Cell response to imaging contrast agents suggested for atherosclerotic plaque imaging

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To my family

“Even if I am a minority of one, truth is still the truth”

Mohandas Karamchand Gandhi
Oxysterols are the major cytotoxic components of oxidized low-density lipoprotein (OxLDL) that accumulate in atherosclerotic plaques. Their uptake by macrophages ensue foam cell formation, atherogenesis and plaque progression. Magnetic resonance imaging (MRI) has grown as a modality to track such intra-plaque developments by using intracellular contrast agents. The focus of this study was to evaluate the effects of two contrast agents; manganese based mangafodipir (Teslascan\textsuperscript{TM}) and iron based super-paramagnetic iron oxide nanoparticles (SPION, Resovist\textsuperscript{TM}) on cell functions and examined their interaction with oxysterol laden cells.

Mangafodipir has antioxidant property and provides protection against oxidative stress. The chemical structure of mangafodipir comprises of organic ligand fodipir (Dipyridoxyl diphosphate, Dp-dp) and Mn (manganese). Mangafodipir is readily metabolized within the body to manganese dipyridoxyl ethyldiamine (MnPLED) after an intravenous injection. MnPLED has superoxide dismutase (SOD) mimetic activity, and Dp-dp has iron chelating effects. The second contrast agent tested in this study is Resovist\textsuperscript{TM}. These SPION are primarily ingested by macrophages and accumulated in lysosomes where they are gradually degraded ensuing increased cellular iron.

In paper I, we examined whether the above-noted effects of mangafodipir could be utilized to prevent 7β-hydroxycholesterol (7βOH) induced cell death. We found that mangafodipir prevents 7βOH induced cell death by attenuating reactive oxygen species (ROS) and by preserving lysosomal membrane integrity and mitochondrial membrane potential.

The second part of this study (paper II) was designed to identify the pharmacologically active part of mangafodipir, which exerts the above-noted effects. We compared the activity of parent compound (mangafodipir) with MnPLED and Dp-dp. We found that mangafodipir; MnPLED and Dp-dp provide similar cyto-protection against 7βOH induced cell death. These results suggest that MnPLED and Dp-dp both contribute to the pharmacologically active part of mangafodipir.
In paper III, we aimed to examine the interaction of SPION with monocytes and macrophages exposed or not to atheroma relevant oxysterols. We demonstrate that SPION loading up-regulates cellular levels of cathepsin and ferritin and induces membranous ferroportin expression. Additionally, SPION incites secretion of ferritin and both pro-inflammatory and anti-inflammatory cytokines. Moreover, exposure to oxysterols resulted in a reduced SPION uptake by cells, which may lead to inefficient targeting of such cells. Although SPION uptake was reduced, the ingested amounts significantly up-regulated the expression of 7βOH induced cathepsin B, cathepsin L and ferritin in cells, which may further aggravate atherogenesis.

The fourth part of the study (paper IV) was designed to examine the interaction of SPION with macrophage subtypes and compare the cellular effects of coated and un-coated iron-oxide nanoparticles. We found that iron in SPION induces a phenotypic shift in THP1 M2 macrophages towards a macrophage subtype characterized by up-regulated intracellular levels of CD86, ferritin and cathepsin L. Differential levels of these proteins among macrophage subtypes might be important to sustain a functional plasticity. Additionally, uncoated iron-oxide nanoparticles induced dose dependent cell death in macrophages, which elucidates the potential cyto-toxicity of iron in iron-oxide nanoparticles.

In conclusion, evidence is provided in this study that intracellular MRI contrast agents have the potential to modulate cell functions. The study reveals a therapeutic potential of mangafodipir, which could be utilized for future development of contrast agents with both diagnostic and curative potentials. Additionally, we found that surface coating in SPION may provide cell tolerance to iron toxicity by modulation of cellular iron metabolism and cell functions. Such alterations in cellular metabolism call for careful monitoring and also highlight new concepts for development of iron containing nanoparticles. A reduced uptake of SPION by atheroma relevant cells justifies development of functionalized SPION to target such cells in atherosclerotic plaques.
POPPULAR SUMMARY

Atherosclerosis is a common vascular disease and its clinical complications, (myocardial infarction, stroke and angina pectoris), cause high morbidity and mortality in western population. These clinical complications are all associated with the blockade in the blood vessel by the fragments of the atherosclerotic plaques. Atherosclerotic plaque formation is initiated by uptake of cytotoxic cholesterol oxidation products by white blood cells of lipid rich foam cells. Thus, along with many other factors, white blood cells and foam cells regulate the development of atherosclerosis.

Magnetic resonance imaging (MRI) is one of the most promising techniques for detecting and tracking such cells in atherosclerotic plaques. For better detection of the plaques by MRI, imaging contrast agents are used, which accumulate in the target cells to enhance the accuracy of detection. Many such contrast agents are currently undergoing pre-clinical and clinical trials. However, their effect on the cell functions is not fully examined. In this study, we evaluated the effects of two contrast agents; manganese based mangafodipir (Teslascan™) and iron based super-paramagnetic iron oxide nanoparticles (SPION, Resovist™) on cell functions and examined their interaction with cells treated with lipid oxidation products; 7βOH (7β-hydroxycholesterol) and 7keto (7 keto-cholesterol).

We found that mangafodipir was non-toxic to cells and pre-treating the cells with mangafodipir prevented 7βOH induced cell death. In addition, a particular component in the chemical structure of this contrast agent may be responsible for this protective effect. However, when white blood cells were exposed to SPION, iron concentration in the cell was increased, which lead to increases in iron regulatory protein levels in the cells, including ferritin, ferroportin and cathepsins. Also, SPION elevates 7βOH and 7keto induced cathepsin levels in the cells. All these proteins are associated with development of atherosclerosis. Equally important, SPION affected immune functions of cells, which is a very critical aspect determining atherosclerotic plaque stability and its progression. In addition, we found that macrophages exposed to 7βOH have a reduced capacity to uptake SPION. We also found that iron in SPION may itself induce toxicity in cells.
In conclusion, our study reveals that imaging contrast agents modulate cell functions which could be an advantage or disadvantage. Mangafodipir provides protection against 7βOH induced toxicity. This property of mangafodipir may be utilized in future development of contrast agents with both detection and treatment abilities. However, we found that SPION with surface modifications gives cell tolerance to iron toxicity by modulation of cellular iron metabolism. Such alterations in cellular metabolism call for careful monitoring and also highlight new ideas for development of iron containing nanoparticles.
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II. **Laskar, A.**, Andersson, R.G.G., Li, W., 2013. Fodipir (Dp-dp) and its dephosphorylated derivative PLED are involved in mangafodipir mediated cytoprotection against 7β-hydroxycholesterol induced cell death. Submitted to *The J of Cardio Pharmcol*.


IV. **Laskar, A.**, Eilertsen, J., Li, W., Yuan, XM., 2013. SPION primes M2 macrophages towards a pro-inflammatory M1 subtype characterized by elevated levels of ferritin and cathepsin L. *Manuscript*.

OTHER PUBLICATIONS NOT INCLUDED IN THIS THESIS


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INTRODUCTION

Macrophages in atherosclerosis

Atherosclerosis is a chronic inflammatory disease in which accumulation of inflammatory cells contributes to atheroma plaque development and vulnerability [1]. Development of atherosclerotic plaque is a complex process and involves activation of endothelium by components of OxLDL found in the intima of the lesion prone areas [2]. OxLDL in the intima is chemotactic for monocytes in the blood. Activated endothelium expresses cell adhesion molecules, chemokine and cytokines and thereby promotes leukocyte recruitment [3-5]. This is a crucial step of atherogenesis in which monocytes infiltrate the sub-endothelial space of larger arteries where they differentiate into intimal macrophages [2, 6-8]. These monocyte derived macrophages that are recruited from the blood account for the majority of the leukocytes in atherosclerotic plaques. These recruited macrophages play reparatory roles in early plaques characterized by phagocytosis of oxidized lipids and apoptotic cells [9]. However, during progression of atherosclerosis, these macrophages secrete inflammatory factors and proteolytic enzymes and contribute to plaque rupture and thrombosis [9]. High macrophage content, apoptosis and reduced clearance of apoptotic macrophages are all associated to vulnerability of atherosclerotic plaques [6]. These intra-plaque macrophages are rich in iron and lipids [10-12] and exhibit functional plasticity in atherosclerotic plaques.

Among others, macrophages are considered the predominant cells which not only promote atheroma plaque development and vulnerability [6, 13] but also play an important role in cardiac damage after infarction [14]. A ruptured atherosclerotic plaque exposes their thrombogenic interior to the luminal blood causing platelet aggregation and thrombus formation [2, 15, 16]. Together these events lead to the major clinical manifestations of atherosclerosis such as myocardial infarction and stroke, which are the most common cause of death among the western society [17-19].
Macrophages and functional plasticity

Macrophages are the highly plastic cell type, which display functional plasticity in atherosclerotic plaques [6, 8, 9, 13, 20, 21]. Both pro-inflammatory (M1) and anti-inflammatory (M2) macrophage subtypes are present in atherosclerotic lesions. In chronic inflammatory diseases like atherosclerosis, macrophage plasticity is determined by immunological microenvironment. Interferon gamma (IFN-γ) and lipopolysaccharide (LPS) or granulocyte macrophage colony-stimulating factor (GM-CSF) or tumour necrosis factor α (TNF-α) direct macrophages towards a classically activated macrophage subtype (M1) which produces pro-inflammatory cytokines and high amounts of reactive oxygen and nitrogen species [8, 21-24]. Macrophages are the main source of pro-inflammatory mediators in the atherosclerotic plaques. In contrast, exposure to interleukin-4 (IL-4) or macrophage colony stimulating factor (M-CSF) polarizes macrophages towards an alternatively activated macrophage subtype (M2) which are associated with secretion of anti-inflammatory cytokines and tissue repair [8]. However, based upon the stimulus to generate these macrophages, the M2 macrophages are further subdivided into M2a (after exposure to IL-4 or interleukin-13 (IL-13)), M2b (after exposure to immune complexes in combination with interleukin-1beta (IL-1β) or LPS) and M2c (after exposure to IL-10, TGFβ or glucocorticoids) [25]. Mox is an additional macrophage subtype that has been proposed [23]. When exposed to 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphorylcholine (ox-PL), bone marrow derived macrophages differentiate to this unique population of Mox macrophages with a unique profile of genes, including Heme-oxigenase-1 (OH-1) and thioredoxin reductase 1 (Txnrd1) [23]. In addition, a fourth subtype of platelet chemokine CXCL4 dependent macrophage (M4) has been proposed [26, 27] which is characterized by a complete loss of CD163 [28] and higher levels of CD86 and mannose receptor [27]. CXCL4 promotes macrophage differentiation in-vitro. These M4 macrophages exhibit reduced phagocytosis of acetylated or OxLDL [28].

As indicated above, diverse methodologies, including cytokine stimulation and growth factor induced differentiation has been used for classical (M1) and alternative (M2) macrophage polarization in-vitro. Growth factors including GM-CSF and M-CSF induces
both macrophage differentiation and macrophage polarization into M1 and M2 macrophages respectively [28]. Overall, these findings suggest that induction of differentiation induces functional polarization in macrophages. However, Human immunoglobulin G (hIgG) or Phorbol 12-myristate 13-acetate (PMA) has been used for induction of differentiation in monocytes [29, 30] but the obtained cell types are insufficiently characterized in terms of functional polarization.

**OxLDL and related oxysterols in atherogenesis**

LDLs produced within the liver carry cholesterol to other cells and tissues of the body, including the arterial wall. LDL undergoes oxidative modification in the intima by ROS probably produced by macrophages [2, 31, 32]. This OxLDL present in the intima is chemotactic to blood monocyte and aids in recruitment of other leukocytes and induces differentiation of monocytes to macrophage foam cells [33]. Furthermore, OxLDL has been shown to induce cell death in smooth muscle cells, endothelial cells and in macrophages [34-37]. Many cytotoxic components of OxLDL have been identified including: oxysterols and oxidized fatty acids [38, 39].

Handling of lipids, especially cholesterol is one of the most important functions of macrophages in the context of atherosclerosis. An imbalance in cholesterol influx and efflux leads to an excessive accumulation of cholesterol in macrophages and subsequent foam cell formation [40-42]. Oxysterols are cholesterol oxidation products resulting from spontaneous or enzymatic oxidation of cholesterol [43]. 7β0H and 7keto-cholesterol (7keto) are the major cytotoxic components of OxLDL, which contribute to development of atherosclerosis [43, 44]. Increased levels of 7β0H and 7Keto are found in atherosclerotic plaques and in plasma of patients with cardiovascular diseases [43]. Thus oxidized lipid rich macrophages are considered an important target for intra-plaque macrophage imaging [45].

**Uptake of OxLDL by macrophages**

Macrophages express a group of scavenger receptors, including SR-A, CD36 and lectin-like oxidized LDL receptor-1 (LOX-1) to interact and uptake OxLDL [9, 46]. LDL and OxLDL are
lysosomotropic and after endocytosis are transferred into lysosomes [47, 48]. Cholesterol esters in LDL and OxLDL are hydrolysed in the lysosomes of these macrophages [9]. After lysosomal processing, some of the un-esterified cholesterol integrates into the plasma membrane, and the rest is stored as lipid droplets [9, 49]. Cholesterol accumulation down-regulates native LDL receptors thus preventing further uptake and accumulation of cholesterol [2, 50, 51]. However, the scavenger receptors in macrophages are unaffected by cholesterol accumulation [51] and thus continue to uptake OxLDL and atherogenic lipoproteins and form foam cells and eventually die by apoptosis or necrosis [2]. The lipid remains of these foam cells form the content of the necrotic core in the intima which is eventually covered with a fibrous cap of collagen rich matrix and vascular smooth muscle cells [2].

**Iron in atherogenesis**

Determining the role of iron in atherogenesis has been an area with continuous focus over decades [11, 52]. The primary reason for that is the presence of catalytic redox-active iron in atherosclerotic vascular tissues which remain trapped in ferritin and hemosiderin within the lesion. High iron concentrations are seen in human atherosclerotic lesions as compared to healthy artery tissue [10, 11].

**Macrophage iron and lysosomes**

Most of the iron in atherosclerotic plaques has been associated with macrophages and foam cells. In advanced plaques, iron derived from erythrophagocytosis within the microhaemorrhagic areas remain the potential source of redox-active iron in atherosclerotic vascular tissues which remain trapped in ferritin and hemosiderin within the lesion. High iron concentrations are seen in human atherosclerotic lesions as compared to healthy artery tissue [10, 11].
activity [57]. Together such developments alone shall be deleterious in atherosclerotic plaques.

In addition, iron excess might incite expression of macrophage scavenger receptors and promote uptake of lipids [58]. Increasing lipid load and increased availability of redox active iron within the lysosomal compartment of the cells results in formation of highly toxic iron laden- ceroid and lipidaceous materials [10, 59]. Release of such cytotoxic materials from these cells may determine viability of nearby existing cellular elements and destabilize plaques. In addition, iron excess in presence of OxLDL has been shown to enhance secretion of ferritin and iron [10, 60], which may regulate immune function in atherosclerotic microenvironment.

**Ferritin and cell functions**

The content of iron in the human body is well controlled by complex mechanisms to maintain homeostasis. Iron in the body is bound to haemoglobin, myoglobin, iron containing enzymes and iron storage proteins; ferritin and hemosiderin [61, 62]. Ferritin is a ubiquitous and highly conserved iron-binding protein. Its heavy-chain also has enzymatic properties, converting Fe(II) to Fe(III) as iron is sequestered in the ferritin mineral core [53, 63]. In cells, ferritin mostly is cytosolic. However, it is known that ferritin also resides in nucleus and mitochondria of mammalian cells [64]. Apart from being an iron storage protein ferritin is immuno-modulatory [65], associated with inflammation, and regulates ROS production and apoptosis [66]. In addition, apoptotic macrophage foam cells of human atheroma are rich in iron and ferritin [67]. Both apoptotic and anti-apoptotic activities of ferritin are documented. Cytosolic ferritin regulates ROS production and apoptosis while nuclear ferritin may protect cells against oxidative stress and DNA damage [64]. In addition, ferritin may slowly release iron when exposed to reducing agents such as cysteine and reduced glutathione [53].

**Immuno-modulatory iron and macrophage phenotype**

Macrophages play an important role in iron homeostasis by phagocytosing erythrocytes degrading them and rendering iron available for erythropoiesis. Iron has immuno-
regulatory properties, and any alterations in cellular iron levels may affect the cellular immune response and inflammatory signalling [68, 69]. In addition functional polarization in macrophages has been associated with differential regulation of iron metabolism [22]. Iron handling by macrophages may determine their functional polarization [22, 24]. Iron regulatory proteins; ferritin and ferroportin have been shown to be differentially expressed in macrophage subtypes. M2 macrophages are distinctively characterized by an iron release with high expression of ferroportin (only known non-heam iron exporter) and low ferritin expression [22, 24, 70]. This iron export mode in M2 macrophages promotes matrix remodelling and cell proliferation. In contrast, M1 macrophages hold intracellular iron and are distinctively characterized by high ferritin expression and low ferroportin [22, 24, 70]. M1 macrophages release pro-inflammatory cytokines that promote atherogenesis [71]. Both M1 and M2 macrophages are demonstrated in atherosclerotic plaques. Thus, differential iron status in intra-plaque macrophages seems to be important to sustain functional polarization. Elevation in arterial iron as a consequence of increased erytrophagocytosis by macrophages or by utilizing an imaging modality involving iron nanoparticles may eventually determine functional plasticity of macrophages and stability of atherosclerotic plaques.

**Imaging atherosclerotic plaques**

Identification and characterization of vulnerable atherosclerotic plaques in asymptomatic individuals remains a major focus of research pertaining to diagnostic imaging. Currently, imaging techniques used for atherosclerotic plaque imaging are broadly classified as invasive imaging techniques and non-invasive imaging techniques respectively [72]. Presently employed invasive imaging techniques include intravascular ultrasound (IVUS), optical coherence tomography (OCT) and near-infrared (NIR) spectroscopy [72]. However, their usage is limited, barely 1% in daily clinical practise pertaining to their invasive nature and growing evidence that plaque activity rather than plaque morphology determines plaque stability [72, 73]. Evolution of non-invasive imaging for evaluation of atherosclerotic plaques may enable improved detection of vulnerable plaques and allow early therapeutic intervention. At present, the available non-invasive imaging techniques to
visualize vessel wall and atherosclerosis are; ultrasound, computed tomography, MRI and nuclear medicine.

**Magnetic resonance imaging**

MRI is considered to be the safest and promising imaging technology which provides a high degree of special resolution and excellent anatomic information [2]. Among others, MRI emerged as a leading non-invasive imaging modality that provides direct assessment of plaque burden and has been shown to provide a platform for interrogating biological characteristics of atherosclerotic plaques [74]. Additionally, with the development of target specific MRI contrast agents a new modality of molecular MRI has emerged with abilities to track cells and molecular pathways of a disease [2, 75-77]. These developments within the field of MRI have been achieved with the usage and unceasing development of imaging contrast agents.

Protons are abundant in human body, and they possess magnetic properties. When exposed to strong magnetic fields, protons in the exposed tissue orient themselves within the magnetic field. The orientation and energy of the proton nuclei changes, when a pulse of radio wave of certain frequency is applied for a specific duration. Eventually, as the nuclei relax, it emits MRI signal. Longitudinal (T1) or transverse (T2) relaxation times of proton and the magnetic susceptibility are the main contrast determinants of MRI. These relaxation times are different in one tissue to another and influence signal intensity. Signal intensity of a tissue is directly proportional to its proton density. A shorter T1 corresponds to a high signal intensity, whereas a shorter T2 corresponds to low signal intensity of a tissue [78, 79].

MRI provides high-resolution imaging of atherosclerotic plaques. However, low sensitivity is the inherent limitation with this technique. Thus, MRI often requires use of contrast agents which enhance the image contrast of the target tissues by increasing or decreasing the signal intensity. MRI contrast agents modify the amplitude of the signals generated by protons in the presence of a magnetic field. MRI contrast agents are broadly classified as T1-shortening (positive) or T2- shortening (negative) contrast agents [79]. Additionally,
the dose of such contrast agents may also determine their contrast effects. Gadolinium (Gd) or manganese (Mn) containing paramagnetic chelates are typically positive contrast agents [80, 81]. At a lower dose, their predominant effect is T1 shortening and thus the uptake of such agents by the tissue makes them hyperintense and bright. However, these agents also produce T2 shortening effects when their concentration rises in the tissue. On the other hand, iron-oxide nanoparticles are typical negative contrast agents [80, 81]. These super-paramagnetic particles generate local magnetic-field gradient disturbing the primary magnetic field on the tissue causing T2 relaxation and darkening of the tissue.

**Biomarkers and atherosclerotic plaques**

Plaque vulnerability is dependent on plaque composition and most vulnerable plaques exhibit high macrophage density, necrosis and lipid rich cores [18, 82-85]. These biological characteristics of atherosclerotic plaques are considered the clinically relevant key feature beyond size and degree of stenosis and may serve as imaging targets. Macrophages and foam cells are excellent imaging targets as they play an important role in plaque formation and its progression. Additionally, selectins, integrins, vascular cell adhesion molecule-1 (VCAM-1) are considered important imaging targets as they modulate the leukocyte recruitment process and thereby favour atherogenesis [73]. Cytokine secretion and release of proteases by the macrophages, including metalloproteinase and cathepsins, destabilize the plaque by damaging extracellular matrix and thinning of the fibrous cap [86, 87]. Thus, these inflammatory mediators are also attractive imaging biomarkers.

Phagocytic function of the macrophages has been exploited for imaging intra-plaque macrophages by using a variety of intracellular imaging contrast agents, including iron-oxide nanoparticles (INPs) and Mn-based chelates. Although, targeting of macrophages in human atherosclerotic plaques by INPs has shown promise [88-91], functionalization of these INPs by chemical attachment of an affinity ligand, such as a peptide or an antibody has emerged as a molecular imaging platform. Most of these functionalized nanoparticles have been mainly validated in animal models for cellular and molecular imaging of intra-plaque macrophages based upon their scavenger function and receptors [2, 92, 93].
The current focus in the field of cardiovascular imaging is to develop agents with both a diagnostic and therapeutic potential. It has been soon realized that even currently existing contrast agents might have this potential. Mangafodipir is such an example. It is a Mn-based imaging contrast agent that exhibits pharmacological effects, including anti-oxidant and cyto-protective activities [94-98]. As these intracellular contrast agents are phagocytosed and remain within the cells for longer durations, they are bound to modulate cell functions. Another group of contrast agents that have been shown to modulate cell functions are iron-oxide nanoparticles. These INPs affect vital cell functions, including cellular immune response [99, 100].

**Mangafodipir**

Mangafodipir was clinically used as an intravenous paramagnetic contrast media for liver MRI (tumour and metastasis) and was marketed as Teslascan™. It was supplied as a sterile aqueous solution. The recommended adult dose of mangafodipir was 5 µmol/kg body weight [80]. Mangafodipir trisodium is the active constituent of Teslascan™, but it also contains ascorbic acid, sodium chloride, sodium hydroxide and water for injection. Mangafodipir trisodium [manganese (II)-N,N-dipyridoxylethlenediamine-N,N'-diacetate-5,5'-bis(phosphate) sodium salt] consists of organic ligand fodipir (Dp-dp) and Mn (Manganese) [101]. After intravenous administration, mangafodipir is dephosphorylated to MnPLED followed by transmetallation by zinc [98, 101]. The released manganese is rapidly cleared from the blood and is taken up by the hepatocytes and thereby increases the signal intensity of the normal liver tissue. The excretion of manganese is through the biliary route whereas the fodipir metabolites are renally excreted.

Besides its clinical application for imaging of liver conditions, mangafodipir has also been shown to exert pharmacological effects. Mangafodipir has been used as a viability marker in patients with myocardial infarction [102] and showed vascular relaxation effect. Mangafodipir also conferred myocardial protection against oxidative stress [96, 103]. These effects may be attributed to the SOD-mimetic activity of mangafodipir and its metabolite MnPLED and also high iron binding capability of Dp-dp and dipyridoxyl ethyldiamine (PLED) [98].
Super-paramagnetic iron-oxide nanoparticles (SPION)

SPION possesses magnetic properties and has been extensively used in many bio-applications, including magnetic resonance imaging, cell separation and magnetic drug and gene delivery [100]. SPION has been widely used as a contrast agent to target macrophages. Low toxicity profile and ease of functionalization have made them a perfect selection for targeted imaging [100]. SPION composes of a crystalline iron core coated with carboxydextran [104, 105]. Based on their size, SPION are broadly categorized as standard (60 - 150 nm), large (>200 nm), ultrasmall (USPIO; 10 – 40 nm), monocrystalline (MION; 10 – 30 nm) and very small (VSOP; 4 – 8 nm) [106, 107]. SPION used in this study is Resovist™, with a hydrodynamic diameter of 60 – 62 nm [106]. It was clinically used for diagnostic imaging of cirrhosis and liver cancer. Resovist™ is composed of a Fe₃O₄ (magnetite) and Fe₂O₃ (maghemite) core, and coated by carboxy-dextran [104]. Both Fe₃O₄ and Fe₂O₃ were found to be cytotoxic to a variety of cells [108-112]. These findings indicate the presence of potentially cytotoxic iron in SPION. The carboxy-dextran coating not only increases the uptake of SPION by macrophages but also prevents aggregation of the iron core [106, 113, 114] and may be important to prevent iron induced cyto-toxicity by increasing biocompatibility.

SPION are intracellular contrast agents with enhanced duration of cellular residence following phagocytosis. Clathrin receptors contribute to internalization of a variety of nanoparticles, and its potential role in SPION uptake has been suggested [115]. After internalization SPION accumulates in lysosomes, where they are gradually degraded [113, 115-117], marked by co-localization with dextranases and resulting in iron accumulation [118].

Increased cellular iron levels in macrophages following SPION degradation may pose critical consequences in iron rich atherosclerotic plaques. Iron excess accounts for observed cellular ferritin up-regulation [119], altered immune response in macrophages [120] and iron-oxide induced delayed cytotoxicity [104, 118, 121]. However, these iron-oxide nanoparticles have been shown to be phagocytosed by intra-plaque macrophages in both animal models and in humans [122-124]. Thus these nanoparticles are unceasingly
developed and their potential to target intra-plaque macrophages is extensively examined in pre-clinical trials in atherosclerotic settings. Recent studies mainly focus on cytotoxicity induced by these nanoparticles, which may leave modulation of important cellular processes by these nanoparticles go unnoticed.
AIMS

Imaging contrast agents are unceasingly developed to target macrophages in atherosclerotic plaques. However, little is known about their impact on normal cellular and physiological or patho-physiological functions. The objective of our study was to evaluate effects of two such magnetic resonance contrast agents; manganese based mangafodipir (Teslascan™) and iron based super-paramagnetic iron oxide nanoparticles (SPION, Resovist™) on cell functions and their interaction with oxysterol laden cells. The specific aims were:

- To determine whether mangafodipir could affect 7βOH induced cell death and identify the underlying mechanisms that might be involved in the process.
- To identify the active moiety in mangafodipir exerting pharmacological effects.
- To examine the interaction of SPION with monocytes and macrophages exposed or not to atheroma relevant oxysterols, focusing on cell function and cellular iron handling.
- To examine the interaction of SPION with functionally polarized macrophages and compare the cellular effects of coated and un-coated iron-oxide nanoparticles.
MATERIALS AND METHODS

Materials

Cysteine protease inhibitor

Trans-epoxysuccinyl-L-leucylamido-(4-guanidino) butane (E64d) is an irreversible cysteine protease inhibitor, including cathepsin B and cathepsin L and also inhibits calpains. Upon pinocytosis, E64d localizes in lysosomal compartment. (N-(trans-carbamoyloxyrane-2-carbonyl)-L-phenylalanine-dimethylamide) (CLIK-148) is a specific cathepsin L inhibitor.

Iron chelators

In paper III and IV, Desferrioxamine (DFO) was used. It is a potent iron chelator, which when endocytosed accumulates in acidic vacuolar compartment. It binds to low molecular weight iron, thereby preventing iron mediated redox-reactions. In paper III, Ammonium Chloride (NH₄Cl) was used, which is a lysosomotropic alkalizing agent, and has been shown to exhibit intra lysosomal iron chelation [125].

Oxysterols

Oxysterols are oxidation products of cholesterol. Oxysterols mimic negatively charged LDL (LDL-), which is an oxidatively modified form of LDL and has been shown to induce endothelial dysfunction and cholesterol accumulation in vascular wall cells. 7keto and 7βOH are the major cytotoxic components of OxLDL, and it has been shown that macrophages exposed to 7keto exhibit cholesterol accumulation and eventually lead to foam cell formation. In this project, 7βOH was used in papers I, II, III and IV, whereas 7Keto was used in paper III and IV.
Methods

Cell culture

**Human monocyte derived macrophages**

Human blood monocytes were isolated from buffy coats density gradient centrifugation using Optiprep™. To induce differentiation, the monocytes were plated on 16 mg/mL hIgG coated air-dried glass coverslips in petri dishes. The cells were maintained in RPMI supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 u/mL penicillin G and 100 µg/mL streptomycin. The cells were cultured at 95% air and 5% CO2 in a humidified atmosphere at 37°C. Medium was replaced every alternate day, and the macrophages were used on day 7 for experiments.

**U937 and THP1 monocytes**

U937 and THP1 cells were maintained in RPMI with 10% fetal bovine serum, 2 mM L-glutamine, 100 µg/ml penicillin G and 100 µg/ml streptomycin. The cells were cultured at 95% air and 5% CO2 in a humidified atmosphere at 37°C. Subdivided cells at approximately 3×10^5 cells/ml were used in the experiments [126].

**THP1 macrophages**

THP1 monocytes were treated with 300 nM PMA for 24 h, to generate THP1 macrophages [63]. After 24 h, PMA was withdrawn and the cells were washed with RPMI culture media and kept in the same media for another 24 h before experiment.

**Macrophage subtypes**

PMA-treated THP1 monocytes were exposed to IFN-γ and LPS or IL-4 and IL-13 to generate M1 or M2 macrophages by following a standardized protocol [30]. Briefly, THP1 cells were treated with 320 nM PMA for 6 hours and then either exposed to 100 ng/ml lipopolysaccharide (LPS) and 20 ng/ml interferon-gamma (IFN-γ) for 18 hours to generate M1 macrophages or exposed to 20 ng/ml interleukin-4 (IL-4) and 20 ng/ml interleukin-13 (IL-13) for 18 hours to generate M2 macrophages.
Figure 1: Generation of M1 and M2 macrophages. PMA induced THP1 macrophages (PMA THP1 M2); THP1 derived M1 macrophages (THP1 M1 MØ) and THP1 derived M2 macrophages (THP1 M2)

**Cell viability**

**Plasma membrane integrity:** Plasma membrane integrity can be assessed by adding a cell permeable dye, which can only stain cells with damaged or porous plasma membrane [37]. In paper I, III and IV cell morphology and plasma membrane integrity was assessed by trypan blue dye exclusion test and observed by light microscopy.

**Apoptosis:** In paper I, apoptosis was assessed after Wright-Giemsa staining or following Annexin V/propidium iodide staining [127]. For Wright-Giemsa staining, cells in suspension were cyto-centrifuged, washed in PBS and spun down onto glass slides using cyto-spin. The cells were then fixed in 4% formaldehyde for 10 minutes followed by Wright-Geimsa for 2 minutes at room temperature. Cell morphology was examined by light microscopy. Apoptotic cells were characterized as shrunken cells with fragmented nuclei.

In paper I, II, III and IV apoptosis was assayed by detection of phosphatidylserine exposure using flow-cytometry following Annexin V/propidium iodide staining. Cells scored as early apoptotic when they were positive to Annexin V, while when cells were positive to both Annexin V and PI, they were scored as post-apoptotic or necrotic cell death. In brief, control and treated cells were collected, washed once with phosphate-buffered saline (PBS), and stained for 10 min on ice with Annexin V and propidium iodide and analyzed by
**Reactive oxygen species**

Intracellular reactive oxygen species was assayed by flow-cytometry following dihydroethidium (DHE) staining. DHE enters the cells and interacts with superoxides to form oxyethedium which interacts with nucleic acids and emits bright-red colour and is detected by a fluorescence microscope [128]. The cultured cells were collected, washed once with PBS, incubated for 15 min at 37°C with 10 µM DHE and analysed with flow-cytometry. Percentages of cells with increased DHE red fluorescence were gated and considered as increased reactive oxygen species.

**Lysosomal membrane integrity**

The integrity of the lysosomal membrane was assessed using the acridine orange (AO) uptake technique as established previously [127]. AO is a lysosomotropic weak base which accumulates in the acidic vacuolar compartment or lysosomes, and is protonated and trapped in the vacuole, thereby imparting red granular fluorescence upon excitation with blue light. An increase in lysosomal membrane permeability results in leakage of AO from the lysosomes which shift emission fluorescence to green. In brief, the cells following different treatments were collected, stained with 5 µg/ml AO (15 min, 37 °C), and analysed with fluorescence microscopy or flow-cytometry. Percentages of cells with decreased lysosomal AO red fluorescence were gated and considered as increased LMP.

**Mitochondrial membrane potential**

The mitochondrial membrane potential (Δψm) was measured using the fluorescent probe JC-1 [129]. JC-1 enters mitochondria and reversibly changes colour from red to green with decrease in membrane potential. In healthy cells with high membrane potential, JC-1 forms complexes j-aggregates and emits red fluorescence [130]. While in apoptotic cells with low mitochondrial potential, JC-1 remains in monomeric form and emits green fluorescence. In brief, following different treatments control and treated cells were collected, incubated with JC-1 (5 µg/ml for 10 min at 37 °C) and analysed with flow-cytometry. The ratio of JC-1 red and green fluorescence intensities was used to calculate mitochondrial membrane
Phagocytic activity was measured by assay of dextran up-take (FITC conjugated dextran, FD40, molecular weight 40 000) and yeast up-take assay (FITC conjugated yeast).

Dextran uptake assay: Following treatment cells were exposed to FD40 (1 mg/mL) at 37°C or 4°C for 1 h. After washing with phosphate-buffered saline (PBS), the up-take of FD40 was determined by flow-cytometry [131, 132].

Yeast uptake assay: For FITC-yeast assay, cells were exposed to FITC conjugated yeast (1.4 x 10^6/ml) for two hours and then washed with PBS. The uptake of FITC-yeast was determined by fluorescence microscopy [132].

Cellular iron

Intracellular iron oxide was localized by Perls’ Prussian blue staining. In brief, the cells were incubated for 30 min with potassium hexacyanoferrat in 10% HCl, washed and counter-stained with nuclear fast red. Ferric ions (Fe^3+) in the cells react with potassium ferrocyanide to form a blue pigment (Prussian blue).

Immuno-cytochemistry

Following treatment the cells were collected, immuno-stained for surface or intracellular proteins and analysed by flow-cytometry or fluorescence microscopy [127].

Intracellular protein: In brief, the cells were fixed with 4% paraformaldehyde, permeabilized with permeabilizing buffer (0.1% saponin and 0.5% serum in PBS), and then incubated with primary anti-bodies to protein of interest at 4°C overnight. They were then incubated with Alex 488 conjugated secondary antibodies at 22°C for 1h.

Surface protein: For determining surface proteins, cells were washed with PBS, and then incubated with primary antibody for 30 min at 4°C followed by incubation with Alex 488 conjugated secondary antibodies at 4°C for 30 min. Then the cells were fixed with 4% paraformaldehyde. Fluorescence intensity of immuno-stained cells was determined by
flow-cytometry or fluorescence microscopy.

**Secreted, nuclear and cytosolic protein**

**ELISA:** Secreted protein in culture media was measured by an ELISA kit according to the company's protocol. The protein concentrations in the culture media were assayed by bicinchoninic acid protein assay (BCA) according to manufacture's instruction (R & D Systems, MN, USA), and normal media was measured as background.

**Western blot:** Cytosolic and nuclear proteins were extracted according to manufacture's instruction by using an extraction kit (Cayman Chemical, Michigan, USA). The extracted cytosol and nuclear fractions were saved at -80°C until usage. Secreted proteins were precipitated using nine volumes of absolute ethanol at -70°C for 2 h. Precipitated proteins were collected by centrifugation (12,300 relative centrifugal force) and were resuspended in the sample buffer with dithiothreitol and bromophenol blue and boiled for 4 minutes at 95°C. The protein concentrations from extracts and in culture media were assayed by BCA protein assay and normal media was measured as background.

Secreted proteins in culture media, whole cells, cytosolic and nuclear extracts from different groups of cells were loaded onto a 12% sodium dodecyl sulphate-polyacrylamide gel and transferred onto a nitrocellulose membrane. Membranes were then incubated with antibodies against protein of interest at 4°C; overnight after being blocked with 5% nonfat milk in tris-buffered saline with 0.1% Tween® 20. Finally, membranes were incubated with horseradish peroxidase-conjugated secondary antibodies at 20°C for 1h and assayed by an enhanced chemiluminescence plus western detection kit. Ponceau S staining or b-actin served as loading control.

**Statistics**

For statistical analysis, unpaired student's t test for two groups or one-way ANOVA followed by posthoc Newman-Kuels test for multiple groups were performed. Results are given, as means ± SEM. To study correlation, Pearson correlation coefficient test or Spearman correlation coefficient test and linear regression test was used. P ≤ 0.05 was considered statistically significant.
RESULTS

Paper I and II

Mangafodipir, MnPLED and Dp-dp prevents 7βOH-induced cell death

In these studies, we investigated whether mangafodipir its metabolite MnPLED and its constituent Dp-dp with anti-oxidant effects could affect 7βOH induced oxidative stress and subsequent cell death. We found that mangafodipir was non-toxic to cells even at concentrations as high as 800 µM. Furthermore, mangafodipir at concentrations between 100 and 400 µM inhibited 7βOH-induced cell death in a dose-dependent manner, while it had no protective effect when the concentrations were less than 100 µM as assayed by trypan blue staining followed by analysis with light microscopy or by annexine V propidium iodide staining followed by analysis with flow-cytometry. Identical to the parent compound, mangafodipir metabolite MnPLED (100 µM) and its structural constituent Dp-dp (100 µM) also provide similar cyto-protection against 7βOH induced cell death.

Involvement of lysosomal pathways

Oxysterol induced apoptosis involves permeabilization of lysosomal membranes and redistribution of their contents to cytosol [127, 133]. To determine whether regulation of LMP was involved in mangafodipir mediated cyto-protection against 7βOH induced cell death, we examined the effect of mangafodipir on LMP by using the AO uptake method. 7βOH induced destabilization of the lysosomal membrane was characterized by a decreased red lysosomal AO red fluorescence with an increased cytosolic green fluorescence as analysed by fluorescence microscopy or flow-cytometry. Pre-treatment with mangafodipir and its metabolite MnPLED and its structural constituent Dp-dp significantly prevented lysosomal membrane permeability induced by 7βOH. No differences at the level of protection against 7βOH induced LMP was observed between mangafodipir, MnPLED and Dp-dp. These results indicate that mangafodipir mediated cyto-protection against 7βOH involves prevention of lysosomal membrane damage.
**Involvement of mitochondrial pathways**

Mitochondria are both the source and the target for ROS. It is known that ROS regulates the release of cytochrome C from mitochondria and thus regulates cell death [134, 135]. Moreover, increased intracellular ROS and mitochondrial membrane permeability (MMP) are involved in 7-oxysterol mediated cell death [136]. To determine whether MMP and ROS were also involved in mangafodipir mediated protection against 7βOH induced cell death, the effect of mangafodipir on 7βOH induced MMP and ROS were evaluated by using JC-1 and DHE staining respectively. As analysed by flow-cytometry, in 7βOH exposure for 18h resulted in decreases in JC-1 induced orange/red fluorescence and increases in JC-1 green fluorescence, which indicates an increase in MMP in cells exposed to 7βOH. Additionally, 7βOH exposure for 18h resulted in significant increases in DHE induced red fluorescence indicating increases in cellular ROS. However, pre-treatment with mangafodipir preserved mitochondrial membrane potential. In addition, mangafodipir, Mn-PLED and Dp-dp prevented 7βOH induced ROS production. No differences at the level of protection against 7βOH induced ROS was observed between mangafodipir, MnPLED and Dp-dp. These results indicate that mangafodipir mediated cyto-protection against 7βOH involves modulation of mitochondrial pathways, including suppression of ROS production and preserving mitochondrial membrane potential.

**Paper III and IV**

**Monocyte differentiation induces functional plasticity in macrophages and simultaneous increases in ferritin and cathepsin L**

We found that induction of differentiation of human monocytes induces functional polarization in macrophages. Although, it is known that PMA induced differentiation in THP1 monocytes induces a M2 macrophage subtype secreting anti-inflammatory cytokines, we further validated this result in our study. We found that PMA stimulation induced M2 macrophages (THP1 PMA M2) characterized by an up-regulation of endogenous CD163 levels. Human monocyte derived macrophages (HMDMs) were also examined for their functional polarization. We found that hIgG induced HMDMs exhibit CD68 and CD86 immuno-positivity and these macrophages did not express CD163 as confirmed by
fluorescence microscopy. In addition, we found up-regulated levels of endogenous ferritin and cathepsin L in THP1 PMA M2 macrophages upon induction of differentiation. Further, polarizing THP1 macrophages into distinct M1 and M2 subtypes raised intracellular ferritin and cathepsin L levels. As expected, we did not observe significant differences in CD163 levels between PMA induced THP1 macrophages (THP1 PMA M2) and macrophages further treated with IL-4 and IL-10 (THP1 M2), which indicate that the M2 macrophages cannot be further influenced by combined cytokine treatment. However, significant differences were observed in the levels of cellular ferritin and cathepsin L between THP1 M1 and THP1 M2 macrophages which indicates that cellular levels of these proteins may be representative of important functional orientation in the macrophages.

**SPION up-regulates ferroportin, ferritin and cathepsin L levels, incites cytokine and ferritin secretion and modulates cell phenotype**

SPION are unceasingly developed to target various biological characteristics of atherosclerosis, including foam cells and macrophages, which exhibit functional plasticity in atherosclerotic microenvironment [2, 23, 124]. However, their interactions with such targets are completely unknown. In these studies, we investigated their interactions with such targets in-vitro. We found that SPION loading affects cell functions without affecting cell viability. In addition, we found that oxysterols reduce the phagocytic activity in macrophages, thereby reducing the uptake of SPION.

**Iron export**

SPION are primarily ingested by monocytes and macrophages. They accumulate in the lysosomes and are gradually degraded resulting in iron accumulation [118]. Cellular viability was un-altered following SPION exposure for 6 - 48 h as assayed by trypan blue staining or by flow-cytometry following Annexin V/PI staining. SPION loading had no effect on cellular ROS as analysed by flow-cytometry following DHE staining. However, SPION loading significantly enhanced intracellular iron concentrations as confirmed by Perls’ Prussian blue staining for iron. This increase in cellular iron levels could be potentially detrimental. Perhaps to reduce iron load, cellular iron export pathways were activated in the cells upon SPION loading. We found that iron loading induced membranous ferroportin
expression in the cells as analysed by florescence microscopy. Ferric ammonium citrate (FeAC) induced ferroportin was used as a positive control. Ferroportin expression was not the only measure taken by the cells to reduce cellular iron levels. A dose and time dependent increase in ferritin secretion was also observed in the culture supernatants following SPION loading. Ferritin is an ubiquitous and highly conserved iron-binding protein, and its secretion by the cells would result in release of iron load. Interestingly, ferritin secretion was considerably up-regulated in untreated controls of Thp1 monocytes at 72 h. Similar ferritin secretion was also observed in THP1 monocytes following LPS exposure.

**Phenotypic shift**

SPION loading increases cellular iron concentrations. Potentially iron regulates macrophage plasticity and has immuno-modulatory properties. We found a significant increase in TNF-α secretion following SPION loading, as measured by ELISA. A dose-dependent IL-10 secretion was also observed following SPION loading, which declined over 72 h. In these experiments cells treated with LPS were positive controls. LPS stimulation significantly augmented TNF-α secretion. Additionally, we found that iron in SPION induces a phenotypic shift in THP1 M2 macrophages marked by elevated CD86 levels in these macrophages which is a characteristic feature of M1 macrophages. This phenotypic shift can be detrimental in various immunological disorders and may aggravate inflammation.

**Ferritin and cathepsin L up-regulation in macrophage subtypes**

M1 macrophages hold intracellular iron, whereas M2 macrophages are distinctively characterized by an iron release. To examine the effect of iron in SPION on cellular iron storage, ferritin levels were examined by flow-cytometry. Since SPION is degraded in the lysosomes and thereby increases lysosomal iron levels, we evaluated the potential of iron in SPION to modulate lysosomal functioning by focusing on lysosomal enzymes. We found that iron in SPION simultaneously up-regulates ferritin and cathepsin L levels in monocytes and in both M1 and M2 macrophages. Furthermore, the subcellular location of SPION induced ferritin was both nuclear and cytoplasmic as confirmed by western blot of the nuclear and cytosolic fractions of the monocytes after indicated treatments. FeAC was used
as a positive control in the model. DFO significantly diminished SPION as well as FeAC induced ferritin and cathepsin L in the cells which confirms the role of iron in the process. These results indicate that SPION elevates ferritin and cathepsin L levels in monocytes and macrophages irrespective of their functional polarization.

**Cathepsin in ferritin degradation**

Lysosomes not only contain abundant hydrolytic enzymes but are also rich in iron. Lysosomal enzymes are pivotal to ferritin proteolysis to maintain ferritin levels and balance redox-active iron. We examined the possibility that the elevated ferritin level upon SPION loading was the driving force for up-regulated cathepsin L. Cells were pre-treated with or without E64 (cathepsin B/L inhibitor) or CLIK 148 specific cathepsin L inhibitor followed by incubation with SPION. Both E64 and CLIK not only elevated SPION and FeAC induced ferritin, but also elevated basal levels of ferritin in monocytes. These results indicate a functional role of lysosomal cathepsins in ferritin degradation.

**Ferritin, cathepsin L and macrophage plasticity**

Iron regulates macrophage plasticity and thus levels of iron-related proteins among macrophage subtypes might be important to sustain a polarized state. We found that induction of differentiation in monocytes imparts functional polarization in resulting macrophages and is associated with up-regulation of endogenous ferritin and cathepsin L levels. Up-regulation in the endogenous levels of ferritin and cathepsin L were observed in hlgG induced CD68+/CD86+/CD163- HMDMs and PMA induced THP1 macrophages. Furthermore, polarizing THP1 macrophages into distinct M1 and M2 subtypes also raised ferritin and cathepsin L levels in these macrophages. However, significant differences were observed in the levels of cellular ferritin and cathepsin L between THP1 M1 and THP1 M2 macrophages, which indicates that cellular levels of these proteins may be representative of macrophage phenotype.
**Oxysterols reduce phagocytic activity in macrophages thereby reduce uptake of SPION**

Foam cells and lipid rich macrophages are biomarkers of atherosclerotic plaques. Such cells are important imaging targets. SPION are unceasingly developed to target such macrophage foam cells in atherosclerotic plaques. We found that oxysterol exposure reduces phagocytic activity and thereby reduces SPION uptake by both THP1 monocytes and THP1 macrophages and HMDMs. SPION uptake was confirmed by Perls' Prussian blue staining. In contrast to control cells, most cells exposed to SPION were Perls' positive. However, pre-treating cells with atheroma relevant 7βOH or 7keto and then exposure to SPION resulted in decreased numbers of Perls' -positive cells, indicating reduced intracellular iron and reduced SPION uptake. This reduced uptake of SPION by cells exposed to oxysterols resulted from a decline in phagocytic activity. Phagocytic activity was measured by assaying uptake of FITC conjugated dextran (FD40) or FITC conjugated yeast. Oxysterol exposure significantly declined FD40 uptake by monocytes and macrophages. Moreover, in comparison to unexposed cells, a significantly low uptake of FITC-yeast was observed in macrophages exposed to oxysterols. These results indicate a decline in phagocytic activity in monocytes and in macrophages, which eventually lead to reduced uptake of SPION by such macrophages. We also found an additive effect of SPION on 7βOH induced cathepsin and ferritin. Although SPION uptake was reduced, the ingested amounts significantly up-regulated the expression of cathepsin B, cathepsin L and ferritin in both monocytes and macrophages, which may further aggravate atherogenesis.

**Surface coating promotes uptake of iron-oxide nanoparticles and potentially prevents iron induced cytotoxicity**

Uptake INPs by macrophages was observed, irrespective to the presence or absence of a surface coating, which resulted in increases of intracellular iron. However, the amounts of INPs taken up by macrophages were dependent on the presence of a surface coating. Carboxy-dextran coating in SPION enhances its solubility and uptake by macrophages [100]. In the lysosomes, this outer dextran coating is degraded and the contents of SPION
are released, which are Fe$_2$O$_3$ and Fe$_3$O$_4$. Despite such dramatic increases in cellular iron levels upon SPION loading, cell viability was unaltered. We compared the effects of SPION with uncoated INPs (Fe$_2$O$_3$ and Fe$_3$O$_4$) on cell viability and functions and observed many differences. We found that, intra-cellular iron levels in macrophages exposed to uncoated INPs were considerably lower compared to macrophages exposed to SPION. SPION are non-toxic to cells even at concentrations as high as 400 µg Fe/ml. In contrast, exposure to uncoated INPs induced dose dependent cell death in macrophages. Additionally, uncoated INPs affected phagocytic activity in macrophages. Cells exposed to sub-lethal concentrations of Fe$_2$O$_3$ and Fe$_3$O$_4$ showed a reduced phagocytic activity compared to cells exposed or unexposed to SPION as analysed by FITC-yeast assay. This reduction in phagocytic activity may be early indications of subsequent loss of cell viability.
GENERAL DISCUSSION

Mangafodipir mediated cyto-protection involves modulation of lysosomal and mitochondrial pathways

Our study substantiates the existing knowledge about underlying mechanisms behind cyto-protection conferred by mangafodipir. The anti-oxidant activity of mangafodipir is well documented [96, 98, 103] which ascertains its role in decreasing cellular ROS levels and thereby protecting cells against oxidative stress. We found that mangafodipir not only prevents 7βOH induced ROS production but also preserves the mitochondrial membrane potential and thereby preventing cell death. By preserving mitochondrial membrane potential, mangafodipir may stop the potential release of cytochrome C and subsequent cell death. These results elucidate that the preservation of mitochondrial membrane potential is an additional effect of mangafodipir on the mitochondrial compartment apart from preventing ROS generation. The role of LMP and related cathepsins as apoptotic initiator is now regarded as important apoptotic pathways [137, 138]. Lysosomal pathways are also involved in cyto-protection conferred by mangafodipir. Our notion is based upon the results where mangafodipir prevented LMP induced by oxysterols, which may prevent the potential release of lysosomal cathepsins and subsequent activation of cell death pathways.

A conserved structure in mangafodipir may attribute to its cyto-protective effects

We compared the parent compound, mangafodipir with its primary metabolite MnPLED and its constituent Dp-dp. Mangafodipir, MnPLED and Dp-dp provide similar cyto-protection against 7βOH induced cell death. These results suggest that the active part of mangafodipir, which imparts the above-noted effects, is conserved among Dp-dp and MnPLED. As Mn is rapidly released and cleared from the blood on intravenous administration. The most likely conserved domain shall be Dp-dp and its dephosphorylated derivative PLED, which have been shown to possess high iron binding capacity [98]. These intriguing results lead us to question whether modulation of cellular iron is involved in cyto-protection mediated by mangafodipir and its metabolites. We tested this hypothesis
by measuring the levels of ferritin in cells exposed to MnPLED and Dp-dp. We did not observe any alterations in ferritin levels upon treatment with MnPLED and Dp-dp, which does not exclude the possible involvement of other iron regulatory proteins or modulation of labile iron pool in MnPLED and Dp-dp mediated cyto-protection.

**Monocyte differentiation induces macrophage phenotype with elevated levels of ferritin and cathepsin L**

Induction of differentiation in monocytes induces functional polarization in acquired macrophages. This notion is supported by our results where HMDMs induced by hlgG were characterized as a distinct CD68+, CD86+ and CD163- macrophage subtype and PMA induced THP1 macrophages were characterized as M2 macrophages which exhibit higher levels of CD163. Additionally, cellular levels of ferritin and cathepsin L might be important in processes like macrophage differentiation and their functional polarization. We found that induction of differentiation in THP1 monocytes by PMA and in human blood monocytes by hlgG up-regulated endogenous levels of ferritin and cathepsin L in resulting macrophages. We further confirmed our observations by polarizing THP1 macrophages into distinct M1 and M2 subtypes following an established protocol [30]. These treatments raised endogenous ferritin and cathepsin L levels in both THP1 M1 and THP1 M2 macrophages. However, significant differences were observed in the levels of cellular ferritin and cathepsin L between THP1 M1 and THP1 M2 macrophages.

**Iron in SPION primes THP1 M2 macrophages towards a high CD86+ macrophage subtype with elevated levels of ferritin and cathepsin L**

Iron in SPION governed the cellular levels ferritin and cathepsin L, as indicated by the actions of DFO which significantly down-regulated SPION induced ferritin and cathepsin L in our model. It is suggested that M1 macrophages hold intracellular iron whereas M2 macrophages release iron [24, 139]. However, we found similar handling of iron in SPION by THP1 monocytes, THP1 M1 macrophages, THP1 M2 macrophages, THP1 PMA M2 macrophages and HMDMs. Iron in SPION simultaneously up-regulated ferritin and
cathepsin L in all cell types tested in this study. However, the levels of SPION induced ferritin and cathepsin L was significantly higher in THP1 M1 macrophages in comparison to THP1 PMA M2 macrophage and THP1 monocytes. Higher endogenous levels of ferritin [24, 139] and cathepsin L in M1 macrophages compared to M2 macrophages may reason the highest levels of SPION induced ferritin and cathepsin L in THP1 M1 macrophages. In addition iron in SPION induced a phenotypic shift in THP1 M2 macrophages towards a macrophage subtype characterized by high levels of CD86, ferritin and cathepsin L, which we found as a characteristic hallmark of M1 macrophages. This phenotypic shift can be detrimental in atherosclerosis and in various immunological disorders and may aggravate inflammation.

**Lysosomal cathepsins have a functional role in ferritin degradation**

We examined the reasons behind simultaneous up-regulation of ferritin and cathepsin L in monocytes and macrophages exposed to SPION. Lysosomal cathepsin is pivotal to ferritin proteolysis to maintain ferritin levels and balance redox-active iron [140, 141]. Inhibition of lysosomal cathepsins resulted in an increase in endogenous and SPION induced ferritin, which is likely due to the prevention of ferritin degradation by lysosomal cathepsins. These results indicate that lysosomal cathepsins have a functional role in cellular ferritin metabolism and are pivotal to maintain ferritin levels following SPION degradation. However, we cannot exclude the possibility that cathepsin inhibition may also interfere with ferritin synthesis as demonstrated by Zhang et al [31].

**SPION loading incites secretion of both pro-inflammatory and anti-inflammatory cytokines**

In atherosclerosis, immunological microenvironment determines the plaque progression and its vulnerability [23]. Iron-oxide nanoparticles are mostly ingested by immune cells, which increase their intra-cellular iron concentrations. Iron overload or its deficiency may incite deleterious physiological effects [10, 142]. Iron overload has been also shown to impair production of nitric oxide (NO) by macrophages [143]. Interestingly, iron-oxide
nanoparticles has been shown to induce immune response characterized by secretion of both pro-inflammatory [144] and anti-inflammatory cytokines [145] by macrophages. Similarly, in our study, we find that SPION loading induces secretion of both (TNF-α) pro-inflammatory and (IL-10) anti-inflammatory cytokines. Additionally, at low concentration of SPION (5µg Fe/ml) this response was noticed, which indicates the potency of these iron-oxide nanoparticles. While a pro-inflammatory response may be the direct effect of iron excess, an anti-inflammatory cytokine IL-10 secretion may correspond to a cellular defence mechanism activated by cellular iron overload. Such immuno-modulatory effects of iron-oxide nanoparticles must be strictly considered in future development of these particles.

**Non-functionalized SPION may be inefficient to target intraplaque macrophages**

Macrophages are determinants of plaque progression and stability. Clearance of debris, lipid and apoptotic and necrotic cells is one of the vital functions of macrophages in atherosclerotic plaques [87, 146]. Attenuation of phagocytic functions in these macrophages may enhance the rate of plaque progression. INPs are increasingly developed to target such macrophages and foam cells in atherosclerotic plaques. Instead of conventional targeting of such macrophages by INPs, molecular targeting by functionalization of these INPs is the preferred approach today. Functionalization of INPs includes modifying surface coating material and tagging them with specific antibodies and peptides, which increases their target specificity [2, 100].

Reduced clearance indicates reduced phagocytic activity of macrophages in atherosclerotic plaques, which may reason the development of such an approach. We observed reduced uptake of INPs by macrophages exposed to atheroma relevant oxysterols, which can be directly attributed to reduction in their phagocytic activity. In this study, we did not examine whether oxysterols activated cell death pathways in macrophages. Thus we do not exclude the possibility that initiation of apoptosis in macrophages by atheroma relevant oxysterols may cause the reduction in phagocytic activity of these macrophages.
These results emphasize the necessity to develop functionalized INPs, which can increase intracellular iron levels in such cells without affecting their viability and functions. And such INPs should be able to distinctively identify macrophages and foam cells for effective determination of plaque severity and vulnerability.

Although, macrophages exposed to oxysterols display a poor uptake of INPs, the uptake does not entirely stop as indicated by up-regulation of oxysterol induced cathepsin L and B by SPION in our cell model. 7-oxysterols induced cell death involves activation of lysosomal pathways, including the release of lysosomal cathepsins. In chronic inflammatory disorders like atherosclerosis lysosomal cathepsins may modulate apoptosis and cellular immune functions. Our results for the first time show that iron in SPION up-regulates both endogenous cathepsin L, and oxysterol induced cathepsin L and cathepsin B in monocytes and macrophages. While up-regulation of oxysterol induced cathepsins by SPION is indicative of an additive effect, the up-regulated cathepsins may further aggravate atherogenesis.

**Functional coating of iron-oxide nanoparticles gives cell tolerance to potential cytotoxicity by regulation of cell functions**

Concentration of iron at atherosclerotic plaques exceeds that found in healthy arterial tissue [12], and it is potentially redox active [11]. Erythrophagocytosis stands as an important source of redox active iron in atherosclerotic plaques [11, 12]. In addition, reduction and removal of stored iron by systemic iron chelation or dietary iron restrictions has been shown to reduce lesion size and increased plaque stability in animal studies [7, 11]. These findings illustrate the importance of critical iron balance in atherosclerotic plaques. Increasing cellular iron concentration by iron-oxide nanoparticles in macrophages to image vulnerable and iron rich atherosclerotic plaques is certainly a major concern.

Iron catalyses the formation of ROS and OxLDL and potentiate OxLDL mediated macrophage cell death [147], which may result in plaque formation and increase severity of atherosclerosis [148, 149]. Although in our study, we found that carboxy-dextran coated
SPION is well tolerated by monocytes and macrophages, we also noticed the potential of uncoated iron oxide nanoparticles to induce cell death. These results clearly indicate that iron in iron oxide nanoparticles is potentially cytotoxic. However, coating their surface by dextran makes them more compatible in biological compartments. Indeed, the biological response generated by these nanoparticles has been attributed primarily to their size and surface coating [2, 100]. The cytotoxic activity of uncoated iron-oxide nanoparticles, including oxidative stress and DNA damage is well documented [2, 100, 104, 112]. In contrast, high compatibility of dextran coated nanoparticles is also documented [100]. These iron-oxide nanoparticles have a long cellular residence time. Although, we could not detect ROS production or cell death on exposure to SPION for as long as 72 h, there is evidence that uncoated iron-oxide nanoparticles and ultrasmall iron-oxide nanoparticles (USPION) induce ROS production and cell death [100, 150]. In addition, we and others have shown that iron-oxide loading up-regulates ferritin in the cells [100, 151, 152]. Iron in iron-oxide nanoparticles may be sustained in ferritin and hemosiderin and remain as a reservoir of potentially reactive iron, which may be detrimental in plaque microenvironment. However, releasing potentially reactive iron is not the only way by which iron-oxide nanoparticle induced ferritin could determine the fate of plaque. Apart from being an iron storage protein ferritin is immuno-modulatory [65, 153], associated with inflammation, and has been shown to regulate ROS production and cell death [64] which may increase the severity and progress of atherosclerosis. Additionally, iron released from iron-oxide nanoparticle loaded cells could trigger apoptotic and necrotic cell death. We found that SPION loading incites membranous ferroportin expression and ferritin secretion which might be a cellular response to reduce elevated intracellular iron levels. However, this action could be potentially unsafe to nearby phagocytes as it is suggested that endocytosis of extracellular ferritin increases the level of free ferrous iron in the lysosomal compartment inducing oxidative stress, LMP and trigger apoptosis and necrosis [142].

One major concern in the field of nano-toxicity is that SPION gradually increases cellular iron concentrations in cells, which may ascertain the adverse effects of iron excess. However, we observed differences in biological response incited by uncoated INPs and carboxy dextran coated SPION. While, uncoated INPs induced dose dependent cell death,
carboxy dextran coated SPION used in our study affected vital cell functions, including cell phenotype, immune response and altered the levels of iron dependent protein. No alteration in cell viability was observed in cells exposed to SPION at doses as high as 400 µg Fe/ml. Our results complied with previously published data, where SPION loading induced minimal or no toxicity [100]. Toxicity induced by SPION, if any, is dependent on factors such as surface coating, break-down products and oxidation state of iron in the SPION [100]. In fact, the surface properties including surface coating of SPION determines the efficiency of their cellular uptake, metabolism and potential toxicity. Masking the iron core in SPION with carboxydextran, its lysosomal accumulation and degradation and gradual release of iron may very well be the underlying reasons behind excellent cellular iron tolerance. However, a recent study indicates significant cyto-toxicity induced by SPION [104]. The authors in this study suggest that degradation of the carboxydextran shell and exposure of the iron oxide core are delayed phenomena, and thus they employed a longer period (five days) of exposure with SPION in their study. However, in our study, we find that SPION loading significantly up-regulates cellular ferritin levels within 24 h, which reaches its peak at 48 h and gradually declines at 72 h. These results are indicative of the fact that degradation of the carboxy dextran shell, iron release and its entrapment in ferritin is a much faster process than anticipated in the above study. We do not exclude the possibility that incubating cells with SPION for as long as five days would have yielded us different results. Although, we consider that this would be a good approach in animal studies, but the results from in vitro study like in ours and from Lunov, et al., 2010 [104] shall be governed by other additional factors. In our study, we observed an increased level of ferritin and IL-10 in the culture medium of untreated control cells after 72 h of incubation. This may indicate immune response by cells in nutrient deprived culture media. The phenomenon was previously observed in melanoma cells, where the cells secreted H-chain rich ferritin to stimulate IL-10 production [154]. This extracellular protein accumulation might trigger or inhibit the observed differences between our studies.

Overall, increases in cellular iron concentration have been attributed to the observed alteration in cell functions and loss of cell viability. In our study, we highlighted several
pathways that the cell could employ to reduce iron burden and thus may escape iron induced cyto-toxicity. SPION induces membranous ferroportin expression, which is by far the only discovered iron export pathway [155]. Membranous ferroportin may reduce the INPs induced cellular iron burden by exporting intracellular iron. Up-regulation of ferritin could be employed as an initial step by the cells to accommodate iron released from lysosomal degradation of SPION. It is suggested that cellular ferritin stores have the capacity to bind to 2.6 folds of excess iron. By doing this cells entrap potentially active iron in ferritin cage thereby reducing cellular free iron concentrations. Additionally, SPION induced nuclear ferritin up-regulation in the cells which may protect cells against oxidative stress and DNA damage [64]. Ferritin secretion by cells exposed to SPION might also release the iron burden. Membranous ferroportin expression and ferritin secretion may together help reducing the cellular iron load in cells exposed to SPION. However, the impact of iron released by ferroportin and secreted ferritin on the adjacent cells has to be evaluated as it has been suggested that released ferritin may also contribute to immunological signalling events [10, 60]. Additionally, SPION loading also resulted in an anti-inflammatory cytokine IL-10 secretion which is indicative of a cellular defence mechanism activated by cellular iron overload and this may also protect the cells.

**A need for better characterization of macrophage subtypes**

Diverse methodologies, including cytokine stimulation and growth factor induced differentiation have been used for classical (M1) and alternative (M2) macrophage polarization from monocytes in-vitro. We characterized hIgG induced macrophages as a distinct CD68+, CD86+ and CD163- macrophage subtype. This phenotype closely resembles CXCL4 induced M4 macrophages characterized by a complete loss of CD163 [28] and higher levels of CD86 [27]. However, we do not exclude the possibility that procedure used in this study to generate HMDMs may also be utilized to generate DC (dendritic cells). Both macrophages and dendritic cells may differentiate from monocytes depending on culture conditions and use of additional growth factors. To differentiate them is an intriguing and much discussed issue [156]. Thus, based on the fact that in our study we did not use additional growth factors and the obtained HMDMs phenotype closely resemble M4
macrophages, we claim these cells as macrophages. However, these macrophages have to be further characterized based upon their cytokine secretion response and a larger panel of macrophage phenotypic markers [156-158] can be utilized to verify our results.

Additionally, we followed an established protocol from a well cited study [30] to generate both M1 and M2 macrophages from THP1 cells. These macrophages have been functionally characterized as M1 and M2 macrophages previously. However, we further validated the phenotype of these macrophages by using widely employed macrophage phenotype markers CD86 and CD163, which mark M1 and M2 macrophages respectively [139, 159-161]. Although, it is known that DC are also CD86 positive cells, and may be differentiated from THP1 monocytes by a similar protocol, it is accepted that THP1 cells have already developed too far along the monocyte/macrophage differentiation pathway to allow redirection toward DC [162]. Additionally, differentiating THP1 monocytes to dendritic cells in-vitro requires additional supplements such as GM-CSF and serum free medium. Such conditioning is lacking in our experimental model. In addition, it is also documented that even after fulfilling most of the above requirements, the capacity of THP1 cells to differentiate to DC is merely 5 % and they fail to acquire DC functions [163-165].

In addition, as indicated in the introduction section of this thesis, M2 macrophages are further subdivided into M2a, M2b and M2c macrophages based upon their stimulus to generate these macrophages [25]. In this study, we used IL-4 and IL-13 to generate M2 macrophages. Thus, our results from THP1 M2 macrophages may be confined to a specific M2a phenotype of macrophages and may be verified in other M2 macrophage phenotypes in future studies.
CLINICAL AND FUTURE PERSPECTIVES

Mangafodipir and SPION based particulate agents such as Resovist™ have been shown to possess similar accuracy in detecting human liver lesions and hepatic metastasis [166-169]. However, SPION based contrast agents have grabbed most attention pertaining to the ease of their functionalization directed for targeted imaging in various imaging modalities, including cardiovascular imaging. Nonetheless, these advancements and benefits of SPION accompany the risk and concerns associated with their exposure. It is of particular concern in the field of atherosclerotic plaque imaging where iron excess may eventually determine the plaque vulnerability [170]. In contrast, Mangafodipir with proven pharmacological effects, including our current findings that it prevents oxysterol induced cytotoxicity, stands a better option in the field of atherosclerotic plaque imaging. In fact, new manganese doped iron-oxide nanoparticles are currently been developed with the intensions to reduce toxicity and intolerance of INPs [80]. Such developments in the future may produce a safe contrast agent with both therapeutic and diagnostic potential.

The results from paper III and paper IV suggest that cellular immune functions can be modulated by SPION pertaining to change in macrophage phenotype. Although, we observed a SPION induced phenotypic shift in macrophages, the phenomenon was examined by analysing the levels of macrophage phenotype marker protein in these macrophages. It would be interesting to observe an altered cytokine secretion response by M2 macrophages exposed to SPION. In addition, future studies should further examine the influence of iron in SPION on the immune-functions of atheroma relevant macrophages. It is known that exposure to OxLDL or oxysterols induces a pro-inflammatory macrophage subset [171-173]. Thus it would be interesting to examine the effects of SPION induced additional iron load on immune response of such macrophages. However, elevation in pro-inflammatory response of such macrophages might be the most probable outcome.

Results from paper III and IV indicate that oxysterols reduce phagocytic activity in macrophages and thereby reduce the uptake of iron-oxide nanoparticles. Cells uptake nanoparticles by diverse mechanisms, including; phagocytosis, micropinocytosis, clathrin
mediated endocytosis and caveolae mediated endocytosis. Recently, clathrin mediated uptake of SPION (Resovist™) has been suggested [115]. Thus, it would be of interest to examine the levels of clathrin in cells exposed to oxysterols. Perhaps, a decline in the level of clathrin in macrophages exposed to oxysterols may reason the observed reduction in SPION uptake by such cells. However, affecting clathrin mediated endocytosis might not be the only way by which oxysterols may affect nanoparticle uptake. It has been also shown that oxysterols down-regulate mRNA levels of caveolin [174], which may very well affect endocytic function of cells. These findings suggest that oxysterols may affect endocytic pathways in macrophages in multiple ways. These results also emphasize the necessity for further investigation of the effects of oxysterol on endocytosis as it may directly correlate to lipid accumulation in atherosclerotic plaques.
CONCLUSIONS

- Mangafodipir mediated cyto-protection against 7βOH induced apoptosis involves modulation of lysosomal and mitochondrial pathways.
- This cyto-protective activity of mangafodipir is conserved within its primary metabolite MnPLED and its structural constituent Dp-dp. These results reveal a therapeutic potential of mangafodipir, which could be utilized for future development of contrast agents with both therapeutic and diagnostic potential.
- Iron in SPION, up-regulates cellular levels of ferritin and cathepsin L. Lysosomal cathepsins have a functional role in cellular ferritin metabolism and are pivotal to maintain ferritin levels following SPION degradation. Atheroma relevant oxysterols reduce phagocytic activity in macrophage subtypes and thereby reduce uptake of SPION. These results indicate that targeting by SPION may be inefficient to detect lipid rich intra-plaque macrophages and foam cells and justifies the development of functionalized iron-oxide nanoparticles to target such macrophages.
- Iron in SPION induces a phenotypic shift in M2 macrophages towards a CD86+ macrophage subtype with elevated levels of ferritin and cathepsin L. Cellular levels of ferritin and cathepsin L among macrophages might be important to sustain a functionally polarized state. Iron in SPION is potentially cytotoxic. In addition, SPION up-regulate oxysterol induced cathepsins in cells, which may promote atherogenesis. Thus, their clinical usage and future development should be strictly monitored.
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