Can neutrophils be manipulated \textit{in vivo}?


Neutrophils are important effector cells of the innate immune system that are capable of responding rapidly to infectious organisms to which the immune system may be naive. However, in certain circumstances their actions can be inappropriate or ineffective. A lack of effective neutrophil function increases the number or persistence of infections. This is well illustrated by inherited disorders that impair neutrophil effector function. In chronic granulomatous disease, deficient activity of NADPH oxidase (usually the membrane-bound gp91 subunit) results in the absence of a respiratory burst and the production of reactive oxygen species necessary for microbial killing, resulting in frequent infection. Defects in the leucocyte adhesion molecules (\(\beta_2\) integrins or selectin molecules) necessary for neutrophil-endothelial interactions lead to poor recruitment to sites of infection and hence an inability to effectively fight infection [1].

In some patients the opposite situation occurs, where inappropriate and prolonged neutrophil activation leads to tissue damage. There is evidence that in the presence of anti-neutrophil cytoplasmic antibodies (ANCA), which develop in some forms of autoimmune vasculitis, neutrophils adhere to endothelium and undergo a respiratory burst leading to endothelial damage. Dysregulated apoptosis and inefficient removal of these neutrophils by macrophages leads to chronic inflammation and organ damage [2–4]. Such events contribute to the tissue injury associated with the inflammatory disease.

Therefore, a potential goal of therapy for diseases involving aberrant neutrophil function in the context of inflammation and autoimmunity would be to manipulate the function of the neutrophil \textit{in vivo}. There are several places along that chain of events, starting with neutrophil activation and ending in the release of reactive oxygen species, that may be amenable to intervention:

- Preventing neutrophil recruitment across vascular endothelium.
- Down-regulating neutrophil activation at the level of receptor-mediated events or intracellular signalling pathways.
- Targeting cytotoxic products of neutrophil activation.
- Manipulating neutrophil life span and/or promoting non-phlogistic removal.

### Neutrophil recruitment

The paradigm of the leucocyte adhesion cascade is now well established, and many, albeit not all, of the details of neutrophil recruitment are established [5]. Included within the cascade are selectin and integrin adhesion molecules and chemoattractant agents. The selectin family are important in neutrophil capture and rolling adhesion. Locally released chemokines act on neutrophil chemokine receptors to provide directional and activating signals, while neutrophil \(\beta_2\) receptors, interacting with their endothelial cell ligands, are important during development of firm adhesion and migration.

Neutrophil recruitment can be investigated using \textit{in vitro} flow assays, \textit{in vivo} intravital microscopy and various animal models.

### Selectins as targets for manipulating neutrophil recruitment

The selectin family contains three members: P-selectin (on platelets and endothelial cells), E-selectin (on endothelial cells) and L-selectin (on leucocytes, including neutrophils). All have a lectin domain, an epidermal growth factor (EGF)-like domain and a variable number of cysteine-rich repeats. Ligands comprise several glycoproteins; a major ligand comprises the mucin-like protein P-selectin glycoprotein ligand-1 (PSGL-1) which can bind all three selectins and which is expressed by platelets and leucocytes [5]. The selectins are attractive targets for manipulating neutrophil recruitment since P- and E-selectin are only expressed on endothelial cells at sites of inflammation, expression being induced by cytokines and some inflammatory mediators.

In flow models, both P- and E-selectin need to be inhibited in order to inhibit capture of neutrophils by cytokine-activated selectin-expressing endothelial cell monolayers [6]. The selectin dependence of neutrophil binding to endothelial cells can be shown \textit{in vivo} using intravital microscopy of the mouse cremaster muscle: further, by using models where neutrophil rolling adhesion is dependent on a single selectin (P-, E- or L-selectin), both recombinant PSGL-1 fusion protein and specific antiselectin antibodies are inhibitory [7]. Development of small molecule inhibitors that will block capture and rolling mediated by all three family members is under way. One such that has been tested in animal models is bimosiamose. This molecule is capable of reducing the size of a myocardial infarction in a rat model following transient occlusion of the left coronary artery [8].

### Inhibition via neutrophil integrins or their endothelial ligands

While selectins are important for neutrophil recruitment in post-capillary venules, some vascular beds where recruitment occurs via capillaries, as in the lungs or renal glomeruli, may be less dependent on selectins. Other mechanisms for manipulating recruitment may be necessary here. However, the constitutive and ubiquitous expression of other adhesion ligands, including \(\beta_2\) integrins, by cells other than neutrophils and the propensity...
for infection when such ligands are non-functional, as witnessed by leucocyte adhesion deficiency states, suggests that targeting such pathways for control of inflammation may be problematical. Effective use of antibodies against, for example, the CD18 common β chain of neutrophil β2 integrins in animal models of injury attains to the potency of this approach [9], but it is likely that it will only be translatable, at best, to confined short-term inflammatory insults in humans. Disappointingly, an anti-CD18 antibody given in association with recombinant tissue plasminogen activator did not limit myocardial infarction in patients [10].

The chemokine receptors CXCR1 and CXCR2 as targets for neutrophil manipulation

Neutrophils express two CXC chemokine receptors, CXCR1 and CXCR2, which are seven-transmembrane-domain G-protein-coupled receptors. Interleukin (IL)-8 or CXCL8 is the main ligand for CXCR1, whilst CXCR2 binds a variety of ELR+ CXC chemokines, including IL-8, growth-related oncogene (GRO)-α/CXCL1 and endothelial cell-derived neutrophil-activating peptide (ENA)-78/CXCL5. Activation via CXCR1 and CXCR2 promotes neutrophil chemotaxis into sites of inflammation and induces neutrophil degranulation with the release of enzymes such as human neutrophil elastase and protease-3. CXCR1 is involved in production of superoxide via NADPH [11], while CXCR2 is responsible for initiating neutrophil migration [12].

Manipulation of neutrophil recruitment to sites of inflammation may be achieved by inhibiting CXCR1 and CXCR2 ligand binding competitively by the use of small molecules or blocking antibodies, or by inducing receptor phosphorylation. In a flow-based adhesion model of ANCA-associated vasculitis, CXCR2 blockade inhibited the ANCA-induced migration of neutrophils across tumour necrosis factor (TNF)-activated endothelium [13]. In animal models of inflammation, engineered CXCR-1 and -2 antagonists abrogated neutrophil chemotaxis by blocking IL-8 binding to the receptors on neutrophils [14]. N,N'-diallylureas, small-molecule antagonists of CXCR2, have been shown to compete with both IL-8 and GRO-α for binding to CXCR2 but not to CXCR1. In vivo, in rabbit models of inflammation, they were able to block neutrophil recruitment to sites of inflammation [15, 16]. It is possible to block the function of CXCR1 in vitro with heterologous desensitization using cell-binding fragments of fibronectin which caused a time-dependent phosphorylation of CXCR1 but not CXCR2 [17]. Interestingly, opiates receptor agonists as well as antibodies directed against specific epitopes of CD45RB on neutrophils, also cause down-regulation of receptor-mediated events or intracellular signalling pathways.

Down-regulating neutrophil activation at the level of receptor-mediated events or intracellular signalling pathways

Receptor-mediated events

Neutrophils display a host of cell surface receptors that mediate communication with the extracellular environment. In physiological circumstances some provide inhibitory and others activating signals.

Inhibitory receptors include adenosine A2A receptors which trigger ‘off’ signals after binding of adenosine which may be released following tissue damage [20]. The A2A receptor-triggered generation of intracellular cAMP then inhibits neutrophil effector functions including up-regulated expression of β2 integrins, adhesion, oxygen radical production, degranulation and production of TNFα. In experiments on neutrophil-endothelial cell interactions, removal of adenosine by the enzyme adenosine deaminase induces enhanced neutrophil-dependent cytotoxicity towards endothelial cells [21].

Key activating receptors such as proteinase-activated receptor (PAR)-2 [22], Fcy receptors (human neutrophils are not thought to express an inhibitory Fcγ receptor) [23], Toll-like receptors [24], integrin and chemokine receptors (see above), as well as many others, have the potential for their effects to be blocked by small-molecule antagonists or antibodies. Proteinase-activated receptors (PARs) are a family of four, seven-transmembrane-domain proteins. They are activated by serine proteinases which cleave the protein to reveal a tethered ligand which binds intramolecularly, activating the receptor [25]. The PAR-2 receptor can also be activated by short peptides corresponding to the ligand region, PAR-2-activating peptides (PAR2AP). PAR-2 is expressed widely in tissues including endothelium and epithelium of airways, kidney and liver, mast cells, eosinophils, T cells and some neurons [25]. PAR-2 on human neutrophils can be activated by trypsin, leading to neutrophil shape change, increased CD11b expression and Ca2+ influx [26]. Neutrophil motility in a collagen matrix can be increased by PAR2AP, which also up-regulates Mac-1 and VLA-4 expression on the neutrophil surface and L-selectin shedding. Production of interleukins-1β, -6 and -8 increase in response to both trypsin and PAR2AP [22].

In a rat model, in vivo treatment of mesenteric venules with PAR2AP led to an increase in adhesion of leucocytes to endothelium. Intraperitoneal injection of PAR2AP led to the migration of leucocytes into the peritoneum. However, as endothelium also expresses PAR-2 these responses may be due to the effect of PAR2AP on the endothelium rather than the leucocytes [27]. A mouse PAR-2 −/− knockout model failed to develop arthritis in response to intra-articular injections of Freunds complete adjuvant or λ carrageenan and kaolin. In the same study PAR-2-expressing mice developed arthritis and hypergammacous mice developed an intermediate phenotype [28].

Taken together these studies show that PAR-2 may play an important role in neutrophil-endothelial interactions and the development of chronic inflammation. PAR-2 may be a target for intervention in inflammation, although no specific PAR-2 antagonists are available as yet.

Intracellular signalling pathways

Intracellular signal transduction pathways provide a rich source of potential points for intervention in many cellular responses. Neutrophils are no exception, but finding pathways that are relevant to inflammation or to specific pathophysiological processes is more problematical. Approaches might include the use of non-specific agents that affect multiple effector pathways as well as design of specific small-molecule inhibitors for highly targeted pathways.

Therapeutics available that affect multiple neutrophil effector pathways include pentoxifylline (a phosphodiesterase inhibitor), non-steroidal anti-inflammatory compounds (cyclo-oxygenase type 1 inhibitors) [29] and statins (HMG Co-A reductase inhibitors) [30, 31]. Pentoxifyllin has been used therapeutically in vasculitic disorders such as Behcet’s disease to modulate neutrophil function and inflammation. In vitro it does have effects on neutrophil recruitment, inhibiting adhesion of activated neutrophils to endothelial cells in static assays, and inhibiting TNF-induced neutrophil migration under flow [32].

Potential specific targets for signal transduction manipulation might include NF-κB or its control elements [33], phosphatidylinositol 3-kinases and Ras GTPases. Phosphatidylinositol 3-kinases (PI3K) are a family of lipid kinases that are capable of phosphorylating phosphoinositides on the D3 position of the inositol ring to form PI(3)P1, PI(3,4)P2 or PI(3,4,5)P3. They can be classified into three classes depending on their preferred substrate and structure. Class I are by far the most studied and are further subdivided into two groups, IA and IB. Type IA consist

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of a 85 kDa and a 110 kDa protein whereas IB comprise a 101 kDa and a different 110 kDa protein (gamma). The IB form has a restricted pattern of distribution, being found predominantly in leucocytes, and is believed to play a fundamental role in trafficking [34]. With ANCA stimulation of neutrophils, PIP3 is generated from non-class IA forms of PI3K, believed by inference to be PI3Kgamma [35]. Due to this molecule’s restricted pattern of expression it could provide a useful target for therapeutic intervention. There are currently no class-specific inhibitors reported [36], but undoubtedly modifications of current inhibitors such as LY294002 will yield molecules capable of selected inhibition, as has been described for the other forms.

p21ras is a small GTPase known to play a pivotal role in a plethora of physiological functions. This molecule may be a switch at which various signals converge and culminate in order to reach a threshold whereby the cell can produce a functional output. ANCA is able to activate p21ras, and this is essential for the production of superoxide by the neutrophil [37]. This is dependent on both the G-protein-coupled pathway activated by F(ab)2 fragments and the tyrosine kinase pathway activated by Fcγ receptors, thus providing a target whereby both arms of activation could be blocked. The most interesting p21ras inhibitor to date is farnesylsobutylamine (FTS). This directly interferes with the insertion of p21ras into the plasma membrane, leading to its degradation in the cytosol [38]. FTS has been shown in vivo in a mouse model of experimental autoimmune encephalomyelitis to selectively act upon highly activated cells such as the autoreactive T lymphocytes, without effects on the unstimulated growth of normal cells [39]. This compound was also purported to be useful in later stages of the disease process. Additionally FTS has been used in a model of Thy-1 nephritis giving decreased glomerular proliferation, monocytic infiltration and proteinuria [40]. We have also shown that a particular isoform of p21ras is selectively activated by ANCA [41] and this may provide an opportunity for intervention by even more specific types of inhibitors.

Targeting cytotoxic products of neutrophil activation

Neutrophils produce many mediators that facilitate their antimicrobial effects, including reactive oxygen species, nitric oxide, proteases, myeloperoxidase, cytokines and lipids. However, the same agents can, on occasion, cause collateral tissue damage. ANCA are able to activate cytokine-primed neutrophils to generate a respiratory burst, and nitric oxide production in a superoxide-dependent manner [42], mediators that play key roles in vasculitic damage. At low levels nitric oxide is cytoprotective, inhibiting platelet aggregation and leucocyte adhesion to the endothelium. However, nitric oxide may also react with superoxide anions, yielding the highly reactive compound peroxynitrite. Peroxynitrite may initiate lipid peroxidation and induce nitration of tyrosine residues, leading to loss of protein structure and function. Indeed nitrotyrosine-positive cells are present in damaged renal tissue of patients with ANCA-associated vasculitis [43]. Peroxynitrite has also been shown to act as a priming agent for neutrophils potentially acting in an autocrine loop enhancing inflammation in ANCA-associated vasculitis [44].

In vitro basal superoxide production by patients with ANCA-associated vasculitis is increased compared with healthy blood donors, even in remission [45]. This may have important consequences for the increased cardiovascular risk present in these individuals, contributing to oxidation of lipoproteins [46] and the development of endothelial dysfunction which characterizes ANCA-associated vasculitis [47]. Treatment of patients with antioxidants, vitamins C and E, reduced the production of superoxide by neutrophils in a small study [45]. Further randomized studies are required to validate the use of these agents. HMG-coA-reductase inhibitors (statins) have also been suggested to have immunosuppressive activities independent of lipid reduction and may be useful adjuvant therapy in ANCA-associated vasculitis. Statins are able to reduce superoxide production by neutrophils following ANCA activation, an effect mediated by inhibition of translocation of ANCA antigens, proteinase 3 and myeloperoxidase, to the cell surface [30].

Manipulating neutrophil life span and/or promoting non-phlogistic removal

Neutrophils normally live for less than 24 h within the peripheral circulation. Ageing neutrophils undergo apoptosis and are removed in a non-phlogistic manner by phagocytic cells, including macrophages. Neutrophil life span can be attenuated or prolonged by various cytokines and growth factors. At inflammatory sites, the process of transendothelial cell migration is associated with prolonged neutrophil survival [48, 49]. Conversely, in ANCA-associated vasculitis, the antibodies induce an accelerated pathway of apoptosis which is dependent on reactive oxygen species; further, the uncoupling of the nuclear and membrane components of apoptosis means that the cells are less readily recognized by phagocytic cells, mitigating against their safe disposal and increasingly the likelihood that the neutrophils will undergo pro-inflammatory secondary necrosis [50].

Encouraging the safe disposal of neutrophils is paramount in acute inflammation. Some compounds appear to be able to promote this, including lipoxins [51], annexin 1 [52] and prostaglandin D2 [53]. Arachidonic acid metabolism can lead to formation of leucotrienes or lipoxins by 5-lipoxygenase or 15-lipoxygenase, respectively, these eicosanoids are pro- and anti-inflammatory, respectively. Recently it was demonstrated that lipoxin B4 and 15-epi-lipoxin B4 (a lipoxin whose formation is induced by aspirin inhibition of cyclooxygenase 2), can stimulate the non-phlogistic removal of apoptotic neutrophils by macrophages. Both lipoxins B4 and 15-epi-B4, can induce increased uptake of apoptotic neutrophils by macrophages. During this process the anti-inflammatory cytokine TGF-β is released [54].

Summary

Neutrophils are important effector cells during inflammatory responses. Further, their many functions, controlled by cell surface receptors and intracellular signalling pathways, provide multiple opportunities for modulating unwanted responses. Potential mediators and drugs to achieve these ends are under development and will likely provide new insights into neutrophil biology.

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