a tumor-like pannus composed of activated synovial fibroblasts and immune cells. The pannus grows and invades into the cartilage and bone of the joint causing joint deformity and destruction (17). Neutrophils are the primary cell type found in the synovial fluid of joints affected by rheumatoid arthritis as well as at the junction of the pannus and cartilage, where invasion occurs (1). Neutrophils are thought to be critical for disease pathogenesis as a lack of neutrophils blocks the development of two different models of the innate, effector arm of rheumatoid arthritis, the K/B×N (18) and collagen antibody-induced arthritis (19) models.

Neutrophils likely contribute in many ways to rheumatoid arthritis. Neutrophils from patients with rheumatoid arthritis are typically more activated (20) with high baseline intracellular reactive oxygen species production compared to control neutrophils. Furthermore, rheumatoid neutrophils appear to be primed even in patients in remission (21). These activated neutrophils likely cause tissue destruction, leading to joint damage as well as immune response amplification. In addition to, or possibly due to, their destructive effects, neutrophils may be involved in increasing the vasopermeability

of the joint (22) to allow antibodies to enter and deposit in the joint (23). Antibody deposition, particularly as part of immune complexes, is thought to be a major contributor to joint inflammation. Neutrophils may also contribute to autoantibody formation since netting neutrophils, i.e. neutrophils making NETs, can be found in rheumatoid synovium. These NETs display some of the citrullinated antigens that are known to be targets of the pathologic anti-citrullinated protein antibodies (ACPAs) in rheumatoid arthritis (24). Thus, as shown in *Fig.* 1, there may be a positive feedback loop in rheumatoid arthritis where ACPA immune complexes accelerate inflammation, which attracts neutrophils, which present citrullinated proteins to be incorporated into immune complexes, which amplify inflammation. Neutrophils and their NETs also enhance synovial fibroblast cytokine production (24), chemotaxis, matrix metalloproteinase production, and invasiveness (25) as well as affect B-cell behavior through release of B-lymphocyte stimulator upon activation with TNFα in the rheumatoid joint (26).

To exert many of their pathologic effects, neutrophils need to enter the joint and there are many factors that contribute to this process. There is a role for P-selectin, E-selectin, CD18 (the β2 integrin), CXCR2, IL-6, Duffy antigen receptor for chemokines, and CXCL5 (27, 28). Moreover, recruitment may be biphasic and related to the presence of immune complexes. Immune complexes lead to activation of the complement cascade with production of C5a and can also bind to Fcγ receptors. Activation of neutrophils via the C5a receptor leads to the release of leukotriene B (4) which attracts neutrophils to the joint early in disease. Later, engagement of Fcγ receptors on neutrophils leads to IL-1β release and subsequent neutrophil-attracting chemokine production, which promotes chronic inflammation (29). Neutrophil migration to the joint appears to be a critical point in disease pathogenesis as many of the treatments for rheumatoid arthritis decrease recruitment of neutrophils into the inflamed joint including leflunomide, methotrexate (30), methylprednisolone (31), and anti-TNFα agents (32).

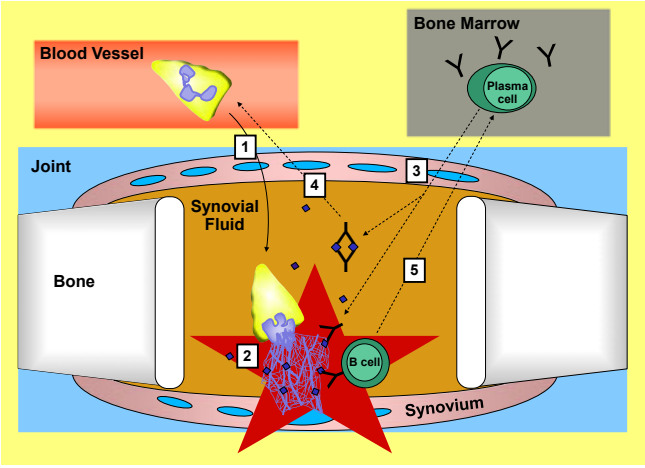

***Fig.* 1. Model for how neutrophils may contribute to rheumatoid arthritis.** This model is numbered, but which step is actually first in disease is not clear. 1. Neutrophils enter the joint space. They (2) become activated and (3) increase synovial permeability to allow antibodies to enter the joint, including anti-citrullinated protein antibodies (ACPAs). 2. Activated neutrophils can die by NETosis releasing their NETs, which are studded with citrullinated proteins (purple diamonds). ACPAs bind to these proteins increasing inflammation. 4. Immune complexes composed of ACPAs and citrullinated proteins attract more neutrophils to the joint amplifying inflammation. 5. Also, naive and/or memory B cells, which are attracted to inflammation and have B-cell receptors that recognize citrullinated proteins, become activated upon recognizing citrullinated antigens in NETs and ultimately home to the bone marrow where they reside as ACPA-secreting plasma cells. Those plasma cells make more ACPAs, which enter the joint (3) to amplify inflammation.

It is interesting that neutrophils are such a large presence in the chronically inflamed joints in rheumatoid arthritis, as they are classically thought of as early responders and short-lived. It is not known how much of the neutrophil presence is due to continued recruitment versus long-lived cells. However, there are some data suggesting that neutrophils in rheumatoid arthritis may be longer lived. For example, synovial fluid from rheumatoid arthritis patients inhibits both spontaneous and immune complex-mediated neutrophil apoptosis (33). This effect is thought to be mediated by adenosine, but IL-17, TNFα, and granulocyte-macrophage colony-stimulating factor may also contribute to increasing the lifespan of the neutrophils (34). The lifespan of neutrophils may be important to disease pathogenesis as methotrexate blocks the neutrophil longevity seen in rheumatoid arthritis (35). Thus, the neutrophil is a critical cell in rheumatoid arthritis with many neutrophils recruited to the inflamed joint with a longer life and a more activated phenotype.

Small vessel vasculitis

Vasculitis is a life-threatening disorder caused by inflammation of the blood vessels. There is a subset of vasculitis called small vessel vasculitis that includes granulomatosis with polyangiitis (previous Wegener's granulomatosis), microscopic polyangiitis, and Churg-Strauss disease. In all of these disorders, there is inflammation of the small vessels primarily in the lungs and kidneys although vessels can also be affected in the skin, upper airways, peripheral nerves, brain, and heart depending on the specific small vessel

vasculitis. Although not detected in every case of small vessel vasculitis, anti-neutrophil cytoplasmic antibodies (ANCAs) can be found in the majority of patients. These antibodies target myeloperoxidase (MPO) and proteinase 3 (PR3), both components of the primary granules in neutrophils (1), and may also target other unknown antigens. Thus, in small vessel vasculitis, neutrophils appear to be the main targets of the auto-antibodies, intricately linking them to the pathophysiology of disease.

ANCAs are thought to drive small vessel vasculitis. In support of this theory, an intravenous infusion of anti-MPO antibodies can drive a glomerular nephritis in mice that is histologically similar to human small vessel vasculitis (36). However, the data are not as clear in humans, as small vessel vasculitis can occur without detectible ANCAs and ANCA levels do not always correlate with disease activity. Regardless, ANCAs, through binding to Fc receptors and recognizing targets with their antigen-binding domains (37), do have many pathologic effects on neutrophils. ANCAs stimulate neutrophils resulting in degranulation, production of oxygen radicals (38) and IL-1β (39), and NET formation (40). ANCAs also increase the chemotactic response of neutrophils (41) and increase their ability to adhere to endothelial cells in a manner dependent on β2 integrins and CXCR2 (42, 43). Thus, neutrophils appear to be both activated by ANCAs and stimulated by them to migrate into the vessel walls where they can cause destruction (Fig. 2).

In addition to a role for ANCAs, neutrophil recruitment into the vessels in vasculitis appears to involve CXCL8 and IL-1β (44). Neutrophil recruitment is key for pathology as the necrosis of blood vessels in small vessel vasculitis is thought to be due to neutrophil infiltration and activation (45). Damage could be occurring through any number of the cytotoxic neutrophil products. Furthermore, NETs are present in the inflamed kidneys in vasculitis and display PR3 and MPO, providing a target for ANCA binding (40). Thus, a feedback loop of inflammation may be occurring where ANCAs bind to the netting neutrophils causing further inflammation and the PR3 and MPO displayed on the neutrophils in the inflammatory milieu may be inducing further ANCA production (Fig. 2).

In human disease, the data for neutrophil involvement are primarily correlative or derived from *in vitro* experiments, but there is evidence for a critical pathologic role for neutrophils in vasculitis in rodent models. In mouse models of small vessel vasculitis, neutrophils are detected at sites of glomerular necrosis and depletion of neutrophils completely blocks disease (46). A separate model of lung disease has been made where infusion of TNFα-primed neutrophils and ANCAs together cause increased pulmonary endothelial permeability and lung edema that requires reactive oxygen species and neutrophil elastase (47). Neutrophil MPO can alter endothelial cell function and close contact of neutrophils via integrins can transfer MPO to endothelial cells (48). Furthermore, migration of neutrophils into the vessel walls is likely critical for disease as a synthetic retinoic acid receptor agonist ameliorates a murine model of vasculitis (induced by *Candida albicans*) through the suppression of neutrophil migration and activation (49). Thus, similar to rheumatoid arthritis, neutrophils are present in the sites of inflammation in vasculitis and likely contribute to disease.

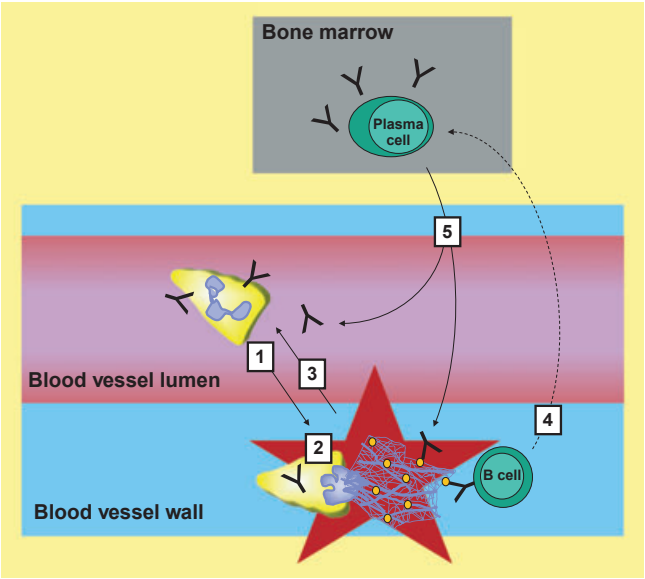

Fig. 2. Model for how neutrophils may contribute to small vessel vasculitis. 1. Peripheral blood neutrophils are activated by circulating anti-neutrophil cytoplasmic antibodies (ANCAs) and stimulated by them to enter the vessel wall. 2. In the vessel wall, activated neutrophils can degranulate and generate free radicals to cause damage and undergo NETosis. The NETs contain myeloperoxidase (MPO) and proteinase 3 (PR3), which are represented as orange ovals and are targets of ANCAs. ANCA immune complexes and damage by neutrophils increase inflammation and (3) attract more neutrophils. 4. Naive and/or memory B cells, which are attracted to the vessel inflammation and have B-cell receptors that recognize MPO, PR3, and potentially other antigens, become activated and ultimately differentiate into plasma cells. The plasma cells migrate to the bone marrow and continue to secrete ANCAs, which (5) enter the bloodstream to promote disease.

Lupus

Lupus is a systemic autoimmune disease that presents with a constellation of symptoms that can be different for each individual. Some of the more severe manifestations of lupus

include lupus nephritis, lupus cerebritis, and lupus vasculitis, but many other manifestations can occur including pericarditis, pleuritis, skin rashes, cytopenias, hair loss, and oral ulcers. Indeed, almost any organ system can become affected in lupus, making this an amorphous and unpredictable disorder. Like rheumatoid arthritis and small vessel vasculitis, patients with lupus have autoantibodies, classically antinuclear antibodies in addition to others.

The role of neutrophils in lupus may be different than in rheumatoid arthritis and small vessel vasculitis. In both rheumatoid arthritis and vasculitis, neutrophils are thought to migrate to the joint or blood vessel and create local inflammation and damage. However, in lupus, a more diffuse systemic disease, pathology due to neutrophils may be more complex and involve more indirect effects (2). For example, there is evidence for increased activation of neutrophils in rheumatoid arthritis, but in lupus the data are mixed. Neutrophils from lupus patients have been shown to have decreased phagocytosis, chemotaxis, and oxidative burst in response to IL-8 (50) and neutropenia is often seen in lupus. In contrast, others have reported that neutrophils in lupus are more activated intravascularly (51). One possible explanation for the conflicting data about lupus neutrophils is the presence of a subset of neutrophil-like cells in lupus patients called low density granulocytes (LDGs), which have enhanced NETosis, increased ability to kill endothelial cells, and increased ability to stimulate plasmacytoid dendritic cells to secrete type I interferon (52), one of the major cytokines involved in lupus. Perhaps these hyperactive LDGs are distinct from the hypo-activated neutrophils seen in some studies and the LDGs are the main contributors to lupus nephritis, accelerated atherosclerosis, or other manifestations of lupus (53). Alternatively, there is a large range of clinical presentations of lupus and the diverse findings with neutrophils may reflect differences in underlying pathogenesis.

Neutrophil NETs have also attracted significant attention in lupus. Lupus neutrophils have been shown to have increased NET formation (54) and impaired NET breakdown (55). NETs can activate plasmacytoid dendritic cells to secrete type I interferon (54). Furthermore, netting neutrophils can infiltrate tissues and cause endothelial damage in lupus (52). Similar to small vessel vasculitis and rheumatoid arthritis, NETs have been hypothesized to be a source of autoantigens in lupus (2), but there is contradictory data for this theory. In lupus-prone mice deficient in the NADPH oxidase, Nox2, neutrophils cannot make NETs and the mice have worsened lupus instead of the predicted improved lupus (56).

Despite the fact that some neutrophil effects in lupus may be indirect, neutrophils do migrate to sites of inflammation, and there is some literature on the regulators of this recruitment. In a strain of lupus-prone mice, neutrophils have higher levels of CXCR4, kidneys have high levels of CXCL12 and high numbers of infiltrating CXCR4-expressing cells, and a peptide antagonist of CXCR4 improves glomerulonephritis and survival (57), suggesting that kidney damaging neutrophils depend on the CXCR4/CXCL12 axis for recruitment. This study also suggests that lupus neutrophils, through elevated levels of CXCR4 may have enhanced recruitment to inflamed sites. In contrast to this study, others have shown that in a mouse model of peritonitis involving thioglycolate, two different strains of lupus mice showed normal recruitment of neutrophils to the peritoneum, but delayed resolution of the neutrophil infiltrate (58). Other factors are also at play. For example, IL-8 may be important in recruiting neutrophils to the inflamed kidney, as there are high levels of IL-8 in the urine in patients with lupus nephritis (59). However, as mentioned above, neutrophils in lupus have been observed to have impaired migration toward IL-8. VCAM-1 is also elevated in the urine of patients with active lupus glomerulonephritis (60), and neutrophils from patients with active lupus express higher levels of the β2 integrin CD11b/CD18 (51), suggesting that integrin signaling is important for neutrophil recruitment in lupus. Thus, there are many conflicting reports regarding the phenotype and functions of neutrophils in lupus, however, the bulk of the evidence supports a pathologic role, the details of which are still poorly defined.

Behcet's disease

Although it is more of an autoinflammatory disorder than an autoimmune disorder, Behcet's disease is worth mentioning as neutrophils are found in the inflammatory lesions (61), and there is some interesting literature regarding their phenotype and Behcet's treatment. The classic symptoms of Behcet's disease are oral and genital ulcers, but patients also have at least some of the following symptoms: pathergy, a variety of skin rashes, eye inflammation, arthritis, vascular thrombosis, and vasculitis. Indeed Behcet's may be caused by inflammation of the blood vessels.

Neutrophils have been evaluated in active Behcet's disease and have increased *in vivo* migration compared to normal controls and patients whose Behcet's is in remission.

However, no differences were seen in reactive oxygen species production or adhesion (62). Furthermore, sera from Behcet's patients increase neutrophil migration, a phenotype that can be inhibited by colchicine (63). Treatment of Behcet's often involves colchicine, a microtubule inhibitor, which is discussed below.

Neutrophil studies in zebrafish and implications for human autoimmune disease

As discussed above, neutrophils play prominent roles in autoimmunity, and their migratory function is often critical for their pathogenic roles. However, it is challenging to study neutrophil motility in humans or even mice. Moreover, observations from *in vitro* migration studies do not always parallel what occurs in three dimensions *in vivo*. Thus, studies in zebrafish may enable us to better understand neutrophil motility with implications for autoimmunity.

One of the major recent observations is that following recruitment to a wound, neutrophils can reverse migrate away from the wound to distant locations in the zebrafish (15), as discussed in detail above. This observation could impact our understanding of the pathophysiology of autoimmunity as reverse migrated neutrophils might influence progression of disease. For example, neutrophils might reverse migrate away from areas of inflammation like the rheumatoid joint or inflamed blood vessel. Those neutrophils could migrate to lymphoid organs to present antigens from the inflamed site to T cells or B cells and contribute to the diversification of the auto-antibody repertoire. Perhaps, even subclinical inflammation, in the joint for example, could lead to neutrophil recruitment. Some neutrophils become activated and die by NETosis and expose citrullinated antigens that become targets for ACPAs. Other neutrophils may reverse migrate to the lymph node to diversify the antibody response. Indeed, it has been shown that the ACPA repertoire expands in preclinical rheumatoid arthritis even before symptoms develop (64). In these scenarios, long-lived, inflammation-sensitized neutrophils would be pathologic, but the opposite might also be true if previously educated neutrophils act as a tolerizing force for T cells. At this time, all of the ways in which neutrophils affect autoimmunity are unknown. Using the zebrafish model to answer questions regarding the regulators of cytoskeletal dynamics in neutrophil movements, we hope to better understand inflammation and autoimmunity. Below, we discuss some of our recent findings and how these observations may contribute to improving our understanding of autoimmune disease.

Rac2 in Neutrophils

Rac2 is a member of the Rho family of guanosine triphosphatases (Rho GTPases), whose expression is restricted to the hematopoietic lineage (65). Rho GTPases are a group of small signaling molecules that activate various cellular signaling pathways leading to events such as actin polymerization and the formation of a reduced NADPH oxidase complex (66). In leukocytes, Rho GTPases participate in the tightly controlled process of transendothelial migration as well as chemotaxis in response to immune system stimulation. Rho GTPases integrate signals from integrins and chemokine receptors and then propagate these signals through the reorganization of the actin cytoskeleton into a motile structure (67).

Among all Rho GTPases, *in vitro* studies reveal a specific role for Rac2 in neutrophil chemotaxis (68). Following fMLP stimulation of neutrophils, Rac2 appears to be a key factor for actin polarization and assembly of a pseudopod. Moreover, Rac2-deficient mice display mild neutrophilia and defective host defense (69), suggesting a defect in neutrophil transendothelial migration and chemotaxis. However, how Rac2 regulates neutrophil directed migration *in vivo* during inflammation was not known. Taking advantage of zebrafish to investigate neutrophil migration *in vivo* (70), we generated two transgenic zebrafish lines that specifically express Rac2 and Rac2 D57N point mutation in neutrophils (71). The Rac2 D57N mutant corresponds to an identified mutation in two infants suffering from a new type of leukocyte adhesion deficiency (LAD), LAD IV (72). We had previously shown that neutrophils from those patients have impaired polarization and directed migration *in vitro* (73). Using zebrafish, we were then able to confirm the impaired polarization and directed migration phenotype of Rac2 D57N neutrophils *in vivo* as well as show that Rac2 not only drives actin architecture but is also required for proper activation of phosphoinositide 3-kinase (PI3K). This finding is of particular interest, because PI3K was previously considered to be an upstream regulator of Rac2 (74), and thus these findings suggest a more complex feedback interaction (*Fig.* 3). Rac2 D57N mutants also exhibit increased release of neutrophils from hematopoietic tissue into the vasculature mainly due to a disruption of the SDF1-CXCR4 neutrophil retention signal in hematopoietic tissue (75). Therefore, using zebrafish and photoconversion cell fate

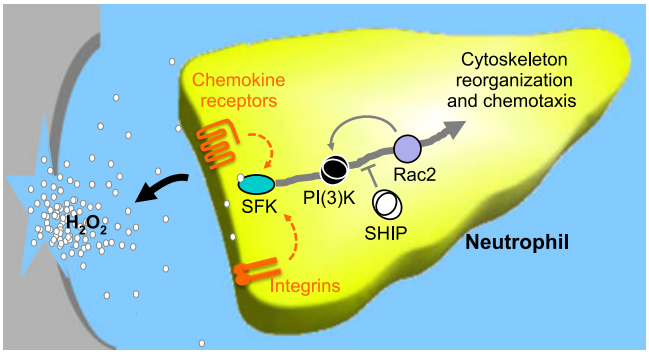

Fig. 3. Model for signaling involving SFKs, Rac2, SHIP, and PI3K in neutrophil recruitment. Upon tissue injury or infection, neutrophils are the first responders of the innate immune response. They are attracted to sites of inflammation by various stimuli including binding of integrin ligands, chemokines, or hydrogen peroxide (H_2O_2), which can lead to signaling through Src family kinases (SFKs). In the case of a wound, H_2O_2 is released by damaged epithelial cells and oxidizes a cysteine in the SFK family member, Lyn, which thus acts as a H_2O_2 gradient sensor. Oxidation of that cysteine leads to increased kinase activity of Lyn. Once activated, Lyn can drive the cytoskeleton reorganization that ultimately leads to neutrophil chemotaxis through the activation of PI3K which induces Rac2. Interestingly, Rac2 can also activate PI3K in what may be a positive feedback loop enhancing migration. Neutrophil chemotaxis is inhibited by SHIP via regulation of PI3K signaling.

mapping, this disease model helped to explain how the specific D57N mutation seen in patients was responsible for the observed neutrophilia.

Rac2 is thus a critical molecule for neutrophil motility. As touched upon above (76–79), inhibitory mutations in Rac2 lead to a severe human neutrophil immunodeficiency syndrome with neutrophils displaying defects such as disorganized actin polymerization, defective oxidative burst, and abnormal chemotaxis depending on the specific mutation. It is clear that when Rac2 is impaired, immunity is impaired as well. However, decreased immune response due to deficient or defective Rac2 perhaps could be capitalized upon to treat autoimmune disease. If Rac2 could be partially inhibited or temporarily inhibited, there could be benefits in autoimmune diseases like rheumatoid arthritis or vasculitis. In support of this idea, in a murine model of infective arthritis, mice deficient in both Rac1 and Rac2 had reduced arthritis and reduced synovial infiltration of neutrophils in the acute phase, but arthritis was more severe in the chronic phase with decreased pathogen clearance from the joint (80). It seems likely that the more severe chronic arthritis was related to poor pathogen clearance, so if one were to extrapolate to an autoimmune arthritis like rheumatoid arthritis, where there is no pathogen to clear, inhibition of Rac1 and Rac2 might be beneficial leading to decreased neutrophil infiltration and improved arthritis. Furthermore, inhibition of Rac1 reduces paw swelling in collagen-induced arthritis, a model of rheumatoid arthritis (81). Therefore, the Rac GTPases may be potential therapeutic targets in rheumatoid arthritis.

The involvement of Rac2 in neutrophil migration *in vivo* also suggests that Rac2 might be abnormally regulated in autoimmune disorders. Indeed, one of the three major RAC2 haplogroups in humans is associated with multiple sclerosis and Crohn's disease, two chronic inflammatory disorders (82). Thus, Rac2 may be important in diseases that involve recruitment of neutrophils to an area of inflammation like the joint in rheumatoid arthritis or the vessels in vasculitis, potentially making it a target for treatment.

Lyn in neutrophils

Src family kinases (SFKs) are signaling proteins that have long been recognized to regulate critical cellular processes such as leukocyte proliferation, survival, and migration (83). One essential role of SFKs in the inflammatory response is regulation of neutrophil activation and recruitment to inflamed tissue (84, 85). SFKs are activated in response to stimulation of a variety of cell surface receptors such as tumor necrosis factor receptor (86), P-selectin glycoprotein ligand 1 (85), interleukin receptors, integrin receptors, and chemotactic sensitive G-protein-coupled receptors (87). Once activated, SFKs contribute to both chemotactic recruitment and endothelium adhesion of neutrophils (84). Although SFK involvement in selectin-mediated neutrophil adhesion is widely accepted, the precise role of SFKs in chemotactic movement and recruitment is still under debate. Indeed, some studies claim that neutrophil recruitment to liver (88, 89), lung (90), peritoneal cavity (85), skin and inflamed organs (91) requires the activity of SFKs while others (92, 93) argue for a SFK-independent recruitment of neutrophils into the peritoneal cavity and skin. However, these apparent discrepant observations may be due to differences in which SFK was evaluated or which modality was used to trigger inflammation.

Among SFKs, emerging evidence suggests that Lyn is the main contributor to endothelial adhesion (86, 94) and directed migration of neutrophils (95). Like all SFKs, Lyn is composed of several domains: an N-terminal unique domain, SH3 and SH2 domains, and a C-terminal kinase domain (83, 96). At the N-terminus, Lyn is myristoylated and palmitoylated causing its association with the plasma membrane. The SH3 and SH2 domains mediate interactions

with proline-rich motifs and with phosphotyrosine-containing sequences, respectively (97). Lyn is in an inactive conformation involving an intramolecular interaction between a phosphotyrosine residue and the SH2 domain. Upon dephosphorylation of Y508, Lyn adopts an open conformation in which the activation loop tyrosine (Y397 in humans) is phosphorylated and the kinase is fully activated (83). A lysine at position 275 is required for full enzymatic activity of the C-terminal kinase domain (96).

SDF1-CXCR4 can trigger the Lyn/PI3K signalling pathway in the neutrophil-like HL-60 cell line and SDF1 induced cell migration is defective in Lyn-deficient bone marrow cells (98). Lyn then has many downstream effects. A recent report from He *et al.* (99) depicts a Lyn-dependent mechanism that governs neutrophil adhesion in chemotaxis gradients. This pathway depends upon G_i-protein-mediated Lyn activation and leading edge recruitment. Next, Lyn relieves the inhibition of and promotes the recruitment of the CrkL-C3G (an adapter protein-guanine exchange factor protein) complex at the leading edge, which leads to localized activation of the small GTPase Rap1 and its downstream target, β2 integrin (CD18). Ultimately, β2 integrins mediate interactions with fibrinogen in the extracellular matrix of endothelial cells and initiates cell migration. In neutrophils, Lyn also controls Rac2 dependent chemotaxis through ELMO-Dock2 (adapter protein-guanine exchange factor protein) complex activation in CXCR8-stimulated cells (100), and Lyn is known to activate the mitogen-activated protein kinase (MAPK) extracellular signal-regulated kinase (ERK) in human neutrophils (101), which represents a critical factor in neutrophil migration (102). Lyn can also drive neutrophil chemoattraction via the PI3K signalling pathway (103). However, others studies call into question the role of Lyn in neutrophil migration. Pereira *et al.* (104) have shown that neutrophils from Lyn-deficient mice display a hyper-adhesive integrin dependent phenotype, and Moscai *et al.* (93) demonstrate that Lyn-deficient neutrophils have normal migration *in vitro* and *in vivo*. These opposing observations highlight the need to investigate more precisely the role of Lyn in the regulation of neutrophil chemotaxis *in vivo*.

Despite the conflicting findings, these studies raise the possibility that Lyn could be a key component in neutrophil inflammation. It had recently been shown that H_2O_2 mediates rapid recruitment of neutrophils to a wound, and reactive oxygen intermediates (ROIs) can increase Lyn kinase activity (86, 105). Thus, we speculated that Lyn could be the neutrophil's detector molecule for the H_2O_2 gradient formed after tissue injury. We took advantage of an ROI-insensitive Lyn, due to C245A and C487A mutations (106), to explore the possibility that these particular cysteines could be key factors for Lyn activation following tissue injury and subsequent H_2O_2 release *in vivo*. We generated a transgenic zebrafish carrying these cysteine mutations in neutrophils and tested if neutrophils with ROI-insensitive Lyn could reach inflammatory sites (107). We discovered that, in zebrafish, cysteine 466 of Lyn (corresponding to human C487) is targeted by H_2O_2 oxidation and this event is necessary for neutrophil recruitment to wounded tissue, showing for the first time *in vivo* that a SFK could act as a redox sensor and drive directional migration through a H_2O_2 gradient (107). This finding sets the stage to determine the molecular mechanism that supports Lyn-mediated directed migration. Lyn could link SDF1-CXCR4 activation to the Rac2 signalling pathway in zebrafish hematopoietic tissue to regulate neutrophil retention and release into the circulation. Furthermore, Lyn could regulate neutrophil polarization and migration through a PI3K/Rac/Rho and/or a MAPK/ERK dependant signalling pathway. The ways in which Lyn could interact with Rac and PI3K and other molecules to regulate migration is outlined in Fig. 3.

How Lyn might contribute to autoimmune disease is more complex than how Rac2 might be involved. However, Lyn and other Src family kinases are thought to be important and are a focus of drug development (89). There are clear relationships between Lyn in B cells and lupus. For example, Lyn-deficient mice have reduced B-cell tolerance and a lupus-like glomerulonephritis (108). Also, lupus patients have decreased Lyn expression and translocation in B cells (109). However, for neutrophils in autoimmune disease, a role for Lyn has not been described. One could hypothesize that Lyn might act as a redox sensor in neutrophils in autoimmune disease similar to wound healing. That said, reactive oxygen species may not play the same role in wound recruitment as in inflamed joint recruitment. H_2O_2 attracts neutrophils to the wound in zebrafish (110). In contrast, treatment with exogenous H_2O_2 or superoxide dismutase was associated with decreased neutrophilic infiltrate and increased neutrophil apoptosis secondary to PI3K/Akt activation in a murine model of inflammatory arthritis (111). Furthermore, mice deficient in gp91phox, a NADPH oxidase subunit, had unaltered neutrophil recruitment to the inflamed joint but delayed resolution (111). These data are somewhat difficult to interpret. It is possible that the treatment with H_2O_2 or superoxide dismutase was supraphysiologic and simply resulted in the observed neutrophil death. Also, there may be sources of reactive oxygen species in the

inflamed joint unrelated to gp91phox resulting in the unimpaired neutrophil recruitment in the absence of gp91phox. Alternatively, the role of H_2O_2 may be different in zebrafish wound response and mammalian arthritis. Lyn may act as a redox sensor in both situations, but with different outcomes. Much additional work is needed in this area, but the clear role for Lyn in mediating neutrophil recruitment in zebrafish identifies it as a target for future investigations in human autoimmunity.

SHIP in neutrophils

SH2-domain-containing inositol 5-phosphatase (SHIP) is a 5′ inositol polyphosphatase that balances PI3K activity by converting PI(3–5)P_3 and PI(1–3,5)P_4 into PI(3,4)P_3 and PI(1–3)P_4, respectively. Related to this specific enzymatic function, SHIP is at the nexus of this intracellular signaling pathway and its activation is considered to be the termination of PI3K-mediated signaling (112). In innate immune cells, PI3K and SHIP act in concert to control cell polarization in chemotaxis gradients (113). PI3Ks generate the intracellular second messengers PI(3–5)P3 and PI(3,4)P2, which are critical regulators of a wide variety of cellular processes including cell migration (114). More specifically, PI3K regulates forward protrusion of the leading edge of a migrating cell by activating Rac through a Rac guanine exchange factor (74). PI3K is considered to be a central regulator of gradient sensing during chemotaxis of neutrophils *in vitro* (115). However, little is known about how polarized cell migration is regulated in three-dimensional (3D) tissue environments *in vivo*, because few systems are amenable to high resolution imaging. Recent advances uncoupled two roles for PI3K in regulating neutrophil migration in live zebrafish: a Rac-mediated protrusion at the leading edge and a Rho-dependent tail contraction (116). This two-tiered regulation of motility by PI3K is indispensable to coordinate F-actin anteroposterior polarization and subsequent directed migration of neutrophils toward injured tissue. Given the importance of SHIP in regulating PI3K activity, we focused further studies on the role of SHIP in neutrophil behavior in live zebrafish.

Although PI3K-mediated chemotaxis is well understood, the role of SHIP is less studied. SHIP-deficient mice show robust leukocyte infiltration into the lung and their bone marrow-derived mast cells display increased PIP3 levels and Akt activation, suggesting a suppressor role of SHIP in inflammation (117). Contradicting these observations, an *in vitro* study showed that SHIP1-null neutrophils have impaired polarization and motility (118). These divergent observations required deeper investigation to clarify the impact of SHIP on PIP3 subcellular distribution and the effect on neutrophil response to acute inflammatory signals. To this end, we investigated the role of SHIP using high resolution real time imaging in live zebrafish. In contrast to PI3K, whose activation is concentrated at the leading edge of motile neutrophils, our findings suggest that SHIP is active both at the leading edge and at the tail, possibly to avoid any rear Akt activation and consequently favor unidirectionality in migratory neutrophils (119). We also show that SHIP acts as a repressor of inflammation as higher numbers of neutrophils accumulate at a wound when SHIP is downregulated using a morpholino. Furthermore, the increased recruitment of neutrophils in SHIP-deficient zebrafish could be reversed by treatment with a PI3Kγ inhibitor, suggesting that SHIP restricts neutrophil attraction via PI3K. These observations in live zebrafish are consistent with the mouse studies noted above (117) and not the *in vitro* studies, suggesting that (1) SHIP limits neutrophil motility by modulating PI3K signalling and that (2) the 3D environment is critical for the physiologic regulation of the phosphatidyl inositol signalling pathway.

Of interest, as mentioned above, Lyn can regulate Rac2 dependent chemotaxis through ELMO-Dock2 and Lyn can mediate neutrophil chemoattraction via PI3K. Lyn can also drive both pro- and antichemotaxis signals and consequently repress or activate SHIP (120). Thus, these molecules may all intersect in controlling neutrophil migration (*Fig.* 3).

There are several ways in which SHIP may be important for autoimmune disease. There is the most evidence for a role of SHIP in rheumatoid arthritis. SHIP-1 levels are reduced in monocyte lineage cells in the synovium in rheumatoid arthritis compared to osteoarthritis, a degenerative arthritis. Interestingly, the microRNA-155 (miR-155) is upregulated in synovial fluid macrophages and in the synovial membrane and miR-155 downregulates expression of SHIP-1 and causes increased production of TNFα. Furthermore, mice that lack miR-155 are resistant to collagen-induced arthritis with reduced autoantibody production and joint inflammation (121). Thus, these studies suggest that reduced SHIP-1 activity, at least in macrophages, may play a role in rheumatoid arthritis by leading to increased TNFα and likely other effects. Given our findings regarding SHIP in limiting neutrophil motility, the lack of miRNA-155 in mice (and thus upregulation of SHIP) may lead to reduced arthritis due to poor neutrophil recruitment as well as reduced macrophage related inflammation. Thus, novel

treatments for rheumatoid arthritis could involve activators of SHIP. There have been small molecule activators of SHIP1 generated that stimulate SHIP-1 activity in macrophages and mast cells and have protective effects in endotoxemia and anaphylaxis (122). Thus, although the effects are not specific for neutrophils or arthritis, these activators may be beneficial in autoimmune diseases in which neutrophils play a role.

As noted above, SHIP is a negative regulator of PI3K activity and PI3K has numerous roles in neutrophils. Furthermore, hyperactivation of PI3K signaling predisposes to autoimmunity (123). The reverse may also be true with reductions in PI3K function reducing inflammation, specifically due to reduced neutrophil function. For example, in the K/B×N serum transfer model of inflammatory arthritis, loss of PI3Kδ or PI3Kγ reduces arthritis and correlates with decreased neutrophil migration into tissue in response to leukotriene B(4) (124). Also, a PI3Kγ inhibitor ameliorates collagen-induced arthritis and reduces neutrophil infiltration into the joint (125). Thus, molecules inhibiting PI3K or activating SHIP may be novel therapeutic strategies in rheumatoid arthritis.

In vasculitis, there are no studies involving SHIP, but there is evidence that PI3K plays a critical role. For example, neutrophil activation by ANCAs leads to PI3K activation (126). Furthermore, bone marrow from PI3Kγ-deficient mice protects wildtype mice from the development of glomerulonephritis in a mouse model of vasculitis in which MPO-deficient mice are immunized with MPO (127). Also, a PI3Kγ inhibitor can suppress glomerulonephritis when wildtype bone marrow is transplanted in this model, likely due to reduced ANCA-induced superoxide production, degranulation and GM-CSF-induced migration of neutrophils (127). Thus, activation of SHIP and inhibition of PI3K could improve both rheumatoid arthritis and vasculitis. There is less known about the role of SHIP and PI3K in neutrophils in lupus, making this disease even more in need of research into PI3K and SHIP function.

Microtubules in neutrophils

Microtubules have essential roles during directed cell migration. They play an important role in focal adhesion turnover (128), cell polarity, and regulation of actin-generated force (129) – three critical components of cell migration. Microtubules had been shown to inhibit neutrophil random motility *in vitro* but are required for directional migration (130, 131). We found that upon depolymerizing microtubules in live zebrafish with nocodozole, neutrophils exhibit impaired directional migration to a wound (132). Microtubule depolymerization activates RhoA *in vitro* (130) and both microtubule polymerization and depolymerization appear to activate Rac *in vitro* (132, 133). Furthermore, microtubule depolymerization inhibits PI3K activation at the leading edge of neutrophils in zebrafish (132). Thus, the assembly status of microtubules regulates Rac and PI3K activity, which contributes to directional motility.

The role of microtubules in inflammatory disease is most obvious in Behcet's disease. As mentioned above, colchicine, a microtubule polymerization inhibitor, is first line treatment for Behcet's and can reduce disease flares. Neutrophils from patients with active Behcet's have more microtubules than controls (134) with altered distribution (135), perhaps consistent with why Behcet's is responsive to colchicine. As colchicine inhibits microtubule assembly and microtubule disassembly reduces recruitment of neutrophils to wounds in zebrafish, colchicine may be acting in Bechet's by limiting neutrophil recruitment to inflamed areas. However, colchicine may have other effects such as reducing the microtubule-regulated endocytosis of inflammatory mediators into neutrophils and thus reducing neutrophil activation (136).

Colchicine is generally not used as a treatment in lupus, vasculitis, or rheumatoid arthritis, but there have been no large studies investigating its use. Thus, there could be an unidentified role for colchicine in these disorders. Alternatively, it is possible that there is a unique role for colchicine in Behcet's disease for reasons that are not completely clear. Perhaps abnormalities in microtubules in Behcet's lead to the effectiveness of colchicine. However, colchicine is also effective in gout, an acute inflammatory arthritis due to monosodium urate crystals, where no microtubule abnormalities have been reported. Another possibility is that perhaps neutrophil recruitment or activation of Rac or PI3K signaling occurs in a microtubule-independent way in some autoimmune diseases, and thus colchicine would not be effective. More studies are needed in this area to clarify the role of microtubules and colchicine in autoimmunity.

Conclusion

There is a clear role for neutrophils in autoimmunity although the extent of neutrophil involvement and various ways that neutrophils contribute to disease are still being uncovered. By exploring the cytoskeletal regulators of neutrophil migration in zebrafish *in vivo*, we hope to shed light on how neutrophils impact human autoimmunity.

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