Innate Alloimmunity

1 Prologue: The Evolution of Oxygen Toxicity and its Role in Host Defense

1.1 Introduction

From an evolutionary point of view, the immune defense systems in mammals have evolved over hundreds of millions of years. In view of this immense period, the 50-year existence of organ transplantation is just a blink of an eye. There is no reason to assume that during this short time, nature has created an immune system against allografts different from that operating as an efficient host defense against infectious pathogens. Therefore, it is tempting to conclude that the innate immune response must be involved in allograft rejection as well (Figure 1.1.1). We as transplant surgeons and physicians must search for the mechanistic links between host defense against pathogens on one side and rejection of allografts on the other side. Indeed, the cardinal link seems to be the initial tissue injury, which is: (1) mediated by a large variety of pathogens associated with the generation and appearance of pathogen-associated molecular patterns (the PAMPs in terms of exogenous ligands of pattern recognition receptors [PRRs] such as Toll-like receptors [TLRs]) on one hand, and (2) mediated by any injurious nonpathogenic factor associated with the generation and appearance of damage-associated molecular patterns (DAMPs) in terms of host endogenous ligands of PRRs on the other hand—just to a term analogous to the PAMPs. The reader, for the first time, encounters here the PAMPs, DAMPs, PRRs, and TLRs, that is, terms that will accompany her or him throughout the entire book and which will be dealt with in more detail below.

There is abundant evidence suggesting that in the center of tissue injury, regardless whether it is induced by infectious, toxic, physical or other injurious events, reactive oxygen species (ROS) play a dominant role. Therefore, I devote to these toxic molecules their own chapter as a prologue. In subsequent chapters, I will try to refer to mechanistic links

between innate immune host defense on one hand and innate alloimmune graft rejection on the other hand.

In Part 2 of this book, I will present the concept that ROS play a major role in mechanisms of innate alloimmunity, leading to acute rejection and contributing to the development of chronic rejection. Thus, the main question that characterizes this monograph throughout is: Why does an ROS-injured allograft lead to an innate and adaptive host response, which then leads to its destruction? Why are both the innate immune and the adaptive alloimmune systems so efficiently prepared to combat against ROS-mediated allograft injury?

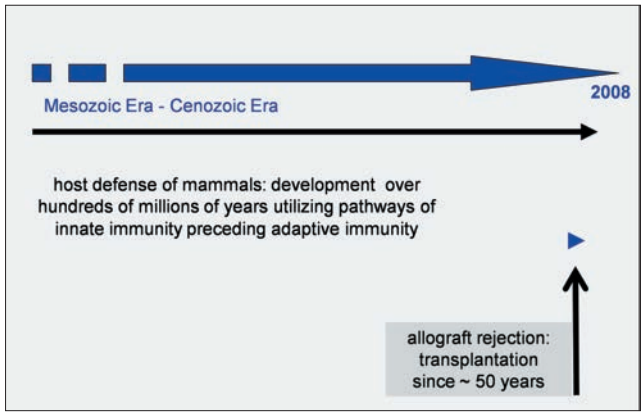

Figure 1.1.1. Host defense and allograft rejection in mammals - seen from an evolutionary perspective: common innate and adaptive immune pathways.

From an evolutionary point of view, the immune defense systems in mammals have evolved over hundreds of millions of years. In view of this immense period, the 50-year existence of organ transplantation is just a blink of an eye. There is no reason to assume that, during this short time, nature has created an immune system against allografts different from that operating as an efficient host defense against infectious pathogens. Therefore, it is tempting to conclude that the innate immune response must be involved in allograft rejection as well.

I will address this question in detail later. In this first chapter, I will briefly mention some aspects of the role these "biological devils" played during evolution, particularly the role they play in microbial pathophysiology and host defense. In doing so, a seminal work lies on my desk and accompanies me: the textbook on *Free Radicals in Biology and Medicine, Third Edition,* edited by Halliwell and Gutteridge and published by Oxford University Press in 2000 [1]. The editors point out in the preface to the first edition of their book: *"The book is aimed mainly at biologists and clinicians. It*

assumes virtually no knowledge of chemistry and attempts to lead the reader as painlessly as possible into an understanding of what free radicals are, how they are generated, and how they can react."

1.2 Oxygen Toxicity and Reactive Oxygen Species

1.2.1 The history of oxygen: a major air pollutant

The element oxygen exists in air as a diatomic molecule, oxygen, strictly speaking, should be called *dioxygen*. Except for certain anaerobic and aerotolerant unicellular organisms, all animals, plants, and bacteria require oxygen for efficient production of energy by the use of oxygen-dependent electron-transport chains, such as those in the mitochondria of eukaryotic cells. In fact, mitochondria make more than 80% of the adenosine-5'-triphosphate (ATP) needed by mammalian cells, and the lethal effects of inhibiting this process, for example, by cyanide, show how important mitochondria are. However, this need for oxygen obscures the fact that oxygen is a toxic mutagenic gas as well as a serious fire risk; aerobes survive because they have antioxidant defenses to protect against it. Oxygen appeared in significant amounts in the earth's atmosphere over 2.5×10^9 years ago; geological evidence suggests that this was due to the evolution of photosynthesis by blue-green algae (cyanobacteria). As they split water to obtain the hydrogen needed to drive metabolic reductions, these bacteria released tons of oxygen into the atmosphere. The inexorable rise in atmospheric oxygen concentrations was advantageous because it led to the formation of the ozone (O_3) layer in the stratosphere. The ability of O_3 and oxygen to filter much of the intense solar ultraviolet radiation helped living organisms leave the sea and colonize the land, but oxygen itself must have placed a severe stress on the organisms present. (Today, the reduction of the O_3 layer is believed be an essential element in global warming, which may lead to an environmental catastrophe.)

1.2.2 Oxygen toxicity in aerobes

Oxygen is now the most prevalent element in the earth's crust, and the percentage of oxygen in the atmosphere has reached 21%. The barometric pressure of dry air at sea level is 760 mm mercury, giving an oxygen partial pressure of about 159 mm Hg. Despite its many advantages, it is known that even 21% oxygen causes damage to aerobes [2]. Indeed, oxygen supplied at

concentrations greater than those in normal air has been known for decades to be toxic to plants, animals, and bacteria such as *Escherichia coli*. For example, studies of bacterial chemotaxis to oxygen (aerotaxis) show that several strains swim away from regions of high oxygen concentration and tend to settle in regions of optimal "redox state" for their growth (Box 1.2). *Escherichia coli* also moves away from solutions containing low levels of hydrogen peroxide or hypochlorous acid, which might enable it to avoid being engulfed by phagocytes [3].

Box 1.2. Redox State.

Redox reaction describes all chemical reactions in which atoms have their oxidation number (oxidation state) changed.

The term *redox* comes from the 2 concepts of reduction and oxidation. It can be explained in simple terms:

Oxidation describes the loss of electrons by a molecule, atom, or ion

Reduction describes the gain of electrons by a molecule, atom, or ion.

However, these descriptions (though sufficient for many purposes) are not truly correct. Oxidation and reduction properly refer to a change in oxidation number—the actual transfer of electrons may never occur. Thus, "oxidation" is better defined as *an increase in oxidation number*, and "reduction" as a *decrease in oxidation number*. In practice, the transfer of electrons will always cause a change in oxidation number, but there are many reactions that are classed as "redox" even though no electron transfer occurs (such as those involving covalent bonds).

When exposed to high-pressure oxygen, the growth of *E. coli* is immediately inhibited. Of note, plots of the logarithm of survival time against the logarithm of the oxygen pressure have shown inverse, approximately linear relations for protozoa, mice, rats, rabbits, fish, and insects [4]. The toxicity of oxygen to humans has been of interest in relation to diving, underwater swimming, design of the gas supply in spacecraft and submarines, and in the use of hyperbaric oxygen to treat cancer, infections, multiple sclerosis, and lung diseases. Increases in the partial pressure of oxygen to which an organism is subjected can be caused not only by an increase in the percentage of oxygen in the air, but also, as in diving, to an increase in the total pressure. It is known, for example, that exposure of humans to pure oxygen at 1 atmosphere for 24 hours leads in all cases to injury to the alveoli of the lungs, associated with lung edema.

1.2.3 What causes the toxic effects of oxygen?

In all of these observations, the crucial question, of course, is: What causes the toxic effects of oxygen? Perhaps the earliest suggestion was that oxygen inhibits cellular enzymes. In addition, in 1954, Gershman and Gilbert, as quoted by Halliwell and Gutteridge, drew a parallel between the effects of oxygen and those of ionizing radiation and proposed that most of the damaging effects of oxygen could be attributed to the formation of free oxygen radicals [5].

To understand any discussion on a role of reactive oxygen species in host defense or allograft rejection, one should define *free radicals*. According to Halliwell and Gutteridge, "a free radical is any species capable of independent existence that contains 1 or more unpaired electrons." An unpaired electron is one that occupies an atomic or molecular orbital by itself. Radicals can be formed by the loss of a single electron from a nonradical, or by the gain of a single electron by a nonradical. In this sense, superoxide anions ($O_2^{\cdot -}$), hydroxyl radicals ($\cdot OH$), peroxyl radicals ($RO_2\cdot$), and alkoxyl radicals ($RO\cdot$) are oxygen radicals. Of note, *ROS* is a collective term often used by scientists to include not only the oxygen radicals but also some nonradical derivatives of oxygen such as hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl), ozone (O_3), and singlet oxygen ($^1O_2$). Nitrogen-containing oxidants, such as nitric oxide ($NO\cdot$) are called *reactive nitrogen species* (RNS). ROS generation is generally a cascade of reactions that starts with the production of superoxide anions. Superoxide rapidly dismutates to hydrogen peroxide either spontaneously, particularly at low pH, or it is catalyzed by superoxide dismutase (SOD). Other elements in the cascade of ROS generation include the reaction of superoxide with nitric oxide to form the very toxic peroxynitrite, the peroxidase-catalyzed formation of hypochlorous acid from hydrogen peroxide, and the iron-catalyzed Fenton reaction, leading to the generation of hydroxyl radical.

There are several recently published review articles on ROS that are recommended to the interested reader [6,7]. More details of ROS and RNS biology and pathology in the context of innate alloimmunity will be described in Part 2.

1.3 Role of ROS in Microbial and Viral Pathophysiology: the Battle Between Pathogens and Host Innate Immune Cells

1.3.1 Introductory remarks

ROS play a crucial role during the evolution of innate immunity. In the course of hundreds of millions of years of evolution, the struggle for life included killing of multicellular macro-organisms by microorganisms and vice versa. Many different weapons for defense were used in this battle, but both sides used the killer machines of ROS. Thus, phagocytes such as neutrophils and macrophages evolved to specialized cells able to generate toxic ROS, which then enabled them to exhibit a broad spectrum of biotoxicity and guaranteed a host defense owing to their optimal microbicidal activities. For many years, it was believed that the reduced form of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase multisubunits complex (Box 1.3.1) represents the dominant machinery of phagocytes, enabling them to generate ROS in the course of host defense; recent evidence, however, suggest that this enzyme system is also active in nonphagocytic cells.

Conversely, microorganisms and viruses also developed the capacity to generate ROS, viruses by using the infected host cells. Thus, during evolution, the battlefield was always full of toxic ROS, which, in terms of "collateral damage," not only killed the target but also injured the surrounding cells, tissues, and organs of the host. Thus, to counterattack ROS and limit the collateral damage and maintain homeostasis, both competitors—microbes and host phagocytes—have evolved antioxidative defense weapons against excessive production of ROS. If, however, the "oxidative burst" predominates, the surrounding tissue will suffer. Over time, ROS-injured tissue and molecules arising from this damaged tissue have become danger signals for the host defense system. It is tempting to speculate that it is this scenario that has led to the development of further defense weapons such as the formation of innate immune cells and mediator substances (eg, macrophages and cytokines) in nonvertebrates and the generation of the more sophisticated T cells and B cells and humoral antibodies of the adaptive immune system in vertebrates. Some data and observations in support of this concept are discussed in this subchapter.

1.3.2 Sources of reactive oxygen species caused or encountered by pathogens in vivo

1.3.2.1 General remarks

As a typical feature of evolution, pathogens continuously quarrel with ROS, which may be generated by the microbes themselves or by host phagocytes in their attack against microbes. Thus, we must distinguish between endogenous and exogenous sources of ROS, a scenario that deserves a brief description. In addition, it is worth mentioning that recent evidence suggests that ROS, whether derived from host phagocytes or from infected cells, also play a role in the pathogenesis of viral infections.

Box 1.3.1. What is NADPH and what are NOX- and DUOX enzymes?

NADPH is the abbreviation for nicotinamide adenine dinucleotide phosphate, defined as a coenzyme of many oxidases (dehydrogenases), in which the reaction $NADP^+ + 2H^+ \rightarrow NADPH^+ + H^+$ takes place; the third phosphoric group esterifies the 2´-hydroxyl of the adenosine moiety of NAD^+. Authors use to use the abbreviation NADPH without definition because it represents a so-called standard abbreviation. In phagocytes, the enzyme complex is called phagocyte NADPH oxidase (PHOX).

NADPH-oxidases (NOX) and related Dual oxidases (DUOX) play varied biological and pathological roles via generation of ROS, the prototype being NOX2 , also called $gp91^{phox}$.

A new family of homologues of NOX2, the NADPH oxidase (NOX)/dual oxidase (DUOX) family, which now contains 7 members, has been described; these are expressed in various cell types, including the epithelium, smooth-muscle cells, and the endothelium. All these enzymes "deliberately" generate superoxide and secondarily produce other ROS, including hydrogen peroxide (H_2O_2).

The NOX enzymes have been proposed to generate ROS as mediators of signal transduction related to growth, angiogenesis, and apoptosis. In addition, circumstantial evidence indicates that NOX enzymes might in some cases function in innate immunity in barrier cells, such as the colon epithelium, in a manner analogous to PHOX.

The DUOX enzymes are dual function enzymes, containing not only an ROS-generating domain homologous to $gp91^{phox}$, but also a peroxidase domain that can use the H_2O_2 produced by the $gp91^{phox}$-homology domain to carry out oxidation of other substrates. A DUOX enzyme in the thyroid has been shown to participate in thyroid-hormone biosynthesis

(Source: Lambeth JD. Nox: enzymes and the biology of reactive oxygen. Nat Rev Immunol 2004; 4: 181).

1.3.2.2 Endogenous sources in bacteria of reactive oxygen species

Like eukaryotic cells, aerobic microorganisms are continuously exposed to endogenous sources of toxic ROS as a consequence of aerobic metabolism. As reviewed elsewhere [8], several microorganisms, including *Enterococcus faecalis*, *E. coli, Lactobacillus* species, *Streptococcus pneumoniae,* and several *Mycoplasma* species, also generate extracellular superoxide radicals and hydrogen peroxide.

Additional studies have shown that these ROS can exert several toxic effects to the host. For example, superoxide radicals produced by *Mycoplasma pneumoniae* can inactivate host cell catalase, resulting in progressive oxidative damage to infected cells in vitro. *S. pneumoniae*-derived H_2O_2 may play a role in host cellular injury in pneumococcal pneumonia, as it has been shown to be toxic to rat alveolar epithelial cells in an in vitro model [9]. The formation of dental plaque and the subsequent development of gingivitis and periodontitis also are related to the balance of H_2O_2-producing and H_2O_2-degrading organisms in the oral microenvironment [10]. In addition, a recent study showed that cleavage of human transferrin by *Porphyromonas gingivalis* gingipains promotes growth and formation of hydroxyl radicals associated with host tissue destruction during periodontitis. In fact, this study indicated that degradation of human transferrin provides sources of iron and peptides. The iron-containing transferrin fragments or the release of iron itself may contribute to tissue destruction by catalyzing the formation of toxic ·OH [11].

1.3.2.3 Exogenous sources of reactive oxygen species for bacteria

The primary source of exogenous oxidative stress for pathogenic bacteria during the process of active infection is their attack by host phagocytic cells. Indeed, host phagocytes use the cytotoxic effects of many members of ROS as a component of their defense mechanisms. As reviewed [8], there are several mechanisms of phagocyte-derived ROS involved in microbicidal activities. For example, when a phagocyte encounters a microorganism, the latter is surrounded by a portion of the phagocyte membrane, which then invaginates, forming a discrete phagosome. This process leads to increased phagocyte oxygen consumption and initiates a complex biochemical signaling system, which then activates a unique membrane-associated NADPH-dependent oxidase (NOX) and a related Dual (DUOX) oxidase

complex. As described in more detail below, these enzymes univalently reduce oxygen to superoxide radicals, which are then secreted into the phagosome; there, superoxide radicals dismute to H_2O_2. Importantly, these toxic compounds also may leak extracellularly as the phagosome is closing.

As further reviewed [8], there are other mechanisms of phagocyte-derived ROS involved in microbicidal activities including (1) intracellular and extracellular conversion of phagocyte-derived H_2O_2 into HOCl, (2) catalyzation by myeloperoxidase (MPO) of the reaction of superoxide and HOCl to form hydroxyl radicals, (3) iron catalyzation of the intracellular and extracellular reaction of superoxide radical and H_2O_2 to form hydroxyl radicals, and (4) generation of nitric oxide via inducible nitric oxide synthase (iNOS), which has been demonstrated in murine cells and also may be seen in human phagocytic cells. The primary microbicidal effect of phagocyte-derived NO· appears to involve intracellular pathogens in particular.

As discussed in detail below, ROS formation against invading microbes is an evolutionarily highly conserved defense mechanism that is also observed in insects. For example, in *Drosophila*, natural infections with bacteria also induce rapid ROS synthesis in the gut, and the dynamic cycle of ROS generation and elimination appears to be vital [12].

In this context, plants also must be briefly mentioned. Plants also use the formation of ROS as an innate defense mechanism. The current understanding of ROS generation in the defense response of *Arabidopsis thaliana* has recently been reviewed [13]. Interestingly, considerable evidence suggests that the apoplastic oxidative burst generated during basal resistance is peroxidase dependent. The ROS generated during this basal resistance may activate NADPH oxidase during the R gene-mediated hypersensitive response (for information on R genes, see Subchapter 3.2.5). The processes involved in the production of ROS in *A. thaliana* cell suspension cultures in response to an elicitor from *Fusarium oxysporum*, were investigated; they revealed that an early calcium influx into the cytosolic compartment, a rapid efflux of K(+) and Cl(-), and extracellular alkalinization of elicited cell cultures appears to be important. However, the alkalinization is not sufficient to stimulate the apoplastic oxidative burst by itself, unlike that in French bean, although vectorial ion fluxes are needed. A secretory component that is sensitive to monensin and N-ethylmaleimide and insensitive to brefeldin A also may be necessary for the release and provision of substrates for peroxidase-dependent generation of H_2O_2 [13].

1.3.2.4 Sources of reactive oxygen species induced by viruses

There is recent evidence clearly indicating that ROS, whether derived from host phagocytes or from infected cells, play a role in the pathogenesis of viral infections. In fact, not only microbes but also viruses can generate ROS in vivo by inducing them in infected host cells. In fact, oxidative stress, primarily due to and associated with increased generation of ROS and RNS, is a feature of many viral infections such as human immunodeficiency virus (HIV)-1, hepatitis C virus (HCV), cytomegalovirus (CMV), and herpes simples virus-1 (HSV-1) infection [14-23]. Oxidative stress in virus-infected cells is accompanied by accumulation of ROS, oxidant damage, and depletion of reduced thiols. The sources of ROS in virus-infected cells are unclear although certain NOXes might be involved. The majority of work has centered around HIV-1, hepatitis B virus, and, recently, HCV and HSV-1 [14, 22, 23].

In humans, infection by HIV causes persistent chronic inflammation, probably influenced by oxidative stress, as indicated by elevated serum levels of products of lipid peroxidation, including malondialdehyde. Patients infected with HIV also show decreased plasma levels of the reduced thiols glutathione and cysteine, both of which participate in antioxidant defense. There is evidence suggesting that the viral Tat protein plays a role in the intracellular increase of ROS, thus increasing the apoptotic index, particularly that mediated by Fas/CD95 (cluster of differentiation 95[CD95]), and depleting $CD4^+$ T lymphocytes [15, 16, 23].

HCV infection is characterized by a systemic oxidative stress that is most likely caused by a combination of chronic inflammation, iron overload, liver damage, and protein encoded by HCV; these events are associated with continuous generation of ROS and RNS, which may be explained by NADPH oxidase activity of Kupffer cells and polymorphonuclear cells in the liver [17, 18]. CMV infection of smooth muscle cells also has been found to generate intracellular ROS, which is associated with activation of NF-kB binding to DNA [19]. Moreover, recent studies suggest that the pathogenesis of influenza virus infection as well as hantavirus-mediated cardiopulmonary syndrome involves direct ROS-induced cellular injury in the infected organs [20, 21].

1.3.2.5 Antioxidative defenses

To counterattack ROS and maintain homeostasis, microbes and host

phagocytes have evolved antioxidative defense weapons against excessive production of ROS. Some of those antioxidative defense molecules are briefly outlined here. They comprise the following [1]:

(1) Agents that catalytically remove free oxygen radicals and other "reactive species." Examples are the enzymes SOD, catalase, peroxidase and "thiol-specific antioxidants."

(2) Proteins that minimize the availability of pro-oxidants, such as iron ions, copper ions, and heme. Examples are transferrins, haptoglobins, hemopexin, and metallothionein. This category includes proteins that oxidize ferrous ions, such as ceruloplasmin.

(3) Proteins that protect biomolecules against damage (including oxidative damage) by other mechanisms, for example, heat shock proteins.

(4) Low-molecular-mass agents that scavenge ROS and RNS. Examples are glutathione, α-tocopherol, and (possibly) bilirubin and uric acid. Some low-molecular-mass antioxidants come from the diet, especially ascorbic acid and α-tocopherol. There is an intimate relation between nutrition and antioxidant defense.

In discussing antioxidative enzymes and nonenzymes, it is again appropriate to vaguely quote from excerpts out of the book of Halliwell and Gutteridge [1]:

a) Superoxide dismutases

Among the most important regulators of ROS levels are the SOD enzymes: Cu/ZnSOD in the cytoplasm and outer mitochondrial space, and MnSOD exclusively in the inner mitochondrial space. Superoxide is converted to hydrogen peroxide (H_2O_2) and oxygen ($O_2{\cdot}^- + O_2{\cdot}^- + 2H^+ \rightarrow H_2O_2 + O_2$) by SOD. Peroxiredoxins and abundant catalase enzyme then scavenge the hydrogen peroxide, converting it to molecular oxygen and water (Figure1.3.1).

The complete amino acid sequences of Cu/ZnSOD from several plants and animals have been determined, and they are all very similar (Figure 1.3.2). Each of the 2 subunits is composed primarily of 8 antiparallel strands of β-pleated sheet structure that form a flattened cylinder plus "loops." The copper ion is held at the active site by interactions with the nitrogens in the imidazole ring structures of 4 histidine residues, and the zinc ion is bridged to the copper by His78 and the carboxyl group of Asp81.

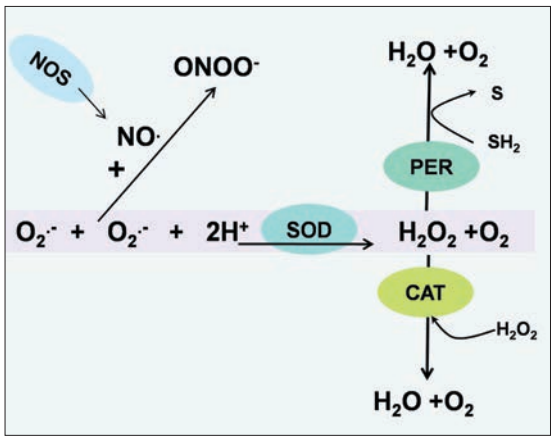

Figure 1.3.1. Action of superoxide dismutase and catalase to eliminate toxic ROS.

Superoxide radical ($O_2^{\cdot-}$) together with nitric oxide synthetase (NOS) - catalyzed nitric oxide radical ($NO^{\cdot}$) form the toxic peroxynitrite (ONOO). Superoxide is converted to hydrogen peroxide (H_2O_2) and oxygen ($O_2^{\cdot-} + O_2^{\cdot-} + 2H^+ \rightarrow H_2O_2 + O_2$) by superoxide dismutase. Peroxiredoxins (PER) and abundant catalase (CAT) enzyme then scavenge the hydrogen peroxide, converting it to molecular oxygen and water.

Source: Figure adapted from: Halliwell B, Gutteridge JMC. Free Radicals in Biology and Medicine. 3rd Edition. Oxford, UK: Oxford University Press; 2000.

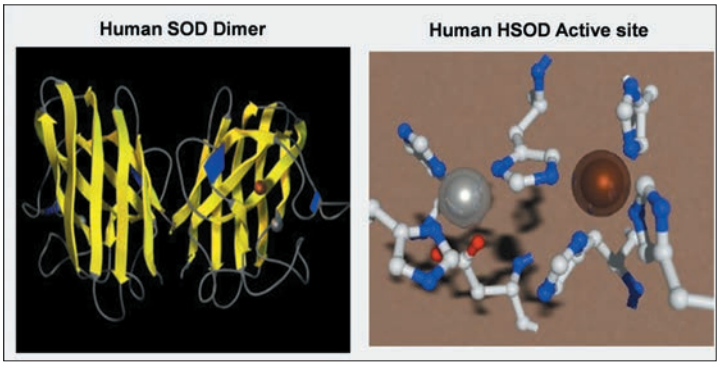

Figure 1.3.2. Human superoxide dismutase.

Cu,Zn superoxide dismutase (CuZnSOD) is a dimeric enzyme consisting of 2 identical subunits of approximately 16 kDa each. Each monomer is composed of 8 antiparallel β-strands arranged in a Greek-key fold. Each monomer binds 1 copper and 1 zinc atom using 1 aspartic acid residue and 6 histidine residues. One histidine acts as a bridge between the metal sites ligating to both the copper and zinc atoms.

Source: Figure reproduced with permission from M. DiDonato,

http://www.scripps.edu/~didonato/current.shtml

Notably, purification of enzymes has proved the existence of 2 slightly different forms of Cu/ZnSOD in wheat seeds, and isoenzymes also have been shown to exist in several other organisms, such as *Drosophila*. In fact, a variant of human Cu/ZnSOD known as SOD-2 has been found to occur in northern Sweden and northern Finland. Most of the population is

homozygous for "normal" SOD (SOD-1), but there are some heterozygotes with both SOD-1 and SOD-2, and a very few SOD-2 homozygotes.

Other SODs are manganese superoxide dismutases (MnSODs). They are widespread in bacteria, plants, and animals. In most animal tissues and yeast, MnSOD is largely (if not entirely) located in the mitochondria [24]. The relative activities of MnSOD and Cu/ZnSOD depend on the tissue and on the species; one obvious variable is the number of mitochondria.

b) Catalases

Dismutation of $O_2^{\cdot -}$ generates H_2O_2, a species also generated by several oxidase enzymes in vivo, including xanthine, urate, and D-amino acid oxidases [25]. Hydrogen peroxide is usually removed in aerobes by 2 types of enzymes: (1) the catalases that directly catalyze decomposition of H_2O_2 to ground state oxygen ($2H_2O_2 \rightarrow 2\ H_2O + O_2$); and (2) peroxidase enzymes that remove H_2O_2 by using it to oxidize another substrate (written SH_2 here): $SH_2 + H_2O_2 \rightarrow S + 2\ H_2O$. Notably, most aerobic cells contain catalase activity [25]. The catalase reaction mechanism is like that of SOD, essentially a dismutation (disproportionation); one H_2O_2 is reduced to H_2O and the other is oxidized to oxygen. The catalase activity of animal and plant tissues is largely or completely located in subcellular organelles, known as peroxisomes, bounded by a single membrane. Peroxisomes contain many of the cellular enzymes that generate H_2O_2, such as glycolate oxidase, urate oxidase, and the flavoprotein dehydrogenases involved in the β-oxidation of fatty acids (a metabolic pathway that operates in both mitochondria and peroxisomes in animal tissue).

c) The glutathione peroxidase family

Glutathione peroxidases (GPX) remove H_2O_2 by coupling its reduction to H_2O with oxidation of reduced glutathione (GSH): $H_2O_2 + 2GSH \rightarrow GSSG + H_2O$ (oxidized form of glutathione [GSSG]) [25]. GPX was first discovered in animal tissue in 1957. Glutathione peroxidases are not generally present in higher plants or bacteria, although they have been reported in a few algae and fungi. GSH, their substrate and cofactor, is a low-molecular-mass thiol-containing tripeptide. It is present in animals, plants, and many aerobic bacteria at intracellular concentrations that are often in the millimolar range; it is rarely present in anaerobic bacteria. Glutathione peroxidases consist of 4 protein subunits, each of which contains 1 atom of the element selenium

at its active site. Selenium-containing peroxidases comprise a family of enzymes, the glutathione peroxidase superfamily, of which at least 4 types exist; 1 is the "classic" glutathione peroxidase (the GPX).

d) Thioredoxin

Thioredoxin (TRX) is a polypeptide, relative molecular mass about 12 000, found in both prokaryotes and eukaryotes. It is widely distributed in mammalian cells, being particularly concentrated in the endoplasmic reticulum, but also found on the cell surface. Thioredoxin contains 2 adjacent –SH groups in its reduced form, which are converted to a disulphide in oxidized thioredoxin. Notably, it can undergo redox reactions with multiple proteins using the reaction:

Thioredoxin-$(SH)_2$ + protein $–S_2$ $\leftrightarrow$ thioredoxin $–S_2$ + protein $–(SH)_2$.

Thioredoxin binds to its target protein, and, via intermediate formation of a mixed disulphide, reduces the protein disulphide bridge while oxidizing its 2 cysteine-SH groups to a cystine (disulphide).

Oxidized thioredoxin can be re-reduced in vivo by thioredoxin reductase enzymes, which contain FAD and show similarities to glutathione reductases, including use of NADPH. Thioredoxin is a member of a group of enzymes often called *thiol-disulphide oxidoreductases*. Another member is glutaredoxin.

Thioredoxin has appeared in many guises in biology, and only now is its central importance to metabolism in animals being appreciated. In vivo, thioredoxin acts as a hydrogen donor for ribonucleotide reductase. It also supplies electrons to methionine sulfoxide reductase, an enzyme that repairs oxidative damage to methionine residues in proteins. Thioredoxin can react directly with H_2O_2, although the metabolic significance of this is unknown.

There is growing evidence indicating that TRX-1, a stress-inducible protein, has an important role in protecting host cells from various types of stresses, including viral infection, ischemic insult, and H_2O_2 exposure. Indeed, TRX-1 has scavenging activity for a variety of ROS such as singlet oxygen, hydroxyl radicals, and H_2O_2. Thus, TRX-1 has an important role in maintaining the redox environment of the cell. Moreover, TRX-1 has potent antiinflammatory effects through suppression of neutrophil infiltration in the inflammatory site [26-28].

e) Heme oxygenase, biliverdin, and bilirubin

Heme oxygenase (HO) is an enzyme found in the endoplasmic reticulum. Two isoforms of the enzyme have been characterized: a constitutive isoform (HO-2) predominant under normal physiological conditions and a stress-induced isoform (HO-1), which is identical with the heat shock protein 32. HO-2 is present at high levels in the spleen, presumably to destroy heme from the processing of worn-out red blood cells (for review, see Maines [29] and Morse and Choi [30]). The overall effect of heme oxygenase is to remove a pro-oxidant (heme) while generating a putative antioxidant, bilirubin. Heme oxygenase can be inhibited by tin protoporphyrin, which has been used to study the biological role of this enzyme (Figure 1.3.3).

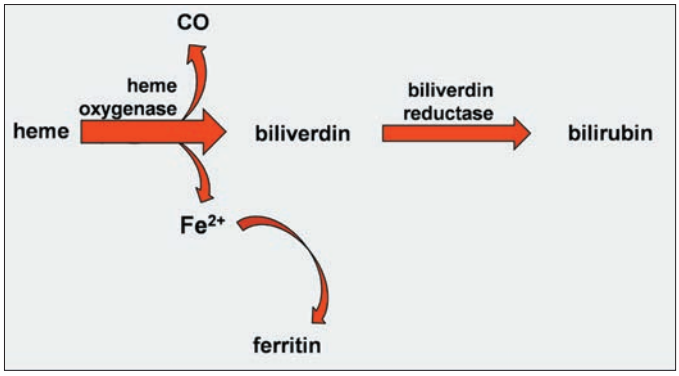

Figure 1.3.3. Catalysis of heme by carbon monoxide (CO).

Heme oxygenase catalyzes the oxidative cleavage of heme to yield equimolar amounts of CO, iron (Fe^{2+}), and biliverdin. Biliverdin is subsequently converted to bilirubin through the action of biliverdin reductase, and iron induces increased ferritin synthesis.

Source: Figure adapted from: Halliwell B, Gutteridge JMC. Free Radicals in Biology and Medicine. 3rd Edition. Oxford, UK: Oxford University Press; 2000.

Heme oxygenase catalyses the breakdown of heme to biliverdin, with the release of iron ions and carbon monoxide (CO). NADPH-cytochrome P450 reductase acts as an electron donor to heme oxygenase. The biliverdin produced is converted to bilirubin by the enzyme biliverdin reductase in the cytosol. Each of these products of enzymatic activity plays a unique and often protective role in the human body [29, 30], as described in the following:

Carbon monoxide: Carbon monoxide, a colorless, odorless diatomic gas, is best known in the medical community for its lethal properties. For more

than 10 years, several investigators have shown that low concentrations of CO can exert biological functions as diverse as neurotransmission, protection against cell death, antiinflammation, protection against oxidative injury, inhibition of cell proliferation, and tolerance of organ transplant. Analogous to NO, some activities of CO have been shown to be cyclic guanosine monophosphate (cGMP) dependent, such as inhibition of platelet aggregation and smooth muscle relaxation.

Bilirubin: Bilirubin is an end-product of heme degradation in mammals and is now recognized as a potent antioxidant manufactured by the body. For example, in vitro, bilirubin is a powerful scavenger of singlet oxygen and peroxyl radicals: in neural cell culture, a minute amount of bilirubin is capable of protecting cells from 10 000-fold higher concentrations of the oxidant hydrogen peroxide. Normal human serum bilirubin concentrations are high enough to provide a substantial portion of the total known antioxidant capacity of serum.

Iron: Free iron is capable of participating in deleterious oxidation reactions, and for this reason, it is carefully sequestered in biological systems. By releasing iron from heme, HO-1 is potentially contributing to a pro-oxidant state within the cell. However, the iron released by HO activity also increases the synthesis of ferritin, which stores free iron and has well-known cytoprotective properties. It has been argued that some of the protective effect of HO-1 induction is attributable to the increase in cellular ferritin that rapidly follows. In addition, HO-1 can repress the expression of a highly active ferrous iron-ATPase transporter involved in iron efflux from cells. In the absence of HO-1, iron efflux from cells can be decreased and can potentially contribute to cell death. Mice with a deletion of the HO-1 gene display increased tissue and intracellular accumulation of iron, supporting the thesis that coregulation of ferritin and the iron transporter may contribute to the cellular protection conferred by HO-1.

f) Compounds derived from the diet

A huge range of dietary constituents has been suggested to exert antioxidant effects in vivo including ascorbic acid (vitamin C), tocopherols (with vitamin E activity), for example, α-tocopherol, particularly carotenoids.

Ascorbate predominantly scavenges superoxide anions ($O_2^{\cdot -}$) and hydroxyl radicals ($^{\bullet}OH$), but also thiol and oxysulfur radicals. Tocopherols

inhibit lipid peroxidation largely because they scavenge lipid peroxyl radicals much faster than these radicals can react with adjacent fatty acid side chains or with membrane proteins. Indeed, vitamin E, as a scavenger of peroxyl radicals, is probably the most important (but not the only) inhibitor of the free radical chain reaction of lipid peroxidation in animals.

Carotenoids (of which the first to be isolated was from carrots, in 1831) are a group of colored pigments (usually yellow, red, or orange) that are widespread in plant tissue (Figure 1.3.4).

Figure 1.3.4. Carotenoids.

Carotenoids in nature soothe our eyes and lift our spirits, whether in the petals of flowers, in the plumage of birds, or in the delicate hues of some fruits and vegetables.

Sources: Figure and text of the legend, adapted from: Olson JA, Krinsky NI. Introduction: the colorful, fascinating world of the carotenoids: important physiologic modulators. FASEB J 1995; 9: 1547-1550, and www.newsdesk.umd.edu/.../images/carotenoid.jpg, www.science.kennesaw.edu/.../images/veggies.jpg.

Carotenoids are also found in some animals and certain bacteria. In plants, carotenoids play a key antioxidant role helping to prevent the formation of, and quench, ROS (especially singlet oxygen) formed during photosynthesis. In vitro studies have shown that β-carotene inhibits peroxidation of simple lipid systems at low oxygen concentrations, in addition showed the potential of carotenoids to act as free radical scavengers.

1.3.2.6 Concluding remarks

All of these reports make clear that we must accept a beneficial function of ROS production, namely the importance of ROS in host defense. The danger of ROS generation, however, is its excessive production ("respiratory

oxidative burst") during phagocytosis which—I repeat—may be associated with the development of collateral tissue damage. The action of the antioxidative defense system may counterattack and limit ROS, but may fail so in those situations of excessive ROS production. On the other hand, the existence of an endogenous "innate" antioxidative defense system may be harnessed by clinicians to treat clinical disorders characterized by excessive ROS production, as encountered in tissue reperfusion injury; transplant clinicians, as did they in Munich in 1994, may harness innate antioxidative agents such as SOD to treat allograft reperfusion injury. The question now arises: How do innate immune cells dedicated to host defense generate these molecules? In fact, the NOX family of NAPDH oxidases has already been mentioned. It has a central role in the generation of ROS, as described in the next section.

1.3.3 The NOX family of ROS-generating NADPH oxidases

1.3.3.1 General remarks

Phagocytes generate large amounts of free oxygen radicals as part of their microbicidal activity, which results from activation of a membrane-associated (NOX/DUOX) NADPH oxidase complex (phox system); several nonphagocytic cells reportedly possess such an enzyme system as well. The NOX family of NADPH oxidases is a family of proteins that transfers electrons across biological membranes. In general, the electron acceptor is oxygen, and the product of the electron transfer reaction is superoxide. The biological function of NOX enzymes is therefore the generation of ROS. Interestingly, in the history of the discovery of the phagocyte NADPH oxidase, clinical research played an important role. In 1957, a new clinical syndrome was recognized in young boys with recurrent pyogenic infections, which was accompanied by granulomatous reactions, lymphadenopathy, and hypergammaglobulinemia—the inherited disorder now referred to as chronic granulomatous disease (CGD). Ten years later, the genetic defect of this disease was further identified, and it was recognized that the respiratory burst, that is, the generation of ROS, was absent in the phagocytes of CGD patients, resulting in the inability to destroy certain bacteria and fungi. In the following section, more aspects of this remarkable enzyme system operating in host defense will be presented, summarizing several comprehensive review articles on this topic [31-36].

1.3.3.2 ROS-generating NAPDH oxidase (NOX/DUOX) family of enzymes in phagocytes

The NADPH oxidase found in phagocytic cells is a multisubunit complex consisting of membrane-bound and cytosolic components. Thus, all NOX family members must be regarded as transmembrane proteins that transport electrons across biological membranes to reduce oxygen to superoxide. In accordance with this preserved function, there are conserved structural properties of NOX enzymes that are common to all family members. Starting from the C-terminus, these conserved structural features include (1) an NADPH-binding site at the very C-terminus, (2) a flavin adenine dinucleotide (FAD)-binding region in proximity of the most C-terminal transmembrane domain, (3) 6 conserved transmembrane domains, and (4) 4 highly conserved heme (Fe)-binding histidines, 2 in the third and 2 in the fifth transmembrane domain (Figure 1.3.5). Given the additional N-terminal transmembrane domain, the histidines are in the fourth and sixth transmembrane domains in DUOX proteins [31, 33, 35].

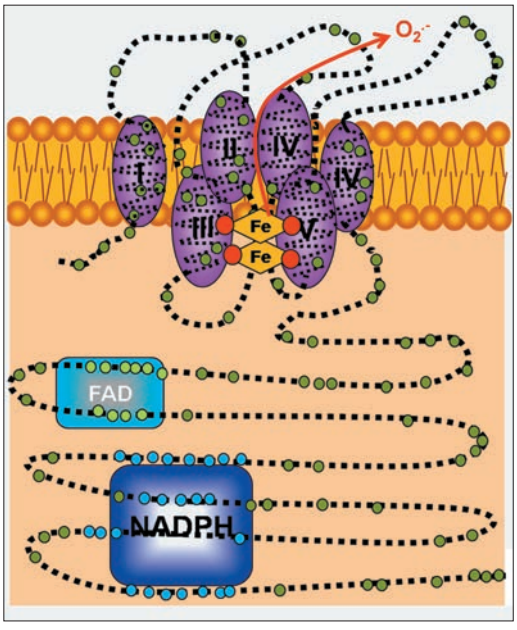

Figure 1.3.5 Schematic and proposed structure of the core region of NADPH oxidase (NOX) enzymes.

The NOX domain possesses 6 highly conserved transmembrane domains (I through VI in *boxes*), domains III and V each contain 2 histidines, spanning 2 asymmetrical membrane-imbedded heme moieties. The cytoplasmic COOH terminus contains conserved FAD and NADPH binding domains. NOX enzymes are thought to be single electron transporters, passing electrons from their cytosolic source, ie, (reduced) NAPDH (ie, electron donor) to FAD, to the first heme, to the second heme, and finally to some extracytoplasmic compartments, ie, oxygen as the electron acceptor–by generating superoxide radicals.

Abbreviations: FAD, flavin dinucleotide; NADPH, nicotinamide adenine dinucleotide phosphate (reduced form).

Source: Figure and text of the legend, adapted from Bedard K, Krause KH. The NOX family of ROS-generating NADPH oxidases: physiology and pathophysiology. Physiol Rev 2007; 87: 245-313.

Much of what is known about the topography and structure of the NOX isoforms is derived from studies on the prototypical NADPH oxidase, the NOX2, also known as gp91^{phox}. In resting cells, the NADPH oxidase complex, that is, the phox system, is maintained in a latent disassociated state, as reflected by the membrane-bound flavocytochrome b_{558}, which is composed of gp91^{phox} (also called NOX2) and p22^{phox} subunits as well as of cytosolic regulatory subunits p47^{phox}, p67^{phox}, p40^{phox}, and the small guanosine-5'-triphosphate hydrolase enzymes (GTPases) Rac1 and Rac2. In other words, in resting cells, NOX2 alone is inactive and must associate with p22^{phox} to form the noncovalent heterodimer flavocytochrome b_{558}.

Box 1.3.1. What are Rac1 and Rac2, GTPase, and small GTPases?

GTPases: GTPases (singular GTPase) are a large family of hydrolase enzymes that can bind and hydrolyze GTP (guanosine-5'-triphosphate). The GTP binding and hydrolysis takes place in the highly conserved G domain common to all GTPases. They help GTP binding proteins hydrolyze GTP and be converted to their ground state.

In biology, small GTPases are small (20-25 kDa) proteins that bind to GTP. This family of proteins is homologous to Ras GTPases and also called the Ras superfamily GTPases. There are more than 100 proteins in the Ras superfamily. Based on structure, sequence, and function, the Ras superfamily is divided into 8 main families, each of which is further divided into subfamilies: Ras, Rho, Rab, Rap, Arf, Ran, Rheb, Rad, and *Rit*. Together with heterotrimeric G-proteins, small GTPases constitute the G-proteins. They are all GTPases and share common features, but small GTPases have slightly different structures and mechanisms of action.

Rac1 and Rac2 are small GTPases of the Rho-family, which have been implicated in the control of the actin cytoskeleton, signal transduction, cell proliferation, and apoptosis. Rac1 and Rac2 GTPases also have multiple overlapping as well as distinct roles in hematopoietic cells.

Activation of NOX2 in host defense has been extensively studied and requires the association of essential cofactors [33, 36]. When phagocytes encounter and engulf microbes, several receptor types are stimulated that trigger overlapping signaling pathways, causing the assembly of oxidase components on the membrane and activation of the oxidases.

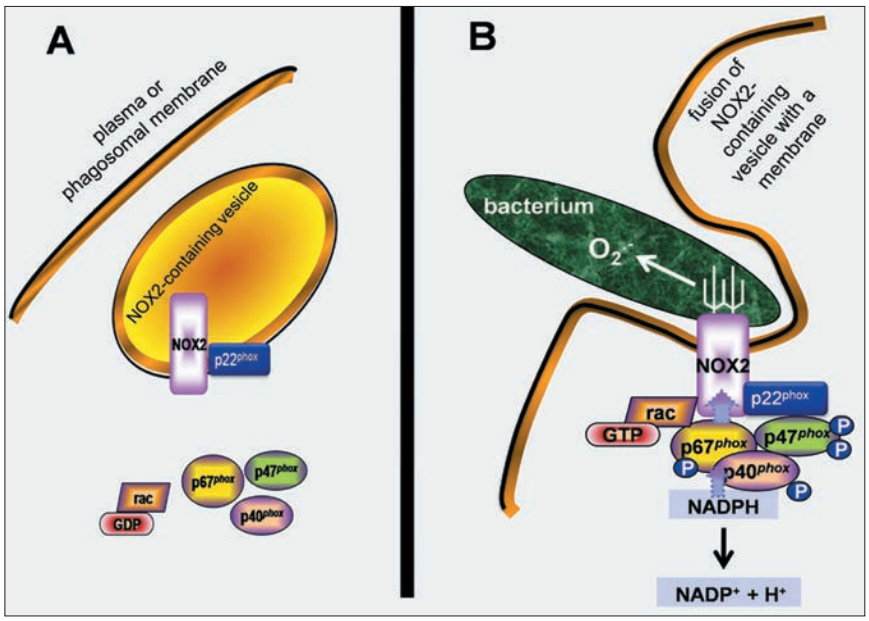

Figure 1.3.6. Model of the assembly of phagocytic NADPH oxidase NOX2 aiming bacterial killing.

In resting neutrophils, NOX2 and $p22^{phox}$ are found primarily in the membrane of intracellular vesicles. They exist in close association, co-stabilizing one another (A). Upon activation, there is an exchange of GDP for GTP on Rac leading to its activation. Phosphorylation of the cytosolic $p47^{phox}$ subunit leads to conformational changes allowing interaction with $p22^{phox}$. The movement of $p47^{phox}$ brings with it the other cytoplasmic subunits, $p67^{phox}$ and $p40^{phox}$, to form the active NOX2 enzyme complex. Once activated, there is a fusion of NOX2-containing vesicles with the plasma membrane or the phagosomal membrane. The active enzyme complex transports electrons from cytoplasmic NADPH to extracellular or phagosomal oxygen to generate superoxide that can enter a bacterium.

Two different sources of the figure and the text of the legend were used, adapted, and partially combined, from: (1) Leto TL, Geiszt M. Role of Nox family NADPH oxidases in host defense. Antioxid Redox Signal 2006; 8: 1549-1561, and (2) Bedard K, Krause KH. The NOX family of ROS-generating NADPH oxidases: physiology and pathophysiology. Physiol Rev 2007; 87: 245-313.

This assembly and activation process occurs in a carefully coordinated manner (see also Figure 1.3.6; for further explanations see also Box 1.3.1). As a first step, flavocytochrome-containing vesicles fuse with newly forming phagosomes. Next, the cytosolic regulators undergo phosphorylation-dependent conformational changes and are thereby targeted to specific membranes, whereby they can assemble, interact with, and activate flavocytochrome b_{558} at the cell or phagosomal membrane. Rac1 or Rac2 is independently activated and translocated to assemble with the oxidase

effector system. Rac endows the enzyme with guanine nucleotide sensitivity as it interacts with the activator component, p67^{phox}, in a GTP-dependent manner. Together these proteins control the flow of electrons through the flavocytochrome. The p47^{phox} regulatory subunit acts as a critical phosphorylation-dependent adaptor molecule that bridges interactions between p67^{phox} and the flavocytochrome. Both priming and adherence of phagocytes also affect the assembly of Rac and other oxidase components [37]. The NOX2 component of the phagocyte oxidase contains all the known electron-carrier (catalytic) functions of the enzyme, as it has the binding sites for cytosolic NADPH, FAD, and 2 membrane-embedded heme molecules. Based on topographic models, the NOX oxidases are thought to use these cytosolic sources of electrons (NADPH or NADH), pass them through a flavin intermediate, and then transfer electrons through the membrane to molecular oxygen within some extracytoplasmic compartments to form superoxide [38].

Thus, mammalian homologues of the NOX2 were identified and now constitute the NOX/DUOX family. In humans, besides NOX2, there are 4 functionally distinct NOXes: NOX1 , NOX3, NOX4, and NOX5, plus DUOX1 and DUOX2, distributed in various cell types (see also Table 1.3.1). All members of the human NOX/DUOX family contain a flavocytochrome moiety that is referred to as the NOX domain [32]. In addition, homologues of the cytosolic regulatory subunits, namely NOX Organizer 1 (NOXO1) and NOX Activator 1 (NOXA1) also have been recently described [31, 33, 35].

A few more words about NOX2: NOX2 was first described in neutrophils and macrophages and is often referred to as the phagocyte NADPH oxidase. NOX2 is still widely considered to have a limited, essentially phagocyte-specific tissue expression, yet when tissue distribution of total mRNA from various organs is investigated, NOX2 appears to be among the most widely distributed among the NOX isoforms. Thus, it has been reported as being expressed in many organs including the thymus, small intestine, colon, spleen, pancreas, ovary, placenta, prostate, and testes [39]. There is increasing evidence at both the message and the protein level for expression of NOX2 in nonphagocytic cells, including endothelial cells, cardiomyocytes, hepatocytes, neurons, and skeletal muscle myocytes [33].

In phagocytes, NOX2 localizes to both intracellular and plasma membranes in close association with the membrane protein p22^{phox}. In resting neutrophils, most of the NOX2 localizes to intracellular compartments, particularly in secondary and tertiary granules. Upon

phagocyte stimulation, there is a translocation of NOX2 to the surface as the granules fuse with the phagosomal or the plasma membrane. This fusion is thought to be the key event for the microbicidal activity of NOX2. However, NOX2 also can be activated within the granules without a need for fusion with surface membranes. The resulting intracellular ROS generation might be involved in signaling functions of NOX2.

Table 1.3.1. Tissue distribution and regulatory components of some NOX enzymes.

ENZYME	HİGH-LEVEL EXPRESSİON	INTERMEDİATE/LOW EXPRESSİON	REGULATORY COMPONENTS
NOX1	colon	endothelial cells, smooth muscle cells, osteoclasts, uterus, placenta, prostate, retinal pericytes	$p22^{phox}$, NOXA1, NOXO1, Rac1
NOX2	phagocytes	endothelial cells, smooth muscle cells, lymphocytes, neurons, cardiomyocytes, hepatocytes, skeletal muscle cells, others	$p22^{phox}$, $p47^{phox}$, $p67^{phox}$, $p40^{phox}$, Rac1, Rac2
NOX4	Kidney, blood vessels	endothelial cells, smooth muscle cells, osteoclasts, fibroblasts, keratinocytes, neurons, others	$p22^{phox}$
DUOX1	thyroid	airway epithelial cells, tongue epithelial cells, cerebellum, testes	Ca^{2+}
DUOX2	thyroid	salivary and rectal glands, gastrointestinal epithelial cells, airways epithelial cells, others	Ca^{2+}

Sources: adapted (simplified) from: Bedard K, Krause KH. The NOX family of ROS-generating NADPH oxidases: physiology and pathophysiology. Physiol Rev 2007; 87: 245-313

Abbreviations: NOXA1, NOX Activator 1; NOXO1, NOX Organizer 1.

NOX1 and NOX4 also play roles in innate host defense. The NOX1 isoform is the first NOX2 homologue that was cloned. The molecule contains 564 amino acids and exhibits 56% homology to NOX2. Similar to NOX2, $p22^{phox}$ and Rac1 are essential for the activity of NOX1 (Table 1.3.1). Extensive analyses of NOX1 mRNA and protein expression suggest that NOX1 is considerably involved in mucosal innate immunity [35,36].

NOX4 is composed of 578 amino acids. NOX4 exhibits 39% sequence identity with NOX2. Like NOX2 and NOX1, NOX4 requires the association with $p22^{phox}$ to form an active complex. Functional data support the notion that NOX4 also may be involved in innate immunity [36]; it is of particular interest in the generation of ROS during reperfusion injury, particularly renal allograft reperfusion injury (which will be discussed in more detail in Part 2). The most abundant NOX4 expression is detected in the kidney, as it was originally designated a renal-specific oxidase called Renox. In addition, NOX4 is detected in vascular endothelial and smooth muscle cells, indicating that it may play a role in reperfusion injury [36,40].

1.3.3.3 Some aspects of the physiological function of ROS and NOX family NADPH oxidases in host defense

Recent studies have revealed important roles of the NOX NADPH oxidase complex in both normal and clinical conditions [33]. Members of this enzyme system have been discovered in a large panoply of biological and pathological events, including host defense and inflammation, cellular signaling, gene expression, regulation of cell growth, oxygen sensing, biosynthesis and protein cross-linking, regulation of cellular redox potential, reduction of metal ions, regulation of matrix metalloproteinases, angiogenesis, and cross-talk with the nitric oxide system. In particular, NOX-generated ROS can participate in immune functions in a variety of ways, which are not mutually exclusive. First, as mentioned, ROS can directly oxidize biomolecules in invading microbes in a fairly nonspecific manner, ultimately resulting in molecular damage and microbial cell death. High abundance NOX enzymes and those that are coexpressed with cooperating enzymes such as peroxidases and nitric oxide synthase are the most likely candidates for this sort of mechanism. Second, ROS, operating as second messenger molecules, can participate in molecular signaling cascades associated with innate immune and inflammatory responses. This occurs through the selective oxidation of specific signaling enzymes/proteins that are linked to processes, such as secretion of cytokines or the activation of other killing mechanisms. At several points in this book, we will deal with this processes in terms of posttranslational modifications in more detail.

Of note, host defense is a key function of NOX2, as evidenced by the severe infections observed in patients with chronic granulomatous diseases. Host defense also might be an important role of other NOX family members [33]. Obviously, however, the situation is complicated, and ROS-dependent

killing mechanisms are still debated. In fact, several mechanisms appear to cooperate to achieve successful oxygen-dependent killing of microorganisms. Thus, for example, superoxide itself could kill under special conditions; hydrogen peroxide (derived from superoxide) has been suggested as being responsible for bacterial killing; and peroxynitrite (as a product of superoxide reaction with nitric oxide) has also been discussed as playing a role in phagocyte-induced bactericidity. Other indirect modes of action of NOX enzymes in microbial killing are also being discussed, for example, their action through inactivation of microbial virulence factors or NOX2-dependent induction of changes of phagosomal pH and ion concentrations.

The consensus of opinion is that probably both direct and indirect effects are operating. Of particular interest in the context of my topic is the recent recognition of the crucial role of NOX-derived ROS in innate immune events mediated by neutrophils and macrophages. These cells express large amounts of NOX2 along with its regulatory subunits. This multicomponent enzyme, then, associates, on stimulation, with the membrane catalytic subunits (NOX, $p22^{phox}$) to facilitate superoxide generation (reviewed in [33]). According to calculations, the concentration of ROS produced in the phagosome is extremely high, probably in the molar range. In addition, MPO is secreted into the phagosome, where it converts H_2O_2 (produced by NOX2) plus chloride into HOCl the latter has a direct microbicidal effect. Moreover, macrophages (but possibly not neutrophils) produce large amounts of nitric oxide during phagocytosis. When nitric oxide reacts with superoxide radicals, it generates the highly cytotoxic chemical species peroxynitrite (as will be described in detail later in this book). Of note, neutrophil-derived ROS, including superoxide and H_2O_2 generated by NOX2, HONO generated from superoxide and nitric oxide, and HOCl generated by MPO, have been implicated in the tissue damage seen in acute and chronic inflammatory conditions in which there is at some stage in the disease a neutrophil or macrophage infiltrate [33].

Of major interest in regard to the function of NOXes and DUOXes in innate immune-mediated host defense is their role in macrophages and dendritic cells (DCs), that is, classic cells of the innate immune system. In fact, it appears that all types of macrophages express the phagocyte NADPH oxidase NOX2 and its subunits $p47^{phox}$, $p67^{phox}$, $p40^{phox}$, and $p22^{phox}$ [33,41]. Obviously, one of the key functions of NOX2 in macrophages is killing of phagocytosed microorganisms, as described for neutrophil granulocytes.