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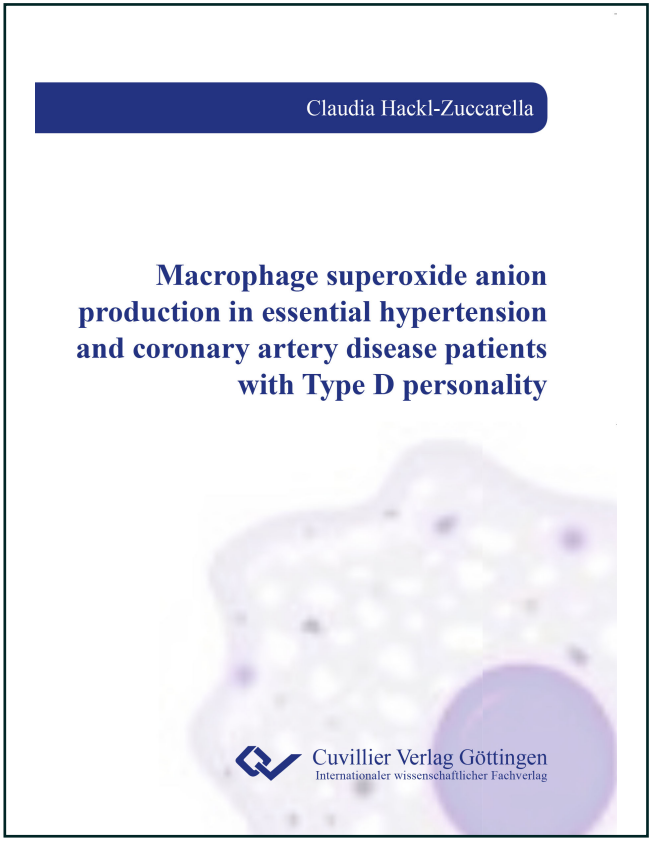
Macrophage superoxide anion production in essential hypertension and coronary artery disease patients with Type D personality



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1 INTRODUCTION

Essential hypertension – defined as a chronic medical condition characterized by a sustained elevation of blood pressure (BP) in the arteries of unknown cause (Mancia et al., 2013, Messerli, Williams & Ritz, 2007; Nandhini, 2014) – is a major risk factor for atherosclerosis and related coronary artery disease (CAD; Alexander, 1995; Hajjar, Kotchen & Kotchen, 2006; Mancia et al., 2013; Perk et al., 2012). Type D personality (Type D) – characterized by a combined expression of high negative affectivity (NA) and high social inhibition (SI; Denollet, Sys & Brutsaert, 1995) – is suggested to be an independent psychosocial risk factor for poor cardiac prognosis in CAD patients (Denollet, Sys, Stroobant, Rombouts, Gillebert & Brutsaert, 1996; Denollet, 2000; Denollet, Pedersen, Ong, Erdman, Serruys & van Domburg, 2006a; Denollet, Pedersen, Vrints & Conraads, 2006b). However, the mechanisms that link hypertension with increased risk of atherosclerosis, and thus CAD and Type D with poor cardiac prognosis in CAD patients, respectively, are not fully understood.

Atherosclerosis, as the underlying process of CAD, is defined as a progressive chronic inflammatory process of arterial wall thickening, and is characterized by intense immunological activity (Hansson & Libby, 2006). The innate immune system plays a pivotal role in the initiation and progression of the inflammatory process in atherosclerosis, with inflammatory macrophages (i.e. tissue-based phagocytic immune cells) derived from circulating peripheral blood monocytes (Davies, Jenkins, Allen & Taylor, 2013; Epelman, Lavine & Randolph, 2014) being key cells in this process (Ghattas, Griffiths, Devitt, Lip & Shantsila, 2013; Ley, Miller & Hedrick, 2011; Moore & Tabas, 2011). A key innate immune effector function of inflammatory macrophages, in particular classically activated (M1) inflammatory macrophages, is microbicidal activity, i.e. the killing of microbes (Colin, Chinetti-Gbaguidi & Staels, 2014; Mosser & Edwards, 2008; Zhang & Wang, 2014). Microbicidal activity, in turn, is largely due to the production of reactive oxygen species (ROS), particularly superoxide anions (de Oliveira-Junior, Bustamente, Newburger & Condino-Neto, 2011; Halliwell, 2006), derived from the activated multisubunit enzyme nicotinamide adenine dinucleotide phosphate (NADPH) oxidase located in the phagolysosomal and plasma membrane of macrophages (De Oliveira-Junior, 2011; Cathcart, 2004).

The emphasis on the inflammatory nature of atherosclerosis raised the possibility to examine new immunological pathways in order to understand the relationship between hypertension and atherosclerosis as well as Type D and poor cardiac prognosis in CAD. Indeed, research in hypertension and CAD indicated that NADPH oxidase-derived superoxide anions likely play a crucial role in the pathogenesis and progression of atherosclerosis, and thus CAD. However, in these studies, NADPH oxidase-derived superoxide anion production was analyzed either in vascular wall cells (Guzik et al., 2000a; Guzik et al., 2006; Vendrov, Hakim, Madamanchi, Rojas, Madamanchi & Runge, 2007) or in circulating macrophage precursor cells (Fortuño, Oliván, Beloqui, San José, Moreno & Diez, 2004; Moreno, San José, Fortuño, Beloqui, Diez & Zalba, 2006; Moreno et al., 2014; Watanabe, Yasunari, Nakamura & Maeda, 2006). Therefore, the possible contribution of NADPH oxidase-derived superoxide anion production by inflammatory M1 macrophages to atherosclerosis in hypertension and CAD is unclear.
Although no studies have examined the role of NADPH oxidase-derived superoxide anion production in CAD patients with Type D, there is evidence for a dysregulation of the immune system as a potential underlying mechanism that may explain the association between Type D and poor cardiac prognosis in CAD. Studies in patients with chronic heart failure (CHF) have shown that Type D independently predicts increased circulating levels of tumor necrosis factor-alpha (TNF-α) and TNF-α soluble receptors (Conraads, Denollet, De Clerck, Stevens, Bridts & Vrints, 2006; Denollet, Conraads, Brutsaert, De Clerck, Stevens & Vrints, 2003). Notably, TNF-α is a proinflammatory cytokine that has been shown to be involved in atherosclerosis by inducing the production of ROS (Zhang et al., 2009). Furthermore, Type D has been associated with increased oxidative stress (Kupper, Gidron, Winter & Denollet, 2009) and endothelial dysfunction (van Dooren et al., 2016), where superoxide anions play an important role. It should be emphasized that endothelial dysfunction accelerates atherosclerosis and has been shown to be an independent predictor of poor cardiac prognosis in CAD patients (Halcox et al., 2002; Heitzer, Schlinzig, Krohn, Meinertz & Münzel, 2001; Suwaidi, Hamasaki, Higano, Nishimura, Holmes & Lerman, 2000).

Given the importance of NADPH oxidase-derived superoxide anion production in essential hypertension as well as superoxide anions and Type D in CAD, we aimed to investigate NADPH oxidase-derived superoxide anion production by human inflammatory M1 macrophages – as pivotal inflammatory cells in the atherosclerotic process – in individuals with essential hypertension and CAD patients with Type D, in order to provide new insights into the potential mechanisms that link both hypertension with increased risk of

atherosclerosis and Type D with poor cardiac prognosis in CAD. Before addressing these research questions, our first aim was to implement and validate an appropriate method for the measurement of NADPH oxidase-derived superoxide anion production by human inflammatory M1 macrophages.

The presentation of the thesis is organized as follows: The theoretical background briefly reviews the main research areas of macrophages, essential hypertension, CAD with its underlying process of atherosclerosis, and Type D. In all research areas, a specific emphasis is placed on the role of NADPH oxidase-derived superoxide anions. Following this, summaries of the empirical studies and their results are presented. The detailed manuscripts of the studies are provided in the Appendix. Finally, the thesis concludes with a general discussion of the results and directions for future research.

2 THEORETICAL BACKGROUND

The purpose of this chapter is to review the main research areas and their association with NADPH oxidase-derived superoxide anion production in order to understand the nature of our empirical studies reported in chapter 4.

This chapter includes five main sections: Section 2.1 provides an understanding of macrophages; section 2.2 presents the cardiovascular risk factor essential hypertension with an emphasis on the role of NADPH oxidase-derived superoxide anions; section 2.3 includes CAD and the underlying process of atherosclerosis with an emphasis on the role of macrophages and NADPH oxidase-derived superoxide anion production in this process. Section 2.4 describes Type D as a psychosocial risk factor for poor cardiac prognosis in CAD patients and elucidates potential biological mechanisms underlying this association, while section 2.5 briefly summarizes the theoretical background.

2.1 Macrophages

Macrophages are tissue-based immune cells (i.e. white blood cells or leukocytes) and were first discovered by the Russian zoologist Ilya Illyich Mechnikov in the late 19th century (Davies et al., 2013; Epelman et al., 2014), who described their phagocytic nature (Nathan, 2008; Tauber, 2003). Macrophages are present in lymphoid and non-lymphoid tissues of the body (Geissmann, Manz, Jung, Sieweke, Merad & Ley, 2010) and are known to play an essential role in both the innate and the adaptive immune system (Abbas, Lichtman & Pillai, 2007; Biswas, Chittezhath, Shalova & Lim, 2012; Dale, Boxer & Liles, 2008).

Macrophages are multi-functional cells and exhibit functional diversity based on their microenvironment (Biswas et al., 2012; Haldar & Murphy, 2014). Beside their hallmark function of phagocytic ability in order to engulf and kill microbes, tumor cells, and other invaders, macrophages also have the ability to present antigen to T lymphocytes, and thus activate specific defense mechanisms of the adaptive immune system (Adams, 1994; Woods, 2000). Furthermore, macrophages are potent secretory cells (Adams, 1994). The secreting molecular products are involved in inflammation, growth regulation, and hematopoiesis. In addition, macrophages influence lymphocyte function, affect tissue repair, act as autoregulatory factors, or are microbicidal (Woods, 2000). This functional diversity of macrophages is first and foremost required to maintain homeostasis (Gordon & Taylor, 2005;

Wynn, Chawla & Pollard, 2013). Figure 1 provides an overview of the major effector functions of macrophages.

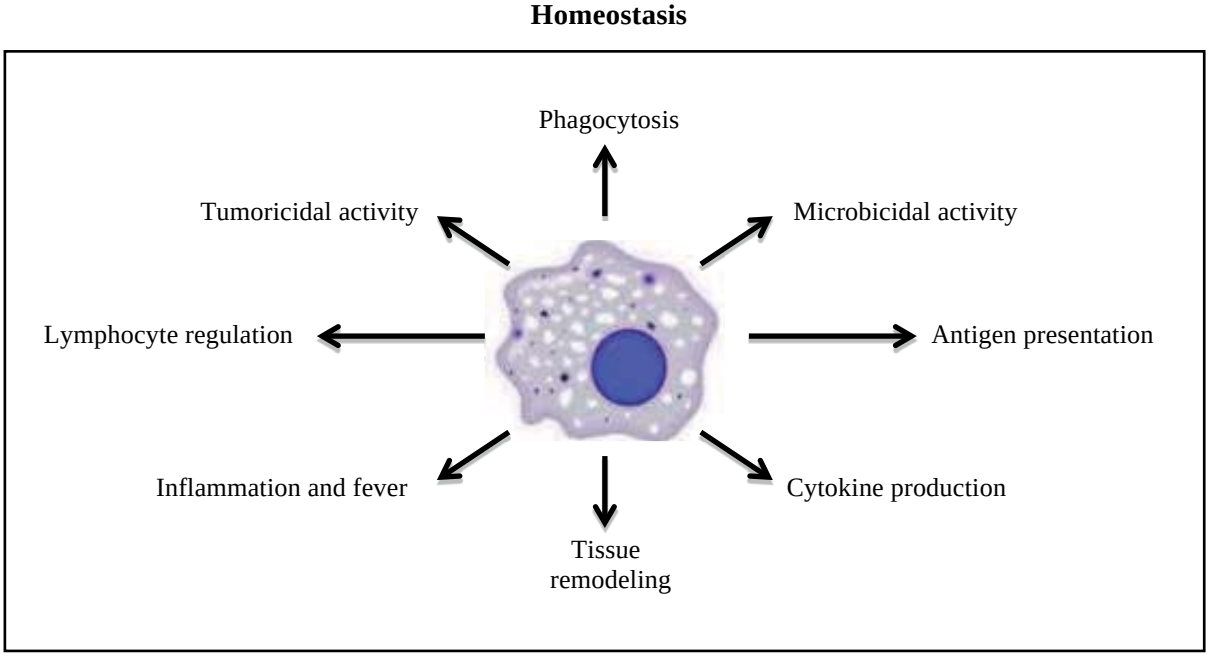

Figure 1: Major effector functions of macrophages. Adapted from Woods (2000).

2.1.1 Origin of macrophages

Macrophages are mononuclear phagocytes and thus cells of the *mononuclear phagocyte system* (MPS). Van Furth and colleagues first introduced the concept of the MPS in the late 1960s and early 1970s (van Furth, Cohn, Hirsch, Humphrey, Spector & Langevoort, 1972). This system is defined as a hematopoietic cell lineage derived from progenitor cells in the bone marrow, the so-called hematopoietic stem cell (HSC; Cao, Harris & Wang, 2015; Hume, 2006; van Furth et al., 1972), and encompasses cells with similar morphology, origin, and biology including promonocytes and their precursors in the bone marrow, circulating blood monocytes, tissue macrophages, and dendritic cells (Hume, 2008; van Furth et al., 1972).

The differentiation of macrophages in the MPS is described as follows (see Figure 2): HSC as progenitor cells give rise to monoblasts, promonocytes, and finally monocytes, which enter into the peripheral blood, where they circulate for several days. Afterwards, monocytes migrate from the blood into tissues, where they further mature into tissue-specific macrophages of the bone (osteoclast), lung (alveolar macrophages), central nervous system (microglial cells), connective tissue (histiocytes), gastrointestinal tract, liver (Kupffer cells),

spleen, and peritoneum (Abbas et al., 2007; Mosser & Edwards, 2008). Notably, tissue-specific macrophages are continually present in the tissue. Both the development of monocytic cells (i.e. monoblast, promonocyte, and monocyte) and the differentiation of monocytes into macrophages are driven by a protein called macrophage colony-stimulating factor (Mosser & Edwards, 2008; Takahashi, 2001). It should be emphasized that macrophages are differentiated from circulating blood monocytes under non-inflammatory and inflammatory conditions (Takahashi, 2001).

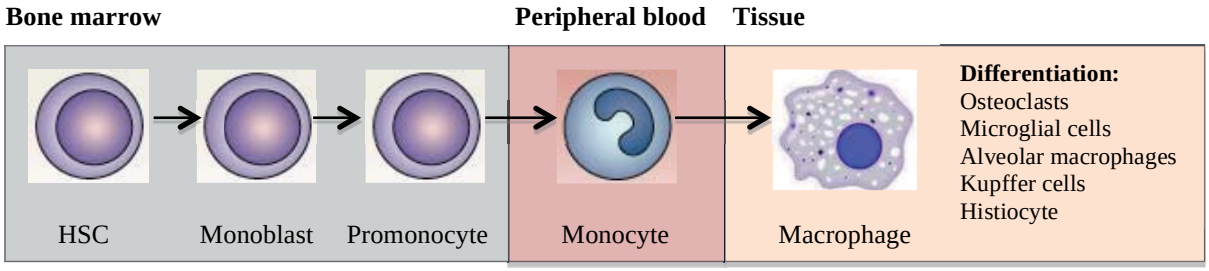

Figure 2: Mononuclear phagocyte system. Adapted from Mosser & Edwards (2008).

2.1.2 Classification of macrophages under inflammatory conditions

Inflammatory macrophages derived from circulating peripheral blood monocytes represent key cells in inflammatory processes (Duffield, 2003; Gordon & Taylor, 2005) and are termed as monocyte-derived inflammatory macrophages (Italiani & Boraschi, 2014). In contrast to tissue-specific macrophages, inflammatory macrophages are only present in the inflamed tissue under inflammatory conditions (Abbas et al., 2007; Mosser & Edwards, 2008).

During inflammation, the migration of circulating monocytes from the bloodstream into inflamed tissue is extremely enhanced and leads to an increase of macrophages at inflamed tissue sites (Mosser & Edwards, 2008) in order to optimize the immune defensive firepower (Italiani & Boraschi, 2014). To increase their competence for host defense under inflammatory conditions, monocyte-derived inflammatory macrophages additionally need to be activated by signals from their microenvironment (i.e. interferon from lymphocytes, tumor cells, bacteria, foreign particles, or environmental toxins). Hence, macrophage activation is an essential cellular process underlying innate immunity (Adams, 1994; Barish, Downes, Alayick, Yu, Ocampo, Bookout, Mangelsdorf & Evans, 2005; Mosser & Edwards, 2008; Wynn et al., 2013).

Depending on the type of activating signals, monocyte-derived inflammatory macrophages can be polarized in functional phenotypes, and thus differ in terms of receptor expression, cytokine production, effector function, and chemokine repertoires (Mantonvani, Sica, Sozzani, Allavena, Vecchi & Locati, 2004; Zhang & Wang, 2014). Two well-established phenotypes are commonly referred to as *classically activated (M1) macrophages* and *alternatively activated (M2) macrophages* (Gordon & Taylor, 2005; Zhang & Wang, 2014). This M1 and M2 terminology reflects the T-helper 1 (Th1) / T-helper 2 (Th2) lymphocytes polarization scheme (Biswas et al., 2012; Hume & Freeman, 2014; see Figure 3) and differs in terms of polarizing signals, cytokine production, and function (Biswas et al., 2012).

2.1.2.1 Classically activated macrophages (M1 phenotype)

Classically activated macrophages are characterized by a high expression of the surface molecules major histocompatibility complex (MHC) class II and B7-2 (CD86; i.e. a costimulator for T-cell activation) and a low expression of mannose receptors (Mosser, 2003). Mannose receptor is a type I transmembrane glycoprotein that binds mannose and fucose residues on microbial walls and mediates phagocytosis (Abbas et al., 2007). Furthermore, classically activated macrophages show a change in their secretory profile pattern by secreting higher levels of proinflammatory cytokines such as interleukin (IL)-6, IL1ß, TNF-α, IL-12 and IL-23 as well as higher levels of chemokines such as CCL15, CCL20, CXCL9, and CXCL10 (Colin et al., 2014; Martinez & Gordon, 2014; Mosser & Edwards, 2008; Zhang & Wang, 2014). Notably, a higher expression of the different chemokines promotes the recruitment of Th1 cells, natural killer (NK) cells, and different leukocytes in order to drive cell inflammatory response forward (Colin et al., 2014). In addition to the higher proinflammatory cytokine and chemokine secretion, classically activated macrophages produce high amounts of highly oxidizing agents (i.e. reactive oxygen species (ROS) and nitrogen species (RNS)), which play a mediating role in microbicial activity, that is, the killing of microbes (Martinez, Sica, Mantovani & Locati, 2008).

Classically activated macrophages are formed in response to interferon-gamma (IFN-γ), alone or in combination with microbe or microbial product such as lipopolysaccharide (LPS), and other inflammatory cytokines like TNF-α (Colin et al., 2014; Mosser, 2003).

IFN-γ is known as the main cytokine associated with the conversion into classically activated macrophages (Mosser & Edwards, 2008) and is secreted by activated CD4+ Th1 cells, CD8+

T cytotoxic 1 cells, and NK cells (Martinez, Helming & Gordon, 2009). The binding of IFN-γ to its receptors (i.e. IFNGR-1 and IFNGR-2 subunits of the IFN-γ receptors) on the surface of macrophages leads to recruitment of Janus kinase (Jak) 1 und Jak2, in turn leading to the phosphorylation of signal transducers and activators of transcription 1 (STAT1). Phosphorylated STAT1 translocates to the cell nucleus and stimulates transcription of STAT1 target genes (Hu & Ivashkiv, 2009; Martinez & Gordon, 2014). In addition, IFN-γ enhances macrophage responsiveness to LPS by an up-regulation of the LPS receptor termed as Toll-like receptor 4 (TLR4; Hu & Ivashkiv, 2009; Schroder, Sweet & Hume, 2006). Notably, the secretion of IFN-γ during inflammation primes macrophages to synthesize proinflammatory cytokines (e.g. IL-6, IL1ß, and TNF-α) and to secrete increased amounts of ROS and RNS in order to enhance their killing ability (Colin et al., 2014; Mosser & Edwards, 2008; Zhang & Wang, 2014). For a sustained M1 population and thus a constant host defense, a sustained IFN-γ production by Th1 lymphocytes is needed (Mosser & Edwards, 2008; Mosser).

LPS is an important component of the cell membrane of Gram-negative bacteria (Hunter, Wang, Eubank, Baran, Nana-Sinkam & Marsh, 2009; Lu, Yeh & Ohashi, 2008) and is recognized by a cell surface receptor complex consisting of the TLR4 and its accessory protein MD-2 (Guha & Mackman, 2001). The activation of the TLR4-MD-2 complex induces the activation of several intracellular signaling pathways such as IkappaB kinase (IKK)-nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), mitogen-activated protein kinase (MAPK), and STAT1 (Guha & Mackman, 2001; Martinez & Gordon, 2014), which in turn lead to the activation of different transcription factors. The activation of these transcription factors coordinates the induction of numerous genes encoding inflammatory mediators (e.g. TNF-α; Guha & Mackman, 2001).

The proinflammatory cytokine TNF-α is secreted by macrophages themselves in response to stimulation with LPS (Takashiba, van Dyke, Amar, Murayama, Soskole & Shapira, 1999) and IFN-γ (Wynn et al., 2013). TNF-α also contributes to the formation of classically activated macrophages through the activation of intracellular signaling pathways (e.g. IKK-NF-κB, MAPK) indicated above (Takashiba et al., 1999). Furthermore, TNF-α has been shown to increase ROS production and thus enhance the microbicidal activity of classically activated macrophages supposedly by the activation of the NF-κB pathway (Gauss et al., 2007).

Notably, other cytokines (e.g. IL-10, transforming growth factor beta (TGFß)) inhibit the activation of classically activated macrophages (Abbas et al., 2007; Martinez & Gordon, 2014).

Functionally, classically activated macrophages are characterized by cellular immunity, proinflammatory cytokine production, and ROS/RNS-mediated microbicidal activity (Biswas et al., 2012; Cao et al, 2015; Dale et al., 2008; Gordon & Taylor, 2005). Thus, M1 macrophages are responsible for anti-microbial responses and tissue damage (Hunter et al., 2009). Notably, a chronic induction of M1 macrophage activation can cause chronic inflammation and tissue damage (Colin et al., 2014; Mosser, 2003; Mosser & Edwards, 2008).

2.1.2.2 Alternatively activated macrophages (M2 phenotype)

As this thesis focuses on M1 macrophages, this section only briefly describes M2 macrophages and their sub-groups.

In contrast to classically activated macrophages, alternatively activated macrophages or M2a macrophages are primarily formed in response to the cytokines IL-4 and IL-13 produced by Th2 cells, mast cells, eosinophils, basophils, NK cells, and even macrophages themselves (Colin et al., 2014; Gordon & Martinez, 2010; Martinez & Gordon, 2014). IL-4 and IL-13 down-regulate intracellular signaling pathways (e.g. NF-κB and STAT1) in order to inhibit the induction of inflammatory chemokines associated with the development of inflammation (Mantovani et al., 2004). M2a macrophages are characterized by the production of anti-inflammatory cytokines such as IL-10 (high production) and IL-12 (low production; Colin et al., 2014; Mantovani et al., 2004). In addition, they express high levels of scavenger, mannose, and galactose receptors (Biswas et al., 2012) and secrete pro-fibrotic factors (e.g. fibronetic), insulin-like growth factor (IGF), and TGFß (Colin et al., 2014; Zhang & Wang, 2014).
Depending on the activating stimuli, M2a macrophages are further classified into two sub-groups (Martinez et al., 2008): (1) M2b macrophages are induced by exposure to immune complexes in combination with LPS and IL-1ß, and (2) M2c macrophages are induced by IL-10, TGFß, or glucocorticoids. M2c macrophages display high expression levels of the Mer receptor tyrosine kinase (MerTK) to provide phagocytosis of apoptotic cells (Colin et al., 2014; Hunter et al., 2009).

Generally, alternatively activated macrophages are involved in the suppression of inflammation, tissue remodeling, wound healing, parasite clearance, tumor progression, and

immunoregulation (Biswas et al., 2012; Sica & Mantovani, 2012), and promote allergic responses (Hunter et al., 2009).

Figure 3 provides a simplified overview of the M1- and M2-polarized macrophages. However, M1 and M2 macrophages only represent two extremes of a linear scale (Biswas et al., 2012; Mosser & Edwards, 2008). This binary classification cannot represent the nuances that exist between macrophage populations (Wynn et al., 2013).

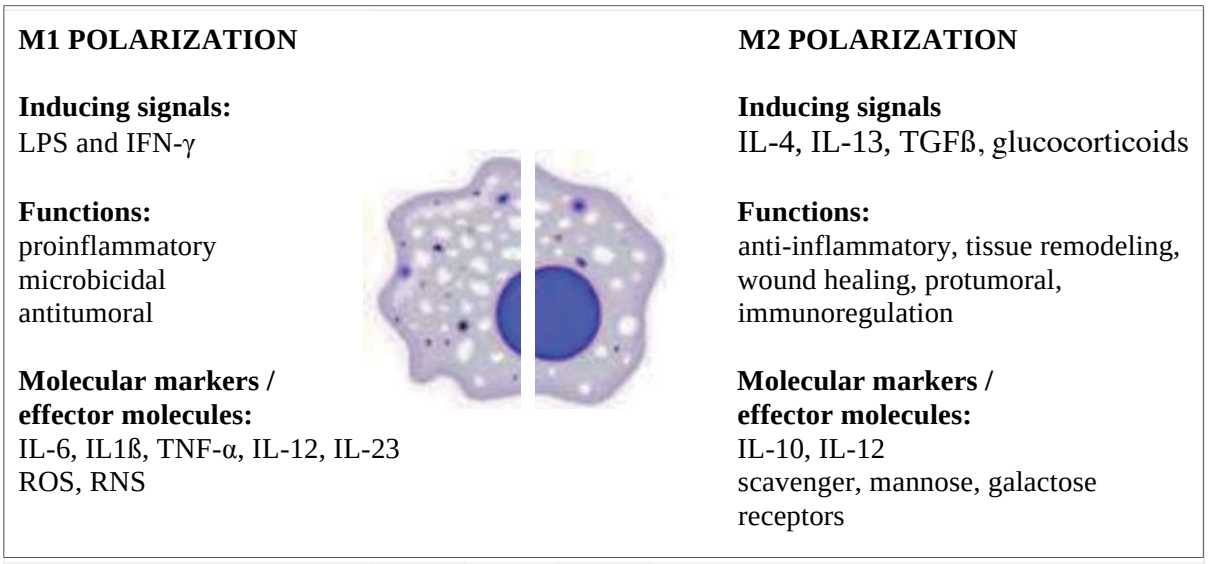

Figure 3: M1- and M2-polarized macrophages. Adapted from Biswas et al., (2012) and Mantovani, Sica & Locati (2007)

2.1.3 Microbicidal activity

Microbicidal activity is a key innate immune effector function of classically activated M1 macrophages (Colin et al., 2014; Mosser & Edwards, 2008; Zhang & Wang, 2014; see section 2.1.2.1). The killing of microbes, and thus microbicidal activity, is primary mediated by oxygen-dependent mechanisms. These mechanisms include the production of free radicals (Paulnock, 2000).

2.1.3.1 Free radicals

Free radicals are molecules containing one or more unpaired electrons, and include ROS and RNS (Halliwell, 2006; Hunter et al., 2009). Both ROS and RNS produced by M1 macrophages play a critical role in host defense (Nathan & Siloh, 2000).
ROS and RNS are collective terms and include both free radical and non-radical compounds (Halliwell, 2006). Notably, due to the unpaired electron, free radicals are highly reactive, unstable and short-lived, whereas non-radical derivatives are less reactive, more stable and long-lived (Paravicini & Touyz, 2008). Free oxygen radicals of ROS comprise, for example, superoxide (O_2^-), hydroxyl (OH), hydroperoxyl (HO_2), and carbonate (CO_3^-), whereas the non-radical oxygen derivatives include hydrogen peroxide (H_2O_2), hypobromous acid (HOBr), hypochlorous acid (HOCl), and ozone (O_3). Nitric oxide (NO), nitrogen dioxide (NO_2), and nitrate radical (NO_3) are known as free radical nitrogen compounds of RNS, whereas nitrous acid (HNO_2), dinitrogen tetroxide (N_2O_4), and dinitrogen trioxide (N_2O_3) are representatives of the non-radical nitrogen compounds (Halliwell, 2006).

Notably, two free radicals are able to join their unpaired electrons and form a covalent bond. Hence, ROS and RNS have common non-radicals such as peroxynitrite ($ONOO^-$) formed by the reaction of NO and O_2, peroxynitrate (O_2NOO^-) formed by the reaction of $ONOO^-$ and CO_2^-, and peroxynitrous acid (ONOOH) formed by the reaction of OH and NO_2 (Halliwell, 2006). Table 1 lists selected types of free radicals and non-radicals of ROS and RNS.

Table 1

Selected types of free radicals and non-radicals of ROS and RNS. Adapted from Halliwell (2006).

	Reactive oxygen species (ROS)	**Reactive nitrogen species (RNS)**
Free radicals	Superoxide (O_2^-) Hydroxyl (OH) Hydroperoxyl (HO_2) Carbonate (CO_3^-) Peroxyl (RO_2) Alkoxyl (RO) Carbon dioxide radical (CO_2^-) Singlet ($O_2{}^1\sum g^+$)	Nitric oxide (NO) Nitrogen dioxide (NO_2) Nitrate radical (NO_3)
Non-radicals	Hydrogen peroxide (H_2O_2) Hypobromous acid (HOBr) Hypochlorous acid (HOCl) Ozone (O_3) Organic peroxides (ROOH) Peroxomonocarbonate ($HOOCO_2$). Peroxynitrite ($ONOO^-$) Peroxynitrate (O_2NOO^-) Peroxynitrous acid (ONOOH)	Nitrous acid (HNO_2) Nitrosyl cation (NO^+) Nitroxyl anion (NO^-) Dinitrogen tetroxide (N_2O_4) Dinitrogen trioxide (N_2O_3) Nitronium cation (NO_2^+) Peroxynitrite ($ONOO^-$) Peroxynitrate (O_2NOO^-) Peroxynitrous acid (ONOOH)

Although both ROS and RNS are involved in oxygen-dependent mechanisms of microbicidal activity, the microbicidal effectiveness of M1 macrophages is largely due to their production

of ROS (de Oliveira-Junior et al., 2011; Halliwell, 2006). Therefore, in the following, the main source of ROS will be described.

2.1.4 Sources of reactive oxygen species

ROS are generated through a variety of sources, which are classified into intracellular and extracellular (Hunter et al., 2009). Intracellular sources comprise the mitochondria electron transport chain, cytochrome p450 (hemoproteins), lipoxygenase and cyclooxygenase pathway, xanthine oxidase complex, peroxisomes, and NADPH oxidase complex (Inoue, Sato, Nishikawa, Park, Kira, Imada & Utsumi, 2003). Extracellular sources include oxidative stress and chemical agents (Ueda, Masutani, Nakamura, Tanaka, Ueno & Yodoi, 2002).

The NADPH oxidase complex is a well-documented and major intracellular source of ROS (Iles & Forman, 2002). Notably, the classic NADPH oxidase was first described and characterized in phagocytes such as neutrophils, eosinophils, or macrophages and is known as *phagocytic* NADPH oxidase. However, studies indicated that similar NADPH oxidase systems are present in non-phagocytic cells such as fibroblasts, endothelial cells, or vascular smooth muscle cells (VSMCs). This NADPH oxidase type is called *non-phagocytic* or *vascular* NADPH oxidase (Quinn & Gauss, 2004).

2.1.4.1 Phagocytic NADPH oxidase

The NADPH oxidase is a multi-component enzyme complex (Babior, 2004) that plays an essential role in killing microbes by generating superoxide anions and other types of ROS (El-Benna, Dang, Gougerot-Pocidalo & Elbim, 2005). This multi-component enzyme is located in the plasma membrane and cytosol of unstimulated phagocytic cells (Vignais, 2002), and consists of two membrane-bound elements ($gp91^{phox}$ and $p22^{phox}$; **phox** for **ph**agocyte **ox**idase), three cytosolic components ($p67^{phox}$, $p47^{phox}$, and $p40^{phox}$) and a low-molecular-weight G protein (Rac1 in monocytes/macrophages or Rac2 in neutrophils; Babior, 2004; El-Benna et al., 2005).

Notably, in unstimulated cells, the NADPH oxidase is unassembled and inactive and its components are divided into plasma membrane and cytosolic locations (DeLeo, Allen, Apicella & Nauseef, 1999), i.e. $gp91^{phox}$ and $p22^{phox}$ are located in the plasma membrane,

whereas $p40^{phox}$, $p47^{phox}$, $p67^{phox}$, and Rac are located in the cytosol as a complex (Babior, 1999).

2.1.4.1.1 Phagocytic NADPH oxidase components

$gp91^{phox}$ and $p22^{phox}$

The two membrane-bound components $gp91^{phox}$ (also termed as Nox2) and $p22^{phox}$ compose the cytosolic core of the enzyme (Cachat, Deffert, Hugues & Krause, 2015). $gp91^{phox}$ is the most important element of NADPH oxidase (Babior, 2004) and is localized in intracellular and plasma membranes in close association with $p22^{phox}$ (Bedard & Krause, 2007). $gp91^{phox}$ contains the catalytic site and the NADPH-binding site, whereas $p22^{phox}$ is responsible for both $gp91^{phox}$ stability and the docking of $p47^{phox}$ (Cachat et al., 2015) in order to bring the cytosolic oxidase complex to the membrane to assemble the active oxidase (Babior, 2004). In contrast to $gp91^{phox}$, $p22^{phox}$ is not directly involved in the electron transfer (Nobuhisa Takeya, Oguras, Ueno, Kohda, Inagaki & Sumimoto, 2006). Both $gp91^{phox}$ and $p22^{phox}$ represent subunits of the heterodimer flavocytochrome b_{558} (Babior, 1999; Orient, Donko, Szabo, Leto & Geiszt, 2007). Flavocytochrome b_{558} comprise one flavin adenine dinucleotide (FAD) and two hemes and is the electron transfer chain of NADPH oxidase (Babior, 1999; El-Benna et al., 2005). Interestingly, $p22^{phox}$ was found in all major cellular components of the human vessel wall, i.e. endothelial cells, VSMCs, macrophages, and fibroblasts, indicating that $p22^{phox}$ is a common component of the *phagocytic* and *vascular* NADPH oxidase system (Azumi et al., 1999; Sorescu et al., 2002).

$p67^{phox}$

$p67^{phox}$ interacts with both Rac1/Rac2 and flavocytochrome b_{558} and regulates its catalytic activity (El-Benna et al., 2005). In addition, $p67^{phox}$ is suggested to be involved in the transfer of electrons directly from NADPH to oxygen to form superoxide (Babior, 2004).

$p47^{phox}$

$p47^{phox}$ binds to flavocytochrome b_{558} during activation and is responsible for transporting the cytosolic $p40^{phox}$- $p47^{phox}$- $p67^{phox}$ complex from the cytosol to the membrane during oxidase activation (Babior, 1999; El-Benna et al., 2005).

$p40^{phox}$

$p40^{phox}$ forms a part of the cytosolic oxidase subunit complex $p40^{phox}$- $p47^{phox}$- $p67^{phox}$. This component is not required for NADPH oxidase activation, and thus its function is still unclear (Babior, 2004; El-Benna et al., 2005; Quinn & Gauss, 2004). However, there are indications that $p40^{phox}$ plays a potential role in stabilization of the $p47^{phox}$- $p67^{phox}$ complex and in facilitating membrane recruitment of this complex during NADPH oxidase activation (Roos, van Bruggen & Meischl, 2003). Furthermore, $p40^{phox}$ is suggested to be both an activator and an inhibitor of NADPH oxidase (Babior, 1999; Groemping & Rittinger, 2005).

Rac

Rac belongs to the Rho-family of small GTPases, which is responsible for the regulation of a variety of signaling pathways (Groemping & Rittinger, 2005). As Rac interact with flavocytochrome b_{558} and $p67^{phox}$ and thus modulate the function of more than one NADPH oxidase protein (Quinn & Gauss, 2004), its presence is essential for optimal NADPH oxidase activation (El-Benna et al., 2005; Groemping & Rittinger, 2005).

2.1.4.1.2 Activation and assembly of the phagocytic NADPH oxidase

The role of NADPH oxidase is to catalyze the production of superoxide anions by transferring electrons from cytoplasmic NADPH to extracellular or intraphagolysosomal oxygen molecules (Babior, 2004; Cachat et al., 2015; Roos et al., 2003) according to the following reaction (Babior, 2004; de Oliveira-Junior et al., 2011; El-Benna et al., 2005; Roos et al., 2003):

$$NADPH + 2O_2 \rightarrow NADP^+ + H^+ + 2O_2^-$$

The superoxide anions formed in this reaction are further converted into hydrogen peroxide (H_2O_2) and other types of ROS (Babior, 2004; Cachat et al., 2015; de Oliveira-Junior et al., 2011). Thus, superoxide anions serve as a precursor to other, more reactive ROS (Babior, 1999; de Oliveira-Junior et al., 2011; Nobuhisa et al., 2006; Sheppard, Kelher, Moore, McLaughlin, Banerjee & Silliman, 2005).

In order to generate and provide agents to kill microbes, the unassembled and inactive NADPH oxidase needs to be activated (DeLeo et al., 1999). The NADPH oxidase activation is accompanied by two events: (1) protein phosphorylation and (2) translocation of cytosolic components to the plasma membrane (El-Benna et al., 2005).