INFLUENCE OF NATURAL PRODUCTS AND NANOPARTICLES ON THE SIGNAL TRANSDUCTION IN HUMAN CELL SYSTEMS

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by

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Eidesstattliche Erklärung

„Ich erkläre hiermit an Eides statt, dass ich die vorliegende Dissertation selbstständig angefertigt habe. Die aus fremden Quellen direkt oder indirekt übernommenen Gedanken sind als solche kenntlich gemacht.

Die Arbeit wurde bisher weder in gleicher noch in ähnlicher Form einer anderen Prüfungsbehörde vorgelegt und auch noch nicht veröffentlicht.“

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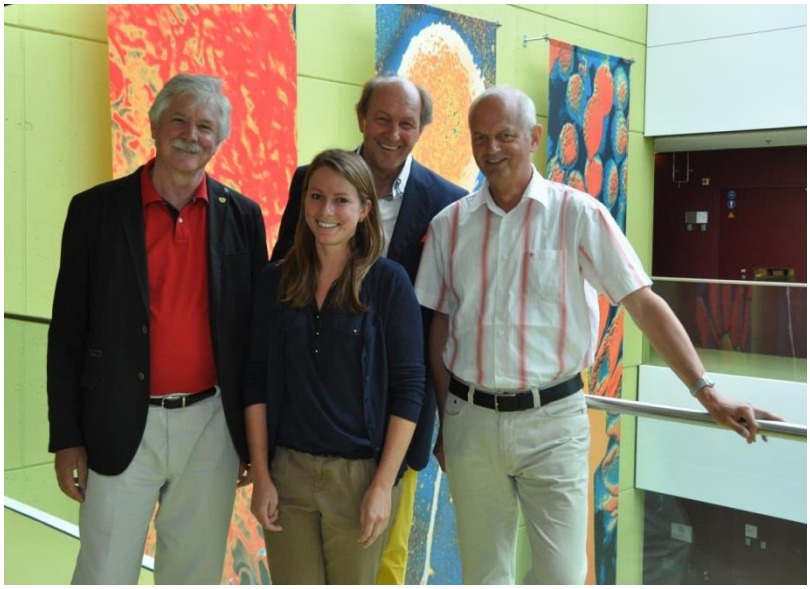

Freude am Schauen und Begreifen
ist die schönste Gabe der Natur.

Albert Einstein

Kurzfassung

Die menschliche Gesundheit wird durch viele Faktoren aus der Umwelt und dem Lebensstil beeinflusst, wobei das Immunsystem für die Gesundheit eine wichtige Rolle spielt. Ein ausgewogenes und gut funktionierendes Immunsystem kann chronischen Erkrankungen vorbeugen. Exogene Faktoren wie Ernährung, Medikamente, Umweltschadstoffe, Nanopartikel oder auch andere neue Materialen können immunologische Signale und Reaktionen beeinflussen. Daher ist es von großer Wichtigkeit, solche Substanzen bezüglich ihres immunologischen Einflusses mit geeigneten Messmethoden zu charakterisieren, um eine Risiko- oder Nutzen-Abschätzung zu garantieren.

Erstes Ziel dieser Arbeit war die Charakterisierung von ausgewählten Pflanzenstoffen auf ihre antioxidativen und immunmodulierenden Eigenschaften. Einflüsse von Naturstoffen auf zentrale Signalwege der Typ1-T-Helferzell (Th1) Immunantwort , wie Bildung des Entzündungsmarkers Neopterin, Abbau von Tryptophan durch das Enzym Indolamin-2,3-Dioxygenase (IDO) und die Aktivierung des Transkriptionsfaktors Nuklear Faktor kappa B (NF-κB), wurden bestimmt und verglichen. Die Bildung und Neutralisierung von reaktiven Sauerstoffspezies (ROS) und die Einstellung des Gleichgewichtes stellt eine große Herausforderung dar. ROS wird in großen Mengen während der Immunabwehr gebildet. Ein Ungleichgewicht und der Zustand von oxidativem Stress können schädliche Auswirkungen haben. Ein zellbasierter und zellfreier Test wurden verwendet, um die ROS-Neutralisierungseigenschaft von Naturstoffen zu überprüfen. Zwei Zellmodelle, wie mononukleare Zellen des peripheren Blutes und isolierte Monozyten, wurden etabliert, um den Einfluss auf das Immunsystem zu testen.

Aufgrund der immer häufigeren Anwendung von Nanopartikel in der Nahrungsmittelindustrie und auch in alltäglichen Produkten bestand der zweite Teil dieser Arbeit darin, herauszufinden ob die oben genannten Testmethoden auch für die Charakterisierung von Nanopartikel geeignet sind.

Die getesteten Stoffe zeigten unterschiedliche Effekte auf die Neopterin Bildung, den Tryptophan Abbau, die NF-κB Aktivierung und/oder die ROS Konzentrationen.

Chloroquin, ein Anti-Malaria Medikament isoliert aus *Brucea javanica*, besitzt entzündungshemmende Eigenschaften, die durch eine verminderte Tryptophan Abbau-Rate und NF-κB Aktivierung bewiesen wurde. Globularifolin, ein Iridoid isoliert aus *Globularia cordifolia*, bewirkte einen Anstieg von NF-κB in unstimulierten Zellen. Behandlung mit Titandioxid, in unterschiedlichen Kristallstrukturen, resultierte in einem zweiphasigen Effekt. Auch das getestete Bulk-Material, mit größeren Partikeln, zeigte immunologische Einflüsse. Unterschiedliche Struktureigenschaften und Partikelgrößen können die Stärke und auch die Art der Wirkungseffekte beeinflussen.

Um den Einfluss von Nanopartikeln auf das Immunsystem zu überprüfen, ist der Einsatz von beiden Zellmodellen von Vorteil, um die komplexen T-Zell und Makrophagen Interaktionen besser zu verstehen.

Mit den verwendeten Methoden können unterschiedlichste Substanzen auf ihre immunmodulierenden Eigenschaften und Zelltyp-spezifischen Einflüsse analysiert werden.

Abstract

The immune system is a major target of many environmental and lifestyle factors that are known to function collaboratively in order to influence human health. A well-balanced immune system can help to prevent chronic diseases. Exogenous compounds originating from distinct sources including those acquired through nutrition, drug ingestion, environmental pollutants, nanoparticles and other new materials etc. are potential antecedents to altered immune function/immune dysfunction. In order to better characterize the immunomodulatory effects of such substances, it is essential to have adequate model systems that allow the prediction of potential risks or beneficial effects.

The first aim of this thesis was to characterize selected phytocompounds according to their attendant antioxidative and immunomodulatory properties. Central pathways of the T helper type 1 (Th1) immune response were used as readout: formation of the central inflammation marker neopterin, tryptophan breakdown via the enzyme indoleamine 2,3-dioxygenase (IDO) and activation of nuclear factor kappa B (NF-κB) represent suitable markers for gauging how natural compounds are able to influence immune responses. Reactive oxygen species (ROS) are generated in huge amounts after immune activation as a defense strategy, but prolonged oxidative stress can result in deleterious consequences. The capacity of compounds to reduce ROS stress via radical scavenging was assessed in cell-free as well as in cell-based assays. The cell models used in this study were human peripheral blood mononuclear cells (PBMC) and isolated myelomonocytic monocytes (THP-1), both of which were stimulated in order to activate immune responses.

Despite an increasing propensity to utilise nanoparticles in the preparation of everyday products and also in the food industry there remains a paucity of data

concerning the cellular pathways that are impinged on by these agents. Therefore a second aim of this thesis was to investigate whether the aforementioned assays for phytochemicals can be applied to assess the possible effects of nanosized-materials on immune cells.

The different compounds and materials tested showed distinct effects on neopterin formation, tryptophan breakdown, NF-κB activation and/or ROS levels. Briefly, chloroquine, an antimalarial drug derived from *Brucea javanica*, was shown to possess prominent anti-inflammatory activities. This was demonstrated by its ability to suppress tryptophan breakdown and to limit NF-κB activation predominantly in activated immune cells. In contrast, globularifolin, an iridoid compound isolated from *Globularia cordifolia*, led to an increase of NF-κB expression in unstimulated cells. Nanoparticles were found to be able to interfere with the investigated signaling cascades. Interestingly, titanium dioxide, which is present in the form of the two crystal structures rutile or anatase, showed a biphasic effect on IDO activity in PBMC. Different structural properties and particle sizes impact on not only the strength but also the direction of the effect. Of note, bulk material, which contains in addition larger-sized particles, was also found to be active. The use of both PBMC and isolated monocytes turned out to be of particular importance for evaluating the mode of action of nanoparticles as it facilitated a better understanding of the complex T-cell macrophage interaction.

In summary, the methods developed in this study are appropriate metrics for investigating the effects of different materials on central immunobiochemical pathways and furthermore they permit cell type specific responses to be determined and for immunotoxicological effects to be analyzed.

LIST OF CONTENTS

1. Introduction

In modern society we place great emphasis on the pursuit of a salutary lifestyle with exemplary nutrition and a well-functioning immune system being amongst our primary concerns. Poor air quality and pollution are currently rife in urban environments and this coupled with unhealthy lifestyles and exposure to chemicals during daily life is the cause of significant health problems. Maintaining healthiness of the body and the prophylaxis of diseases constitute basic needs and at the same time represent a major challenge for the human body. An efficient, well balanced immune system is essential for human health yet we are constantly exposed to factors with potential to modulate the immune system in unpredictable ways. These variables include pathogens, components of the environment, nutrition, drugs, natural products, phytochemicals and other newly applicable substances such as nanoparticles. An improved understanding of the influences of such frequently encountered exogenous factors on immune function is vital in order to ensure the proper functioning of the immune system and to promote a more salubrious lifestyle.

For many years, research at our department focuses on signal transduction and cell-to-cell communication. Moreover, there is a long-lasting experience in the application of *in vitro* systems for the risk-benefit assessment of natural products, botanical drugs as well as phytochemicals.

During my PhD study, I could participate in several projects (listed below) that aimed to establish new strategies for the estimation of signaling changes activated by exogenous compounds.

FFG 818111 Establishment of *in-vitro* models for a risk-benefit assessment of phytochemicals. *ao.Univ.-Prof. Mag.Dr.rer.nat. Florian Überall,*

FFG 834251 Analysis of bioactive extracts – cascading use of waste from plant harvest and processing. *ao.Univ.-Prof. Mag.Dr.rer.nat. Florian Überall,*

FFG 834169 Cellular and molecular risk assessment of volatile organic compounds from wood-based materials on human cell models using a new type of emission and exposure chamber. *ao.Univ.-Prof. Mag.Dr.rer.nat. Florian Überall,*

FWF P24168 Abiotic stress in extremophiles - strategies and utilization. *Univ. Doz. Dr. Markus Ganzera,*

FWF P25150 Characterization of immunomodulatory effects of nanoparticles *in vitro*. ao.Univ.-Prof. *Mag.rer.nat.Dr.phil. Dietmar Fuchs*

The topic of the doctoral thesis comprises the establishment of appropriate test methods and systems to validate risks and benefits of exogenous factors on the function of the human immune system.

1.1. Influence on the immune response

During human immune response several cell types play a major role to protect the human body against pathogens. T- and B-lymphocytes, natural killer (NK) cells, monocytes/macrophages and dendritic cells (DC) are the most prominent representatives during immune activation responses. A coordinated interplay between innate and adaptive immune system is necessary for an efficient defense mechanism. Mostly T- and B-cells are accountable for the adaptive response, whereas T cells and macrophages are mainly responsible for cell mediated (=Th1-type) immune response, on the contrary B-cells are involved in the humoral

response, where antigen specific antibodies are produced. Different types of immune responses are named by different subsets of T-helper (Th) cells. The differentiation of T-cells into subtypes is suggested to depend on the redox environment and evoke a specific cytokine milieu (Zhou et al., 2009). In general, during oxidative stress conditions, T-cells mainly differentiate into Th1-type cells, whereas under “antioxidative stress” T cells shift towards Th2-type ones (Zaknun et al., 2012, Gostner et al., 2013). Th2-type cells secrete different cytokines such as interleukin-4 (IL-4), IL-5 and IL-13, which are all essential for B-cell activation as well as against helminth infections (Mowen et al., 2004).

Additionally to these two groups, T-cells can differentiate into Th17-cells, which combine adaptive and innate immunity and are involved in the defense strategies against bacteria or fungi (Yu and Gaffen et al., 2008). Th9 subsets are responsible for growth control, activation of mast cells and the production of IL-9 (Schmitt et al., 2013). Th22 cells infiltrate into inflamed skin and produce cytokine IL-22 (Eyerich et al., 2009). A newly identified lineage, namely follicular helper T (Tfh) cells are known to help B-cells (Crotty S 2011). Furthermore, regulatory T-cells (Tregs) are responsible for the maintenance of self-tolerance (Hori et al., 2003). Beside the different subtypes of Th cells, herein we are focusing on Th1-type (cellular) immune response.

1.2. Th1-type immune reaction

During the adaptive defense mechanisms against invading pathogens, Th1-type cells release large amounts of the most prominent Th1-type cytokine interferon-gamma

(IFN-γ), which can induce pro-inflammatory signaling cascades and is able to activate immune responses (Romagnani 2006).

The relevant function of Th1-type cells describes the protection of the host against intracellular infections including viruses or toxoplasma (Abbas et al., 1996, Aliberti et al., 2004). Th1 responses are primary observed after viral or microbial infections, malignant tumor disease as well as allograft rejections after transplantation.

IFN-γ is the main stimulatory cytokine for monocytes and macrophages, which can induce the production of cytocidal ROS as well as induction of pro-inflammatory cytokines (Romagnani 2006, Nathan 1986). IFN-γ induces biochemical pathways by the induction of the enzymes GTP-cyclohydrolase-I (GTP-CH1) and indoleamine 2,3-dioxygenase (IDO). IDO is expressed in macrophages, microglia, neurons and astrocytes (Guillemin et al., 2007) and is a rate-limiting enzyme in the conversion of the essential amino acid tryptophan into kynurenine. IDO can act as a part of an anti-proliferative strategy for immunocompetent cells to inhibit the growth of infected and malignant cells, by the depletion of tryptophan or/and by the production of toxic metabolites (Samelson-Jones et al., 2006). The second enzyme GTP-CH1 leads to the formation of the central inflammation marker neopterin, which can be measured in body fluids like urine, blood and cerebrospinal fluid with an ELISA assay (Fuchs et al., 1992). Furthermore, high neopterin levels are observed with lower levels of antioxidants in human serum (Murr et al., 2009) and high neopterin levels are associated with H_2O_2 release (Nathan et al., 1986) which confirms neopterin to be an indirect marker for oxidative stress.

Neopterin derivates seem to interfere with redox-regulated pathways such as transcription factor nuclear factor-kappa B (NF-κB) signaling. Activation of the central inflammation marker NF-κB is highly inducible by ROS and can further lead to the production of pro-inflammatory cytokines and in parallel induce cell death in target

cells (Asehnoune et al., 2004). A close relationship between NF-κB and the production of neopterin and tryptophan degradation has been observed in the human myelomonocytic cell line THP1-blue *in vitro* (Schroecksnadel et al., 2010).

To summarize, tryptophan breakdown rate, neopterin formation and NF-κB activation represent useful and stable biomarkers to monitor an activated immune system. Therefore appropriate methods have to be explored, which can monitor the above mentioned markers to allow the validation of different natural substances or nanoparticles regarding their immune modulatory and antioxidative properties.

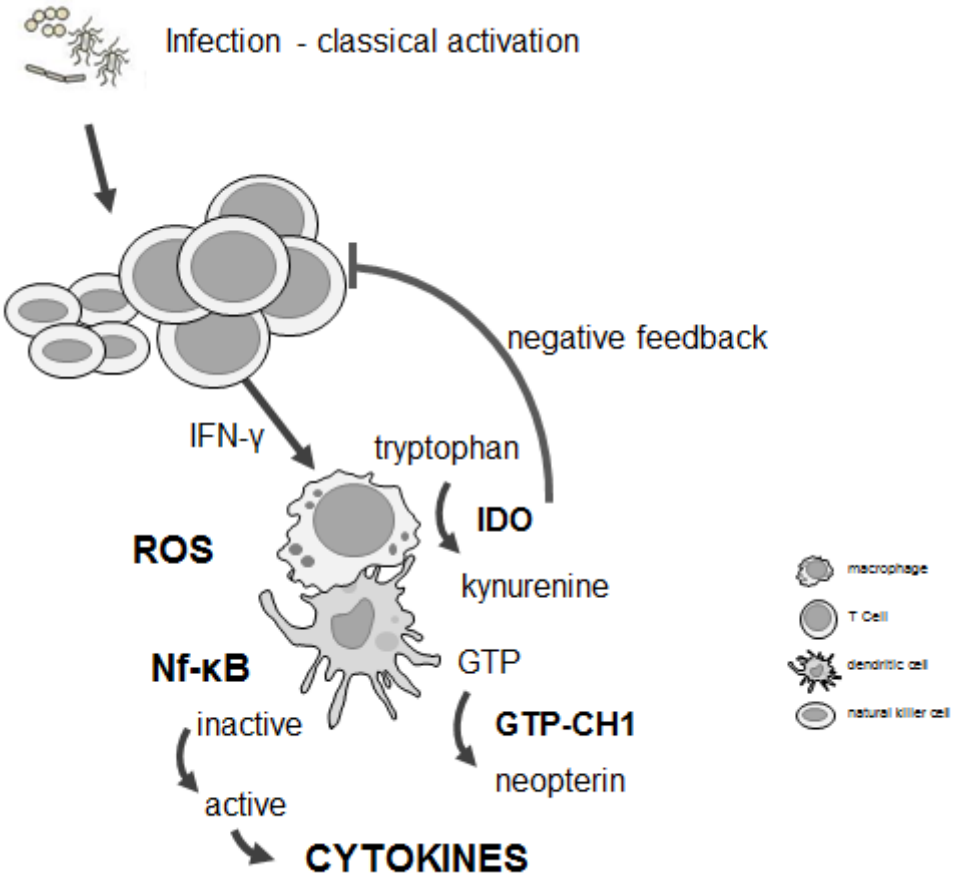

Figure 1: After infection and antigen presentation, T cells produce large amounts of IFN-γ to activate macrophages and dendritic cells. This activation leads to an induction of the enzymes indoleamine 2,3 dioxygenase (IDO) and GPT-cyclohydrolase 1 (GTP-CH1) and cytocidal reactive oxygen species

(ROS). IDO can act also anti-inflammatory by a negative inhibition of T-cell responses. Additionally the central inflammation marker nuclear factor kappa B (NF-κB) gets activated and boosts the production of pro-inflammatory cytokines to promote Th1-type immune reaction (adapted from Schröcksnadel et al., 2006, Gostner et al., 2014b in press, Becker et al., 2013)

1.3. Antioxidants and reactive oxygen species

Antioxidants are reducing agents, which can inhibit or prevent oxidation processes. The function of such an antioxidant is to be a radical scavenger or transform itself into a rather inert radical, which can terminate a radical relevant chain reaction (Brigelius-Flohé et al., 2005). Such an agent can be absorbed from dietary intake or can be produced within the body. They can be obtained from diet since many normal food compounds exhibit antioxidative properties and are especially abundant in fruits or vegetables like bananas, cranberries, apples, dates, red grapes, potatoes, tomatoes as well as in brewed beverages such as coffee, cacao or tea (Zaknun et al., 2012). In the last years ascorbic acid (vitamin C), carotenes (vitamin A), melatonin, glutathione, tocopherols and tocotrienols (vitamin E) have been under certain investigation to neutralize free radicals and exhibit antioxidant capacities to be potent agents for nutritional supplementation (Winkler et al., 2007, Balsano et al,. 2009).

However, a clear beneficial demonstration in disease prevention of antioxidant supplements is not yet available (Stanner et al., 2004). Such preparations are already widely used, most also without any medical indication.

Many clinical trials did not result in an association between antioxidant supplementation and disease preventions, however high dose of antioxidants, which derive from diet or medications could even increase the risk of disease e.g. for

cardiovascular disorders or age-related disease (Zheng et al., 2013, Myung et al., 2013, Bjelakovic et al., 2007) and allergy (Zaknun et al., 2012, Milner et al., 2004).
This can explain why several antioxidant therapies in clinical studies have poor or adverse outcomes and other or further therapeutic approaches should be taken into account (Kris-Etherton et al., 2004, Bjelakovic et al., 2004, Dotan et al., 2009).

Cardiovascular disease, cancer, neurological disorders, diabetes, ischemia/reperfusion or even normal aging processes are characterized by a dysbalance between radical generation and neutralization (Dalle-Donne et al., 2006, Dhalla et al., 2000, Jenner P., 2003). Various enzymatic and non-enzymatic antioxidant defense systems can protect the cell against reactive oxygen species and reactive nitrogen species (ROS/RNS) attacks (Cadenas E., 1997). (I) Endogenous antioxidant enzymes, such as catalase (CAT), gluthathione reductase (GSR) or superoxide dismutase (SOD) as well as (II) non-enzymatic systems, such as glutathione (GSH), urate and coenzyme Q, can neutralize ROS. In addition, (III) exogenous molecules, like vitamins or various free amino acids, peptides and proteins serve as low molecular weight antioxidants (Valko et al., 2006). All these enzymes and molecules are able to convert ROS into stable and harmless compounds via redox-based mechanisms.
Low levels of ROS, which occur under normal conditions in the human body, are a byproduct of biological reactions. The main intracellular sources of ROS (O2•-) are the mitochondrial respiratory chain (Le Bras et al., 2005), xanthine oxidase (McNally et al., 2003), lipid peroxidases (Zhang et al., 2002), cytochrome P450 enzymes (Fleming et al., 2001) and the uncoupled endothelial NO synthase (Vasquez-Vivar et al., 1998).

At high concentrations radicals can be hazardous for cells, while at moderate concentrations ROS play an important role in many signaling processes as regulatory mediators such as induction of stress responses or detoxification or immune activation (Gostner et al. 2013, Valko et al., 2007). Furthermore ROS are utilized in the induction and maintenance of signaling pathways, which are involved in cell growth and differentiation. A high level of ROS/RNS, produced by respiratory burst, has also an important role in the defense strategy of pathogens and invading agents (Decoursey et al., 2005). However high concentrations of ROS can be hazardous and damage not only target cells. Thus ROS generation, release and neutralization has to be tightly regulated during immune response (Valko et al., 2007).

Elevated oxidative stress levels are suggested to be involved in the development as well as in the progression of chronic diseases. To support the cellular antioxidant machinery in detoxification and neutralization of ROS/RNS, an uptake of antioxidant therapeutics, supplements but also antioxidant rich diet was suggested to be beneficial. However it has to be regarded with suspicion due to the possible adverse effects.

Extra uptake of antioxidants for protecting the body´s defense mechanisms might not only support the development of allergies but also disturb a successful immune response, by neutralizing ROS/RNS and thereby disturb the normal negative feed-back loops of anti-proliferative mechanisms, which are induced during the Th1-type immune responses. Several studies result in an influence of naturally derived antioxidants on Th1-type immune response, by focusing on tryptophan breakdown and neopterin production *in vitro* (Jenny et al., 2011, Becker et al., 2013).

1.4. Natural substances in general:

Botanical drugs, defined as clinically validated pharmaceuticals of plant origin, consist mainly of several constituents and are named as multi-components. Defined by the Food and Drug Administration (FDA), they contain ingredients from fresh or dried plants, isolated or combined chemical components of plant origin, algae, macroscopic fungi, or combinations thereof. They are widely used for diagnosis, cure, mitigation, treatment or prevention of diseases in humans. Since ancient times, plant-derived remedies are frequently used in folk medicine and traditional medicinal systems, used as therapeutic agents for pathologies like several inflammatory diseases such as infections, fever, or pain (Schmidt et al. 2008, Zvetkova et al., 2001). Natural products have long been contributed to the development of modern therapeutic drugs (Bellik et al., 2012). Approximately 25% of the modern medications are developed from plants (Liu et al., 2008). Much interest has been generated for phytochemicals, like phenols, alkaloids and terpenoids, with a role in the modulation of inflammatory responses.

Botanical drugs are multicomponent preparations, which are composed of many different compounds originated from different structural classes (Gostner et al., 2012b). The advantage of such multicomponent mixtures represents the observation that some mixtures possess prominent pharmacological properties at low or non-toxic concentrations. The potent activity results from the combination of the effects on different cellular targets (Gostner et al., 2012b). The summarized effect can be due to addition, synergism or antagonism of the single compounds (Wagner et al., 2009).

In order to give some insight into the bioactivity of botanical products, it is important to differentiate between the most important structural classes of pharmacologically active substances from plants. These substances are mostly secondary metabolites

of different structural classes synthesized as chemical signals enabling the plant to respond to environmental signals or function in the defense against herbivores, pathogens, bacteria fungi or competitors (Wink M., 2008).

One can differentiate between two big groups of secondary metabolites: (I) nitrogen containing metabolites and (II) metabolites without nitrogen (Wink M., 2008). The biggest groups with nitrogen are alkaloids, which are a very prominent class with underlying effects ranging from anticholinergics, pain-relieving properties, antiparasitic to anticholinesterase activities.

The most prominent classes without nitrogen are terpenoids and steroids, which contribute equally to human health and are used in medicine and toxicology. They are able to inhibit the most important ion pump Na^+, K^+-ATPase in animal cells (Wink M., 2008). Several anti-inflammatory compounds and antineoplastics are representatives of this group (Han Y., 2005, Paduch R., 2007, Quintans-Junior L., 2011, Hirota et al., 2010, Chen et al., 2013).

Phenols (mostly phenylpropanoids) like aspirin and podophyllotoxin contributed to more application of phytochemicals in modern medicine. It is important to note, unlike the mono-compound drugs, the pharmacological activity cannot be assigned to a single secondary metabolite in multi-component botanical drugs and extracts (Wink M., 2008). The fact that extract´s activity may be the result of potentiating effects of several compounds became clear, as it happened to be lost or reduced during the isolation or fractionation processes (Schmidt et al., 2007). Thus, compounds exert their bioactivities by interacting with other molecules rather than by acting alone. Another important point is that the characteristic and function of some phytochemicals is still not fully explored. Developing innovative scientific methods for validation, characterization and standardization of multicomponent botanical therapeutics is of utmost importance to gain acceptance into the mainstream

medicine (Gostner et al. 2012b). Thus, the effect of a single molecule in multicomponent mixtures can be only small, but the summation of all effects leads to a potent outcome of the mixture.

A major goal is the discovery of novel anti-inflammatory and anti-allergic agents (Bellik et al., 2012). Several drugs of plant origin are in clinical use or in Phase II and III clinical trials. Examples are the green tea derived epi-gallocatechin-3-gallate, which is currently in clinical trials for treatment of Alzheimer´s disease (Bleiholder et al., 2013). Furthermore, clinical trials are running for resveratrol and its anti-carcinogenic effects for treatment and prevention of cancer (Gescher et al., 2013) and curcumin with predicted effects on several pathologies such as artheriosclerosis, inflammatory bowel disease, psoriasis as well as colon or pancreas cancer (Goel et al., 2008).

Plant derived secondary metabolites persist of various classes of chemical structures and can therefore interact with different biological targets, which e.g. can be involved in inflammation or inflammatory processes. The matter of importance for the search of inflammation inhibiting compounds represents a significant work.

Therefore it is of major importance to establish appropriate screening methods to explore possible immune modulating actions of plant-derived compounds and extracts, to open new advantages for the usage as therapeutic options as well as additive treatments in broad range of immunological disorders (Becker et al., 2013).

Finding safer and more effective botanical application forms for several diseases represents a major challenge of researchers worldwide. One possible strategy is the use of nanostructured form of drugs, vaccines, herbs or natural products (Bell et al., 2014)

The common ground of the naturally occurring components of plants presents their antioxidant and anti-inflammatory properties. Antioxidants are known to inhibit or prevent oxidation processes and are able to disturb radical driven chain reactions in the human body. Such antioxidant compounds can be produced within the body or can be absorbed from dietary intake. In general antioxidants are moreover considered to exert anti-inflammatory, anti-aging and health promoting effects in the human body (Young et al., 2001. Halliwell B., 1996) However, a prevalence of antioxidants can also promote adverse effects such as the development of allergies or asthma (Zaknun et al., 2012, Milner et al., 2004).

The newly designed nanoparticles, which are currently often used e.g. in therapeutics as drug delivery agents, in food for optical purposes, in surgical implants for corrosive protection or occurring in air as environmental pollutant, however the so far consequences for the immune system and the eventually negative impacts, are still unclear and too little investigated. The lack of adequate test systems poses the question if the established methods, which are still used for natural products, can be applied to validate nanoparticles?

1.5. Nanoparticles

The use of nanoscale materials has grown enormously in the last years, and the engineered nanoparticles are commonly used for economic, scientific and therapeutic purposes, however the hazards to humans cannot be ignored and represent new health risks upon interacting with the biological system. Nanotechnology is associated with engineering, information technology or diagnostic applications and is

used for different objects like electronics, computers, cosmetic or food and consequently penetrated deeply into our lives (Roy et al., 2013).

Nanoparticles are small sized particles in the range of 1-100 nm with a huge increase in their surface area to their surroundings and exhibit high catalytic properties (Roy et al., 2013). Due to the small size, nanoparticles are able to penetrate cells and interact with vital cellular mechanisms or macromolecules such as DNA (Singh et al., 2009) and can easier move through the body after inhalation, ingestion, injection or dermal contact compared to bigger size particles. Many of the particles are chemical inert in their macro-scale form, however in nano-scale they are highly reactive (Hutter et al., 2010). For example silver particles exert antimicrobial properties as well as gold particles possess high efficient catalyst properties (Hutter et al., 2010, AshaRani PV., 2009).

Humans are consistently exposed to particles in the atmosphere, e.g. diesel exhaust particles, fly ashes or carbon black (Evelyn et al., 2003). Therefore, inhalation and uptake via the respiratory tract represents the most frequent route of airborne nanoparticles. Other ways of penetration illustrates entering via skin pores, debilitated tissues, injection, olfactory or intestinal tracts (Yah et al., 2012).

Due to insufficient toxicity studies and their unknown implication on humans regarding their different physicochemical properties, more information about their effect patterns is needed.

Nanoparticle toxicity is strongly related to oxidative stress generation, alteration of calcium homeostasis, gene expression modification, production of pro-inflammatory cytokines and changes in cellular signaling events (Roy et al., 2013). Various studies confirmed that nanoparticles are able to generate ROS and produce pro-inflammatory cytokines and thus potentially increase disease development.

ROS generation and oxidative stress is involved in various pathologies, thus nanoparticles are able to induce cellular damage by oxidative stress overbalance or single strand breaks (Rothen-Ruthishauser et al., 2007). Therefore nanoparticles probably represent a potential risk for human health.

Another aspect of nanoparticle toxicity illustrates the surface properties of the different types of particles. The functionalization and intentional modification of engineered particles can influence the toxicity of nanoparticles and the interaction with the biological system. Due to their small size, nanoparticles are able to cross cell membranes and pass the pulmonary epithelial barrier and are able to reach the bloodstream and finally get in contact with immune cells (Rothen-Ruthishauser et al., 2007). Interaction and binding with biomolecules such as DNA, small peptides or proteins, the physicochemical properties such as size, surface area and charge are of major importance. The binding of nanoparticles to proteins, due to electrostatic, hydrophobic and specific chemical interactions, is accountable for the final distribution inside the body (Roy et al., 2013). Cell membranes with large negatively charged domains can be a potent barrier for negatively charged nanoparticles to interact with each other. Furthermore surface properties of the particles can be crucial for the interactions with cells. Particles with negative zeta potential seem to have a barrier for cellular uptake and negatively charged cells could repel them. However particles with positive zeta-potentials can bind to several molecules including DNA and proteins and may influence the distribution of nanoparticles throughout the body. When they are bound to proteins, they may be quickly cleared by macrophages and cannot reach other immune cells (Patil et al., 2007).

These unique properties can open possibilities for researchers to design nanoparticles for drug delivery and therapeutic approaches due to their immune

stimulatory property (Aggarwal et al., 2009). Currently, nanoparticles are tested for different therapeutic approaches, e.g. *in vitro* tests with curcumin loaded silica nanoparticles for breast cancer therapy (Ma´mani et al., 2014), and zinc oxide as delivery substance of various cancer treatment agents (Sardar et al., 2014). Silva et al., reported anti-bacterial activity of encapsulated nanoparticles with tropical fruit products against listeria or E. coli strain K12 (Silva et al., 2014).

Due to the huge diversity of different available types of nanoparticles, we selected an important representative, which is commonly used in various daily life products.

TiO_2 nanoparticles are one of the best studied nanomaterials; it is present in many food products, chemicals, electrical or electronic goods. TiO_2 particles are mostly used as white pigments in many foods and interestingly the most commonly use of these particles is as photosensitizers in a so called photodynamic therapy for cancer patients (Zhang et al., 2014). Actually TiO_2 is used in various consumer products such as sunscreens for UV protection, white dye in food or tooth paste or in dental and surgical implants and are used as heat generator agent in magnetic hyperthermia therapy (Lee et al., 2010). The diverse crystal structure (anatase, rutile and brookite) represents a specialty of titanium dioxide. However, only rutile and anatase are commonly used for TiO_2 applications and are biological active (Diebold U., 2003). Until now several studies indicated influences on inflammatory responses as activation of toll like receptors (TLR) and NF-κB (Zhu et al., 2014, Cui et al., 2011) as well as release of the pro-inflammatory cytokine IL-1B (Yazdi et al., 2010)
From this point of view, the established assays can estimate the possible effects of nanostructured particles, and as a result more insights in immunobiochemical pathway changes can be observed.

2. Specific Aims and Results

In order to know more about the risks, benefits and the mode of action of exogenous factors of distinct origin such as chemicals, food, drugs, and dietary supplement it is of utmost importance to establish several methods and model systems to validate the properties and allow the prediction of potential risks or beneficial effects regarding their antioxidant and anti-inflammatory activities. The tests should include the characterization of the radical scavenging ability and the possible property to influence central pathways of the Th1 immune responses.

Plant constituents are able to interfere with regulatory mechanisms, which are part of the inflammatory signaling cascade and have an influence on immunological responses. Therefore the characterization of phytochemicals and their interaction with the immune system is of utmost importance to estimate the potential harmful consequences.

Beside the neutralization and/or formation of ROS, several plant-constituents can have an influence on further signaling pathways and gene expression of e.g. cytoprotective enzymes (Sakar et al., 2009) or activate the central inflammation marker NF-κB.

Due to the participation in different projects with natural substances and the general increasing focus on healthy nutrition and food the first aim of the thesis represents the question

2.1. Is it possible to find appropriate methods for the characterization of natural substances regarding their antioxidant and immunmodulating properties?

With this part the mode of action of single or multi-components as well as newly designed drugs and chemicals regarding their influences on redox-regulated signaling cascades can be determined. Furthermore several food and dietary supplements, which are declared as preventive and “healthy” can be distinguished in a convenient point of view.

Different methods were established and able to verify antioxidant and immunomodulatory properties of natural substances.
In combination of all these assays every type of chemical and substance can be monitored regarding their influence on redox regulated pathways.

The published review will give an overview about the appropriate methods, which can be used to screen compounds on cell-free as well as cell based models. Peripheral blood mononuclear cells (PBMC) can be used to have a closer look on human immune response. The human myelomonocytic leukemia cell line (THP-1) can show influences on isolated monocytes directly. These cell model systems can indicate how natural compounds can influence the complex T-cell - macrophage interaction. To determine the antioxidant capacity of natural products a cell based as well as cell-free assay are applied.

2.1.1. Comparison of *in vitro* tests for antioxidant and immunomodulatory capacities of compounds

Becker et al., 2014a

Phytomedicine. 2014 Jan 15;21(2):164-71.

doi: 10.1016/j.phymed.2013.08.008.

Epub 2013 Sep 14.

The published methods have been used to characterize the naturally occurring acylated iridoid glucoside globularifolin and the antimalarial drug chloroquine. The effects of the two compounds on immune responses and also the ability to scavenge free radicals have been published in two following peer-reviewed papers.

2.1.2. Effects of globularifolin on cell survival, nuclear factor-κB activity, neopterin production, tryptophan breakdown and free radicals *in vitro*

Sipahi et al., 2014

Fitoterapia. 2014 Jan;92:85-92.

doi: 10.1016/j.fitote.2013.10.012.

Epub 2013 Nov 1.

2.1.3. Antimalarial drug chloroquine counteracts activation of indoleamine (2,3)-dioxygenase activity in human PBMC

Gostner et al., 2012a

FEBS Open Bio. 2012 Aug 17;2:241-5.

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Antimalarial drug chloroquine counteracts activation of indoleamine (2,3)-dioxygenase activity in human PBMC

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ABSTRACT

Antimalarial chloroquine is also used for the treatment of immune-mediated diseases. The interference of chloroquine with interferon-γ-induced tryptophan breakdown and neopterin production has been investigated in human peripheral blood mononuclear cells (PBMC) *in vitro*. Micromolar concentrations (2–50 μM) of chloroquine dose-dependently suppressed mitogen-induced tryptophan breakdown in PBMC but not in the myelomonocytic THP-1-Blue cell line, after 48 h of treatment. In stimulated PBMC, neopterin production was super-induced by 10 μM chloroquine, while it was significantly suppressed at a concentration of 50 μM. These anti-inflammatory effects may relate to the therapeutic benefit of chloroquine in inflammatory conditions and may widen the spectrum of its clinical applications.

1. Introduction

Since the 1930s, chloroquine [(RS)-N′-(7-chloroquinolin-4-yl)-*N*,*N*-diethyl-pentane-1,4-diamine] is a widely used antimalarial drug, mainly due to its relatively good tolerability and the cost-efficient synthesis [1,2]. Upon cellular uptake, chloroquine accumulates in acidic organelles such as endosomes, lysosomes and in Golgi vesicles, where it interferes with the activity of enzymes and posttranslational protein modification steps [3,4]. Furthermore, drug accumulation decreases the intracellular iron concentration and induces oxidative stress [5,6]. Accumulated in the digestive vacuoles of parasite affected cells, chloroquine inhibits the malaria parasite's digestive pathway for hemoglobin [7]. The development of resistant *Plasmodium* strains has now led to the replacement of the first-line treatment and prophylaxis with chloroquine and its derivatives with other therapeutics such as artesimin [8,9]. However, chloroquine and analogs became interesting as treatment options for other immune-related disorders, due to their immunomodulatory properties, e.g. by interfering with pro-inflammatory cytokine secretion [10,11]. On the basis of its anti-inflammatory properties, chloroquine and hydroxychloroquine are used for the treatment of rheumatoid arthritis, discoid lupus erythematosus, amebic hepatitits and chronic Q fever [2,12–15].

The pro-inflammatory cytokine interferon-γ (IFNγ) plays a central role in the cellular immune response, as it induces several immune-regulatory pathways and cellular responses [16,17]. Inflammation is further characterized through the activation of the redox-sensitive transcription factor nuclear factor-kappa B (NF-κB). NF-κB regulates a variety of genes that control immune responses such as the pro-inflammatory cytokines [18]. IFNγ stimulates also the production of neopterin by guanosine triphosphate (GTP)-cyclohydrolase-I (GTP-CH-I, EC 3.5.4.16) in macrophages [19]. Likewise, neopterin was found to support oxidation processes by reactive oxygen and chloride metabolites [20] as well as their formation in inflammatory cells like neutrophils [21]. In turn, in target cells redox-sensitive signaling cascades such as nuclear factor-κB are triggered by neopterin [22]. IFNγ signaling is also involved in the activation of indoleamine 2,3-dioxygenase (IDO, EC 1.13.11.52), the enzyme that catalyses the rate-limiting step in the conversion of tryptophan to kynurenine [23]. Neopterin levels, tryptophan concentrations and IDO activity have been successfully used to monitor cell-mediated immune activation and to reveal prognostic information in a variety of diseases, including rheumatoid arthritis [24–26].

Elevated concentrations of urine and serum neopterin have been detected in patients infected with *Plasmodium falciparum* and *Plasmodium vivax* from epidemiologically distinct populations [27–30]. Similarly, increased breakdown of tryptophan has been reported in a murine malaria model [31].

A more detailed analysis of potential interactions of chloroquine with interferon-γ-induced tryptophan breakdown and neopterin

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production might help to explain the beneficial effects for the treatment of rheumatic diseases and might introduce new therapeutic regimen for disorders that are associated with increased immune activation.

Therefore, the aim of this *in vitro* study was to investigate the immunomodulatory properties of chloroquine in human peripheral blood mononuclear cells (PBMC) and in THP-1-Blue cells. Mitogen-stimulated PBMC represent a widely used model to evaluate pro- and anti-inflammatory properties of compounds, where neopterin production and tryptophan degradation can be used as read-outs [32]. The THP-1-Blue cell line is transfected with a NF-κB/AP-1-inducible reporter system that allows the monitoring of NF-κB activity in cell supernatants. In this cell line, lipopolysaccharide (LPS)-induced NF-κB expression has been reported to correlate with neopterin production and IDO activity [33]. Further, the production of soluble interleukin 2 receptor alpha (sIL-2Rα) was used to monitor the influence of chloroquine on the inflammatory process [32,34].

2. Materials and methods

2.1. Chemicals

Lipopolysaccharide (LPS) of *Escherichia coli*, phytohemagglutinin (PHA) and chloroquine were obtained from Sigma–Aldrich (Vienna, Austria). The latter was dissolved in RPMI 1640 medium (MedPro, Vienna, Austria) before each experiment.

2.2. Isolation and culture of human PBMC

PBMC were isolated from whole blood obtained from healthy donors by density centrifugation (Lymphoprep, Nycomed Pharma AS, Oslo, Norway). After isolation, PBMC were washed three times in phosphate buffered saline containing 0.5 mM EDTA. Cells were maintained in RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum (FCS, Biochrom, Berlin, Germany), 2 mM L-glutamine (Serva, Heidelberg, Germany) and 50 μg/ml gentamicin (Bio-Whittaker, Walkersville, MD) at 37 °C with 5% CO_2.

2.3. THP-1-Blue cell culture

THP-1-Blue cells (Invivogen, San Diego, USA) were incubated at 37 °C with 5% CO_2 in RPMI 1640 medium supplemented with 10% FCS and 200 μg/ml zeocin (Invivogen, San Diego, USA).

2.4. Cell treatment

PBMC were seeded at a density of 1.5×10^6 cells/ml in supplemented RPMI 1640, preincubated for 30 min with or without different concentrations of chloroquine (2.0–50 μM) and stimulated, or not, with 10 μg/ml PHA for another 48 h. THP-1-Blue cells were seeded at a density of 5×10^5 cells/ml in supplemented RPMI 1640. Cells were preincubated for 30 min with different concentrations of chloroquine (6.25–50 μM) and stimulated or not with 1 μg/ml LPS.

Supernatants for Kyn/Trp determination were collected after 48 h, after this period the accumulated tryptophan breakdown and neopterin formation reaches a plateau [32]. Supernatants from THP-1-Blue cells for NF-κB activity measurement were collected for both, 24 and 48 h treatment duration. However, in agreement with earlier observations, the read-out at 24 h was able to better discriminate results obtained with different concentrations of compounds [33].

Table 1
Concentrations of tryptophan, kynurenine, kynurenine to tryptophan ratio (Kyn/Trp) and neopterin in the supernatant of unstimulated PBMC and in cells stimulated with 10 μg/ml PHA for 48 h. Results shown are the mean values ± SEM of three independent experiments run in duplicates.

	Tryptophan (μM)	Kynurenine (μM)	Kyn/Trp (μmol/mmol)	Neopterin (nM)
Unstimulated	29.9 ± 6.2	2.4 ± 0.9	99.2 ± 46.6	3.8 ± 0.1
PHA (10 μg/ml)	1.9 ± 1.1*	14.3 ± 0.7*	12408 ± 4379*	20.1 ± 1.4*

* $p < 0.05$, compared to unstimulated cells.

2.5. Measurement of tryptophan, kynurenine, neopterin and sIL-2Rα concentrations

Tryptophan, kynurenine, neopterin and cytokine measurements were performed in centrifuged supernatants. Tryptophan and kynurenine concentrations were measured by high performance liquid chromatography (HPLC) using 3-nitro-L-tyrosine as an internal standard [35]. To estimate IDO activity, Kyn/Trp was calculated and expressed as μmol kynurenine/mmol tryptophan.

Neopterin concentrations were determined by ELISA (BRAHMS, Hennigsdorf, Berlin, Germany) with a detection limit of 2 nM, IL-2Rα concentrations were measured by ELISA obtained from R&D (Biomedica, Vienna, Austria), both according to the manufacturer's instructions.

2.6. Measurement of NF-κB activity and cell viability in THP-1-Blue cells

THP-1-Blue cells are stably transfected with a reporter plasmid expressing the secreted embryonic alkaline phosphatase (SEAP) under the control of a NF-κB/AP-1 inducible promoter. Upon NF-κB activation, SEAP is expressed and released into the media, where the enzyme activity has been measured in a colorimetric assay at 635 nm (Bio-Tek Instruments, Bad Friedrichshall, Germany) by incubating 10% (v/v) of cell supernatant with 90% (v/v) of Quanti-Blue reagent (Invivogen, San Diego, USA).

Cell viability was measured by CellTiter-Blue® assay (Promega, Vienna, Austria) according to the manufacturer's instructions.

2.7. Statistical analysis

For statistical analysis, the Statistical Package for the Social Sciences (version 19, SPSS, Chicago, Ill, USA) was used. Because not all data sets showed normal distribution, for comparison of grouped data non-parametric Friedman test and Wilcoxon signed-ranks test were applied. *p*-Values below 0.05 were considered to indicate significant differences.

3. Results

3.1. Effect of chloroquine on tryptophan metabolism and neopterin formation in PBMC

After an incubation period of 48 h, neopterin concentrations (±SEM) in culture supernatants of unstimulated PBMC were 3.8 ± 0.1 nM and the mean Kyn/Trp ratio was 99.2 ± 46.6 μmol/mmol. Upon stimulation of cells with the phytohemagglutinin (PHA), neopterin production increased to 20.1 ± 1.4 nM and the mean Kyn/Trp was increased approximately 100-fold. The concentrations of neopterin, tryptophan, kynurenine and IDO activity, indicated by Kyn/Trp, in unstimulated and PHA-stimulated PBMC are listed in Table 1.

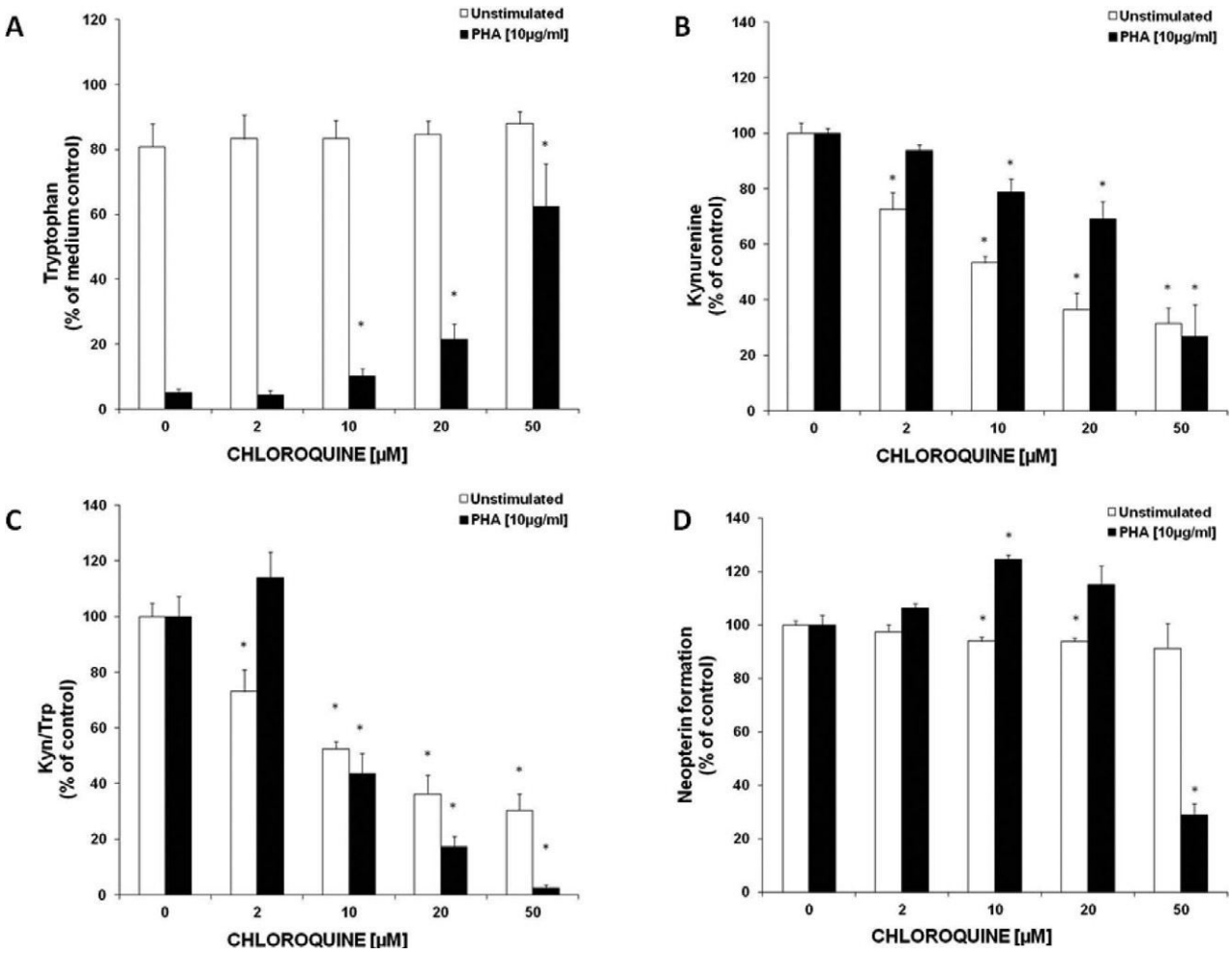

Fig. 1. Effect of 48 h of chloroquine treatment on tryptophan (A) and kynurenine (B) concentrations, on IDO activity expressed as kynurenine to tryptophan ratio (Kyn/Trp) (C) and on neopterin production (D) in unstimulated (white bars) and PHA-stimulated PBMC (black bars), expressed as % of baseline (control cells treated with or without PHA, respectively). Results shown are the mean values ± SEM of three independent experiments run in duplicates (*$p < 0.05$, compared to cells without added chloroquine).

In unstimulated cells, tryptophan concentrations were not affected by chloroquine addition (2.0–50 μM of chloroquine, Fig. 1A), but kynurenine levels decreased in a dose-dependent manner (Fig. 1B). In PHA-stimulated PBMC, chloroquine suppressed tryptophan breakdown in a dose-dependent manner (Fig. 1A) and in parallel kynurenine levels declined (Fig. 1B). A reduction of the Kyn/Trp upon chloroquine treatment was dose-dependent in both, unstimulated and PHA-stimulated PBMC, with a more pronounced effect in PHA-stimulated cells (Fig. 1C).

The addition of chloroquine to unstimulated PBMC resulted in an only slight suppression of neopterin concentrations in culture supernatants, e.g. 20 μM chloroquine suppressed neopterin production (±SD) to 93.8 ± 3.5% of baseline. In PHA-stimulated cells, the neopterin formation was significantly increased at a concentration of 10 μM chloroquine (124.5 ± 4.0%), while 50 μM of chloroquine resulted in a strong decrease of neopterin concentrations down to 29.0 ± 10.4% compared to PHA-stimulated control cells (Fig. 1D).

3.2. Effect of chloroquine on sIL2Rα secretion in PHA-stimulated PBMC

IL2Rα concentrations in cell culture supernatants were increased more than 100-fold in PHA-stimulated PBMC in comparison to unstimulated cells ($p < 0.005$), additional chloroquine treatment (10 and 50 μM) reduced this effect in a dose-dependent manner ($p < 0.05$ and 0.005 for 10 and 50 μM chloroquine- and PHA-stimulated PBMC in comparison to PHA-stimulated control, details not shown).

3.3. Effects of chloroquine in THP-1-Blue cells

Cytotoxicity of chloroquine was evaluated in THP-1-Blue cells treated with increasing doses for 24 h, with or without additional stimulation by lipopolysaccharide (LPS). Treatment resulted in a dose-dependent decrease of cell viability with IC_{50} values of 63.16 μM in unstimulated and 54.35 μM in stimulated cells.

After an incubation of 48 h, the Kyn/Trp (±SEM) was significantly increased in LPS-stimulated THP-1-Blue cells (96.4 ± 12.6 μmol/mmol) in comparison to unstimulated cells (20.4 ± 1.1 μmol/mmol). There was no effect on Kyn/Trp upon treatment of THP-1-Blue cells with chloroquine for both, LPS-stimulated and unstimulated cells (data not shown).

Upon 24 h of LPS stimulation, the activation of NF-κB according to SEAP activity was increased (9.60 ± 1.18 (SEM)-fold) in comparison to unstimulated cells ($p < 0.005$). The additional treatment with chloroquine decreased SEAP activity in a dose-dependent manner in LPS-stimulated cells (SEAP activity expressed as fold of unstimulated control: 12.5 μM = 6.77 ± 0.84, 50 μM = 4.24 ± 0.63, $p < 0.005$), while unstimulated cells were not affected (Fig. 2).

4. Discussion

In this study, the capacity of chloroquine to modulate immune responses in human PBMC and in myelomonocytic THP-1-Blue cells was investigated *in vitro*. In PBMC, both stimulated or not with the

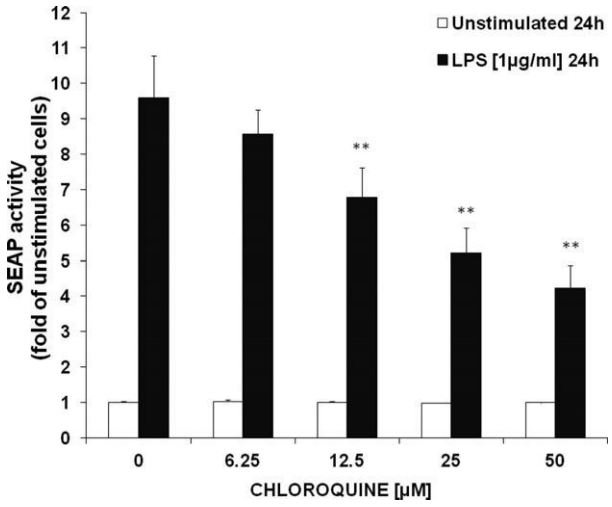

Fig. 2. Effect of chloroquine on the enzyme secreted embryonic alkaline phosphatase (SEAP) as a measure of nuclear factor-kappa B (NF-κB) activation in unstimulated (white bars) and lipopolysaccharide (LPS)-stimulated (black bars) THP-1-Blue cells, after 24 h of treatment. Results shown are the mean values ± SEM of six independent experiments performed in duplicates ($^{**}p < 0.005$, compared to baseline = control cells treated with or without LPS, respectively).

mitogen PHA, chloroquine reduced Kyn/Trp effectively and in a dose-dependent manner already at low concentrations. Also, IL-2Rα secretion was decreased in mitogen-stimulated cells upon chloroquine treatment.

In agreement with our study, a variety of effects of antimalarial drugs on immune-mediated mechanisms have been described such as the suppression of pro-inflammatory cytokine production. Chloroquine has been shown to decrease tumor necrosis factor-α (TNF-α), IFNγ, interleukin-1 (IL-1) and 6 (IL-6) secretion PBMC and malaria patient blood samples [9,11], to reduce monocyte receptor expression [36] and to regulate monocyte GTP-cyclohydrolase-1 expression [37].

Only recently, He et al. reported a new mechanism underlying the anti-inflammatory activity of chloroquine, whereby glucocorticoid signaling is activated and thereby promotes the transrepression of pro-inflammatory cytokines in a mouse collagen-induced arthritis model [38]. Our finding, that chloroquine interferes with T-helper cell type-1 immune response *in vitro* by repressing tryptophan breakdown in PHA-stimulated PBMC at low micromolar concentrations, represents an additional strategy to inhibit inflammation. Other nonsteroidal anti-inflammatory substances, such as acetylsalicylic acid, and corticosteroid drugs, such as prednisolone, have been reported to inhibit tryptophan breakdown and also neopterin production [39,40]. Interestingly, in our study, neopterin production decreased significantly only at the highest dose of 50 μM chloroquine in PHA-stimulated PBMC, while it was significantly increased with 10 μM.

Of note, chloroquine had no effects on the tryptophan metabolism in unstimulated as well as LPS-stimulated THP-1-Blue cells at the tested concentrations. In LPS-stimulated THP-1-Blue cells, chloroquine was able to reduce NF-κB/AP-1 driven reporter gene expression in a dose-dependent manner. The decrease of NF-κB activation goes in parallel with a decrease in cell viability. Cepika et al. reported that chloroquine had no effect at low micromolar concentrations (2 μM) in monocyte populations of PBMC upon LPS challenge, and suggested that chloroquine regulation of cytokine secretion in monocytes is exerted only at high toxic concentrations, while lower doses affect other signaling pathways and upregulation of costimulatory molecules [41]. On the contrary to monocytes, in human astroglial cells, chloroquine alone, without additional stimulus, was able to induce activation of NF-κB and subsequent expression of pro-inflammatory genes [42]. Therefore, the effect of chloroquine on NF-κB activation remains elusive.

In conclusion, our *in vitro* study shows that chloroquine treatment results in the suppression of distinct mechanisms in mitogen-stimulated PBMC in comparison to toll like receptor (TLR) stimulated monocytic THP1-Blue cells. Data indicates that chloroquine might have stronger influence on IDO activity by acting on cytokine secretion of T-cells than by acting on THP-1-Blue monocytes. Although THP-1-Blue cells are at an advanced stage of myelomonocytic development and their responsiveness to LPS has been extensively examined, they represent an undifferentiated phenotype [33,43]. The use of macrophages that have an exaggerated response to LPS and act differently than monocytes, might give more insight into chloroquine's mode of action.

However, our findings might be of importance for the discussion about the use of chloroquine for the treatment of other disorders associated with overwhelming immune response. The application of chloroquine and its analogs has already been proved efficient in the treatment studies of autoimmune disease, e.g. in systemic lupus erythomatosus [12,13]. Furthermore, there are clinical trials ongoing with chloroquine and its derivatives that explore their potential for the treatment of viral infections and cancer, with human immunodeficiency virus (HIV) infection being the most studied disease [44–47]. Our results support the view that a more detailed analysis of chloroquine activities in further *in vitro* and *in vivo* studies will be of central importance to explore additional potential therapeutic regimes in other chronic inflammatory conditions such as coronary heart disease or neurodegeneration.

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By reasons of poor air quality and higher pollution and due to the newly and rapid increasing application of nanoparticles in modern food and life, and the so far poorly performed risk and toxicological studies of nanoparticles regarding their influences on immunobiochemical signaling cascades the second part of the thesis consist the question:

2.2. Are the established methods moreover useful for the risk and benefit assessment of nanoparticles?

This part contributes to an important task for classification and characterization of nanoparticles regarding their pro- or antioxidant as well as pro- or anti-inflammatory properties.

The part was composed of the implementation of the published assays with regard to different types of nanoparticles. The part should confirm the feasibility of the established assays to monitor the potential influences on immune responses and ROS dependent cascades.

Therefore different types of nanoparticles (titanium dioxide, silver, aluminiumoxide, zinkoxide, carbon nanotubes), which are frequently used in daily life products, were tested with the previously published methods. The first results of TiO_2, which were utilized in three different types of particles sizes and formulations (bulk material with random particle size, 10 nm size and 23 nm size), on PBMC and isolated monocytes have already been published. The different tested particle sizes can determine, whether the chemical structure or solely the size have an influence on the immune modulatory properties of the particles.

The findings of this part will be explained in the two following publications either on PBMC or THP1 cells.

2.2.1. TiO_2 nanoparticles and bulk material stimulate human peripheral blood mononuclear cells

Becker et al., 2014b

Food Chem Toxicol. 2014 Mar;65:63-9.

doi: 10.1016/j.fct.2013.12.018.

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2.2.2. Effects of TiO2 nanoparticles on human myelomonocytic cell line THP-1

Becker et al., 2014c

J Nanomater Mol Nanotechnol 2014; S2:005.

doi:10.4172/2324-8777.S2-005

The increase of neopterin production after nanoparticle treatment implies that the compounds are able to induce pro-inflammatory pathways in T-cells and macrophages. However higher concentrations of nanoparticles did not lower neopterin concentrations compared to lower IDO activity with highest treatment concentrations of nanoparticles on PBMC. After induction of inflammatory cascades, like formation of neopterin, the production of ROS can be favored and contribute to negative effects due to generated oxidative stress. Further studies on PBMC regarding ROS formation confirm that nanoparticles are able to produce ROS in a dose dependent manner.

Furthermore, also other types of natural substances have been tested. The traditional polyherbal composition *Triphala* is commonly used for internal purification and digestion improvement. The composition can dose dependently inhibit triggered immune cascades in the THP-1 cell model (data not published yet). The naturally occurring polyphenol thymol, known for antioxidative, anti-inflammatory and anti-bacterial properties, can inhibit tryptophan breakdown and IDO activity significantly. However with high concentrations (> 500 µM) cell viability was already highly influenced (data not published).

Moreover campherol, a naturally occurring flavonoid and a commonly used compound in traditional medicine, is also able to diminish generated inflammatory responses in a dose dependent way. A lower neopterin formation and a reduced IDO activity as well as an inhibition in tryptophan breakdown after campherol treatment can be observed (data not published).

Several terpens have been tested whether they possess the ability to influence inflammatory responses. Additionally also different food preservatives, which are

extremely implemented in our society, have been tested and showed high antioxidant activities (unpublished).

Also other types of nanoparticles, namely elementary silver, zinkoxide (ZnO), carbon nanotubes and aluminiumoxide (Al_2O_3) were tested, if they have similar effect patterns compared to TiO_2 particles and if they are also able to interfere with redox-regulated signaling pathways. Silver, which is known to be antibacterial, can indeed inhibit inflammatory markers such as neopterin formation and the tryptophan breakdown rate in PBMC. Treatment with silver particles result in stronger inhibiting responses compared to Al_2O_3 particles, where lower but also significant anti-inflammatory results can be observed (data not published).

ZnO as well as the currently used drug delivery substances like carbon nanotubes showed no significant changes on inflammatory signaling cascades. However higher concentrations of ZnO ($\geq$ 18.25 µg/mL) already influenced cell viability significantly (data not published).

3. Conclusions

Finding appropriate methods to test different types of natural substances is of utmost importance; therefore this thesis represents an important work for a risk and benefit assessment for substances from diverse origins. Appropriate methods will help to validate the potential antioxidative and immunomodulating capacities of phytochemicals and other new materials. The findings can help to elucidate the so far healthy and preventive declared property of phytochemicals and food supplements. So far, antioxidants represent the key player in oxidative stress related disease prevention. However, plenty of *in vivo* studies manifest that antioxidants exhibit no convincing evidence of beneficial effects regarding preventing cancer, cardiovascular diseases or age-related diseases (Bjelakovic et al., 2014). Antioxidant supplements do not longer possess positive or preventive effects, however, in contrast they may possess adverse effects, like favoring allergy or asthma development (Zaknun et al., 2012), or an increased risk for prostate (Lawson et al., 2007) or lung cancer (Slatore et al., 2008) or even may result in an increased risk for diabetes mellitus (Bleys et al., 2007, Stranges et al., 2007).

Since antioxidants are present in almost every food, beverage or are concealed as food preservatives, the established assays can monitor the potentiating immunological activities of all different compounds. The assays are able to illustrate unexpected characteristics of natural substances, environmental pollutants and new materials, like nanoparticles, regarding influences on Th1 specific- and redox regulated pathways.

The results can help to prevent unwanted side-effects of eventually abundant uptake of antioxidants. Furthermore the findings of the established methods can help to

change the mindset for an additional uptake of antioxidants. They are declared as disease preventing and health promoting agents, however an excess of antioxidants can result in oxidative stress and gets damaging for the cell or even increase the risk of allergy.

The first aim of this thesis, which was covered by the establishment of methods, which can indicate immunomodulating and ROS scavenging properties, was implemented with success. The PBMC cell model can monitor T-cell specific interactions, as well as influences on monocytes and macrophages, although with lower effect due to smaller quantity in PBMC. The THP-1 cell model can investigate specific effect on monocytes directly. These two cellular systems can monitor the complex interplay between immune cells from different points of view. In summary, all tested natural substances and nanoparticles are able to quench the simulated inflammatory milieu and decrease the level of biomarkers in the PBMC and THP-1 models *in vitro*.

The established cell based and cell free assays for testing antioxidant capacity of the compounds can manifest that all tested substances exhibit scavenging properties, except the tested nanoparticles, which on the contrary were able to induce radical generation. The cell based assay (cellular antioxidant activity CAA assay) is able to show cellular uptake of the compound and the scavenging ability within the cell. The simpler radical absorbance capacity (ORAC) assay can be conducted without cells, however with these two assays only the interaction with peroxyl-radicals can be detected and other insights into the transcriptional induction of antioxidant or detoxifying enzymes are lacking.

Therefore a combination of all the assays can reveal different properties of tested compounds; this underlies the fact, that there are substantial differences between

antioxidants and their mechanism of action or capacity to interact with the immune system and other cellular signaling processes.

All tested naturally occurring substances, like flavonoids, polyphenols, traditional medicinal mixtures or the commonly used preservatives are able to interfere with immune responses, predominantly in the anti-inflammatory way. Even preservatives possess high ORAC values, which indicate high antioxidant ability. The highly increasing use of preservatives in food contributes to a huge increase of antioxidant uptake. This can be one reason, why allergy development is highly increasing. The importance of this thesis illustrates the identification and characterization of so far unknown risks and benefits of natural substances from different origins. This illustration can avoid and soften the general opinion regarding positive effects of antioxidant supplements, which are able to inhibit Th1 immune reactions and therefore favor Th2 responses.

The results of the first part of the thesis contribute to a better activity monitoring in the field of natural substances, and can therefore promote phytopharmaceutical usage in our medicine and can raise the application and medication of natural substances for minor disorders.

Due to the proper application of the mentioned methods, the second part consisted of the testing of nanoparticles with the aid of the above mentioned assays. Highly increasing applications of nanoparticles and the insufficient toxicity attracts the interest to do more research on nanoparticles and their potential immunotoxicological effects.

The assays were indeed able to determine the effects of nanoparticle treatment on immune responses. Exposure to TiO_2 resulted in biphasic effects on PBMC, however

silver and Al_2O_3 showed anti-inflammatory results, which were comparable to those from tested phytochemicals as Triphala, thymol or campherol.

TiO_2 bulk material, which contains also bigger-sized particles, was tested to differentiate, whether the size of the particles or solely the chemical structure is responsible for the toxicity. Interestingly, also bulk material showed effects on PBMC. Donaldson et al., discussed that ultrafine particles (<20 nm) had a deleterious effect on phagocytosis of macrophages, compared to larger particles (ca. 200 nm) (Donaldson et al., 2001). Based on those facts and the results of Donaldson it seems to be a relationship between surface area and the capability to generate ROS and activate inflammatory signaling cascades.

The observation that zeta-potentials are of major importance for the particle reactivity, one can explain why OCTi60, with a negative zeta potential, have achieved more influence on mitogen-stimulated PBMC compared to P25 (slightly positive zeta-potential).

The data shows that not only size of the particles is important for their toxicity the greater extent has to be on their surface properties.

These results will give a fast impression how nanomaterials are interacting with immune cells. However, for final conclusions, nanoparticles have to be tested with further cell types to discover the complex interaction with immunobiochemical pathways.

In summary, nanoparticles and natural substances are nontoxic agents for humans; however we need to find the right balance and the citation of Paracelsus: "*the dose makes the poison*" should be taken into account.

All these described methods represents *in vitro* data, though the PBMC model represents a more *in vivo* like system to monitor the biological responses of

immunomodulating agents, however it has to be kept in mind, that isolated PBMC cannot entirely reflect the *in vivo* situation.

Additionally further studies have to be done to get a more precise overview about the effect pattern. An important contribution in the dose effect relation of natural substances as well as nanoparticles can be the microarray technology. With this unbiased trial one can gain better and deeper insights into the resulting effects regarding the genome wide transcriptional gene expression profiles. Eventually unknown effects on other pathways can widen the application and usage of the tested compounds.

4. Methods

4.1. Preparation of nanoparticles

The particles were mixed with water and were sonicated for a distinct time to get a better dispersion of particles and were finally diluted in culture medium with final concentration of 10 mg/mL. To differentiate between the different particles the average hydrodynamic size, size distribution and zeta potential were determined. Detailed protocol can be found in Becker et al., 2014b

4.2. Preparation of natural substances

Different types of natural substances were diluted in distilled water to have a harmless solvent. Additional sterile filtration (0.22 µm) will exclude potential contaminations. Further details can be found in Sipahi et al., 2014, Klein et al., 2013.

4.3. Cell viability Assay

To measure viability of THP-1 cells and PBMC under the influence of the nanoparticle or natural substance exposure, we used the CellTiter-Blue^{TM} Cell Viability Assay (Promega, Germany), which provides a fluorometric method using the indicator dye resazurin to estimate the number of viable cells. Metabolically active cells retain the ability to reduce resazurin into resorufin, which is highly fluorescent. Non-viable cells do not reduce the indicator dye and thus do not generate a fluorescent signal. A detailed protocol can be found in Sipahi et al., 2014.

4.4. Measurement of tryptophan, kynurenine and neopterin

The assay relies on stimulated immune competent cells, which indicate an active immune response. The cells can be treated with different compounds to monitor the ability to influence immunochiochemical pathways.

Cells were stimulated to trigger the production of pro-inflammatory cytokines, which result in a higher readout of neopterin formation and IDO activity.

The different tryptophan and kynurenine concentrations of cell culture supernatants were determined with reversed phase HPLC and the kynureine to tryptophan ratio can be calculated for the activity of IDO. Neopterin concentrations were measured using an ELISA assay. Further details on procedures can be found in Becker et al., 2014a, b, c,

4.5. ORAC Assay

This assay relies on the ability of tested compounds to scavenge free radicals (peroxyl-radical). This radical is able to destroy the fluorescence reagent and as a result a lower value can be detected. The final ORAC values can be compared with other results of different substances. Further details can be found in Becker et al., 2014a, Ou et al., 2001).

4.6. CAA Assay

The assay is based on the fluorescent probe 2',7'-dichlorofluorescin diacetate (DCFH-DA), which is able to diffuse through cell membranes and gets hydrolyzed by intracellular esterases to non-fluorescent 2',7'-dichlorofluorescin (DCFH), DCFH is rapidly oxidized by radicals to highly fluorescent 2'-7'-dichlorofluorescein (DCF), whose intensity is proportional to the amount of intracellular ROS (Bass et al., 1983, Wolfe et al., 2007). If the added substances possess antioxidant properties, the substance can neutralize radicals and thus oxidation of DCFH is not any longer possible and a lower fluorescence signal can be detected. Detailed protocols can be found in Becker et al., 2014a, Klein et al., 2013.

4.7. Measurement of NF-κB activity

This assay is based on THP-1 blue cells, which are stable transfected THP-1 cells. They express a secreted embryonic alkaline phosphatase (SEAP) gene under control of a promoter encoding for NF-κB and AP-1 transcription factor binding sites. Upon stimulation with lipopolysaccharide (LPS), THP-1 blue cells activate NF-κB and finally secrete SEAP in the media, which results in a color change of the media. Produced SEAP levels are proportional to NF-κB activation and can be detected by a spectrophotometric plate reader. Detailed protocol can be found in Gostner et al., 2012a.

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