UNIVERSITY of the
WESTERN CAPE

THE EFFECTS OF TITANIUM OXIDE NANOPARTICLES ON CULTURED CELLS AND THE IMMUNE SYSTEM

By

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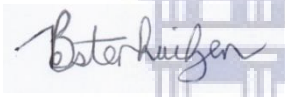
DECLARATION

I declare that:

The effects of titanium dioxide on cultured cells and the immune system is my own work, that it has not been submitted for any degree or examination at any other university, and that all the sources I have used or quoted have been indicated and acknowledged by complete references.

Bevan Peter Esterhuizen

Signature: 

Date: March 2021

DEDICATION

For my late grandparents; Elizabeth and William O'Brien, and Francis and Pieter Esterhuizen who always believed in me, and who would have been so proud.

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ABSTRACT

Engineered nanomaterials derived from various bulk materials are being developed in ever larger quantities and with very diverse chemical compositions. The physical and chemical properties of the smaller nanoparticles are very different compared to their larger bulk chemicals. Titanium dioxide nanoparticles (TiO_2NPs) are an example of such an engineered nanomaterial. Titanium dioxide nanoparticles are mainly used as a pigment in many applications such as glazes, enamels, plastics, pharmaceuticals, cosmetics, and it is widely used in sunscreens. Human exposure to TiO_2NPs can occur both during manufacturing and use. Although studies have shown no significant penetration of TiO_2NPs in sunscreens beyond the stratum corneum (SC) of individuals with intact barrier function, there is cause for concern that compromised barrier integrity might facilitate entry of these TiO_2NPs into deeper underlying tissue and the circulatory system and cause adverse effects. The potential toxicity of TiO_2NPs under conditions where it has passed the skin barrier needs to be assessed.

The murine macrophage cell line RAW 264.7 was selected to assess the effects of TiO_2NPs on the immune system. Due to the fact that this cell line expresses several biomarkers characteristic of macrophages, it is used very often in studies investigating the impact of various chemicals, environmental pollutants, and pharmaceuticals on the immune system. Cellular parameters monitored upon TiO_2NPs exposure included cytotoxicity, and cytokine and chemokine synthesis by the cells.

The aim of the study was to monitor the effects of TiO_2NPs on the immune system, and secondly to investigate whether these NPs are taken up through clathrin-mediated endocytosis using chlorpromazine (CPZ) as a clathrin-mediated endocytosis pathway

inhibitor. Results showed that TiO_2NPs upregulated RAW cell viability at a concentration of 250 µg/ml. TiO_2NPs and CPZ also stimulated cytokine and chemokines associated with inflammation, namely: MIP-1α, MIP-1β, MIP-2, and G-CSF. The cytokine/chemokine production by the RAW cells was NP and CPZ dose-dependent.

Although not found to be cytotoxic in this study, TiO_2NPs increased cytokine and chemokine release, demonstrating that it has immunomodulatory effects and may be detrimental to human health. This study also demonstrated that TiO_2NPs are not taken up through the clathrin-mediated endocytosis pathway. Much remains to be elucidated regarding transportation into cells, bioaccumulation, and the health effects of TiO_2NPs.

Keywords: Titanium dioxide nanoparticles, cytotoxicity, cytokines, chemokines, RAW 264.7 murine macrophages, chlorpromazine, clathrin-mediated endocytosis.

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LIST OF ACRONYMS AND ABBREVIATIONS

ANOVA	analysis of variance
AP	alternative pathway
ATCC	American Type Culture Collection
atm	atmosphere
BSA	bovine serum albumin
CCL3/MIP-1α	macrophage inflammatory protein 1 alpha
CCL4/MIP-1β	macrophage inflammatory protein 1 beta
CCL5/RANTES	Regulated on Activation, Normal T cell Expressed and Secreted
CCV	clathrin-coated vesicles
CO_2	carbon dioxide
CP	classical pathway
CVD	chemical vapor deposition
CXCL2/MIP-2	macrophage inflammatory protein 2
DAS ELISA	double antibody sandwich enzyme-linked immunosorbent assay
DLS	dynamic light scattering

DMEM	Dulbecco's modified Eagle's medium
FBS	fetal bovine serum
g/l	gram per liter
G-CSF	granulocyte colony-stimulating factor
GDP	guanosine diphosphate
GTP	guanosine triphosphate
H^+	hydrogen
hrs	hours
hsc70	heat shock protein 70
i.v.	intravenous
kbar	kilobar
LacCer	lactosylceramide
LDH	Lactate dehydrogenase
LP	lectin pathway
mg/kg	milligram per kilogram
mg/l	milligram per liter
mg/ml	milligram per millilitre
mins	minutes
mm	millimetre

MSNs	mesoporous silica nanoparticles
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
n	sample size
NAD^+	nicotinamide adenine dinucleotide
NADH	nicotinamide adenine dinucleotide + hydrogen
NF-κβ	nuclear factor kappa beta
nm	nanometer
NPs	nanoparticles
O_2^-	superoxide
PAMPs	pathogen-associated molecular patterns
PVD	physical vapor deposition
rcf	relative centrifugal force
ROS	reactive oxygen species
SC	stratum corneum
SD	standard deviation
secs	seconds
TEM	transmission electron microscopy
Ti	titanium

TiO_2	titanium Dioxide
TiO_2NPs	titanium dioxide nanoparticles
TLRs	Toll-like receptors
TNF-α	tumour necrosis factor alpha
UV	ultraviolet
XTT	2,3-Bis-(2-Methoxy-4-Nitro-5-Sulfophenyl)-2H-Tetrazolium-5-Carboxanilide
ZnO	zinc oxide
v/v	volume per volume
µg/day	microgram per day
µg/g	microgram per gram
µg/l	microgram per liter
µg/ml	microgram per millilitre
µl	microliter
µM	micromolar
µm	micrometer

CHAPTER 1:

Background, Aims, and Hypothesis

1.1. Background

Engineered nanomaterials are designed and produced to have special structure-dependent properties (e.g., chemical, mechanical, electrical, optical, magnetic, and biological), which make them desirable for application in commercial and medical industries. Engineered nanomaterials include many different forms which are labelled according to their shape, for example, nanoparticles, nanowires, nanotubes, nanofibers, and nanorings. All of these engineered nanomaterials are derived from bulk materials [1]. As the dimensions of solid material become smaller, its physical and chemical properties become very different from those of the same material in larger bulk form [2]. Nanomaterials are used in various applications, including cosmetics, electronics, and coatings, as well as automobile technology, to name a few [3]. However, these special properties which make nanomaterials so desirable may lead to structure-dependent biological activity that differs from and is not directly predicted by the bulk properties of the constituent chemicals and compounds, as it is released into the environment [1]. The increasing production of engineered nanomaterials increases the risk of human exposure [3] through various environmental routes (inhalation, ingestion, dermal contact, and parenteral) or intentional administration [4].

Once inside the body, engineered nanomaterials can translocate to tissues [5], where it then interacts with various biological components (such as cells, receptors, and proteins) of the immune system [4]. The immune system is an organization of cells and molecules with specialized roles in protecting the body against infection. The immune system comprises two categories, innate immunity (present at birth) and adaptive or acquired

immunity, which develops due to exposure to a foreign substance, e.g., bacteria, viruses [6]. The substances that affect the immune system are not only limited to pathogens but also environmental and therapeutic engineered nanomaterials [7]. An example of such an engineered nanomaterial is titanium dioxide nanoparticles (TiO_2NPs), which are produced from the hydrogeochemical and industrially important transition metal, titanium (Ti) [8]. The potential hazard of TiO_2NPs, as a new emerging environmental and pharmaceutical engineered nanomaterial, needs to be assessed. Cell cultures are considered a reliable model for this purpose. The results obtained from such an *in vitro* study will indicate potential biomarkers that can be used for further studies on uptake and intracellular transport mechanisms involved with emerging engineered nanomaterial toxicity.

1.2. Aims

- To evaluate the use of mammalian cell cultures to monitor the effects of TiO_2NPs on the immune system *in vitro*.
- Identify molecular biomarkers that can be used in rapid bioassays to monitor the effects of TiO_2NPs.
- Investigate whether TiO_2NPs are taken up by the immune cells through clathrin-mediated endocytosis, using *in vitro* techniques.

1.3. Objectives

- Determine if TiO_2NPs have an effect on the immune system
- Investigate whether TiO_2NPs are taken up into cells via the clathrin-mediated endocytosis pathway.

1.4. Hypothesis

H_0: TiO_2NPs do not affect the immune system *in vitro*.

H_1: TiO_2NPs affect the immune system *in vitro*.

CHAPTER 2:

Literature Review

2.1. Nanotechnology

Nanotechnology can be described as the science and engineering involved in the design, synthesis, characterization, and application of nanomaterials. The prefix "nano" is derived from the Greek word for "dwarf" [9]. The British Standards Institution, the American Society for Testing Materials, and the Scientific Committee on Emerging and Newly-Identified Health Risks define nanomaterials as materials with at least one dimension under 100 nanometers (nm). Within this group of materials, there is a sub-group of materials called nanoparticles (NPs), which are defined as materials with at least two dimensions between 1 and 100 nm [10]. One nanometer is equal to one billionth of a meter. To relate this size to real life, a red blood cell is approximately 7000 nm wide while a human hair is approximately 80 000 nm wide. Furthermore, biological units such as atoms are smaller than 1 nm, while molecules such as proteins range between 1 nm and 10 nm [9]. Figure 2.1. [11] provides a graphic illustration of the scales of various biological and technological objects.

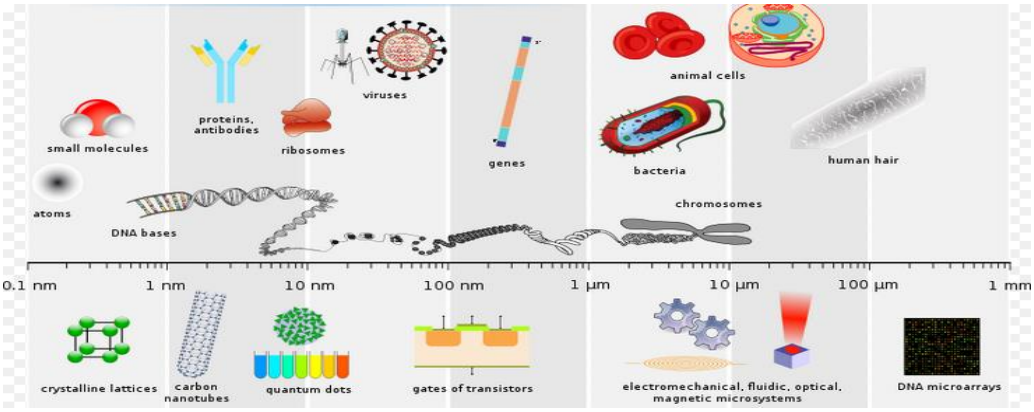

Figure 2.1. A comparison of the scales of various biological and technological objects [11].

Naturally occurring nanoparticles and nanomaterials, although not classified as such, have been known and used by humans for a long time. Naturally occurring NPs have been used in artwork and other human-made materials [12]. The rapid development of nanotechnology has led to increasing numbers of consumer products and industries based on nanomaterials [13]. Nanomaterials, with unique properties and applications, have been developed and are currently used in commercial products such as wound dressings, cosmetics, detergents, food packaging, drug delivery, biosensors, and antimicrobial coatings. The smaller size of NPs results in a higher surface-to-volume ratio, thus a higher percentage of the total molecules on the surface compared to the inside of the NP [14]. Therefore the NPs physicochemical properties, as well as reactivity to biological and inorganic environmental systems is different compared to that of bulk products [8]. TiO_2 NPs have been widely used in industrial and consumer products in recent years [14].

2.2. Titanium, Titanium dioxide, and Titanium dioxide nanoparticles

2.2.1. Titanium

Titanium (Ti), the ninth most abundant element in the earth's crust, is widely distributed. The average Ti concentration in the earth's crust is 4400 milligrams per kilogram (mg/kg). In nature, Ti does not exist in the metallic state due to its strong affinity for oxygen and other elements. Ti's most common oxidation state is +4, but there are also +3 and +2 states. Metallic Ti, titanium dioxide (TiO_2), and titanium tetrachloride ($TiCl_4$) are the most commonly used compounds in industry. Ti exists in ordinary animal tissues but only in trace quantities. There is no proof that Ti is an essential element for humans or animals. A typical diet will contribute between 300 – 400 micrograms per day (μg/day) [14].

2.2.2. Titanium dioxide (TiO_2)

The naturally occurring oxide of Ti is TiO_2, also known as titanium (IV) oxide, titanic acid anhydride, Titania, or Ti powder. TiO_2 is a non-combustible, odourless, white powder with a molecular weight of 79.9 g / mol, 2972 °C boiling point, 1843 °C melting point, and a relative density of 4.26 g / cm^3 at 25 °C [14]. TiO_2 is a particulate that is poorly soluble and is commonly used as a white pigment. Naturally, TiO_2 occurs in 3 crystalline structures: rutile, anatase, and brookite [15]. Figure 2.2. [16] provides a graphic illustration of the different forms of TiO_2.

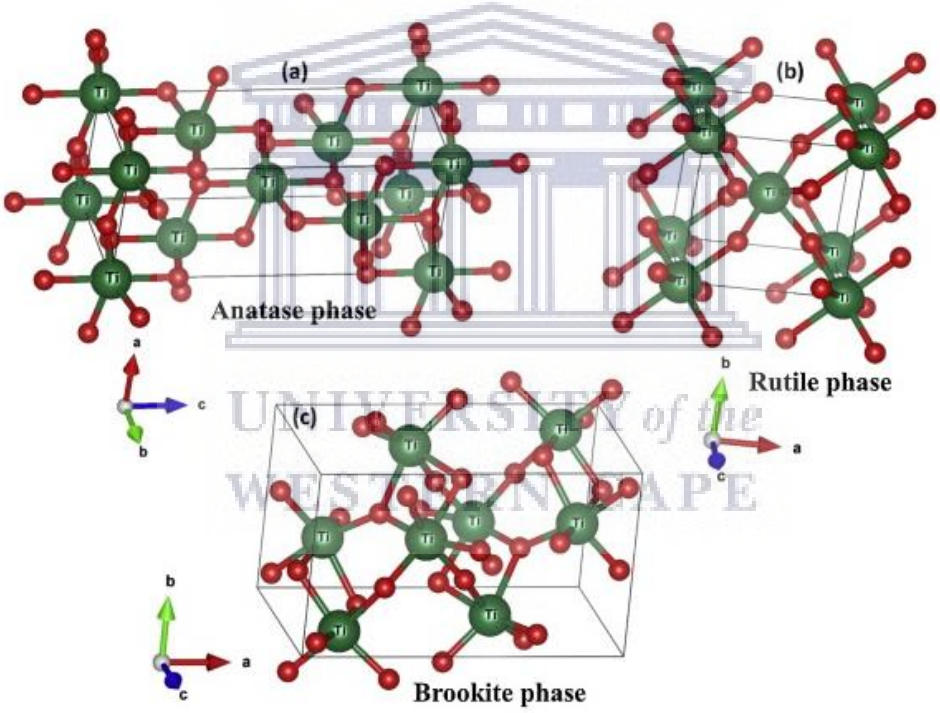

Figure 2.2. Different forms of titanium dioxide [16].

Upon heating, both anatase and brookite convert to rutile which, according to thermodynamic calculations, is more stable at any temperature and pressure below 60 kilobar (kbar) [17]. Rutile is the most common and stable form of this pigment. The refractive indices in the ultraviolet (UV) range and visible wavelength spectrum are

important optical properties of this crystal. Such high refractive indices are partly responsible for the whiteness of the TiO_2 pigments. It is a semi-conductive substance with an electronic structure distinguished by several orbital bands separated by an energy bandgap for which there are no molecular orbitals [15].

TiO_2, as a bulk material, is mainly used as a pigment due to its brightness, high refractive index, and discoloration resistance. Global production for all uses of TiO_2 is in the millions of tons per annum. Almost 70% of all TiO_2 produced is used as a pigment in paints but is also used as a pigment in glazes, enamels, plastics, paper, fibers, foodstuffs, pharmaceuticals, cosmetics, and toothpaste. Other uses of TiO_2 include antimicrobial applications, air- and water purification catalysts, medical applications, and energy storage [18].

2.2.3. Titanium dioxide nanoparticles (TiO_2NPs)

TiO_2NPs are normally a blend of the crystal forms anatase and rutile. TiO_2 particles are produced and used in different fractions of particle size, including fine (approx. 0.1-2.5 μm) and nanosized (< 0.1 μm, primary particles) [14]. Techniques for preparing TiO_2NPs can be divided into five general groups, namely: sol-gel, deposition methods, sonochemical and microwave-assisted methods, hydro/solvothermal methods, and oxidation methods [17]. Historically, sol-gel methods were initially developed and TiO_2NPs of high yield and crystallinity were produced after dried gels underwent thermal treatments. However, hydrothermal and solvothermal methods were applied afterwards because of the poor properties of the as-prepared materials [19].

Briefly, in deposition methods, materials in a vapor state are condensed to form a solid phase material. The method is usually conducted in a vacuum chamber and if there is a

chemical reaction, it is called chemical vapor deposition (CVD), and if there is no reaction it is called physical vapor deposition (PVD) [17]. A reactant gas mixture is brought into contact with the surfaces to be coated during CVD processing, where it decomposes, depositing a thick, pure coating of a metal or compound. The deposit may be formed by a reaction in the vapor phase between the precursor gases or by a reaction between vapor and the substrate surface itself. A difficulty with this method is that it needs high temperatures, 800 °C, or more [20].

In PVD, a thin layer of a material is deposited, usually a metal, from a vapor onto the surface to be coated. The vapor is produced by direct heating or electron beam heating of the metal in a vacuum chamber, from which it condenses onto the cold substrate [20].

The sol-gel method is a wet-chemical technique that can be characterized as converting a precursor solution to an inorganic solid through water-induced polymerisation reactions. Hydrolysis produces a sol that is basically a dispersion of colloidal particles into a liquid, and condensation contributes to gel formation. The possibility to shape the resulting material into desired forms such as fiber, film and monodispersed powder is one of the most desirable features of the sol-gel process [17].

The hydrothermal method is a process of crystallizing a substance using an aqueous solution of the material at a high temperature and high vapor pressure. It is usually described as crystal synthesis or the production of crystals from substances that are insoluble at the customary temperature of 100 °C and pressure of < 1 atm. The process is run under controlled temperature and pressure in autoclaves [17]. Typically, bases such as ammonia or alkaline hydroxides are used to create an intermediate titanium hydroxide, which is then dehydrated to titanium at relatively high temperatures (usually between 150

°C and 250 °C). Many factors such as pH, temperature, and stirring are crucial in controlling the morphology and phase of the crystallites [19].

The solvothermal method uses a non-aqueous solvent compared to the hydrothermal method, has greater control of the properties of TiO_2 and the temperature can be raised much higher, therefore allowing the use of high boiling point solvents [17]. In comparison to the hydrothermal method, the form and morphology of crystallites can be affected by a greater variety of surfactants or structure-directing agents in solvothermal methods. The choice of solvent has also been found to have a dramatic effect on the shape and size of the crystallites produced. Titanium formation can occur under solvothermal conditions even in the absence of any additional compounds other than an oxygenated titanium precursor such as titanium alkoxide. This is because certain titanium alkoxides can thermally decompose into titanium and an unsaturated organic compound (alkene) [19].

TiO_2NPs are among the top five NPs used in consumer products and are the most frequent nanomaterial used for dermal application. Large quantities of TiO_2NPs are used in sunscreens [14]. Older sunscreen formulations using zinc oxide (ZnO) or TiO_2 did not use nanoparticles, but rather material of a micron dimension. Micron-size materials' optical properties improve reflection and have a fairly low refractive index relative to nano-sized materials [12]. These large molecules which reflect/scatter UV light can cause skin whitening [21]. Contemporary sunscreens using nano-sized ZnO or TiO_2 are therefore fairly opaque and transparent compared to older formulations that have stayed bright white following application. It dramatically increased these products' cosmetic appeal without losing their efficacy [12].

2.3. Sunscreen lotion and TiO_2 nanoparticles

Sunscreens are used to protect the skin from the harmful effects of UV solar radiation. Much of this radiation with wavelengths of 100–290 nm, referred to as UVC rays, are filtered off from the atmosphere mainly because stratospheric molecular oxygen absorbs wavelengths smaller than 242 nm to produce ozone. This stratospheric ozone is capable of partially absorbing UVB rays (290–320 nm). However, together with UVA (UVA-2, 320–340 nm; and UVA-1, 340–400 nm), most of the remaining UVB rays reach our skin and cause biological and metabolic reactions [15]. Short-term reactions to sunlight can be largely ascribed to UVB radiation. These include the synthesis of cholecalciferol (vitamin D) and the possibility of developing skin redness (erythema) at higher UVB doses. Long-term sunlight effects involve various degenerative changes in the skin. Known examples include the formation of actinic keratoses and skin cancer from epidermal cells [22].

Titanium dioxide and zinc oxide nanoparticles have recently become common as inorganic physical sunscreens because they can reflect and disperse UVA and UVB radiation while preventing skin irritation and disruption of the endocrine system usually caused by chemical UV filters [13]. The advantages of sunscreens based on inorganic compounds include the absence of skin irritation and sensitization, product inertness, minimal skin penetration, and wide-spectrum protection [21].

2.4. Data available on potential adverse effects of TiO_2NPs

Human exposure to TiO_2NPs can occur both during manufacturing and use. TiO_2NPs may be found as aerosols, suspensions, and emulsions. Inhalation and dermal exposure are the major routes of TiO_2NP exposure that have toxicological relevance in the workplace [14].

Research on exposure to TiO_2NPs using excised human tissue, animals, and cultured cells has been done. In one comprehensive review based on studies and position statements from 1980 to 2008, there was no proof of substantial penetration of sunscreens containing nanosized particles of titanium dioxide and zinc oxide beyond the stratum corneum (SC) [23]. Another study examined the *in vivo* penetration of TiO_2 through human skin transplanted to immunodeficient mice, and also measured the *in vitro* effects of nanoparticles on the specific functional properties of various epidermal and dermal cells in culture. Using different methods of nuclear microscopy, evidence is given that TiO_2 nanoparticles *in vivo* do not penetrate through the intact epidermal barrier. However, TiO_2 was found to exert major cell type-dependent effects on such cellular functions as viability, proliferation, apoptosis, and differentiation when exposed directly to *in vitro* cell cultures [24]. Another article [25] reported that *in vitro*, TiO_2NPs cannot penetrate through SC after 24 h exposure to isolated porcine skin. *In vivo,* the results were very different. After 30 days of topical application to the pig ear, TiO_2 nanomaterials (4nm and 60 nm) can penetrate through a horny layer and be located in the deep layer of the epidermis. Furthermore, TiO_2NPs can penetrate through the skin after 60 days of dermal exposure in hairless mice, enter different tissues, and cause various pathological lesions in several major organs [25].

Other experimental studies on animals include inhalation [26], and nasal [27] in mice, inhalation [28, 29], and injection [29] in rats. TiO_2NP with a primary particle size of 2 – 5 nm was used to assess the toxicity of these NPs using a murine model of inflammation and damage to the lungs. The results showed that mice subacutely exposed to 2 – 5 nm TiO_2NPs showed significant but mild, post-exposure inflammatory response among animals at weeks 0, 1, or 2 following exposure that resolved by week 3 [26]. In another

study, two crystalline phases (anatase and rutile) of TiO_2NPs were intranasally instilled in female mice for 30 days. The findings presented tentative evidence that nasally instilled TiO_2NPs could translocate into the central nervous system and potentially trigger brain damage and that the hippocampus will be the main brain target [27]. Research done on Sprague-Dawley rats aimed to identify changes in the production of reactive species and endogenous nitric oxide (NO) after exposure to NPs. The findings of this study indicate that exposure to NPs in combination with microvascular dysfunction decreases NO bioavailability which can mutually enhance local reactive species [28]. In another study, after inhalation exposure or intravenous (i.v.) injection, the fate of TiO_2NPs in Wistar rats was examined and compared with pigmentary TiO_2 and quartz. Most of the TiO_2NPs inhaled were collected in the lung. Translocation to the mediastinal lymph nodes was also reported, albeit to smaller amounts than after pigmentary TiO_2 inhalation, but far higher amounts than after quartz exposure. TiO_2NPs systemically accessible, as simulated by the i.v. injection was mostly concentrated in the liver and spleen [29].

Studies with anatase phase TiO_2NPs show that the nanoparticles produce reactive oxygen species (ROS) and destroy cultured melanoma cells when exposed to UV light [30]. Another article investigated TiO_2NPs as a source of free radicals under UV irradiation by means of electron paramagnetic resonance spectroscopy. This study found that significant amounts of ROS were produced when a standard concentration of TiO_2NPs was exposed to UV radiation on a glass coverslip. Nonetheless, in the sense of total skin-generated ROS, this amount of ROS was negligible when the experiment was performed on *in vitro* excised porcine skin [31].

A study was done to determine the acute lung toxicity of intratracheally instilled pigment-grade TiO_2NPs in rats (rutile-type particle size = ~300 nm) versus nanoscale TiO_2 rods

(anatase = 200 nm x 35 nm) or nanoscale TiO_2 dots (anatase = ~10 nm) compared to a positive control particle form, quartz. The results show that the toxicity of TiO_2NPs does not depend solely on the size and surface area, but rather on the surface properties as shown in the comparison of mineral forms of TiO_2NPs (anatase and rutile) and particle types (nanorod and nanodot) [32]. These findings were corroborated in another study that evaluated the lung toxicity of ultrafine-TiO_2 particles in rats and compared them with TiO_2 samples in two different size ranges and surface modifications. The findings show that, depending on their composition and crystal structure, exposures to ultrafine-TiO_2 particle types can cause differential pulmonary effects [33]. Reports have suggested that TiO_2NPs through oxidative stress cause cytotoxicity [34, 35].

Topical application of TiO_2NPs on the skin with compromised barrier integrity due to a physiological condition (e.g., Vulgaris psoriasis), burning, or physical abrasion, is a source of legitimate concern. TiO_2NPs have not currently been found to have entered the skin through normal control and psoriatic lesions [36].

2.5. Immune system

The immune system can be considered a multi-layered system consisting of three main defence mechanisms: (i) external barriers including physical barriers (such as skin, ciliated epithelia, mucous membranes) and chemical barriers (such as destructive enzymes in secretions, stomach acids); (ii) innate and (iii) acquired immune response [37]. The immune system is a very complex and regulated network of organs that requires the cooperation and interaction of different types of cells, cell products, tissues, and organs [38] with specialized roles in defending against infection [6]. The immune response uses a vast array of defence mechanisms to regulate the entry of pathogenic organisms into the body and typically eliminate them [39]. Invading bacteria encounter

two fundamentally different types of responses, the innate and acquired immune response [6]. The innate and acquired immune systems are often identified as contrasting separate arms of the host response; however, they usually act together, with the innate response providing the first line of host defence and the acquired response becoming prominent as antigen-specific T and B cells have undergone clonal expansion after several days. In addition, the antigen-specific cells amplify their responses by recruiting innate effector mechanisms to fully control invasive microbes. Thus although the innate and acquired immune response and their mechanisms of action are fundamentally different, cooperation between them is necessary for an intact and completely successful immune response [39]. While the innate response is rapid but sometimes, due to a wider range of specificity, damages normal tissues, the acquired response is precise, but it takes several days or weeks to develop. The acquired response has memory so that subsequent exposure results in a more aggressive and swifter response, but this is not immediate [40].

2.5.1. Barrier system

The skin consists of the epidermis and dermis, and the upper epidermal layer, the stratum corneum, provides its primary protection against the percutaneous penetration of chemicals [15].

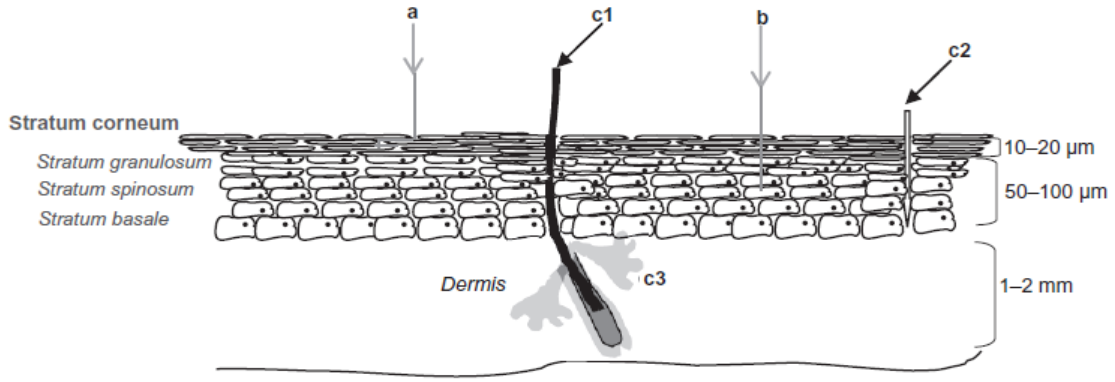

Figure 2.3. The viable epidermis, underlying the SC, contains three layers, the stratum basale, the stratum spinosum, and the stratum granulosum. The SC consists of approximately 15 layers of corneocytes. The main cell type in the viable epidermis is the keratinocyte. Pathways for cutaneous penetration include the paracellular (a), transcellular (b), and the transappendagael route, which includes the transport along hair follicles (c1), sweat pores (c2), and sebaceous glands (c3) [15].

The densely packed structure of the SC consists of dead corneocytes trapped within regions of lipids [41]. A cell membrane protects the corneocytes, which are mostly packed with keratin, water, and various enzymes. This cornified envelope reduces the division of substances into corneocytes and is therefore important for the function of the skin barrier. A crucial role of the skin barrier is played by the unique intercellular SC lipid organization. The intercellular lipids form two lamellar phases with repeated distances of approximately 6 and 13 nm. In addition to the importance of SC for skin barrier function, other factors contributing to the same purpose include tight junctions, skin-related immune functions, and hair follicle (sebaceous) gland secretions [42, 43]. The thickness of the SC partially determines the probability of a particle entering viable skin cells. This thickness is different for various parts of the body and varies with age, gender, and skin type [15].

Damaged skin, however, is often distinguished by a decreased barrier function due to altered tight junctions [44] or changes in lipid composition and organization [45]. In addition, UVB-induced disruption of the skin barrier may also lead to increased epidermal permeability, likely associated with defective layers of lipid lamellar in the SC [46]. Penetration of the SC may occur through various routes: (1) The transappendagael route that involves transportation through sweat pores, hair follicles, and skin (sebaceous) glands; (2) the transcellular SC route; and the paracellular SC route (see Figure 2.3) [15]. Other skin-damaging factors such as skin flexing movements, erosions, and ulcers can also promote the penetration of substances that usually cannot pass the skin barrier. This can lead to unwanted local and systemic reactions [45, 47, 48].

Pathogens must first overcome multiple surface barriers, such as enzymes and mucus, which either are directly antimicrobial or inhibit microbe attachment, in order to cause an infection. For most microbes neither the keratinized skin surface nor the mucus-lined body cavities are suitable environments, therefore microbes need to breach the ectoderm. Every organism breaking through this first barrier faces two stages of defence namely, the innate and acquired immune responses [6].

2.5.2. Innate immunity

Innate immunity is the first line of host defence against pathogenic micro-organisms entering the body. The term innate immunity is sometimes used to include physical, chemical, and microbiological barriers, but more commonly includes the immune system elements (neutrophils, monocytes, macrophages, complement proteins, cytokines, and acute-phase proteins) [40]. This innate mechanism of defence lacks memory and is based mostly on a limited set of microbial determinants shared by a large number of pathogens

[37]. Because the recognition molecules used by the innate system are broadly expressed on a large number of cells, this system is ready to act quickly after an invading response from the host. Thus, it provides the initial response from the host [39]. The innate immune system is comprised of all the immune defences without immunological memory. A feature of innate responses is that they remain unchanged however often the antigen is encountered. These kinds of responses evolved earlier in evolution than the acquired responses [6].

2.5.2.1. Pathogen recognition by the innate immune system

The innate immune system initially recognizes micro-organisms that invade a vertebrate host via germline-encoded pattern-recognition receptors (PRRs). Various PRR types, including Toll-like receptors (TLRs) and cytoplasmic receptors, identify distinct microbial components referred to as pathogen-associated molecular patterns (PAMPs) and stimulate immune cells directly [49]. Toll-like receptors have emerged as crucial components in innate immunity among PRRs. These molecules can detect a wide spectrum of organisms, from viruses to parasites. It has been shown that the founding member of the TLR family, Toll, initially involved in the development of polarity in the Drosophila embryo, is responsible for anti-fungal responses in adult fly [50]. This finding led to 10 human equivalents being identified for pathogen recognition [51]. Based on their location and the type of PAMPs they recognize, TLRs can be classified into different groups. TLRs 1, 2, 4, 5, and 6 are expressed primarily on the cell surface, where they mostly recognize bacterial products, whereas TLRs 3, 7, 8, 9 are located in intracellular compartments and mostly recognize viral products and nucleic acids [49]. TLRs are found especially on macrophages and dendritic cells but also on neutrophils, eosinophils, epithelial cells, and keratinocytes [39].

2.5.2.2. Effector mechanisms of the innate immune system

Phagocytosis is an essential effector mechanism of the innate immune response. Virtually all cells of the innate immune system are phagocytes, whether resident in tissue or in the circulatory system [52]. Macrophages, neutrophils, and monocytes are the principal phagocytic cells [39]. Pathogens are engulfed upon contact with a phagocyte, trapped in an intracellular vesicle, and targeted for destruction by a complex set of digestive enzymes or reactive oxygen species formed within the cell [52]. Efficient phagocytic removal of pathogens requires the rapid mobilization of effector cells to the site of infection; a process sometimes referred to as the inflammatory response [53].

2.5.2.3. Complement system

The complement system consists of more than 30 proteins found in plasma and on cell surfaces, amounting to more than 3g/L and containing more than 15% of the plasma's globular fraction [54]. Activated complement produces three main types of effectors: (1) anaphylatoxins (C3a and C5a), which are strong pro-inflammatory molecules that attract and activate leukocytes; (2) opsonins (C3b, iC3b, and C3d), which bind to target surfaces to promote the transportation and removal of target cells or immune complexes; and (3) terminal membrane attack complex (MAC, C5b-9) which directly lyses targeted (opsonized) pathogens or damaged cells [55]. The C3a and C5a anaphylatoxins produce a multitude of effects in inflammatory reactions. C3a and C5a act as powerful chemoattractants for phagocytes to sites of injury or inflammation, it acts as vasodilators and induces smooth muscle contraction, it causes histamine release from mast cells and induces oxidative bursts (consumption of O_2) from neutrophils [56]. Initially, complement was thought to play a major role in innate immunity where a robust and rapid response to invading pathogens is mounted. However, it has become apparent that

complement also plays an important role in the acquired immunity of T and B cells that aid in the removal of pathogens [54].

Activation of the complement system occurs through three distinct pathways, namely; the classical pathway, lectin pathway, and alternative pathway. The initiation of these pathways occurs through the binding and activation of each pathway's recognition unit to ligands largely unique to each pathway [57]. All the pathways converge at C3 (the most abundant protein complement found in the blood), resulting in activation products, C3a, C3b, C5a, and membrane attack complex (C5b-9) being formed [56] (see Figure 2.4).

2.5.2.3.1. The Classical Pathway

Classical pathway (CP) initiation occurs when the C1 complex (C1q, in complex with C1r and C1s serine proteases), binds to the Fc region of complement-fixing antibodies (generally IgG and IgM) attached to pathogenic surfaces [54]. This interaction results in a conformational change resulting in C1r and C1s activation. The activated C1s cleaves C4 and C2 into small, inactive fragments (C4a and C2b) and larger active fragments (C4b and C2a). C4b binds to cell surface glycoproteins and binds noncovalently to C2a to produce the classical pathway C3 convertase, C4bC2a [55]. C3-convertase generation, which cleaves C3 into the anaphylatoxin C3a and the opsonin C3b, is the point at which all cascades of complement activation converge. When C3 is cleaved into C3b, an internal thioester bond is revealed which allows for stable covalent binding of C3b to hydroxyl groups on nearby carbohydrates and proteins. This process effectively 'tags' microorganisms as foreign, resulting in further complement activation on and around the opsonized surface and culminating in anaphylatoxin development and MAC assembly [54]. Additionally, C3b complexes with the C3 convertase to form the C5 convertase:

C4bC2aC3b. The C5 convertase cleaves C5 into C5a and C5b. The MAC (C5b-9), is then initiated by C6 and C7 binding to C5b and then C8 and C9 molecules binding to the C5bC6C7 complex. The MAC complex forms a pore by inserting itself into pathogen cell membranes, which results in lysis of the pathogen cells [56].

2.5.2.3.2. The Lectin pathway

The lectin pathway (LP) functions in a manner similar to that of the classical pathway, but independent of immunoglobulins [54]. The LP uses members of the collectin family of plasma proteins, namely mannose-binding lectin (MBL) and ficolins [55]. The LP is activated when either MBL or ficolin binds to pathogen surfaces via carbohydrate PAMPs. MBL and ficolin circulate in the serum as complexes with MBL-associated serine proteases (MASPs) similar to the C1 complex of the CP. Four structurally similar MASPs exist; 1, 2, 3, and a truncated MASP2 called MAP19. When binding to pathogens, conformational changes are induced resulting in MASP2 being autoactivated, which cleaves C4 to form C4a and C4b. When C4b attaches, it induces C2 to bind, which is then cleaved by MASP2 to form C2b and C2a. C4b together with attached C2a has enzymatic activity resulting in the formation of the LP C3 convertase, C4bC2a [56].

2.5.2.3.3. The Alternative pathway

Whereas the classical and lectin pathways are normally triggered upon the detection of exogenous materials, the alternative pathway (AP) is constitutively active in the normal host at low levels. At a low level, C3 (which is abundant in the plasma) is constantly hydrolysed to form the C3b analogue, C3(H_2O), which binds to pathogen targets. It is this spontaneous hydrolysis that is thought to initiate the AP [55]. C3(H_2O) (which also has an active thioester bond), binds to Factor B which then allows for the cleavage of Factor

B by Factor D into Bb and Ba and forms the initial AP C3 convertase, C3(H_2O)Bb. This C3 convertase forms the basis of an amplification loop in which C3(H_2O)Bb begins transforming C3 into C3b and C3a, in an analogous fashion to the classical and lectin pathways C3 convertase (C4bC2a). The C3b produced in this way can bind to nearby surfaces and associate with Factor B, which in turn can be activated by Factor D to form C3bBb, the predominant AP C3 convertase [54]. Properdin is a protein released by activated neutrophils that stabilizes the convertase by binding to C3b and preventing its cleavage by Factors H and I [56]. The incorporation of C3b in the C3 convertase results in the formation of the C5 convertase: C3bBbC3b. This C5 convertase cleaves C5 into C5a and C5b, ultimately resulting in the formation of MAC (C5b-9) [55].

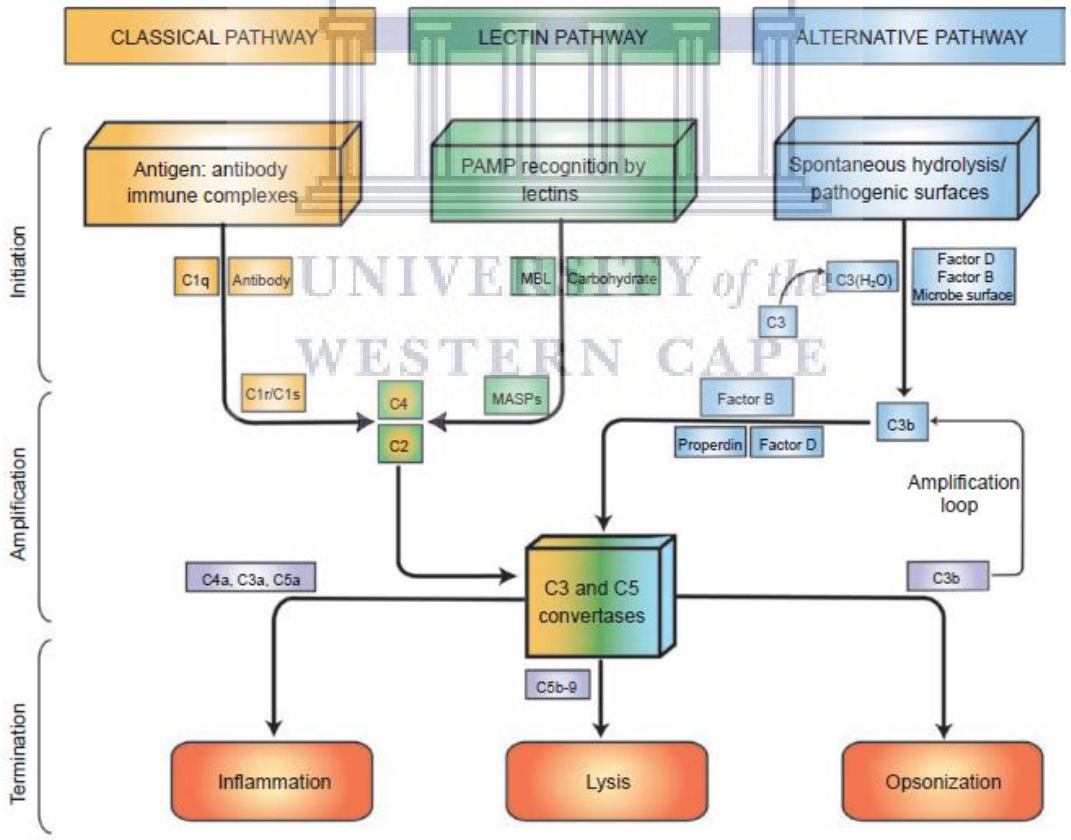

Figure 2.4. Activation pathways of the complement system [54].

2.5.2.4. Inflammation

This prototypical innate response is triggered by the detection of pathogens by innate receptors, mostly expressed by non-lymphoid cells (such as endothelial cells) or macrophages residing near the infection site. After pathogen recognition, these cells secrete a number of chemokines (defined as small soluble proteins that serve as chemotactic factors by guiding cell migration) such as Regulated on Activation, Normal T cell Expressed and Secreted (CCL5/RANTES), that attract phagocytes from blood circulation to the site of infection [58]. Activated resident cells and phagocytes also release soluble mediators called cytokines (defined as proteins released by cells that affect the activity of other cells) such as tumour necrosis factor (TNF- α) and interleukins that further enhance the phagocytic ability of the innate immune system cells. Elevated cytokine and chemokine secretion result in the mobilization of cells and plasma proteins at the tissue infection site through increased vessel permeability, resulting in classical signs of inflammation (increased swelling, redness, pain, and heat) [59]. The inflammatory process includes a variety of signalling pathways, such as the nuclear factor kappa B (NF-κB), which is an essential signalling molecule that increases the development of inflammatory-related effector molecules [60]. The inflammatory response not only contributes to the recruitment of cells and soluble mediators with anti-microbial activity to the infection site but also plays an important role in damaged tissue healing [59].

2.5.3. Acquired immunity

Acquired immunity offers a second line of defence, often at a later stage of infection [37]. This response consists of specific antigen reactions by T lymphocytes and B lymphocytes

[40]. Generally speaking, molecules recognized by lymphocyte receptors are referred to as antigens and can range from simple chemical structures to highly complex molecules. Specialized cells, called antigen-presenting cells, display and interact with lymphocytes in response to the antigen. B cells secrete immunoglobulins, which are the antigen-specific antibodies that eliminate extracellular microorganisms. T cells help B cells by producing antibodies, and by stimulating macrophages, and destroying virally infected cells [6]. The acquired immune responses depend on processes generated by previous antigen exposure and include both cell-mediated and humoral immunity [38]. The acquired immune system initially produces only small numbers of cells with specificity for any particular pathogen, cells that encounter and identify a pathogen need to proliferate to acquire sufficient numbers to mount an effective response [39]. Thus the acquired response usually expresses itself temporarily in host defence after the innate response [39]. After the pathogen has been eliminated, the acquired immune response establishes a "memory" state, characterized by the ability to effectively protect the body from re-infection with the same agent [37]. The body can respond to almost anything that can be bound by the receptors of either the innate or the acquired immune system [6]. The substances that affect the immune system are not only limited to pathogens but also environmental and therapeutic engineered nanomaterials (see Figure 2.5.). Research has shown that by binding to proteins in the blood, nanoparticles can stimulate and/or suppress immune responses [7].

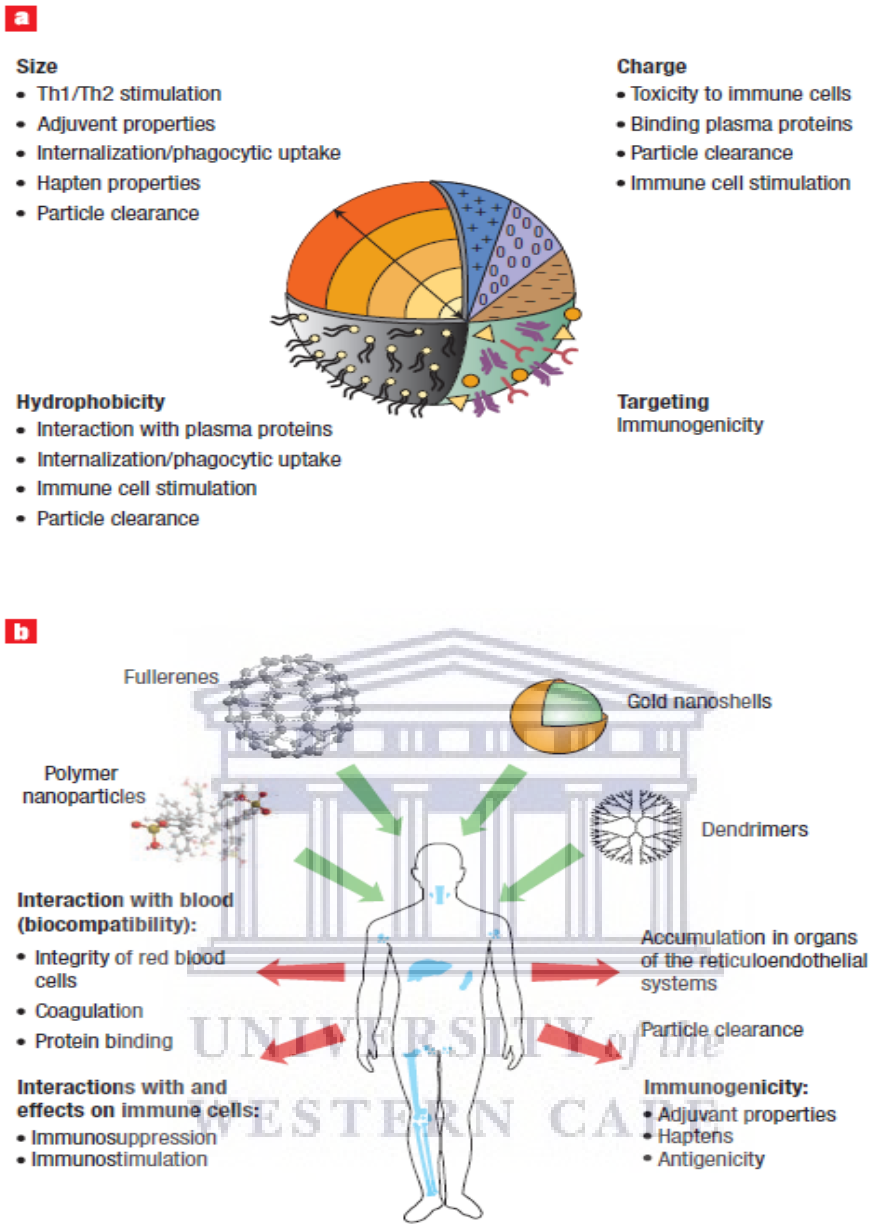

Figure 2.5. Nanoparticle properties determine their interaction with the immune system. a, the effect of nanoparticle size, charge, hydrophobicity, and targeting on immunotoxicity. b, some nanoparticles (shown schematically) can trigger certain immune responses as listed here. A characterization scheme for nanoparticles intended for biomedical applications must include testing for these responses. Such tests may exclude a potentially harmful drug candidate from the development pipeline and inform future studies relevant to the immunomodulatory properties of nanoparticles [7].

2.6. Plasma membrane and endocytosis

The plasma membrane is a dynamic structure that acts to distinguish the chemically distinct intracellular environment (cytoplasm) from the extracellular environment by controlling the entry and exit of small and large molecules and by coordinating them. Due to the action of integral membrane protein pumps or channels, important small molecules such as amino acids, sugars, and ions may traverse the plasma membrane. Macromolecules must be carried into the cell in membrane-bound vesicles resulting from the invagination and pinching-off of parts of the plasma membrane in a process called endocytosis. Endocytosis occurs by various pathways falling into two broad categories, namely, ' phagocytosis ' or cell eating (taking up large particles) and ' pinocytosis ' or cell drinking (taking up fluid and solutes). Phagocytosis is typically limited to specialized mammalian cells, while pinocytosis occurs through at least four basic mechanisms in all cells: macropinocytosis, clathrin-mediated endocytosis (CME), caveolae-mediated endocytosis, and clathrin- and caveola-independent endocytosis (see Figure. 2.6) [61].

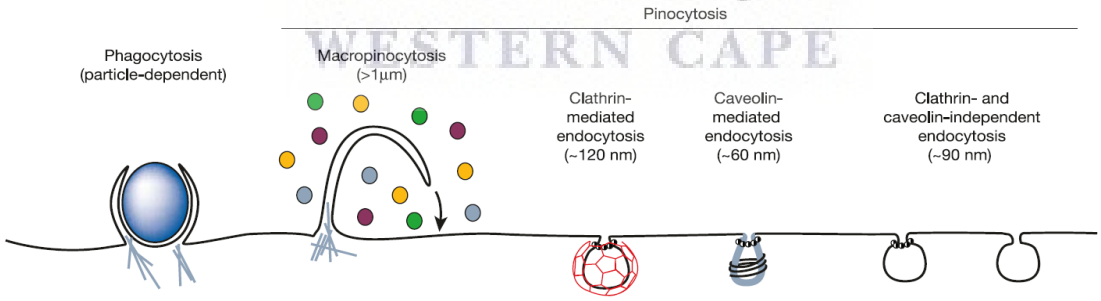

Figure 2.6. Multiple portals of entry into the mammalian cell. The endocytic pathways differ with regard to the size of the endocytic vesicle, the nature of the cargo (ligands, receptors, and lipids), and the mechanism of vesicle formation [61].

2.7. Clathrin-mediated endocytosis

Clathrin-mediated endocytosis is the uptake of material from the surface into the cell through clathrin-coated vesicles. While clathrin-coated vesicles can also be produced from other membrane compartments, the term clathrin-mediated endocytosis only refers to intake via plasma membrane-formed vesicles [62].

Adaptor and accessory proteins coordinate clathrin nucleation at plasma membrane sites that are intended for internalization [63]. This nucleation facilitates the polymerization of clathrin into curved lattices, thereby stabilizing the deformation of the attached membrane. Clathrin polymerization (coupled with the action of other proteins) helps to shape and constrict the neck of the vesicle, thereby helping to bring the membranes that surround the neck into proximity. The membrane scission protein dynamin is a large GTPase. GTPases are a broad family of hydrolase enzymes binding to and hydrolyzing the nucleotide guanosine triphosphate (GTP) to guanosine diphosphate (GDP) [64]. GTPase forms a helical polymer around this constricted neck and mediates vesicle fission from the plasma membrane upon GTP hydrolysis [65], releasing the clathrin-coated vesicles (CCV) irreversibly into the cell's interior. The basket of clathrin is then released by auxiline and heat shock protein 70 (hsc70) from the vesicle (see Figure 2.7). The naked vesicle then undergoes further trafficking within the cell before delivery of its cargo by fusion with an intracellular compartment.

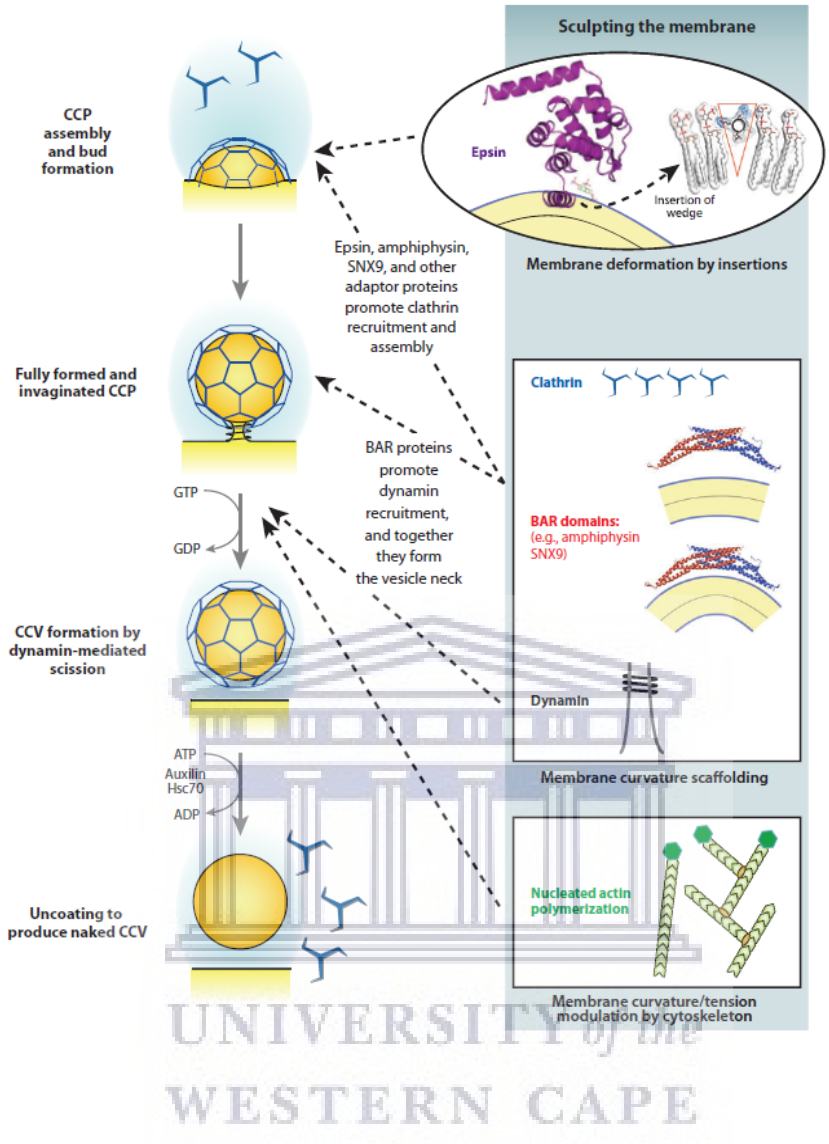

Figure 2.7. Membrane curvature modulation during endocytosis. Schematic diagram (left panels) demonstrating the stages of, and some of the key protein players in, the budding and scission of clathrin-coated pits (CCPs). After budding to form a clathrin-coated vesicle (CCV), the clathrin basket is removed by uncoating proteins to form a naked vesicle. On the right are illustrations of different mechanisms of membrane curvature generation and stabilization [66].

2.8. Nanoparticle uptake and Chlorpromazine Hydrochloride

Cells use various processes for the internalization of extracellular materials. Several endocytosis mechanisms have been established, and the field is very active and constantly being updated. It is generally accepted that nanomaterials and nanomedicines usually enter cells via active processes and, therefore some form of endocytosis [67]. These nanomaterials are, in fact, too large to simply diffuse inside cells. Several studies have attempted to determine how the uptake mechanisms change with the type of nanoparticles (NP) depending on their size charge, shape, and other NP parameters [68-71].

Overall, there are several methods available for researching transportation into cells. Many classic approaches are based on methods to block a certain entry portal and thus assess its role in the uptake of the material of interest [67]. Among all these approaches, the majority of studies involving nanomaterials use pharmacological inhibitors [68, 70]. They are often chosen because they have a very fast action, thereby reducing the likelihood of adaptation for cells, and are easy to use.

Chlorpromazine hydrochloride (CPZ) is a compound used to inhibit clathrin-mediated endocytosis. Chlorpromazine is a cationic amphiphilic drug that inhibits the role of AP-2, one of the main adapter proteins that acts on the cell membrane in clathrin-mediated endocytosis. Chlorpromazine is also known to trap receptors inside endosomes and therefore prevent their recycling. Hence this compound interferes at multiple levels with clathrin-mediated endocytosis [72, 73].

This study aimed to monitor the effects of TiO_2NPs on the immune system by using *in vitro* exposures to murine RAW 264.7 macrophages. Furthermore, the study also aimed to determine whether the TiO_2NPs are taken up by the murine RAW 264.7 macrophages

through the clathrin-mediated endocytosis pathway by using the clathrin-mediated endocytosis pathway inhibitor, chlorpromazine. Cytotoxicity, inflammatory biomarkers, and cytokines of the immune system were assessed in this study.

CHAPTER 3:

Materials and Methods

3.1. Characterization of titanium dioxide nanoparticles

The manufacturer (Evonik Degussa Corporation) produced aeroxide P25 TiO_2 nanoparticles. As mentioned by the manufacturer, P25 is a hydrophilic fumed TiO_2, a mixture of rutile and anatase forms with an average primary particle size of 21 nm [74]. The nanoparticles were further characterized by [75] and noted to have a semi-spherical shape. While the primary size of the nanoparticles are 21nm, the particles aggregate in distilled water and reach sizes of 194 ± 7 nm [75].

3.2. Preparation of titanium dioxide nanoparticles

Stock concentrations (10 mg/ml) of TiO_2NPs, obtained from Evonik Degussa Corporation, were prepared using distilled water and after that sonicated in short bursts for 5 minutes using a tip sonicator (QSonica, LLC. Misonix sonicators, XL-200 Series). This procedure was performed before every experiment.

3.3. Preparation of chlorpromazine hydrochloride inhibitor

Stock concentrations (50 mg/ml) of the chlorpromazine hydrochloride inhibitor, obtained from Sigma (catalogue number: C8138-5G), were prepared using distilled water. The stock concentration of the inhibitor was then filtered through a 0.45 µm (Starlab scientific) sterile filter. The sterile filtered chlorpromazine hydrochloride inhibitor was aliquoted and stored at -20°C until used.

3.4. RAW 264.7 Cells

3.4.1. Cell culture

The cell line RAW 264.7, murine macrophages, was obtained from American Type Culture Collection (ATCC TIB-71). The cells were cultured in tissue culture treated flask (Greiner Bio-one) and maintained in Dulbecco's modified Eagle's medium (DMEM) (Lonza) supplemented with 10 % v/v heat-inactivated fetal bovine serum (FBS) (Hyclone), 0.1 % v/v glutamax (Sigma-Aldrich), 0.1% v/v antibiotic/antimycotic (Sigma-Aldrich) and gentamicin (Sigma-Aldrich). The cells were incubated in a humidified atmosphere of 5 % CO_2 at 37 ºC, and the cells were sub-cultured every 2-3 days.

3.4.2. Chlorpromazine Hydrochloride Inhibitor and Nanoparticle exposure

The RAW 264.7 cells were seeded and cultured at a density of 5 x 10^5 cells/ml in treated 96 well plates and incubated in a humidified atmosphere of 5 % CO_2 at 37 ºC for approximately 48 hrs until the cells reached 80-90 % confluence. The cells were then pre-exposed for 1 hr to various concentrations (5 µg/ml and 10 µg/ml) of the chlorpromazine hydrochloride inhibitor in complete medium. Thereafter, the cells were exposed to various concentrations (31.25 µg/ml and 250 µg/ml) of TiO_2 nanoparticles prepared in serum-free medium and left overnight (~18 hrs) in complete medium with a final FBS concentration of 5% under standard tissue culture conditions. The control was left untreated (0 µg/ml TiO_2NPs, 0 µg/ml CPZ). Culture supernatants were collected, centrifuged at 12 000 relative centrifugal force (rcf) for 1 min and diluted accordingly, and aliquoted (60 µl assigned to each assay) for mouse macrophage inflammatory protein 1 α (MIP-1α), MIP-1β, MIP-2, and granulocyte colony-stimulating factor (G-CSF) determination.

3.4.3. Cytotoxicity Assay (XTT)

After the removal of the supernatants, cells were washed twice with Phosphate Buffered Saline (PBS) (constituents: KH_2PO_4, NaCl, and Na_2HPO_4; catalogue number: BE17-517Q; Lonza), and cytotoxicity was measured by adding 150 µl of a 1/3 dilution of 2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium (XTT) (Roche) reagent in complete medium to each well. Formazan formation was determined by reading the plate at 450 nm (Multiskan Ex, Thermo Electron Corporation) immediately after XTT addition and again after an incubation period of 30 min and 1hr respectively at 37 °C. The increase in absorbance at 450 nm is proportional to formazan formation. The developed formazan amount is directly proportional to the viability of cells.

3.4.4. Mouse MIP family chemokines (MIP-1α, MIP-1β, and MIP-2) Double Antibody Sandwich (DAS) Enzyme-Linked Immunosorbent Assay (ELISA)

The mouse MIP-1α-, MIP-1β, and MIP-2 ELISA (e-Bioscience, Ready-Set-Go) kits were used to measure MIP family chemokine levels in the diluted cell culture supernatants (MIP-1α and MIP-1β: 1/50 v/v, MIP-2: 1/10 v/v). Assays were performed in 96 well Nunc maxisorb plates. The kits contained all the reagents for the assays and were performed as per the manufacturer's instructions.

3.4.5. Mouse G-CSF Double Antibody Sandwich (DAS) Enzyme-Linked Immunosorbent Assay (ELISA)

The mouse G-CSF ELISA (e-Bioscience, Ready-Set-Go) kits were used to measure G-CSF cytokine levels in the diluted cell culture supernatants (1/2 v/v). Assays were

performed in 96 well Nunc maxisorb plates. The kit contained all the reagents for the assay and was performed as per the manufacturer's instructions.

3.5. Statistical Analysis

All experiments were performed in triplicate, and the data was calculated using Microsoft Excel. Data is presented as mean ± standard deviation (SD). One-way analysis of variance (ANOVA) using SigmaPlot 12.0 was used to determine statistical differences with $P < 0.01$ being deemed significant.

CHAPTER 4:

Results

4.1. The effects of TiO_2NPs and CPZ on RAW 264.7 cell viability

TiO_2NPs without CPZ at a concentration of 250 µg/ml significantly upregulated ($P < 0.001$) cell viability of RAW cells compared to the culture control (Figure 4.1). The other TiO_2NPs and CPZ concentrations had no significant effect on the viability of RAW cells.

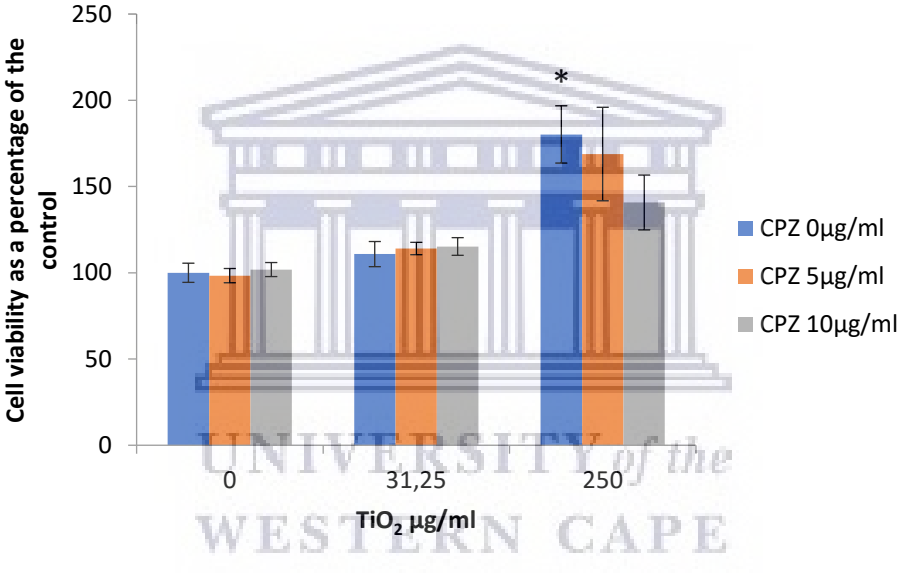

Figure 4.1. Cell viability of RAW 264.7 macrophage cells exposed to various concentrations of TiO_2NPs and CPZ. Data represents mean ± SD with n = 8. Bars marked with a * indicate significantly different ($P < 0.001$) compared to the negative control (0 µg/ml of TiO_2 and CPZ).

4.2. The effects of TiO_2NPs and CPZ on the secretion of MIP family chemokines (MIP-1α, MIP-1β, and MIP-2) by RAW 264.7 cells.

4.2.1. MIP-1α

TiO_2NPs without CPZ at the concentration of 250 µg/ml significantly upregulated ($P < 0.001$) the secretion of the chemokine MIP-1α from RAW cells compared to the culture control. Furthermore, at 250 µg/ml TiO_2NPs, in the presence of CPZ at concentrations of 5µg/ml and 10µg/ml, the secretion of MIP-1α was also upregulated (Figure 4.2). At concentrations lower than 250 µg/ml TiO_2NPs there were no significant effects on MIP-1α secretion by the RAW cells. At these lower TiO_2NPs concentrations, CPZ at 5µg/ml and 10µg/ml did not affect MIP-1α secretion by the RAW cells.

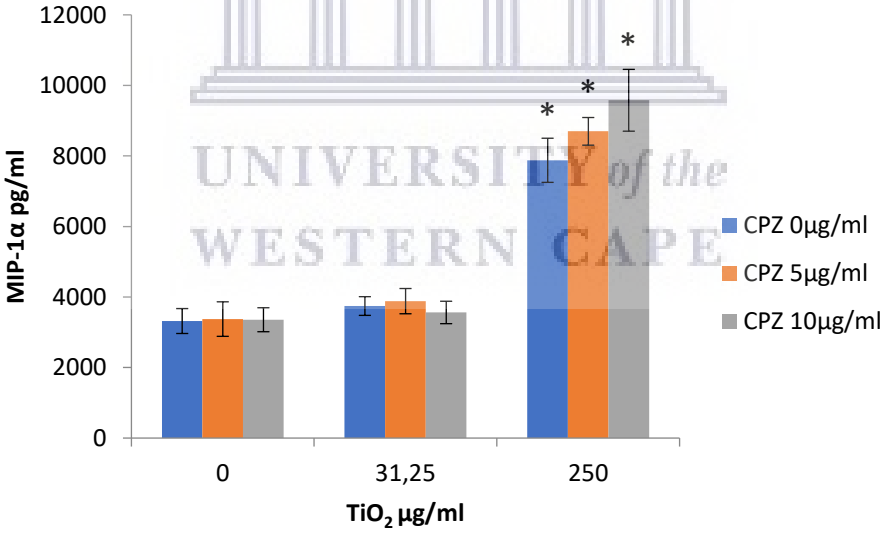

Figure 4.2. The effect of TiO_2NPs and CPZ on MIP-1α secretion by RAW 264.7 cells. Data represents mean ± SD with n = 8. Bars marked with a * indicate significantly different ($P < 0.001$) compared to the negative control (0 µg/ml of TiO_2 and CPZ).

4.2.2. MIP-1β

TiO_2NPs without CPZ at the concentration of 250 µg/ml significantly upregulated ($P < 0.001$) the secretion of the chemokine MIP-1β from RAW cells compared to the culture control. Furthermore, at 250 µg/ml TiO_2NPs, in the presence of CPZ at concentrations of 5µg/ml and 10µg/ml, also resulted in the upregulation ($P < 0.001$) of MIP-1β secretion (Figure 4.3). At concentrations lower than 250 µg/ml TiO_2NPs there were no significant effects on MIP-1β secretion by the RAW cells. At these lower TiO_2NP concentrations, CPZ at 5µg/ml and 10µg/ml did not affect MIP-1β secretion by the RAW cells. This chemokine followed the same trend as the MIP-1α data (Figure 4.2).

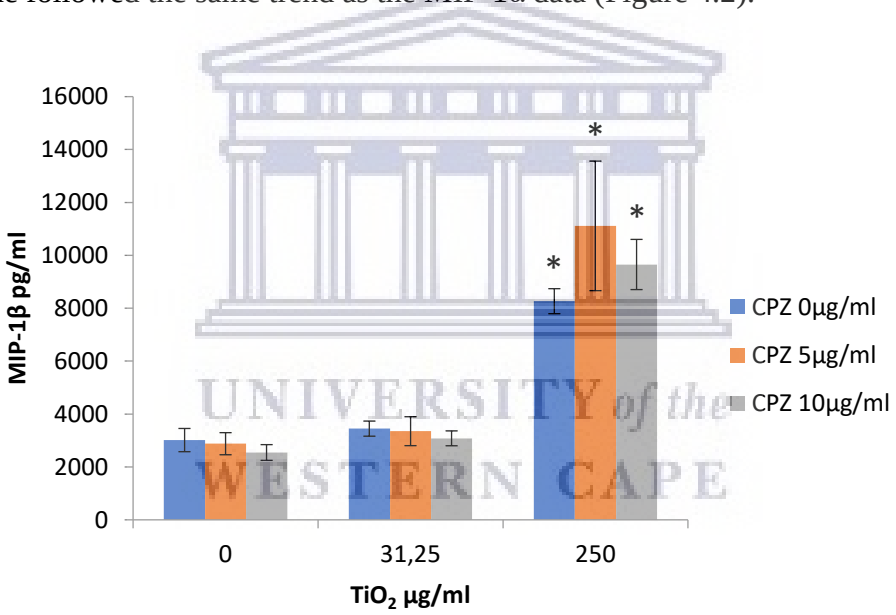

Figure 4.3. The effect of TiO_2NPs and CPZ on MIP-1β secretion by RAW 264.7 cells. Data represents mean ± SD with n = 8. Bars marked with a * indicate significantly different ($P < 0.001$) compared to the negative control (0 µg/ml of TiO_2 and CPZ).

4.2.3. MIP-2

CPZ at 10 µg/ml, without any TiO_2NPs, significantly upregulated ($P < 0.001$) MIP-2 secretion compared to the culture control. In the presence of 31.25 µg/ml TiO_2NPs, and 10 µg/ml CPZ, MIP-2 secretion was also upregulated ($P < 0.001$). At 250 µg/ml TiO_2NPs, together with either of 0µg/ml, 5µg/ml or 10µg/ml CPZ, MIP-2 secretion was significantly upregulated ($P < 0.001$) by RAW cells (Figure 4.4). The other TiO_2NPs and CPZ concentrations had no significant effect on the secretion of MIP-1α from the RAW cells.

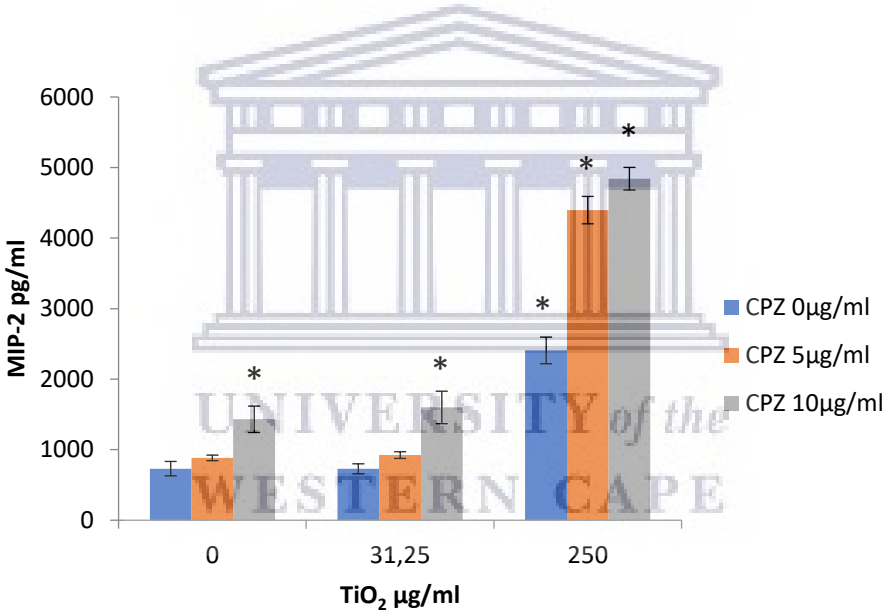

Figure 4.4. The effect of TiO_2NPs and CPZ on MIP-2 secretion by RAW 264.7 cells. Data represents mean ± SD with n = 8. Bars marked with a * indicate significantly different ($P < 0.001$) compared to the negative control (0 µg/ml of TiO_2 and CPZ).

4.3. The effects of TiO_2NPs and CPZ on the secretion of the cytokine Granulocyte colony-stimulating factor (G-CSF) by RAW 264.7.

TiO_2NPs without CPZ at the concentration of 250 µg/ml significantly upregulated ($P < 0.001$) the secretion of the cytokine G-CSF from RAW cells compared to the culture control. Furthermore, 250 µg/ml TiO_2NPs, in the presence of CPZ at concentrations of 5µg/ml and 10µg/ml, the secretion of G-CSF was also upregulated ($P < 0.001$) (Figure 4.5). At concentrations lower than 250 µg/ml TiO_2NPs there were no significant effects on G-CSF secretion by RAW cells. At these lower TiO_2NPs concentrations, CPZ at 5µg/ml and 10µg/ml did not affect G-CSF secretion by RAW cells.

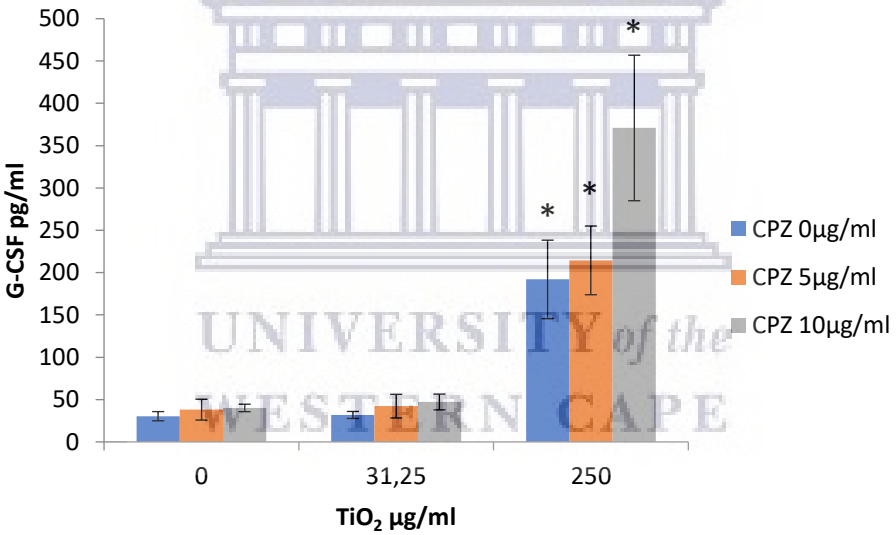

Figure 4.5. The effect of TiO_2NPs and CPZ on G-CSF secretion by RAW 264.7 cells. Data represents mean ± SD with n = 8. Bars marked with a * indicate significantly different ($P < 0.001$) compared to the negative control (0 µg/ml of TiO_2 and CPZ).

CHAPTER 5:

Discussion and Conclusion

5.1. Discussion

Titanium dioxide nanoparticles represent a scientific breakthrough in biological and medicinal applications. Titanium dioxide accounts for 70 percent of the worldwide total pigment production volume. Ultrafine grades of titanium dioxide (i.e. 10–50 nm) are used for blocking ultraviolet light in plastics, catalysts, and especially in sunscreens applied to the skin [76]. The skin consists of the epidermis and dermis, and its primary defence against the percutaneous penetration of chemicals is provided by the upper epidermal layer, the stratum corneum [15]. A comprehensive review of nanosized particulate sunscreens containing TiO_2 found no evidence of substantial penetration beyond the stratum corneum [23]. However, what is unclear is what happens when there is a breach in the skin barrier which then allows these particles to enter the circulatory system.

Macrophages, the sentinels of the immune system, play a key role in identifying the particles that have been introduced into the system [77]. Physicochemical features of particles can affect these interactions and can also potentiate toxicological mechanisms. Macrophages identify opsonized proteins, different surface chemistries, and other surface and biological characteristics that mark certain nanoparticles for clearance and/or toxicological fates [78]. Given the extent of TiO_2NP use, monitoring the effects of TiO_2NPs on macrophages and the immune system is essential. As such, using a murine RAW 264.7 macrophage cell culture system as a representative of the immune system was the most appropriate method for this study. The aim of the current study was to monitor the effects of TiO_2NPs on the immune system and to investigate whether these TiO_2NPs are taken up by the immune cells through the clathrin-mediated endocytosis

pathway by using the clathrin-mediated endocytosis pathway inhibitor, chlorpromazine. Chlorpromazine is a cationic amphiphilic drug that is believed to inhibit clathrin-mediated endocytosis by preventing clathrin-coated pit formation by reversible translocation of clathrin from the plasma membrane to the intracellular vesicles and its adapter proteins [72].

5.1.1. The effects of TiO_2NPs and CPZ on RAW 264.7 cell viability

Cell culture systems are considered an alternative to using animals. Cell culture systems are less ethically ambiguous compared to using animals, it is easier to monitor and replicate, and it is less costly. Cell viability assays are useful in screening engineered nanomaterials for their cytotoxicity to various cells [79]. Assays based on tetrazolium determine cellular viability based on mitochondrial activity. Mitochondrial activity is a crucial determinant of cell viability because mitochondrial dysfunction or damage is one of the main drivers of programmed cell death [80]. In active mitochondria, the tetrazolium ring is cleaved by dehydrogenase enzymes, thus the reaction occurs only in living cells [81]. Mitochondria transform nicotinamide adenine dinucleotide + hydrogen (NADH) to nicotinamide adenine dinucleotide (NAD^+) and release hydrogen (H^+) through the electron transport chain. The free H^+ atom binds to the salt of tetrazolium leading to a colorimetric reaction that can be measured with a spectrophotometer [80]. Colour intensity is representative of the number of active mitochondria present and indirectly viable cells. Determining the cellular viability of RAW 264.7 murine macrophages using the XTT assay was considered the most appropriate method for this study.

A section of this study was to examine if the TiO_2NPs are taken up by the RAW cells via clathrin-mediated endocytosis by using the clathrin-mediated endocytosis pathway

inhibitor, chlorpromazine. Chlorpromazine interrupts the clathrin-mediated endocytosis pathway by inhibiting AP-2, one of the key adapter proteins that act on the cell membrane [73]. Before it could be determined whether or not the TiO_2NPs were taken up by the clathrin-mediated endocytosis pathway, it was important to first determine whether or not the CPZ was toxic to the cells. As it has been previously reported that the effects of endocytic pathway inhibitors such as CPZ are extremely cell type-dependent [72]. In this study, CPZ was used at concentrations of 5 µg/ml and 10 µg/ml and was shown to have no significant effect on cell viability at any of the concentrations used (Figure 4.1). It was therefore deemed appropriate to use these concentrations in the subsequent experiments.

The levels at which TiO_2NPs exist within consumer products range from 6.8×10^{-6} to 5.7×10^{-3} µg/ml [82]. In this study, much higher concentrations (31.25 and 250 µg/ml) were used and therefore the findings could be argued irrelevant. However, numerous studies have found that these nanoparticles have the potential to bioaccumulate in organisms over time [83, 84], and thus such high concentrations could be considered appropriate for *in vitro* analysis. In addition, based on previous research [77, 85, 86] utilizing different forms of TiO_2, the concentrations used in this study fall within the ranges used.

The cell viability assay results indicate that 31.25 µg/ml TiO_2NPs had no effect on the cells and seem similar in effect as at 0 µg/ml TiO_2NPs (negative control). However, there is a significant increase in cell viability at 250 µg/ml TiO_2NPs in comparison to 0 µg/ml (negative control), as can be seen in Figure 4.1. Traditionally, *in vitro* toxicity research focuses on whether cell death occurs from exposure to a potentially toxic agent or not. However, while there may be no cell damage or death after exposure to nanoparticles, changes in cellular function may result. Therefore, when determining the effects of

nanoparticle exposure on cells, cellular modifications, and sub-lethal effects should also be considered and investigated.

Previous research has shown that some NPs could induce ROS production and oxidative stress, resulting in inflammation and even cell death [87, 88]. Oxidative stress is a condition of redox disequilibrium in which the development of ROS overwhelms the cell's antioxidant defence capability, resulting in adverse biological consequences [89]. However, some studies have shown that some NPs are capable of decreasing the intracellular ROS levels and thereby decreasing oxidative stress and improve the viability of cells [90-92]. Huang et al. (2010) reported that mesoporous silica nanoparticles (MSNs) significantly promoted human malignant melanoma cell (A375) proliferation and accelerated cell cycle progression *in vitro*. The investigators suggested that the mechanism behind this promotion effect was induced by MSNs which caused the reduction of endogenous ROS in cells. Further findings showed that upregulation of the anti-apoptotic molecules, Bcl-2, and inhibition of NF-κβ activation by MSNs may promote cell proliferation in a redox-sensitive signal pathway [92].

Certain comparisons can be drawn between the current study and that of Huang et al. (2010) [92]. In both studies, the NPs used significantly upregulated cell viability and promoted cell proliferation respectively. Viability and proliferation are two distinct characteristics of cells. Viability is a measure of a population's number of living cells, while proliferation is a measure of the division of cells. It is important to note that not all viable cells divide. In the current study, it would seem that viability and proliferation are linked as the number of viable cells significantly increased at 250 µg/ml TiO_2NPs when compared to 0 µg/ml TiO_2NPs (the negative control), as seen in Figure 4.1. However, further investigation is required to establish whether the mechanism behind this

upregulation seen in the current study is similar to that which has been suggested by Huang et al. (2010) [92]. Based on the findings of the above-mentioned studies [87, 88, 90-92], it would seem that ROS and oxidative stress are important biomarkers to consider when studying the effects of NPs on cells, in addition to the XTT assay. Together, these assays would provide a more holistic picture of the effects of TiO_2NPs on RAW cells and might explain the upregulation in viability seen in the current study.

The results found in the current study differ from what has been previously reported regarding the toxic effects of TiO_2NPs on RAW 264.7 murine macrophages. One study indicated that cell viability was not significantly altered over a concentration range of 0 – 200 µg/ml TiO_2NPs [85]. Another study reported a dose-dependent increase in cytotoxicity leading to a significant decrease in cell viability over a concentration range of 0 – 80 µg/ml TiO_2NPs [86]. In another study, the investigators reported that cell viability was not significantly altered from low concentrations of 0.001 to 10 µg/ml TiO_2NPs, but acute toxicity was displayed at 100 µg/ml and 1000 µg/ml TiO_2NPs [77].

It is important to note is that in each of the aforementioned studies [77, 85, 86], the TiO_2NPs used were different, either in size, functionalization, or synthesis method. Dinesh et al. (2017) synthesized their TiO_2NPs by sol-gel combustion using glycine and $_L$-alanine amino acids as reducing agents and calcium nitrates as oxidizing agents and reported particle sizes of ~85.5 nm (glycine-TiO_2NPs) and ~72.8 nm ($_L$-alanine-TiO_2NPs) [77]. Kang et al. (2008) made use of ultrafine TiO_2NPs with a size of 21 nm obtained from the Degussa Corporation (Parsippany, New Jersey), as well as fine TiO_2 with a particle size of 1µm that was purchased from Sigma-Aldrich (Atlanta, Georgia) [85]. Further variation occurs as nanoparticles are functionalized. The purpose of functionalization is to add a characteristic that was previously unavailable or to improve

an available characteristic. Dhupal et al. (2018) used TiO_2NPs with a size of 20 nm, purchased from Sigma-Aldrich (Darmstadt, Germany), which were then modified with bovine serum albumin (BSA) as a coating agent to have a negative charge [86]. These new characteristics allow the nanoparticles to behave differently from their macro-sized constituents. Therefore, it is important to take into account synthesis methods and physicochemical characteristics of nanoparticles when comparing toxicity data across different studies.

It is also important to note that in each of the above-mentioned studies different endpoints to indicate cytotoxicity were monitored. In the study done by Kang et al. (2008), cell viability was measured with a Lactate dehydrogenase (LDH) assay. LDH is an abundant intracellular enzyme and is considered a marker of lytic cell death when it is released into cell culture supernatants [85]. Dhupal et al. (2018), observed cytotoxicity under an inverted microscope, and viability was assessed using Cell Counting Kit-8 (CCK-8) [86]. Whereas, Dinesh et al. (2017), measured cytotoxicity using LDH and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays [77]. In light of this, efforts are required to improve the standardization of assays used to test nanomaterials *in vitro* and *in vivo*. [93].

5.1.2. The effect of TiO_2NPs and CPZ on MIP family chemokine production

Inflammation is a potential adverse effect of exposure to nanoparticles. The inflammatory response is modulated by cytokines and chemokines released by activated macrophages [94]. One of the aims of this study is to identify a cytokine and chemokine profile that could be indicative of TiO_2NPs exposure. The MIP family chemokines (MIP-1α, MIP-1β, and MIP-2), known for being hallmarks of inflammation, were chosen for further

analysis. The various MIP proteins function as chemokines that attract monocytes and neutrophils [95]. These cytokines are detected using ELISAs and can be quantified by measuring the absorbance of the streptavidin-horseradish peroxidase labelled antibodies at 450 nm.

Although MIP-2 presented at much lower levels than MIP-1α and MIP-1β, the trend of up-regulation remained constant at 250 µg/ml TiO_2NPs throughout the three figures (4.2, 4.3, and 4.4). This indicates that the TiO_2NPs at the concentration of 250 µg/ml are inflammatory. However, when taking the cell viability results (Figure 4.1) into account, this upregulation can't be conclusively attributed to inflammation only, as there are more cells present in this concentration group, and therefore it stands to reason that more MIP family chemokines would also be produced in relation to the number of cells. Giovanni et al. (2015) reported that the treatment of RAW 264.7 macrophages with very low concentrations of TiO_2NPs (10^{-7} µg/ml) with a size of 25-30 nm, as used in consumer products have been found to induce NF-κβ activation and the consequent upregulation of the expression of pro-inflammatory genes. Notably, cytotoxicity was only observed at a significantly higher concentration of nanoparticles (10 µg/ml), indicating that TiO_2NPs might exert immunomodulatory effects independent of cell viability [82]. Therefore, it is important to consider that even though the TiO_2NPs used in the current study showed no apparent cytotoxic effects, it might still have immunomodulatory effects.

Furthermore, 250 µg/ml TiO_2NPs in the presence of 5 µg/ml and 10 µg/ml CPZ upregulated the secretion of MIP-1β (Figure 4.3), and MIP-1α and MIP-2 (Figures 4.2, and 4.4). Interestingly, 10 µg/ml CPZ upregulated the secretion of MIP-2 at 0 µg/ml and 31.25 µg/ml TiO_2NPs (Figure 4.4). Upregulation of MIPs occurs at 250 µg/ml TiO_2NPs in the absence and presence of CPZ, which would suggest that the TiO_2NPs were not

taken up through the clathrin-mediated endocytosis pathway. This assumption is based on the expectation that if the TiO_2NPs are internalized through this pathway, the CPZ should decrease the amount of chemokines produced. The exact mechanism behind this upregulation trend remains unclear. As mentioned earlier, it is unclear whether the increased chemokine release is in response to the TiO_2NPs, or whether it is due to an increased number of cells. However, when looking at the MIP-2 results (Figure 4.4) it appears that CPZ is inflammatory, as it increased the amount of MIP-2 released in the absence of TiO_2NPs. These results further add to conflicting reports on endocytic pathway inhibitors such as CPZ, which suggests that the inhibitory effect is extremely cell type-dependent, as is the concentration for maximizing inhibitory effect [72]. One study assessed the specificity of CPZ for inhibiting clathrin-mediated endocytosis by looking at the effects it had on lactosylceramide (LacCer) uptake. LacCer is a fluorescent endocytic probe. Treatment with CPZ had no inhibitory effect on the uptake of LacCer and also resulted in a marked increase in the internalization of this marker in human retinal pigment epithelial cells (ARPE-19) and human hepatocellular carcinoma cells (HuH-7) [72]. This highlights the importance of thoroughly characterizing the effects of endocytic pathway inhibitors on any particular cell line before attempting to define the uptake pathway for any particular nanoproduct that is considered to reach cells by endocytosis.

5.1.3. The effect of TiO_2NPs and CPZ on G-CSF production

Pro-inflammatory cytokines, such as granulocyte colony-stimulating factor (G-CSF), cause both acute and chronic inflammatory responses. G-CSF directly controls neutrophilic G granulocyte production and improves the functionality of mature neutrophils [96]. Performing an ELISA for G-CSF served as confirmation of the

significant differences in the effect of TiO_2NPs and CPZ exposure on RAW 264.7 murine macrophages.

The results show upregulation at 250 µg/ml TiO_2NPs, similar to that of the MIP family chemokines. As mentioned earlier this possibly indicates that the TiO_2NPs at the concentration of 250 µg/ml are inflammatory. However, when considering the cell viability results (Figure 4.1), this upregulation can't be conclusively attributed to inflammation only, as there are more cells present in this concentration group, and therefore it stands to reason that more G-CSF would also be produced in relation to the number of cells.

Furthermore, 250 µg/ml TiO_2NPs in the presence of 5 µg/ml and 10 µg/ml CPZ also upregulated the secretion of G-CSF (Figure 4.5). This would suggest, similar to that of the MIP family chemokine data, that the TiO_2NPs were not taken up through the clathrin-mediated endocytosis pathway.

5.2. Conclusion

Titanium dioxide nanoparticles used in sunscreens are considered safe when applied to the skin, as previous studies have found no evidence of substantial penetration beyond the stratum corneum, the most upper layer of the epidermis. However, what is unclear is what happens when there is a breach in the skin barrier, and the TiO_2NPs traverse the epidermis and dermis to enter the circulatory system. This eventuality was investigated in this study using a RAW 264.7 murine macrophage cell culture system.

Under basal conditions, TiO_2NPs increased the viability of RAW cells according to the XTT assay results. In addition to the effects on cell viability, the TiO_2NPs also upregulated the release of various cytokines and chemokines at a concentration of 250

μg/ml. In this study, the cytokines and chemokines used were CCL3/MIP-1α, CCL4/MIP-1β, CXCL2/MIP-2, and G-CSF. In cultures, these cytokines and chemokines were upregulated by TiO_2NPs and may theoretically be used to determine the effects of TiO_2NPs on macrophages. When taking the cell viability results into account, this upregulation in cytokines and chemokines caused by the TiO_2NPs does not seem to be an inflammatory response by the RAW cells. It seems more plausible that the TiO_2NPs upregulated cell viability, which had a direct effect on the amount of cytokines and chemokines produced by the RAW cells. Further investigations are required into the exact mechanism behind these upregulation effects. Another way to determine the sub-lethal effects of nanoparticles is to perform genomic and proteomic array experiments to investigate alterations in the expression of certain genes and proteins within the cell. Although the TiO_2NPs concentrations used in this study are much higher than the concentrations used in consumer products, it is important to note that these nanoparticles have the potential to bioaccumulate, and therefore continued use over an extended period may cause adverse health effects.

The secondary aim of the study was to determine whether the TiO_2NPs were internalized by RAW cells via clathrin-mediated endocytosis by using the clathrin-mediated endocytosis pathway inhibitor, chlorpromazine. The results showed CPZ to have no inhibitory/down-regulatory effects on the secretion of the various chemokines and cytokines used in this study. These findings suggest that the TiO_2NPs were not taken up via clathrin-mediated endocytosis in RAW 264.7 macrophages as downregulation effects were expected. Therefore, further investigations into alternative uptake mechanisms of TiO_2NPs by RAW 264.7 cells are required.

Although TiO_2NPs have been increasingly studied in recent years, much remains to be elucidated regarding their transportation into cells and consequent effects. Based on all of the above findings the null hypothesis which states that TiO_2NPs do not affect the immune system *in vitro* is refuted. Although not found to be cytotoxic in this study, TiO_2NPs increased cytokine and chemokine release, demonstrating that it has potential immunomodulatory effects, therefore the alternative hypothesis which states that TiO_2NPs affect the immune system *in vitro* is accepted. However, further analysis into the mode of action of TiO_2NPs and CPZ and its immunomodulatory effects on RAW 264.7 murine macrophages is required.

CHAPTER 6:

Future Perspectives and Recommendations

- The TiO_2NPs were characterized at room temperature and in aqueous solutions. In addition, the nanoparticles should be defined in the culture medium and at the temperature of incubation in which they will be used. Characterization techniques should include Dynamic Light Scattering (DLS) as well as microscopy to get a more holistic understanding of the possible effects of the TiO_2NPs.

- In this study, the XTT assay was used to determine cell viability. In addition, assays that examine plasma membrane integrity such as Trypan blue exclusion, neutral red, and LDH should also be considered. ROS and oxidative stress should also be considered as possible markers to monitor. Together these assays would provide a more holistic picture of the effects of TiO_2NPs on cell viability.

- This research examined only acute exposures. Chronic exposure to TiO_2NPs should be addressed, as this could potentially alter extracellular biomarkers.

- Only extracellular cytokines and chemokines have been monitored in this study. In future studies, a proteome profile of intracellular proteins indicative of cell stress and apoptosis should be considered as this would give an overall view of how the nanoparticles interact with the cells and possible pathways, they may affect.

- In this study, chlorpromazine was used as an inhibitor to determine whether the TiO_2NPs were taken up by clathrin-mediated endocytosis. The results indicate that this was not the case. However, there are various other uptake pathways, such

as caveolin-mediated endocytosis, and macropinocytosis to name a few. Each with different inhibitors such as Genistein, and Amiloride which could be used to study uptake mechanisms. In future studies, these different uptake pathways and inhibitors should also be considered.

- The current study explored only the effects of TiO_2NPs and CPZ mediated *in vitro* under basal conditions. Future studies should include LPS stimulated cultures, that would simulate a diseased state, to investigate whether the TiO_2NPs and CPZ have the same effects as found in this study.

- It has been suggested that endocytic pathway inhibitors exhibit cytotoxic and inhibitory effects that depend on the type of cell being used. Therefore, in the future, it is important to thoroughly characterize their effects on any particular cell line before attempting to define the uptake pathway for any particular nanoproduct that is considered to reach cells by endocytosis.

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