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Integration of inflammatory signals by rolling neutrophils

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Summary: In inflammation, neutrophils roll along the endothelial wall of postcapillary venules and sample inflammatory signals. Neutrophil activation is required to generate β_2 integrin bonds with the endothelium that are strong enough to withstand the flow forces and thus achieve arrest from the rolling state. Unlike naïve T cells, neutrophils are not only activated by ligation of G-protein coupled receptors with chemokines and other chemoattractants but also receive signals from engagement of adhesion molecules including the selectins and β_2 integrins. Rolling neutrophils integrate the sum total of inputs received while scanning the inflamed endothelium. In this process, the velocity of rolling neutrophils systematically decreases as a function of their contact time with the inflamed endothelium. If an activation threshold is reached, β_2 integrins switch to the high-affinity conformation, redistribute on the cell surface, and trigger arrest and adhesion. Rolling cells that do not reach the activation threshold detach from the endothelium and are released back into the circulation. The role of chemokines, adhesion molecules, and other activating inputs involved in this response as well as signaling pathways are the subjects of ongoing investigations. This review provides a conceptual framework for neutrophil recruitment from the flowing blood.

This review focuses on the molecular mechanisms of neutrophil recruitment in the inflammatory process. In most tissues, including skeletal muscle, kidney, various connective tissues, mucosal membranes, the tissues of the eye, and most lymphatic organs, neutrophils roll along the walls of postcapillary venules after even the slightest disturbance by mild surgical trauma (1), heat (2), ischemia and reperfusion (3), histamine (4, 5), or mast cell-degranulating agents (6). This rolling is mostly mediated by P-selectin, a highly conserved carbohydrate-binding molecule expressed on the endothelium and on activated platelets (7). P-selectin binds fucosylated and sialylated O-glycans, most of which are presented in the context of sulfated tyrosine residues on a single glycoprotein, P-selectin glycoprotein ligand-1 (PSGL-1). PSGL-1 is constitutively expressed on all neutrophils, eosinophils, monocytes, and lymphocytes (8). PSGL-1 activity is regulated

by a set of glycosyl transferases that decorate the glycoprotein with core-2 structures, fucose residues, and a terminal sialic acid residue (9, 10). In the skin, P-selectin is constitutively expressed on the endothelial surface, and P-selectin-dependent leukocyte rolling is present even without trauma (11). In some vascular beds, including the alveolar capillaries in the lung and the sinusoids of the liver, leukocyte rolling is not commonly observed, and neutrophil recruitment mechanisms are largely selectin-independent. Little is known about the molecular mechanisms of neutrophil entry into these organs (12, 13). They will not be considered further in this review.

Leukocyte adhesion cascade

The classical leukocyte adhesion cascade comprises the elements of tethering (or capture), rolling, activation and adhesion (14, 15). In the simplest case, neutrophil capture and rolling can be mediated by P-selectin binding PSGL-1, activation may occur through a chemokine like interleukin-8, and adhesion requires only intercellular adhesion molecule-1 (ICAM-1) binding β_2 integrins on the neutrophil. In an elegant, reconstituted system, these minimal requirements have recently been demonstrated to be sufficient for neutrophil arrest under shear flow (16). Exposure of the rolling neutrophil to immobilized interleukin (IL)-8 is thought to induce a pro-adhesive conformational switch in β_2 integrins, analogous to enhanced neutrophil adhesion seen after exposure to soluble IL-8 (17). Experiments with isolated blood neutrophils rolling on cytokine-activated cultured endothelial cells, for example of human umbilical vein origin, suggest a similar mechanism (18). By their very nature, these cell–cell interaction systems can only demonstrate that certain molecules are required for the cascade to proceed, but they can rarely show that these elements are indeed sufficient. The situation in inflamed venules of the living organism is even more complicated, because all cell–cell interactions occur in the presence of red cells and plasma proteins, and because the endothelial cells lining post capillary venules are more highly differentiated than cultured endothelial cells.

Arrest chemokines for T cells, B cells, and monocytes

The paradigm of rolling followed by rapid activation by a single chemokine was recently demonstrated for naïve T lymphocytes traveling through high endothelial venules of the iliac lymph node (19). In this system, almost all T-cell rolling requires L-selectin, and all activation requires the chemokine SLC and its receptor CCR7 (19). Blocking or eliminating either the chemokine or the receptor results in an inability of naïve T cells to reach peripheral lymph nodes. Based on these data, SLC can be termed an arrest chemokine because:

- it is expressed on the endothelial surface of high endothelial venules;
- blocking or removing the chemokine or the receptor removes the arrest function;
- adding the chemokine back to a mouse lacking SLC expression in high endothelial venules (plt/plt mouse) restores the arrest function.

More recently, two arrest chemokines have been described that trigger arrest of rolling monocytes. Blocking either RANTES or KC, the mouse chemokine most closely resembling human IL-8, results in an about 50% reduction of monocyte arrest on cultured endothelial cells (20) or in the atherosclerotic carotid artery (20, 21). In these experiments, expression of RANTES and KC was demonstrated on the endothelial surface, blocking the chemokine or its receptor removed the arrest function, and adding the chemokine back restored monocyte arrest. Very recent data suggest arrest chemokines for B cells. Apparently, SLC, BLC and SDF-1 all contribute to the arrest of rolling B cells under physiological conditions (J. Cyster, Keystone Symposium 'Molecular Mechanisms of Leukocyte Trafficking', 2002). These chemokines were identified as being involved in B-cell recruitment to secondary lymphoid organs in short-term homing assays, so there is a possibility that one or more of them may act downstream from arrest, for example during transmigration.

Arrest chemokines for neutrophils

Arrest chemokines for neutrophils have been much harder to define. Although many chemokines including IL-8 (22) and other chemoattractants including formyl peptides (23), complement C5a (23), leukotriene LTB4 (24), and platelet activating factor (PAF) (25) all cause rapid neutrophil adhesion, their role in the activation of rolling leukocytes in inflamed venules has not been established. In certain *in vitro* systems, neutrophil arrest from rolling can be attributed to a single chemoattractant. For example, human umbilical vein endothelial cells (HUVEC) treated with histamine or thrombin support P-selectin-dependent neutrophil rolling and PAF-dependent activation (26). However, in most inflammatory models *in vivo*, PAF synthesis or receptor antagonists have little or no effect.

Similarly, IL-8 has been shown to promote neutrophil and monocyte arrest on IL-1β–treated HUVEC (27, 28). In humans, two neutrophil receptors recognize IL-8, CXCR-1 and CXCR-2. Mice express no ortholog of CXCR-1 and no true ortholog of IL-8. Mice lacking CXCR-2 show a severe reduction of neutrophil recruitment in a model of peritonitis and grossly altered hematopoiesis (29). As expected, these mice show no leukocyte adhesion response to the CXCR-2 ligand, macrophage inflammatory protein-2 (MIP-2), but a normal response to formyl peptide (30). However, when CXCR-2 null mice were treated with tumor necrosis factor (TNF)-α to induce a local inflammation in the cremaster muscle, their neutrophils arrested just as efficiently as those of wild-type control mice (Dunne, Forlow, Ley, unpublished). This suggests that other neutrophil activators exist under these conditions that make CXCR-2 dispensable for neutrophil arrest.

Although LTB4 causes rapid neutrophil arrest when administered locally to post capillary venules (24), mice lacking the LTB4 receptor ($BLTR^{-/-}$ mice) have no major defect in neutrophil arrest (Dunne, Luster and Ley, unpublished). To date, no arrest chemokine or chemoattractant (as defined above) for neutrophils has been defined for any inflammatory situation. This is surprising, given that neutrophil rolling and adhesion have been studied longer and by more groups than rolling and adhesion of T cells or monocytes.

Time course of neutrophil arrest

The arrest process, or conversion from rolling to firm adhesion, has only been studied quite recently (28, 31, 32). *In vitro* reconstitution experiments have shown that rolling T lymphocytes can arrest on a substrate of P-selectin and ICAM-1 very rapidly, within one video frame (0.03 s) (31). More recent investigations performed with high temporal resolution on Jurkat cells, a T-cell line, have shown that local activation may proceed on a timescale of milliseconds (33). Rolling neutrophils exposed to a large amount of extravascular IL-8 or other chemoattractants (23, 24, 34) also stop rolling immediately, within 1 s.

To study neutrophil arrest in inflamed venules *in vivo*, a new technique called leukocyte tracking was developed that follows each rolling leukocyte from its exit from a capillary to its eventual arrest or detachment (32). This method has so far only been applied to a TNF-α-induced model of inflammation in the cremaster muscle microcirculation. Here, neutrophils roll for long distances and spend more than 1 min rolling before they actually arrest (32). In TNF-α-treated cremaster muscle venules, P-selectin, E-selectin and L-selectin all contribute to rolling (35), and the β_2 integrins LFA-1 and Mac-1 regulate neutrophil rolling velocity (36) and, eventually, arrest. During the protracted rolling process, the rolling velocity is not constant, but systematically decreases from about 10 μm/s to a complete stop (32). Interventions that elevate leukocyte rolling velocities result in moderate (mice lacking E-selectin) to severe (mice lacking CD18) reductions of neutrophil arrest in this system. The transit time of neutrophils through the inflamed venules correlates strongly with the arrest efficiency (37, 38). These observations suggest that neutrophils may sample the endothelial surface and possibly also soluble mediators and integrate the signal to reach a threshold level of integrin activation that allows them to stop and eventually transmigrate.

Network model of neutrophil adhesion

The linear cascade model of rolling, activation and adhesion has helped elucidate the general principles of leukocyte recruitment. However, this model does not account for the fact that these processes are indeed overlapping and intertwined with each other. Recently, a network-like model has been proposed to allow for multiple pathways of rolling, activation and adhesion for neutrophils (39). This model accounts for observations that suggest that specific activators result in adhesion through specific adhesion molecules. For example, blockade of L-selectin shedding (for details see below) results in enhanced leukocyte adhesion that is LFA-1 dependent and ICAM-1 dependent (40). By contrast, increased adhesion induced by the chemokine MIP-2 is completely ICAM-1 independent in inflamed venules (41). Recent evidence suggests that P-selectin-dependent rolling results in preferential neutrophil adhesion through $\alpha_4\beta_1$ integrin, a molecule expressed at a low level on blood neutrophils (Forlow & Ley, unpublished). Many more such pathways may exist, resulting in a number of possibilities for a neutrophil to reach a site of inflammation.

Certain bottlenecks of the inflammatory response cannot be bypassed when as few as two molecules are blocked (39). As an example, neutrophil recruitment to inflammatory stimuli is severely impaired in mice lacking both E- and P-selectin (42, 43), but not either one alone (44, 45). Apparently, neutrophil activation through chemokine receptors and other 7-spanning G-protein coupled receptors does not constitute such a bottleneck, because no chemoattractants that would be absolutely required for neutrophil arrest have been defined. The weakness of the pathway model is that many of the ele-

ments have not been identified and the missing pieces are necessarily hypothetical. Its strength is that it is internally consistent and also consistent with published observations of neutrophil arrest.

Evidence for neutrophil activation through multiple inputs

The neutrophil seems to be quite different from the naïve T cell in that it does not rely on a single input for activation. As part of the innate immune system, the neutrophil has evolved to respond to many stimuli from bacterial (formylated peptides, lipopolysaccharides (LPS)), endogenous (oxygen free radicals), and immunologic (chemokines, cytokines) sources. Inappropriate neutrophil activation in the bloodstream is potentially perilous, because such neutrophils are less deformable and may lodge in capillary beds distant to the site of inflammation (46). The concept of multiple inputs in the context of engagement of adhesion receptors rather than a single arrest chemokine seems to be a much better description of neutrophil function. Indeed, there is evidence suggesting that neutrophils receive signals not only through G-protein-coupled receptors, but also through Fc receptors (47) and inflammatory adhesion molecules (see below). Although the signals generated by ligation of each of these receptors may be small and far from triggering complete neutrophil activation, the signaling pathways overlap and can be additive or even synergistic. This concept was introduced earlier as 'priming' of neutrophils by one agent facilitating activation by another (48). Although the original priming concept implies a temporal sequence rather than concomitant signaling, there is evidence that the rolling neutrophil integrates signals received simultaneously through G-protein coupled receptors and adhesion molecules.

Neutrophil activation through G-protein-coupled receptors

Receptors for chemoattractants have long been recognized as powerful activators of all aspects of neutrophil function. One theory, formulated by Marco Baggiolini, holds that a single chemokine, IL-8, can trigger responses at all levels, depending on the dose and duration of exposure (49). A small dose of IL-8 causes proteolytic removal of L-selectin from the cell surface and increased surface expression of the β2 integrin Mac-1, secondary to the release of secretory granules. A higher dose of IL-8 can trigger elaboration of oxygen-free radicals through the NADPH oxidase system, and yet higher levels induce release of azurophilic granules. Alternatively, neutrophil activation may require a priming agent and a second stimulus that then causes full activation at a low dose that would be insufficient to cause activation without priming. A third idea was recently developed in Eugene Butcher's lab, who discovered that signals from certain chemoattractants can be integrated by migrating neutrophils, based on a hierarchy of receptor cross-desensitization (50). All G-protein coupled receptors desensitize to their respective ligands (51), i.e. the receptor protein is internalized or phosphorylated in response to ligation so that responses to a second dose of agonist are absent or much attenuated. In addition, some but not all neutrophil chemoattractants show the phenomenon of heterologous or cross-desensitization (52). For example, fMLP not only desensitizes the fMLP receptor, but also the LTB4 receptor, although LTB4 does not cause desensitization of fMLP receptors (53). This concept results in a hierarchy of chemoattractants. It is important to note that this model was developed in migration assays and does not necessarily hold for integrin activation assays (neutrophil arrest from rolling), calcium flux assays, or the regulation of degranulation or superoxide production (54).

In vivo studies have demonstrated an elevation of intracellular free calcium in rolling neutrophils (32), but it is not known whether this response is caused by engagement of G-protein coupled receptors. Pertussis toxin (PT) induces adenosine 5'-diphosphate (ADP) ribosylation of Gαi, the G-protein responsible for most responses to chemoattractants, and is therefore an excellent tool to determine whether this signaling pathway is involved in a given physiological response. Incubation with pertussis toxin inhibits arrest of naïve lymphocytes perfused through high endothelial venules of Peyer's patches (55). Monocyte arrest to atherosclerotic arteries is significantly inhibited by pertussis toxin treatment (21). However, systematic *in vivo* studies using PT on neutrophils have not been reported. Since neutrophils appear to behave fundamentally differently from naïve T cells or monocytes, it remains unclear whether pertussis toxin would significantly inhibit neutrophil arrest under inflammatory conditions.

Induction of neutrophil arrest in simplified experimental systems

There is little doubt that conformational changes of the β_2 integrins LFA-1 and Mac-1 are responsible for causing neutrophil arrest from rolling. Local addition of chemoattractant leads to rapid arrest of rolling leukocytes, and blocking CD18 integrins eliminates this response (56). The conformational

changes of integrins have recently been defined in detail by Tim Springer's group (57, 58). The apparent difference in signal requirements for integrin activation and other neutrophil responses may be a consequence of exposure of a limited number of G-protein coupled receptors to a chemoattractant by engagement of receptors with ligands presented on the vessel wall. Indeed, recent evidence has shown that exposing monocytes to a solution of MCP-1 induces monocyte arrest through an α_4 and β_2 integrin-dependent pathway (28), whereas exposure of rolling neutrophils to surface-expressed MCP-1 does not produce this effect (21). These findings make it likely that neutrophil arrest is differentially triggered by surface-bound vs. soluble chemoattractants, but direct evidence for this triggering has yet to be presented. Ronen Alon has recently studied lymphocyte-like cell lines in a flow chamber system. His data suggest that engagement of the chemokine receptor CXCR4 by SDF-1 immobilized on a glass surface can trigger subsecond activation of α_4 integrins (33). It is unclear whether similar localized events occur in neutrophils, but in the absence of contrary evidence, it is reasonable to hypothesize that they may occur.

Neutrophil activation through L-selectin

Several lines of evidence suggest that L-selectin/ligand interactions can lead to activation of leukocytes. As early as 1994, it was proposed that L-selectin engagement by antibodies or ligand mimetics elevates intracellular free calcium levels and potentiates the oxidative burst, associated with mitogen-activated protein (MAP) kinase phosphorylation (59, 60). Cross-linking of L-selectin on neutrophils initiates signaling through a variety of pathways, but it is not known how relevant these antibody cross-linking experiments are for the situation of rolling cells. In some of these systems, ligation and cross-linking of L-selectin results in β_2-integrin-dependent adhesion (61), but L-selectin-dependent rolling is not sufficient to arrest neutrophils in flow chamber systems (62) or *in vivo*. L-selectin cross-linking is associated with phosphorylation of p38 MAP kinase at 1 min and is followed by ERK1/2 phosphorylation at 10 min (63). L-selectin shedding, which is also induced by neutrophil activation, is inhibited by blocking ERK or p38 MAP kinases (64). Many of the studies showing such effects were conducted using intact monoclonal antibodies to L-selectin that may engage Fc receptors and induce neutrophil activation through a combination of L-selectin and Fc receptor-mediated signaling. A few studies use F(ab′)2 fragments of L-selectin antibodies cross-linked by secondary F(ab′)2 fragments (40), thus eliminating the input through Fc receptors. Protein tyrosine phosphorylation can also be increased by monovalent Fab fragments of L-selectin antibodies (65).

The concept that signaling through L-selectin may enhance leukocyte–endothelial interaction and promote subsequent firm adhesion was introduced by Tedder's group (66). In that study, antibodies to certain epitopes in the lectin domain of L-selectin or engagement of the lectin domain by a ligand mimetic, PPME, induced signaling, while antibodies to other epitopes did not. In a flow chamber system, cross-linking of L-selectin has been shown to promote β_2-dependent neutrophil arrest on cultured human umbilical vein endothelial cells (18). Other data were obtained in model systems like homotypic or heterotypic neutrophil aggregation under shear (61, 63), which are more removed from the physiologic situation.

The importance of L-selectin-mediated neutrophil activation was recently confirmed *in vivo*, using modulation of L-selectin shedding as a tool to alter the signal input through L-selectin (40). When L-selectin shedding was blocked by a hydroxamic acid-based protease inhibitor, increased neutrophil arrest resulted, and this was completely abolished in mice lacking L-selectin. However, inhibition of L-selectin shedding also reduced neutrophil rolling velocity (67) and increased the 'smoothness' of rolling (reduced microjumps) (40). Therefore, it is unclear whether the effect of blocking L-selectin shedding is direct (through signaling) or indirect (through increased exposure of neutrophils to the surface of the inflamed venule). Experiments designed to directly assess of the contribution of L-selectin-dependent signaling will be necessary to fully establish the role of L-selectin engagement in neutrophil activation during rolling. The preponderance of the evidence would suggest that this route of activation is important. A signaling role of L-selectin in neutrophil arrest would help explain the substantial neutrophil recruitment defects seen in L-selectin-deficient mice (68) that far exceed any defects expected from the observed relatively minor contribution of L-selectin to neutrophil rolling (69, 70).

Neutrophil activation through ligation of PSGL-1

The idea that engagement of P-selectin may lead to β_2 integrin activation and neutrophil arrest was first proposed by Guy Zimmerman (26). In these early experiments, platelet activating factor (PAF) was proposed as a signaling intermediate. Later, P-selectin glycoprotein ligand-1 was discovered as a major ligand for P-selectin on neutrophils and a contributing ligand to E-selectin binding. In a flow chamber rolling assay, neutrophil adhesion to cells transfected with both E-selectin

to provide a rolling substrate and ICAM-1 to provide an adhesion substrate was partially blocked by an antibody to PSGL-1 (71). The physiological importance of this potential signaling through PSGL-1 is not known, because the contribution of PSGL-1 to E-selectin-dependent neutrophil capture and rolling may be limited, at least in some inflammatory conditions (72). Assays in which neutrophils are rolling on a cellular substrate cannot test whether signaling is direct or whether other cell surface molecules or soluble mediators may be involved. For example, it is possible that disrupting PSGL-1 binding to P-selectin or E-selectin removes receptors for chemoattractants sufficiently far from the cellular surface, so that effective activation through a surface-bound chemoattractant becomes impossible. In cell–cell adhesion assays, it is very difficult to demonstrate direct activating effects of adhesion molecule engagement. Therefore, data from the experiments mentioned above are hard to interpret. This includes reports of P-selectin-dependent induction of MCP-1 and IL-8 biosynthesis in monocytes incubated with activated platelets (73).

An alternative approach is the use of fixed cells that presumably cannot produce soluble mediators, although they still express cell surface glycoproteins that may continue to be functional as activators. Adding paraformaldehyde-fixed platelets to resting neutrophils induces tyrosine phosphorylation of a 100-kDa protein through a PSGL-1- and β_2-integrin-dependent pathway (74). In human neutrophils, engagement of PSGL-1 can activate p42/p44 MAP kinase (75). This effect required intact antibodies, suggesting that PSGL-1 cross-linking or coengagement of Fc receptors may be involved. PSGL-1 engagement may induce transcription of the urokinase receptor, CD87 (76), which in turn may regulate ligand specificity of β2 integrins (see below). Certainly, PSGL-1 engagement by P-selectin during rolling is not sufficient to arrest rolling neutrophils (16, 69, 77). Neutrophil activation through PSGL-1 engagement remains an attractive possibility, but the physiologic role of this pathway in neutrophil arrest remains to be explored.

Neutrophil activation by engagement of β_2 integrins

There is no doubt that engagement of β_2 integrins like LFA-1 or Mac-1 provides powerful outside-in signals that help activate neutrophils and other leukocytes. Experiments in CD18 knockout mice suggest that he engagement of β_2 integrin is required to produce a respiratory burst and oxygen free radicals (78). It is also clear that β_2 integrins are involved in leukocyte rolling under conditions of TNF-α-induced inflammation, because CD18-, Mac-1- or LFA-1-deficient mice or mice treated with a function-blocking mAb to CD18, LFA-1 or Mac-1 all have elevated rolling velocities (32, 36, 37). However, it is much less clear what β_2 integrins actually do during slow rolling. Do they engage 'classical' ligands such as ICAM-1 on endothelial cells? Does such engagement provide costimulatory signals? Or do β_2 integrins somehow strengthen selectin-dependent bonds through indirect mechanisms? Unfortunately, almost all published work on β_2 integrin outside-in signaling uses antibody or ligand cross-linking experiments to provide an activating signal, and the findings may be of limited relevance to the situation of rolling leukocytes. Cross-linking β_2 integrins causes activation of nonreceptor tyrosine kinases (79), which trigger downstream effects such as degranulation or oxygen radical production. Engagement of β_2 integrins on human neutrophils activates the small G protein RhoA, which is involved in organizing the actin cytoskeleton (80). Glucosylphosphatidyl inositol (GPI)-linked membrane proteins can interact with β_2 integrins like Mac-1 to alter their ligand-binding specificity and affinity. In particular, urokinase receptor (CD87) has been reported to associate with Mac-1 (81) and modulates leukocyte adhesion, locomotion and activation (82).

Some of the signaling through β_2 integrins seems to proceed along distinct pathways. As an example, intracellular ceramide accumulation abolishes agonist-induced neutrophil adhesion to protein-coated surfaces but not β_2-integrin-dependent homotypic aggregation (83). Ceramide causes increased intracellular free calcium, degranulation, and increased Mac-1 expression but not increased cell adhesion, because ceramide fails to activate β_2 integrins. It is attractive to speculate that activating signaling pathways might be triggered through β_2 integrin engagement during rolling, but there is no experimental evidence for this at this time.

One of the most striking effects of ligating β_2 integrins is the induction of expression of β_1 integrins on neutrophils. β_2 integrin engagement appears to be required for expression of at least $\alpha_4\beta_1$ (84) and $\alpha_2\beta_1$ (85); both integrins have important functions in neutrophil adhesion and migration through the extracellular matrix space. It is not known whether β_1 integrin expression is induced during slow rolling, or whether transendothelial migration is required for β_1 integrin expression on neutrophils. In fact, such an experiment is difficult to design.

β_2 integrin ligation has also been associated with induction of neutrophil apoptosis (86) through activation of cytoplasmic tyrosine kinases and c-Jun N-terminal kinase (JNK) (87). Cross-linking Mac-1 on neutrophils induces production

of IL-8 and IL-1β (88), and other forms of activation can induce similar responses. β_2 integrin engagement appears to be required for the release of heparin-binding protein (HBP), which is responsible for the neutrophil-dependent increase in endothelial permeability (89, 90). Again, it is not known whether transient β_2 integrin engagement during slow rolling is sufficient to induce release HBP, or whether firm adhesion is required.

Reactive oxygen species (ROS) such as H_2O_2 can trigger activation of β_2 integrins through a pathway that can be blocked by tyrosine kinase inhibitors (91). Since ROS are released from endothelial cells in areas of inflammation, it is plausible that such signaling could contribute to neutrophil arrest.

Neutrophil activation through adhesion to E-selectin

This line of thought was pioneered by Sam Wright's group more than 10 years ago (92), but the initial results were not confirmed for many years. Recently, Scott Simon showed that neutrophil adhesion to E-selectin-expressing cells in a flow chamber rolling assay activates β_2 integrin avidity, and this adhesion can be blocked by MAP kinase inhibitors (71). This effect was in part mediated through PSGL-1. This finding, if confirmed, could be of key importance for our understanding of neutrophil recruitment. Since the major ligand(s) mediating E-selectin-dependent neutrophil rolling is not known, it remains attractive to speculate that this molecule may have an important signaling function. On a cautionary note, the evidence for E-selectin-mediated neutrophil activation stems from cell–cell adhesion assays, which are subject to the caveats mentioned above.

Integration of activating signals by rolling neutrophils

Neutrophils rolling in inflamed venules engage P-selectin (69) mainly through PSGL-1 (72, 93), E-selectin through PSGL-1 and unknown ligands (72), L-selectin through poorly defined endothelial ligands (69, 70), chemoattractants through their cognate G-protein-coupled receptors, and LFA-1 and Mac-1 by binding to unidentified endothelial ligands, possibly including ICAM-1 (36, 41). This scenario provides ample opportunities for neutrophil activation. Apparently, under inflammatory conditions, no single stimulus dominates so much as to be required for neutrophil arrest. Blocking each individual signaling pathway leads to no or at most partial reductions in the efficiency of converting rolling to adherent neutrophils. Based on these observations, it seems most likely that the rolling neutrophil scans the endothelial surface for inflammatory adhesion molecules, chemokines, other chemoattractants, and cytokines. It is unclear how this integration mechanism works; especially in view of the long time (2 min or more) required to sample enough endothelial surface to come to arrest. Many of these ob-

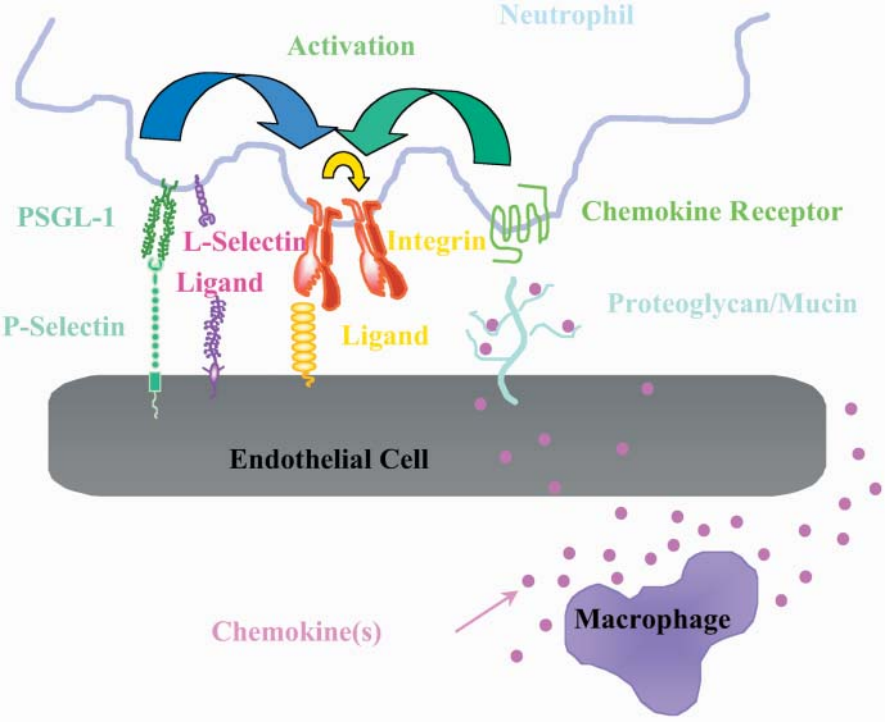

Fig. 1. Neutrophil (top) rolling on an inflamed endothelial cell. During rolling under inflammatory conditions, neutrophils engage all three selectins. Integrin activation for firm arrest may occur through 1) P-selectin engaging PSGL-1; 2) L-selectin engaging an endothelial ligand; 3) chemokines (and other chemoattractants) presented on the endothelial cell surface and activating their cognate receptors; and 4) β_2 integrins binding endothelial ligand(s) during slow rolling.

servations are based on intravital microscopy, where the exposed tissue is superfused by a saline solution that may dilute soluble chemoattractants and therefore cause an artifactual lengthening of the required exposure time and vessel length. However, experiments conducted under an impermeable mylar film showed very similar rolling times and distances (Dunne & Ley, unpublished data), suggesting that dilution of chemoattractants is not a leading cause of protracted neutrophil arrest.

Importance of prolonged contact of rolling neutrophils with the inflamed endothelium

We have previously proposed that the transit time of rolling cells through an inflamed area determines whether neutrophil arrest and recruitment can occur (37). This proposition was initially based on observations that the transit time of rolling leukocytes correlates with the number of adherent neutrophils in wild-type mice, E-selectin-deficient mice, and CD18-integrin-deficient mice. Since transit time is inversely related to rolling velocity, it is attractive to propose that a certain slowness of rolling is required to allow neutrophil activation from all these different inputs. Indeed, mice lacking both E-selectin and CD18 show grossly elevated rolling velocities and dramatically reduced neutrophil recruitment (38). Similarly, reduced neutrophil recruitment was seen in LFA-1-deficient mice and in Mac-1-deficient mice, both of which show elevated rolling velocities (36). Conversely, reducing leukocyte rolling velocity by inhibiting L-selectin shedding increases neutrophil adhesion (40). It is unclear whether the time a leukocyte spends rolling and the amount of physical contact with the endothelial surface are important in determining arrest. On a cautionary note, all evidence for regulation of neutrophil arrest by rolling velocity is correlative in nature, and a causative relation has yet to be demonstrated. Also, the tight correlation between leukocyte rolling velocity and adhesion described in studies from this laboratory has so far not been confirmed by other laboratories.

Recent evidence suggests that slow rolling may not be absolutely required for neutrophil adhesion. Mice lacking both P-selectin and CD18 integrins show almost normal rolling velocities, yet neutrophil adhesion is drastically reduced (94). Conversely, mice lacking fucosyl transferase IV, an enzyme involved in the generation of E- but not P-selectin ligands on neutrophils, show elevated rolling velocities but no defect in leukocyte recruitment in thioglycollate-induced peritonitis (95). In this model, E-selectin function is largely redundant with P-selectin function (45), suggesting that remaining P-selectin ligands are sufficient to ensure neutrophil recruitment. In support of this notion, fucosyl transferase IV and VII double knockout mice with deficient P- and E-selectin ligands show very substantial restriction of neutrophil recruitment (95).

The importance of leukocyte rolling velocity as a determinant of adhesion is further supported by biomechanical studies. Leukocytes are viscoelastic bodies (96), which means that their deformation in response to an applied force is not instantaneous but time-dependent. Many studies have shown that slowly rolling leukocytes can deform substantially, especially under conditions of high wall shear stress (97, 98). As a consequence of this deformation, the contact area between the rolling leukocyte and the endothelium can be more than doubled (99). Since leukocyte deformation is time-dependent, this increase in contact area will only be achieved when rolling is sufficiently slow to allow deformation to proceed. Increased contact area may increase the chances of sampling a sufficient amount of activating stimuli present on the endothelial surface and also increase the number of adhesive bonds. Both effects would help explain why slow rolling promotes firm adhesion.

Conclusion

The evidence presented above suggests that neutrophil activation under relevant *in vivo* conditions is fundamentally different from that of naïve T cells or monocytes. Neutrophil activation appears to be gradual, but the cell biological mechanisms and signaling pathways responsible for this activation are not known. They may include G-protein coupled receptor signaling, selectin- and selectin ligand-mediated signaling, and engagement of β_2 integrins during the conversion from rolling to firm adhesion (*Fig. 1*), which lead to cytoskeletal changes, possibly activation of NADPH oxidase and the respiratory burst, release of some but not all types of granules, and other aspects of neutrophil activation. The ability of the neutrophil to integrate activating signals from diverse sources is poorly understood. *In vivo* experiments suggest that this integration occurs during the rolling process and may take between 10 s up to 2 or 3 min. These observations suggest that neutrophils are capacitated as they progress from a resting state in the bloodstream through the states of being rolling and adherent cells to a fully activated cell next to a pathogen. Breaking this sequence, for example, by flooding the blood with cytokines and chemoattractants in sepsis, causes inappropriate neutrophil activation associated with a paradoxical inability to recruit neutrophils to the site of infection.

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