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REVIEW

Priming and de-priming of neutrophil responses in vitro and in vivo

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Abstract

The activation status of neutrophils can cycle from basal through primed to fully activated ("green-amber-red"), and at least in vitro, primed cells can spontaneously revert to a near basal phenotype. This broad range of neutrophil responsiveness confers extensive functional flexibility, allowing neutrophils to respond rapidly and appropriately to varied and evolving threats throughout the body. Primed and activated cells display dramatically enhanced bactericidal capacity (including augmented respiratory burst activity, degranulation and longevity), but this enhancement also confers the capacity for significant unintended tissue injury. Neutrophil priming and its consequences have been associated with adverse outcomes in a range of disease states, hence understanding the signalling processes that regulate the transition between basal and primed states (and back again) may offer new opportunities for therapeutic intervention in pathological settings. A wide array of host- and pathogen-derived molecules is able to modulate the functional status of these versatile cells. Reflecting this extensive repertoire of potential mediators, priming can be established by a range of signalling pathways (including mitogen-activated protein kinases, phosphoinositide 3-kinases, phospholipase D and calcium transients) and intracellular processes (including endocytosis, vesicle trafficking and the engagement of adhesion molecules). The signalling pathways engaged, and the exact cellular phenotype that results, vary according to the priming agent(s) to which the neutrophil is exposed and the precise environmental context. Herein we describe the signals that establish priming (in particular for enhanced respiratory burst, degranulation and prolonged lifespan) and describe the recently recognised process of de-priming, correlating in vitro observations with in vivo significance.

KEYWORDS
de-priming, degranulation, neutrophils, priming, respiratory burst, signaling

1 | INTRODUCTION

Circulating neutrophils are quiescent, and in health the vast majority undergo final disposal without encountering an activating signal. However, as first responders to infection or injury, neutrophils need the functional versatility to enable appropriate responses in the face of varied and evolving threats (Figure 1). Unprimed neutrophils exposed to activating agents such as fMLF, LTB4 or C5a secrete little or no granule contents or reactive oxygen species (ROS). However, neutrophils that have been exposed to a range of agonists enter a state of high alert, with the capacity to respond aggressively (eg, through degranulation, respiratory burst activity and increased lifespan) if a further activating stimulus is encountered. This pre-activation or primed state was first recognised in vitro,[1] and shortly afterwards shown to occur in vivo in the setting of systemic infection.[2] A wide variety of priming agents (chemokines, cytokines, alarmins, integrins, pathogen-derived molecules and mechanical forces) have been identified, and primed neutrophils circulate in vivo in many inflammatory diseases (eg, rheumatoid arthritis[3] and cystic fibrosis[4]) and pathological states (eg, trauma or cardiopulmonary bypass[5,6]). In keeping with the requirement for dynamic regulation of neutrophil activation (to ensure that maximal activation occurs only when and where required), primed cells also have the flexibility to spontaneously "de-prime" or revert back to the unprimed quiescent state[7]; notably the retention of primed cells in vascular beds may even facilitate this de-escalation (Ref[8]; Figure 1). Increased levels of circulating primed neutrophils correlate with adverse outcomes,[6,9] while defective neutrophil priming has been associated with recurrent infections.[10] This delicate balance between augmented microbicidal function and increased risk of tissue injury is reflected by the complex signalling processes that regulate neutrophil priming. Herein we describe the signals that establish priming and describe how neutrophils can de-prime, correlating in vitro observations with in vivo significance.

2 | DIVERSITY OF NEUTROPHIL PRIMING IN VITRO AND IN VIVO

Primed neutrophils that are subsequently activated display enhanced phagocytosis, ROS generation, degranulation, release of bioactive mediators and NETosis.[11] Numerous primer/activator combinations induce these changes, but the precise nature and duration of the primed phenotype varies; for example priming induced by PAF is transient, while growth factors such as GM-CSF establish a more durable primed state. Multiple molecular pathways including mitogen-activated protein kinases (p38 and ERK1/2) and phosphoinositide 3-kinases (PI3Ks) are required for priming to occur; however, subtle differences between priming agents are apparent even if convergence to common effector

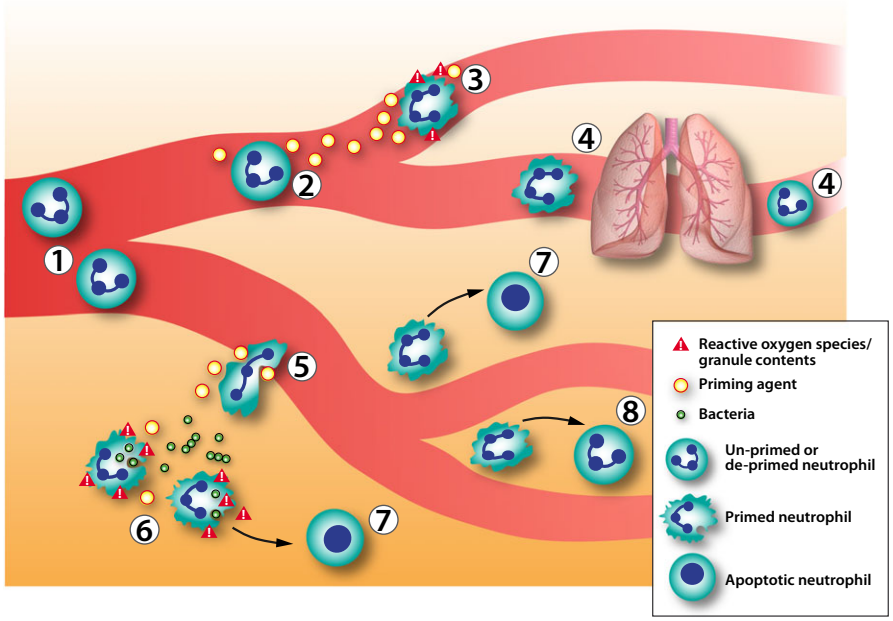

FIGURE 1 Neutrophil priming and de-priming in vivo: potential sites and consequences. Circulating quiescent neutrophils (1) may encounter intravascular priming agents (2) with the potential for local damage (3); if sequestered in the pulmonary capillary bed neutrophils have the potential to gradually de-prime and be released back in to the systemic circulation (4). Circulating neutrophils may become primed on egressing the circulation both by engagement of adhesion receptors and by encountering endothelial-bound or tissue-associated priming agents (5). Primed neutrophils ingest and kill bacteria, with the risk of collateral tissue damage (6). Primed neutrophils are also "primed to die" in response to cues at the inflammatory site (7) or may de-prime spontaneously (8)

mechanisms occurs. For example, GM-CSF and TNF both promote p47^{phox} phosphorylation to prime the respiratory burst, but rely on ERK and p38 respectively[12]; RNAseq confirms that they activate both cytokine-specific and shared pathways.[13] Identically primed neutrophils exposed to different activating agents also initiate diverse signals; TNF-primed neutrophils treated with either the bacterial tri-peptide fMLF or with bacteria display enhanced ROS production; both employ p38 signalling, but only fMLF also entrains ERK.[14] Other activating agents such as C5a and LTB4 may also elicit different responses, but as most studies have used fMLF our review focuses on this agonist.

Multiple priming influences co-exist in disease states in vivo. Different proportions of primed cells are seen to circulate in individual patients,[15] perhaps related to differences in priming signal intensity, neutrophil maturation or the clearance of primed cells.[16] Likewise, cells isolated from pathological sites may display some, but not all of the hallmarks of priming.[17] Because of this in vivo complexity, our understanding of priming and the signalling processes that underlie it comes mostly from in vitro studies with isolated human neutrophils exposed to single primer/activator combinations (eg, TNF/fMLF; Figure 2). However, neutrophil isolation itself may initiate partial priming and isolated cells are not subject to the modifying effects of other blood constituents on this process.[18] Despite their limitations, in vitro approaches have provided valuable insights, with the potential to progress to settings recapitulating the complex in vivo environment, for example co-culture systems and animal models.

3 | SIGNALLING PRIMING RESPONSES IN VIVO AND IN VITRO

3.1 | Reactive oxygen species production

Generation of phagosomal ROS by the multi-component NADPH oxidase is vital for pathogen killing, whereas the secretion of extracellular ROS (which may be augmented dramatically by priming, Figure 3) can cause major tissue injury both in vitro and in vivo.[19,20] Multiple molecular pathways and cellular events must be activated for priming of the respiratory burst to occur, with the timing and contribution of each step being context dependent.

3.1.1 | Oxidase assembly at the cell membrane

The production of ROS is mediated by the flavocytochrome b_{558} (cytb$_{558}$) oxidase, which is partly assem-

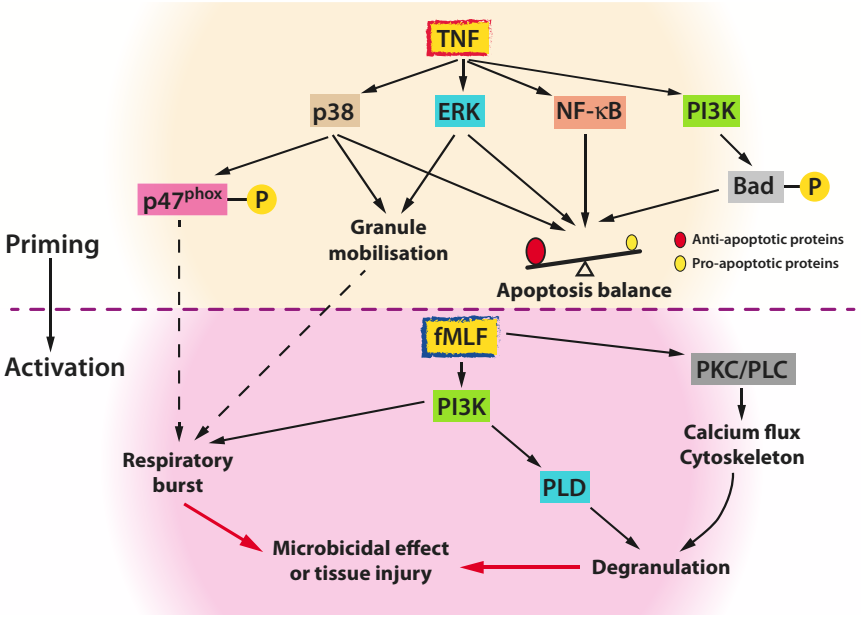

FIGURE 2 Signalling events underlying TNF priming of fMLF-mediated neutrophil activation. Priming with TNF activates multiple signalling pathways, including p38 and ERK1/2, PI3K and NF-κB, with both linear and overlapping downstream consequences. TNF-mediated phosphorylation of p47^{phox} is largely dependent on p38, while both ERK1/2 and p38 mobilise secretory vesicles to the cell surface. Both of these actions promote and enhanced the respiratory burst on subsequent exposure to an activating agent such as fMLF. Simultaneously, multiple pathways influence the balance of pro- and anti-apoptotic proteins such as Bad and Mcl1. On exposure to fMLF, enhanced PI3K activation acts on the primed cell to enhance assembly and activation of the multi-component NADPH oxidase. PI3K-stimulated PLD activation, together with calcium transients and changes in the cytoskeletal architecture, promote enhanced degranulation

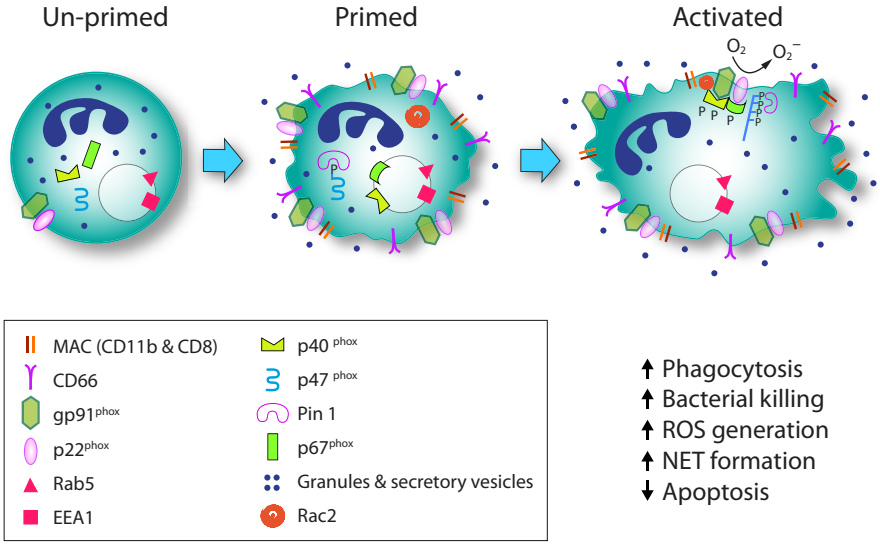

FIGURE 3 Cellular and molecular events involved in neutrophil priming. Circulating or unstimulated neutrophils are largely spherical and express a limited number of receptors on their surface; $p47^{phox}$, a cytosolic component of the NADPH oxidase, is present in the cytosol in a nonphosphorylated state. Priming induces neutrophil shape change with polarisation, and secretory vesicle exocytosis increases the expression of NADPH oxidase subunits $gp91^{phox}$ and $p22^{phox}$, as well as other molecules such as integrins and FPR1. $p47^{phox}$ becomes partially phosphorylated, and following interaction with Pin1, undergoes a conformational change facilitating interactions with membrane phospholipids and other oxidase components. NADPH oxidase components such as $p67^{phox}$ and $p40^{phox}$ are recruited to Rab5/EEA1 positive endosomes in preparation for delivery to the plasma membrane. Subsequent activation of the primed neutrophil enables a fully functional NADPH oxidase complex to become assembled and activated, associated with enhanced granule protein exocytosis

bled at membranes following specific phosphorylation and cellular trafficking events upon priming. Components of the cytb558 oxidase $gp91^{phox}$ and $p22^{phox}$ are predominantly sited on granule membranes in resting cells, while $p47^{phox}$, $p67^{phox}$, $p40^{phox}$ and Rac2 are cytosolic. Priming mobilises $cytb_{558}$-containing granules and vesicles to the plasma membrane[14]; in LPS priming this was shown to increase expression of other relevant proteins such as fMLF receptors (FPR1) and CD11b,[21] although this may not occur with all priming agents. The precise nature of the trafficking processes and their signalling controls are still debated and likely depend on the priming agent used. For example, LPS priming requires cytoskeletal changes and NADPH oxidase pre-assembly at Rab-5 positive endosomes[22]; subtly different findings were reported for PAF, which causes $p40^{phox}$ and $p67^{phox}$, but not $p47^{phox}$ or $gp91^{phox}$ to re-locate to Rab5-positive endosomes (Ref[23]; Figure 3). However, the fundamental importance of granule trafficking to the priming process is shown by experiments blocking exocytosis either directly or indirectly (by the inhibition of clathrin-mediated endocytosis), which prevents priming of the respiratory burst by either TNF or PAF.[24,25] The importance of the subtle differences between priming agents and the precise in vivo dependence of priming on endocytosis/exocytosis remain to be elucidated.

3.1.2 | Phosphorylation of oxidase components

In resting cells, $p47^{phox}$ is nonphosphorylated; on cellular activation it becomes highly phosphorylated (Figure 3) and undergoes conformational changes, facilitated by the proline isomerase Pin1 (reviewed in ref[26]), unmasking multiple binding domains for interaction with other oxidase components and membrane phospholipids. Several priming agents induce $p47^{phox}$ phosphorylation at residue S345 via the JAK/STAT, p38 or ERK1/2 signalling cascades,[12,27-28] with the precise pathway depending on the priming agent. Subsequent exposure to activators leads to enhanced phosphorylation at further sites.[11]

The in vivo relevance of these events is demonstrated by neutrophils from patients with myeloproliferative disease due to a gain-of-function JAK2 mutation (V617F); such neutrophils exhibit constitutive S345 $p47^{phox}$ phosphorylation and are basally primed.[29] A similar phenotype has been observed in neutrophils from inflamed rheumatoid joints, where a competitive inhibitory S345-based peptide abolishes ROS production by these inflammatory cells, suggesting that this phosphorylation is critical for primed NADPH oxidase activity in vivo.[12]

NF-κB signalling also contributes to $p47^{phox}$ phosphorylation in LPS-primed mouse neutrophils.[30] Decreased

neutrophil p47^{phox} phosphorylation induced by LPS has been reported patients carrying mutations in NF-κB-modulating proteins.[31] This resulted in reduced (NEMO-deficient cells) or absent (IRAK4-deficiency) LPS priming of the respiratory burst.[31] Phosphorylation events involving other oxidase components are important for NADPH oxidase activation,[26] but their relevance to priming is yet to be established.

3.1.3 | Interactions with membrane phospholipids

We have previously shown that PI3K-dependent PtdIns (3,4,5)P_3 (PIP_3) generation correlates with ROS release in vitro, and TNF-primed fMLF-stimulated human neutrophils display biphasic PI3K activation, with the second (PI3Kδ isoform-dependent) phase governing primed ROS generation.[32] The mechanism(s) by which PI3K regulates the primed respiratory burst appear context dependent, but interactions with PI3K-generated 3-phosphorylated lipids promote membrane translocation of oxidase components (reviewed in ref[33]). Additionally, PI3K activates the Rac-GEF P-Rex1 and hence Rac, a critical NADPH oxidase-activating small GTPase; mouse neutrophils lacking P-Rex1 exhibit a defect in LPS-ROS formation.[34] Interestingly, P-Rex1 has been identified as a potential anti-inflammatory target in a mouse model of pulmonary fibrosis,[35] although whether and how this relates to its impact on neutrophil priming is unclear.

3.2 | Priming of neutrophil degranulation

Neutrophil granules and secretory vesicles comprise a reservoir of antimicrobial products, NADPH oxidase components and receptors. Primary (azurophilic) granules incorporate histotoxic contents such as myeloperoxidase (MPO) and neutrophil elastase (NE). Secondary (specific) granules and tertiary (gelatinase) granules include adhesion molecules such as CD11b and FPR1. Secretory vesicles contain FPR1, CD11b and cytochrome b_{558}. Priming alone leads to exocytosis of secretory and gelatinase vesicles, with subsequent increased release of specific and azurophilic granule contents on exposure to activating agents.[36-37] Several signalling pathways regulating primed ROS production also influence degranulation. Cadwallader and colleagues[38] found that TNF-induced PI3K-dependent phospholipase D (PLD) activation is required for enhanced MPO and ROS secretion, and PLD activation likewise signals GM-CSF priming of NE release.[39] Potera et al[37] noted involvement of p38 and ERK1/2 in TNF-augmented granule release, but ROS suppressed the magnitude of this response, suggesting a negative feedback role. Consistent with this, our group has shown that hypoxia sufficient to suppress NADPH oxidase function enhances primed (GM-CSF or PAF) neutrophil degranulation in a PI3Ky-dependent fashion.[40]

Primed granule exocytosis is tightly regulated and hierarchical; in an in vivo skin blister model, complete mobilisation of secretory vesicles was accompanied by discharge of 38% of tertiary, 22% of secondary and just 7% of primary granule proteins.[41] A similar rank order of degranulation was observed in response to calcium ionophore, and calcium chelation abolishes secretion of all but secretory vesicles.[42] Many priming agents induce calcium transients and enhance activation-induced calcium flux,[39] influencing dynamic changes in the actin cytoskeleton required for granules mobilisation to the plasma membrane.[43] Phosphoproteome analysis of primed neutrophils[36] supports the importance of cytoskeletal reorganisation in priming for granule protein exocytosis. How these and other priming-relevant pathways might be manipulated in vivo is an important question. Of note, enhanced NE release associated with priming and tissue injury has been detected in several diseases,[5,44] and inhibition of neutrophil exocytosis was protective in rodent models of lung injury[45-47] demonstrating the in vivo relevance of this process and its potential value as a therapeutic target.

3.3 | Delayed apoptosis

Most priming agents also delay the ability of neutrophils to undergo constitutive apoptosis, extending the time frame over which these cells may combat infection or cause bystander injury. We and others have shown the effect of TNF to be biphasic, with an early increase in cell death followed by extended survival; TNF and other pro-survival priming agents such as GM-CSF induce changes in the expression, stability and function of an array of both pro- and anti-apoptotic proteins,[48] with the balance between these pathways determining neutrophil lifespan (Figure 2). Thus, even when priming promotes survival, neutrophils encountering subsequent stimuli at the inflammatory site are also primed to die (eg, through enhanced expression of Bim[48]).

Priming agents such as GM-CSF delay apoptosis predominantly by increasing the stability of the anti-apoptotic protein Mcl-1, which normally has a very fast intracellular turnover time. Mcl-1 levels are down-regulated by proteasomal degradation and caspase activation but preserved by sustained activation of the PI3K effector Akt.[49] Neutrophil Mcl-1 levels have been reported to be higher in neutrophils isolated from patients with COPD[50] and sepsis.[51] In an animal model, attenuating Mcl-1 down-regulation inhibited neutrophil apoptosis and delayed the resolution of endotoxin-mediated lung inflammation,[52] highlighting the potential for manipulating these pathways in vivo. PI3K-dependent signalling also plays a role in the pro-survival

effects of GM-CSF and TNF, in part via Akt-mediated phosphorylation of the pro-apoptotic Bcl-2 family member Bad.[53] Neutrophils treated with PAF are dependent on both ERK1/2 and PI3K survival signals, with ERK- and PI3K-dependent Bad phosphorylation on residues S112 and S136, respectively.[54]

Understanding and elucidating these varied signalling pathways in the context of disease is important, as modulation of neutrophil activation status and apoptosis represents an emerging therapeutic strategy in chronic "granulocyte-dominated" inflammatory disease.[55]

4 | THE INTERPLAY OF PRIMING AND ADHESION

Most in vitro experiments are performed on neutrophils in suspension culture, stimulated with soluble agonists; while this may be relevant to circulating neutrophils exposed to priming agents in the bloodstream (Figure 1), adherent or postmigratory neutrophils display significantly altered functional properties. Priming agents impact greatly on the biophysical and adhesive properties of neutrophils and likewise the process of adhesion modifies neutrophil responses in a manner very akin to priming.

Priming up-regulates the surface expression of neutrophil adhesion receptors such as CD11b (Figure 3) by mobilisation of secretory vesicles, both in vitro[56] and in vivo[57] and further increases the affinity (or avidity) of such integrins via "inside-out" signalling. Engagement of integrins during transmigration stimulates "outside-in" signalling, which together with exposure to endothelial-bound mediators such as PAF ensures that neutrophils are primed as they egress (Ref[58]; Figure 1). The engagement of such receptors can alter neutrophil responsiveness dramatically; for example cells adherent to fibronectin release substantial ROS on exposure to TNF (normally regarded as a priming agent rather than an activating stimulus)[59] and display sequential PI3K and δ-PKC activation, with δ-PKC-dependent phosphorylation of $p47^{phox}$.[60] Of note, these effects are agonist specific, as treatment of adherent cells with fMLF resulted in δ-PKC-independent ROS generation.[61] Reminiscent of the biphasic PI3K activation seen in neutrophils primed in suspension,[32] stimulation of adherent mouse neutrophils induced a biphasic NADPH oxidase response, the initial phase being signalled by PI3Kγ, with a role for PI3Kα/δ in the subsequent prolonged phase; src-family kinases were critical for both phases in this setting.[61]

Neutrophil L-selectin (CD62L) mediates initial rolling interactions with the endothelium, and its rapid, often near-total shedding provides a sensitive marker of neutrophil priming both in vitro[56] and in vitro.[57] Circulating neutrophils in mice expressing an L-selectin point mutation (N138G), which modifies force-catch bonds induced by mechano-stimulation, form unstable aggregates and are primed in vivo[62]; this priming status is associated with enhanced bacterial clearance, but increases inflammatory injury and enlarged venous thrombi, again emphasising the precarious tightrope between host defence and tissue injury. These primed neutrophils do not adhere via β2-integrin differently from control neutrophils, suggesting an alternative form of priming by mechanotransduction.[63]

5 | DE-PRIMING

It was initially assumed that neutrophil priming was an irreversible process. However, proof of concept experiments with PAF demonstrated the potential for near-complete recovery back to a quiescent or de-primed state. With PAF this was shown to be a spontaneous event, with a re-setting of fMLF-triggered NADPH oxidase response and near-complete recovery from a polarised to a rounded morphology.[7] Our group has recently shown the potential for this process to occur in vivo in humans, with immediate (first pass) trapping of injected exogenously (GM-CSF)-primed neutrophils within the very narrow pulmonary capillaries, followed by their subsequent gradual release back into the systemic circulation, a finding that we propose to reflect in situ de-priming.[8] Furthermore, the ability of repeated mechanical deformation of neutrophils (achieved by optical lasers or by passage through a series of multiple 5-μm micro-channels) highlights the mechano-sensitive nature of neutrophils and the potential for active and rapid de-priming of these cells.[63] This recovery option for primed neutrophils fits with mathematical modelling of the number of circulating primed neutrophils found in disease settings[64] and may play a role in the now well-recognised capacity of murine neutrophils to return from sites of inflammation back to the systemic circulation (retro-transmigration)[65] or to the bone marrow and lymph node.[66] Mechanistically, however, very little is known about the effects of de-priming on cell signalling events or the restoration of neutrophil cell surface receptors and molecules involved in adhesion and other immune functions. Likewise, whether the de-primed neutrophil is entirely indistinguishable from the unprimed neutrophil and the true physiological role and fate of these cells also needs to be determined.

In summary, priming appears to be one of the key processes designed to regulate neutrophil function, with the capacity to either restrain (unprimed/basal state) or greatly enhance (primed state) their repertoire of functional responses: this process therefore acts as a "volume switch" for the functional reactivity of these cells to any activating agonist they subsequently encounter. As part of the priming process, this causes a dramatic change in cell contour and

deformability, and re-programmes the susceptibility of these cells to undergo apoptotic cell death. Understanding the signalling processes involved in priming and de-priming should allow therapeutic opportunity to limit granulocyte-mediated tissue damage.

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