

# Biological Perspectives

## Mechanisms and Consequences of Neutrophil Interaction with the Endothelium

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**Leukocyte recruitment into inflamed tissue proceeds in a cascade-like fashion. The first contact of neutrophils with the endothelium is mediated by selectins and their counterreceptors, followed by rolling of neutrophils along the endothelial wall of postcapillary venules and integrin-mediated arrest. While rolling, neutrophils collect different inflammatory signals that can activate several pathways. In addition to activation of neutrophils by ligation of G-protein-coupled receptors with chemokines and other chemoattractants, integrins and selectin ligands are also able to signal into the cell, where they initiate neutrophil extravasation, promote cytoskeletal rearrangement, and ultimately induce superoxide production and degranulation. These signaling pathways may be targeted by therapeutic interventions to inhibit specific functions of neutrophils without affecting others. This Review is focused on the signaling events during the interaction of neutrophils with the endothelium. (*Am J Pathol* 2008, 172:1–7; DOI: 10.2353/ajpath.2008.070502)**

Neutrophils are produced in the bone marrow and circulate in the blood for a few hours before they are selectively recruited to tissues that have been injured by infection, trauma, or an autoimmune reaction. Neutrophils constitute the first line of defense against invading bacteria. A defect in neutrophil recruitment, such as seen in leukocyte adhesion deficiency, is associated with recurrent bacterial infections that can be lethal.<sup>1</sup> On the other hand, inhibition of neutrophil recruitment has positive effects on ischemia-reperfusion injury,<sup>2</sup> abacterial inflammation,<sup>3</sup> and autoimmune disease.<sup>4</sup> Understanding neutrophil activation, signaling, and recruitment can help identify therapeutic targets to selectively inhibit the specific functions of neutrophils without affecting others.

At sites of inflammation, neutrophils roll along the endothelium of postcapillary venules, collect inflammatory signals, arrest, and transmigrate. The first contact between neutrophils and endothelium of postcapillary venules is known as capture or tethering and is mediated by selectins and their counterreceptors.<sup>5</sup> Selectin binding and the presentation of chemokines by endothelial cells induce activation of signaling pathways in neutrophils that cause changes in integrin conformation (inside-out signaling). Depending on the resulting integrin conformation, binding of activated integrins to their counterreceptors causes either slow rolling or arrest. Slow rolling of neutrophils along the wall of inflamed venules is mediated by the  $\beta_2$ -integrins  $\alpha_L\beta_2$  [lymphocyte function antigen (LFA)-1] and  $\alpha_M\beta_2$  [macrophage antigen (Mac)-1].<sup>6</sup> Partial activation of LFA-1 exposes the ligand binding site of the integrin leading to LFA-1-dependent rolling.<sup>7</sup> Efficient conversion from rolling to firm adhesion is dependent on the time a leukocyte spends in close contact with the endothelium.<sup>8</sup> Upon arrest, integrins bound to their ligands can signal into the neutrophil (outside-in signaling), stabilize the adhesion (postadhesion strengthening),<sup>9</sup> activate different signaling pathways, and initiate transmigration. During leukocyte interaction with the endothelium, leukocytes receive signals from P-selectin glycoprotein ligand (PSGL)-1, L-selectin, G-protein-coupled receptors (GPCRs), and integrins, which can activate different pathways and subsequently lead to activation of the leukocyte with actin polymerization, crawling, transmigration through the endothelium, respiratory burst, and degranulation.

This Review focuses on the molecular mechanisms involved in neutrophil interactions with the endothelium, signaling events that are induced during this process, and functional consequences of their activation.

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## PSGL-1

PSGL-1 is a disulfide-bonded homodimer highly expressed on microvilli of leukocytes.<sup>10</sup> Based on biochemical analysis, most of the PSGL-1 is thought to be located in lipid rafts.<sup>11</sup> PSGL-1 binds L-selectin and P-selectin through a sialyl Lewis x (sLe<sup>x</sup>)-containing O-glycan and a nearby tyrosine sulfate residue<sup>10</sup> and E-selectin through the N-terminal region as well as other binding sites on PSGL-1. Binding of E-selectin to PSGL-1 requires sialylated and fucosylated O-glycans but not tyrosine sulfatation.<sup>10</sup> Antibody blockade of the N-terminal region of PSGL-1 inhibits rolling of leukocytes on P-selectin and L-selectin *in vivo*.<sup>12,13</sup> Elimination of PSGL-1 by gene targeting and homologous recombination reduces the number of neutrophils interacting with the endothelium,<sup>14,15</sup> alters the rolling velocity on P-selectin and on E-selectin,<sup>14,16</sup> and diminishes neutrophil recruitment into inflamed tissue.<sup>15</sup>

In addition to its adhesive function, PSGL-1 is also known to initiate intracellular signaling events upon ligand engagement. This downstream signaling leads to activation of neutrophils, including activation of  $\beta_2$ -integrins,<sup>17</sup> tyrosine phosphorylation,<sup>18</sup> secretion of cytokines,<sup>18</sup> transcriptional activation,<sup>19</sup> and cytoskeletal rearrangement.<sup>20</sup> The cytoplasmatic tail of human PSGL-1 consists of 63 amino acids and is highly conserved among species.

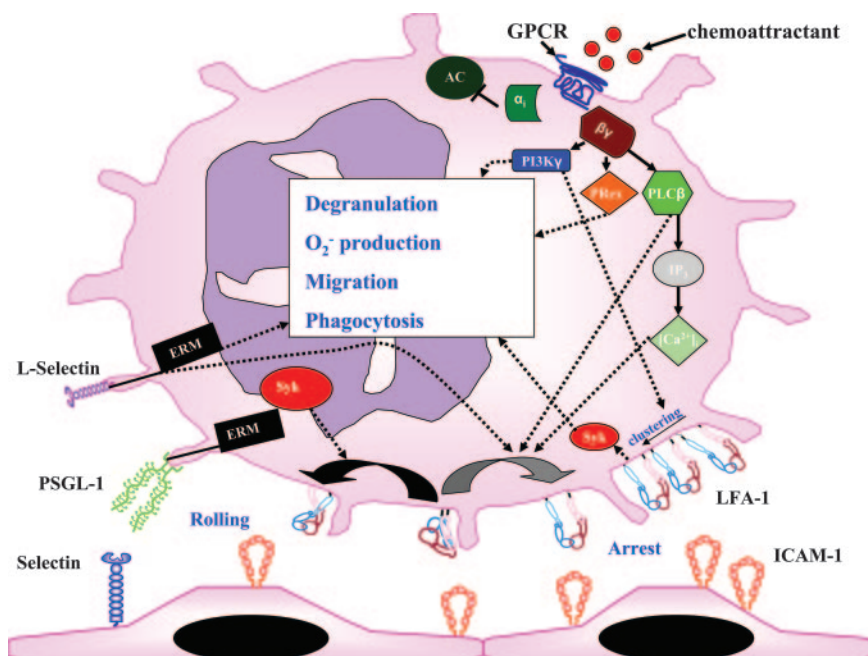
PSGL-1 can interact with the cytoskeleton. For this interaction, a juxtamembrane region of 18 amino acids of the PSGL-1 cytoplasmatic tail is necessary,<sup>19</sup> which interacts with the proteins moesin and ezrin, which belong to the ezrin-moesin-radixin (ERM) family. ERM proteins function as linking proteins between the plasma membrane and the actin cytoskeleton and play an important role in the formation of protrusive plasma membrane structures.<sup>21</sup> The C-terminal domain of the ERM proteins can bind F-actin, whereas the N-terminal domain binds to

the cytoplasmatic tail of PSGL-1.<sup>19</sup> Unless activated, the N- and C-terminal domains of ERM proteins are associated in a manner that prevents F-actin binding.<sup>22</sup> The interaction of PSGL-1 with the cytoskeleton is required for rolling; the destruction of the cytoskeleton or the elimination of the cytoplasmatic tail of PSGL-1 is associated with an increase in rolling velocities on P-selectin, reduced number of adherent cells, and a reduced capability to stabilize rolling.<sup>23</sup> Decoupling of PSGL-1 from the cytoskeletal network also reduces its ability to cluster on microvilli.<sup>24</sup>

ERM proteins contain an immunoreceptor tyrosine-based activation motifs-like motif in their N-terminal region,<sup>25</sup> which can directly interact with spleen tyrosine kinase (Syk).<sup>19</sup> PSGL-1 engagement induces tyrosine phosphorylation of Syk. Syk is involved in PSGL-1-dependent signaling and therefore in regulating rolling on P-selectin and E-selectin (Figure 1).<sup>11,16</sup> Slow rolling on E-selectin and intercellular adhesion molecule-1 (ICAM-1) is abolished by pharmacological blockade of Syk and is absent in Syk-deficient bone marrow chimeric mice. Blocking of p38 mitogen-activated protein kinase also increases the rolling velocity and reduces the number of neutrophil-interacting cells with E-selectin and ICAM-1.<sup>16,17</sup>

## L-Selectin

L-selectin is expressed by all blood neutrophils and is a type I transmembrane glycoprotein with an N-terminal C-type lectin domain, followed by an epidermal growth factor-like domain, two short consensus repeats, a transmembrane domain, and a cytoplasmatic tail. L-selectin interacts with sialylated ligands expressed by the endothelium and is involved in rolling and activation of leukocytes. The expressed levels, cytoskeletal anchoring, and



**Figure 1.** Activation of signaling pathways during the contact of neutrophils with the endothelium. E-selectin binding to PSGL-1 leads, through Syk, to partial LFA-1 activation (black curved arrow) that mediates rolling on ICAM-1 (shown as a dimer). L-selectin engagement may activate, through ERM proteins, the neutrophil and induce integrin activation. Activation of GPCRs leads to dissociation of the G protein in a  $\alpha$ -subunit and a  $\beta\gamma$ -complex. The  $\alpha$ -subunit inhibits some, but not all, adenylyl cyclase isoforms. The  $\beta\gamma$ -complex interacts directly with PI3K $\gamma$ , PLC $\beta$ , and P-Rex-1. The activation of these enzymes can induce degranulation, O<sub>2</sub><sup>-</sup> production, migration, phagocytosis, integrin clustering, and full activation of the integrin (gray curved arrow). LFA-1 interaction with its counterreceptor triggers outside-in signaling and further activates the neutrophil. **Arrows** indicate signaling pathways, but interactions may be indirect.  $\alpha$ ,  $\beta$ ,  $\gamma$ , G-protein subunits; AC, adenylyl cyclase; [Ca<sup>2+</sup>]<sub>i</sub>, intracellular calcium; GPCR, G-protein-coupled receptor; IP<sub>3</sub>, inositol triphosphate; P-Rex, PtdIns(3,4,5)P<sub>3</sub>-dependent Rac exchanger.

the distribution of L-selectin determine the tethering efficiency and rolling velocity.<sup>26–28</sup>

Cross-linking of L-selectin on neutrophils can also initiate  $\beta_2$ -integrin activation,<sup>29</sup> but L-selectin-dependent rolling does not induce neutrophil arrest in flow chamber systems.<sup>30</sup> L-selectin surface expression on neutrophils can be rapidly down-regulated by proteolytic cleavage of L-selectin near the cell surface by ADAM-17 (TACE) and at least one other “shedase.”<sup>31</sup> L-selectin-mediated neutrophil activation is of physiological importance, as shown by modulating L-selectin shedding. Blocking of L-selectin shedding *in vivo* increases signal input through L-selectin and results in an increase in neutrophil arrest, reduced neutrophil rolling velocities,<sup>32</sup> and more stable rolling.<sup>33</sup> It remains unclear whether these *in vivo* results are the consequences of increased L-selectin-mediated signaling or are due to increased exposure of neutrophils to the surface of activated endothelial cells when L-selectin shedding is inhibited.

Human and murine L-selectin cytoplasmic tails are 100% conserved between residues Arg-356 and Ser-364 and have similar lengths (17 amino acids). The cytoplasmic tail of L-selectin is constitutively associated with calmodulin<sup>34</sup> and the actin-binding protein  $\alpha$ -actinin<sup>27</sup> and can also interact with proteins of the ERM family.<sup>35</sup> The C terminus of the cytoplasmic tail of L-selectin directly interacts with  $\alpha$ -actinin and forms a complex with vinculin and talin.<sup>27</sup> The presence of vinculin and talin increases the binding affinity of  $\alpha$ -actinin for L-selectin.<sup>27</sup> The binding site of L-selectin for  $\alpha$ -actinin is located within the 53-kDa domain of  $\alpha$ -actinin,<sup>27</sup> suggesting that  $\alpha$ -actinin functions as a direct linker between L-selectin and the cytoskeleton. Truncation of the  $\alpha$ -actinin binding site, the 11 residues at the COOH-terminal end of the cytoplasmic tail, or treatment with cytochalasin B disrupt the connection between L-selectin and the cytoskeleton and lead to reduced tethering and rolling *in vivo* and *in vitro*.<sup>26,28</sup> Despite the reduced functionality of the truncated form of L-selectin lacking most of cytoplasmic domain (11 of 17 amino acids), the mutant possesses an unchanged ability to recognize carbohydrates<sup>28</sup> and is still localized on microvillar tips, suggesting that other cytoskeletal proteins are required to anchor L-selectin to microvilli.<sup>27</sup>

Calmodulin interacts with the cytoplasmic tail of L-selectin in resting leukocytes.<sup>34</sup> Activation of leukocytes leads to a release of calmodulin from the cytoplasmic tail of L-selectin and causes the concomitant shedding of L-selectin. Abrogation of the interaction of calmodulin with the cytoplasmic tail of L-selectin by mutagenesis or an antagonist leads to a higher turnover of cleaved L-selectin.<sup>34</sup> These data show that calmodulin negatively regulates L-selectin shedding, likely by conformational change in the extracellular domain that renders the cleavage site inaccessible.

Two members of the ERM family, ezrin and moesin, can bind to the cytoplasmic tail of L-selectin, but the binding properties are regulated differently. Moesin binds to the cytoplasmic tail of L-selectin after stimulation, whereas ezrin is constitutively associated with the cytoplasmic tail.<sup>35</sup> Two residues (Arg-357 and Lys-362)

in the ERM-binding domain of the human L-selectin tail contribute to the interaction with the N-terminal domain of moesin.<sup>36</sup> Mutation of these two amino acids results in a reduction of induced shedding, lower levels of L-selectin in microvilli, and decreased tethering efficiency.<sup>36</sup>

L-selectin engagement of leukocytes by antibody or glycomimetics results in increased intracellular  $\text{Ca}^{2+}$ -levels, cytoskeleton-independent, Src-kinase (p56<sup>ck</sup>)-dependent tyrosine phosphorylation of the cytoplasmic tail of L-selectin and concomitant activation of downstream signaling pathways.<sup>37–39</sup> Tyrosine phosphorylation of the cytoplasmic tail of L-selectin leads to an association of L-selectin with the guanine nucleotide exchange factor SOS/Grb2.<sup>38</sup> This signaling cascade also activates Ras, p38 mitogen-activated protein kinase, extracellular signal-regulated kinase 1/2 phosphorylation, and Rac and leads to increased  $\text{O}_2^-$  production<sup>38</sup> (Figure 1).

## Integrins

Integrins are type I transmembrane cell adhesion molecules consisting of two noncovalently associated subunits  $\alpha$  and  $\beta$  that mediate cell-pathogen, cell-cell, and cell-extracellular matrix interactions.<sup>40</sup> Neutrophils express the  $\beta_2$ -integrins  $\alpha_L\beta_2$  (LFA-1),  $\alpha_M\beta_2$  (Mac-1),  $\alpha_X\beta_2$ , and low levels of  $\alpha_4\beta_1$ . These integrins are involved in slow rolling,<sup>6</sup> adhesion,<sup>40</sup> postadhesion strengthening,<sup>9</sup> neutrophil migration,<sup>41</sup> respiratory burst,<sup>42</sup> phagocytosis,<sup>43</sup> and polarization and are a linkage between extracellular matrix and cytoskeleton.<sup>44</sup> Binding of integrins to their ligands induces outside-in signaling with a concomitant activation of several intracellular signaling pathways. Mac-1 engagement is necessary for oxidative burst<sup>42</sup> and phagocytosis.<sup>43</sup> The extracellular domain of the  $\alpha$ -chain of LFA-1 ( $\alpha_L$ ) contains seven N-terminal homogeneous repeats organized into a  $\beta$ -propeller structure. The I domain is inserted in the third repeat and consists of two distinct sites: the metal ion-dependent adhesion site and the I-domain allosteric site. These sites require divalent cations for ligand binding and regulate the affinity of the integrin for their ligands.<sup>45</sup> The membrane-proximal repeats contain a calcium-binding site that may be important for the orientation of the  $\beta$ -propeller as well as the association with the  $\beta$ -chain.<sup>46</sup> The extracellular domain of the  $\beta$ -chain includes an I-like domain, which corresponds to the I domain of the  $\alpha$ -chain, and both together represent the integrin binding site for the ligands. The legs of the  $\alpha$ - and  $\beta$ -chain are formed by a series of globular domains that incorporate a knee or “genu.”<sup>47</sup> In the “bent position”, the genu is folded, and the integrin assumes a compact structure in which the ligand-binding site is close to the membrane (<5 nm). In the “extended conformation state”, the integrin legs straighten, and the ligand-binding site is projected about 25 nm from the membrane.<sup>47</sup> The cytoplasmic tail of the  $\alpha$ -chain of LFA-1 contains a GFFKR motif that is involved in the  $\alpha/\beta$  association and in ligand recognition.<sup>48</sup> The  $\alpha/\beta$  association is also important in keeping the integrin in a low-affinity state. Mutational changes<sup>49</sup> or complexes that interact with the cytoplasmic domain break the  $\alpha/\beta$

association, although details of this are not known for LFA-1. The cytoplasmatic tail of the  $\beta$ -subunit is important for the linkage with the cytoskeleton.

The  $\beta_2$ -integrins can dynamically regulate their adhesiveness by altering their affinity and avidity. "Affinity" is defined as a measure of the equilibrium interaction of monovalent receptors and ligand in solution or on a surface. "Avidity" describes the interaction of two surfaces in which receptors and ligands come together to form many molecular interactions. Stimuli received by cell surface receptors for cytokines, adhesion molecule receptors, and chemokines initiate intracellular signals that lead to a transient change of the integrin conformation. In addition, activation by phorbol esters may enhance lateral mobility in the plasma membrane and change integrin anchorage with the cytoskeleton.<sup>50</sup> The affinity states of LFA-1 are regulated by the conformation of the I domain. The position of the seventh  $\alpha$ -helix controls the conformation of the magnesium-containing ligand binding site in the I domain.<sup>51</sup> The different positions are associated with the three distinct affinity states (low-, intermediate-, and high-affinity state),<sup>51</sup> but additional states may exist, and the equilibrium is dynamic.

The main component of integrin avidity regulation is based on the postligand binding interaction of the integrin with the cytoskeleton. Lateral mobility leads to an accumulation of integrins in areas where ligands are available, a process called "clustering," which regulates cell avidity for surface-presented integrin ligands. These features assure specific binding of leukocytes at sites of inflammation.

LFA-1 can control the rolling velocities of leukocytes *in vivo*,<sup>6,16</sup> as well as their adhesion<sup>52</sup> and migration<sup>52,53</sup> (Figure 1). During neutrophil migration through the endothelium, LFA-1 forms ring-like clusters at the neutrophil-endothelial junction.<sup>53</sup> These clusters move from the leading edge to the neutrophil uropod, always in contact with their major ligand ICAM-1.<sup>53</sup>

Mac-1 is stored in neutrophil secretory, secondary, and tertiary granules, ready to be released after cell activation.<sup>54</sup> In addition to ICAM-1, Mac-1 has several other ligands, including different bacterial and fungal glycoproteins, heparin, coagulation factor Xa, fibrinogen, and complement C3bi.<sup>55</sup> Like LFA-1, Mac-1 can also regulate the rolling velocity of leukocytes *in vivo*<sup>6</sup> and, depending on the model, participates in neutrophil migration. Mac-1 is also involved in neutrophil-platelet interactions<sup>56</sup> and intravascular crawling of monocytes<sup>57</sup> and neutrophils<sup>58</sup> and is critical for certain forms of phagocytosis.<sup>43</sup>

### G Protein-Coupled Receptors

Neutrophils can be activated by soluble stimuli including *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine, complement C5a, platelet-activating factor, leukotriene B<sub>4</sub>, and the chemokines CXCL1, 2, 3, 4, 5, 6, 7, and 8.<sup>59</sup> Chemoattractants can induce arrest from rolling, cytoskeleton rearrangement, cell polarization, migration along a concentration gradient, degranulation, and respiratory burst.<sup>60</sup>

Chemoattractants bind to GPCRs, which undergo a conformational change after ligand binding.<sup>60</sup>

G proteins consist of an  $\alpha$ -subunit and a  $\beta\gamma$ -complex.<sup>61</sup> G proteins are classified according their  $\alpha$ -subunit, where neutrophils express G<sub>s</sub>, G<sub>i</sub>, and G<sub>q</sub> family members. G<sub>i</sub> proteins are the most abundant and important G proteins in neutrophils, because they mediate almost all pro-inflammatory effects of chemoattractants. The G $\alpha_i$  family consists of the subunits G $\alpha_z$ , G $\alpha_o$ , G $\alpha_{i1}$ , G $\alpha_{i2}$ , and G $\alpha_{i3}$ <sup>62</sup> and can be blocked by pertussis toxin with the exception of G $\alpha_z$ . G $\alpha_{i2}$  and G $\alpha_{i3}$  are expressed abundantly in leukocytes.<sup>63</sup> Five different  $\beta$ -subunits and 12  $\gamma$ -subunits exist, forming a number of G $\beta\gamma$ -complexes.<sup>61</sup>

The binding of a chemoattractant to its receptor results in the activation of the associated G protein, which dissociates into the GTP-bound G $\alpha$ -subunit and the G $\beta\gamma$ -complex<sup>60</sup> (Figure 1). The majority of neutrophil responses induced by chemoattractants except platelet activating factor can be inhibited by pertussis toxin, which shows that the G $\alpha_i$  class of G protein is associated with relevant chemoattractant receptors in neutrophils. The G $\beta\gamma$ -subunit is able to activate phosphatidylinositol 3-kinase (PI3K)  $\gamma$  and phospholipase C (PLC)  $\beta_2$  and, to a lesser extent, PLC  $\beta_3$ .<sup>64,65</sup> PLC  $\beta$  hydrolyzes phosphatidylinositol 4,5-bisphosphate to produce inositol triphosphate and diacylglycerol. Inositol triphosphate mobilizes Ca<sup>2+</sup> from nonmitochondrial stores, and diacylglycerol activates Ca<sup>2+</sup>-dependent and Ca<sup>2+</sup>-independent protein kinase C isoenzymes. Protein kinase C isoenzymes are important for activating cytotoxic effector function of neutrophils such as O<sub>2</sub><sup>-</sup> formation.<sup>66</sup> Neutrophils from mice lacking PLC  $\beta_2$  show deficient chemoattractant-induced Ca<sup>2+</sup> release and Mac-1 up-regulation but enhanced chemotaxis and increased leukocyte recruitment in response to fMet-Leu-Phe.<sup>67</sup> PLC  $\beta_2$ - and PLC  $\beta_3$ -deficient mice show a complete deficiency of chemokine-stimulated O<sub>2</sub><sup>-</sup> formation.<sup>68</sup> A recent study showed that PLC is involved in chemokine-induced  $\alpha_4\beta_1$ -integrin affinity up-regulation and arrest of monocytes.<sup>69</sup>

Certain G $\beta\gamma$ -subunits can also activate PI3K $\gamma$ ,<sup>70</sup> which catalyzes the phosphorylation of phosphatidylinositol-3,4-bisphosphate to phosphatidylinositol-3,4,5-trisphosphate. Phosphatidylinositol-3,4,5-trisphosphate binds to proteins containing pleckstrin homology domains, resulting in downstream signaling. For example, protein kinase B (also known as Akt) is activated by PI3K $\gamma$  after exposure to chemoattractants. Protein kinase B translocates to the leading edge of migrating neutrophils.<sup>71</sup> PI3K $\gamma$  also regulates other effectors including low-molecular mass GTP binding proteins of the Rho family.<sup>71</sup> Elimination of PI3K $\gamma$  leads to reduced O<sub>2</sub><sup>-</sup> formation,<sup>65</sup> failure of postadhesion strengthening,<sup>72</sup> and reduced chemotaxis<sup>73</sup> but is not involved in arrest.<sup>72</sup>

Rac is one of the GTPases of the Rho family that is activated after GPCR activation. Rac1 and Rac2 are both involved in regulation of phagocyte migration, whereas Rac2 also regulates activation of neutrophil respiratory burst.<sup>74,75</sup> Guanine nucleotide exchange factors activate Rac by exchanging GDP for GTP. Two other guanine nucleotide exchange factors, Vav1 and P-Rex-1, are involved in Rac2 activation.<sup>76,77</sup> Vav protein activity is reg-

ulated by tyrosine kinases, including Src family kinases and Syk,<sup>77</sup> whereas P-Rex1 is activated in a synergistic fashion by phosphatidylinositol-3,4,5-triphosphate and the G $\beta\gamma$ -complex.<sup>78</sup> Vav guanine nucleotide exchange factors are also involved in adhesion strengthening.<sup>79</sup> Full activation of Rac is required for NADPH oxidase activation, whereas partial activation of Rac is sufficient for neutrophil chemotaxis. Rac1 and Rac2 can also be regulated by dedicator of cytokinesis 2.<sup>80</sup> Dedicator of cytokinesis 2 deficiency leads to an inhibition of cell polarity and translocation speed.<sup>80</sup> p21-activated kinase is downstream from Rac, and after activation, p21-activated kinase can induce phosphorylation of extracellular signal-regulated kinase 1/2, p38 mitogen-activated protein kinase, and c-Jun N-terminal kinase.<sup>81,82</sup>

After activation of the GPCR and the release of the G $\beta\gamma$ -complex, the G $\alpha_i$ -subunit inhibits some, but not all, adenylyl cyclase isoforms (Figure 1).<sup>83</sup> This results in reduced cAMP levels and cAMP-dependent protein kinase activity. Small GTPases are also effectors of the G $\alpha_i$ -subunit after activation.<sup>60</sup> Ras, one of the GTPases downstream of the G $\alpha_i$ -subunit, can activate PI3K by binding directly to the 110-kDa catalytic subunit.<sup>84</sup> A recent study using G $\alpha_{i2}$ -deficient mice demonstrated that G $\alpha_{i2}$  in nonhematopoietic cells is involved in migration of leukocytes into the lung in a model of allergy and after lipopolysaccharide stimulation, whereas G $\alpha_{i2}$  in leukocytes regulates chemotaxis in response to CXCL2/3.<sup>85</sup> G $\alpha_{i2}$  in neutrophils is also required for chemokine-induced arrest.<sup>86</sup>

### Functional Consequences

In addition to the described signaling inputs, the adhesion of neutrophils to biological surfaces by integrins is another powerful activator (outside-in signaling).<sup>87</sup> Depending on the magnitude of signaling inputs, neutrophils may become fully activated and manifest a number of functional responses like spreading, transmigration, phagocytosis, superoxide production, and degranulation. Among the most important functional response of activated neutrophils is the ability to rearrange their actin cytoskeleton and chemotax toward inflammatory stimuli.<sup>88</sup> Activated neutrophils have the ability to arrange the subunits of the NADPH oxidase at the phagosome membrane and undergo respiratory burst, resulting in release of oxygen radical intermediates.<sup>42</sup> Neutrophils spreading over biological surfaces or transmigrating into inflamed tissue release their granule components, including metal-binding proteins, peroxidases, and hydrolytic enzymes.<sup>89</sup> During degranulation, granule membranes fuse with the cytoplasmic membrane, and this process provides a rich source of new cell surface receptors that can interact with the environment.<sup>90</sup> In addition to these functions, adhesion of neutrophils augments the ability to phagocytose foreign particles and pathogens,<sup>91,92</sup> and also induces signaling events that regulate neutrophil apoptosis.<sup>93</sup> Stimulation of neutrophils in suspension with inflammatory mediators does not induce significant granule secretion or sustained respiratory burst. To initiate functional responses of cell spreading, migration, respiratory burst, and granule secretion, neutrophils must

be co-stimulated with both an inflammatory mediator and an adhesive surface<sup>44</sup> or pharmacological intervention.

### Conclusions

Selectin- and selectin ligand-induced signaling, G-protein-coupled receptor signaling, and outside-in signaling of integrins control activation of neutrophils and their functional responses (spreading, migration, respiratory burst, and degranulation), but the precise signaling pathways and the points of convergence are poorly understood. Many different clinical trials tried to modulate leukocyte trafficking by blocking molecules that are involved in distinct steps of the multistep trafficking cascade. Clinical trials blocking  $\beta_2$ -integrins to block neutrophil trafficking in the context of ischemia-reperfusion injury have not shown any benefits. Antibody blockade of ICAM-1 had negative effects on strokes in a phase II trial.<sup>94</sup> However, the treatment with Natalizumab, a monoclonal antibody against  $\alpha_4\beta_1$ , is very efficacious in patients with Crohn's disease<sup>95</sup> and multiple sclerosis.<sup>96</sup> Inhibition of the  $\alpha_L$ -integrin has beneficial effects in treating graft-versus-host disease, transplant rejection, and psoriasis. Different antagonists of chemokine receptors are now being tested in preclinical and early clinical settings. Blocking of adhesion molecules or GPCRs is a "double-edged-sword" approach, because this treatment can lead to impaired host response. Uncovering the proximal and distal signaling pathways and their interconnections may help to develop better therapeutics that modulate neutrophil function in inflammatory disease.

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