

REACTIVE OXYGEN SPECIES (ROS) – A FAMILY OF FATE DECIDING MOLECULES PIVOTAL IN CONSTRUCTIVE INFLAMMATION AND WOUND HEALING

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Abstract

Wound healing requires a fine balance between the positive and deleterious effects of reactive oxygen species (ROS); a group of extremely potent molecules, rate limiting in successful tissue regeneration. A balanced ROS response will debride and disinfect a tissue and stimulate healthy tissue turnover; suppressed ROS will result in infection and an elevation in ROS will destroy otherwise healthy stromal tissue. Understanding and anticipating the ROS niche within a tissue will greatly enhance the potential to exogenously augment and manipulate healing.

Tissue engineering solutions to augment successful healing and remodelling of wounded or diseased tissue rely on a controlled balance between the constructive and destructive capacity of the leukocyte secretome, including ROS.

This review comprehensively considers leukocyte derived ROS in tissue repair with particular interest in surgical intervention with inclusion of a biomaterial. The article considers ROS fundamental chemistry, formation, stimulation and clearance before applying this to discuss the implications of ROS in healing tissue with and without a biomaterial. We also systematically discuss ROS in leukocyte signalling and compare and contrast experimental means of measuring ROS.

Keywords: Reactive oxygen species; inflammation; wound healing; leukocytes; pattern recognition receptors; cell signalling; biomaterials.

Introduction

The survival and longevity of any animal requires an active vigilant set of defence mechanisms to combat infection, efficiently repair damaged tissue and remove debris associated with apoptotic/necrotic cells. Compromised tissue rapidly results in decreased mobility, organ failures, hypovolaemia, hypermetabolism, and ultimately infection and sepsis. Therefore, mammals have evolved an array of physiological pathways and mechanisms that enable damaged tissue to return to a basal homeostatic state. In an ideal scenario this occurs without compromise of tissue mechanics, scarring or incorporation of microbial material.

Leukocytes are a fulcrum in the positive and negative feedback systems of the multivariate wound healing response (Wilgus, 2008). Through a highly complex extracellular signalling network coupled with a dynamic array of surface receptors these cells drive the host through tissue imbalances to stabilisation. At the most fundamental level, professional phagocytes – macrophages and neutrophils – debride compromised tissue, internalising and destroying pathogens in a series of controlled cytoplasmic explosions. As the wound site is cleared, building blocks are assembled for repair, and the same cells take on a more constructive role, orchestrating proliferation of stromal cells to repair the damaged area. Away from the wound, systemically some of these phagocytes drain and migrate through the lymphatic system to begin the unimaginable sophistication of antigen presentation, introducing into the healing milieu exquisite specificity and targeted microbial eradication from T and B lymphocytes (Witko-Sarsat *et al.*, 2000; Park and Barbul, 2004; Szpaderska and DiPietro, 2005; Nathan, 2006; Tsirogianni *et al.*, 2006; Emming *et al.*, 2009; Kumar and Sharma, 2010).

To further compound the complexity of wound healing, inflammatory cells may also be presented with the challenge of a foreign body in the form of a medical device or implanted biomaterial. A large acute leukocyte interaction can have significant effects on the longevity and efficacy of the material itself; it can cause a vastly accelerated degradation profile, loss of mechanical integrity and poor integration with healthy tissues. Therefore, understanding the mechanisms and pathways by which leukocytes interact with implantable materials is a paramount deliverable during their engineering and fabrication phases (Devereux *et al.*, 1990; Sutherland *et al.*, 1993; Babensee *et al.*, 1998; Nygren *et al.*, 2001; Mizuki *et al.*, 2005; Kader and Coyle, 2007; Anderson *et al.*, 2008;

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Jeannin *et al.*, 2008; Brown and Griendling, 2009; Franz *et al.*, 2011).

Leukocytes of the professional phagocyte class (monocytes, neutrophils and dendritic cells) possess numerous molecular bactericidal mechanisms which occur both inside and outside of the cell, with potential for catastrophic damage to otherwise healthy native tissue without appropriate regulation. During phagocytosis of invading organisms leukocytes rapidly increase their consumption of molecular oxygen, in a process referred to as the respiratory burst. This arms the most destructive weapons in the phagocyte's arsenal: reactive oxygen species (ROS) (Nathan and Shiloh, 2000). This short-lived group of incredibly cytotoxic molecules form an integral part of the phagosome. By means of a controlled generation on the inner phagosomal membrane the ROS rapidly degenerate internalised pathogens. However, ROS are also secreted into interstitial fluids where their beneficial role in focal sterilisation of the extracellular environment may be over-shadowed by their destructive influence to otherwise healthy tissue.

ROS is an umbrella term, comprising radicals and unstable oxygen containing molecules with the propensity to easily form radicals. Collectively, this group of molecules contain one or more unpaired electrons making them extremely susceptible to interaction with biological polymers such as protein, DNA, lipids and carbohydrates (Buonocore *et al.*, 2010). ROS have a fundamental function in innate immunity and form the first line of defence against invading organisms. Indeed, no ROS review article would be complete without paying homage to chronic granulomatous disease (CGD) – an inherited condition in which sufferers lack a respiratory burst response. CGD patients are massively susceptible to bacterial and fungal infections during the acute phase of inflammation and before the advancement of antibiotics, typically died within the first year of life. Studying cells isolated from CGD patients allowed huge leaps to be made in elucidating the molecular mechanisms of ROS generation in leukocytes (Henderson and Chappell, 1996; Cooper *et al.*, 2000).

Human disease states may also arise downstream of ROS production. Pathologies as diverse as joint diseases, asthma, cancer, obesity and neurodegeneration can all be influenced by the improper functioning of the ROS processing enzyme superoxide dismutase (SOD), the functions and significance of which will be discussed in detail throughout this review (Janssen-Heininger *et al.*, 2005; Johnson and Giulivi, 2005; Afonso *et al.*, 2007).

ROS play a role in a number of physiologic processes throughout an organism; their ubiquitous nature throughout all mammalian tissues has highlighted malfunctions in their production as a potential route of tumourigenesis (Storz, 2005), and lead to considering molecules which target various components of the ROS production system as candidate anti-cancer chemotherapeutic agents (Ferguson *et al.*, 2005). Further to fundamental host defence, ROS also play pivotal roles in cell signalling, wound debridement and clearance of apoptotic/necrotic cells throughout tissue remodelling. Typically considered as brutal, vigilante molecules during inflammation, the cell

signalling effects of ROS are often overlooked in favour of the much more elegant cellular proteinaceous language of chemokines and growth factors. However, ROS also play an important role in conveying the progression of wound healing throughout the inflammasome by means of both paracrine and second messenger roles.

This comprehensive review will focus on defining the role of ROS in healing tissue, discuss in detail the main route of ROS formation in phagocytes and how ROS are able to execute their physiologic functions as both anti-microbial and cell signalling molecules, with particular interest to their impact in tissue regeneration in conjunction with the inclusion of a biomaterial.

ROS Synthesis

Although ROS can be produced by a number of cytoplasmic enzymes – including xanthine oxidase, lipoxygenase, cyclooxygenase and nitric oxide synthase – in addition to numerous aspects of mitochondrial physiology, most of the phagocyte ROS derive from superoxide generated by NADPH oxidase. This multimeric membrane bound electron-transfer complex is the main site of ROS formation in phagocytes and undergoes multiple modifications to its intercellular component to functionalise during leukocyte activation.

The two membrane spanning proteins of the NADPH complex; p22^{phox} and gp91^{phox} (NOX2) are the catalytic components of the enzyme, ever present on the phagocyte cell membrane, collectively referred to as cytochrome b₅₅₈. Upon phagocyte activation, the cytosolic regulatory elements p47^{phox}, p67^{phox} and p40^{phox} translocate and associate with cytochrome b₅₅₈. Simultaneously to the phox (phagocyte oxidase) moieties, the GTPase cofactor rac2 also translocates to the plasma membrane after dissociation from its repressor RhoGDI by Rac guanine nucleotide exchange factor and interacts directly with p67^{phox}. The association of rac2 in combination with phosphorylation of multiple activation loci on p47^{phox} by the MAPKs (mitogen associated protein kinases) ERK (extracellular signal regulated protein kinase) and p38 produces the actively reducing NADPH complex with suitably intricate biochemistry to ensure that ROS are only synthesised in the presence of appropriate stimuli (Henderson and Chappell, 1996; Dahlgren and Karlsson, 1999; Diebold and Bokoch, 2001; Nygren *et al.*, 2001; Groemping *et al.*, 2003; Mizuki *et al.*, 2005; Bokoch and Zhao, 2006; Lam *et al.*, 2010).

The NADPH oxidase complex is not specific to phagocytes with many mammalian cell types expressing various forms. Tissue specific NADPH oxidases differ with respect to their NOX/DUOX (dual oxidase) isoform of which 7 exist with varying tissue distributions. Interestingly, a single cell can possess multiple NADPH complexes containing differing NOX components suggesting different downstream signalling of alternative NOX containing NADPH complexes (Brown and Griendling, 2009). NADPH assembly signals may include classical PAMPs (pathogen associated molecular patterns) such as LPS, soluble peptides with specific recognition

receptors such as f met-leu-phe (fMLP), complement, lectins, chemotaxins or through interaction with humeral immunity *via* F_c receptors activated by opsonised pathogen or pathogenic membrane constituents. NADPH oxidase produces superoxide *via* a two-step process. The initiation reaction occurs when an electron is transferred from NADPH to flavin adenine dinucleotide (FAD) located at the C terminus of gp91^{phox}. This electron transfers to a haeme moiety contained within p22^{phox} which then interacts with molecular oxygen to result in superoxide. This reaction can be summarised as:



Cytochrome b_{558} is an exclusively membrane bound complex. However, its presence is not specifically restricted to membranes that contact the extracellular environment. Cytochrome b_{558} also occurs in the membranes of numerous intracellular structures.

Neutrophils, possess at least 4 different types of granules within their cytoplasm. These can be subcategorised into azurophilic, gelatinase, secretory and specific granules and contain an array of bactericidal molecules. Granules can fuse with the phagosome to secrete their payload directly onto captured pathogens or integrate with the plasma membrane to elute their contents into the extracellular space. In this instance, granule membranes also act as a sink for surface receptors, incorporating a new series of activation receptors onto the cell surface during their fusion (Nathan, 2006).

Neutrophil granules, particularly those of the specific and gelatinase subclasses, possess abundant cytochrome b_{558} within their membranes (total neutrophil cytochrome b_{558} ; plasma membrane: granule membrane 5 %:95 %). This compounds the granules' cytotoxicity to internalised pathogens. When a granule fuses with the phagosome to incorporate its lytic payload, the membrane fusion also increases the phagosomal NADPH oxidase concentration and resultant ROS release (Dahlgren and Karlsson, 1999).

Further ROS Formation

The formation of superoxide by NADPH oxidase is the initiation reaction for the formation of further ROS within the cytoplasm and extracellular spaces. Hydrogen peroxide (H_2O_2) is formed by spontaneous dismutation of superoxide in addition to enzymatically regulated conversion by superoxide dismutase (SOD). H_2O_2 is a weak oxidising agent. However, a Fenton reaction with iron yields hydroxyl ($\cdot\text{OH}$) radicals, the most oxidising of all cellular ROS.

H_2O_2 is converted into hypochlorous acids such as hypochlorite (OCl^-) by reaction with Cl^- catalysed by the lysosomal haeme containing enzyme myeloperoxidase stored inside neutrophil azurophilic granules. The result of this can be increased phagosome cytotoxicity as a result of granule/phagosome fusion, OCl^- containing granules by transmembrane diffusion of H_2O_2 directly into azurophilic granules, or elution of OCl^- into the extracellular inflammatory milieu by granule fusion with the plasma

membrane. Hypochlorite can then undergo secondary reactions with H_2O_2 and amines to produce singlet oxygen and chloramines.

Reaction of superoxide with nitric oxide produced by inducible nitric oxide synthase (iNOS) yields peroxynitrite (ONOO^-) which can undergo further spontaneous reaction with carbonyl compounds to produce a number of organic peroxy radicals. The components of the nitrogenous arm of additional ROS reactions are referred to broadly as reactive nitrogen species (RNS) (Brown and Griendling, 2009).

ROS Detoxification

The lack of specificity of ROS towards particular bacterial molecules, coupled with their ability to be secreted into the extracellular milieu rather than solely retained in the phagosome, means that these molecules can exert their cytotoxic properties on healthy native tissue if appropriate systems are not in place to safely detoxify them. Furthermore, generation of reactive oxygen metabolites is an unavoidable by-product of respiration. Therefore, biochemical systems by which ROS can be disposed are vital to the survival of an organism. ROS detoxification can occur in a number of ways and genomically is controlled by a diverse series of transcription factors including peroxisome proliferator-activated receptor- γ (PPAR γ), p53, activating transcription factor 4 (ATF4) and nuclear factor-erythroid 2 related factor 2 (Nrf2) (Maher and Yamamoto, 2010). There are enzymatic systems in place which decrease the potency of particular reactive species, or render them completely harmless. Alternatively, ROS can interact with antioxidants such as vitamins, glutathione or proteins, particularly those with metal ion moieties capable of oxidation/reduction reactions – such as the iron present in transferrin and ferritin (Limon-Pacheco and Gosebatt, 2009).

SOD, by virtue of the fact it converts O_2^- into less potent H_2O_2 , is considered a ROS detoxification enzyme. Three isoforms of SOD exist, intracellular Cu/Zn SOD (SOD1), extracellular Cu/Zn SOD (SOD3) and MnSOD (SOD2) which is predominantly located in the mitochondria. In addition to detoxification, SOD is an important enzyme in facilitating the signalling properties of ROS. This because H_2O_2 , due to its relatively long half-life compared to other cellular oxidants and its ability to traverse membranes, is the optimal signalling candidate derived from NADPH oxidase (Fattman *et al.*, 2003; Zelko *et al.*, 2002).

Catalase 'picks up the baton' from SOD in the race to rid tissues of damaging ROS. Catalase is a haeme-containing enzyme which is expressed throughout numerous tissues of the body, it decomposes H_2O_2 into water and molecular oxygen. This enzyme is most often found within intracellular organelles, such as peroxisomes, and therefore relies on the diffusion of H_2O_2 into these compartments to undergo detoxification (Nishikawa *et al.*, 2009).

H_2O_2 can also be decomposed into water by enzymes of the glutathione peroxidase (GPx) family. In humans, there are 8 GPx isoforms with varying tissue and substrate specificities. Typically, GPx enzymes are selenoproteins

(GPx1-4 and GPx6, depending on species). In the context of NADPH derived oxidant detoxification, GPx catalyses the removal of H⁺ from H₂O₂ to oxidise glutathione (GSH) to glutathione disulphide (GS-SG) and reduce the H₂O₂ to water. Glutathione reductase then converts GS-SG back to reduced GSH *via* NADPH (Toppo *et al.*, 2009). Absence of this enzyme renders cells *in vitro* hyper-susceptible to H₂O₂ induced cell death (De Haan *et al.*, 2004; Johnson and Giulivi, 2005).

Peroxiredoxins are an additional class of 6 ROS detoxification enzymes, with the propensity to convert H₂O₂ to water. To a lesser extent, these enzymes also possess the ability to break down ONOO⁻. The peroxiredoxins are a prevalent group of enzymes, found mainly in the cytosol; these proteins comprise 0.1-0.8 % of the total soluble protein found in mammalian cells (Wood *et al.*, 2003).

ROS Triggers

Phagocytes can be induced to release extra ROS – and intracellularly, by a number of activation signals both with and without accompanying phagocytosis. These triggers are an amalgamation of molecular binding events which may be encountered as a consequence of infection, wounding, disease or administration of a foreign material during surgery. An unavoidable component of surgery is the mechanical manipulation and associated trauma to healthy and/or previously compromised tissues. This results in the secretion of numerous damage associated signals from stroma, in addition to release of the intercellular components of mechanically lysed cells into the wound milieu. Immediately after delivery, an implanted device will adsorb serum proteins onto its surface including Ig and complement – which also have the propensity to compound the overall inflammatory reaction elicited by the surgical procedure. All of these factors represent potential pathways for ROS stimulation whilst also highlighting the importance of considering ROS throughout the fabrication stages of a biomaterial. Both of these aspects will be discussed in further detail in the sections which follow.

F_c Receptors (F_cRs)

In vivo, materials and foreign particles become coated with immunoglobulins (Igs); this opsonisation process is part of the system which identifies substances and cells as being non-host and flags them for destruction and removal by cytotoxic inflammatory cells. F_cRs are a complex class of molecules designated broadly into three classes (F_cα, F_cγ and F_cε) based the antibody class which they bind. They are further categorised into subtypes based on the precise antibody isotype with which they interact and the cell type on which they are found. F_cγ receptors (of which there are three subclasses F_cγRI, F_cγRII, and F_cγRIII) are the most abundant F_cR on the surface of phagocytes and bind IgG. Collectively, phagocytes possess a huge complement of F_cRs – making them excitable by almost any form of opsonisation. In most cases F_cR binding causes phagocyte activation, with the exception of F_cγRIIB which inhibits leukocyte activation. Typically (although not exclusively, neutrophil F_cγRIIIB

is GPI anchored), F_cγR receptors are membrane spanning with an immunoreceptor tyrosine-based activation motif (ITAM) on the intercellular arm. ITAM phosphorylation, as a consequence of F_c/F_cγR binding, triggers activation of numerous kinases and downstream signalling cascades including p38, ERK and JNK (c-jun N-terminal kinase) which mediate phagocytosis, NADPH oxidase assembly and ROS production (Indik *et al.*, 1995; Nimmerjahn and Ravetch, 2008; Higurashi *et al.*, 2012).

The downstream ROS elicited effects of F_cR binding can also present as a disease phenotype, for example in the condition Wegener's granulomatosis (WG). WG patients possess anti-neutrophil cytoplasmic antibodies (ANCA) typically towards the neutrophil serine protease PR3, which in WG is expressed on the neutrophil surface. The population of the neutrophil surface by ACNAs against PR3 causes cell activation by F_cγRII, degranulation and subsequent vasculitis as a result of secreted ROS (Schilder, 2010).

Complement

Complement proteins are a small family of molecules which typically exist as pro-proteins in systemic circulation. Upon activation, they are cleaved into active forms by proteases such as C3-convertase which act in unison with F_c mediated interactions with leukocytes to amplify the inflammatory response. Leukocytes possess surface receptors for numerous different complement components, binding of which results in cellular activation. Mo/MΦ lineage phagocytes possess the complement receptors; C5aR, C3aR, CR1, CR3/4, cC1qR, C1qRp. Neutrophils possess an identical receptor fingerprint with the inclusion of gC1qR. The genomic and transcriptomic events which precede complement receptor binding are less well defined than their F_cR equivalents, although possibly include the influence of protein kinase C and MAP kinases. Consequently, complement receptor activation results in pro-inflammatory signal transduction cumulating in phagocytosis, NADPH oxidase activation, ROS generation and neutrophil degranulation. It has been demonstrated that different complement receptors stimulate respiratory burst activity to varying amounts; one study in particular showed that C3a stimulated a production of H₂O₂ at a rate 100-fold less than if stimulated with C5a (Ehrengruber *et al.*, 1994; Klegeris and McGeer, 1994; Leslie, 2010).

Pattern Recognition Receptors (PRRs)

Phagocytes possess a number of receptor classes, termed pattern recognition receptors (PRRs), which recognise and bind numerous highly conserved biomolecules. These include PAMPS (pathogen associated molecular patterns) that are associated with microbial threats, DAMPS (danger-associated molecular patterns) that are associated with tissue damage or apoptotic cells and RAMPS (resolution-associated molecular patterns) associated with wound healing (Shields, 2011). This PRR system allows specific and rapid destruction and antigen presentation of broad classes of infectious organisms or infected self, whilst the genomic recombination to create antigen specific lymphocyte support is underway in the thymus.

Indeed, numerous pattern recognition receptors are dual functional – recognising both exogenous PAMP stimuli and endogenous DAMPs also (Jeannin *et al.*, 2008). An example of this occurs during apoptosis, when macrophages specifically recognise apoptotic cells by their shuttling of ‘eat me’ signals to the cell surface – including phosphatidyl serine (PS) which is acknowledged by specific PS receptors (Wu *et al.*, 2006; Savill *et al.*, 1993). Additional apoptotic cell signals also involve the modification of membrane lipids and proteoglycans which mimic PAMPs enabling PAMP receptor dual functionality, termed apoptotic cell associated molecular patterns (ACAMPs). However, apoptotic cell clearance *via* PAMP style endocytosis is typically associated with anti-inflammatory cytokine release such as IL-10, rather than pro-inflammation and ROS synthesis.

It is paramount to consider the role of PRRs in the activation of leukocytes by tissue based/biologic biomaterials such as the decellularised xenogeneic and allogeneic tissues used widely throughout surgery. Cellular remnants such as DNA and membrane fragments which remain in these materials, as a function of insufficiently stringent processing control, will play a part in cellular activation in response to their administration (Daly *et al.*, 2012).

PRRs can be subdivided into numerous classes; for the purpose of this review focus will be on those specifically involved in phagocyte ROS production; Toll-like receptors, Nod-like receptors and C-type lectin receptors.

Toll-like receptors (TLRs)

These receptors are designated Toll-like receptors (TLRs) due to their homology with the *Drosophila* protein Toll. Phagocytes of the innate inflammatory system exhibit a conserved ability to recognise a diverse class of microbial molecules based on their TLR profile, with several TLRs demonstrating specificity with more than one microbial antigen.

TLRs have the ability to bind a diverse class of PAMP and DAMP antigens which include proteins, carbohydrates and nucleic acids. TLRs recognise extracellular PAMP antigens and intracellular antigens such as nucleic acids as part of the phagocytic process or during release from virally infected cells. Examples of PAMPs and their respective TLRs include lipoteichoic acid and mannan on gram negative bacteria and fungi respectively (TLR2+TLR6), prions (TLR2), lipopolysaccharide (LPS) (TLR4), flagellin (TLR5) and viral RNA (TLR7/8) (Kawai and Akira, 2007; Kumagai and Akira, 2010).

TLRs can recognise bacterial epitopes directly or through interaction with chaperones and accessory proteins. LPS recognition, for example, relies on a shuttling of soluble LPS by seroma LPS binding protein onto CD14 which then presents to and activates TLR4 (Defranco *et al.*, 2007).

TLR stimulation results in a storm of intercellular transcription events which generally focus on promoting inflammatory cytokine production through NF- κ B. However, TLR activation also activates MAPKs, including p38, ERK and JNK pathways critical in NADPH oxidase assembly and function. *In vitro* studies have shown that

LPS stimulates ROS synthesis *via* TLR4-mediated Rac1 and NOX activation. This compounds the intracellular inflammatory signalling cascade by acting as second messengers for the classic redox dependent NF- κ B pro-inflammatory pathway (Kasahara *et al.*, 2011). Phagocyte killing of ubiquitous *Staphylococcus aureus* is contributed to an interaction with TLR2 which activates ROS production by trigger of p47^{phox} phosphorylation, through a yet to be defined mechanism. This killing is independent of the classical p38 MAPK pathway and is thought to involve the interleukin-1 receptor kinase; IRAK4, highlighting the intracellular convergence of numerous extracellular binding events with the common outcome of ROS production (Jann *et al.*, 2011).

MCP-1 (monocyte chemoattractant protein-1) is secreted by macrophages to recruit continuing waves of myeloid cell support to a wounded or healing tissue. Binding of TLR9 by bacterial nucleotide motifs stimulates macrophage MCP-1 release which has been shown to be *via* a ROS-dependant mechanism involving phospholipase A₂, arachidonic acid and JNK, which ceases when NADPH oxidase is inhibited.

Nod-like receptors

Nucleotide binding oligomerisation domain (Nod)-like receptors are a family of intracellular PAMPs associated with infected self and endosomal microbial degeneration. NOD activation is typically associated with driving NF- κ B and pro-caspase-1 inflammasomes and reports linking NOD-like receptor activation to specific ROS release are rare, although NLRX1 over-expression has been shown to enhance ROS production. NOD1 and NOD2 bind elements of peptidoglycan which is present in almost all bacteria. It has been demonstrated that stimulation of NOD2 leads to activation of p38 and ERK MAPKs which both play a function in NADPH oxidase activation, therefore making it reasonable to assume that NOD receptor stimulation by intracellular PAMPs could potentially result in ROS generation (Kanneganti *et al.*, 2007; Philpott and Girardin, 2010).

C-type lectin receptors

C-type lectin receptors (CLRs) are a group of extracellular receptors expressed on Mo/M Φ lineage phagocytes and some subsets of stromal cells which typically, although not exclusively, recognise conserved prokaryotic carbohydrate motifs. These receptors can be broadly categorised into 2 classes, based on their ability to bind mannose or galactose type ligands. CLRs include the macrophage mannose receptor (CD206) which has the ability to bind numerous microbial membrane constituents including mannose (viruses, fungi bacterial), fucose (helminths, bacteria) and N-acetyl glucosamine. Dectin-1 is possibly the most studied CLR on Mo/M Φ cells, which recognises specific β -glucan motifs expressed on the surface of fungi (Herre *et al.*, 2004). Binding of dectin-1 has been shown to result in ROS generation through syk (spleen tyrosine kinase) signalling. The inflammatory consequences of mannose receptor CD206 binding are much less defined with both pro- and anti-inflammatory events occurring as a consequence of the specific carbohydrate with which it

interacts (McGreal *et al.*, 2005; Osario and Reis e Sousa, 2011; Vautier *et al.*, 2012).

Nucleotide receptors

Free nucleotides, particularly ATP are a classic DAMP; they are abundant in wound sites, by virtue of tissue damage resulting in cell lysis or are secreted by numerous cells throughout inflammation. Studies have demonstrated the capacity of extracellular nucleotides to stimulate ROS release from both macrophages and neutrophils. This occurs as a result of nucleotide interaction with P2 nucleotide surface receptors. The P2 receptors are a large family of surface molecules; broadly designated into P2X (ligand gated ion channels) and P2Y (G-protein coupled receptors). Particular interest has been designated to the P2X₇ receptor which has been shown to modulate a diverse set of inflammatory functions by activation of MAPKs which includes ROS release. Interestingly ADP has also been shown to trigger a respiratory burst in neutrophils through a mechanism which is independent of the MAPK ERK, in this instance implying PKC (protein kinase C) as the NADPH activating kinase (Gozal *et al.*, 2001; Di Virgilio *et al.*, 2001).

Chemokine receptors

The inflammatory milieu is abundant with soluble cytokines which are the primary language by which leukocytes and stromal cells communicate to manage the way in which a wound heals. Therefore, it is no surprise that the effects inflammatory cytokines exert on professional phagocytes often include a respiratory burst.

The catalytic gp91^{phox} (NOX2) subunit of phagocyte NADPH oxidase is up-regulated in response to inflammatory cytokines. IFN γ regulates gp91^{phox} gene expression through both interferon regulatory factor1 (IRF1) and STAT1 binding of the interferon stimulated response elements located in the gp91^{phox} promoter region. NF- κ B, a major downstream product of a multitude of leukocyte activation stimuli has also been demonstrated to up-regulate gp91^{phox} transcription (Lambeth *et al.*, 2007). Additional to ROS, previous studies have also demonstrated the ability of human Mo/M Φ cells to increase their NOS synthesis in response to IFN γ (Bose and Farina, 1995).

Cytokine activation of phagocytes does not necessarily involve classical inflammatory soluble molecules. Insulin has been demonstrated to increase phagocyte O₂ production through an NADPH oxidase dependent pathway which involves activation of ERK, p38 and NF- κ B (San Jose *et al.*, 2009).

In addition to the direct stimulation and upstream transcriptional regulation of NADPH by inflammatory molecules, cytokines also have the ability to 'prime' the respiratory burst. In this instance, the cytokine will not directly stimulate ROS release but will hypersensitise the cell to stimulation by a microbial ligand, resulting in a more rapid and amplified antimicrobial respiratory burst. This is the case with IL-8, which amplifies ROS release from neutrophils in response to stimulation with the bacterial peptide fMLP, however alone does not stimulate ROS release unless used in stochastically high concentrations (Brechard *et al.*, 2008). It was shown that this is a

consequence of IL-8 mediating an influx of intracellular Ca²⁺, although the precise molecular mechanism of NADPH activation was not defined. This priming role is also true of IL-12 which has been shown to augment the ROS response of primary leukocytes to opsonised zymosan (Yano *et al.*, 2003). TNF α and GM-CSF have also been shown to prime neutrophils in a manner which involves phosphorylation of p47^{phox} resulting in conformational changes required for NADPH oxidase activation. Interestingly, these two chemokines have completely different surface receptors but both prime by the same mechanism. G-CSF and IL-1 β do not cause p47^{phox} phosphorylation and prime to a much smaller extent (Dewas *et al.*, 2003; Tarr *et al.*, 2003).

TNF α is also a potent primer of phagocyte reparatory burst. In polymorphonuclear cells this is through a p38 MAPK mediated process in which intracellular granules containing cytochrome b₅₅₈ exocytose, increasing the expression of in-tact NADPH oxidase on the cell surface (Ward *et al.*, 2000b).

The interaction of cytokines with inflammatory cells may also inhibit or suppress ROS production. IL-10, the major anti-inflammatory interleukin, has been shown to suppress ROS release. It has been hypothesised that the method of suppression varies as we age. In younger people it was shown that IL-10 inhibited ROS production in response to IFN γ stimulation, through interaction with the protein kinase A (PKA) pathway which switches to the p38 pathway towards middle age (Chaves *et al.*, 2009). In the elderly IL-10 showed no suppressive effects on ROS release. Similar results were also obtained considering leukocytes from type II diabetic patients who showed that IL-10 had relinquished its typical ROS inhibitory properties in this disease state (Nogueira-Machado, 2003).

Although not regarded as a pro-inflammatory cytokine TGF β has been implicated in directing the assembly of functional NADPH oxidase on the membrane of pre-monocytic cells (Basoni *et al.*, 2006).

Measuring ROS Production

A number of techniques exist for monitoring and measuring cellular ROS which are typified by ROS causing excitation or a spectroscopic shift of one of a multitude of reporter molecules. This can then be quantified using luminometry, flow cytometry or microscopy. ROS reporting assays can be tailored to investigate the role of particular oxidative species in a cell response by using probes with specific interactions with target oxidants or by inclusion of scavenger molecules such as formate (OH⁻), deferoxamine (Fe²⁺), diethyldithiocarbamic acid (SOD inhibitor) and salicylhydroxamic acid (myeloperoxidase inhibitor) to ablate defined ROS or ROS precursors from the reaction milieu.

Pholasin[®]

The authors have used Pholasin[®], a luminescent protein, as a ROS probe with great success for quantifying the respiratory burst of leukocytes as a component of pre-clinical evaluation of implantable biomaterials. Pholasin[®] is a promiscuous ROS excitable photo-protein derived

from the marine mollusc *Pholas dactylus*, which emits measurable photons in the presence of most intra and extra cellular derived ROS – including superoxide anion, hydroxyl radical and possibly ferryl radical as well as singlet oxygen and the non-radical oxidants: hypochlorous and hypobromous acids and peroxyxynitrite. Therefore Pholasin[®] provides a sensitive measure of all cellular secreted ROS without species specific discrimination. Pholasin[®] is not actively internalised into cells therefore is considered a specific measure of ROS released as a function of phagocyte degranulation (Knight *et al.*, 1996; Knight, 1997; Muller *et al.*, 1998; Reichl *et al.*, 2000; Reichl *et al.*, 2001; Glebska and Koppenol, 2005; Bryan *et al.*, 2012a; Bryan *et al.*, 2012b).

Luminol

Luminol (5-Amino-2,3-dihydro-1,4-phthalazinedione) is a synthetic molecule of which the ability to oxidise producing N₂ and light emitting aminophthalate is widely exploited in molecular biology. Despite this, in the context of cellular ROS its interactions with phagocyte derived oxidants are poorly understood and the precise species which stimulate its excitation are debated. Reports vary as to the specificity of luminol for precise species. However, it is generally regarded that myeloperoxidase activity is crucial for luminol excitation by leukocytes suggesting a rate limiting role of hypohalous acids in its fluorescence emission. Whilst the influence of the MPO arm of the leukocyte ROS system on luminol is reasonably well agreed upon, studies often contradict one another with respect to the ability of additional ROS to excite this probe. Conflicting reports have hypothesised that luminol can also be excited by H₂O₂, OH⁻, O₂⁻, and ONOO⁻. Luminol has the ability to diffuse across cell membranes, which means that it reports both intra and extracellular ROS and can also be used microscopically to visually track ROS producing cells. Recently, luminol has also been used to track reactive ROS *in vivo* by means of delivery into the peritoneum and subsequent observation of ROS in response to subcutaneously delivered biomaterials using whole animal imaging of hairless mice (Vilim and Wilhelm, 1989; Samuni *et al.*, 1991; Faulkner and Fridovich, 1993; Dahlgren and Karlsson, 1999; Myhre *et al.*, 2003; Rinaldi *et al.*, 2007; Liu *et al.*, 2011).

2,7-Dichlorodihydrofluorescein (DCFH)

DCFH is oxidised in the presence of ROS to 2,7-dichlorofluorescein (DFH), a fluorescent compound which emits light at 520 nm. A precursor molecule, DCFH diacetate, can traverse cell membranes readily and become de-acetylated into bioactive DCFH by intracellular esterases. This molecule is therefore suitable for monitoring of both intracellular and extracellular ROS. It is another promiscuous reporter molecule; excited by superoxide, hydroxyl, and peroxy radicals in addition to reactive nitrogen species. Reports have also suggested that DFH-mediated fluorescence may be a product of enzymatic oxidation of DCFH by intracellular oxidases such as SOD and xanthine oxidase or interaction with haeme and haemoproteins, which is of paramount consideration when utilising this probe to monitor leukocyte ROS emission in

a whole blood environment. Additionally, the succinimidyl ester of 2',7'-dichlorodihydrofluorescein diacetate is amine reactive and therefore can be conjugated to a number of substrates and proteins which has allowed study of material uptake by phagocytes and monitoring of ROS release occurring directly within the phagosome (Ohashi *et al.*, 2002; Myhre *et al.*, 2003; Gomes *et al.*, 2005; Rinaldi *et al.*, 2007; Tlili *et al.*, 2011; Kalyanaraman *et al.*, 2012).

Lucigenin

Lucigenin (10-Methyl-9-(10-methylacridin-10-ium-9-yl)acridin-10-ium dinitrate) is another synthetic ROS probe; however, in contrast to luminol it is not membrane permeable. Lucigenin is typically considered to be specific to superoxide in extracellular fluids (Rathakrishnan and Tiku, 1993; Faulkner and Fridovich, 1993; Myhre *et al.*, 2003).

Cytochrome C

The spectroscopic shift caused by the reduction of cytochrome c (cyt.Fe³⁺) can be used to quantify production of superoxide. Iron bound to cytochrome C becomes reduced (cyt.Fe²⁺) in the presence of this radical which causes a measurable absorbance maxima at 550 nm (Sanders *et al.*, 1994; Hadjur and Jardon, 1995; Rinaldi *et al.*, 2007).

Electron spin resonance (ESR)

ESR utilises a one of a family of 'spin trapping' molecules. In this technique, ROS binds to a synthetic molecule causing a measurable shift in the ESR of that molecule. The shift will vary as a function of the numerous different ROS species which could bind the reporter resulting in a series of potential molecular configurations. The result is a quantifiable ESR spectroscopic fingerprint simultaneously interrogating multiple radical species in a single assay. Typically, spin traps are nitron or nitroso compounds. Nitron compounds react with most cellular ROS however the nature of the physicochemical interaction between ROS and these reporters makes resolving the precursor radical species difficult. Nitroso compounds react with a more limited range of ROS, however their origins are easier to resolve. Spin trap molecules include MNP (2-methyl-2-nitrosopropane), 3,5-dibromo-4-nitrosobenzene sulfonic acid, DBNBS (3,5-dibromo-4-nitrosobenzene sulfonic acid) and DMPO (5,5-dimethyl-1-pyrroline N-oxide) (Sanders *et al.*, 1994).

ROS Considerations in Wound Healing and Biomaterials

Wound healing

Wound healing is a complex and challenging cellular and biochemical event which requires the uniting of numerous cells to synergise and remodel the compromised tissues. Within a wound healing niche many cell types exist, whose signals to one another in both endocrine and paracrine fashions ensure a return to normal homeostasis of the damaged area. Wound healing involves an optimal combination of the constructive and destructive roles of

cells and their associated ROS to remodel tissue in parallel to eradicating infectious organisms and non-self-material.

Leukocytes possess a vast number of systems to influence the healing of a tissue. Arguably, most researched are the interleukin family of proteins which are the major language with which cells in a healing wound converse. Interleukins have the ability to mediate leukocyte chemotaxis (IL-8), promote (IL-1, IL-6, IL-17, IL-23) and demote (IL-10) inflammation in addition to controlling the development and specificity of immunological memory (IL-2, IL-4, IL-15, IL-7). Additional protein signals are secreted by leukocytes and stroma into the wound healing milieu and play a pivotal role in driving appropriate cell populations within the tissue space, including TGF β , TNF α , MCP-1 and IFN γ .

Further to their signalling roles, proteins secreted by leukocytes also have destructive properties. Typically, in the form of enzymes which possess the ability to degrade or reduce the integrity of bacterial extracellular matrices (perforin, defensins). This results in stasis, lysis or creates pores through which further leukocyte secreted compounds can enter and corrupt the intercellular husbandry of an infected cell to induce apoptosis (granzyme) or deplete extracellular fluids of vital trace minerals required for bacterial proliferation (lactoferrin) (Gutteridge *et al.*, 1981; Okutomi *et al.*, 1997; Nathan, 2006; Pham, 2008).

Similarly to proteins, ROS have the ability to act in both signalling and host defence capacities. Also similar to proteins such as matrix metalloproteinases, ROS have the potential to cause catastrophic damage to otherwise healthy tissue if left un-harnessed, therefore in the healing wound it is imperative that a balance is struck between the antimicrobial and tissue degenerative effects of phagocyte ROS. Indeed, it is this ROS-mediated tissue damage which plays a significant role in ageing and it has been hypothesised that the reliance on ROS during inflammation becomes greater throughout ageing (Khodr and Khalil, 2011).

ROS has also been implicated in causing fibrosis and scar formation. In a murine model of hepatic fibrosis, inclusion of the antioxidant α -lipoic acid and an α -lipoic acid derivative was shown to reduce fibrotic/cerotic effects by inhibiting ROS production and subsequent signalling by hepatic stellate cell activated by PDGF and TGF β (Poli, 2000; Foo *et al.*, 2011). ROS have also been implicated in pulmonary fibrosis, also involving the actions of TGF β (Van der Vliet, 2008).

Chronic wounds are often characterised by the presence of excessive ROS or the absence of antioxidant ROS scavenger molecules such as vitamins E, C and glutathione. Interestingly, levels of wound antioxidants have been shown to decrease as we age, which corresponds to the delayed wound healing responses seen in the elderly. This suggests that reduced or delayed wound healing occurs as a consequence of lower concentrations of antioxidants allowing the wound ROS reaction to proceed unchecked, progressively compounding tissue damage (Schafer and Werner, 2008). This phenomenon has been shown in tissues as diverse as brain, demonstrating increased ROS synthesis in the absence of vitamin C (Kondo *et al.*, 2008).

The use of antioxidants to enhance healing of chronic wounds has also been shown to be advantageous in conjunction with hyperbaric oxygen therapy. In this instance inclusion of α -lipoic acid to restrict ROS mediated tissue damage was shown to enhance chronic wound healing whilst also decreasing the production of pro-inflammatory IL-6 (Alleva *et al.*, 2005).

Similarly, in a mouse model of protein malnutrition (PM), known to delay wound healing the dietary inclusion of N-acetylcysteine, an antioxidant which causes an increase in cellular glutathione was shown to enhance tissue repair. Furthermore, the study also used transgenic mice expressing varying amounts of SOD. Up-regulating this enzyme in PM was shown to also enhance wound healing however when animals were not fed a protein restricted diet this up-regulation impeded wound healing, suggesting a basal level of ROS is critical in appropriate and rapid tissue repair (Gordillo and Sen, 2003; Lim *et al.*, 2006; Sen and Roy, 2008). Studies using knockout mice in which the gp91^{phox} and p47^{phox} subunits of NADPH were ablated concluded that in response to LPS, ROS actually play a key role in inflammatory resolution with knockout animals displaying greater production of inflammatory cytokines in response to LPS than wild-type equivalents (Zhang *et al.*, 2009).

Despite the obvious cytotoxic effects of ROS, a basal level within the wound is advantageous outside of their role in sterilisation. For example, H₂O₂ is chemotactic to numerous cells involved in wound healing, particularly keratinocytes in which low levels of H₂O₂ promote cell migration and proliferation through activation of the ERK pathway (Loo *et al.*, 2011). A low level of H₂O₂ (10 μ M) has also been shown to be chemotactic to neutrophils (Klyubin *et al.*, 1996). Interestingly, a further study utilised the antioxidant enzyme thioredoxin to inhibit neutrophil chemotaxis by LPS and also adhesion to epithelium, which would be reasonable to speculate was *via* a ROS-dependant mechanism (Nakamura *et al.*, 2001).

In addition to phagocytes, stromal cells also have the ability to respond to ROS to orchestrate inflammation. A previous study showed that epithelial cells secreted the pro-inflammatory molecules ICAM1, TNF α , MCP-1, IL-8 and IFN α in response to H₂O₂ in a dose dependant manner. It was hypothesised that this was due to H₂O₂ induction of NF- κ B (De Oliveira Marques, 2007). Interestingly, this study also observed the synergistic expression of haeme oxygenase-1 (HO-1) which is anti-inflammatory, synthesising carbon monoxide which inhibits IL-1 β and TNF α expression in macrophages.

Fibroblasts, whose proliferation, matrix deposition and potential differentiation play an important role in the sealing of a wound by secretion of collagens and other extracellular matrix proteins, which form a physical barrier to impede passage of microbes either into or through tissues of an organism, are also influenced by ROS. NADPH oxidase-derived ROS have been demonstrated to mediate the phenotype of fibroblasts by acting downstream of angiotensin II to stimulate differentiation into myofibroblasts through an influence on p38 and JNK signalling cascades. Indeed, ROS is implicated in

several instances of fibrosis, diseases characterised as abnormalities in fibroblast number and phenotype in a diverse number of tissues from lung to heart (Shen *et al.*, 2006; Lambeth, 2007).

Biomaterials

Intercellular ROS play a role downstream of extracellular growth factor receptor activation to induce transcriptional modification to coordinate several proliferative and remodelling effects, demonstrated studying the mitogenic effects of EGF on corneal epithelial cells (Huo *et al.*, 2009). Highlighting the fine dose dependency of the beneficial and detrimental effects of ROS, the cytotoxic effects of 2-hydroxy-ethyl methacrylate (HEMA) and triethylene glycol dimethacrylate (TEGDMA) degradation products of resins, adhesives and cements used commonly in dental engineering have been contributed to an increase in intracellular ROS by oral epithelial cells (Chang *et al.*, 2005; Demirci *et al.*, 2007).

In response to excessive ROS within a wound, leukocytes and stromal cells possess a number of both intracellular, extracellular and membrane bound antioxidant systems to scavenge ROS before healthy tissue can become compromised. SOD may itself be considered a detoxification enzyme by dismutating the highly toxic superoxide radical into less destructive hydrogen peroxide. However, left undisposed of hydrogen peroxide has the potential to form the most reactive of all the radicals, $\cdot\text{OH}$. Therefore, the enzymes glutathione peroxidase and catalase are produced, which both deconstruct hydrogen peroxide into water and oxygen. In addition to proteinaceous ROS control, fundamental antioxidant metabolites such as ascorbate, α -tocopherol, NADPH, urate and lipoic acid all possess ROS-scavenging abilities. Indeed, it has been demonstrated that up-regulations occur in ROS scavenging enzymes during cutaneous wound healing, particularly at the wound edge possibly with the goal of protecting proliferative cells from the harmful oxidants contained within the compromised interstitial fluids (Steiling *et al.*, 1999).

A previous study also implicated the ROS scavengers SOD and catalase as playing a major role in healing of periodontal wounds, whilst also implicating ROS in mediating the resorption profile of PLA implanted materials (Aliyev *et al.*, 2004).

The importance of understanding the influence of biomaterials on wound healing ROS is widely acknowledged. Phagocytes interact directly and intimately with biomaterial surfaces and *via* receptor mediated activation through opsonisation of the material surface with endogenous protein or integrin remodelling. Foreign surfaces will almost inevitably lead to phagocyte ROS production. Indeed, previous research has demonstrated that the surface contact by the $\beta 2$ integrin chain (CD18) is a rate-limiting step in the release of phagocyte ROS stimulated by TNF α (Abram and Lowell, 2009).

The potential of a biomaterial to undergo phagocytosis greatly enhances its ability to stimulate cellular ROS as it is incorporated into the phagosome. Studies have demonstrated, using zinc nanoparticles, that macrophages produce more significant ROS in response to smaller

particles (10-30 nm) which corresponds to an optimal size to phagocytose (Song *et al.*, 2010).

Previous studies have demonstrated the ability of biomaterials to quench ROS release, demonstrating the intrinsic antioxidant capacity of several biomaterials which may be advantageous particularly in chronic wounds.

Previous work using hyaluronan based biomaterials has demonstrated their ability to quench superoxide released from polymorphonuclear phagocytes *in vitro* in a dose dependant manner. It was hypothesised in this study that the polymer forms a meshwork around individual cells making it difficult for ROS to escape into extracellular spaces (Moseley *et al.*, 2003). Research has also suggested coenzyme Q10 (CoQ10), a naturally occurring quinone endogenous to most cells, as a biomaterial component due to its native role in oxidative stress protection. CoQ10 has been grafted onto PLGA microspheres which demonstrated an ability to quench ROS greater than CoQ10 alone and has been hypothesised for use in fields associated with ROS elevation (Swarnakar *et al.*, 2011).

Metals and metallic compounds have also been highlighted as excellent candidate materials for managing and manipulating ROS. Platinum-ferritin substrates have been demonstrated to act in a manner analogous to catalase and peroxidase in ROS detoxification. The widely acknowledged biocompatibility of titanium has been hypothesised to be due to the ability of this metal to scavenge ROS onto its surface during titanium oxide formation *in vivo* (Taylor *et al.*, 1996; Fan *et al.*, 2011).

Additionally, studies have hypothesised fullerenes as biomaterial constituents due to their ability to scavenge ROS and protect primary cells from oxidative stress (Markovic and Trajkovic, 2008; Yin *et al.*, 2009).

The ability of biological polymers to scavenge ROS has also been considered in the instance of wound dressings (Schonfelder *et al.*, 2005). Research has shown that dressings synthesised from oxidised regenerated cellulose (ORC) and ORC:collagen co-polymers quench ROS more than collagen alone. However, collagen did demonstrate some ROS quenching capacity. A wound dressing with ROS scavenging capability would be particularly advantageous in the instance of chronic non-healing wounds, where ROS contribute massively to the non-healing nature of the damaged tissue. ROS quenching is not an implied characteristics of a biopolymer though, chitosan and chitosan/soy copolymers have been demonstrated not to quench ROS produced by fMLP/PMA leukocyte induced activation, although these polymers were shown themselves not to exhibit any intrinsic ROS stimulating potential (Santos *et al.*, 2007).

It may also be possible to manage and manipulate ROS in a biomaterial context by inhibition of the ROS synthesis pathway. It has been suggested that antibodies against ROS synthetic enzymes such as myeloperoxidase and components of NADPH oxidase may play a future role in eliminating the products of these enzymes from the inflammatory milieu by cross-linking and subsequent deactivation. Conversely, it may also be possible to include recombinant or purified ROS processing enzymes such as SOD and catalase and synthetic mimetics of these proteins as part of a material delivery strategy to control ROS

damage during material integration (Cheung *et al.*, 2008; Nishikawa *et al.* 2009).

The ROS stimulating capabilities of a material come not only from the material itself but also as a consequence of the material becoming coated with proteins the instant it contacts blood (Nygren *et al.*, 2001). Often, particularly in the case of complement and immunoglobulins, these proteins have the capacity to stimulate ROS production. In response to this, much research is targeted on the production of anti-fouling surfaces which inhibit protein adhesion, or the generation of surfaces which include molecules such as nitroxide radical containing polymers, akin to those used for spin trap ROS characterisation which ablates ROS by conversion into a stable non radical product on the material surface (Yoshitomi *et al.*, 2012). Similar techniques using nitroxide radicals grafted onto biopolymer nanoparticles has been suggested as therapeutic devices to control the inflammatory damage occurring during acute tissue injury, with a particular interest in renal damage (Yoshitomi *et al.*, 2011).

ROS and Cell Signalling

Outside of their destructive role, ROS play a part in coordinating inflammation through their signalling capacity. The change in focal cellular Redox which accompanies ROS release is an important messenger system both inside and outside the cell. The molecular criteria of a successful signalling molecule suggest that it should be enzymatically generated and the signal should have the propensity to be ceased also by enzymatic degradation. This set of boundaries is fulfilled by several ROS including NO, O₂⁻ and H₂O₂. The destructive role of ROS is attributed to their deforming properties on biological polymers, which may also contribute to their signalling capacity allowing them to irreversibly modify proteins and transcription factors to influence cell fate and phenotype (Fialkow *et al.*, 2007).

Intracellular ROS Signalling

The redox state which accompanies a change in intracellular ROS plays a pivotal role in translating the binding of extracellular receptors into functional transcription, this has been implied in multiple signalling cascades including MAPK/ERK, PKC and NF-κB inducible kinase involving transcription factors such as NF-κB, AP-1 and NRF-2 which combined have the ability to mitigate a diverse range of inflammatory cellular outcomes (Gupta *et al.*, 2011). ROS mediated activation of NF-κB alone may result in the production of MCP-1, IL-6, TNFα and IL-1α/β (Millar *et al.*, 2007; Naik and Dixit, 2011).

In cells, NF-κB is repressed by its inhibitory molecule, IκB. Phosphorylation of IκB by IκB kinase releases NF-κB allowing translocation to the cell nucleus and transcription of numerous NF-κB mediated genes which in phagocytes includes several cytokines (IL-1, IL-6, IL-8, MIP-2), cytokine receptors, acute phase proteins, MHC and adhesion molecules rate-limiting in inflammation and wound healing (Clark and Valente, 2004; Abram and Lowell, 2009). This dissociation can occur as a result of

an increase in intracellular H₂O₂ concentration which in monocytic cells stimulated with IL-1β has been shown to be a downstream product of NADPH oxidase activation.

H₂O₂ has been demonstrated to act as a second messenger during dendritic cell antigen presentation to T-lymphocytes. This process is complex and requires precise and intricate communication between the two inflammatory cells. Increase in dendritic cell ROS was shown to increase expression of CD86, directly corresponding to an increase in antigen presentation. However, inhibition of cellular redox signalling reduced secretion of IFNγ, IL-2 and IL-4 in the T-lymphocytes and IL-6 and IL-12 in the antigen presenting cells. H₂O₂ has also been implicated as a second messenger in Fc receptor mediated phagocytosis, possibly through ROS interaction with the tyrosine kinase syk.

NADPH oxidase produced ROS also act as second messengers to external proliferative signals, demonstrated using ceramide-1-phosphate. In this instance the signal transduction was elucidated to be *via* activation of the enzymes phospholipase A₂ and PKC (Arana *et al.*, 2012).

ROS also has the ability to positively feedback to NADPH oxidase to regulate further O₂⁻ production. H₂O₂ has been shown to be a potent mediator of NADPH oxidase activation through a mechanism which involves an influx of Ca²⁺ *via* store-operated Ca²⁺ (SOC) channels, which are activated by emptying of intracellular Ca²⁺ stores as a consequence of the change in intracellular redox. Calcium influx activates the Ca²⁺ dependant PKC which phosphorylates and activates p47^{phox} (Jamali *et al.*, 2010). Contrary to H₂O₂ feedback, NO has been shown to decrease ROS production by inhibiting NADPH oxidase activity through a mechanism which involves deactivation of PKC, demonstrating two feedback loops targetting the same kinase with totally opposing cellular consequences (Klink *et al.*, 2009).

Intracellular phagocyte ROS signalling events also directly influence the fate of alternative inflammatory cells. Intracellular H₂O₂ has been demonstrated to decrease transcription of the IL-12, a potent mediator of T_H1 T cell polarisation *via* inhibiting translocation of its transcription factor c-rel to the cell nucleus (Kahn *et al.*, 2006).

During inflammation the intracellular ROS signalling by non-haematopoietic cells also plays its part. In endothelial cells, omnipresent in vasculature, increase in intracellular ROS causes a release of stored Ca²⁺ from the endoplasmic reticulum into the cytosol. This activates Ca²⁺ dependent MAPK signalling and extracellular P-selectin expression, which causes neutrophil cells to adhere to endothelium (Millar *et al.*, Phan and Tibbles, 2007). H₂O₂ has also been demonstrated to up-regulate intercellular adhesion molecule (ICAM) expression in endothelial cells, which plays an important role in leukocyte transmigration and extravasation during inflammation (Hubbard and Rothlein, 2000).

In addition to H₂O₂ and the well-documented signalling roles of RNS, despite its short lived nature O₂⁻ has also been demonstrated to have a signalling capacity in addition to its disinfectant properties. O₂⁻ directly stimulates PKC activity through interaction through liberation of zinc from its zinc finger region. PKC activation has the propensity

to mediate multiple downstream cellular events including proliferation, transcription and differentiation (Knapp and Klann, 2000).

Extracellular ROS signalling

As stated previously, ROS are ideal signalling molecules due to the intricacy and control with which they can be synthesised and degraded. Coupled with this, the fact that several ROS – particularly H_2O_2 – are membrane permeable, make them excellent candidate paracrine signalling molecules that could influence multiple other haematopoietic and non-haematopoietic cells within the inflammatory milieu.

Exogenous oxidants have been shown to increase the rate of F_cR mediated phagocytosis by professional phagocytes. This was a consequence of H_2O_2 amplifying phosphorylation of intracellular F_cR ITAM motifs. The downstream signalling associated with F_cR stimulation involves activation of the transcription factor *syk*, the rate of which was increased in H_2O_2 treated leukocytes, confirming the downstream functionality of ROS enhanced F_cR efficiency (Pricop *et al.*, 1999).

Myeloid cell ROS has been shown to directly influence the role of the adaptive arm of the immune system in inflammation through NADPH oxidase produced ROS. Macrophages can be categorised into two classes based on their inflammatory (M1) or anti-inflammatory (M2) cytokine release profile. M1 macrophages interact with aggressive T_h1 CD4 T lymphocytes which maintain the inflammatory response in an acute phase. M2 macrophages cross-talk with T_h2 CD4 T-lymphocytes that drive inflammation towards a humeral phase. Studies have demonstrated that ROS secreted from M2 anti-inflammatory macrophages polarise the CD4 T-lymphocyte subset towards a T_{reg} phenotype *in vitro*. T_{reg} cells are actively involved in repressing the inflammatory response, therefore utilising a classically destructive group of molecules as an inflammatory repressor *via* their paracrine activation of anti-inflammatory cells (Kraaij *et al.*, 2010). The molecular mechanism for this polarisation is undefined, although it has been postulated that this may be a result of interaction of ROS with membrane bound $TGF\beta$ on the lymphocytes.

Additional studies have also concluded an endocrine effect of ROS on lymphocytes. Incubating mixed populations of T-lymphocytes with granulocytes resulted in a decrease of $IFN\gamma$ secretion from the T-cell population. It was concluded that this phenomenon was dose dependant on H_2O_2 . In this study, H_2O_2 was shown to suppress the pro-inflammatory nature of T-cells by additionally to $IFN\gamma$ also repressing $TNF\alpha$, IL-2 and IL-4 production. This was implied as a potential mechanism for the reduction in T-cell response observed towards tumours in advanced cancer patients (Schmielau and Finn, 2001). Reduction of T-cell cytotoxic potency by H_2O_2 has also been contributed to its ability to influence the TCR, reducing the expression of CD3 ζ chain and also CD16 ζ in Natural Killer (NK) cells (Kono *et al.*, 1996; Kumar and Sharma, 2010).

Apoptosis, unlike necrosis is an anti-inflammatory event critical in the resolution of inflammation. During this process cells undergo programmed death without

elution of their cytotoxic intracellular compartments into the inflammatory milieu. Further anti-inflammatory ROS roles may be implicated in the ability of exogenous NO and ONOO⁻ to cause neutrophil apoptosis. Although the apoptotic influence of NOS on phagocytes is well known, contrasting reports exist over the finer points of the phenomenon, which numerous dose dependant relationships suggest for windows in which NOS exhibit anti- rather than pro-apoptotic effects and, additionally, these observations may vary as a function of species (Ward *et al.*, 2000a; Taylor *et al.*, 2003).

Although yet to be demonstrated in a mammalian model, zebra fish studies have elegantly demonstrated a chemotactic role of H_2O_2 in recruiting phagocytes to wounded tissue. The H_2O_2 released from damaged tissue termed the 'wound burst' may play a role in recruiting the first wave of inflammatory cells to the wound. Phagocytes have been shown to chemotax up H_2O_2 gradients and a redox sensitive kinase; Lyn of the Src kinase family has been implicated in the process. However, the precise mechanism by which activation of this kinase translates to cellular directional motility is yet to be defined (Yoo and Huttenlocher, 2009; Yoo *et al.*, 2011).

Exogenous H_2O_2 has also been demonstrated to stimulate dendritic cell maturation from immature precursor cells. This was characterised by an increase in molecules associated with T-cell activation (HLA-DR, HLA-DQ, CD86, CD40) and a decrease in molecules associated with antigen capture (CD32) (Rutault *et al.*, 1999). H_2O_2 stimulated DCs also demonstrated a greater capacity to promote T-cell proliferation during antigen presentation (Matsue *et al.*, 2003).

Conclusion

Leukocytes are pivotal in orchestrating debridement, disinfection ultimate repair of compromised tissue. In order to achieve this, leukocytes possess an exquisite array of non-specific signalling and defensive molecules capable of undertaking constructive and destructive roles during wound healing. Crucially, leukocytes have the ability to rapidly and precisely produce a number of ROS which are incredibly cytotoxic molecules with potential to cause catastrophic damage to healthy tissue, whilst ensuring sterilisation of infected and damaged tissues. ROS also play a critical role in signalling both inside and outside of the cell. Indeed, generation of biomaterial surfaces with the ability to manage the ROS response within a healing tissue and maintain this potentially cytotoxic cellular process within a constructive window presents an extremely tantalising future prospect in medical device design. This level of coordination within a healing tissue by a material will provide a powerful antimicrobial effect during infiltration of host cells; accelerate the degradation of dead or dying cells and increasing the turnover and integration of healing stromal tissues with minimal fibrosis, scar formation and loss of mechanical strength.

Future research required in this area will involve characterisation and quantification of the species and amounts of ROS involved in normal wound healing. This

will drive fabrication of biomaterials into an exciting generation of devices which ensure that ROS levels remain within a window of normalcy to reduce the consequences of ROS exacerbation, particularly in chronic non-healing wounds.

Future understanding of ROS in wound healing will also be heightened by the stem cell field, which is currently producing fascinating data suggesting that adult stem cells are exquisitely sensitive to changes in tissue redox with the potential to greatly alter their viability, plasticity and lineage commitment (Jang and Sharkis, 2007; Kim *et al.*, 2007; Mouche *et al.*, 2007; Carriere *et al.*, 2009; Valle-Prieta and Conget, 2010; Kanda *et al.*, 2011; Kim *et al.*, 2011; Daly *et al.*, 2012). Therefore, we must ensure that the biomaterials and stem cell communities unite to make one another acutely aware of the impact of material modifications of wound site ROS in the differentiation and proliferation of stem cells during tissue repair.

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Editor's Note: All comments by the reviewers were answered by text changes, hence there is no **Discussion with Reviewers** section.