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# Hypoxic regulation of neutrophil function and consequences for *Staphylococcus aureus* infection

Katharine M. Lodge<sup>a</sup>, A.A. Roger Thompson<sup>b</sup>, Edwin R. Chilvers<sup>a,\*</sup>, Alison M. Condliffe<sup>b</sup>

<sup>a</sup> Department of Medicine, University of Cambridge School of Clinical Medicine, Cambridge University Hospitals, Hills Road, Cambridge, CB2 0QQ, UK

<sup>b</sup> Department of Infection, Immunity and Cardiovascular Disease, University of Sheffield Medical School, Beech Hill Road, Sheffield, S10 2RX, UK

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## Abstract

Staphylococcal infection and neutrophilic inflammation can act in concert to establish a profoundly hypoxic environment. In this review we summarise how neutrophils and *Staphylococcus aureus* are adapted to function under hypoxic conditions, with a particular focus on the impaired ability of hypoxic neutrophils to effect *Staphylococcus aureus* killing.

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## 1. Principal effector functions of neutrophils

Neutrophils are the major cellular arm of the innate immune system and the first line of defence against invading micro-organisms. They recognise and eliminate pathogens rapidly and effectively by a range of cytotoxic mechanisms, and also modulate the wider host response, recruiting other immune cells and amplifying inflammatory cascades. Neutrophils comprise 50%–70% of circulating leukocytes but have a short circulating half-life, necessitating a bone marrow generation rate of  $10^{11}$  per day, increasing to up to  $10^{12}$  per day during bacterial infections [1]. Neutrophil homeostasis is maintained through a delicate balance of granulopoiesis, bone marrow release, margination in intravascular pools, tissue recruitment, and cell death and destruction [2].

In health, circulating neutrophils are quiescent but, in disease states, exposure to priming agents, such as platelet activating factor, granulocyte-macrophage colony-stimulating factor (GM-CSF) or bacterial lipopolysaccharide (LPS),

renders them more responsive to recruitment and activation signals. Priming also augments pathogen entrapment and killing mechanisms, including chemotaxis, phagocytosis, granule exocytosis, production of reactive oxygen species (ROS) and release of neutrophil extracellular traps (NETs). Primed neutrophils in the systemic circulation have been identified in disease states, such as bacterial sepsis, and in addition to their augmented bactericidal capacity they may contribute to disease pathogenesis [3].

Extravasated neutrophils migrate towards sites of inflammation and infection down chemoattractant concentration gradients, a process termed chemotaxis. Chemoattractant control of neutrophil migration is complex, not least because the effect on chemotaxis may depend on agonist concentration and context, but also the in vivo milieu is a dynamic environment comprising multiple chemokine signals. There is intracellular signalling hierarchy, with end-target chemoattractants signalling predominantly through p38 MAPK [4].

Neutrophils are avid phagocytes, which recognise and rapidly ingest bacteria. Target particles are engulfed into the phagosome, a plasma membrane-derived vacuole formed by extension of neutrophil pseudopods. Phagocytic receptor ligation initiates phosphorylation cascades, enabling pseudopod extension by means of dynamic changes in the actin

\* Corresponding author. Department of Medicine, University of Cambridge School of Clinical Medicine, Box 157 Addenbrooke's Hospital, Hills Road, Cambridge, CB2 0QQ, UK.

E-mail address: [erc24@cam.ac.uk](mailto:erc24@cam.ac.uk) (E.R. Chilvers).

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cytoskeleton (reviewed in Ref. [5]). As phagosomes mature, they acquire microbicidal activity by fusion of additional components, including cytosolic granules which contain abundant proteases and antimicrobial peptides.

Although they comprise a spectrum, granules are classified by their protein content: azurophilic granules are rich in myeloperoxidase (MPO), defensins and serine proteases including neutrophil elastase (NE), cathepsin G and proteinase 3; specific granules contain abundant lactoferrin and matrix metalloproteinase-8 (MMP-8); whilst the exemplar protein of gelatinase granules is MMP-9. Specific and gelatinase granules also contain p22<sup>phox</sup> and gp91<sup>phox</sup>, the membrane subunits of NADPH oxidase, which enable ROS production [6]. As well as having antimicrobial effects, external release of proteases can degrade the extracellular matrix, enabling neutrophil transit through host tissues but also contributing to tissue injury.

Generation of ROS through activation of the NADPH oxidase electron transport chain plays a critical role in the killing of several bacterial and fungal pathogens. NADPH oxidase is an electron donor, which reduces molecular di-oxygen to form superoxide anion, yielding an array of antimicrobial ROS. The dramatic increase in oxygen consumption associated with ROS production is termed the respiratory burst. Patients with chronic granulomatous disease, a rare genetic disorder caused by a defective NADPH oxidase complex, are unable to mount an effective respiratory burst and consequently suffer severe recurrent infections with fungi, such as *Aspergillus*, and several species of bacteria, including *Staphylococcus aureus* [7]. There is a complex interplay between ROS, granule-derived proteases and proteins, and pH in the phagosome, and the contribution of each component to pathogen killing varies between organisms.

NETs are expulsions of decondensed chromatin, beaded with antimicrobial proteins and proteases, into the extracellular space; NET formation is stimulated by pro-inflammatory mediators, including tumour necrosis factor- $\alpha$  (TNF- $\alpha$ ) and pathogen-associated molecular patterns [8]. NETs adhere to various pathogens in vivo, and may facilitate killing of organisms that are too large to be ingested [9], but it has been postulated that this attachment may be utilised by certain organisms to form biofilms, and may induce direct tissue damage [10]. There is also conflicting evidence for a direct microbicidal effect of NETs [8,11].

Neutrophils undergo constitutive apoptosis, resulting in short survival times; however, apoptosis can be delayed at sites of inflammation by both signals from the host, e.g. GM-CSF, and bacteria, e.g. LPS [12]. In order to limit host tissue damage from dying cells, efferocytosis safely disposes of potentially histotoxic neutrophil contents and also inhibits macrophage pro-inflammatory cytokine production, hastening the resolution of inflammation (reviewed in Ref. [13]).

Apoptosis can be initiated through the extrinsic pathway (ligation of cell surface death receptors such as FAS), or through the mitochondrial-driven intrinsic pathway. Electron microscopy studies identify comparatively few mitochondria in neutrophils, but fluorescent dyes have revealed a complex

mitochondrial network which controls cell fate by releasing pro-apoptotic proteins, such as cytochrome *c*, into the cytosol [14]. Bioenergetic profiles and inhibitor studies have demonstrated that neutrophils rely almost entirely on glycolytic respiration for energy production, rather than oxidative phosphorylation, and that the respiratory burst is independent of mitochondrial respiration [15]. Hence, these organelles in neutrophils contribute very minimally to ROS production and molecular oxygen consumption, with the predominant function being regulation of cell death.

Neutrophils can also influence other immune cell populations. They can release both pro- and anti-inflammatory cytokines in an agonist-dependent manner [16], secrete products such as defensins and cathelicidins which induce CD4<sup>+</sup> and CD8<sup>+</sup> T cell chemotaxis [17], and acquire certain properties of antigen presenting cells [18]. Although these attributes confer a more complex, flexible and environment-specific role than previously appreciated, the key neutrophil function remains host defence against invading pathogens, and when this function is significantly compromised, severe infection is more likely.

## 2. Relevance of hypoxia to neutrophils

Neutrophils are generated within the bone marrow, a significantly hypoxic microenvironment even under healthy physiological conditions, with murine in vivo measurements of local oxygen tension recorded as low as 1.3 kPa [19]. Indeed, within the bone marrow structure, haematopoietic stem cells are found sequestered in regions staining most strongly for the hypoxia probe pimonidazole, a 2-nitroimidazole compound, which forms covalent bonds with cellular macromolecules at oxygen levels below 1.3 kPa. Taken together with the evidence that low oxygen tensions favour the maintenance of haematopoietic stem cells in culture [20], hypoxia appears to play a critical role in neutrophil development.

Once released from the bone marrow, mature circulating neutrophils are exposed to a wide range of oxygen tensions, transiting rapidly from a pO<sub>2</sub> of 13 kPa in main systemic arteries, to 7 kPa in arterioles and 3–4 kPa in capillaries and venules. Given the oxygen diffusion limit from capillaries of 80–140  $\mu$ m, the oxygen tension in normal tissues is often even lower, generating so called “physiological hypoxia”. Along with the bone marrow, physiological hypoxia has been demonstrated in tissues such as healthy muscle and connective tissue [21], colonic epithelium [22] and, intriguingly, even in the skin [23], despite being in such close proximity to air. This relative lack of molecular oxygen can be further amplified in pathological conditions, such as organ inflammation or ischaemia, and within solid tumours, due to damaged vasculature, compartmentalisation of infection, and high metabolic activity and oxygen requirements of pathogens and host cells. Hypoxia has been demonstrated in numerous pathological environments through in vitro and in vivo sampling: by microelectrode pO<sub>2</sub> measurement of wounds and venous ulcers [24]; by blood gas analysis of abscesses, a characteristic

feature of staphylococcal infection [25]; by staining for hypoxia inducible factor (HIF), which increases exponentially below 6% oxygen, in chronic obstructive pulmonary disease [26]; by pimonidazole staining in pulmonary infection [27]; and by luminescence-based in vivo optical imaging in skin infection [28]. Interestingly, in a murine model of acute colitis, neutrophils actively contributed to the hypoxic microenvironment by depletion of molecular oxygen through NADPH oxidase activity and, hence, induced stabilisation of epithelial HIF [29]. Furthermore, *Staphylococcus aureus* was shown to deplete oxygen in a skin infection model, and biofilm-induced oxygen demand made the underlying dermal tissue anoxic [30]. Hypoxia impedes wound healing, and has been shown to impair clearance of inhaled *S. aureus*, though not *Proteus mirabilis*, in a mouse model of lung infection [31]. Moreover, supra-physiological levels of oxygen can promote resolution of certain infections [32].

### 3. Oxygen sensing by neutrophils

As neutrophils are the frontline cells involved in host defence in most hypoxic environments, it is vital that these cells have the capacity to function effectively at low oxygen levels. Neutrophil adaptation to hypoxia is critically dependent upon the HIF/PHD pathway (Fig. 1). HIF is a heterodimeric protein comprising  $\alpha$  and  $\beta$  subunits, with degradation of HIF- $\alpha$  subunits mediated by prolyl hydroxylase domain-containing enzymes (PHDs) [33]. These oxygen-sensitive hydroxylase enzymes are inhibited under hypoxia and, thus, hypoxia facilitates the accumulation of HIF- $\alpha$  subunits, which dimerise with HIF- $\beta$  (ARNT) and bind to hypoxia response elements on

target genes. Further regulation of HIF transcriptional activity is provided by factor inhibiting HIF (FIH), which hydroxylates an asparaginyl residue, preventing interaction with HIF co-activators and further repressing HIF-mediated transcription [34] (Fig. 1).

Of the 3 known HIF- $\alpha$  subunits, neutrophils express both HIF-1 $\alpha$  and HIF-2 $\alpha$  mRNA, and both proteins are stabilized in hypoxia and when these cells are exposed to inflammatory stimuli, such as LPS and streptococci [35]. HIF-1 $\alpha$  and HIF-2 $\alpha$  regulate distinct but overlapping target gene sets, with HIF-1 $\alpha$  having a greater impact upon immediate metabolic targets. In hypoxic culture, neutrophils upregulate glycolytic HIF-1 $\alpha$  target genes, such as glyceraldehyde 3-phosphate dehydrogenase and phosphoglycerate kinase [36]. Indeed, neutrophils deficient in HIF-1 $\alpha$  have reduced ATP levels even under normoxic conditions, reflecting the importance of HIF-1 $\alpha$  in the regulation of neutrophil energetics [37]. It is therefore not surprising that HIF-1 $\alpha$  is essential for extended neutrophil survival in hypoxia [36]. Notably, deficiency of HIF-2 $\alpha$  impairs neither neutrophil survival in hypoxia nor key neutrophil functions, suggesting that HIF-2 $\alpha$  plays a less prominent role in the control of hypoxic neutrophil responses. Nonetheless, HIF-2 $\alpha$  does appear to regulate neutrophil lifespan in the context of inflammation; lower expression of the antioxidant enzyme catalase in HIF-2 $\alpha$  deficient neutrophils compared to wildtype cells was associated with increased apoptosis in response to nitrosative stress, and enhanced inflammation resolution in a model of acute lung injury [35].

Neutrophils express 3 isoforms of PHD enzymes (PHDs1–3). Hypoxia significantly upregulates PHD3 in a HIF-1 $\alpha$  dependent manner. Loss of PHD3 does not alter induction of

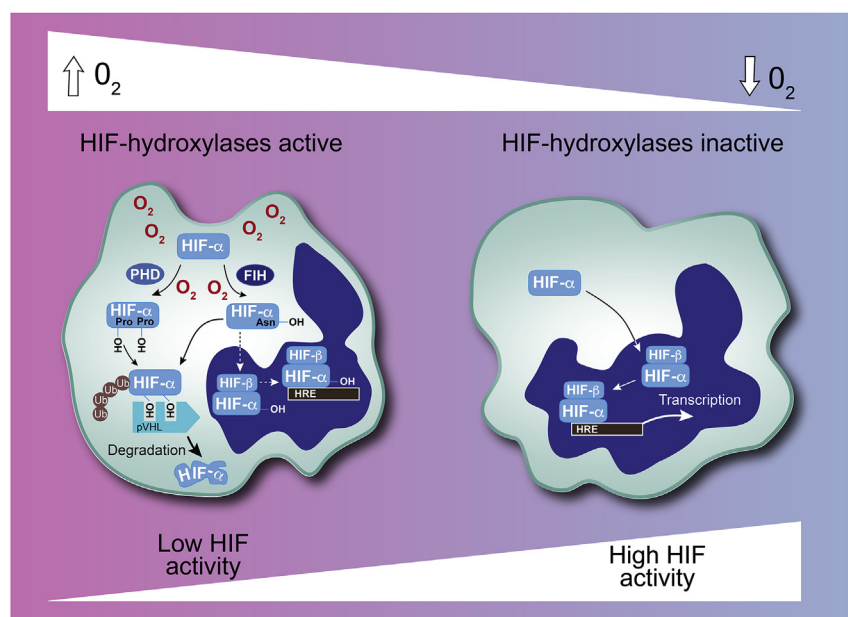


Fig. 1. In normoxic environments, prolyl hydroxylase-containing enzymes (PHDs) mark HIF- $\alpha$  for ubiquitination and proteasomal degradation via the von Hippel Lindau (vHL) protein complex. Factor inhibiting HIF (FIH) inactivates HIF- $\alpha$  transcription by asparaginyl hydroxylation, preventing binding of transcription co-factors. PHDs and FIH display an absolute requirement for dioxygen, Fe(II), ascorbate and 2-oxoglutarate. In hypoxic environments, reduced hydroxylase activity due to the lack of molecular oxygen allows stabilisation of HIF- $\alpha$  which translocates to the nucleus and binds HIF $\beta$ . The HIF heterodimer binds hypoxia response elements (HRE), upregulating transcription of hypoxia-responsive genes.

HIF-1 $\alpha$  target genes in hypoxic neutrophils but is necessary for cell survival in hypoxic conditions, suggesting that these pro-survival effects are mediated independent of HIF-1 $\alpha$  in neutrophils [38]. Interestingly, under hypoxia PHD3 deficiency only impacted on neutrophil apoptosis, with other effector functions, such as phagocytosis, chemotaxis and the respiratory burst, being unaffected. Specific roles of the other PHD enzymes in neutrophils still require elucidation but, given the distinct functions for PHD3 observed in macrophages [39], may well be of interest. PHD enzymes belong to a wider family of 2-oxoglutarate-dependent oxygenases including the Jumonji histone demethylases. In hypoxic macrophages, transcription of specific cytokines was suppressed due to increased methylation of promoter region histone H3 residues as a result of demethylase inhibition [40]; these enzymes have not been studied in the neutrophil.

Crosstalk between the HIF pathway and nuclear factor kappa-light-chain enhancer of activated B cells (NF $\kappa$ B) adds a further layer of complexity to oxygen sensing in neutrophils. *HIF1A* mRNA levels are regulated by members of the NF $\kappa$ B pathway via the binding of NF $\kappa$ B to the *HIF1A* promoter. This is likely to be of particular importance in inflammatory microenvironments as it provides a mechanism by which stimuli such as LPS and TNF- $\alpha$  can influence basal transcription and induction of *HIF1A* mRNA in myeloid cells. Hypoxic induction of NF $\kappa$ B is in turn regulated by HIF-1 $\alpha$ , with evidence of reduced induction of the NF $\kappa$ B components IKK $\alpha$ , IKK $\beta$  and p65 in HIF-1 $\alpha$  deficient neutrophils [36].

## 4. Hypoxic effects on neutrophil functions

### 4.1. Apoptosis

Ingestion of *S. aureus* induces neutrophil apoptosis, which then progresses rapidly to necrosis and may contribute to pathogen virulence [41]. Hypoxia modulates the neutrophil apoptotic threshold, delaying constitutive apoptosis in a concentration-dependent and reversible manner via HIF-1 $\alpha$  mediation of NF $\kappa$ B signalling. This hypoxic survival effect can be prevented by NF $\kappa$ B inhibitors, and is diminished in HIF-1 $\alpha$ -deficient murine neutrophils [36]. Further evidence for a pro-survival effect of HIF-1 $\alpha$  is provided by the delayed apoptosis of human neutrophils observed when HIF is stabilised, either by loss-of-function mutations in von Hippel Lindau (vHL) protein (which targets HIF-1 $\alpha$  for degradation) [42], by exposure of healthy volunteers to acute hypoxia [43], or by pharmacologic or genetic manipulation of HIF-1 $\alpha$  in zebrafish [44]. Similarly, intermittent hypoxia delayed apoptosis of TNF- $\alpha$ -treated human neutrophils [45], and neutrophils isolated from patients with obstructive sleep apnoea, which is characterised by intermittent hypoxia/re-oxygenation, exhibited delayed apoptosis and increased expression of the adhesion molecule CD15 [46]. Hypoxia increases  $\beta_2$  integrin protein expression, which is again mediated by HIF-1 [47], and may contribute to the hypoxic survival effect;  $\beta_2$  integrin clustering or activation with endothelial ligands, such as ICAM-1, delays apoptosis through AKT and

MAPK-ERK signalling, although  $\beta_2$  integrin activation in the presence of death-inducing agonists, such as TNF- $\alpha$ , can also accelerate apoptosis.

Two further mediators of hypoxic neutrophil survival have been identified. Firstly, MIP-1 $\beta$  is secreted by hypoxic granulocytes and confers a survival effect when co-incubated with normoxic neutrophils [36]. Secondly, PHD3 is upregulated by hypoxia and prolongs neutrophil survival independent of HIF-1 $\alpha$  by suppression of the pro-apoptotic factor Siva-1. The impact of hypoxia-mediated neutrophil survival is context dependent; PHD3-deficient mice exhibited accelerated neutrophil apoptosis and enhanced resolution of sterile inflammation (ALI and colitis models) [38], but displayed increased mortality (thought to reflect aberrant macrophage function) when challenged with abdominal sepsis [39].

### 4.2. Adhesion, transmigration, chemotaxis and recruitment

Firm adhesion of neutrophils to endothelium prior to transmigration is mediated by the interaction of neutrophil  $\beta_2$  integrins with endothelial ligands, such as ICAM-1. Multiple studies support a hypoxia-mediated increase in  $\beta_2$  integrin expression (e.g. Ref. [47]) with only one report (where re-oxygenation was permitted after neutrophil isolation) finding no difference [44]. Although reports of modulation of ICAM-1 expression by hypoxia are conflicting [48,49], several studies have shown increased neutrophil adhesion to the endothelium under hypoxia (e.g. Ref. [50]). Studies of neutrophil transmigration under hypoxia have also shown an increase. Hypoxia enhanced neutrophil transmigration *in vitro* in a model of intestinal epithelium ischaemia-reperfusion [51] and *in vivo* in rodent models of acute systemic hypoxia when assessed in multiple organs by intravital microscopy [52] or by quantification of MPO [53]. However, the reported effects of hypoxia on chemotaxis are variable. Extravasated neutrophils undergo shape change to a polarised morphology, which is essential for directional movement. McGovern et al. showed no change in IL-8-induced shape change, or chemotaxis towards IL-8, bacterial formylated peptide (fMLF) or LPS in human neutrophils cultured under hypoxia [54], and, likewise, Peyssonnaud et al. found no difference in chemotaxis towards fMLF through an endothelial monolayer between wildtype, HIF-1 $\alpha$  null and vHL-null murine neutrophils [55]. In contrast, Wang and Liu showed enhanced chemotaxis of neutrophils isolated from hypoxic subjects [56], whereas Rotstein et al. showed reduced chemotaxis of human neutrophils towards fMLF and zymosan-activated serum under hypoxia in an agarose gel migration assay [57]. The different findings in these studies are likely due to variation in assay type, particularly the use of true hypoxia versus HIF-1 $\alpha$  manipulation, and the effects of re-oxygenation of cells prior to assays. It is also unclear how well these *in vitro* assays represent the true physiological environment with its multitude of signals, and studies of true neutrophil recruitment under normoxia or hypoxia, a composite outcome of adhesion, transmigration and chemotaxis, have yielded conflicting results. Tissue neutrophil infiltration



in a murine model of chemical irritant-induced cutaneous inflammation was significantly reduced in a myeloid HIF-1 $\alpha$  knockout mouse model at 24 h, whereas vHL deletion promoted infiltration [37]. Likewise, pharmacological HIF-1 $\alpha$  stabilisation enhanced tissue neutrophil recruitment in mice treated with intradermal LPS [58]. Conversely, pharmacological HIF-1 $\alpha$  stabilisation has been shown either to diminish neutrophil recruitment (in murine uropathogenic *Escherichia coli* bladder infection) [59], or to have no impact (in murine *S. aureus* skin infection) [60]. Recruitment of wildtype, HIF-1 $\alpha$ -null or vHL-null neutrophils in a mouse model of group A *Streptococcus* ulcer was comparable up to 24 h [55]. Similarly, in a mouse model of *Pseudomonas aeruginosa* keratitis, HIF-1 $\alpha$  siRNA knockdown did not affect neutrophil recruitment at 24 h, although by day 5 there was increased tissue neutrophil infiltration [61]. The inconsistencies between these studies may be explained by variations in time, species, induction agent and tissue site, or they may reflect the fact that hypoxia does not equate precisely to HIF stabilisation. Overall, there is currently no clear or consistent picture of how hypoxia affects neutrophil recruitment in vivo, and any effects are likely to be highly context-dependent.

#### 4.3. Phagocytosis

On reaching a site of infection, neutrophils must employ their myriad killing mechanisms. Neutrophils obtained from acutely hypoxic volunteers (whole blood or purified cells) showed enhanced phagocytosis of zymosan [62] and *E. coli*, with enhanced expression of opsonic (FC $\gamma$ IIIbR) and complement (C1qRp and C5aR) receptors [56], which are important for pathogen recognition and ingestion. Interaction with matrix proteins further increased FC $\gamma$ R expression in the setting of hypoxia [63], which may reflect the in vivo environment more accurately. Likewise, phagocytosis of zymosan by neutrophils isolated from rabbits after experimental acute ischaemia [64], phagocytosis of *E. coli* by neutrophils isolated from hypoxic pre-conditioned rats [65] and phagocytosis of *S. aureus* by neutrophils isolated from volunteers exposed to intermittent hypoxia [66] were all increased. These in vitro assays were performed under normoxic conditions; however, similar results were obtained using isolated human neutrophils with in vitro assays performed under hypoxia [67]. Further evidence that hypoxia-induced signalling pathways can increase phagocytosis is provided by neutrophils from patients with heterozygous mutations in vHL protein, which display enhanced phagocytosis of *Streptococcus pneumoniae* under normoxia, further augmented by hypoxia [42]. The same group found that neutrophils from healthy volunteers incubated under hypoxia had enhanced CD11b expression but that phagocytosis of *S. pneumoniae* was not increased [54]. However, the majority of studies suggest that neutrophil phagocytosis is increased by hypoxia.

#### 4.4. Reactive oxygen/nitrogen species

Data regarding the effects of oxygen availability on ROS production are conflicting. Studies of neutrophils isolated from

human volunteers or mice exposed to hypoxia have shown an increase in ROS production [44,56,65], although, of note, all cell isolation and in vitro assays were conducted under normoxic conditions. Whilst HIF-1 $\alpha$  manipulation did not affect ROS release [55,60], in vitro assays conducted under hypoxia have consistently shown decreased intracellular and extracellular superoxide anion production, restored by re-oxygenation [54] and further increased under hyperbaric oxygen conditions. Moreover, intermittent hypoxia and its clinical correlate obstructive sleep apnoea appear to prime neutrophils for augmented superoxide anion generation [68]. Together these data suggest that ROS production depends on oxygen availability, and that the decrease seen under hypoxic conditions is likely due to a lack of molecular oxygen, which is not recapitulated by modulation of HIF-1 $\alpha$  signalling. Microbicidal activity of ROS is highly context-dependent and varies with bacterial species; ROS production contributes significantly to *S. aureus* killing whereas *E. coli* killing is predominantly oxidase-independent [54,69]. In addition to ROS, neutrophils produce antimicrobial reactive nitrogen species, a process which does appear to be under HIF-1 $\alpha$  control. In a zebrafish mycobacterial infection model, stabilisation of HIF-1 $\alpha$  increased production of reactive nitrogen species via inducible nitric oxide synthase (iNOS), decreasing mycobacterial burden [70].

#### 4.5. Granule exocytosis

A clear consensus has emerged that hypoxia increases the release of neutrophil antimicrobial peptides and proteases. As such, hypoxia enhanced the release of multiple granule products, including MMP-9, lactoferrin and active NE and MPO from stimulated human neutrophils [71]. HIF-1 $\alpha$  siRNA knockdown reduced protein levels of murine  $\beta$ -defensins and cathelicidin-related antimicrobial peptide (analogous to human antimicrobial peptide LL-37) in a mouse model of pseudomonal infection [61]. Similarly, HIF-1 $\alpha$  null human neutrophils exhibited reduced NE and cathepsin G activity and displayed a marked reduction in active cathelicidin expression, with the opposite true of vHL-deficient neutrophils [55]. Moreover, pharmacological stabilisation of HIF-1 $\alpha$  upregulated the genes encoding LL-37 in human neutrophils [72]. Enhanced degranulation may promote tissue injury and cavity formation; staphylococcal abscesses are associated with a massive influx of neutrophils, and MMPs have been implicated in mycobacterial cavity formation [73]. However, neutrophil proteases might also enhance phagocyte access to sites of infection and, hence, aid extracellular killing.

#### 4.6. Neutrophil extracellular traps

There have been few studies into the effect of hypoxia on NETosis and, to date, the data are variable. Pharmacological stabilisation of HIF-1 $\alpha$  had no observed effect on NET production but increased *S. aureus* killing in vitro, even in the presence of a phagocytosis inhibitor, an effect which was abrogated by the addition of deoxyribonuclease [60],

suggesting that killing was NET-dependent. Consistent with these results, pharmacological and genetic HIF-1 $\alpha$  knockdown decreased NET production and inhibited extracellular bacterial killing [74]. However, in vitro NET formation under true hypoxia has been shown to be diminished [71]. As NETosis is predominantly dependent on NADPH production of ROS [75], and therefore reliant on availability of molecular oxygen, it seems likely that NET production under hypoxia would be reduced in line with the ROS data, though it appears that HIF-1 $\alpha$  signalling also has a role to play. Furthermore, other cells in a hypoxic environment may also impact on neutrophil function; for example, in a mouse hepatic tumour model, neutrophils incubated with media from hypoxic tumour cells showed increased NETosis, and this was associated with increased metastatic disease [76].

In summary, hypoxia appears to inhibit neutrophil apoptosis, enhance degranulation and the release of antimicrobial products, and promote phagocytosis, but reduce ROS and NET production. However, despite increased adhesion of neutrophils to endothelium in hypoxia, the effect on recruitment to sites of infection is less certain, context-dependent, and requires further investigation.

## 5. Hypoxic effects on *S. aureus* and its killing by neutrophils

*S. aureus* is a virulent and versatile pathogen, which causes significant morbidity. Mortality rates following staphylococcal bacteraemia are increasing, and methicillin resistant *S. aureus* (MRSA) bacteraemia has a higher case fatality record than methicillin-sensitive strains [77]. Approximately 30% of the population is colonised with *S. aureus*, and yet in healthy individuals this usually has no pathological significance; however, in some situations there is a major risk of invasive disease, particularly in vulnerable populations such as the elderly or immunocompromised, and those with skin barrier breaches or impaired mucosal immunity. Prosthetic joints, heart valves and other indwelling devices are a particular risk for the development of deep-seated infection and provide a reservoir of staphylococcal infection that is extremely challenging to eliminate. Clinical manifestations of *S. aureus* infection include local tissue destruction and abscess formation, and haematogenous dissemination, resulting in infections such as osteomyelitis, endocarditis and pneumonia.

The importance of neutrophils in host defence against *S. aureus* infection is well illustrated by patients with defects in neutrophil number and/or function. The critical neutrophil concentration, where bacteria multiply and are phagocytosed at the same rate, has been determined for *S. aureus* as 400,000 neutrophils/ml [78], similar to the clinically relevant concentration of 500,000 neutrophils/ml, below which neutropaenic patients are at high risk of severe pyogenic bacterial infection. Furthermore, patients with severe congenital neutropaenia, leucocyte adhesion deficiency (impaired endothelial transmigration), Chediak-Higashi syndrome (impaired chemotaxis and degranulation) and chronic granulomatous disease (defective ROS production), all suffer from recurrent staphylococcal infections [79].

*S. aureus* has evolved extensive virulence mechanisms, which aim to evade neutrophil killing, including inhibition of neutrophil chemotaxis and extravasation, strategies to evade phagocytosis, disarmament of antimicrobial peptides and proteases, removal of anti-oxidants, degradation of NETs and direct lysis of neutrophils by secreted leukotoxins. This subject has been extensively reviewed [80], and further details are beyond the scope of this review. Despite being considered classically an extracellular pathogen, *S. aureus* has been shown to survive within the phagosome, and transfer of neutrophils containing viable intracellular bacteria to a naïve animal can institute infection [81]. Given the diversity of staphylococcal virulence mechanisms, which are intrinsically linked to neutrophil attack and evasion, it is important to note that there are a large number of *S. aureus* clinical isolates and laboratory strains used experimentally, and commonly used strains frequently carry significant mutational alterations in regulatory genes. For example, *S. aureus* strain NCTC8325, isolated from a historic sepsis patient and now maintained as the laboratory strain RN1, is fully antibiotic sensitive and also defective in two regulatory genes, one of which encodes a transcription activator of virulence factor protein A. In comparison, USA300 is a virulent community-acquired MRSA clinical isolate with high haemolytic activity and leukotoxin secretion [82]. Hence, it is conceivable that variations between strains used experimentally may underlie some of the conflicting results.

*S. aureus* infection often establishes a hypoxic environment; for example staphylococcal biofilms induce hypoxia in dermal tissue, impairing wound healing [30]. A second example relates to osteomyelitis; healthy bone is intrinsically hypoxic, and further decreases in skeletal oxygen concentration upon *S. aureus* infection were revealed by intravital oxygen monitoring [83]. Hence, *S. aureus* must possess flexibility in order to survive in hypoxic environments. Bacteria can adapt to hostile environments via two component histidine kinase systems. Transposon sequencing in a murine model of *S. aureus* osteomyelitis identified the staphylococcal respiratory response two component system SrrAB as essential for hypoxic survival, co-ordinating an increase in quorum sensing-dependent exotoxin production, which enhanced in vitro human osteoblast cytotoxicity [83]. Infection with an SrrA mutant decreased staphylococcal growth in vivo although, interestingly, the growth defect was rescued by depletion of neutrophils, suggesting that *S. aureus* requires SrrAB to resist hypoxic stress imposed by neutrophils. Targeted mutations have shown that SrrAB is activated by hypoxia and required for staphylococcal growth in a hypoxic static biofilm [84]. However, SrrAB control of virulence in hypoxia seems context specific as over-expression of SrrAB in a rabbit model of *S. aureus* endocarditis repressed virulence factors [85].

It is fascinating to consider how hypoxia influences *S. aureus* infection, as both bacteria and neutrophils appear well adapted to function in this environment and both may induce hypoxia in the surrounding tissues. Some studies have suggested that hypoxia restricts *S. aureus* infection. Pharmacological stabilisation of

HIF-1 $\alpha$  with two different agonists increased the bactericidal capacity of human neutrophils against *S. aureus* in vitro, and limited *S. aureus* proliferation and lesion formation in mouse skin infection [60,72]. Hypoxia increased *S. aureus* killing by stimulated human neutrophils in vitro [67], and neutrophils isolated from subjects exposed to intermittent hypoxia had enhanced bactericidal activity against *S. aureus* [66]. However, whether HIF-1 $\alpha$  stabilisation reflects true hypoxia is debatable as *S. aureus* was not subjected to low oxygen tensions in these models and may therefore be more susceptible. Furthermore, it is not clear whether the in vitro experiments were conducted under hypoxia or after re-oxygenation; for example, in one such study [67], neutrophils were rendered hypoxic by circulating blood in a gas-permeable silicon circuit against an anoxic gas mixture, but no mention was made of how (or if) hypoxia was maintained during the subsequent 4 h killing assay.

In fact, the majority of the literature suggests that hypoxia impairs *S. aureus* killing by neutrophils. Hypoxia reduced the ability of neutrophils to kill *S. aureus* in vitro (e.g. Ref. [86]), increased the size of *S. aureus* lesions in dog and rabbit skin infection models [32,87], and impaired clearance of *S. aureus* from the lung in rodent pneumonia models (e.g. Ref. [31]). Furthermore, pharmacological HIF-1 inhibition increased survival in a mouse model of *S. aureus* peritonitis [88].

Effective *S. aureus* killing is predominantly dependent on ROS production [69]. A number of in vitro studies have suggested that impaired ROS generation in hypoxia accounts for decreased bactericidal activity of neutrophils against *S. aureus*; the degree of phagocytosis has not been found to be diminished [54,86]. McGovern et al. showed that hypoxia markedly reduced both ROS generation and *S. aureus* killing, and, interestingly, observed an intracellular bacterial survival advantage when *S. aureus* was incubated with neutrophils under hypoxia [54]. They proposed that reduced ROS production was due to the lack of molecular oxygen as hypoxia did not change the expression of NADPH oxidase subunits; although challenging to interrogate NADPH oxidase assembly directly, addition of pyocyanin (which oxidises intracellular NADPH, NADH and reduced glutathione) did not increase ROS generation under hypoxia, indicating that the hypoxic effect on ROS production was independent of NADPH. Furthermore, in this study, a brief (15 min) period of re-oxygenation restored both ROS formation and bactericidal capacity. Inhibition of neutrophil ROS production by selective inhibitors of p38 and p44/42 MAPK has also been shown to decrease *S. aureus* killing, although these inhibitors additionally reduced mobilisation of  $\beta_2$  integrin to the plasma membrane, which may have contributed to the effect [89]. Neutrophils isolated from patients suffering recurrent pyogenic infections showed a strong correlation between impaired *S. aureus* killing and reduced superoxide anion production [90], and neutrophils from patients with chronic granulomatous disease (where ROS generation is absent) had significantly impaired ability to kill *S. aureus* [32]. Furthermore, neutrophil oxidants were able to inhibit a *S. aureus* quorum-sensing virulence-inducing peptide, a control mechanism which may be lost in hypoxia [91].

The prevailing view of the role of ROS in bacterial killing has been that the generation of highly toxic oxidants in the presence of MPO have a direct microbicidal effect; superoxide generated by NADPH oxidase dismutates to yield H<sub>2</sub>O<sub>2</sub>, a substrate for MPO, which then catalyses the oxidation of halides, the most important and cytotoxic being hypochlorous acid (HOCl). Recently this scheme has been challenged: Segal and colleagues have suggested that the primary role of NADPH oxidase is electron delivery to the phagosome, which is compensated by an influx of potassium ions, alkalising the vacuolar pH so that it is optimal for antibacterial protease activity [92]. They argue that oxidant production is a by-product, with MPO acting as a scavenger to protect proteases from oxidant damage and inactivation, and in fact inhibiting bacterial killing by H<sub>2</sub>O<sub>2</sub> at physiological pH. However, Green et al. still maintain that HOCl is instrumental in bacterial killing, demonstrating that decreased chlorination in the phagosome halved killing of *S. aureus* [93]. HOCl production was predominantly localised to the phagosome and, although a significant amount reacted with phagosomal proteins prior to microbial contact, there was sufficient HOCl to be directly microbicidal. Indeed, modifications of host proteins by HOCl may provide further active species, such as chloramines, which could extend microbial killing. Whether ROS act directly or indirectly in this regard, there is considerable in vitro evidence that the lack of ROS generation in hypoxia substantially reduces *S. aureus* killing.

The role of neutrophil proteases in host defence against *S. aureus* under hypoxia is unclear. Hypoxia enhances neutrophil degranulation, and the granule protease cathepsin G appears to be particularly important for *S. aureus* killing; mice lacking cathepsin G had increased mortality in a peritoneal sepsis model [92]. However, NE did not kill *S. aureus* directly, NE-null neutrophils killed *S. aureus* as well as wildtype cells in vitro, and mice lacking NE actually had a survival benefit in *S. aureus* sepsis [94], possibly due to excess damage of host tissues by NE in wildtype mice, which may be further increased in hypoxia.

Controversy surrounds the role of NETs in *S. aureus* killing. Whether NETs kill [8,95] or merely trap [11] *S. aureus*, it seems likely that a reduction in NETosis under hypoxia (as discussed in Section 4.6.) would allow *Staphylococcus* to thrive. Moreover, NET-associated *S. aureus* had decreased amounts of  $\alpha$  toxin when compared with free bacteria, suggesting that NETs have a potential role in controlling *S. aureus* by degrading virulence factors [8]. However, *S. aureus* has been shown to degrade NETs in vitro and these degradation products can induce macrophage apoptosis [96]. At this stage, the role of NETs in hypoxic environments and their interaction with other cells of the immune system remains speculative.

In summary, the majority of the literature supports defective killing of *S. aureus* under hypoxic conditions, both in vitro and in vivo, predominantly due to impaired ROS production (Fig. 2). The role of antimicrobial proteases and NETosis in hypoxic staphylococcal killing is not well established and invites further investigation.

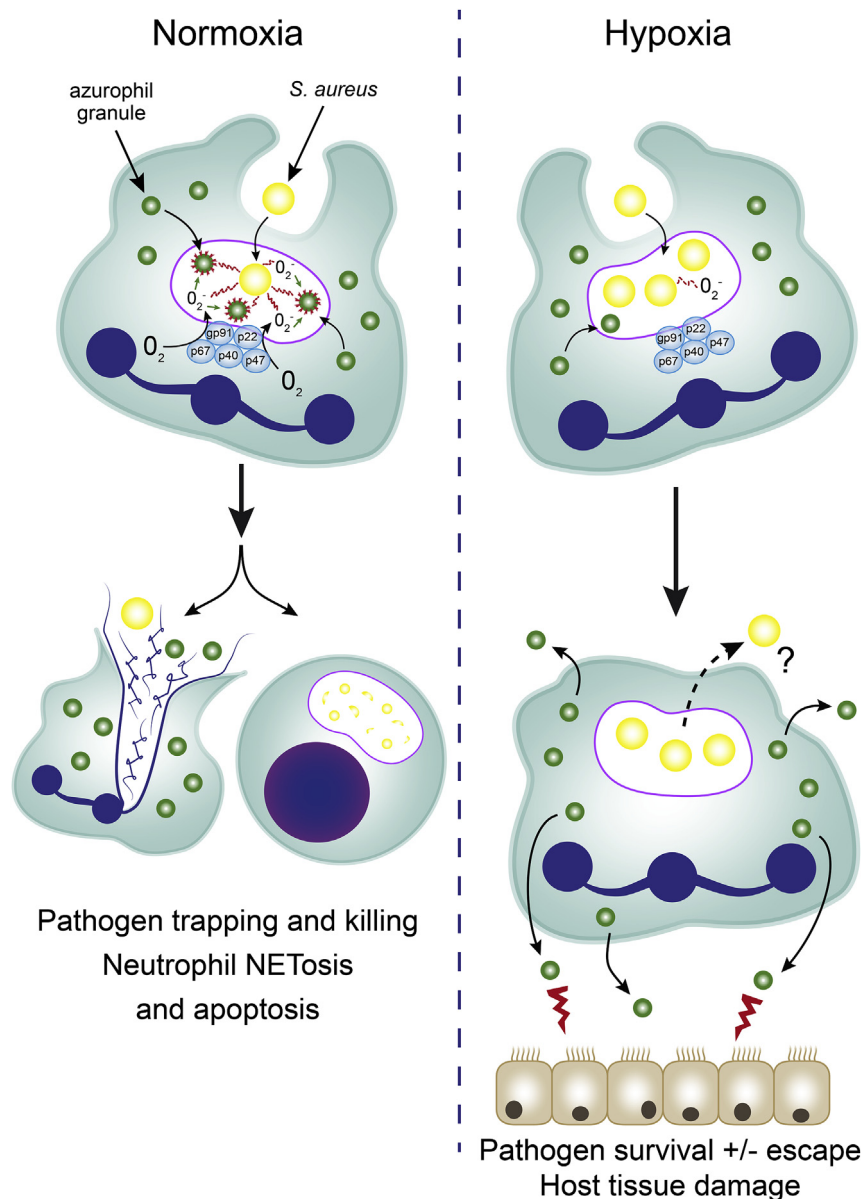


Fig. 2. In normoxic environments, *S. aureus* is ingested into the phagosome; fusion of neutrophil granules releases antimicrobial proteins and proteases into the vacuole, and NADPH oxidase facilitates production of ROS from molecular oxygen, contributing to *S. aureus* killing. Neutrophils may either form NETs, trapping *S. aureus*, or undergo apoptosis and clearance. In conditions of hypoxia, phagocytosis of *S. aureus* is maintained; neutrophil granules release antimicrobial proteins and proteases into the vacuole but there is markedly reduced ROS production due to lack of molecular oxygen. *S. aureus* killing within the vacuole is severely impaired, with the potential for pathogen escape. Hypoxia augments extracellular granule release, with the potential to damage host tissue.

## 6. Conclusion

Neutrophils and *S. aureus* are often found in profoundly hypoxic environments where both must function effectively, each striving for dominance. At first appearance, it seems that neutrophils are well adapted to hypoxia, with reliance on anaerobic energy production, prolonged survival, and augmented phagocytosis and degranulation. However, *S. aureus* has evolved numerous strategies to evade neutrophil killing and this evasion is further enhanced by hypoxia, where lack of molecular oxygen significantly impairs neutrophil ROS production and, hence, staphylococcal killing. Currently, *S. aureus* appears to be winning the hypoxic battle. Modulation

of the hypoxic neutrophil response is certainly worthy of future investigation to see if the balance can be tipped.

## Conflict of interest

None.

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## References

- [1] Dancey J, Deubelbeiss K, Harker L, Finch C. Neutrophil kinetics in man. *J Clin Invest* 1976;58(3):705–15.
- [2] Summers C, Rankin SM, Condliffe AM, Singh N, Peters AM, Chilvers ER. Neutrophil kinetics in health and disease. *Trends Immunol* 2010;31:318–24.
- [3] Bass DA, Olbrantz P, Szejda P, Seeds MC, McCall CE. Subpopulations of neutrophils with increased oxidative product formation in blood of patients with infection. *J Immunol* 1986;136(3):860–6.
- [4] Heit B, Tavener S, Raharjo E, Kubers P. An intracellular signaling hierarchy determines direction of migration in opposing chemotactic gradients. *J Cell Biol* 2002;159(1):91–102.
- [5] Flannagan RS, Jaumouille V, Grinstein S. The cell biology of phagocytosis. *Annu Rev Pathol Mech Dis* 2012;7:61–98.
- [6] Cascão R, Rosário H, Fonseca J. Neutrophils: warriors and commanders in immune mediated inflammatory disease. *Acta Rheumatol Port* 2009;34:313–26.
- [7] Kuijpers T, Lutter R. Inflammation and repeated infections in CGD: two sides of a coin. *Cell Mol Life Sci* 2012;69:7–15.
- [8] Brinkmann V, Reichard U, Goosmann C, Fauler B, Uhlemann Y, Weiss DS, et al. Neutrophil extracellular traps kill bacteria. *Science* 2004;303(5663):1532–5.
- [9] Urban CF, Reichard U, Brinkmann V, Zychlinsky A. Neutrophil extracellular traps capture and kill *Candida albicans* and hyphal forms. *Cell Microbiol* 2006;8(4):668–76.
- [10] Jung C-J, Yeh C-Y, Hsu R-B, Lee C-M, Shun C-T, Chia J-S. Endocarditis pathogen promotes vegetation formation by inducing intravascular neutrophil extracellular traps through activated platelets. *Circulation* 2015;131:571–81.
- [11] Menegazzi R, Declève E, Dri P. Killing by neutrophil extracellular traps: fact or folklore? *Blood* 2012;119(5):1214–7.
- [12] Colotta F, Re F, Polentarutti N, Sozzani S, Mantovani A. Modulation of granulocyte survival and programmed cell death by cytokines and bacterial products. *Blood* 1992;80(8):2012–20.
- [13] Haslett C. Granulocyte apoptosis and its role in the resolution and control of lung inflammation. *Am J Respir Crit Care Med* 1999 Nov;160:S5–11.
- [14] Maianski NA, Geissler J, Srinivasula SM, Alnemri ES, Roos D, Kuijpers TW. Functional characterization of mitochondria in neutrophils: a role restricted to apoptosis. *Cell Death Differ* 2004;11:143–53.
- [15] Chacko BK, Kramer PA, Ravi S, Johnson MS, Hardy RW, Ballinger SW, et al. Methods for defining distinct bioenergetic profiles in platelets, lymphocytes, monocytes, and neutrophils, and the oxidative burst from human blood. *Lab Invest* 2013;93:690–700. Nature Publishing Group.
- [16] Pearl-Yafe M, Fabian I, Halperin D, Flatau E, Werber S, Shalit I. Interferon- $\gamma$  and bacterial lipopolysaccharide act synergistically on human neutrophils enhancing interleukin-8, interleukin-1 $\beta$ , tumour necrosis factor- $\alpha$ , and interleukin-12 P70 secretion and phagocytosis via upregulation of Toll-Like Receptor 4. *Shock* 2007;27(3):226–31.
- [17] Chertov O, Michiel DF, Xu L, Wang JM, Tani K, Murphy WJ, et al. Identification of defensin-1, defensin-2, and CAP37/azurocidin as T-cell chemoattractant proteins released from Interleukin-8-stimulated neutrophils. *J Biol Chem* 1996;271(6):2935–40.
- [18] Takashima A, Yao Y. Neutrophil plasticity: acquisition of phenotype and functionality of antigen-presenting cell. *J Leukoc Biol* 2015;98:489–96.
- [19] Spencer J, Ferraro F, Roussakis E, Klein A, Wu J, Runnels J, et al. Direct measurement of local oxygen concentration in the bone marrow of live animals. *Nature* 2014;508(7495):269–73.
- [20] Jing D, Wobus M, Poitz DM, Bornhäuser M, Ehninger G, Ordemann R. Oxygen tension plays a critical role in the hematopoietic microenvironment in vitro. *Haematologica* 2012;97(3):331–9.
- [21] Nolte D, Steinhauser P, Pickelmann S, Berger S, Roger H, Messmer K. Effects of diaspirin-cross-linked hemoglobin (DCLHb) on local tissue oxygen tension in striated skin muscle: an efficacy study in the hamster. *J Lab Clin Med* 1997;130(3):328–38.
- [22] Zheng L, Kelly CJ, Colgan SP. Physiologic hypoxia and oxygen homeostasis in the healthy intestine. A review in the theme: cellular responses to hypoxia. *Am J Physiol Cell Physiol* 2015;309:C350–60.
- [23] Stewart FA, Denekamp J, Randhawa VS. Skin sensitization by misonidazole: a demonstration of uniform mild hypoxia. *Br J Cancer* 1982;45:869–77.
- [24] Mani R, White JE, Barrett DF, Weaver PW. Tissue oxygenation, venous ulcers and fibrin cuffs. *J R Soc Med* 1989;82:345–6.
- [25] Simmen H, Blaser J. Analysis of pH and pO<sub>2</sub> in abscesses, peritoneal fluid, and drainage fluid in the presence or absence of bacterial infection during and after abdominal surgery. *Am J Surg* 1993;165:24–7.
- [26] Polosukhin VV, Lawson WE, Milstone AP, Egunova SM, Kulipanov AG, Tchuvakin SG, et al. Association of progressive structural changes in the bronchial epithelium with subepithelial fibrous remodeling: a potential role for hypoxia. *Virchows Arch* 2007;451(4):793–803.
- [27] Grahl N, Puttikamonkul S, Macdonald JM, Gamcsik MP, Ngo LY, Hohl TM, et al. In vivo hypoxia and a fungal alcohol dehydrogenase influence the pathogenesis of invasive pulmonary aspergillosis. *PLoS Pathog* 2011 Jul;7:e1002145.
- [28] Mahnke A, Meier RJ, Schatz V, Hofmann J, Castiglione K, Schleicher U, et al. Hypoxia in *Leishmania major* skin lesions impairs the NO-dependent leishmanicidal activity of macrophages. *J Invest Dermatol* 2014;134:2339–46.
- [29] Campbell EL, Bruyninckx WJ, Kelly CJ, GLoover LE, McNamee EN, Bowers BE, et al. Transmigrating neutrophils shape the mucosal microenvironment through localized oxygen depletion to influence resolution of inflammation. *Immunity* 2014;40:66–77.
- [30] Lone AG, Atci E, Renslow R, Beyenal H, Noh S, Fransson B, et al. *Staphylococcus aureus* induces hypoxia and cellular damage in porcine dermal explants. *Infect Immun* 2015;83(6):2531–41.
- [31] Green GM, Kass EH. The influence of bacterial species on pulmonary resistance to infection in mice subjected to hypoxia, cold stress and ethanolic intoxication. *Br J Exp Pathol* 1965;46(3):360–6.
- [32] Knight RD, Halliday B, Hunt TK. Oxygen as an antibiotic. The effect of inspired oxygen on infection. *Arch Surg* 1984;119:199–204.
- [33] Bruick RK, McKnight SL. A conserved family of prolyl-4-hydroxylases that modify HIF. *Science* 2001;294:1337–40.
- [34] Lando D, Peet DJ, Gorman JJ, Whelan DA, Whitelaw ML, Bruick RK. FIH-1 is an asparaginyl hydroxylase enzyme that regulates the transcriptional activity of hypoxia-inducible factor. *Genes Dev* 2002;16:1466–71.
- [35] Thompson AAR, Elks PM, Marriott HM, Eamsamarn S, Higgins KR, Lewis A, et al. Hypoxia-inducible factor 2 $\alpha$  regulates key neutrophil functions in humans, mice, and zebrafish. *Blood* 2013 Jan 16;123(3):366–76.
- [36] Walmsley SR, Print C, Farahi N, Peyssonnaud C, Johnson RS, Cramer T, et al. Hypoxia-induced neutrophil survival is mediated by HIF-1 $\alpha$ -dependent NF- $\kappa$ B activity. *J Exp Med* 2005 Jan 3;201(1):105–15.
- [37] Cramer T, Yamanishi Y, Clausen BE, Förster I, Pawlinski R, Mackman N, et al. HIF-1 $\alpha$  is essential for myeloid cell-mediated inflammation. *Cell* 2003;112(5):645–57.
- [38] Walmsley SR, Chilvers ER, Thompson AA, Vaughan K, Marriott HM, Parker LC, et al. Prolyl hydroxylase 3 (PHD3) is essential for hypoxic regulation of neutrophilic inflammation in humans and mice. *J Clin Invest* 2011;121(3):1053–63.
- [39] Kiss J, Mollenhauer M, Walmsley SR, Kirchberg J, Radhakrishnan P, Dudda J, et al. Loss of the oxygen sensor PHD3 enhances the innate immune response to abdominal sepsis. *J Immunol* 2012;189:1955–65.
- [40] Tausendschon M, Dehne N, Brune B. Hypoxia causes epigenetic gene regulation in macrophages by attenuating Jumonji histone demethylase activity. *Cytokine* 2011;53(2):256–62.
- [41] Kobayashi SD, Braughton KR, Palazzolo-Ballance Amy M, Kennedy AD, Sampaio E, Kristosturyan E, et al. Rapid neutrophil destruction following phagocytosis of *Staphylococcus aureus*. *J Innate Immun* 2010;2:560–75.

- [42] Walmsley SR, Cowburn AS, Clatworthy MR, Morrell NW, Roper EC, Singleton V, et al. Neutrophils from patients with heterozygous germline mutations in the von Hippel Lindau protein (pVHL) display delayed apoptosis and enhanced bacterial phagocytosis. *Blood* 2006;108(9):3176–8.
- [43] Elks PM, Eeden FJ Van, Dixon G, Wang X, Reyes-aldasoro CC, Ingham PW, et al. Activation of hypoxia-inducible factor-1 $\alpha$  (Hif-1 $\alpha$ ) delays inflammation resolution by reducing neutrophil apoptosis and reverse migration in a zebrafish inflammation model. *Blood* 2011;118(3):712–23.
- [44] Tamura DY, Moore EE, Partrick DA, Johnson JL, Offner PJ, Silliman CC. Acute hypoxemia in humans enhances the neutrophil inflammatory response. *Shock* 2002;17(4):269–73.
- [45] Dyugovskaya L, Polyakov A, Ginsberg D, Lavie P, Lavie L. Molecular pathways of spontaneous and TNF- $\alpha$  mediated neutrophil apoptosis under intermittent hypoxia. *Am J Respir Cell Mol Biol* 2011;45:154–62.
- [46] Dyugovskaya L, Polyakov A, Lavie P, Lavie L. Delayed neutrophil apoptosis in patients with sleep apnea. *Am J Resp Crit Care Med* 2008;177:544–54.
- [47] Kong T, Eltzschig HK, Karhausen J, Colgan SP, Shelley CS. Leukocyte adhesion during hypoxia is mediated by HIF-1-dependent induction of  $\beta$ 2 integrin gene expression. *PNAS* 2004;101(28):10440–5.
- [48] Antonova O, Loktionova S, Golubeva N, Romanov YA, Mazurov AV. Damage and activation of endothelial cells during in vitro hypoxia. *Bull Exp Biol Med* 2007;144(10):384–6.
- [49] Yoon C, Hur J, Oh I, Park K, Kim T, Shin J, et al. Intercellular adhesion molecule-1 is upregulated in ischemic muscle, which mediates trafficking of endothelial progenitor cells. *Arterioscler Thromb Vasc Biol* 2006;26:1066–72.
- [50] Baudry N, Danialou G, Boczkowski J, Vicaut E. In vivo study of the effect of systemic hypoxia on leukocyte–endothelium interactions. *Am J Resp Crit Care Med* 1998;158:477–83.
- [51] Colgan SP, Dzus AL, Parkos CA. Epithelial exposure to hypoxia modulates neutrophil transepithelial migration. *J Exp Med* 1996;184:1003–15.
- [52] Wood JG, Johnson JS, Mattioli LF, Gonzalez NC. Systemic hypoxia increases leukocyte emigration and vascular permeability in conscious rats. *J Appl Physiol* 2000;89:1561–8.
- [53] Eltzschig HK, Thompson LF, Karhausen J, Cotta RJ, Ibla JC, Robson SC, et al. Endogenous adenosine produced during hypoxia attenuates neutrophil accumulation: coordination by extracellular nucleotide metabolism. *Blood* 2004;104:3986–92.
- [54] McGovern NN, Cowburn AS, Porter L, Walmsley SR, Summers C, Thompson AAR, et al. Hypoxia selectively inhibits respiratory burst activity and killing of *Staphylococcus aureus* in human neutrophils. *J Immunol* 2011;186:453–63.
- [55] Peyssonnaud C, Datta V, Cramer T, Doedens A, Theodorakis EA, Gallo RL, et al. HIF-1 $\alpha$  expression regulates the bactericidal capacity of phagocytes. *J Clin Invest* 2005;115(7):1806–15.
- [56] Wang J, Liu H. Systemic hypoxia enhances bactericidal activities of human polymorphonuclear leucocytes. *Clin Sci* 2009;116:805–17.
- [57] Rotstein O, Fiegel V, Simmons R, Knighton D. The deleterious effect of reduced pH and hypoxia on neutrophil migration in vitro. *J Surg Res* 1988;45:298–303.
- [58] Leire E, Olson J, Isaacs H, Nizet V, Hollands A. Role of hypoxia inducible factor-1 in keratinocyte inflammatory response and neutrophil recruitment. *J Inflamm* 2013;10:28.
- [59] Lin AE, Beasley FC, Olson J, Keller N, Shalwitz RA, Hannan TJ, et al. Role of hypoxia inducible factor-1 $\alpha$  (HIF-1 $\alpha$ ) in innate defense against uropathogenic *Escherichia coli* infection. *PLoS Pathog* 2015;11(4):e1004818.
- [60] Zinkernagel AS, Peyssonnaud C, Johnson RS, Nizet V. Pharmacologic augmentation of hypoxia-inducible factor-1 $\alpha$  with mimosine boosts the bactericidal capacity of phagocytes. *J Infect Dis* 2008;197:214–7.
- [61] Berger EA, McClellan SA, Vistisen KS, Hazlett LD. HIF-1 $\alpha$  is essential for effective PMN bacterial killing, antimicrobial peptide production and apoptosis in *Pseudomonas aeruginosa* keratitis. *PLoS Pathog* 2013;9(7):e1003457.
- [62] Fritzenwanger M, Jung C, Goebel B, Lauten A, Figulla HR. Impact of short-term systemic hypoxia on phagocytosis, cytokine production, and transcription factor activation in peripheral blood cells. *Mediat Inflamm* 2011;2011:429501.
- [63] Simms HH, D'Amico RD. Hypoxia induces an opsonic mismatch on the polymorphonuclear leukocyte surface-reversal via arg-gly-asp-ser-mediated adhesion. *J Surg Res* 1993;54:299–304.
- [64] Freischlag JA, Hanna D. Neutrophil (PMN) phagocytosis and chemotaxis after 2 hr of ischemia. *J Surg Res* 1991;50:648–52.
- [65] Lu Y, Wu C, Huang Y, Huang C, Yang C, Lee T, et al. Neutrophil priming by hypoxic preconditioning protects against epithelial barrier damage and enteric bacterial translocation in intestinal ischemia/reperfusion. *Lab Invest* 2012;92:783–96.
- [66] Serebrovskaya TV, Nikolsky IS, Nikolska VV, Mallet RT, Ishchuk VA. Intermittent hypoxia mobilizes hematopoietic progenitors and augments cellular and humoral elements of innate immunity in adult men. *High Alt Med Biol* 2011;12(3):243–52.
- [67] Knowles R, Keeping H, Graeber T, Nguyen K, Garner C, D'Amico R, et al. Cytokine control of PMN phagocytosis: regulatory effects of hypoxemia and hypoxemia-reoxygenation. *Am J Physiol Cell Physiol* 1997;272(41):C1352–64.
- [68] Schulz R, Mahmoudi S, Hattar K, Sibelius ULF, Olschewski H, Mayer K, et al. Enhanced release of superoxide from polymorphonuclear neutrophils in obstructive sleep apnea. *Am J Resp Crit Care Med* 2000;162:566–70.
- [69] Ellison CD, Davidson K, Ferguson GJ, O'Connor R, Stephens LR, Hawkins PT. Neutrophils from p40 phox  $-/-$  mice exhibit severe defects in NADPH oxidase regulation and oxidant-dependent bacterial killing. *J Exp Med* 2006;203(8):1927–37.
- [70] Elks PM, Brizee S, Vaart M Van Der, Walmsley SR, Eeden FJ Van, Renshaw SA, et al. Hypoxia inducible factor signaling modulates susceptibility to mycobacterial infection via a nitric oxide dependent mechanism. *PLoS Pathog* 2013;9(12):e1003789.
- [71] Hoenderdos K, Lodge KM, Hirst RA, Chen C, Palazzo SGC, Emerenciana A, et al. Hypoxia upregulates neutrophil degranulation and the potential for tissue injury. *Thorax* 2016;71:1030–8.
- [72] Okumura C, Hollands A, Tran D, Olson J, Daresh S, von Köckritz-Blickwede M, et al. A new pharmacological agent (AKB-4924) stabilizes hypoxia inducible factor (HIF) and increases skin innate defenses against bacterial infection. *J Mol Med* 2012;90(9):1079–89.
- [73] Ong CW, Elkington PT, Friedland JS. Tuberculosis, pulmonary cavitation, and matrix metalloproteinases. *Am J Resp Crit Care Med* 2014;190(1):9–18.
- [74] McInturff AM, Cody MJ, Elliott EA, Glenn JW, Rowley JW, Rondina MT, et al. Mammalian target of rapamycin regulates neutrophil extracellular trap formation via induction of hypoxia-inducible factor 1 $\alpha$ . *Blood* 2012;120(15):3118–25.
- [75] Fuchs TA, Abed U, Goosmann C, Hurwitz R, Schulze I, Wahn V, et al. Novel cell death program leads to neutrophil extracellular traps. *J Cell Biol* 2007;176(2):231–41.
- [76] Tohme S, Yazdani HO, Al-khafaji AB, Chidi AP, Loughran P, Mowen K, et al. Neutrophil extracellular traps promote the development and progression of liver metastases after surgical stress. *Cancer Res* 2016;76(6):1367–80.
- [77] *Staphylococcus aureus: guidance, data and analysis*. Public Health England; 2016. <https://www.gov.uk/government/collections/staphylococcus-aureus-guidance-data-and-analysis>.
- [78] Li Y, Karlin A, Loike JD, Silverstein SC. Determination of the critical concentration of neutrophils required to block bacterial growth in tissues. *J Exp Med* 2004;200(5):613–22.
- [79] Lakshman R, Finn A. Neutrophil disorders and their management. *J Clin Pathol* 2001;54:7–19.
- [80] Foster TJ. Immune evasion by staphylococci. *Nat Rev Microbiol* 2005;3:948–58.
- [81] Gresham HD, Lowrance JH, Caver TE, Wilson BS, Cheung AL, Lindberg FP. Survival of *Staphylococcus aureus* inside neutrophils contributes to infection. *J Immunol* 2000;164:3713–22.

- [82] Herbert S, Ziebandt A-K, Ohlsen K, Schafer T, Hecker M, Albrecht D, et al. Repair of global regulators in *Staphylococcus aureus* 8325 and comparative analysis with other clinical isolates. *Infect Immun* 2010; 78(6):2877–89.
- [83] Wilde AD, Snyder DJ, Putnam NE, Valentino MD, Hammer D, Lonergan ZR, et al. Bacterial hypoxic responses revealed as critical determinants of the host-pathogen outcome by TnSeq analysis of *Staphylococcus aureus* invasive infection. *PLoS Pathog* 2015;11(12):e1005341.
- [84] Kinkel TL, Roux CM, Dunman PM, Fang FC. The *Staphylococcus aureus* SrrAB two-component system promotes resistance to nitrosative stress and hypoxia. *MBio* 2013;4(6). e00696–13.
- [85] Pragman AA, Yarwood JM, Tripp TJ, Schlievert PM. Characterization of virulence factor regulation by SrrAB, a two-component system in *Staphylococcus aureus*. *J Bacteriol* 2004;186(8):2430–8.
- [86] Mandell GL. Bactericidal activity of aerobic and anaerobic polymorphonuclear neutrophils. *Infect Immun* 1974;9(2):337–41.
- [87] Jonsson K, Hunt TK, Mathes SJ. Oxygen as an isolated variable influences resistance to infection. *Ann Surg* 1988;208(6):783–7.
- [88] Werth N, Beerlage C, Rosenberger C, Yazdi AS, Edelmann M, Amr A, et al. Activation of hypoxia inducible factor 1 is a general phenomenon in infections with human pathogens. *PLoS One* 2010 Jan;5(7):e11576.
- [89] Schnyder B, Meunier PC, Car BD. Inhibition of kinases impairs neutrophil activation and killing of *Staphylococcus aureus*. *Biochem J* 1998;331:489–95.
- [90] Wolach B, Gavrieli R, Roos D, Berger-Achituv S. Lessons learned from phagocytic function studies in a large cohort of patients with recurrent infections. *J Clin Immunol* 2012;32:454–66.
- [91] Rothfork JM, Timmins GS, Harris MN, Chen X, Lulis AJ, Otto M, et al. Inactivation of a bacterial virulence pheromone by phagocyte-derived oxidants: new role for the NADPH oxidase in host defense. *PNAS* 2004;101(38):13867–72.
- [92] Reeves EP, Lu H, Jacobs HL, Messina CGM, Bolsover S, Gabella G, et al. Killing activity of neutrophils is mediated through activation of proteases by K flux. *Nature* 2002;416:291–7.
- [93] Green JN, Kettle AJ, Winterbourn CC. Protein chlorination in neutrophil phagosomes and correlation with bacterial killing. *Free Radic Biol Med* 2014;77:49–56.
- [94] Belaouaj A, McCarthy R, Baumann M, Gao Z, Ley T, Abraham SN, et al. Mice lacking neutrophil elastase reveal impaired host defence against gram negative bacterial sepsis. *Nature Med* 1998;4(5):615–8.
- [95] Pilsczek FH, Salina D, Poon KKH, Fahey C, Yipp BG, Sibley CD, et al. A novel mechanism of rapid nuclear neutrophil extracellular trap formation in response to *Staphylococcus aureus*. *J Immunol* 2010;185: 7413–25.
- [96] Thammavongsa V, Missiakas DM, Schneewind O. *Staphylococcus aureus* degrades neutrophil extracellular traps to promote immune cell death. *Science* 2013;342:863–6.