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Úloha NADPH oxidázy při tvorbě volných radikálů a modifikace proteinů

DIPLOMOVÁ PRÁCE

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Role of NADPH oxidase in free radical generation and protein modification

DIPLOMA THESIS

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Abstrakt:

Monocyty hrají klíčovou roli v imunitním systému a jejich diferenciace v makrofágy je podstatným aspektem imunitní regulace. Nikotinamidadenindinukleotidfosfát (NADPH) oxidáza, známá také jako NOX, hraje klíčovou roli při tvorbě reaktivních forem kyslíku (ROS) z molekulárního kyslíku (O_2), které mohou ovlivňovat polarizaci makrofágů. Cílem této studie je porozumět NADPH oxidáze a jejím izoformám v kontextu tvorby ROS a modifikací protein, a jejich vztahu k oxidačnímu stresu a patologickým procesům.

Zkoumali jsme tvorbu ROS a modifikace proteinů během diferenciace makrofágů vyvolané působením forbol-12-myristátu-13-acetátu (PMA) a kyseliny askorbové. PMA je dobře známý silný aktivátor proteinkinázy C (PKC) a kyselina askorbová, která je známým antioxidantem, má prooxidační schopnosti. Zjištěné morfologické změny a exprese NADPH oxidázy naznačují, že monocyty se mohou v reakci na zánět nebo diferenciaci přeměnit na makrofágy produkující ROS. Kromě polarizace monocytů na makrofágy jsme studovali expresi NOX2 během této přeměny a úroveň modifikace proteinů.

Klíčová slova	Buňky U-937, NADPH oxidázy, volné radikály, oxidativní stress, monocyty, makrofágy, konfokální laserová skenovací mikroskopie, malondialdehyd.
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Abstract

Monocytes play crucial roles in the immune system, and their differentiation into macrophages is a significant aspect of immune regulation. Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, also known as NOX, plays a critical role in the generation of reactive oxygen species (ROS) from molecular oxygen (O_2), which can influence macrophage polarization. The present study aims to comprehend NADPH oxidase and its isoforms in the context of ROS formation and protein modifications and their relationship with oxidative stress and pathological processes.

We investigated ROS generation and protein modifications during macrophage differentiation induced by Phorbol-12-Myristate-13-Acetate (PMA) and ascorbic acid (vitamin C). PMA is a well-known potent activator of protein kinase C (PKC), and ascorbic acid, being a well-known antioxidant, has been reported to possess pro-oxidant capabilities. The morphological changes and the expression of NADPH oxidase found suggest that monocytes can transform into ROS-producing macrophages in response to inflammation or differentiation. In addition to the polarization of monocytes to macrophages, we studied the expression of NOX2 during the transition and the level of protein modification during this transition.

Keywords	U-937 cells; NADPH oxidase, reactive oxygen species; oxidative stress; monocytes; macrophages, confocal laser scanning microscopy, malondialdehyde.
Number of pages	54
Number of appendices	1
Language	English

Aims of the Diploma thesis

1. Understanding NADPH oxidase and its isoforms, and its relation with reactive oxygen species/ free radical generation.
2. To learn human cell handling, protocol standardization, and use of different methodologies including but not limited to cell proliferation assay, protein isolation, SDS-PAGE, western blotting, etc.
3. Summarize reaction mechanism leading to the generation of reactive oxygen species and protein modification.
4. Evaluate the measured results and discuss them in relation to the literature.
5. Conclude the role of NADPH oxidase and its isoform in cells.

Contents

1 Introduction

Reactive oxygen species (ROS) are small and highly reactive molecules derived from oxygen metabolism in living organisms. This group includes radicals like the superoxide anion radical ($O_2^{\bullet -}$) and the hydroxyl radical ($HO^{\bullet}$), as well as non-radicals such as hydrochlorous acid and ozone. They have dual roles, acting as stressors implicated in various diseases, such as cardiovascular disease, while also serving as inflammatory mediators, eliminating microorganisms, and playing crucial roles in various physiological processes, including cell signaling, immune response, and regulation of gene expression. However, excessive ROS production can lead to oxidative stress, causing damage to cellular components such as proteins, lipids and DNA (Deoxyribonucleic acid), and contributing to the pathogenesis of numerous diseases (Bardaweel et al., 2018; Collin, 2019; Liu et al., 2023).

One of the main sources of ROS generation in cells is the Nicotinamide Adenine Dinucleotide Phosphate (NADPH) oxidase enzyme system. NADPH oxidases are multi-subunit protein complexes located in the plasma membrane and other cellular organelles. These enzymes catalyze the transfer of electrons from NADPH to molecular oxygen (O_2), resulting in the production of $O_2^{\bullet -}$ and other ROS. Understanding the structure and function of NADPH oxidase and its isoforms is essential for elucidating its role in ROS generation and cellular physiology. This includes exploring the various isoforms of NADPH oxidase and their distinct distribution patterns and expression levels in different tissues throughout the body. Additionally, studying the regulation of NADPH oxidase activity and its interaction with other cellular components provides insights into the mechanisms underlying ROS production.

To investigate the role of NADPH oxidase in ROS generation and its effects on cellular processes, it is imperative to employ a range of experimental techniques and methodologies. This includes mastering human cell handling techniques, standardizing experimental protocols, and utilizing diverse methodologies such as cell proliferation assays, protein isolation, sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE), Western blotting, and other biochemical assays.

Furthermore, elucidating the reaction mechanisms that lead to ROS generation and protein modification is crucial for understanding the molecular basis of oxidative

stress-related diseases. By evaluating the measured results and comparing them with the existing literature, researchers can gain deeper insight into the physiological significance of NADPH oxidase and its isoforms in cellular redox homeostasis.

In this thesis, my aim is to investigate the role of NADPH oxidase and its isoform in ROS generation, as well as the consequent mediation of protein oxidation. By integrating experimental techniques with biochemical and molecular analysis, we seek to elucidate the complex interplay between NADPH oxidase activity and ROS production when cells are subjected to differentiation with chemical inducers such as phorbol-12-myristate-13-acetate (PMA) and ascorbic acid. Through our research, our objective is to contribute to a better understanding of the pathophysiological mechanisms underlying oxidative stress-related diseases and to explore potential therapeutic strategies targeting NADPH oxidase. Furthermore, we address the dual role of ascorbic acid, where our data present a dose-dependent behavior (Ewald et al., 2017; Tarafdar and Pula, 2018; Vermot et al., 2021). The results obtained with ascorbic acid have been reffered as published article in the follow up texts and has been appended at the end of the thesis for reference.

2 Current state of the topic

2.1 NADPH oxidase

The NADPH oxidase (NOX) enzymes are intricate multi-subunit protein complexes embedded in the cell membrane. Their primary role involves facilitating the transfer of electrons across the plasma membrane to O_2. This process leads to the production of $O_2^{\bullet -}$ and, subsequently, the formation of ROS, including hydrogen peroxide (H_2O_2) and $HO^{\bullet}$ (Figure 1) (Suh Y. et al., 1999; Bedard and Krause, 2007; Gao et al., 2012).

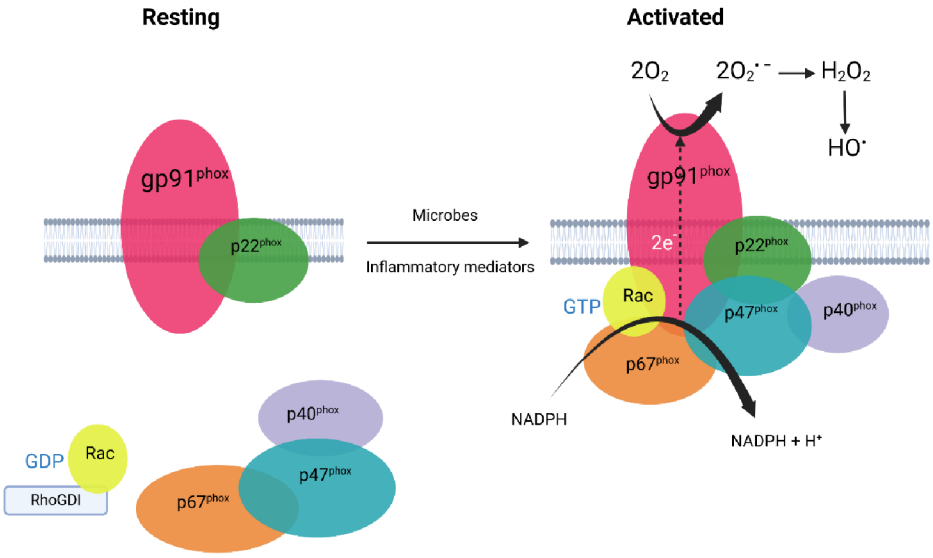

Figure 1: Scheme representing the resting and activated form of phagocytic NADPH oxidase and the reaction mechanism leading to ROS formation (created using biorender.com).

2.2 Isoforms of NADPH oxidases

NOX2, originally named $gp91^{phox}$ during the investigation of respiratory bursts in neutrophils, was the first NOX enzyme to be identified. Subsequently, upon their discovery, other members of the NOX family were cloned (Suh Y. et al., 1999; Geiszt M., 2000; Tarafdar and Pula, 2018). Presently, the NOX family encompasses NOX1, NOX2, NOX3, NOX4, NOX5, DUOX1, and DUOX2 isoforms, according to the latest nomenclature. The seven NOX isoforms (Figure 2) exhibit conserved structural properties and functions (Bedard and Krause, 2007; Brown and Griendling, 2009; Magnani et al., 2017). These include a minimum of six transmembrane domains and cytosolic domains for the binding of flavin adenine dinucleotide (FAD) and NADPH (Van der Vliet A. et al., 2018; Tarafdar and Pula, 2018).

Although the primary function of NOX enzymes is the generation of ROS, their activation mechanism can vary among different isoforms and the type of ROS. In their native state, NOX proteins exist as inactive monomers and rely on interactions with other proteins for their activation, maturation, stabilization, incorporation of heme groups, and translocation to the membrane or site of activity (Brown and Griendling, 2009; Brandes et al., 2014). NOX1 and NOX3 are considered the most closely related isoforms to phagocytic NADPH oxidase, showing a notable 60% sequence identity with NOX2. NOX1 predominates in various tissues, including the colon, prostate, uterus, and vascular cells (Suh et al., 1999; Bánfi et al., 2000; Krause, 2004). In contrast, NOX3 expression is predominantly observed in the inner ear, where it plays a significant role. In the cochlea, NOX3-mediated ROS production has been implicated in hearing loss (Rybak et al., 2012). Although NOX3 expression has also been detected in the brain and lungs, its precise functions in these tissues remain unclear (Cooney et al., 2013; Ruwanpura et al., 2013). Furthermore, similar to NOX2, both NOX1 and NOX3 undergo glycosylation *in vivo* (Nakano et al., 2007; Miyano and Sumimoto, 2014).

Under physiological conditions, the activation of NOX1 and NOX3 depends on the presence of the cytosolic factors NOXO1 and NOXA1, which have similarities to the $p47^{phox}$ and p67phox found in NOX2. However, the findings from *in vitro* studies suggest that $p47^{phox}$ and $p67^{phox}$ can effectively substitute for NOXO1 and NOXA1 during the assembly and activation processes of NOX1 and NOX3. This observation suggests that the interaction between various NOX isoforms and their assembly partners lacks complete specificity (Takeya et al., 2003; Cheng et al., 2004).

Unlike its counterparts, NOX4 exhibits constitutive activity. Although this baseline activity is believed to be modulated by the cellular positioning of NOX4, there have been indications of the involvement of activating agents such as protein disulfide isomerase or Polymerase-δ Interacting Protein 2. This proposition arose subsequent to the observation of these factors co-localizing with the NOX4/$p22^{phox}$ complex (Chen et al., 2008; Lee et al., 2010).

NOX5 has a core architecture similar to other isoforms, exhibiting 27% identity with NOX2, along with a unique N-terminal extension that houses four EF-hand motifs. Unlike other NOX isoforms, NOX5 shows different features, including Ca^{2+}-dependent

activation, independent of $p22^{phox}$ and cytosolic factors, and lack of glycosylation (Kiyohara et al., 2018).

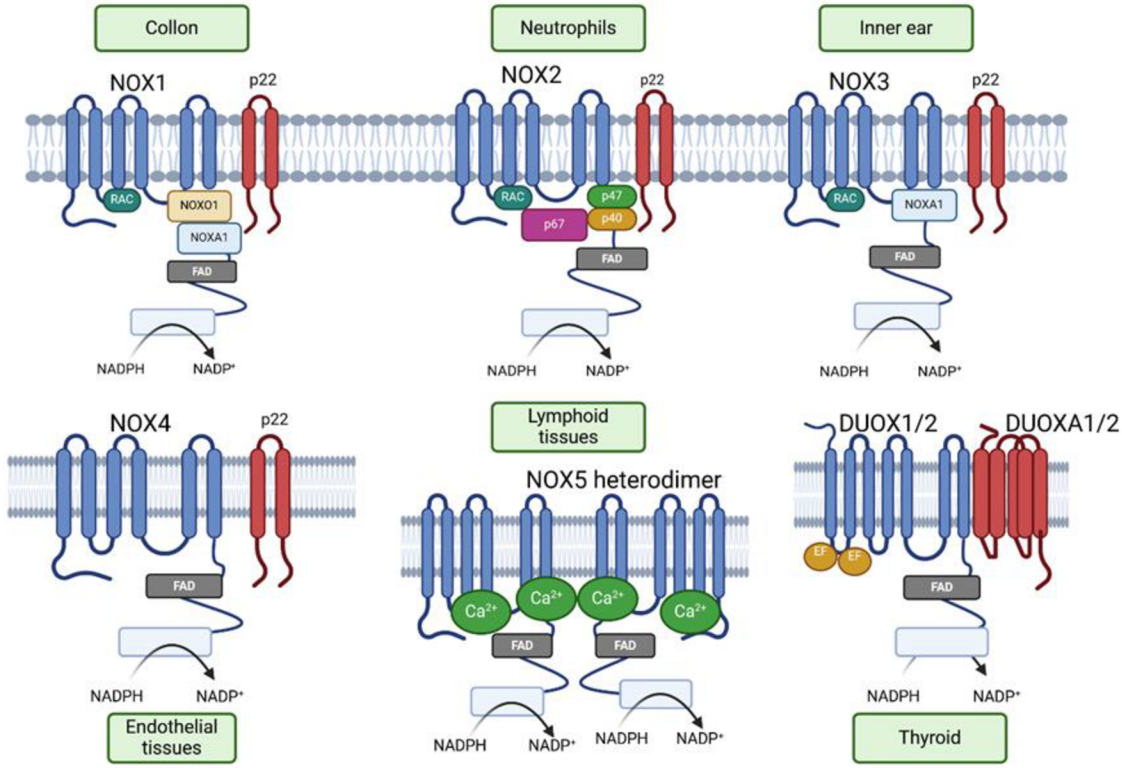

Figure 2: NADPH oxidase isoforms. Despite their similar structural features and enzymatic functions, NOX family enzymes exhibit different activation mechanisms. NOX1 activation requires the presence of $p22^{phox}$, NOXO1, NOXA1 and the small GTPase Rac, while NOX2 requires $p22^{phox}$, $p47^{phox}$, p67phox and Rac. NOX3 activation involves $p22^{phox}$ and NOXO1, and depending on the species, may require NOXA1, with the participation of Rac being uncertain. NOX4 requires $p22^{phox}$ for *in vivo* activity and shows constitutive activation. Activation of NOX5, DUOX1, and DUOX2 is mediated by Ca^{2+} ions, with DUOX1 and DUOX2 necessitating association with maturation factors DUOXA1/DUOXA2 for activation (created using biorender.com).

The discovery of dual oxidases DUOX1 and DUOX2 followed the observation of NADPH and Ca^{2+}-dependent H_2O_2 production in thyroid cells (Dupuy et al., 1999). DUOXes share similarities with other NOX isoforms, featuring the NOX catalytic core akin to NOX1-4, and an EF-hand domain similar to NOX5, albeit with two EF motifs instead of four. In mammalian DUOX, the absence of histidines responsible for heme chelation is associated with a lack of intrinsic peroxidase activity, while *C. elegans* DUOX exhibits heme binding and demonstrates modest peroxidase activity (Meitzler and Ortiz, 2009). The glycosylation status of DUOX correlates with its maturation and the nature of its ROS product.

NOX enzymes are expressed ubiquitously, yet their distribution patterns and expression levels vary across different tissues within the body (Ansari and Scheff, 2011). For example, NOX1 exhibits high expression levels in the colon, while NOX2 predominates in phagocytes. NOX3 is primarily found in the inner ear, NOX4 is abundant in the kidney and blood vessels, and NOX5 is prominent in lymphoid tissue and the testes. Additionally, DUOX1 and DUOX2 are predominantly expressed in the thyroid gland (Bánfi et al., 2001; Bedard and Krause, 2007; Altenhöfer et al., 2015).

For many years, ROS and their reaction products were believed to be just harmful by-products of aerobic metabolism that caused changes at the cellular and molecular levels. This view has been erroneously confirmed by the identification of enzymes such as superoxide dismutase (SOD), catalase, and peroxidase that serve to eliminate these waste products. However, it was later shown that there is a family of enzymes whose function is to deliberately generate ROS (Babior et al., 2002).

In addition to its role in host defense and phagocyte function, extensive evidence provides a significant role for ROS in various cellular processes, including cell proliferation, apoptosis, angiogenesis, endocrine function, and oxidative modification of the extracellular matrix (Davalli et al., 2016) (Figure 3).

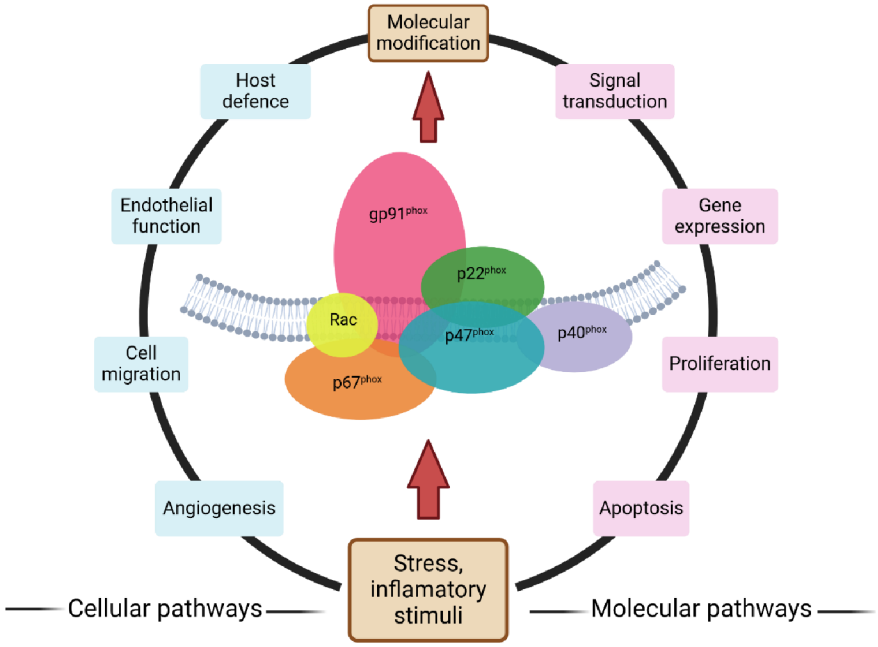

Figure 3: Scheme showing the subunits of the NADPH oxidase complex (NOX2) and the cellular or molecular pathways where its participation has been reported (created using biorender.com).

2.3 NADPH activation leading to ROS generation

Activation of phagocytic NOX is a consequence of various preceding cellular-level events. Tissue-resident macrophages play a pivotal role in the initial response to pathogenic microorganisms by detecting their presence and secreting pro-inflammatory chemoattractant at the infection site. This action triggers the extravasation of neutrophils from the vasculature, guiding them toward the inflammation epicenter through a chemoattractant gradient (Nguyen et al., 2017).

Antigen recognition on the leukocyte membrane involves various membrane receptors, including Toll-like receptor (TLR), C-type lectin receptor, macrophage mannose receptor, scavenger receptors, and opsins. These receptors facilitate direct interactions with microorganism-specific motifs, leading to activation of the immune response (DeLeo et al., 1999; Yang et al., 2012; Sameer and Nissar, 2021). Upon recognition of antigens, phagocytes initiate the engulfment of pathogens, setting off a signaling cascade that activates NOX2. This activation process involves the recruitment of multiple cytosolic partners to form the NOX2 complex, culminating in the release of bactericidal effectors into the phagosome to eliminate the pathogen (Rosales and Uribe-Querol, 2017).

NOX2 activation requires the assembly of a multiprotein complex through intricate inter-protein, protein-lipid, and intra-protein interactions. Phosphorylation-induced conformational changes unmask the autoinhibited tandem Src homology 3 domain of $p47^{phox}$, enabling its translocation and binding to the Pattern recognition receptor region of $p22^{phox}$. This interaction provides a scaffold for the assembly of $p67^{phox}$ and $p40^{phox}$ in the complex. Additionally, phosphorylation-induced conformational changes expose the phox homology domains of $p47^{phox}$ and $p40^{phox}$, facilitating the anchoring of the trimeric $p47^{phox}$-$p67^{phox}$-$p40^{phox}$ complex to membrane phosphoinositides. In the presence of NADPH and Rac, this complex enables electron transfer and $O_2^{\bullet-}$ production (Sumimoto, 2008; Herb, 2024).

In vitro studies have shown that an excess of the $p67^{phox}$ subunit, along with Rac, is necessary and sufficient for NOX2 catalytic activity. Interestingly, $p47^{phox}$ was found to be non-essential for activation in reconstituted systems (Koshkin et al., 1996; Freeman and Lambeth, 1996; Ueyama et al., 2007). This suggests that $p47^{phox}$ primarily serves an organizing role, facilitating the recruitment of different cytosolic partners to

the membrane. Knockout mouse studies have highlighted the essential role of Rac2 in optimal activation of the respiratory burst in neutrophils. Although both Rac1 and Rac2 can activate NOX2 to a similar extent in a cell-free system, Rac2 is required for efficient activation *in vivo* (Abo et al., 1991; Morgan and Liu, 2011; Dickinson and Chang, 2011).

Detailed biochemical and structural analysis of cytosolic factors and Rac protein interactions have led to the development of engineered proteins that mimic the assembly of the NOX complex. These chimeric fusion proteins, comprising specific domains from $p47^{phox}$, $p67^{phox}$, and Rac, serve as valuable tools for studying NOX assembly and activity in living cells (Alloul et al., 2001; Ebisu et al., 2001; Berdichevsky et al., 2007; Valenta et al., 2022).

Within a cell, ROS are generated within cellular compartments such as the cytosol, peroxisomes, and endoplasmic reticulum. However, mitochondria stand out as the primary site responsible for around 90% of ROS production. Within mitochondria, these ROS, known as mtROS, emerge as byproducts during adenosine triphosphate (ATP) via oxidative phosphorylation. Specifically, $O_2^{\bullet -}$ is released by complexes I and III of the electron transport chain, later transforming into H_2O_2 with the aid of mitochondrial SOD2 (Murphy, 2008). Historically viewed as accidental byproducts of aerobic metabolism, ROS have garnered significant attention in the past decade for their roles as mediators in both physiological and pathophysiological processes. Damage to mitochondria can result in oxidative stress and the formation of highly reactive $HO^{\bullet}$ via the Fenton reaction. While mtROS are implicated in various neurological disorders, this thesis directs its focus on ROS generation by NADPH enzymes (Tarafdar and Pula, 2018).

Overproduction of ROS or their generation in inappropriate locations can cause oxidative distress, causing cell damage, tissue and organ malfunction, and ultimately contributing to the development of diseases (Liu et al., 2018).

2.4 Human cell line U-937

The U-937 cells are derived from a human pro-monocytic myeloid leukemia cell line. They were isolated from the pleural fluid of a 37-year-old male with a histiocytic lymphoma in 1976 (Sundstrom and Nilsson, 1976). Pleural effusion can occur in various diseases, such as heart failure, different types of malignant tumors, kidney failure, or

various inflammatory or infectious diseases. In the so-called pleural space, there is an accumulation of fluid in the space between the lungs and the chest wall (more precisely, between the visceral *pleura* and the *pleura parietalis*). This space is usually very small and contains only a tiny amount of fluid. Therefore, increasing this amount limits the extensibility of the lungs and manifests itself, among other things, as difficulty in breathing (Massimo and Yehuda, 2013; Jany and Welte, 2019).

The U-937 cell line exhibits several characteristic features of the resting human monocyte, including high levels of surface receptor expression and phagocytic function. From existing models of cancer cell lines that have been used to study the modulation associated with monocytes and macrophages during inflammatory diseases, U-937 cells are often examined and used for experiments (Suresh et al., 2017).

2.5 Cell differentiation using chemical inducers

The compound PMA (phorbol 12-myristate 13-acetate) used in our study is a tumor-promoting phorbol ester, which can induce the differentiation of U-937 cells. It has been used to induce monocytic differentiation in U-937 cells *in vitro*. Many clinical studies have been conducted in which PMA has been used to study the expression of various cytokines, especially TH1-type cytokines, as well as in Toll-like receptor (TLR) signaling pathways (Valdés López and Urcuqui-Inchima, 2018; Kuno et al., 2020).

This compound was chosen as the chemical stimulant in this thesis. It works as an inducer slowing cell proliferation and triggering the process of differentiation. The PMA is also a tumor promoter and is involved in gene transcription, cell growth and programmed cell death, cell differentiation, immune pathway, and a decrease in receptor sensitivity via protein kinase C (PKC) signalling pathways. This chemical compound has been reported also to significantly increase the production of five cytokines without any significant damage to immune cells. However, it depends on the concentration and duration of exposure. The U-937 cells with PMA express higher levels of CD11b and PKC. Studies with U-937 cells have revealed that PKC is involved in regulating cell proliferation and differentiation, as well as in regulating cell death and apoptosis. The addition of PMA increases cell adherence and arrests the cell cycle after differentiation (Leontieva and Blagosklonny, 2014).

In addition to the well-known PMA as a potent activator of PKC that is commonly used to induce monocyte differentiation into macrophages *in vitro, the* active form of vitamin D3 (1α,25-Dihydroxyvitamin D3) and retinoic acid have been shown to work in similar ways.

2.6 Free radicals

Free radicals are species characterized by the presence of at least one unpaired electron in their outer shell. While they possess the capability of independent existence, their lifespan is typically short, ranging from nanoseconds to minutes, owing to their high reactivity. Radical species tend to interact with nearby stable molecules, seeking to acquire the missing electrons required for stabilization, thus initiating a chain reaction. These reactive species are involved in skin aging and inflammation, yet they also function as crucial regulators of homeostasis within the human body, including the proliferation of epidermal keratinocytes (Phaniendra et al., 2014; Jomová et al., 2023). Free radicals can arise through reduction, the predominant mechanism by which a neutral particle accepts an electron. Oxidation, which involves the loss of an electron, also contributes to their formation. A less common method involves homolytic cleavage of covalent bonds; however, this process is energetically demanding and therefore not prevalent in biological systems (Phaniendra et al., 2014).

In living organisms, numerous radical chain reactions occur continually. Besides radical species, non-radical species can also exist, and the classification is presented below. The imbalance resulting from ROS, reactive nitrogen species (RNS) production, coupled with inadequate antioxidant levels, leads to oxidative, nitrosative, and halogenation stress, consequently facilitating oxidative post-translational modifications of proteins (Stadtman and Levine, 2003; Wall et al., 2012a). To prevent biomolecules from oxidative damage, both enzymatic and non-enzymatic antioxidant mechanisms are activated. Lipids, proteins, and nucleic acids undergo structural and functional changes as a result of oxidative damage caused by $HO^{\bullet}$ when the concentration of ROS exceeds the antioxidant system's capacity (Halliwell and Gutteridge, 2015).

2.7 ROS in human cells

Oxygen plays a crucial role in the sustenance of life for aerobic organisms, including humans. While approximately 95% of O_2 is utilized for energy production, the residual 5% engenders the formation of highly reactive metabolites referred to as

ROS. These ROS encompass a spectrum of compounds, including oxidizing agents and substances prone to radical conversion (Halliwell and Whiteman, 2004; Murai and Matsuda, 2023).

Cell machinery, spanning various organelles such as the Golgi complex, mitochondria, peroxisomes, and the NADPH complex within the cell membrane, orchestrates the generation of ROS in animal cells. Any disruption in the delicate redox equilibrium of cells can cause damage to vital cellular components, including proteins, lipids, and nucleic acids (Phaniendra et al., 2014). Some of these cellular machineries are presented in more detail below.

2.7.1 ROS generation in mitochondria

Mitochondria serve as a primary source of endogenous ROS due to their central role in the production of oxidative ATP production, where O_2 undergoes reduction to water in the electron transport chain. Radical molecules like $O_2^{\bullet -}$ are generated at various sites within the mitochondria, including complex I (site IQ), complex III (site IIIQo), glycerol 3-phosphate dehydrogenase, pyruvate dehydrogenase, and 2-oxoglutarate dehydrogenase (Brand, 2010). These sites release $O_2^{\bullet -}$ into the mitochondrial matrix, with complex III (site IIIQo) and glycerol 3-phosphate dehydrogenase also producing ROS in the intermembrane mitochondrial space. Manganese superoxide dismutase (Mn-SOD) converts $O_2^{\bullet -}$ within the mitochondrial matrix, while copper and zinc superoxide dismutase (Cu- and Zn-SOD) perform this conversion in the intermembrane mitochondrial space or cytosol (Okado-Matsumoto and Fridovich, 2001). H_2O_2 in the mitochondrial matrix can further react via the Fenton reaction catalyzed by mitochondrial aconitase, producing $HO^{\bullet}$ (Vasquez-Vivar et al., 2000). Another source of ROS in mitochondria is the cytochrome (CYP) catalytic cycle, where CYP enzymes metabolize various organic substrates, yielding $O_2^{\bullet -}$ and H_2O_2 as by-products (Yasui et al., 2005). Several members of the CYP family are present in the mitochondrial membrane of steroidogenic organs, liver, and kidney (Omura, 2006). Furthermore, other mammalian proteins such as NADH-cytochrome b5 reductase, dihydroorotate dehydrogenase, complex II (succinate dehydrogenase), and monoamine oxidases have been identified as contributors to ROS generation within mitochondria (Whatley et al., 1998; Zhang et al., 1998; Kaludercic et al., 2014; Hey-Mogensen et al., 2014).

2.7.2 ROS generation in peroxisomes

Peroxisomes play diverse roles in cellular metabolism, including the breakdown of fatty acids, purines, polyamines, and amino acids, as well as the regulation of ROS and RNS, and transition metal ions (Del Río and López-Huertas, 2016). They contribute to the cellular ROS and RNS pool by generating H_2O_2, $O_2^{\bullet -}$, $HO^{\bullet}$, $NO^{\bullet}$, and peroxynitrite ($ONOO^-$) (Di Meo et al., 2016). Initially recognized for their involvement in H_2O_2 production and degradation, peroxisomes remain significant contributors to cellular H_2O_2 levels despite containing catalase (CAT) (De Duve and Baudhuin, 1966; Boveris et al., 1972; Fritz et al., 2007). H_2O_2 is formed during normal peroxisomal enzymatic reactions and through the dismutation of $O_2^{\bullet -}$, with potential to generate $HO^{\bullet}$ via the Fenton reaction (Valko et al., 2005; Fransen et al., 2012).

Also, $O_2^{\bullet -}$ is generated within peroxisomes both in the matrix and membrane. Enzymes like xanthine oxidoreductase and urate oxidase produce $O_2^{\bullet -}$ and H_2O_2 during purine metabolism, while the peroxisomal membrane's electron transport chain is another source (Sandalio et al., 1988; Gutteridge and Halliwell. 1992; Sandalio et al., 2013). Xanthine oxidoreductase additionally facilitates the conversion of nitrates and nitrites to $NO^{\bullet}$ which can react with $O_2^{\bullet -}$ to form $ONOO^-$ (Pacher et al., 2007).

To counteract oxidative stress, peroxisomes are equipped with antioxidant defenses, including enzymes like CAT, SOD, peroxiredoxins, glutathione S-transferases, and epoxide hydrolase 2, along with low-molecular weight antioxidants (Fransen et al., 2012; Sandalio et al., 2013; Del Río and López-Huertas, 2016).

Alterations in redox homeostasis contribute to the development and progression of cancer (Panieri and Santoro, 2016). The peroxisome maintains cellular oxidative balance. The alteration of the balance of its activity is associated with carcinogenesis. Reduced CAT activity has been shown to lead to ROS production, which, in turn, results in DNA damage and genomic instability that promote cancer development. Reduced CAT expression has been demonstrated in hepatocellular carcinoma and colon, lung, kidney, and prostate cancers, as well as in precancerous conditions (Lauer et al., 1999; Bostwick et al., 2000; Frederiks et al., 2009; Cho et al., 2014). Continuing the discussion on peroxisomes and mitochondrion, in the next section, we will dive into various ROS (Figure 4) generated within cells and explore the mechanisms underlying their production.

2.8 Types of ROS and mechanism of generation

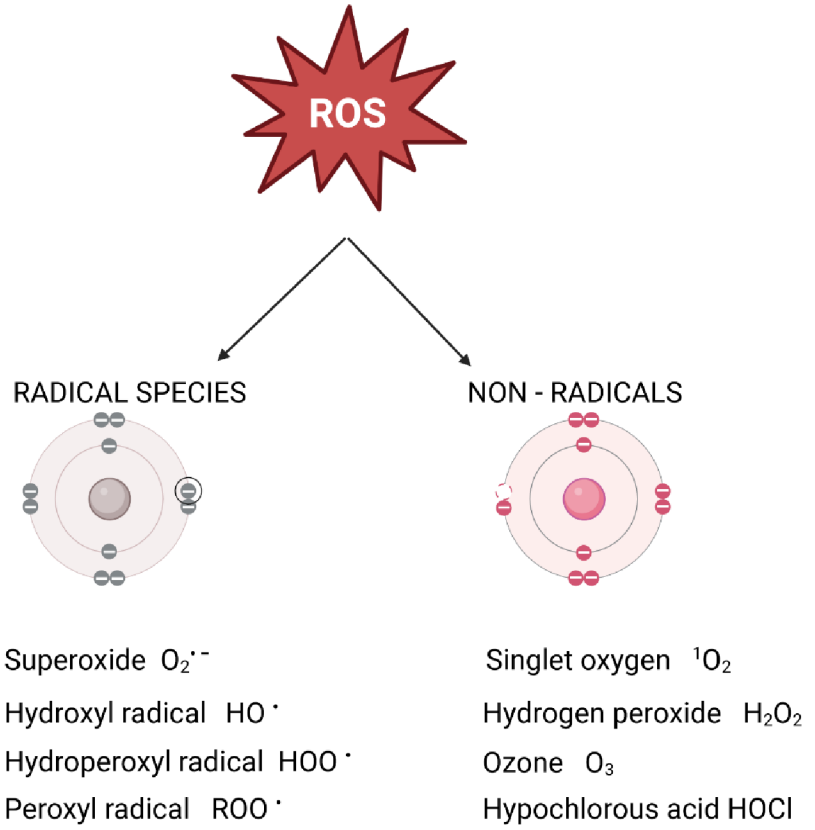

Figure 4: Reactive oxygen species in biological systems categorized as radical and non-radical species (created using biorender.com).

2.8.1 Superoxide anion radical

The superoxide anion radical ($O_2^{\bullet -}$) is formed by the one-electron reduction of O_2. It is the most abundant free oxygen radical in living organisms. It is produced during non-enzymatic reactions, by reducing oxygen by flavins, quinones, and thiols; and by the activity of several enzyme systems (for example, xanthine oxidase, lipoxygenase, and cyclooxygenase), as well as during the respiratory chain on the inner membrane of mitochondria and during photosynthesis in chloroplasts (Jomová et al., 2023; Andrés et al., 2023). It participates in reactions that can result in the production of additional reactive substances, such as the $HO^{\bullet}$, $^1O_2$, peroxynitrite, and perchloric acid. These processes can occur directly or more often, catalyzed by enzymes or transition metal ions (Valko et al., 2005).

2.6.2 Hydroxyl radical

It is considered the most reactive ROS and therefore the most harmful. It can be formed by a variety of processes, the most significant of which is the so-called Fenton reaction, which occurs when H_2O_2 reacts with metal ions. Because of its high reactivity, it normally

interacts with molecules at incredibly fast speeds in the immediate vicinity of production. As a very strong oxidizing agent, it is able to react with most organic and inorganic molecules (Racek, 2003; Igarashi et al., 2022).

2.8.3 Hydrogen peroxide

A molecule of H_2O_2 is formed by the dismutation of $O_2^{\bullet -}$. In addition to SOD, it can be formed by catalases, glutathione peroxidases, and NADPH oxidases. It is not a free radical, but it has oxidizing properties. Compared to radical species, however, it oxidizes molecules slowly (Racek, 2003). Its danger lies mainly in its ability to undergo transformations to form much more reactive forms such as $HO^{\bullet}$ or perchloric acid (Kohen and Nyska, 2002; Racek, 2003).

2.8.4 Hypochlorous acid

It is produced by neutrophil granulocytes using the enzyme myeloperoxidase. This reaction is involved in the killing of bacteria by phagocytic cells (Splettstoesser and Schuff-Werner, 2002). Although hypochlorous acid is a potential oxidant and chlorinating agent, it does not fall into the category of radical species. It has the ability to attack a variety of compounds and contributes, for example, to the chlorination of cholesterol (Carr and Winterbourn, 1998).

2.8.5 Singlet oxygen

The excited state of the dioxygen molecule is termed singlet oxygen ($^1O_2$). It is highly reactive. Since it is involved in the oxidation of lipids, proteins and nucleic acids, $^1O_2$ causes oxidative stress and has harmful and carcinogenic consequences. (Štípek, 2000; Agnez-Lima et al., 2012). In its fundamental state, O_2 possesses an open-shell electronic structure, housing two electrons of equal spin in distinct degenerate molecular orbitals. Its capacity to accept electrons during redox reactions is limited, engaging in slow reactions with most organic compounds. Oxygen exhibits two singlet multiplicity excited states, with the lower energy state designated as $O_2(^1\Delta g)$ or $^1O_2$. In this state, electrons occupy the same orbital with opposite spins, enhancing its oxidative potential compared to O_2. The subsequent singlet level, represented by $O_2(^1\Sigma g^+)$, is characterized by high energy and low stability, resulting in reduced reactivity. Singlet oxygen, which can be originated from the light-activated photosensitizer, is a potent oxidizing agent crucial in solution reactions, emitting phosphorescence in the infrared spectrum. Singlet oxygen's role extends to various oxidation reactions and biological processes, serving as

an activated species. Direct excitation of the ground state to produce $^1O_2$ is improbable due to spin, symmetry, and parity restrictions (María et al., 2024).

2.8.6 Reactive nitrogen species

Reactive nitrogen species constitute a class of nitrogen-derived compounds that are crucial in biological processes. Chief among these is nitric oxide ($NO^{\bullet}$), typically generated through the enzymatic conversion of *L*-arginine by nitric oxide synthase (Wall et al., 2012b; Gonos et al., 2018). They encompass a diverse array of nitrogen-containing molecules, including $NO^{\bullet}$ and its higher oxides such as dinitrogen trioxide (N_2O_3), peroxynitrite (ONOO) and nitroxyl anion (NO^-) which has been summarized in Figure 5 (Smulik et al., 2014).

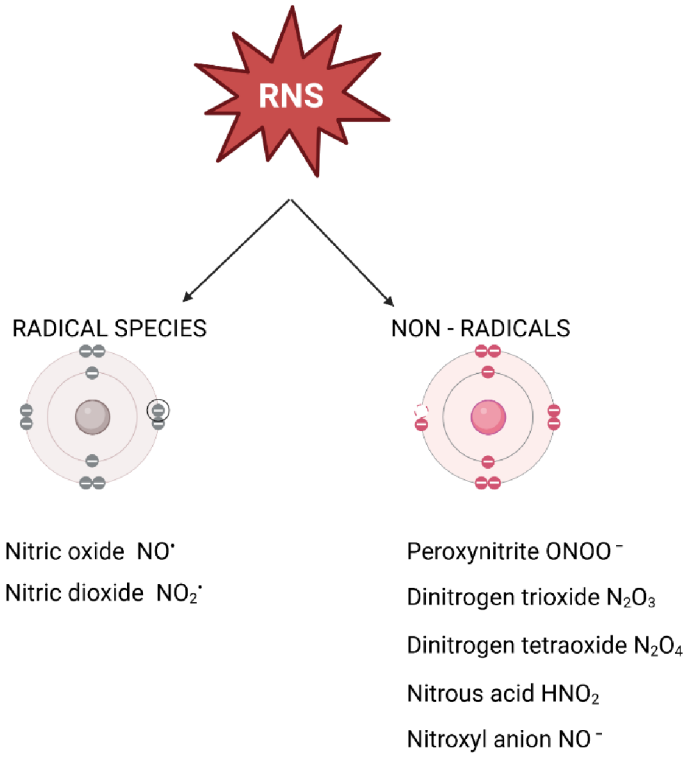

Figure 5: Reactive nitrogen species in biological systems categorized as radical and non-radical species (created using biorender.com).

The imbalance between ROS and RNS and the antioxidant defense, in favor of pro-oxidants, causes oxidative or nitrosative stress, respectively, and facilitates post-translational oxidative modification of proteins or broad biomolecule oxidation (Sadowska-Bartosz et al., 2018). The resultant oxidative modifications adversely affect protein function, imparting multiple deleterious effects on cellular physiology. Accumulating evidence implicates oxidative protein modifications in age-related processes and the pathogenesis of various diseases, including atherosclerosis, neurodegenerative disorders, and cataracts (Sadowska-Bartosz et al., 2018; Vona et al. 2021).

2.9 Oxidative stress

Oxidative stress is a state that refers to a state of imbalance between the production and accumulation of ROS in cells or tissues. Its ratio is determined by a biological system's capacity to detoxify reactive intermediates. These ROS have a variety of physiological functions and can affect the general health of cells or organisms in either a positive or negative way. Enzymes and small molecules, known as antioxidants, interact with individual ROS to decrease oxidative damage and/or modify redox signalling in living

organisms (Halliwell and Gutteridge, 2015; Sies et al., 2022). There are several antioxidants, both enzymatic and non-enzymatic, that can be used by cells as a defense against ROS or free radicals in general (Sharma et al., 2012). However, each antioxidant has a unique specificity and reacts with different types of ROS. Enzymatic systems including SOD for $O_2^{\bullet -}$, peroxidases for H_2O_2, and metal ion sequestration are also important *in vivo* antioxidants (Halliwell and Gutteridge, 2015; Halliwell et al., 2020). Most low-molecular-mass substances used as "antioxidants" are stoichiometric scavengers of specific ROS and frequently exhibit minimal or no reactivity with $O_2^{\bullet -}$ or H_2O_2. N-acetylcysteine, for instance, is a popular "antioxidant" but it also has other mechanisms of action. Some ROS, most notably H_2O_2, cannot be scavenged by N-acetylcysteine *in vitro* (Jomová et al., 2023). Additionally, it can produce hydrogen sulfide, raise levels of glutathione, and directly break protein disulphides by expanding the cellular cysteine pool (Pedre et al., 2021). *In vivo*, low-molecular-mass substances such as vitamin E, which scavenges lipid peroxyl radicals, do function as antioxidants. The SOD, catalase, and glutathione peroxidase are examples of enzyme-based antioxidants, whereas vitamins E, C, and pyridoxine (B6); flavonoids, and polyphenols are examples of non-enzymatic antioxidants (Murphy et al., 2022). According to Joseph et al. (2015), oxidative stress contributes to the emergence of diseases such as cancer, Parkinson's disease, Alzheimer's disease, atherosclerosis, heart failure, myocardial infarction, fragile X syndrome, vitiligo, autism, infection, depression, and possibly Asperger syndrome in humans (Stewart et al., 2006). However, because ROS may target and eliminate infections and are also utilized as a therapeutic strategy to treat cancer, they can be useful (Tönnies and Trushina, 2017).

2.10 ROS and human diseases

ROS play a significant role in various pathologies, with many of these diseases ranking among the leading causes of mortality. There is compelling evidence indicating the involvement of ROS in cancers, cardiovascular diseases, neurological disorders, fibrotic diseases, infectious diseases, chronic inflammation, autoimmune diseasses and sensory impairments (Figure 6).

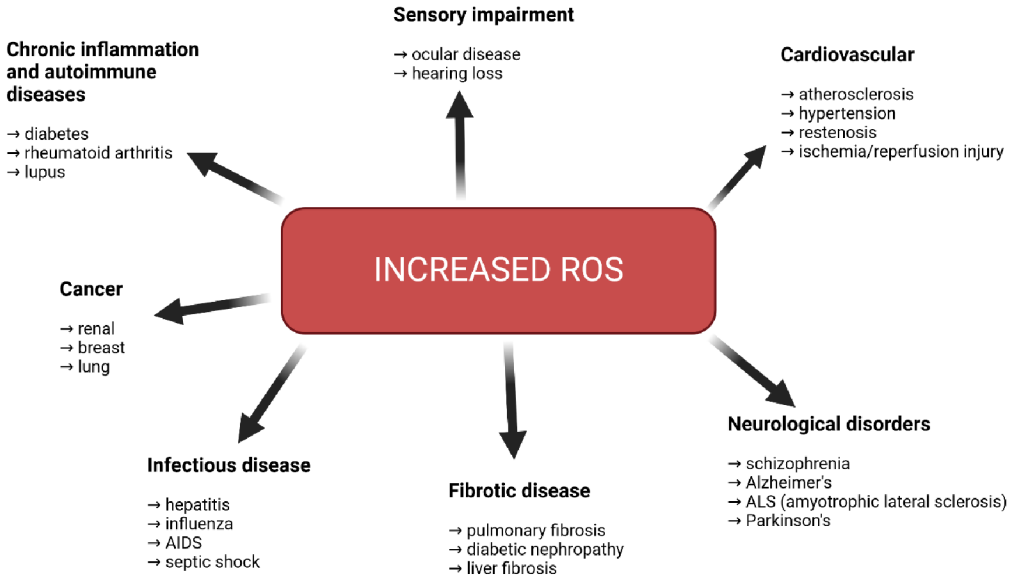

Figure 6: A summary of human diseases where reactive oxygen species (ROS) are implicated (created using biorender.com).

2.10.1 Cancer

DNA damage caused by ROS is a key factor in the initiation and progression of cancer. ROS-induced DNA mutations encompass base alterations, DNA sequence rearrangements, DNA lesions, gene duplications, and oncogene activation of oncogenes (Waris and Ahsan, 2006). In addition to contributing to cancer development through oncogenic mutations, ROS dysregulation also plays a role, as observed in renal cell carcinoma. This condition is characterized by inappropriate upregulation of hypoxia response genes. Elevated levels of the hypoxia-inducible transcription factor (HIF-1α) promote tumor growth, angiogenesis, and metastasis (Liao et al., 2007). The heightened HIF-1α levels in these tumors are partly attributed to the loss of the von Hippel-Lindau protein, which typically facilitates HIF-1α degradation.

2.10.2 Cardiovascular disease

Reactive oxygen species play a central role in numerous cardiovascular diseases, and the underlying causal mechanisms are complex. Vascular cells exhibit the concurrent expression of multiple NOX enzymes, and alterations in the expression of NOX1, 2, 4, and 5 have been linked to various cardiovascular conditions. In vascular smooth muscle cells sourced from large arteries, NOX1 is essential for processes such as migration, hypertrophy, and proliferation, while NOX4 facilitates differentiation. Furthermore,

NOX1 and 2 have been implicated in the pathogenesis of hypertension (Brieger et al., 2012).

2.10.3 Neurological disorders

Neurological diseases advance through complex pathways, and the expression of the NOX enzyme in microglia cells emerge as a pivotal factor. Beyond microglia, other brain cells, such as astrocytes and neurons, also contribute to ROS generation through various mechanisms. Although low levels of ROS are crucial for normal brain function, excessive levels can induce neurotoxicity and disease onset. In Alzheimer's disease, a cascade of neuroinflammatory events exacerbates neurodegeneration and dementia. Microglia activation, particularly triggered by soluble amyloid forms, perpetuates ROS production, leading to neuronal damage and eventual cognitive decline. Similarly, Parkinson's disease pathogenesis involves NOX-derived oxidative stress, which contributes to the death of dopaminergic neurons. In particular, studies hint at NOX2 involvement in this process, evident in models such as MPTP-induced (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) neurotoxicity where NOX2 deficiency mitigates dopaminergic neuron loss. Furthermore, oxidative stress stands central in amyotrophic lateral sclerosis (ALS), a progressive motor neuron disorder. Understanding the multifaceted role of ROS in neurodegenerative diseases underscores the therapeutic potential of targeting NOX enzymes to alleviate oxidative stress-induced neuronal injury (Sorce and Krause, 2009).

2.10.4 Fibrotic diseases

Many chronic inflammatory conditions involve fibrosis, a pathological process characterized by excessive accumulation of components of the extracellular matrix that lead to scarring. This fibrotic progression can ultimately result in organ dysfunction and, in severe cases, death. Fibrosis affects virtually all tissues in the body and is closely associated with chronic inflammation, metabolic balance, and the signaling pathway of transforming growth factor-β1. The delicate balance between the oxidant and antioxidant systems plays a crucial role in regulating these processes. Fibrosis can affect various organ systems, including the lungs, heart, kidneys, and liver, leading to an abnormal buildup of connective tissue components. This tissue remodeling often results in organ dysfunction, contributing to high rates of morbidity and mortality (Antar et al., 2023).

2.10.5 Infectious diseases

Normal immune function relies on specific oxidative states, and ROS playing essential roles in microbial killing, regulating the immune response, and terminating inflammation. Evidence of ROS involvement in immune function is observed in patients with chronic granulomatous disease (CGD), who lack the ROS-producing enzyme NOX2. This deficiency leads to immunodeficiency, resulting in recurrent infections such as pneumonia and abscesses. Phagocytes from patients with CGD do not generate ROS upon stimulation, hindering their ability to efficiently kill bacteria. Hypochlorous acid, a potent microbial killer, is produced from H_2O_2 and chloride ions by myeloperoxidase. While ROS play a crucial role in the combat of bacterial, fungal, and microbial infections, their role in viral protection remains unclear (Mauch et al., 2007; Brieger et al., 2012).

Reactive oxygen species not only contribute to host defense, but also paradoxically limit inflammation and the immune response. Patients with CGD often exhibit non-infectious hyperinflammatory conditions such as granulomas and colitis, and autoimmune diseases are more prevalent in both CGD patients and heterozygous carriers of CGD mutations. Surprisingly, NOX2 deficiency-induced hyperinflammation may sometimes confer protection, as observed in CGD mice showing increased inflammation protecting against influenza infection. Further research is needed to elucidate the complex interplay between NOX2-dependent ROS generation and inflammation (Snelgrove et al., 2006; Schäppi et al., 2008; Tschopp, 2011).

2.10.6 Chronic inflammation and autoimmune diseases

The role of oxidative stress in the development of rheumatoid arthritis (RA) is significant. Staroń and co-workers conducted an analysis comparing erythrocytes from RA patients to those from healthy individuals, revealing elevated lipid peroxidation, reduced activity of antioxidant enzymes, and impaired sodium-potassium ATPase function (Staroń et al., 2012). In the synovial cavity, Mapp and co-workers observed an increased presence of ROS, leading to the oxidation of various molecules, including immunoglobulins, particularly IgM targeting the fragment crystallizable (Fc) region of IgG (known as rheumatoid factor), lipoproteins, lipids, and hyaluronan (Mapp et al., 1995). Oxidatively damaged immunoglobulins are more susceptible to non-enzymatic degradation by sugars, particularly at arginine and lysine residues, resulting in the formation of advanced glycation end products (Hitchon and El-Gabalawy, 2004), akin to processes seen in diabetes mellitus where prolonged hyperglycemia glycoxidates hemoglobin

into hemoglobin A1c. In particular, antibodies against glycoxidized IgG are specifically detected in patients with early synovitis (Newkirk et al., 2003). Furthermore, increased ROS levels contribute to damage to the DNA mismatch repair system, which is known to be impaired in RA, leading to elevated concentrations of 8-oxo-7-hydro-deoxyguanosine (Hitchon and El-Gabalawy, 2004).

2.10.7 Sensory impairments

Several age-related ocular diseases, including cataracts and retinal degeneration, are believed to be linked to oxidative stress. Similarly, age-related hearing loss is hypothesized to be the result of ROS activity. In particular, NOX3 exhibits significant expression levels in the inner ear, where it contributes to the biogenesis of otoliths, critical structures involved in gravity perception. However, emerging evidence suggests a potential association between increased NOX3 activity and hearing impairment. Specifically, cisplatin-induced ototoxicity has been linked to excessive activation of NOX3. These findings underscore the intricate interplay between ROS and auditory function, warranting further investigation into the role of NOX3 in hearing-related disorders (Ueno et al., 2007).

3 Material and Methods

3.1 Cell line and growing condition

The U-937 cell line used in my study is derived from the pro-monocytic myeloid leukemia cell line. It was identified and isolated from the pleural fluid of an adult male (Sundström and Nilsson, 1976). This line exhibits many characteristics like monocytes. The line was obtained from the American Type Culture Collection (ATCC; Rockville, MD, USA). Since it is a human cell line, it was grown in an incubator at 37 °C and 5 % CO_2 saturation. RPMI-1640 with the addition of FBS is considered the optimal culture medium. These conditions stimulate the internal conditions of the human body.

3.2 Cell differentiation

Differentiation inducers, phorbol 12-myristate 13-acetate (PMA) at 250 nM (a light-sensitive chemical compound) and ascorbic acid at concentrations ranging from 0 to 10 μM and were used in this study. Cultures were seeded with a total of $1x10^6$ colony-forming units (CFU)/mL of live cells and incubated for 72 h to allow

differentiation processes to occur. Ascorbic acid, a well-known antioxidant, was dissolved in an extracellular buffer comprising HEPES (15 mM), NaCl (135 mM), KCl (5 mM), $CaCl_2$ (1.8 mM), and $MgCl_2$ (0.8 mM), adjusted to pH 7.4 to maintain optimal conditions for cell culture. This carefully prepared medium ensured the stability of ascorbic acid and facilitated its interaction with the cells under investigation.

3.3 Trypan blue assay for determination of cell viability and proliferation

Cell viability and proliferation were determined using the automated cell counter (Bio-Rad Laboratories, Hercules, CA, United States). To assess cell viability and proliferation, a pipetted volume of 10 µl of cell suspension was mixed with an equal volume of trypan blue (the final concentration of trypan blue was 0.05%) in an Eppendorf tube. The mixture was then loaded onto a counting slide, which is a dual chamber designed for cell counting. The use of trypan blue dye with the TC20 counter facilitates rapid staining and accurate determination of cell viability and proliferation. This dye is crucial for distinguishing between living and dead cells in a cell suspension. Due to its negatively charged chromophore, trypan blue does not permeate the intact membranes of viable cells. Instead, it selectively enters damaged or dead cells, staining them blue, while live cells with intact membranes remain unstained and appear bright. Viability is deemed acceptable for our experimentation if it is equal to or exceeds 70%. Based on the ratio of live (unstained) and death cells (blue stained), we can estimate the level of cell proliferation. A higher ratio of live cells indicates a higher level of proliferation, while a lower ratio may indicate a lower level of proliferation or even cell death.

3.4 MTT assay for cell viability

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) is converted into a purple-coloured formazan product by viable cells with active metabolism which shows an absorbance maximum at 590 nm. As cells undergo cell death, their capacity to convert MTT into formazan diminishes, making color formation a reliable indicator of cell viability. In this diploma thesis, cell viability was assessed through two methods: measuring the metabolism of a tetrazolium substrate MTT and using trypan blue staining. The influence of PMA dissolved in dimethylsulfoxide (DMSO) and ascorbic acid dissolved in EB on U-937 cell proliferation was evaluated by using the MTT assay, following the protocol outlined in the Cell Proliferation Assay Kit (ab211091) and trypan blue staining.

U-937 cells were seeded in replicates (n=3) and cultured with 250 nM concentrations of PMA/ ascorbic acid for 72 hours at 37 ° C. After this incubation period, we measured viability using the trypan blue test. Cells were centrifuged at 2,500 rpm at 4° C for 5 min. The culture medium was then carefully aspirated and 50 µL serum-free medium and 50 µL of MTT reagent were added to each well, followed by a three-hour incubation at 37° C. To prevent damage to cells with formazan crystals after incubation, MTT solvent was added to each well for solubilization of crystals. The 96-well plate was placed on a shaker for 15 minutes. The absorbance of the samples was then read at 590 nm.

3.5 Confocal laser scanning microscopy

To visualize U-937 cells on glass slides, a Fluorview 1000 confocal unit connected to the IX80 microscope (Olympus Czech Group, Prague, Czech Republic) or Andor BC43 benchtop confocal microscope (Oxford Instruments, Belfast, Ireland) was used. Cell membrane integrity presented in Article 1 (Figure 2) was monitored by staining with FM4-64 [15 µM, at room temperature (RT)], a lipophilic dye, while Hoechst 33342 (2 µM, RT) was utilized to visualize the nucleus under the specified experimental conditions (please refer to Section 2.5 of published article). The cells underwent co-staining with FM4-64 and Hoechst 33342 (Sigma Aldrich GmbH, Germany), and after a 5 min incubation, were transferred to a glass slide for visualization. For the immunohistochemical analysis (CD11b) presented in Article 1, after treatment with 5 µM and 10 µM ascorbic acid for 48 hours, the culture medium was removed from the plates and 3.5 % paraformaldehyde was applied for cell fixation for 30 min. Subsequently, the cells were washed three times with phosphate buffered saline (PBS) containing 0.1 % Tween-20 (PBST) for 5 min each. After fixation, cells were blocked with 0.5 % BSA in PBS for 30 min at RT. The blocked samples were then probed with CD11b Monoclonal Antibody-FITC (dilution 1:2500) for 60 min at RT, followed by washes with PBS (3x, 5 min each). Immunofluorescence was measured using excitation achieved by a 488 nm line of an argon laser and the signal was detected by a 505–550 nm emission filter.

3.6 Isolation of cellular protein

The cell pellet was resuspended in 200 µL of RIPA buffer [150 mM NaCl, 50 mM Tris (pH 8.0), 0.5% sodium deoxycholate, 0.1% SDS, 1% NP-40] supplemented with 1% proteinase and phosphatase inhibitors, and kept on ice for 5 min. Subsequently, the suspension was subjected to sonication at 40% amplitude for 1 cycle of 30 seconds,

repeated 6 times, with cells transferred to ice after each cycle to prevent protein damage and degradation. After sonication, the sample was centrifuged at 19,000 rpm for 30 min at 4˚C, leading the cell debris pellet to the bottom of the tube. Cautiously, the protein-containing supernatant was collected into a separate tube without disturbing the pellets. This process ensured the isolation of intact proteins for further analysis.

3.7 SDS-PAGE and western blotting

SDS-PAGE and western blotting are methodologies used to separate and identify proteins. It is often used to detect the presence of a particular protein in a sample. The technique involves running samples of proteins on an electrophoresis gel, which separates proteins based on size and charge. After the proteins have been separated, they are transferred to a membrane [either nitrocellulose or polyvinylidene fluoride (PVDF) membranes]. Antibodies specific to the target protein are then used to detect its presence. The target protein will bind to the antibody and can be visualized using specific detection methods, such as colorimetric or chemiluminescent reactions, which we have also used in our investigation.

3.6.1 SDS-PAGE

The isolated protein in an amount of 10 µg was mixed with 5x Laemmli sample buffer and dithiothreitol (DTT, 100 mM) in an eppendorf tube and then incubated at 70° C for 10 min. After this incubation, the samples were centrifuged at 14,000 rpm for 5 min. The protein samples were then loaded and isolated on 10% 1mm thick polyacrylamide gels (4% stacking gel and 10% resolving gel). For the visualization of molecular weight of proteins, a pre-stained protein ladder [Proteintech (PL00001)] was also used along with protein samples.

3.6.2 Transferring of the proteins to membranes

After electrophoresis, the proteins were transferred from gel to membranes. In experiments in which a PVDF membrane was used, it was charged with methanol. Transfer was achieved using the Trans-Blot Turbo transfer system (Bio-Rad, California, USA). The transfer duration lasted 7-30 min depending on experiments. For confirmation of proper protein transfer and to ensure that the loading of samples is uniform, Ponceau staining was performed where the membranes were incubated with Ponceau stain for 5 min, followed by washing with d/w, and then scanned on Amersham imager 600.

3.6.3 Immunoblotting

The membranes were blocked with blocking reagent containing 5% bovine serum albumin (BSA) in PBS (pH 7.4) and 0.1 % Tween 20 (2 h in RT or overnight at 4 °C). The blocking solution was then removed, and the desired primary antibody [anti-NOX2 mice polyclonal antibody or rabbit polyclonal anti-malondialdehyde (anti-MDA) antibody] was added for 1-2 h. During this incubation, the membranes were placed on a shaker at RT. After incubation with primary antibody, membranes were washed with PBS mixed with 0.1 % Tween 20 for 30 min. Every 10 min, the washing solution was discarded and replaced with fresh one. Following this step, the membranes were incubated with secondary antibody [HRP-conjugated goat anti-mice secondary antibody for anti-NOX2 and horseradish peroxidase (HRP) conjugated antirabbit secondary antibody for anti-MDA] for 1-2 h (dilution 1:10000). The washing steps mentioned above were repeated. When washing was done, secondary antibodies were discarded and Immobilon Western Chemiluminescent HRP (Horseradish peroxidase) substrate (Sigma Aldrich, GmbH, Germany) (GE Healthcare, UK) was added to the membranes right before scanning on Amersham imager 600 (GE Healthcare, United Kingdom).

4 Results

4.1 Cell viability measurement using Trypan blue exclusion test

In this study, we conducted a comprehensive quantitative assessment of viable cells within the U-937 cell line following exposure to PMA and variable concentrations of ascorbic acid. To ascertain cell viability, the widely employed trypan blue exclusion assay was employed. U-937 cells were subjected to treatment with optimized concentrations based on pilot experiments with PMA (250nM) and ascorbic acid (0-10 μM). This approach not only offers insights into the impact of chemicals on cell viability but also provides a dynamic assessment of the recovery and resilience of U-937 cells during the post-treatment phase (Figure 7; Fig. 3 of published article). We also followed the U-937 cell viability for 10 days to determine at which stage of growth it would be best to perform the experiments. (Figure 8).

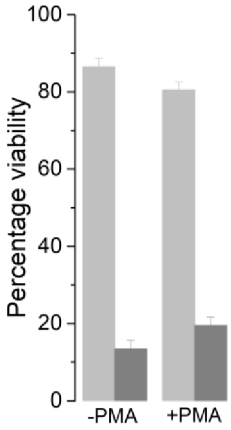

Figure 7: Percentage viability using trypan blue showing live and dead U-937 cells, measured before and after differentiation (250 nM PMA). the same time.

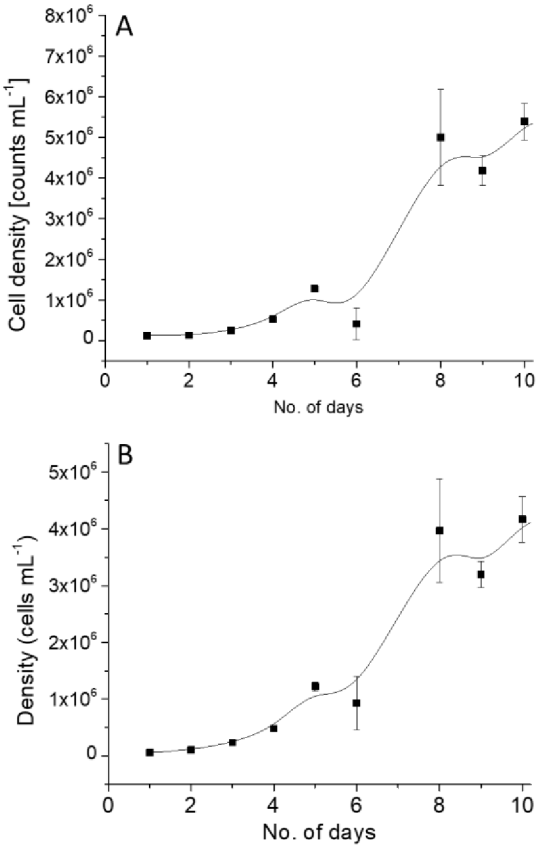

Figure 8: Growth curve of total count (A) and live count (B) of the cells U-937, measured for 10 consecutive days in T-75 flasks. The data points were collected each day at the same time.

4.2 Differentiation of U-937 cells

Visual changes in cellular morphology were meticulously monitored 72 h after the addition of PMA and ascorbic acid using a confocal laser scanning microscope. The dynamic alterations in cell morphology became notably apparent after a 72 h

incubation period. For visualization of cellular morphology, a combination of fluorescent dyes, specifically FM4-64, a lipophilic styryl compound and Hoechst 33342, were used. These dyes are valuable indicators for assessing cell membrane integrity and nuclear structure. The differentiated U-937 cells exhibited distinctive extensions, adopting an amoeboid morphology (Figure 9). Before treatment, the cells displayed a characteristic clear and spherical structure. However, post-treatment, substantial morphological transformations were evident, manifesting as prominent pseudopodia. In particular, the incidence of these pseudopodia was found to increase in cell treated either with PMA or ascorbic acid. In contrast, at higher concentrations of ascorbic acid (10 µM), a reduction was observed in these distinctive morphological structures (Figure 2, published article). This intriguing change in cell morphology at elevated concentrations suggested a complex dose-dependent relationship between exposure to ascorbic acid and the phenotypic changes in U-937 cells. These findings underscore the importance of not only characterizing morphological alterations, but also discerning the nuances of concentration-dependent effects, thus providing a more comprehensive understanding of the impact of ascorbic acid on the cellular dynamics of U-937 cells.

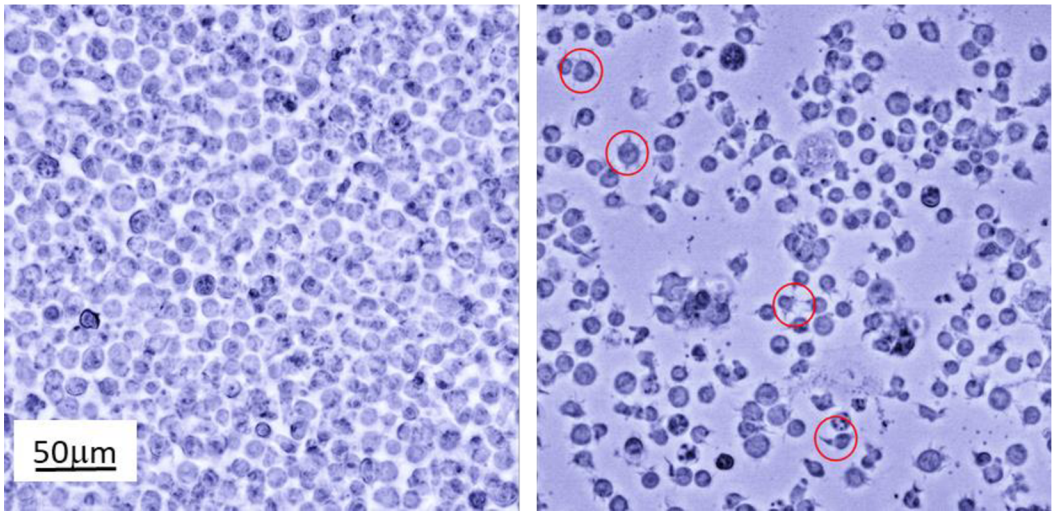

Figure 9: Control cells in the absence (A) and presence of 250nM PMA. The cells were grown for 72 h.

4.3 MTT assay

The MTT assay was used to measure cell viability and cytotoxicity of the chemicals used. Absorbance was measured after 3 h of incubation at 37 °C with MTT. According to the results, the cytotoxicity of the chemicals used could not be calculated as the tested cells showed unexpectedly higher absorbance compared to the control cells.

Background	Control (-PMA)	Test (+ PMA)
0.071	0.596	0.884
0.064	0.625	0.881
Ø = 0.068	Ø = 0.611	Ø = 0.883

Table 1: The background sample contains serum-free medium and MTT reagents; control sample consisted of cells in serum-free medium and MTT reagents while the test sample was 72 h PMA differentiated cells in serum-free medium and MTT reagent. The absorbance was recorded at 590 nm.

The MTT analysis showed a higher absorbance in the tested cells. According to the literature, the method is used as a simple test of cell viability. Thus, its applicability and limitations should be more considered. Since our results were opposite to those expected, it can be assumed that the test cells took up more reagent or their metabolic activity was higher than that of the control cells (Ghasemi et al., 2021). The viability of the cells tested was 45% higher than the control cells according to the MTT assay.

4.4 NOX2 expression during cell differentiation

We utilized Western blot analysis to assess the levels of NADPH oxidases (particularly NOX2). Our findings indicate a subtle increase in NADPH oxidase expression (Figure 10). Specifically, there's a noticeable enhancement in band intensity around 65 kDa. Intriguingly, treated samples exhibit a significant enhancement in band intensity around 42 kDa. This suggests a potential avenue for exploration; perhaps post-translational modifications could be playing a role in this observed phenomenon.

Upon exposure to PMA, U-937 cells undergo a complex cascade of signaling events, orchestrated by activation of PKC. This activation, in turn, triggers downstream pathways associated with cell differentiation and immune responses. As part of this differentiated state, the observed increase in NOX expression is particularly noteworthy. NOX2 plays a crucial role in the generation of ROS, specifically $O_2^{\bullet-}$. Macrophages, as activated by PMA-induced differentiation, require elevated levels of ROS for essential immune functions, including phagocytosis and microbial killing. The observed elevation in NOX2 expression, as revealed by Western blot analysis, underscores the dynamic cellular responses associated with the activation and differentiation of U-937 cells into macrophage-like phenotypes. This finding aligns with the anticipated role of NOX2 in respiratory burst, a strong characteristic of activated macrophages engaged in effective

immune responses. These results provide valuable information on the intricate interplay between signaling pathways, cellular differentiation, and up-regulation of key components involved in the immune response. The increased presence of NOX2 signifies an increased capacity for ROS production, contributing to the cellular defense necessary for effective immune functions.

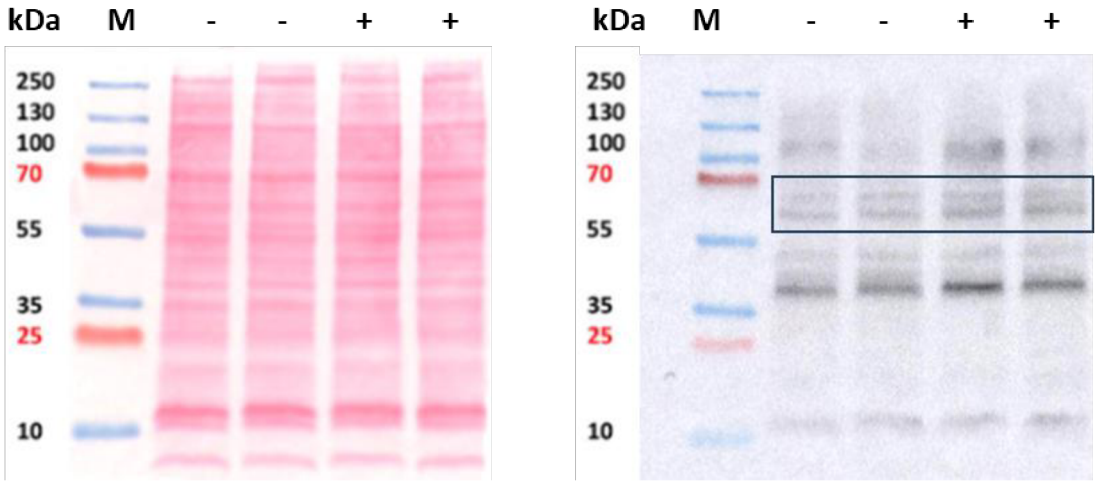

Figure 10: Analysis of NOX2 expression from the whole-cell homogenate of U-937 cells. Untreated (-PMA) and treated (+PMA) for 72 hours. The left side displays a scan from Ponceau staining, while the right side presents the western blot analysis of NOX2.

4.5 CD11b expression in non-differentiated and differentiated cells

In order to confirm the differentiation of monocytes to macrophage under the influence of differentiation inducer, we evaluated the expression profile of the surface marker CD11b. The results obtained from this investigation show the efficacy of the differentiation inducer used in our study. In the untreated U-937 cell population, a baseline examination revealed minimal/ no expression of the surface marker, indicative of the undifferentiated state. Upon subjecting U-937 cells to 250 nM PMA (Figure 11), an enhanced expression of CD11b surface marker became apparent. The figure presented below is representative of several biological and technical replicates.

Similarly in our result presented where ascorbic acid was used as a differentiation inducer, a concentration-dependent upregulation of the CD11b surface marker was also apparent. Specifically, at concentrations of 5 µM and 10 µM, a significant increase in FITC fluorescence was observed, providing a clear visual representation of the enhanced expression of CD11b under both treatment conditions (please refer to Fig. 3 of the published article). This fluorescence-based assessment not only corroborates

the efficacy of differentiating agents in promoting the differentiation of U-937 cells into a macrophage-like phenotype but also demonstrates the sensitivity of CD11b as a reliable marker for tracking and quantifying this transition.

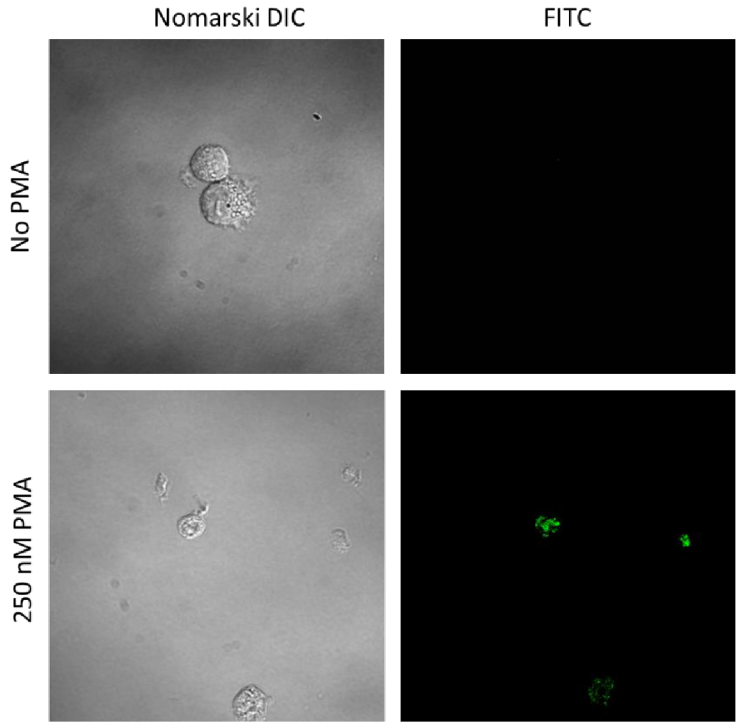

Figure 11: Expression of CD11b surface marker in cell treated without (upper panel) and in the presence of 250nM PMA. The images from left to right represent Nomarski DIC and FITC fluorescence.

4.6 Cell differentiation and protein modification

In our investigation into protein modifications, we focused on the interaction of malondialdehyde (MDA), a reactive aldehyde compound, with amino acid residues of proteins, leading to the formation of MDA-protein adducts. This modification process serves as a pivotal marker for oxidative stress and lipid peroxidation within cellular and tissue environments.

To assess these protein modifications in the context of U-937 cells, whole-cell homogenate from cells treated with differentiation inducer were prepared. Subsequent separation of these homogenates using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting with an anti-malondialdehyde (anti-MDA) antibody enabled us to visualize and quantify the extent of protein modification. In PMA-treated U-937 cells, a pronounced formation of MDA-protein adducts was observed, particularly evidenced by evident protein bands at approximately 40 kDa, as depicted (Figure 12 and Figure 13). The densitometry presented in the form

of bar graph was created on ImageJ and processed in Microsoft Excel. This concentration-dependent effect indicates a robust response to PMA-induced oxidative stress. Similar to this observation, in U-937 cells differentiated using ascorbic acid, the pattern of protein modification was significant. Notably, the formation of MDA-protein adducts was most prominent in protein bands at approximately 20 kDa and 40 kDa, and this effect became increasingly pronounced with increasing concentrations of ascorbic acid (upto 5µM). Beyond this concentration, the ascorbic acid is predicted to have a switch from pro-oxidant activity to antioxidant activity (Figure 5, published article).

Our findings indicate a substantial increase in protein modification in cells subjected to differentiation. In summary, our investigation into MDA-protein adduct formation in U-937 cells treated with PMA and ascorbic acid reveals distinctive patterns of protein modification, shedding light on the intricate interplay between oxidative stress, differentiation, and the concentration-dependent effects of ascorbic acid on protein dynamics.

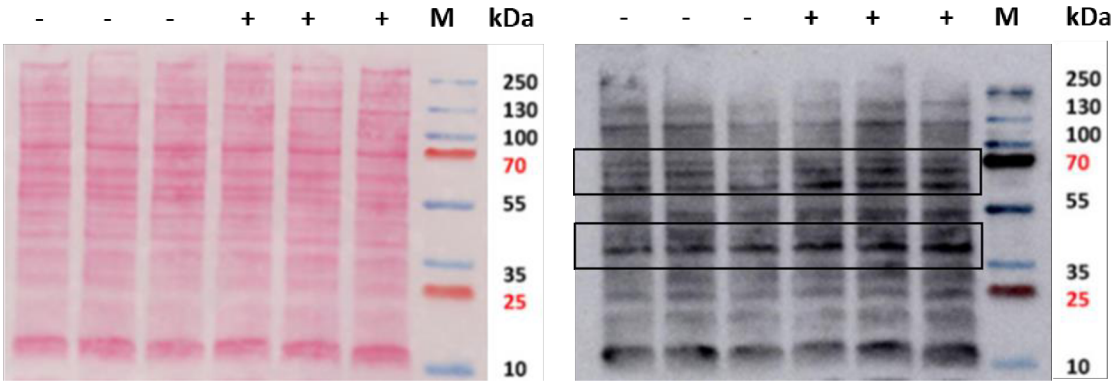

Figure 12: Detection of modified proteins was achieved by identifying MDA-protein adducts in the whole-cell homogenate of U-937 cells. Cells were either untreated (-) or treated with 250nM PMA (+) for 72 h. A Ponceau staining image is displayed on the left, while Western blot analysis of anti-MDA adduct formation is shown on the right.

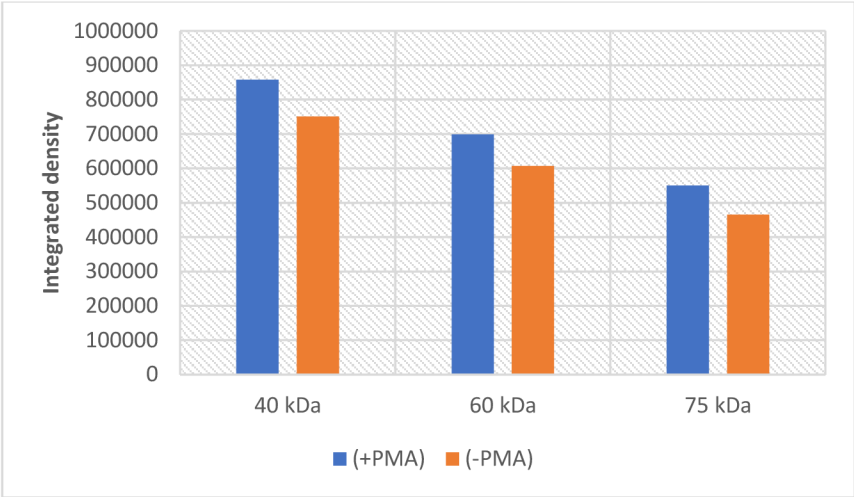

Figure 13: Densitometric analysis (MDA-protein adducts) of lanes presented in Figure 10. A bar graph was presented using ImageJ.

5 Discussion

U-937 cells are known for their high expression of the toll-like receptor 7 (TLR7), making them a useful tool for investigating the immunomodulatory effects of drugs. This receptor is involved in the recognition of viruses, and the activation of TLR7 in U-937 cells can be used to study the effect of various treatments on immunomodulation. It has been used in studies to investigate the effects of various drugs on cytokine expression in U-937 cells and to study the effects of various drugs on TLR signaling pathways. This cell line was also used to study the molecular mechanisms of drug resistance in cancer cells, and how chemotherapeutic agents have been shown to affect gene expression levels and potential signaling pathways (Shindo et al., 2007; Khatua et al., 2022). Treatment of U-937 cells with chemotherapeutic agents has been shown to affect gene expression levels and potential signaling pathways; infection of this cell line with certain viruses has been used to study viral entry and replication (Liu et al., 2019), the role of microglial cells in neurodegenerative diseases (Xu et al., 2020), to study the activity of the NFkB pathway (nuclear factor kappa light chain enhancer of activated B cells), have been used to assess the effects of heavy metals on cytokine production (Dąbek et al., 2010).

Some chemical substances can stimulate and induce differentiation such as PMA, 12-O-tetradecanoylphorbol-13-acetate (TPA), vitamin D3, and retinoic acid. The degree of differentiation depends on the type of substance, the concentration of the stimulus agent, and the length of exposure to the stimuli (Chanput et al., 2014).

PMA also activates the calcium and phospholipid-dependent isoforms of PKC and stimulates AMP-activated protein kinase (AMPK), which is an energy sensor that

regulates cellular metabolism, resulting in macrophage maturation. These macrophages have the potential to be used in clinical trials to investigate cancer immunotherapy (Yang et al., 2018; Yang et al., 2019). The application of PMA exogenously stimulates the NADPH oxidase complex, which can result in the generation of $O_2^{\bullet -}$ within cells. It may produce H_2O_2 and then $HO^{\bullet}$ with the aid of SOD (Auchère and Rusnak, 2002; Halliwell and Gutteridge, 2015; Prasad et al., 2015; Prasad et al., 2016; Pospíšil et al., 2019). Surprisingly, the mechanism of ascorbic acid, which also causes cell differentiation into macrophages similar to PMA, has recently been investigated (Diederich et al., 2023). Using the analyzes carried out, it was shown that cells treated with inducers such as PMA and ascorbic acid were shown to have higher levels of the surface marker CD11b, with an increase in CD11b indicating cell differentiation, as detected by an increase in fluorescence (Starr et al., 2018), which aligns with our results.

When cells produce ROS, they can inflict damage on essential biomolecules such as lipids, proteins, and nucleic acids. ROS have the capacity to directly oxidize proteins, leading to structural and functional disruptions, along with the formation of protein aggregates and cross-linkages. This alteration in protein composition and conformation can impede vital protein-protein interactions and enzyme processes. Among amino acids, cysteine, methionine, and histidine are particularly susceptible to the effects of ROS generation. Lipids, especially due to the presence of PUFA, are highly vulnerable to peroxidation, resulting in the formation of lipid hydroperoxides and subsequent reactive lipid species. Our investigation revealed that MDA, a byproduct of lipid peroxidation, is formed through a series of reactions involving the cleavage of peroxide bonds and rearrangement of resultant radicals. In addition to lipid and protein damage, ROS can interact with DNA/RNA, oxidizing their bases and causing the formation of DNA adducts, strand breaks, and cross-links. Left unrepaired, these alterations can lead to mutations and genomic instability, potentially contributing to disease development (Prasad et al., 2024).

Throughout cellular differentiation, there was a notable rise in the expression of the NADPH oxidase complex, hinting at a potential link to increased ROS production. Previous studies have demonstrated that during the transition of cells from monocytes to macrophages, there is heightened expression of the NOX4 isoform. The findings outlined

in this thesis corroborate this observation, indicating a similar pattern for the NOX2 isoform.

6 Conclusion

The U-937 pro-monocytic cell line was chosen for studying ROS due to its minimal cultivation requirements. Our objective was to explore their proliferation, induction of differentiation, and viability. Viability and proliferation were assessed using trypan blue staining. Chemical inducers promoted the differentiation of monocytes into macrophages, accompanied by ROS production during cell polarization. Blotting results confirmed NADPH expression using a polyclonal NOX2 antibody, while protein modification was verified using an anti-MDA polyclonal antibody. These findings indicated alterations in multiple proteins, suggesting that ROS not only disrupted lipid structures but also modified proteins, potentially contributing to mutations, carcinogenesis, accelerated aging, or autoimmune reactions.

Understanding the regulatory mechanisms of NADPH oxidases is crucial, as members of the NOX family represent important therapeutic targets. Both their activation and regulation are implicated in various degenerative diseases, cancer, cardiovascular disease, diabetes, among others. Detailed knowledge is essential for designing effective therapeutic strategies to address the complexities associated with these pathologies. Progress in the coming years looks promising with the development of new, less time-consuming methods, facilitating comprehensive functional and structural investigations into NOX enzyme activation mechanisms.

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9 Abbreviations

AIDS	Acquired immune deficiency syndrome
ALS	Amyotrophic lateral sclerosis
ATP	Adenosine triphosphate
BCA	Bicinchoninic acid
BSA	Bovine serum albumin
CGD	Chronic granulomatous disease
CLSM	Confocal laser scanning microscopy
Cu-SOD	Copper superoxide dismutase
CYP	Cytochrome catalytic cycle
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
DUOX1	Dual oxidase 1
DUOX2	Dual oxidase 2
FAD	Flavin adenine dinucleotide
Fc	Fragment crystallizable
FBS	Fetal Bovine Serum
FITC	Fluorescein isothiocyanate
H_2O_2	Hydrogen Peroxide
HIF-1α	Hypoxia-inducible transcription factor
$HO^{\bullet}$	Hydroxyl Radical
$HOO^{\cdot}$	Hydroperoxyl radical
HO^{-}	Hydroxyl anion
H_2O	Water
H_2O_2	Hydrogen peroxide

HRP	Horseradish peroxidase
IgG	Immunoglobulin G
IgM	Immunoglobulin M
Mn-SOD	Manganese superoxide dismutase
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
mtROS	Mitochodrial reactive oxygen species
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NO^-	Nitroxyl anion
$NO^{\cdot}$	Nitric oxide
N_2O_3	Dinitrogen trioxide
NOX	NADPH Oxidase
NOX2	NADPH Oxidase 2
NOX3	NADPH Oxidase 3
NOX4	NADPH Oxidase 4
NOX5	NADPH Oxidase 5
$^1O_2$	Singlet oxygen
O_2	Molecular oxygen
$O_2^{\bullet-}$	Superoxide anion radical
$ONOO^-$	Peroxinitrite
PEG	Polyethylene Glycol
PKC	Protein kinase C
PMA	Phorbol-12-Myristate-13-Acetate
POBN	N-tert-butyl-4-pyridylnitrone-1-oxid
PVDF	Polyvinylidene fluoride
RA	Rheumatoid arthritis
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RT	Room temperature
SOD	Superoxide dismutase
SDS-PAGE	Sodium dodecyl-sulfate polyacrylamide gel electrophoresis
TBS	Tris-buffered saline
TLR	Toll-like receptor
Zn-SOD	Zinc superoxide dismutase

Name of chemical	Composition
Gel buffer (tris) 3X	36.3 g Tris base (3M) 0.3 g of SDS 36 g of 6M urea dH_2O to 100 mL
Acrylamide-Bisacrylamide (AB-3) stock solution	48 g of acrylamide 1.5 g of bisacrylamide dH_2O to 100 mL
Separating gel	3 mL of AB-3 5.4 mg of dH_2O 3.5 mL of gel buffer 1.5 mL of glycerol 97 µL of APS 7.5 µL of TEMED
Stacking gel	1 mL of AB-3 7.9 mg of dH_2O 3.3 mL of gel buffer 117 µL of APS 9 µL of TEMED
Anode buffer	121 g of Tris base (1M), 800 mL of dH_2O Adjust pH to 8.9 dH_2O to 1000 mL
Cathode buffer	60.5 g of Tris base (1M) 90 g of tricine 5 g of SDS dH_2O to 500 mL
Transfer buffer	19.35 g of Tris base (300mM) 3 mL of 100mM acetic acid add 450 mL of dH_2O modify pH to 8.6 dH_2O to 500 mL
Phosphate buffer saline (PBS)	80 g of NaCl 2 g 28mM KCl 35.8 g 100 mM $Na_2HPO_4.12H_2O$ 2.45 g of KH_2PO_4 dissolve it in dH_2O adjust pH to 7.4 dH_2O to 1000 mL
Blocking – PBST	100 mL of PBS 1 mL of Tween 20 dH_2O to 1000 mL
Primary antibody solution (amount for 2 membranes)	49.9 mL of PBST-BSA 1 µL of anti-MDA (or NOX2, NOX4)
Secondary antibody solution (amount for 2 membranes)	49.95 mL of PBST-BSA

	5 μL of secondary antibody (Goat anti-rabbit IgG (H+L) Horseradish peroxidase conjugate)

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Differential effects of ascorbic acid on monocytic cell morphology and protein modification: Shifting from pro-oxidative to antioxidant properties

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Reactive oxygen species
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Antioxidants
Pro-oxidant

ABSTRACT

In this study, we investigated the properties of ascorbic acid (vitamin C), which is a naturally occurring water-soluble vitamin. Our goal is to evaluate its pro-oxidative and/or antioxidant capabilities. To do this, we initially used a confocal laser scanning microscope (CLSM) to visualize the differentiation pattern in U-937 cells under the treatment of variable concentrations of ascorbic acid. Prior to induction, U-937 cells showed a spherical morphology. After treatment, significant morphological changes were observed in the form of prominent pseudopodia and amoeboid structures. Interestingly, pseudopodia incidences increased with an increase in ascorbic acid concentrations. In addition, our analysis of protein modification using anti-malondialdehyde antibodies showed changes in more than one protein. The findings reveal the link between the differentiation of U-937 cells into macrophages and the protein modifications triggered by the production of reactive oxygen species when U-937 cells are exposed to ascorbic acid. Furthermore, the transformation of ascorbic acid from a pro-oxidative to an antioxidant property is also demonstrated.

1. Introduction

Ascorbic acid (vitamin C) is a natural water-soluble vitamin and a potent reducing and antioxidant agent [1–3]. It functions in fighting bacterial infections, in detoxification, and, in the formation of collagen in fibrous tissue, teeth, bones, connective tissue, skin, and capillaries, besides absorption of iron [4,5]. Since it is obtained from food and available through a wide range of supplements, severe deficiency caused by ascorbic acid is rather rare. In unusual cases, it can lead to conditions such as weakness, fatigue, joint and muscle aches, and bleeding gums, which are also symptoms associated with scurvy [6].

Ascorbic acid is a powerful antioxidant and helps to protect the body from the harmful effects of free radicals [7] Besides this, ascorbic acid is essential for the synthesis of collagen, helps in stimulating the production of white blood cells, iron absorption, synthesis of neurotransmitters. It also acts as natural antihistamines and promotes the formation of connective tissues [8–12]. Free radicals or reactive oxygen species (ROS) are unstable molecules that can damage cells and are associated with the development of chronic diseases such as cancer, heart diseases, and Alzheimer's disease [13–17]. Ascorbic acid, as an antioxidant, neutralizes free radicals by providing electrons to stabilize them, thus reducing their ability to cause cell damage [1,2]. It also helps regenerate other antioxidants, such as vitamin E, and further improves antioxidant properties [18,19]. It is also known for its anti-inflammatory effect; it is important to note that the body can absorb only limited amount of ascorbic acid at a time and that excessive amounts are excreted in the urine. Ascorbic acid supplements are usually safe, but excessive intake can cause side effects such as diarrhea, nausea, and stomach cramps.

Ascorbate (/ascorbate anion) can induce the transformation of Fe^{3+} into Fe^{2+}, which can lead to the formation of the hydroxyl radical ($HO^{\bullet}$) in the cytoplasmic pool. Due to this property, ascorbate (/ascorbate anion) can also act as a pro-oxidant. Additionally, the oxidation of ascorbate results in the generation of the ascorbyl radical ($AH^{\bullet}$) via monodehydroascorbate radical ($A^{\bullet-}$) [20,21]. Hence, it is essential to approach hypothesis formulation with utmost care to ascertain whether the experimentally obtained results stem directly from ascorbate or ROS

Abbreviations: AA, ascorbic acid; ROS, reactive oxygen species; CLSM, confocal laser scanning microscopy; PMA, phorbol 12-myristate 13-acetate; RIPA, radioimmunoprecipitation assay buffer.

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during cell supplementation.

In the current study, we aimed to evaluate the pro-oxidant and antioxidant capabilities of ascorbic acid. We have used the U-937 cell line, which is a pro-monocytic myeloid leukemia cell line of human origin [22]. Under *in vitro* conditions, an unlimited number of uniform cells can be prepared from U-937 cells [23]. These leukemia cells bear the t(10; 11)(p13; q14) translocation, which results in a fusion between the MLLT10 (myeloid/lymphoid or mixed-lineage leukemia) gene and the Ap-3-like clathrin assembly protein PICALM (clathrin assembly lymphoid myeloid leukemia), which is likely important for the tumorous nature of the cell line [24]. We investigated the relationship of ROS production under the effects of variable concentrations/the length of incubation of ascorbic acid. When the immune reactions are activated, monocytes migrate to different tissues and organs within the body. After reaching the target, depending on the specific signals from local micro-environments, they can be differentiated into diverse types of cells. One of the principal cell types which can be differentiated from monocytes are the macrophages. The monocyte-to-macrophage differentiation process involves several phases: Upon accessing the target tissue, monocytes are exposed to cytokines/chemokines which are secreted by the neighbouring cells. Following this, signalling pathways within the monocytes are initiated leading to changes in gene expression/cellular morphology, which is also a typical characteristic of macrophages. Macrophages acquire enlarged morphology with increased capacity for phagocytosis. Additionally, it has been known that the gene expression profile shifts to support macrophage-specific functions such as the generation of inflammatory mediators and the potential for antigen presentation.

Visualization of differentiation under induction was accomplished utilizing confocal laser scanning microscopy (CLSM). Interestingly, it has been shown that ascorbic acid has the potential as a differentiation agent, i.e., unspecialized cells become specialized in different types of cells with specific functions. Research shows that ascorbic acid can induce differentiation in various types of cells, including stem cells, cancerous cells, and immune cells [25,26]. In immune cells, ascorbic acid has been shown to improve the differentiation and function of certain types of immune cells, including T cells and dendritic cells. Overall, the ability of ascorbic acid to induce differentiation in different types of cells is an interesting field of research for potential therapeutic applications. Thus, instead of inducing differentiation using another chemical stimulant, we evaluated the dependence and relation of three parameters: differentiation induction of monocytes to macrophages in U-937, pro-oxidant concentration, and antioxidant concentration of ascorbic acid. Furthermore, protein immunoblotting was used to understand protein modification. Our results show a correlation between the differentiation of U-937 cells into macrophages and protein modification under the effect of generated ROS.

2. Materials and methods

2.1. Chemicals and antibodies

For blotting, a polyclonal anti-malondialdehyde (anti-MDA) antibody suitable for the measurement of MDA was used. The primary antibody was purchased from Abcam (Cambridge, CB2 0AX, UK) [Anti-Malondialdehyde antibody (ab27642)]; CD11b Monoclonal Antibody-FITC from eBioscience™ (ThermoFischer Scientific, USA) and secondary antibody was purchased from Bio-Rad (Hercules, CA, USA) [Goat Anti-Rabbit IgG-HRP Conjugate (1706515)]. Cell culture medium, antibiotics, and inhibitors used were from Biosera (Nuaillé, France) and Roche (Mannheim, Germany).

2.2. Cell line and growing condition

The U-937 cell line is a human pro-monocytic myeloid leukemia cell line [22] obtained from the American Type Culture Collection (ATCC; Rockville, MD, USA). The TC20 automated cell counter (Bio-Rad Laboratories, Hercules, CA, United States) was used to determine cell density and was monitored using 0.25 % trypan blue dye. Experiments were performed when viability was close to or above 70 %. To growth medium (RPMI-1640) pre-supplemented with 0.05 mM L-glutamine were added 10 % fetal bovine serum (FBS) and 1 % of the antibiotics (penicillin and streptomycin) in v/v ratio.

2.3. Induction of differentiation

The differentiation of U-937 cells was studied under ascorbic acid concentrations ranging from 0.1 to 10 μM. Culture medium with $1x10^5$ cell suspension/mL was supplemented with ascorbic acid at the final concentration of either 0.1 μM, 1 μM, 5 μM or 10 μM for 72h, and followed by a resting phase of 24 h to achieve cell adhesion and express macrophage characteristic cytokine expression. For validation, we also employed a widely recognized differentiation inducer, phorbol 12-myristate 13-acetate (PMA) (Sigma Aldrich, St. Louis, Missouri, United States) at final concentrations of 150 and 250 nM. The timing of the induction protocol was deduced from our pilot experiments focusing on U-937 cell differentiation stages in relation to ascorbic acid treatment.

2.4. Cell viability assay

The TC20 automated cell counter was used to determine the cell viability (expressed as total cell counts mL^{-1}) after incubation of U-937 cells with 0.25 % trypan blue in a ratio of 4:1 for approximately 2 min. Trypan blue is used to determine the ratio of living and dead cells in a cell suspension, and it is because the dye enters only dead cells and stains them blue, while live cells have intact cell membranes and remain bright and unstained [27]. In our experimental condition, cell density and viability were measured after treatment of U-937 cells with ascorbic acid for 72 h and following the 24 h resting period. The data obtained are presented in Fig. 1.

2.5. Confocal laser scanning microscopy

A Fluorview 1000 confocal unit attached to the IX80 microscope was used to image U-937 cells on glass slides (Olympus Czech Group, Prague, Czech Republic). Staining with FM4-64 (15 μM, RT), which is a lipophilic dye was used to monitor cell membrane integrity, and Hoechst 33342 (2 μM, RT) was used to visualize the nucleus under the experimental conditions mentioned. FM4-64 excitation was done using a 543 nm He–Ne laser and its emission was recorded in the range of 655–755 nm. The cells were co-stained with FM4-64 and Hoechst 33342 [Sigma Aldrich GmbH (Germany)]. Following an incubation of 5 min, the cells were transferred to a glass slide for visualization. For immunohistochemistry, after treatments with 5 μM and 10 μM ascorbic acid for 48 h, the culture medium was removed from the culture plates, and 3.5 % paraformaldehyde was added for cell fixation for 30 min. The cells were then washed three times with phosphate buffered saline containing 0.1 % Tween-20 (PBST) for 5 min each.

Afterward, the fixed cells were blocked with 0.5 % BSA in phosphate buffered saline (PBS) for 30 min at room temperature (RT). Subsequently, the blocked samples were probed with CD11b Monoclonal Antibody-FITC (dilution 1:2500) for 60 min at RT, followed by three washes with PBS (5 min each) and Immunofluorescence was measured using excitation achieved by a 488 nm line of an argon laser, and the signal was detected by a 505–550 nm emission filter.

2.6. Protein immunoblotting

Ascorbic acid (72 h, 0.1–10 μM) or PMA (72 h, 150 and 250 nM) pretreated U-937 cells were incubated in serum-free medium for 24 h (resting time), cells were then collected by centrifugation and washed with phosphate buffer saline (PBS) (pH 7.4) twice. Following this step,

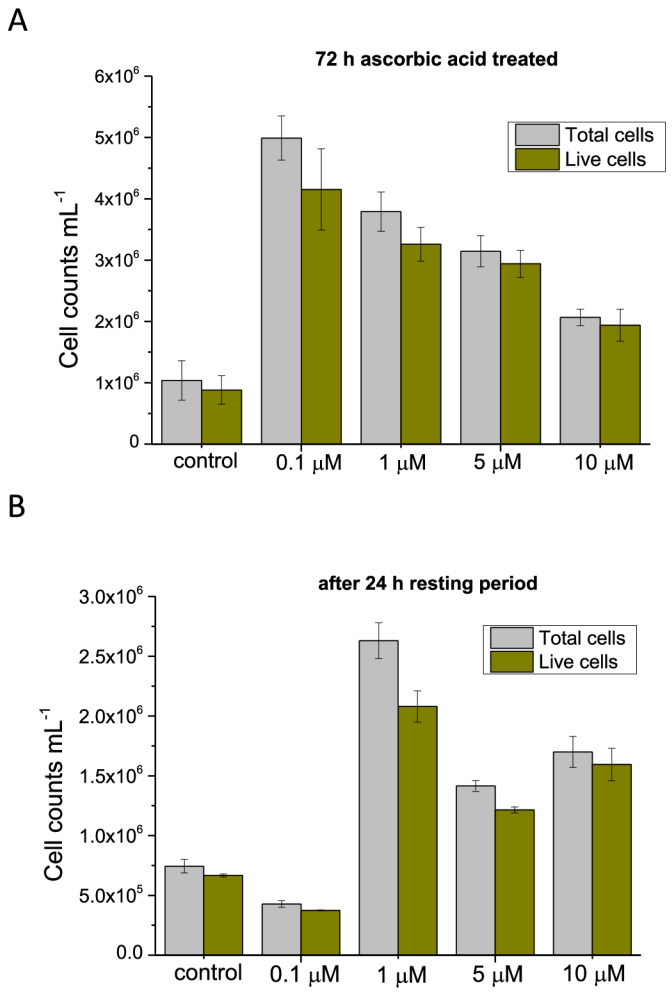

Fig. 1. Cell viability of U-937 cells. U-937 cells at different concentrations of ascorbic acid (0.1 μM, 1 μM, 5 μM and 10 μM). The data are presented as the mean value (±SE) of biological replicates (n = 2).

cells were sonicated in RIPA lysis buffer [150 mM NaCl, 50 mM Tris (pH 8.0), 0.5 % sodium deoxycholate, 0.1 % SDS, 1 % NP-40] containing 1 % (v/v) protease and phosphatase inhibitor. The processed homogenate was centrifuged at 14,000 rpm (30 min, 4 °C) and the collected supernatant fraction was quantified using a Pierce BCA protein estimation kit (Thermo Fisher Scientific, Paisley, UK).

For anti-MDA blotting, samples were prepared with 5× Laemmli sample buffer along with 100 mM Dithiothreitol (DTT); and a protein concentration of 10 μg/lane was used for electrophoresis. The protein samples were then boiled for 10 min at 70 °C. The proteins were seperated on 10% SDS gels and then transferred to nitrocellulose membranes using the Trans-Blot Turbo transfer system (Bio-Rad, California, USA). The nitrocellulose membranes were then blocked for 90 min at room temperature (RT) with 5 % BSA in tris-buffered saline (TBS) (pH 7.4) and 0.1 % Tween 20 (referred to as TBST). The blocked membranes were probed for 90 min at RT with anti-MDA antibody (dilution 1: 5000) followed by 3X washing (10 min each) with TBST; incubated for another 90 min at RT with HRP-conjugated goat anti-rabbit secondary antibody (dilution 1:10000). Following 3 steps of TBST (10 min each), immunocomplexes were imaged using the Amersham imager 600 and Immobilon Western Chemiluminescent HRP Substrate (Sigma Aldrich, GmbH, Germany) (GE Healthcare, UK).

3. Results

3.1. Cell viability using the trypan blue exclusion test

Quantitative estimation of viable cells exposed to varying concentrations of ascorbic acid in U-937 cells was carried out using trypan blue. U-937 cells were treated for 72 h followed by a 24 h of resting period. In Fig. 1A, the gray bar indicates the total cell count, while the green bar indicates the number of live cells within the population (SE, n = 2), while Fig. 1B indicates the cell viability after the resting period. From Fig. 1, it is evident that cell viability under different concentrations used in the experiments is more than 80 % with ascorbic acid treatment. There was a minor/negligible effect noticed in comparison to the control at different concentrations. Therefore, it is considered that the cells are metabolically active in response to the treatments.

3.2. Cell differentiation induced by ascorbic acid and various inducers

Visual changes were monitored 72 h after the addition of ascorbic acid using a confocal laser scanning microscope. Cell morphology was altered after 72 h of incubation with doses of ascorbic acid. When exposed to various differentiation inducers such as PMA, dimethyl sulfoxide (DMSO), retinoic acid, Zn^{2+}, 12-O-tetradecanoylphorbol-13-acetate (TPA), and low concentrations of glutamine, pro-monocytic cells tend to undergo maturation into monocytes or macrophages. To confirm whether the cellular integrity of the U-937 cells under the experimental conditions does not lead to damage of cells, FM4-64 which is a lipophilic styryl compound, and Hoechst 33342 were used. It can be observed that in U-937 cells treated with variable concentration of ascorbic acid (Fig. 2), no obvious cell damage occurred, and cellular integrity (red fluorescence) and nuclear integrity (blue fluorescence) were maintained under all conditions. Differentiated U-937 cells have distinct extensions bearing amoeboid morphology. Before the treatment, the cell morphology exhibited a clear, spherical structure, whereas, after treatment, significant morphological alterations can be observed in the form of prominent pseudopodia, the incidence of which can be seen to be higher with increasing concentration of ascorbic acid from 0.1 μM until 5 μM. A reduction in these morphological structures was observed at higher concentrations of ascorbic acid (10 μM) (Fig. 2).

3.3. Expression of cell surface marker CD11b

To confirm the differentiation of pro-monocytic cells into macrophages, we tracked the expression of the CD11b surface marker. As shown in the results (Fig. 3), it is evident that in untreated U-937 cells, the surface marker expression is minimal. However, in cells treated with ascorbic acid (at 5 and 10 μM concentrations), FITC fluorescence is visibly present under both treatment conditions.

3.4. Effect of ascorbic acid in U-937 cells and associated protein modification

Protein modification occurs when MDA, a reactive aldehyde compound, reacts with amino acid residues of proteins, resulting in the formation of MDA-protein adducts. This is often used as a marker of oxidative stress and lipid peroxidation in cells and tissues. Whole-cell homogenate from U-937 cells treated with PMA and ascorbic acid separated using SDS-PAGE followed by immunoblotting using anti-malondialdehyde (anti-MDA) antibody showed modification of more than one protein. In PMA-treated U-937 cells, the formation of MDA is most pronounced as displayed by protein bands at approximately 40 kDa (Fig. 4A) which is also evident from the densitogram presented in Fig. 4B. In ascorbic acid treated U-937 cells, the formation of MDA is most pronounced in the protein bands at approximately 20 kDa and 40 kDa, and this effect becomes more prominent with increasing concentrations of ascorbic acid (Fig. 5A and Supplementary data 1). A

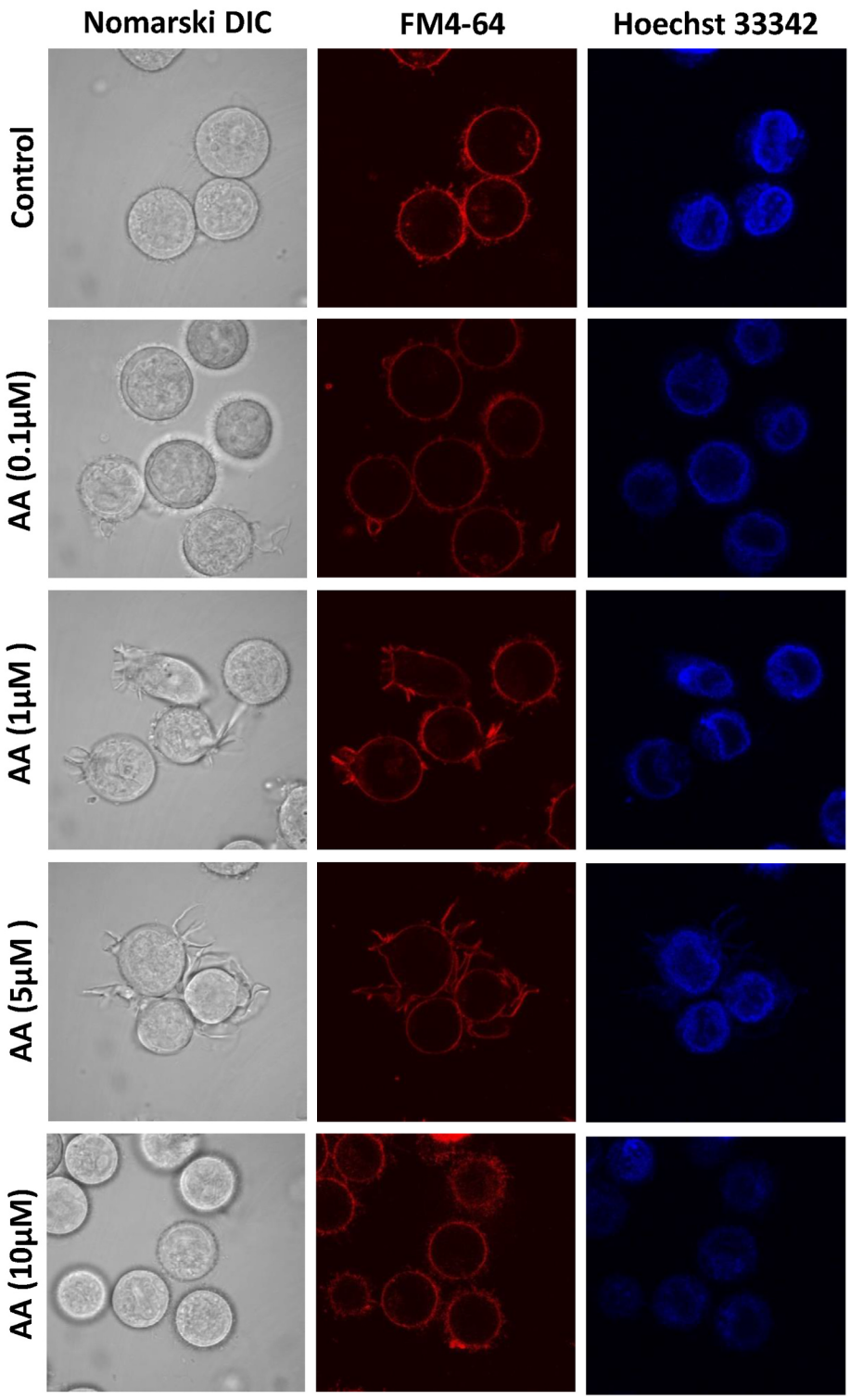

Fig. 2. Double staining using Hoechst 33342 and FM4-64 in 72 h differentiated U-937 cells. Differentiation was induced using variable concentrations of ascorbic acid (0.1–10 μM). Images were captured in various channels at a magnification of 1000 × after staining for 5 min (from left to right are Nomarski DIC, FM4-64 and Hoechst 33342). For each variant, the presented images represent several scans conducted on both biological and technical replicates.

consistent and linear increase in band intensity at this molecular weight range is observed in treated cells, especially with 1 μM and 5 μM ascorbic acid, as indicated in the densitogram shown in Fig. 5B. Notably, in cells differentiated with 10 μM ascorbic acid, a sudden decline in band intensity is evident. We confirmed our findings by employing reverse-phase HPLC to measure the concentration of MDA in cells treated with 1 and 5 μM ascorbic acid, comparing them to the control (indicated as -). A significant increase was observed in case of ascorbic acid differentiated cell (supplementary data 2). These findings can be associated with the differentiation pattern illustrated in Fig. 2. Unexpectedly, the band intensity at 40 kDa exhibits a slight reduction in the 0.1 μM ascorbic acid-treated sample when compared to the control. We attribute this phenomenon to potential non-specific interactions.

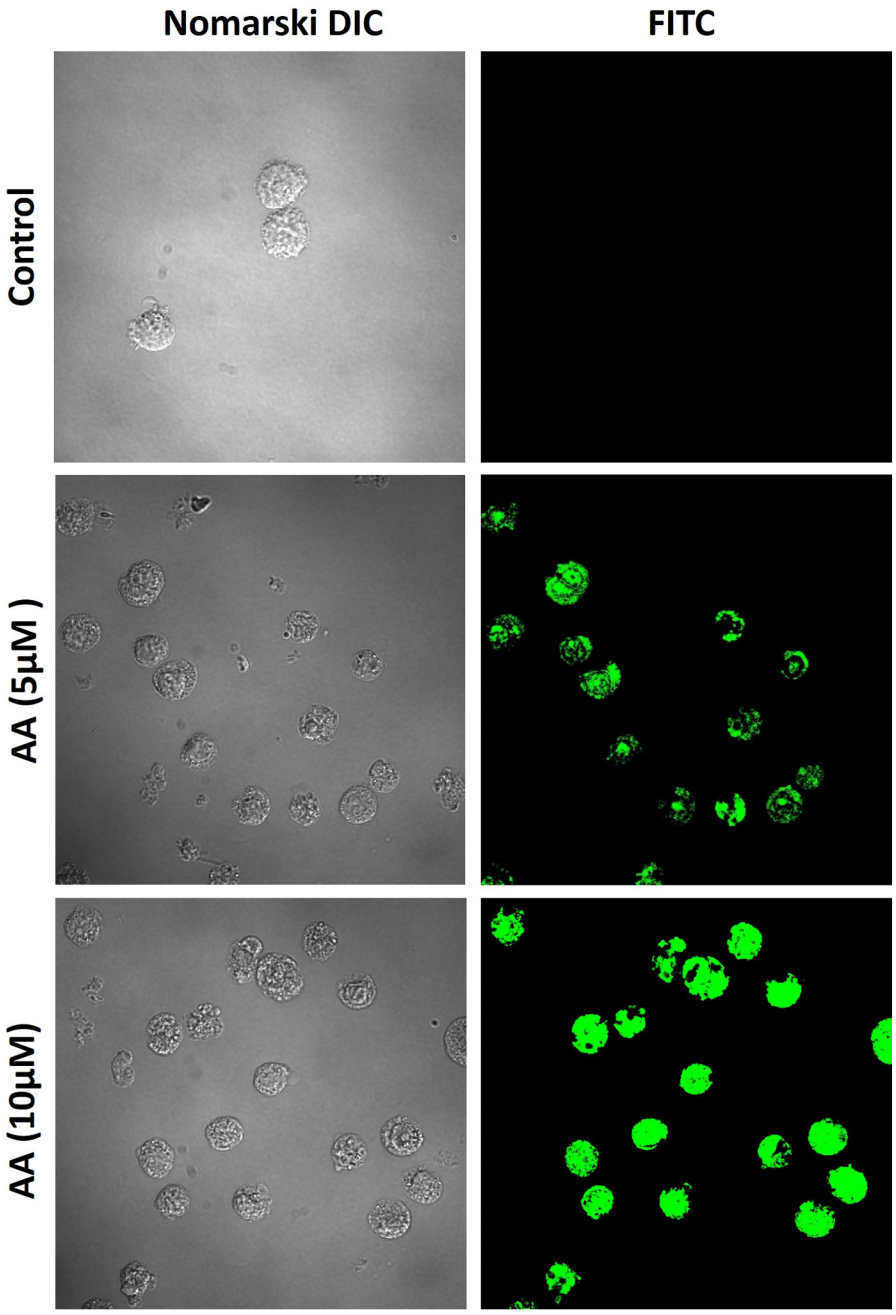

Fig. 3. Expression of CD11b surface marker in cell treated without (upper panel) and in the presence of 5 μM (middle panel) and 10 μM (lower panel) of ascorbic acid for 48 h. The images from left to right represent Nomarski DIC and FITC fluorescence.

4. Discussion

Monocytes are circulatory precursors that originate from a myeloid lineage which can further differentiate into macrophages or dendritic cells after recruitment in tissues and blood stream [28]. Our immune system gains the advantage of phagocytosis, antigen presentation, and cytokine production from these cells. U-937, a pro-monocytic cell lines differentiate into macrophages or into dendritic cells *in vitro* in presence of different inducers [29]. The basic feature of this cell line is the synthesis and secretion of lysozyme with the absence of immunoglobulin production.

When cells such as U-937 and THP-1 cells are exposed to differentiation inducers such as PMA, retinoic acid etc., their proliferation is slowed while the differentiation process is triggered. The monocytes under these treatments are known as "macrophage-like" because of their structure. However, the properties of the transformed cell line are not yet well known. It depends on the dose and time of treatment with inducers. Cells treated with different inducers have been shown to express elevated levels of CD11b and CD14. It also starts to induce adherence accompanied by cell cycle arrest [30]. Cell differentiation is also known to activate the calcium and phospholipid-dependent isoforms of protein kinase C (PKC) thereby inducing AMP metabolism, which leads to its maturation into a macrophage [31,32]. It is now well accepted that the treatment with differentiation inducers exogenously activates the NADPH oxidase complex, which can lead to the formation of $O_2^{\bullet -}$ [33]. In the presence of superoxide dismutase (SOD), it can form H_2O_2 and subsequently $HO^{\bullet}$ in the presence of transition metal ions [34]. During the last decades, differentiation studies were done utilizing PMA and

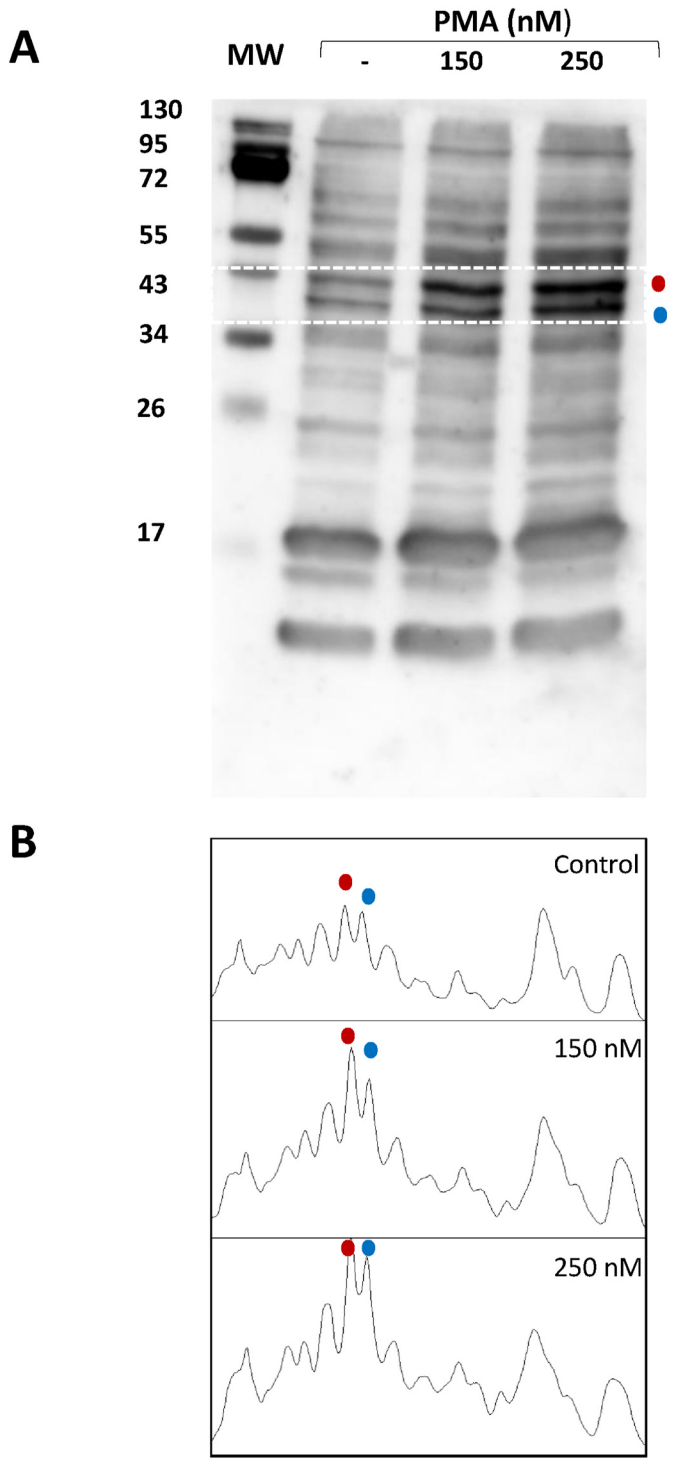

Fig. 4. A. Protein MDA adducts formed in U-937 cells treated with 250 nM PMA. This illustrates protein modification in U-937 cells that were differentiated for 72 h, visualized on blot with anti-MDA antibody. **B.** Quantification of protein bands from an anti-MDA blot by densitogram analysis.

retinoic acid as inducers whereas, more recently, ascorbic acid has been known to relate to cell differentiation [35], the molecular mechanism of which is still unclear. Differentiation studies on dental stem cells have been performed using ascorbic acid in the time range of 24–72 h; however, the stability of ascorbic acid over the period of several days in solution should be taken in account considering the instability of the compound [36].

With the production of ROS in cells, biomolecules such as lipids, proteins, and nucleic acids can be damaged. Reactive oxygen species can directly oxidize proteins, leading to structural and functional impairments, as well as the formation of protein aggregates and cross-linking. Altogether, it can lead to change in normal protein folding and conformation resulting which the protein-protein interaction and enzymatic activity can be hampered. Amino acids, including but not limited to cysteine, methionine, and histidine are known to be most affected by ROS generation. Lipid particularly due to the presence of polyunsaturated fatty acids (PUFA's) are prone to peroxidation leading to the formation of lipid hydroperoxide and subsequently other reactive lipid species. In our study, MDA which is formed as a by-product of lipid peroxidation known to be generated through a series of reaction involving cleavage of the peroxide bonds and rearrangement of the resulting radicals. In addition to most damage to lipids and proteins, ROS is known to react with DNA/RNA, oxidizing its bases, leading to the formation of DNA adducts, chains, and DNA-cross links. If not repaired, this can eventually lead to mutations and genomic instability that can be responsible for disease development.

Our study shows the differentiation behavior of U-937 cells under exogenous supplementation of ascorbic acid. During the process of cell differentiation, it has been observed that there is a higher expression of NADPH oxidase complex, which eventually can be hypothesized with the increase in the production of ROS. Based on our previous study, we observed a higher expression of NOX-4 in differentiated U-937 cells [16]. There might be a direct/indirect role of ROS in the activation of NADPH expression and eventually self-oxidation.

5. Conclusions

Prooxidative *vs.* antioxidative action of ascorbic acid (vitamin C) was evaluated in relation to differentiation of U-937 cell line into macrophages. Analysis of protein modification using anti-malondialdehyde antibodies showed changes in more than one protein. The findings demonstrate the relationship between the differentiation of U-937 cells into macrophages and the protein modification caused by the production of ROS under the influence of ascorbic acid together with a switch from pro-oxidative to the antioxidant property of the compound under investigation. Findings from this study indicate ascorbic acid promotes differentiation of monocytes into macrophages underlying its role in immune response besides its antioxidant activity. Studies focusing on ascorbic acid role in differentiation and development have to be considered.

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CRediT authorship contribution statement

Ankush Prasad: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Validation, Visualization, Writing – original draft, Writing – review & editing. **Deepak Rathi:** Data curation, Formal analysis, Methodology, Writing – original draft, Writing – review & editing. **Michaela Sedlářová:** Data curation, Investigation, Methodology, Writing – review & editing. **Renuka Ramalingam Manoharan:** Investigation, Methodology, Writing – review & editing. **Eliška Průdková:** Data curation, Investigation, Writing – review & editing. **Pavel Pospíšil:** Data curation, Formal analysis, Investigation, Writing – review & editing.

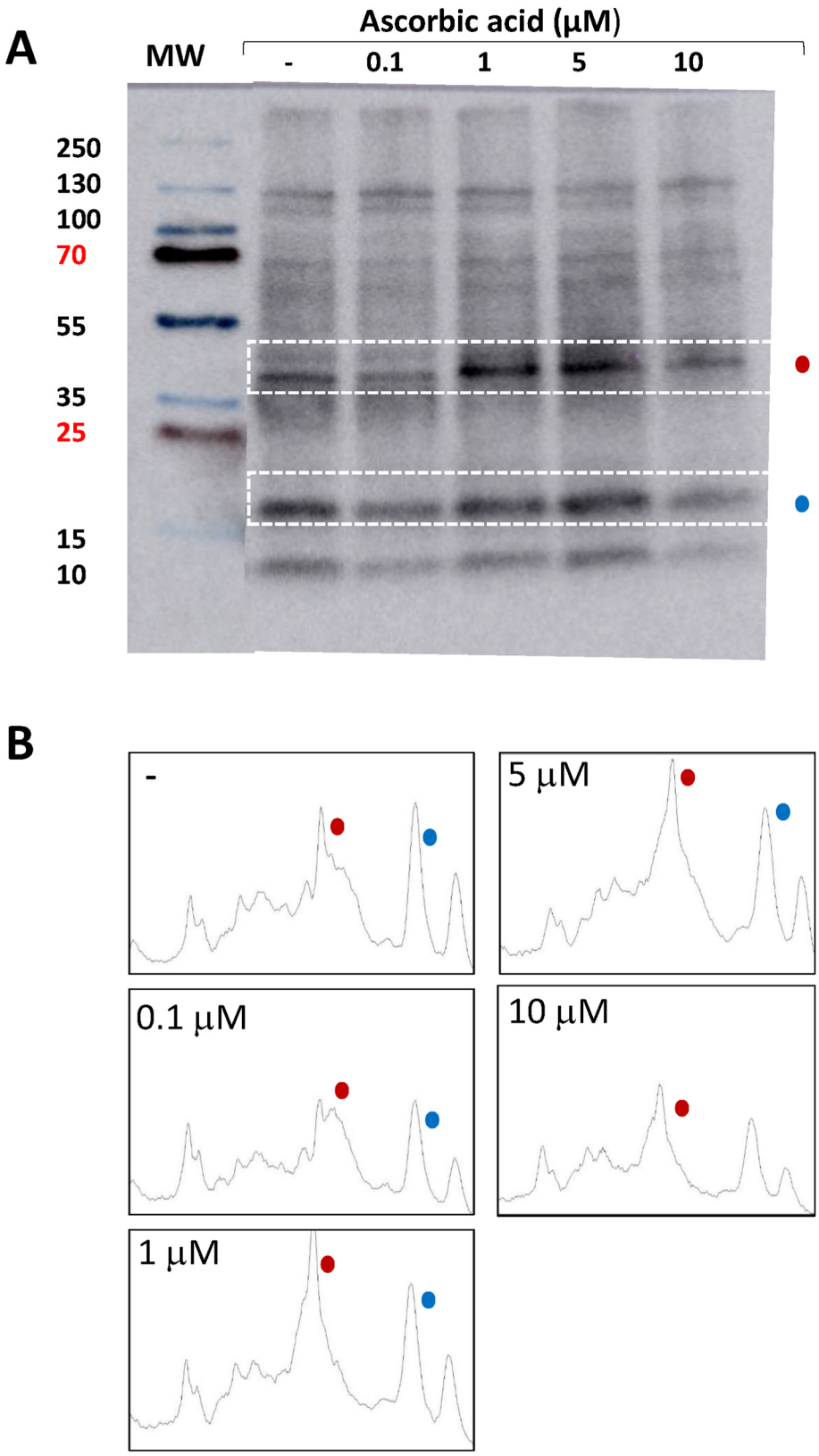

Fig. 5. **A.** Protein MDA adducts formed in U-937 cells treated with 0.1–10 μM ascorbic acid. This illustrates protein modification in U-937 cells that were differentiated for 72 h, visualized on blot with anti-MDA antibody. **B.** Quantification of protein bands from an anti-MDA blot by densitogram analysis.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrep.2023.101622.

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Supplementary dataset 1

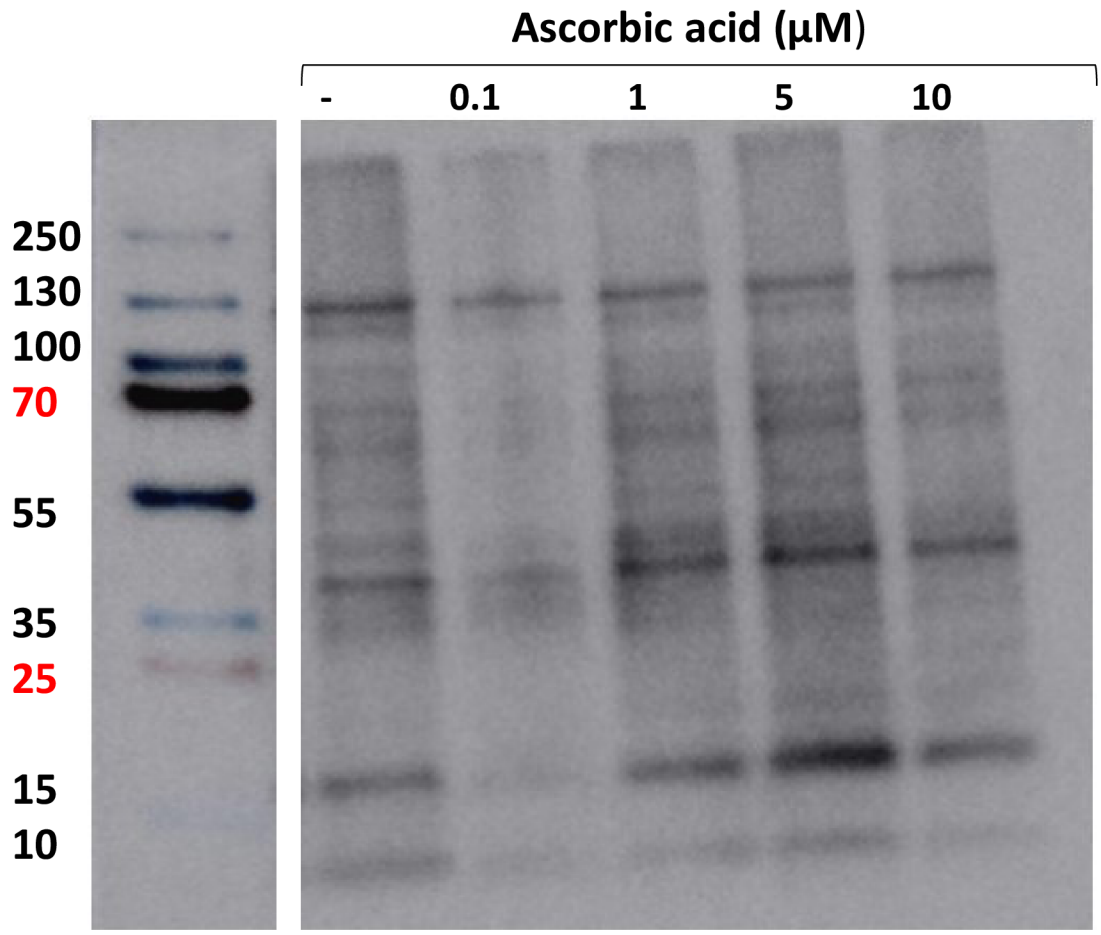

Figure S1: Protein MDA adducts formed in U-937 cells treated with 0.1-10 µM ascorbic acid. This illustrates protein modification in U-937 cells that were differentiated for 72 h, visualized on blot with anti-MDA antibody.

Supplementary dataset 2

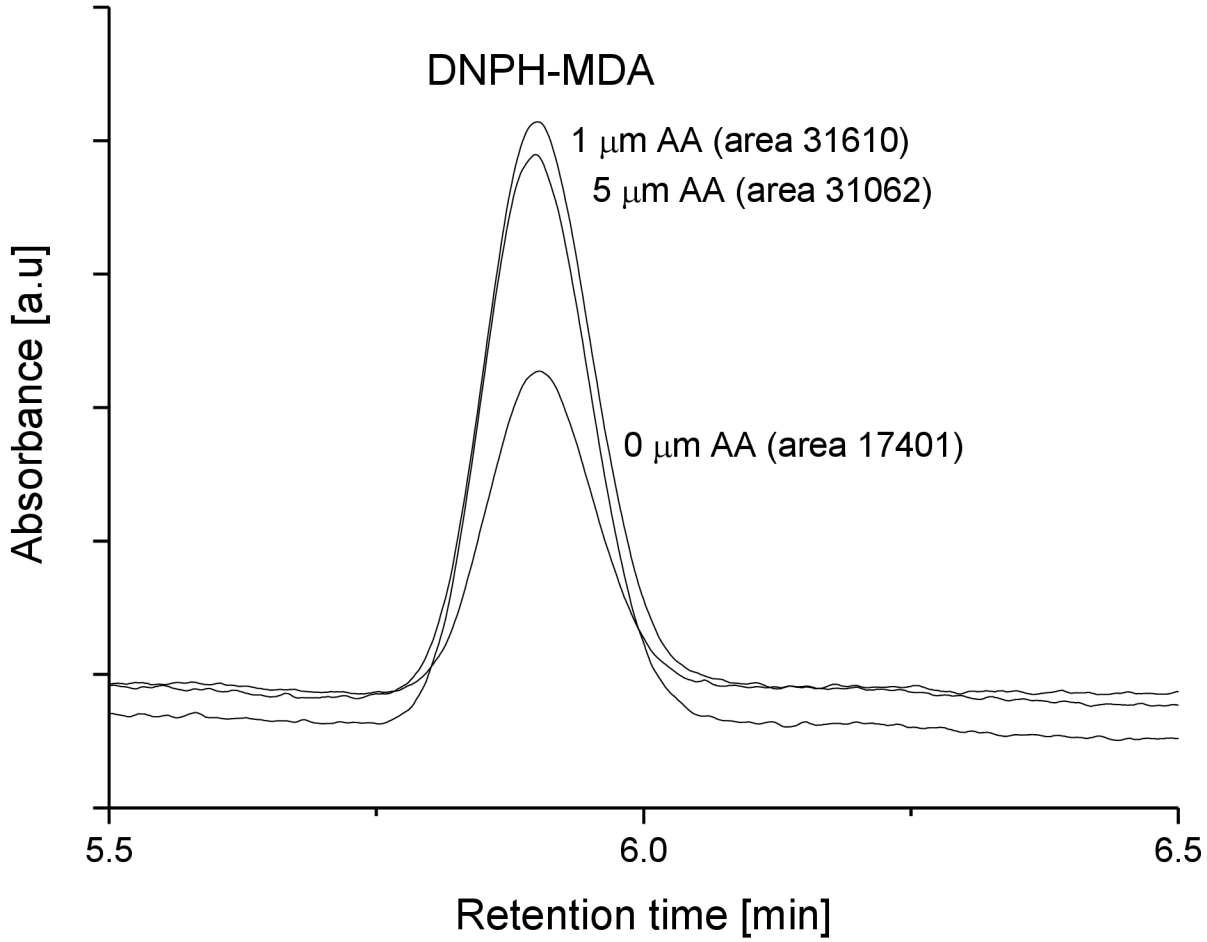

Figure S2: Quantification of MDA in non-differentiated (control) and differentiated U-937 cells (72 h, 1 and 5 μM AA) monitored by the reverse-phase HPLC. The retention time of MDA-DNPH derivative was at 5.8 min.

Experimental protocol used is briefly mentioned below:

The total amount of MDA was assessed through reverse-phase HPLC analysis, following the procedure outlined by Pilz et al. (2000). In summary, HPLC-grade water (LC-MS grade) was added to the fraction samples to reach a total volume of 200 µl. To release MDA from proteins via alkaline hydrolysis, 40 µl of 6 M aqueous sodium hydroxide was added and incubated at 60 °C for 30 minutes. Proteins were precipitated by acidifying the sample using 100 µl of 35% (v/v) perchloric acid, followed by centrifugation at 16,000 g for 10 minutes. The supernatant (125 µl) was transferred to a new Eppendorf tube, and MDA was derivatized by adding 1 µl of 50 mM 2,4-dinitrophenyl hydrazine (DNPH) prepared in 2 M sulphuric acid and incubated at RT for 30 minutes. A 50 µl volume of the derivatized MDA was injected into the HPLC (Alliance e 2695 HPLC System, Waters, Milford, MA, U.S.A.) equipped with a 2998 Photodiode Array (PDA) detector. The isocratic separation was carried out using an Arion ASTRA® C18-HE HPLC column (3.0 µm 150 mm × 4.6 mm) (Chromes s. r.o., Prague, Czech Republic) with acetonitrile: water (50:50 v/v) as the solvent system and a flow rate of 1 ml min^{-1}. The MDA-DNPH derivative was detected in the samples at 310 nm with the PDA detector. Operation and data processing were performed using Empower software.