Institut Pasteur
Microbes and Infection 19 (2017) 166–176
www.elsevier.com/locate/micinf

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

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Received 28 June 2016; accepted 12 October 2016
Available online 24 October 2016

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.

Keywords: Neutrophil; Hypoxia; *Staphylococcus aureus*

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

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http://dx.doi.org/10.1016/j.micinf.2016.10.005
1286-4579/

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 $p22^{phox}$ and $gp91^{phox}$, 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-α (TNF-α) 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 $CD4^+$ and $CD8^+$ 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_2 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 μm, 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_2 measurement of wounds and venous ulcers [24]; by blood gas analysis of abscesses, a characteristic