University of Natural Resources
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MASTER'S THESIS

Working towards an animal testing free future:

Development of a simple ELISA-based test to assess the sensitization potential of substances

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carried out at OFI, Vienna

Master's program: H 066 418 Biotechnology

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Wien, 14. 09. 2016

ABSTRACT

Allergic contact dermatitis (ACD) is a form of T cell mediated delayed type IV hypersensitivity reaction following skin contact with allergenic substances in sensitized individuals. ACD represents one of the most prevalent skin conditions. In particular potentially allergenic chemicals in cosmetics and topically applied pharmaceuticals need to be identified before they become ingredients of products, in order to assure the safety of consumers. Until recently predictive allergenicity testing relied completely on animal assays like the Local lymph node assay (LLNA). Since the EU-wide ban on animal testing for components of cosmetic ingredients became effective in 2013, the development of alternative *in vitro* assays is receiving increased interest. Skin sensitization is a complex process, thus a stand-alone assay is not sufficient to obtain a reliable prediction of the sensitization potential of a substance. Rather, *in vitro* assays targeting different steps of the sensitization process should be integrated into a test battery. Such a battery of assays could comprise the Direct peptide reactivity assay (DPRA), representing haptenation of self-proteins by allergens, ARE, detecting keratinocyte response to sensitizers and human cell line activation test (h-CLAT), targeting dendritic cell activation upon stimulation with allergenic substances. Within the scope of this thesis all three tests were established at the OFI. Additionally, an alternative detection method for h-CLAT was developed, in order to simplify the equipment needed to conduct the assay. A cell-based ELISA was the method of choice for detection of the surface markers CD54 and CD86. FITC-labelled antibodies used in the original protocol were found not suitable for photometrical measurement, therefore purified anti-CD86 and anti-CD54 antibodies and a HRP-conjugated secondary antibody in combination with TMB substrate solution were chosen. The washing steps needed to remove unbound antibodies as well as primary antibody dilutions were optimized. $CoCl_2$ and p-BQ were used as model substances to compare the assays' performance to data from literature as well as experiments using the original protocol, which includes detection of marker expression via flow cytometry. The applied concentrations of selected test substances were determined based on their IC50 values, which were assessed using EZ4U cytotoxicity assay. In terms of discriminating sensitizers and non-sensitizers the h-CLAT/ELISA assay yielded satisfying results, comparable to those obtained by flow cytometric measurement and from literature. The viability data from h-CLAT/ELISA and h-CLAT/flow cytometry as well as viability data from literature varied due to the unstable features of the used cell line THP-1. The developed assay therefore yielded sufficient

sensitivity, but the reproducibility needs to be optimized e.g. by standardizing the cultivation parameters. Validation of the assay and the establishment of the test battery remain to be done.

ZUSAMMENFASSUNG

Allergische Kontaktdermatitis ist eine Form von spezifischer, verzögerter Immunreaktion auf Hautkontakt sensibilisierter Personen mit allergenen Substanzen und ist eine der am weitesten verbreiteten Hauterkrankungen. Zum Schutz von Konsumenten ist es daher besonders wichtig, potentiell allergene Chemikalien zu identifizieren, bevor sie Bestandteil von Kosmetika oder pharmazeutischen Produkten werden, die zur Anwendung auf der Haut bestimmt sind. Bis vor Kurzem beruhte die Bestimmung sensibilisierender Substanzen ausschließlich auf Daten aus Tierversuchen, wie zum Beispiel dem LLNA (local lymph node assay). Seit der 2013 EU-weit in Kraft getretenen Verordnung zum Verbot der Verwendung von Tierversuchen zur Untersuchung kosmetischer Inhaltsstoffe, hat die Entwicklung alternativer *in vitro* Tests hohe Priorität. Da Hautsensibilisierung ein äußerst komplexer Prozess ist, reicht ein einzelner *in vitro* Test nicht aus, um das Sensibilisierungspotential einer Substanz zu bestimmen. Um eine verlässliche Vorhersage treffen zu können, könnten mehrere *in vitro* Tests zu einer Testbatterie zusammengefasst werden. Eine solche Testbatterie könnte zum Beispiel den DPRA (direct peptide reactivity assay), den ARE und den h-CLAT (human cell line activation test) beinhalten, von denen jeder einen anderen Schritt im Sensibilisierungsprozess repräsentiert. Im Rahmen dieser Arbeit wurden DPRA, ARE und h-CLAT am OFI etabliert. Des Weiteren wurde ein Zell-basierter ELISA als alternative Detektionsmethode für den h-CLAT entwickelt, um das zur Durchführung des Tests benötigte Equipment zu vereinfachen. Mithilfe des ELISA werden die Oberflächenmarker CD86 und CD54 detektiert, die laut dem Originalprotokoll mit FITC-markierten Antikörpern gefärbt und mit Durchflusszytometrie gemessen werden. Da die FITC-markierten Antikörper für die Messung mittels Photometer nicht geeignet sind, wurden Antikörper gegen beide Marker, sowie ein Peroxidase-markierter Sekundärantikörper gewählt. Die Detektion erfolgt mittels photometrischer Messung des Farbumschlages, der durch Zugabe von TMB Substratlösung hervorgerufen wird. Die Waschschritte, die benötigt werden, um ungebundene Antikörper zu entfernen, wurden optimiert, sowie die idealen Verdünnungen der Primärantikörper bestimmt. Kobalt(II)chlorid und para-Benzochinon wurden als Modellsubstanzen herangezogen, um die Ergebnisse des entwickelten h-CLAT/ELISA Tests mit Literaturdaten zu vergleichen. Außerdem wurden Ergebnisse aus Durchflusszytometrie Messungen laut Originalprotokoll mit einbezogen. Die verwendeten Konzentrationen der Testsubstanzen wurden anhand von IC50 Werten bestimmt, die mittels EZ4U Zytotoxizitätstest abgeschätzt wurden. Der entwickelte ELISA lieferte zufriedenstellende Ergebnisse bezüglich der

Unterscheidung sensibilisierender und nicht-sensibilisierender Substanzen, welche auch mit den Ergebnissen der Durchflusszytometrie Messung und Literaturdaten übereinstimmen. Die Bestimmung der Zellviabilität war hingegen nicht reproduzierbar, was vermutlich auf die niedrige Stabilität der verwendeten Leukämie-Zelllinie THP-1 zurückzuführen ist. Obwohl die Sensibilität hoch ist, muss die Reproduzierbarkeit, die stark von den Kultivierungsbedingungen der Zellen abhängt, noch optimiert werden. Die Validierung des Tests sowie die Etablierung der Testbatterie bleiben ebenfalls offen.

ACKNOWLEDGEMENT

An dieser Stelle möchte ich die Gelegenheit nutzen, mich bei all jenen Personen zu bedanken, die mich während meiner Arbeit und meines Studiums unterstützt und diese/s erst ermöglicht haben.

Zuallererst möchte ich Assoc. Prof. Dr. Regina Grillari danken, ganz besonders für die, trotz des Zeitdrucks, umsichtige und verständnisvolle Betreuung im Endspurt.

Danke auch an Dr. Christian Kirchnawy, DI Gabriele Ettenberger und das restlich OFI Team (besonders Lisi Mertl, Fiona Laupenmühlen und Fiona Hager), für die Betreuung, den fachlichen Rat, das super Arbeitsklima und die vielen lustigen Abende beim Burgeressen, Public Viewing, usw.

Ein riesen Dankeschön auch an DI Thomas Mohr, für die vielen Tipps, den fachlichen Beistand und ein offenes Ohr für alle Probleme und Problemchen, die sich während des Arbeitens ergeben haben.

Da nun leider sechs wunderbare Jahre an der Boku zu Ende gehen, möchte ich auch allen neu gewonnenen Freunden und Bekannten danken, die diese Zeit unvergesslich gemacht haben.

Last, but not least danke, danke, danke an meine tolle Familie und meinen Freund, die mich immer unterstützt und motiviert haben und sich mit mir über gute (und bestandene) Prüfungen und Ergebnisse gefreut haben.

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ABBREVIATIONS

(D)PBS	(Dulbecco's) phosphate buffered saline
2-MBT	2-Mercaptobenzothiazole
ACD	Allergic contact dermatitis
ACN	Acetonitrile
APC	Antigen presenting cell
ARE	Antioxidant response element
C3	Complement component 3
CA	Cinnamic aldehyde
CD54	Cluster of differentiation 54
CD86	Cluster of differentiation 86
Con A	Concanavalin A
DC	Dendritic cell
DEP	Diethyl phthalate
DMSO	Dimethyl sulfoxide
DNCB	2,4-Dinitrochlorobenzene
DPRA	Direct peptide reactivity assay
EC1.5	Concentration of a sensitizer that causes 1.5-fold enhancement of marker expression
EC3	Concentration of a sensitizer that causes 3-fold enhancement of marker expression
e.g.	Example given
ELISA	Enzyme linked immunosorbent assay
FCA	Freunds complete adjuvant
FcR	Fc receptor
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
GC	Gas chromatography
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GPMT	Guinea pig maximization test
h	Hours
h-CLAT	Human cell line activation test
HRP	Horse radish peroxidase

IC50	50% inhibitory concentration
ICAM	Intercellular cell adhesion molecule
IgG1	Immunoglobulin G1
IL	Interleukin
Keap1	Kelch-like ECH-associated protein 1
LC	Langerhans cell
LLNA	Local lymph node assay
MHC	Major histocompatibility complex
min	Minutes
MMA	Methyl methacrylate
MUSST	Myeloid U937 skin sensitization test
n.d.	no data
NaF	Natriumfluoride
Nrf2	Nuclear factor-erythroid 2-related factor 2
p-BQ	p-Benzoquinone
p-PD	p-Phenylendiamine
PA	Phthalic anhydride
PAMPs	Pathogen-associated molecular patterns
PBT	Polybuthylene therephthalate
PEEK	Polyether ether ketone
PFA	Paraformaldehyde
PI	Propidium iodide
PPSU	Polyphenylen sulfone
RFI	Relative fluorescence intensity
RT	Room temperature
sec	Seconds
T_C	Cytotoxic T cell
TFA	Trifluoroacetic acid
T_H	Helper T cell
TMB	3,3′,5,5′-Tetramethylbenzidine
TNF	Tumor necrosis factor
T_{Reg}	Regulatory T cells

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1. RELEVANCE OF THE SUBJECT

The human skin is a large and complex organ that acts as a first immune defence by providing a physical barrier against chemical, physical, thermal and biological threats. Additionally, it is an immunologic organ that plays an important part in innate immunity. This role is mediated through keratinocytes, which form the epidermis, dendritic cells responsible for processing and presenting antigens to T cells and mast cells. Consequently, the skin is frequently affected by allergic reactions following contact with small molecular weight compounds and allergic contact dermatitis is a common inflammatory skin disease of increasing prevalence and relevance in industrialized countries. In most western countries the increasing complexity of different kinds of allergens, for example in cosmetics, medical products, food, fragrances, jewellery and various synthetic materials inevitably leads to a rising incidence of allergic diseases. Whereas allergic reactions of type X, like anaphylactic shock, may be life-threatening, allergic contact dermatitis is not. Nevertheless, with an incidence of up to 20 % (Honda et al. 2013) in certain parts of the world allergic contact dermatitis has become a significant public health issue. Apart from the fact, that allergic contact dermatitis compromises the quality of life and the social life of the patients concerned, this distressing disease also has a great socioeconomic impact. As allergic contact dermatitis is the most frequent of occupational skin diseases (in particular eczema on the hands), it could be responsible for an increasing number of sick leaves and generates considerable health care expenses. People working in certain jobs are at an increased risk for sensitization, as they are exposed to particular allergens on a regular basis. Examples range from health care workers, who get in contact with rubber compounds, cleaning and disinfection agents, dental personnel and hairdressers, to metal and construction workers, who work with nickel or chromium containing materials. Despite the unpleasant effects of allergic contact dermatitis it is often underestimated by affected employees and often not even a dermatologist is consulted.

Due to the importance of ACD many studies have been performed to get an insight into the epidemiology and mechanisms involved. However the results of various studies are generally difficult to compare, because of geographic, occupational and cultural variations in allergen exposure among the investigated groups of study participants, as well as constantly evolving sources of allergens. Additionally, there are differing legislations for certain allergens in different countries (Bordel-Gómez, Miranda-Romero, and Castrodeza-Sanz 2010; Jurado-

Palomo et al. 2011; Merk et al. 2007; Metz and Maurer 2009; Thyssen et al. 2007; Bock et al. 2003).

Basically the most effective way to evade or fight allergic contact dermatitis is to avoid the offending substance (Cohen and Heidary 2004; Al-Otaibi and Alqahtani 2015). Due to this there is an increasing need to identify potential allergens and evaluate their sensitization hazard (especially in common, everyday items such as personal care products, cosmetics, pharmaceuticals and so on). For the screening for skin sensitizer's different *in vitro* and *in vivo* methods are available, which are described in 2.3.2.

A quite recent example for the currently rising awareness of the topic "allergies" in general in Europe is the implementation of the Regulation (EU) No 1169/2011 of the European Parliament and of the council of the 25th of October, 2011, which regulates the provision of food information to consumers (European Parliament and Council of the European Union 2011). Since the regulation became operative, the customers have to be informed about the fourteen most important allergens contained in unpackaged food in a written form. This also affects gastronomy, as every dish on a menu has to be provided with a list of contained allergens, which is currently a controversial subject in Austria.

2. INTRODUCTION

2.1 IMMUNE SYSTEM

The human immune system consists of the innate immune system, which mediates unspecific, but fast immune responses and the adaptive immune system that protects the body by a slow, but highly specific response.

2.1.1 INNATE IMMUNITY

The innate immunity includes physical and chemical barriers (like the skin, the low pH of gastric acid and mucus) as a first line of defense, separating the interior of the body from the exterior Thereby, access of harmful pathogens is prevented. Cell-intrinsic responses (like the degradation of viral double-stranded RNA or the fusion of pathogen-induced phagosomes with lysosomes) as well as professional phagocytic cells (macrophages and neutrophils), natural killer cells and the complement system are also attributed to the innate immunity. There are two types of innate immune responses, which are triggered upon binding of pathogen-associated molecular patterns (PAMPs) to pattern recognition receptors – inflammation of the affected sites and phagocytosis of the pathogens by phagocytic cells and dendritic cells. Pattern recognition receptors include i.e. soluble components of the complement system in the blood and membrane-bound receptors on host cells. The complement system is a crucial component of the innate immunity and is made up by about 20 soluble proteins. The early complement components, which function as an amplifying proteolytic cascade act locally to activate the essential complement component C3 by proteolytic cleavage. The cleaved C3b fragment forms a proteolytic complex following covalent binding to the surface of pathogens. C3b is recognized by receptors on phagocytes and B cells, which enables the subsequent assembly of the late complement components to form a membrane attack complex. The smaller fragment of C3 (C3a) acts independently through recruiting phagocytes and lymphocytes to the site of infection, thereby promoting inflammation. The innate and the adaptive immune response are linked through dendritic cells (DCs), which are widely distributed in virtually all tissues and organs and resemble the most potent antigen-presenting cells (APCs). Among DCs are i.e. epidermal DCs, so called Langerhans cells (LCs) (Alberts, Wilson, and Hunt 2008; Banchereau and Steinman 1998; Chung et al. 2004; Mellman and Steinman 2001; Steinbrink et al. 2009).

2.1.2 ADAPTIVE IMMUNITY

Whereas the innate immune system mounts general immune responses, the adaptive immune system provides highly specific defense responses and long-lasting protection. The responses are carried out by lymphocytes (white blood cells) and comprise T cell mediated defense reactions as well as reactions carried out by antibodies, which are produced by B cells. Whereas T cells react directly against an antigen presented on an APC (i.e. DC) and therefore act on a quite short range, antibodies circulate in the bloodstream and are also distributed to more distant sites. There they inactivate viruses or toxins and mark pathogens for destruction by specific binding. As already mentioned, an adaptive immune response is brought into action through the innate immune system through DCs. DCs express high levels of pattern recognition receptors on their surface through which they detect and ingest invading pathogens at a site of infection. Upon processing the pathogen by cleaving it into peptide fragments (either in the proteasome or the endosome), these fragments are bound to MHC class I molecules in the ER or to MHC class II molecules in the lysosomes. MHC proteins carrying the peptides are presented on the surface of DCs and presented to naïve T cells in peripheral lymphoid organs (i.e. lymph nodes) to which the DCs migrate after their activation. Maturing DCs show enhanced MHC and co-stimulatory molecule expression as well as secretion of cytokines. Lymphocytes continuously recirculate between blood stream and lymph. They enter lymph nodes through specialized endothelial cells lining postcapillary venules, which is mediated through adhesion via homing receptors (selectins), chemokines and integrins. If the lymphocytes encounter an antigen in the secondary lymphoid organs they remain there to become activated. T cells are activated through the formation of an immunological synapse on the interface between T lymphocyte and DC (binding of T cell receptors to MHC-peptide-complexes). Cell surface co-stimulatory molecules like CD4 (T_H and T_{Reg} cells), CD8 (T_C cells) or CD28 and cell-cell adhesion molecules displayed by DCs and T cells stabilize the binding with otherwise low affinity. The subsequent differentiation, proliferation and migration of the effector T cells depend on the type of MHC molecule (MHC I or MHC II) and on the cytokines secreted by the DCs. Upon activation T cells can differentiate into three types of effector cells that carry out the immune response: cytotoxic (T_C), helper (T_H) or regulatory T cells (T_{Reg}). Each type fulfills its own distinct function. While T_C cells provide protection against intracellular pathogens by killing infected cells, T_{Reg} play a role in tolerance induction (especially to self-antigens) through their ability to suppress the activity of effector T cells. The third type of T cells is called helper T cells, due to its ability to activate macrophages, T_C cells and B cells for fighting intracellular (T_H1) and extracellular pathogens

(T_H2). The type of T_H cell determines the nature of the subsequently elicited adaptive immune response. Some of the effector T cells therefore migrate to the site of infection to combat invading pathogens while others remain in the lymphoid organ to help activate other T cells and B cells. In the lymph nodes also B cells get activated by an antigen to differentiate to antibody-secreting monospecific effector cells. There are five classes of immunoglobulins (IgG, IgA, IgE, IgM and IgD), which are among the most abundant proteins in the blood. Besides binding antigens via their highly specific antigen binding sites, which are located on the CDRs of the Fab fragments, antibodies can activate complement and effector cells through their Fc fragment. Another crucial type of cells is memory cells, which arise after differentiation of naïve T or B cells. They mount a more sensitive, rapid and effective immune response after repeated encounter of the antigen and can provide even life-long protection, whereas effector cells die within days or weeks (Alberts, Wilson, and Hunt 2008; Banchereau and Steinman 1998; Chung et al. 2004; Mellman and Steinman 2001; Steinbrink et al. 2009).

2.2 ALLERGIC CONTACT DERMATITIS

During our daily lives we face a lot of foreign chemicals and in most cases their unconscious ingestion, inhalation or skin contact have no visible consequences. However, some virtually harmless molecules of low molecular weight can interact with self-proteins to form complete antigens, thereby causing skin sensitization (Martin et al. 2011).

Allergic contact dermatitis (ACD) as a result of skin sensitization represents one of the most important occupational and environmental issues. ACD is a form of antigen-specific, T cell-mediated delayed type IV hypersensitivity reaction, following contact with skin sensitizers, mostly haptens, in sensitized individuals. Skin sensitizers are defined as substances with the intrinsic ability to induce an immunologic response in sensitized individuals. Exogenous small molecular weight molecules, which are not immunogenic themselves, but are able to form macromolecular immunogens by penetrating the stratum corneum and binding to self-skin proteins, are referred to as haptens. Following challenge with the same or a cross-reactive skin sensitizer, sensitized individuals are prone to elicit manifestations of ACD (e.g. redness, edema) (Divkovic et al. 2005; Saint-Mezard et al. 2003; Gober and Gaspari 2008).

ACD consists of two distinct phases, a sensitization (also afferent) and an elicitation (also efferent) phase (Erkes and Selvan 2014), an overview of which is shown in the following

figures. During the sensitization phase an innate inflammatory immune response is initiated resulting in the priming of antigen-specific T cells (see Figure 2-1). Upon challenge with the same sensitizer follows the elicitation phase (see Figure 2-2), during which clinical manifestations like skin lesions develop (Martin et al. 2011). A key for both figures is shown in Figure 2-3.

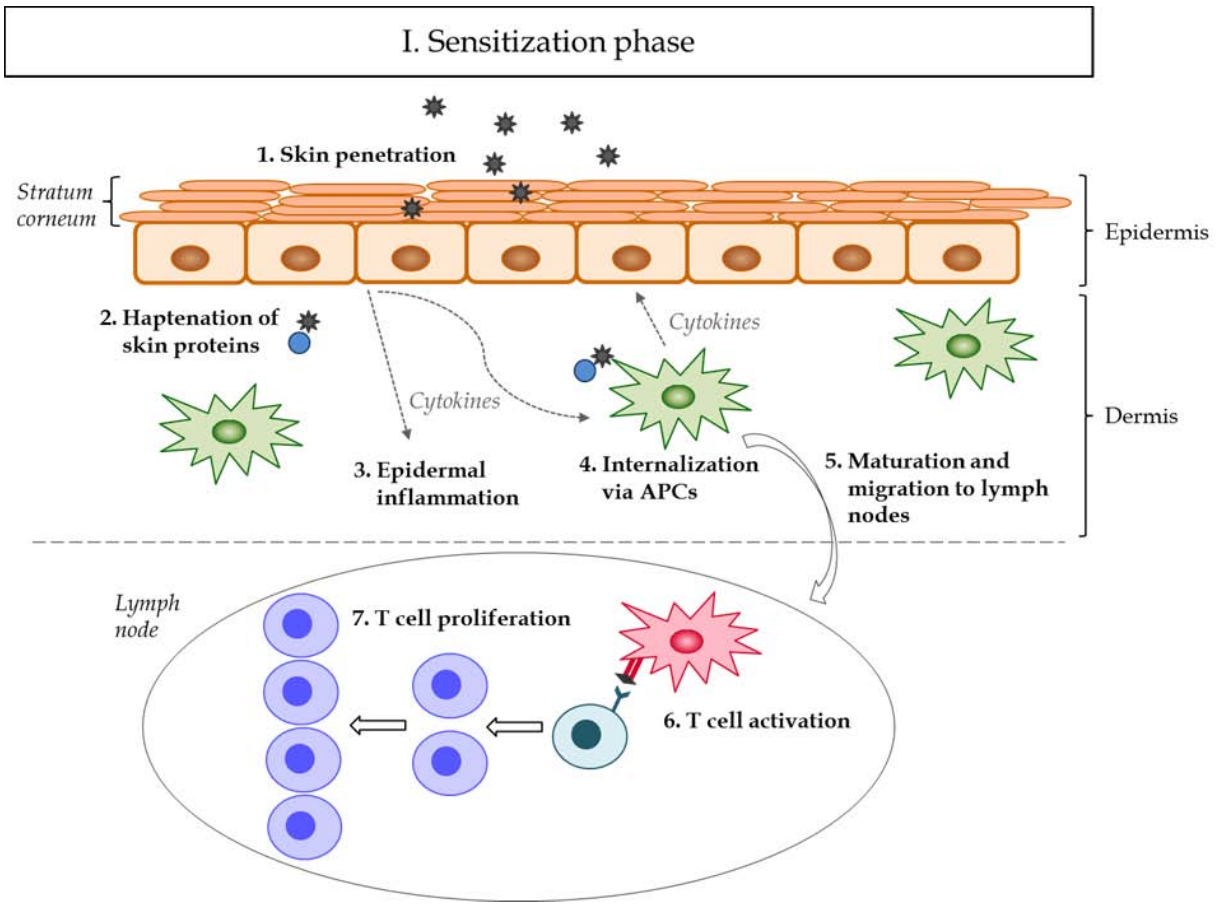

Figure 2-1: Scheme of the sensitization phase of ACD. After crossing the skin barrier (1) the contact allergens bind to endogenous skin proteins (2) via different mechanisms (i.e. covalent binding or cross linking) thereby forming immunogens and activating the immune system. Keratinocytes are stimulated to release cytokines (3) causing APC stimulation. The hapten-protein conjugates are internalized and processed by APCs (e.g. DCs) (4). The DCs mature and migrate to local lymph nodes (5) in response. Within regional lymph nodes the activated DCs present the immunogenic antigen fragments to T cells. The naïve T cells get activated (6) via binding of T cell receptors to MHC molecules on DCs, on which antigen fragments are presented. Additionally, co-stimulatory and cell adhesion molecules are involved in the activation process. Upon proliferation of the activated (primed) T cells (7) they start circulating in the body until renewed encounter of the antigen. The figure was adapted from Smith Pease, Basketter, and Patlewicz 2003.

Upon contact of haptens with the skin, they bind to self-skin proteins covalently or through cross-linking, thereby forming macromolecular immunogens and activating the innate immune system. Other than haptens, which possess electrophilic properties enabling direct reaction with nucleophilic amino acids in skin proteins, pre- and prohaptens require chemical or metabolic activation (e.g. oxidation) to become haptens. It is estimated that pre- and prohaptens contribute one-third to known sensitizers (Chipinda, Hettick, and Siegel 2011; Chipinda et al. 2011). Interaction of reactive haptens with keratinocytes causes the release of various cytokines by the damaged cells, including IL-1 β, IL-18, TNF-α and GM-CSF which in turn stimulate LCs and dermal DCs to take up the antigens. Upon internalizing the hapten-protein conjugates, the antigen presenting cells migrate to draining lymph nodes. During migration the DCs undergo functional maturation, during which they acquire immunostimulatory properties. Mast cells have been shown to play a role during migration and simultaneous maturation of APCs (e.g. enhancement of MHC, co-stimulatory and adhesion molecule expression) through secretion of ICAM and TNF-α. IL-β1 p,roduced by DCs acts on keratinocytes and induces them to produce TNF-α as well as acting as an autocrine signal for migration. Also CCR7 receptor, which binds to CCL21 and CCL19 is responsible for guiding migrant DCs to the lymph nodes. The APCs enter the lymph nodes through the afferent lymphatics. Within the paracortex of draining lymph nodes the APCs activate naïve T cells by presenting the MHC-bound, haptenated peptides. In addition to the binding of MHC molecules to T cell receptors, T cells require secondary signals through binding of co-stimulatory (CD4, CD8) and adhesion molecules (ICAM) to become activated. Activation of naïve T cells results in differentiation and clonal expansion of hapten-specific memory T cells ($CD4^+$ and $CD8^+$), which have the potential to become hapten-specific effector T cells during elicitation phase (Enk 1997; Erkes and Selvan 2014; Kimber et al. 1998; Ryan et al. 2007a; Grabbe and Schwarz 1998; Kimber et al. 1999). The sensitization phase, which is thought to have no clinical consequences typically lasts 8-15 h in humans (Saint-Mezard et al. 2003).

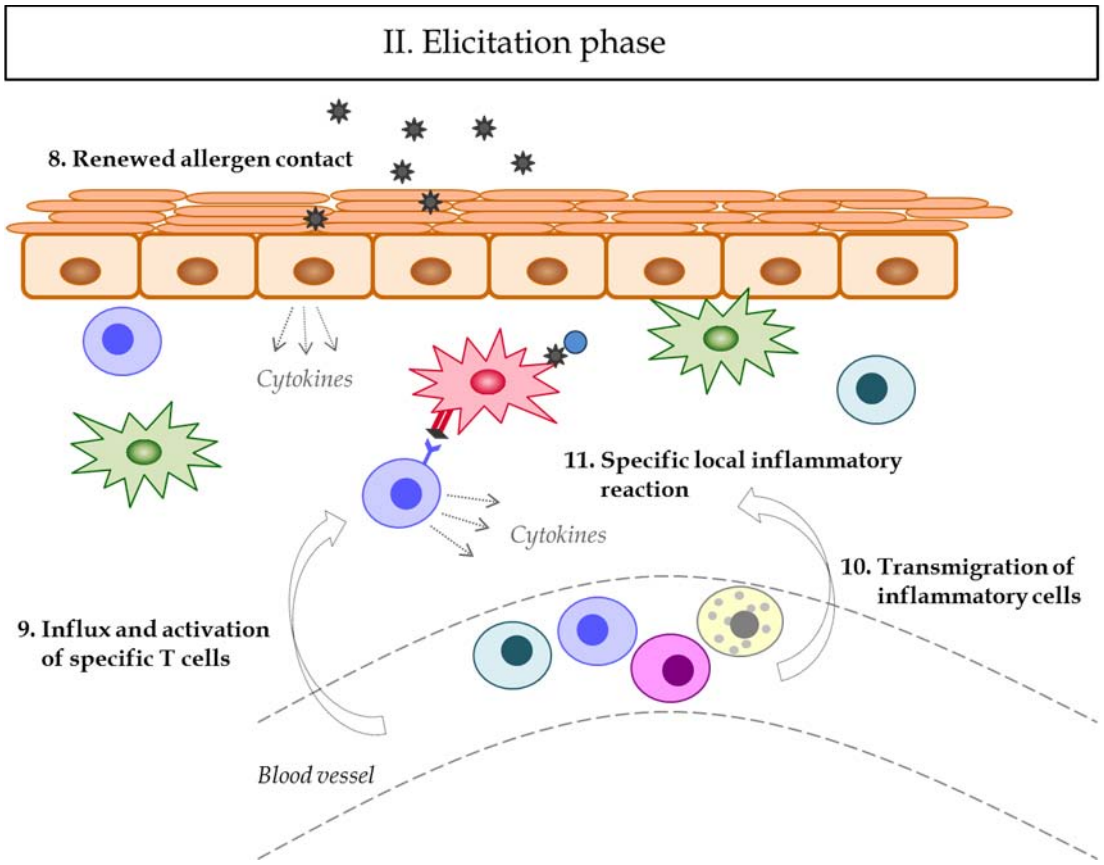

Figure 2-2: Scheme of the elicitation phase of ACD. Upon repeated contact with the allergen (8) keratinocytes secrete cytokines and the local endothelia as well as antigen-presenting cells are activated. Antigen-specific T cells infiltrate the exposed skin area and accumulate (9). A cascade of inflammatory events is induced by the release of cytokines by T cells. Influx of accessory cells (10) enhances the inflammatory response and leads to clinical manifestations of ACD (11). The figure was adapted from Smith Pease, Basketter, and Patlewicz 2003.

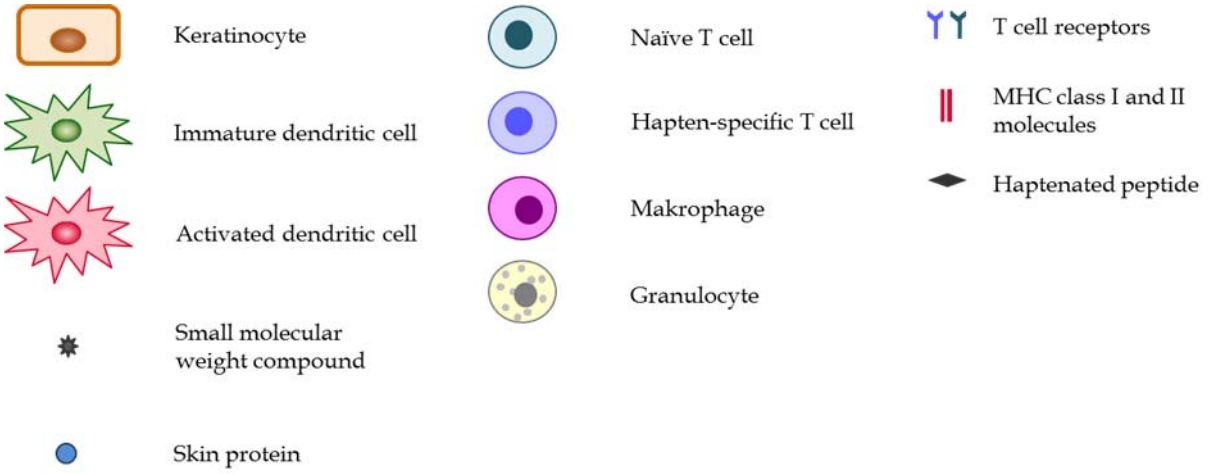

Figure 2-3: Key for Figure 2-1 and Figure 2-2.

Repeated contact with the same antigen in sensitized individuals first leads to non-specific inflammation caused by the release of cytokines by epidermal cells (e.g. keratinocytes). For example the pro-inflammatory cytokines TNF-α and IL-1 are thought to have an impact on elicitation of ACD. APCs as well as local endothelia are activated, leading to the transmigration and accumulation of antigen-specific effector T cells. These in turn secrete mediators that lead to the influx of other inflammatory cells. The resulting enhanced inflammation process of the tissue is responsible for clinically apparent cutaneous lesions (Saint-Mezard et al. 2003). These lesions occur mainly in the area of contact, but can also develop in other areas of the body (Martin et al. 2011). In humans the elicitation phase lasts about 72 h before decreasing progressively due to down regulating mechanisms (e.g. IL-4 and IL-12 are thought to play a role in terminating the adaptive immune response) (Saint-Mezard et al. 2003).

The different steps of the sensitization process can be exploited in various assays (described in 2.3), including the following:

Ad 2. Binding of haptens to self-proteins (haptenation) can be measured by means of DPRA (see 2.3.2.1).

Ad 3. Keratinocyte response to allergens can be measured using assays like KeratinoSens, LuSens and ARE (see 2.3.2.2).

Ad 4. DC activation and maturation provoked through to contact with skin sensitizers can be measured by means of h-CLAT (see 2.3.2.3) and MUSST (see 2.3.2.4).

Ad 7. Proliferation of primed lymphocytes in regional lymph nodes upon skin sensitization can be measured using the murine LLNA (see 2.3.1.2).

METAL ALLERGY

Metal ions play many important roles e.g. in DNA replication or enzymatic reactions and therefore are among the essential nutrients (Wang and Dai 2013). However, certain species of metals pose a serious threat to human health by acting genotoxic, carcinogenic or by causing allergic contact dermatitis (Muñoz and Costa 2012; Nordberg 2007). While nickel, cobalt and chromium are the most prevalent, also aluminium, beryllium, copper, mercury and others are emerging sensitizers (Almpanis et al. 2010). Due to industrialization metals are near-ubiquitously present in jewellery, cosmetics, intracardiac and endovascular devices or dental and orthopedic implants leading to an increased exposure. This has resulted in nickel

hypersensitivity being among the most prevalent allergic conditions (Roediger and Weninger 2011; Wolf et al. 2013) affecting up to 20 % of humans (Schram, Warshaw, and Laumann 2010). Through interaction with saline, blood and proteins and through mechanical stress metals undergo corrosion (leading to elution of metal ions that may activate the immune system). This is a problem when they get in contact with biological systems like the human body (Wolf et al. 2013; Almpanis et al. 2010). The first case of a stainless steel fracture plate causing a dermatitis reaction was described in 1966 (Basko-Plluska, Thyssen, and Schalock 2011). Since then metal allergies have evolved as one of the most common forms of ACD. Nevertheless, the molecular basis and mechanisms underlying metal sensitization are still largely unknown (Yin et al. 2012; Wang and Dai 2013).

Immunological reactions to metals are complex and many factors are involved. Despite the ubiquitous presence of e.g. nickel in the environment the majority of people do not show a reaction after exposure. This suggests that in addition to Ni^{2+} binding to self-peptides on MHC molecules on DCs, co-stimulatory signals mediated by e.g. pattern recognition receptors (like toll like receptors) are required to induce priming and expansion of T cells. Especially human, but not mouse TLR4 was identified as a crucial Ni^{2+} receptor (Schmidt et al. 2010), with Ni^{2+} binding to TLR4 being sufficient for sensitization. On the other hand nickel is capable of independently eliciting an inflammatory response through IL-1 release by keratinocytes following Ni^{2+} exposure (Roediger and Weninger 2011). Therefore, it is difficult to distinguish between irritation and allergic responses which both show similar clinical manifestations. It has been suggested that the cytokines IL-17, IL-22 and IFN-γ produced by $CD4^+$ and $CD8^+$ T cells, T_H17 and other cell types play important roles during the elicitation of ACD and $CD4^+$ cells are the major effector cells to infiltrate skin following Ni^{2+} exposure (Dyring-Andersen et al. 2013).

2.3 METHODS FOR HAZARD IDENTIFICATION OF SUBSTANCES

2.3.1 *IN VIVO* METHODS

2.3.1.1 GUINEA PIG TESTS

For predictive sensitization testing guinea pigs have long been the animals of choice. In guinea pig tests sensitization activity is measured as a function of cutaneous reactions following

challenge of previously sensitized animals. Two different types of tests have been established, non-adjuvant and adjuvant tests, in which sensitization is amplified by injection of Freunds complete adjuvant (FCA). Examples for both test types are the Buehler test (non-adjuvant) developed by Buehler 1965 and the Guinea pig maximization test (adjuvant) developed by Magnusson and Kligman 1969 (OECD 1992; Kimber et al. 1999).

Guinea pig maximization test (GPMT)

In the Guinea pig maximization test the minimum of test animals is 10 in the treatment and 5 in the control group to get reliable results. The test consists of a two-stage induction operation as well as a challenge exposure. For induction the highest test substance concentration to cause mild to moderate irritation is used, while it should still be well tolerated systemically. The concentration of test substance used for the challenge exposure should be the highest not to provoke irritation. Before starting the test, the guinea pigs in the treatment and control groups are shaved/short clipped at the shoulder area. All test animals are given three intradermal injections of 0.1 mL to start the induction exposure. The treatment group is first injected a 1:1 mixture (v/v) FCA and water (or physiological saline) into the area that has been cleaned of hair. The second injection contains the selected amount of test substance in a suitable vehicle while the third injection consists of the test substance formulated in a 1:1 mixture (v/v) FCA and water (or physiological saline). Water-soluble chemicals are dissolved in the aqueous phase before mixing with FCA whereas liposoluble substances are suspended in FCA prior to mixing. The control group animals are given three injections as well, the first being the same as for the treatment group, the second being undiluted vehicle and the third consisting of a 50 % formulation of vehicle in a 1:1 mixture (v/v) FCA and water (or physiological saline). The second part of the induction exposure is conducted at day 5-8 after the injections. Topical application using closed patches is performed at the injection site after shaving the area. The test substance is applied to a 2 x 4 cm filter paper, which then is held in place for 48 h by an occlusive dressing. If the substance itself does not act as an irritant the test area is painted with 0.5 mL of 10 % SDS in Vaseline 24 h prior to patch application to create a local irritation. The control group is provided with patches loaded with vehicle only. Challenge with the same test substance is performed two weeks after topical induction. A patch loaded with test substance is applied to the previously shaved test area and held in contact with the skin for 24 h by an occlusive dressing. The test area is cleaned 21 h after removing the patch and further 3 h later the skin reaction is monitored and recorded. A second observation is made 24 h after the first. The reaction is graded according to a scale: 0 = no visible change, 1 = discrete or patchy

erythema, 2 = moderate and confluent erythema, 3 = intense erythema and swelling (OECD 1992; Magnusson and Kligman 1969).

Buehler Test

For obtaining a reliable result using the Buehler test a minimum of 20 animals in the test group and 10 in the control group are required. As the GPMT the Buehler test consists of an induction and a challenge application, however the procedure differs. In contrast to the GPMT the induction is achieved merely by topical application of the test chemical instead of injection. The flank of the test animal is cleared of hair and a test patch system is applied. The patch is held in place for 6 h. For the control group only vehicle without test substance is used. The application is performed on day 0, days 6-8 and days 13-15. For induction the highest dose of test substance that causes mild irritation is used. On day 27-29 challenge exposure is conducted by applying a patch containing test substance at the highest non-irritant concentration to the untreated flank of the test animal for 6 h. Water-soluble substances are dissolved in water or a non-irritating vehicle while 80 % ethanol (for induction) and acetone (for challenge) are used for other test chemicals. The test area is cleared of hair 21 h after removal of the substance containing patch. The skin reactions are recorded according to the same grades used for the GPMT. A second observation is made 24 h after the first. A second challenge exposure one week after the first can be considered if it is required to clarify the obtained results (OECD 1992).

The drawbacks of the two mentioned methods are mainly the subjective endpoint and the fact that the dose of the test substances largely depends on their irritant properties. Furthermore, colored test substances can inhibit evaluation of the skin reaction. Another problem that arises especially with adjuvant-type methods like the GPMT can be the overestimation of the potential of topically applied weak materials or underestimation of the potential of strong sensitizers (Robinson et al. 1990; Kimber et al. 1999; Maurer 2007).

2.3.1.2 MURINE LOCAL LYMPH NODE ASSAY (LLNA)

The LLNA was developed as an alternative stand-alone method to guinea pig models, since the immune system of the mouse has been studied substantially better than that of the guinea pig. Whereas in guinea pig tests the elicitation phase is measured, the LLNA measures the sensitization phase of ACD. It is currently the standard method of choice for the identification of contact allergens. The LLNA relies on the measurement of the induction of lymphocyte proliferation in draining lymph nodes provoked through topical application of sensitizers,

which is considered a hallmark of skin sensitization (D.A. Basketter et al. 1996; Frank Gerberick et al. 2007; OECD 2002; OECD 1992). Besides the discrimination of sensitizers from non-sensitizers the LLNA also provides a quantitative correlation between the administered dose of the test chemical and the proliferation response. Therefore, measurement of the relative skin sensitization potency is possible which is usable for risk assessment (David A Basketter, Gerberick, and Kimber 2001; De Jong et al. 2002; Frank Gerberick et al. 2007).

The test is conducted using 6-12 weeks old female CBA strain mice, which receive topical applications of the test chemicals once a day on the dorsum of both ears. The application is repeated on three consecutive days. For each chemical at least three different concentrations are tested, each applied to a dose group of at least four animals. A suitable vehicle control (the vehicle being dictated by the solubility of the chemical) such as an acetone-olive oil mixture or DMSO is included. Following exposure all mice receive an injection of radiolabelled thymidine (3H-TdR) into their tail vein. The animals are euthanized five h later and the auricular lymph nodes are excised. The lymph nodes are either pooled for each experimental group or for each mouse individually and are disaggregated mechanically. After being washed the lymph node cells are resuspended in trichloroacetic acid and stored at 4 °C for at least 12 h. The incorporation of 3H-TdR is measured by β-scintillation counting and recorded as disintegrations per minute (dpm) per node. For each chemical dose a stimulation index (SI) is calculated relative to the vehicle control. In order to be considered a contact allergen at least one concentration of a chemical must induce a threefold or more increase in lymph node cell proliferation ($SI \geq 3$). Using the dose-response curve the concentration inducing a threefold increase in proliferation (the EC3 value) is calculated. According to the EC3 values chemicals can be classified as extreme ($EC3 < 0.1$), strong ($0.1 \leq EC3 > 1$), moderate ($1 \leq EC3 > 10$) or weak sensitizers ($10 \leq EC3 \geq 100$) (David A Basketter, Gerberick, and Kimber 2001; Frank Gerberick et al. 2007; OECD 2002).

2.3.2 *IN VITRO* METHODS

Due to increased ethical awareness on animal testing the reduction of the use of animals is widely shared objective, especially with regards to tests carried out outside medical research and drug development. Until now the standard methods of choice to determine if a chemical acts as a skin sensitizer included mice or guinea pigs (Kimber et al. 1999). In particular the EU-wide ban on animal testing for components of cosmetic products, which became effective in 2013 (EC 2008; EC 2006) boosted the development of *in vitro* alternatives for testing the

sensitization potential of substances (Divkovic et al. 2005). Examples for such in vitro assays are given in Figure 2-4.

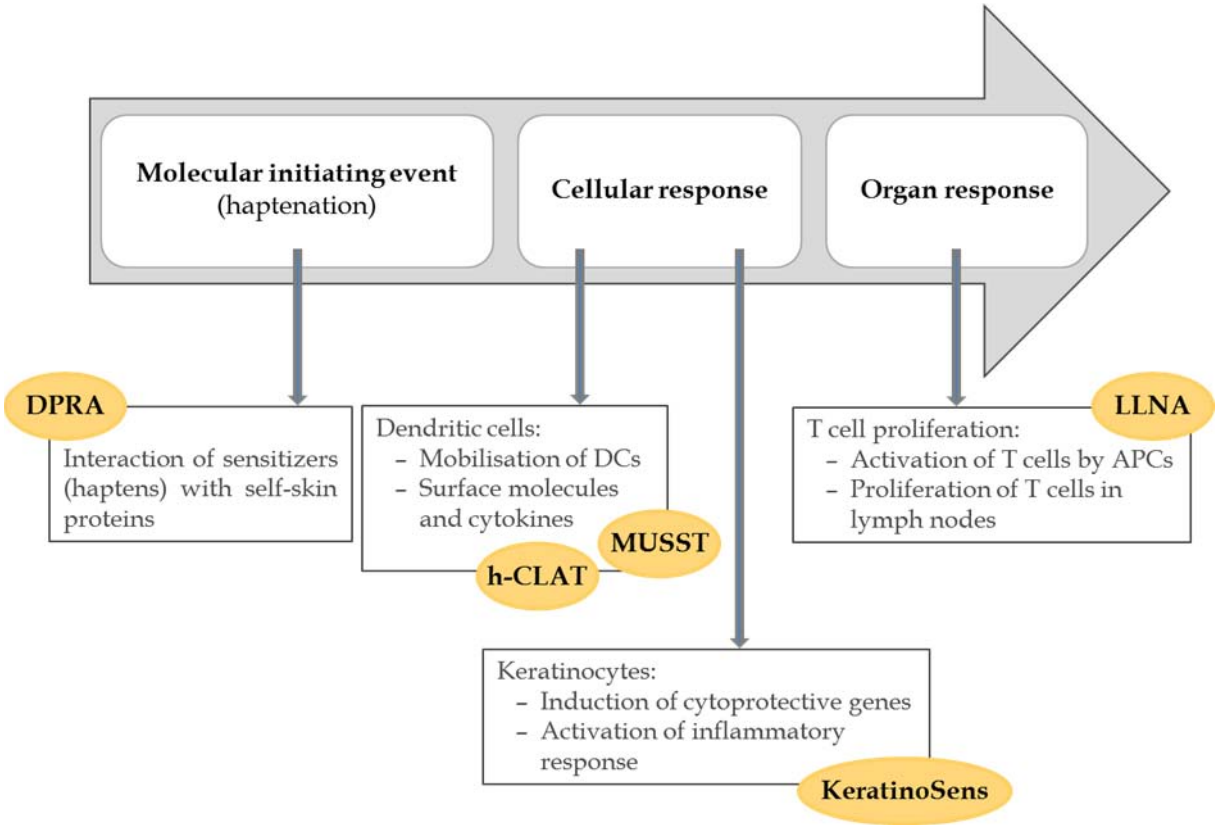

Figure 2-4: Overview of some *in vitro* methods. The mentioned *in vitro* tests (DPRA, h-CLAT, MUSST, KeratinoSens and LLNA) represent different stages of the complex skin sensitization process.

However, *in vitro* methods reflect only parts of the *in vivo* situation and in pharmaceutical and cosmetic industries it is mandatory to identify potential inducers of ACD before they are used in new products (Aeby et al. 2004). Consequently, tests for the predictive identification of allergenicity of ingredients are required (Andersen and Frankild 1997).

To emulate the *in vivo* situation, various *in vitro* methods to determine the sensitizing potential of substances are under development, targeting different stages of the skin sensitization process. *In vivo*, allergic contact dermatitis as the toxicity endpoint of skin sensitization is a complex process involving many steps. Thus, for reliable prediction of the skin sensitizing potential of a substance, the use of only one method is insufficient. Rather than using a single test, a battery of assays, representing different key steps in skin sensitization and allergic contact dermatitis has to be used (Aeby et al. 2004; Emter, Ellis, and Natsch 2010; Python,

Goebel, and Aeby 2007; Yoshida et al. 2003; van der Veen et al. 2014; Nukada et al. 2013; Ramirez et al. 2014).

A typical test battery may encompass following steps:

1. Peptide reactivity assays to determine whether haptenation takes place
2. Stress response of keratinocytes
3. Activation of DCs.

The first step in many sensitization processes is the so-called haptenation. Haptens are able to chemically modify self-proteins thereby forming macromolecular immunogenic complexes (Divkovic et al. 2005). With a few exceptions, formation of hapten-carrier protein complexes is a prerequisite to skin sensitization *in vivo*. Haptenation can be tested e.g. through the Direct Peptide Reactivity Assay (DPRA).

2.3.2.1 DIRECT PEPTIDE REACTIVITY ASSAY (DPRA)

The DPRA is an *in chemico* peptide-based assay, developed by Gerberick et al. (Gerberick et al. 2004; Gerberick et al. 2007) for screening contact allergens. It mimics the reaction of a potential contact allergen with skin proteins after its penetration into the epidermis, as protein reactivity represents the first key step in the induction of skin sensitization and thus the development of allergic contact dermatitis. If and to what extent a chemical reacts towards skin proteins correlates with skin sensitization potential. Therefore, DPRA allows the discrimination between skin sensitizers and non-sensitizers and the classification of sensitizers. The test method is based on the fact, that the majority of chemical allergens are small molecules with electrophilic properties, which react with electron-rich groups in the side chains of nucleophilic amino acids of skin proteins and therefore are able to bind them covalently. This molecular initiating event is addressed by the DPRA, through quantification of the reactivity of chemicals towards synthetic model peptides, which contain either a single lysine (Ac-RFAAKAA-COOH) or a cysteine residue (Ac-RFAACAA-COOH). Besides lysine and cysteine there are other amino acids like histidine, tyrosine and methionine that contain nucleophilic heteroatoms as well and can be bound by contact allergens. However, lysine and cysteine are the most cited ones. The samples are mixed with a defined concentration of the synthetic peptides at a certain ratio (according to the OECD guideline at a 1:50 ratio for lysine and a 1:10 ratio for cysteine) and incubated for 24 h. Preferable solvents for the samples are for example acetonitrile, water, isopropyl alcohol and acetone, whereas cysteine is solubilized in a pH 7.5

phosphate buffer and lysine in a pH 10.2 ammonium acetate buffer. The higher pH is based on the pKa of lysine and the need to deprotonate the primary amine to make it available for reactivity. After incubation the relative peptide concentration is monitored by high performance liquid chromatography (HPLC) using a gradient eluation. The HPLC is coupled with an UV detector, measuring the eluate at 220 nm. The peak area of the sample is determined and the percent peptide depletion value is calculated considering the reference control (a mixture of the respective peptides and the solvent to determine potential interaction). Besides the reference control, a positive control (cinnamic aldehyde) and a co-eluation control are necessary. The test substances are measured in triplicates and are subsequently categorized into four classes of reactivity depending on the percent peptide depletion values – minimal reactivity (mean % depletion ≤ 6.38 %), low reactivity (6.38 % ≤ mean % depletion ≤ 22.62 %), moderate reactivity (22.62 % ≤ mean % depletion ≤ 42.47 %) and high reactivity (42.47 % ≤ mean% depletion). Thus, chemicals with a mean % depletion less than or equal to 6.38% are considered non-sensitizers and substances with a mean % depletion greater than or equal to 6.38 % are regarded as sensitizers. Although the model developed by Gerberick et al. provides a good prediction accuracy of 89 %, a sensitivity of 88 % and a specificity of 90 % (Gerberick et al. 2007), the method also has its limits. For example it is not suitable for testing metal compounds, since their action mechanism on skin proteins is different from that of covalent binding. Furthermore, it is difficult to detect prohaptens, which need to be enzymatically bioactivated to reveal their allergenic potential. Concerning prohaptens there are approaches to incorporate a metabolic component, for example a peroxidase/peroxide oxidizing system. Despite some boundaries the DPRA is a valuable method for the identification and categorization of skin sensitizers, especially if it is combined with additional *in vitro* methods (Gerberick et al. 2004; Gerberick et al. 2007; OECD 2014).

The next major step in the skin sensitization cascade is the cellular response to potential skin sensitizers. Tests that address different cellular responses comprise e.g. assays mimicking keratinocyte (see 2.3.2.2) and DC response to skin sensitizers (see 2.3.2.3 and 2.3.2.4).

2.3.2.2 KERATINOCYTE RESPONSE TESTS

Another approach for the testing of the skin sensitization potential of chemicals was developed by (Emter, Ellis, and Natsch 2010), who investigated the suitability of the Keap1-Nrf2-ARE signalling pathway for hazard evaluation of chemicals, as it is induced by many skin sensitizers. Emter et al. claim that *in vitro* assays for toxicological endpoints (such as skin

sensitization) should emulate the biological system, which they are supposed to model as closely as possible. Although dendritic cells are key players in the immune response to sensitizers, the activation of keratinocytes is among the initial cellular events as they are the dominant cells in the epidermis and therefore are the primary cells to come in contact with skin sensitizing substances. They participate in the immune response through cytoprotective gene pathways and therefore can also be used for the prediction of potential sensitizers. The Nrf2 electrophile sensing pathway used in the KeratinoSens assay involves the repressor protein Keap1 (Kelch-like ECH-associated protein), the transcription factor Nrf2 (nuclear factor-erythroid 2-related factor 2) and the antioxidant response element (ARE) which is located in the promotor regions of many phase II detoxification genes (see Figure 2-5). As the sensor protein Keap1 contains highly reactive cysteine residues and has been shown to be covalently modified by electrophilic molecules, the pathway is considered a cellular marker for skin sensitization. After the covalent modification of Keap1 through chemical compounds, Keap1, which is bound to Nrf2 under normal conditions and acts as its repressor, dissociates. Transcriptional factor Nrf2 translocates to the nucleus, binds to ARE and initiates mRNA transcription. Based on that principle a high-throughput assay (KeratinoSens) based on a novel keratinocyte cell line was developed. The cell line used in the assay is derived from HaCaT cells and contains a reporter construct consisting of a luciferase gene under the control of a single copy of the ARE-element of the human AKR1C2 (human aldoketoreductase) gene. The key advantage of the method is the measurement of gene induction events at subcytotoxic concentrations on one hand and the simplicity of luciferase-induced luminescence measurement on the other hand (Ramirez et al. 2014). A limitation is the cystein content of Keap1, resulting in false negatives for substances exclusively binding to lysine peptides, such as phthalic anhydrides (Emter, Ellis, and Natsch 2010; Natsch and Emter 2008).

The LuSens assay works similar to the KeratinoSens assay as it also addresses the activation of keratinocytes by use of a human keratinocyte cell line containing a reporter gene construct consisting of an antioxidant response element. LuSens assay resembles KeratoSense closely with the difference that ARE of rat Nqo 1 (NADPH:quinine oxidoreductase 1) instead of AKR1C1 is used. Therefore skin sensitizers inducing the Keap1-Nrf2-ARE pathway and the corresponding luciferase activity can be monitored using a photometer (Ramirez et al. 2014).

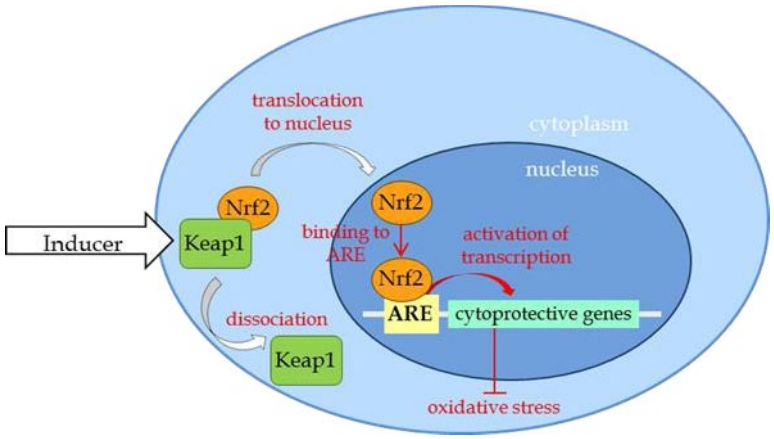

Figure 2-5: The Nrf2-antioxidant response pathway. Upon binding of an inducer (e.g. a skin sensitizer) to the sensor protein Keap-1 the repressor dissociates from the transcriptional factor Nrf2. Nrf2 translocates to the nucleus where it binds to antioxidant response elements (AREs), thereby activating/upregulating the transcription of cytoprotective genes. Figure 2-5 was adapted from Ettenberger-Bornberg et al. 2015.

2.3.2.3 H-CLAT

Since dendritic cells are key players in the cellular immune response to sensitizers resulting in allergic contact dermatitis they have been considered promising for the development of *in vitro* assays replacing animal tests, such as the LLNA. Due to the difficulties and complexities in the isolation and cultivation of native DCs, several cell types displaying DC-like properties have been considered as an alternative. An example are PBMCs (peripheral blood mononuclear cells), however costly isolation, donor-to-donor variability and limited availability restrict the usefulness of these cells. Human myeloid leukaemia cell lines (such as THP-1, U937 and MUTZ-3) also display DC-like properties and have been regarded a valuable alternative to native DCs. Following treatment with cytokines these cell lines have the ability to differentiate towards a dendritic cell-like phenotype. Upon stimulation with skin sensitizers they enhance expression of the co-stimulatory molecules CD86 and CD54 (in THP-1) or CD86 (in U937). These changes in phenotype enable their use for monitoring skin sensitizers. Expression of pro-inflammatory cytokines such as IL-1β or IL-8 may also indicate cell activation by potential sensitizers. Among the currently available cell lines, especially U937 and THP-1 are well suited to test the sensitization potential of substances since these cells do not require pre-treatment with cytokines in order express activation biomarkers. They are therefore considered useful for exploiting phenotypical alterations in response to exposure to sensitizers as predictive endpoints for ACD (Aeby et al. 2004; Emter, Ellis, and Natsch 2010; Python, Goebel, and Aeby 2007; Yoshida et al. 2003; T. Ashikaga et al. 2002; Ryan et al. 2007b).

A practical implementation is the h-CLAT test developed by Ashikaga et al. based on the cell line THP-1. Upon treatment with subtoxic concentrations of sensitizing substances (not irritants), THP-1 cells enhance the expression of the surface molecules CD86 and/or CD54. Both molecules play a role in the activation of naïve T cells by LCs in draining lymph nodes. CD86 (B7-2) acts through binding of the corresponding receptors CD28 and CTLA-4 on T cells. CD54 (ICAM) serves as intercellular cell adhesion molecule to stabilize cell-cell contact via binding to CD11a/CD18 (Sheikh and Jones 2008; T. Ashikaga et al. 2002).

Since the concentration triggering DC activation may differ considerably from substance to substance and may be close to toxic concentrations, the concentration range has to be determined prior to testing CD86 and CD54 expression. An appropriate concentration range is selected based on CV75 values. In the original test protocol, the toxicity is determined by propidium iodide (PI) staining followed by a flow cytometric analysis. After determination of an appropriate concentration range, cells are seeded at a defined density ($5*10^5$ cells per well) into wells and the substances (dissolved in either DMSO or PBS) are added in a way that the DMSO concentration does not exceed 0.2 %. Cells are incubated for 24 h during which sensitizing chemicals cause an increase in expression of CD86 and/or CD54 on the cell surface, whereas non-sensitizers do not. Following stimulation the surface markers are stained with FITC-labelled anti-CD86/anti-CD54 antibodies at 4 °C for 30 min and the fluorescence is measured by means of flow cytometry. Cell viability is measured simultaneously by exclusion of PI. As only cell populations with a viability >50 % are further analysed, antibody binding to dead cells is excluded by applying an appropriate PI gate. The cell count threshold is set at 10,000 cells. Results are expressed as relative fluorescent intensity, i.e. the geometric mean of the log10 transformed fluorescence intensities. A sensitizer is defined as a substance that is capable of upregulating CD86 by a factor of 1.5 or CD54 by a factor of 1.2 at any concentration point at which cells display a viability of larger than 50% (T. Ashikaga et al. 2006; Takao Ashikaga et al. 2008; Ryan et al. 2007b; Yoshida et al. 2003).

2.3.2.4 MYELOID U937 SKIN SENSITIZATION TEST (MUSST)

The MUSST is a DC-like cell based assay that was first described by (Python, Goebel, and Aeby 2007). It is in many ways similar to the h-CLAT with the exception that the human myeloid cell line U937 rather than THP-1 is used and only CD86 serves as a biomarker for cell activation. U937 cell count is adjusted to 5*105 cells/mL and 100 µL are seeded in 96 well plates. The cells are exposed to a concentration range of the test substances (determined as

described above) for 48 h at 37 °C and 5 % CO2. Staining and measurement is done as described above. Substances are considered to have sensitizing potential, if the ratio of RFIs (treatment vs. control) of CD86 exceeds a threshold of 1.5 at any tested concentration. (Bauch et al. 2011; Python, Goebel, and Aeby 2007).

2.4 AIM OF THE THESIS

A major regulatory concern during the development of new products that come in contact with the skin, such as pharmaceuticals for topical application or cosmetics, is the safety of the consumers. Thus the identification of chemicals that pose a health risk by acting as skin sensitizer is a mandatory step in safety assessment (Goebel et al. 2012). Until the EU-wide ban on animal testing for components of cosmetic products became effective in 2013, identifying potential allergens has relied completely on animal studies. Due to regulatory and ethical reasons avoiding the use of animals is of ever increasing importance. Hence alternative *in vitro* assays are being studied intensely. This thesis is conducted to contribute to the development of a battery of assays for the determination of the skin sensitization potential of substances. The test battery (as shown in Figure 2-6) should include assays targeting different key steps of the sensitization process, such as the DPRA, ARE and h-CLAT in order to make a reliable prediction.

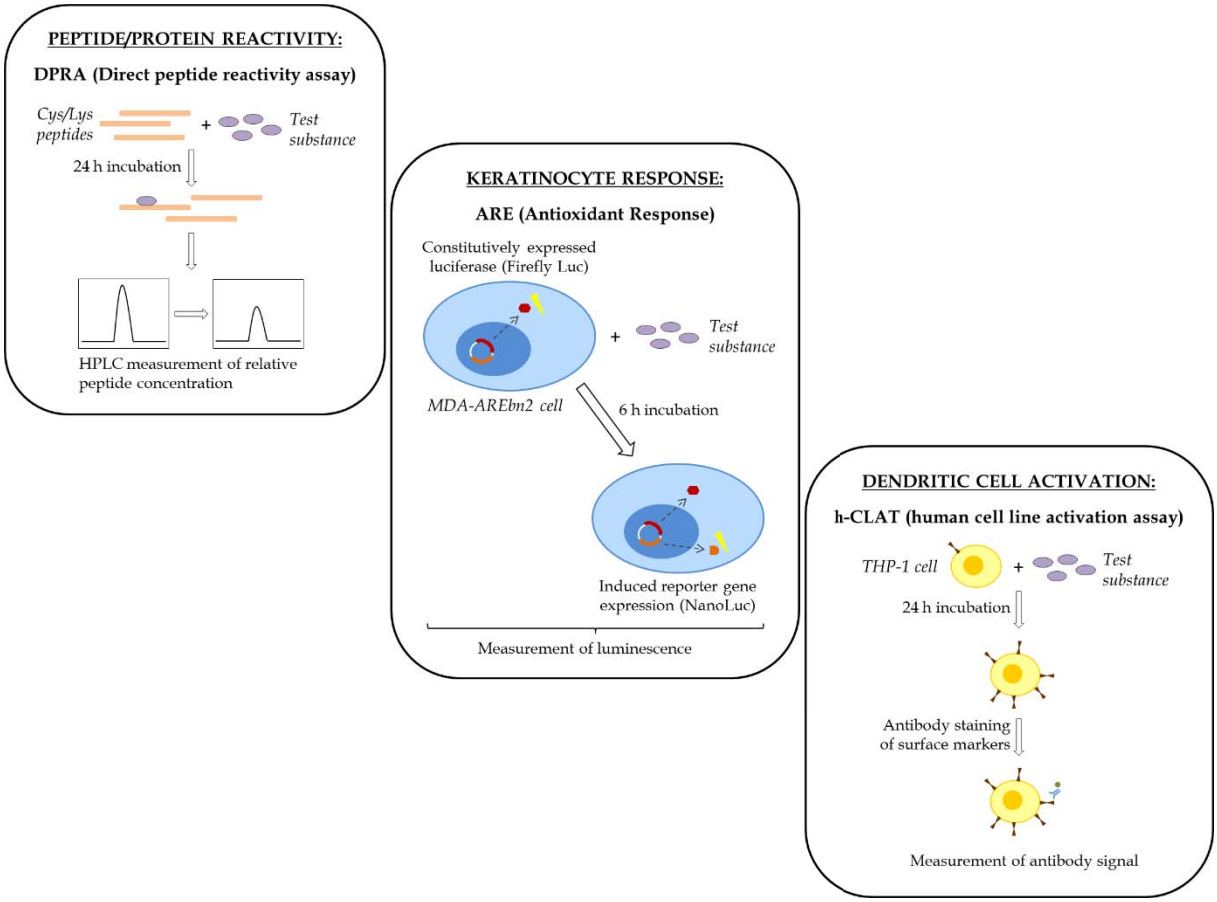

Figure 2-6: Battery of assays for the determination of the sensitization potential of substances. In order to obtain a reliable prediction of the sensitization potential of a substance one *in vitro* assay is not sufficient. Therefore a combination of assays targeting different stages of the sensitization process needs to be used.

Within the scope of this thesis an *in vitro* sensitization test should be developed by combining the principle of the h-CLAT (developed by Ashikaga et al. 2006) with a simple cell-based ELISA as an alternative detection method. The new ELISA-based method like the original h-CLAT exploits the fact that THP-1 cells show enhanced expression of the surface markers CD86 and/or CD54 upon exposure to sensitizing substances. While both assays stimulate the cells in the same way, through incubation with test substances, they differ in the used detection method. Whereas for the h-CLAT a flow cytometer is needed, the ELISA can be performed in every laboratory with standard equipment. Another advantage of the ELISA as detection method would be higher throughput, which is especially useful with multiple determinations. The main challenge compared to a standard ELISA is that the analytes are surface proteins attached to cells. Therefore, they cannot be immobilized by capture antibodies, as it is done e.g. in sandwich ELISA, as the cells may be washed away or get lost. Since a cell-based ELISA will be used, the cell count per well is of crucial importance and cell loss during the assay (e.g. due to washing steps) has to be considered. Additionally, to obtain a detectable signal a suitable antibody setup and appropriate dilutions of the antibodies need to be chosen. Furthermore, a method for normalizing the measured signal to the actual cell count is useful. After the establishment of the h-CLAT and the development of the cell-based ELISA the test results should be validated against h-CLAT flow cytometric data and compared to other methods included in the test battery.

3. MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 CELL LINES

The used cell lines are listed in Table 3-1.

Table 3-1: Used cell lines.

Cell line	Specification	Source
MDA-AREbn2		FH Campus Vienna (Vienna, Austria)
THP-1	ATCC® TIB-202™	ATCC LGC Standards GmbH (Wesel, Germany)
U937	ATCC® CRL1593.2™	ATCC LGC Standards GmbH (Wesel, Germany)

The monocytic cell line THP-1 was originally isolated from the peripheral blood of a one year old boy suffering from acute monocytic leukemia. THP-1 cells express Fc receptors as well as C3b, but no cytoplasmic or surface immunoglobulins. The presence of alpha-naphthyl butyrate esterase activities which could be inhibited by NaF, the phagocytosis of both latex particles and sensitized sheep erythrocytes, lysozyme production and the ability to restore T-lymphocyte response to Con A were described as distinct monocytic markers of the cell line (Tsuchiya et al. 1980). Furthermore THP-1 serves as surrogate cell line for human Langerhans cells, as they show characteristics like MHC class II and CD86 expression, IL-1 secretion and phagocytosis (Ashikaga et al. 2002; ATCC 2015a).

The hematopoietic cell line U937 was derived from malignant cells obtained from the pleural effusion of a 37-year old male patient with generalized histiocytic lymphoma. The histiocytic origin of U937 cell line was shown by its ability for lysozyme production and its strong esterase activity. U937 express C3, but no immunoglobulins, and the cell line is negative for Epstein Barr virus expression. The cell line is sensitive for TNF and anti-Fas antibodies, as it expresses Fas antigen (Sundström and Nilsson 1976; ATCC 2015b).

The cell line MDA-AREbn2 was created through the stable integration of the gene construct pGVL8 4AREb into the human breast cancer cell line MDA-MB-468 (ATTC HTB-132) via PiggyBac transposon system. The construct contains 4 AREb sequences, a constitutively expressed firefly luciferase (Luc) sequence and a sequence of the reporter nanoluciferase

(Nluc). The cell line was constructed and provided by FH campus Vienna (Ettenberger-Bornberg et al. 2015).

3.1.2 MEDIA AND SUPPLEMENTS

The media and supplements used for cultivation of the cell lines are listed in Table 3-2 and Table 3-3.

Table 3-2: Used media.

Media	Supplier
DMEM with high Glucose, with L-Glutamine, with Sodium Pyruvate, with Phenol Red	Gibco Life Technologies (Paisley, UK)
RPMI 1640, HEPES, no Glutamine	Gibco Life Technologies (Paisley, UK)
RPMI 1640, without L-Glutamine and Phenol Red	Lonza (Basel, Switzerland)

Table 3-3: Used supplements.

Supplements	Supplier
Dimethyl sulfoxide ≥ 99,9%	Sigma Aldrich (Saint Louis, USA)
Fetal calf serum (FCS)	Gibco Life Technologies (Paisley, UK)
GlutaMAX™ 100x	Gibco Life Technologies (Paisley, UK)
MEM Non-Essential Amino Acids Solution 100x	Gibco Life Technologies (Paisley, UK)
Penicillin/Streptomycin 200 mmol/L	Gibco Life Technologies (Paisley, UK)
Trypsin-EDTA solution	Gibco Life Technologies (Paisley, UK)

The 500 mL bottles of RPMI 1640 medium were opened under sterile conditions in the laminar flow workbench and 10 % fetal calf serum (50 mL), 2 mmol/L GlutaMAX (5.5 mL), 100 U/mL penicillin and 100 µg/mL streptomycin (550 µL Pen/Strep mix) were added for completion of the medium, which is necessary for the cultivation of the cell line U937. For the cultivation of THP-1 also 0.05 mmol/L 2-mercaptoethanol (1.77 µL) were added to the medium, which is a potent reducing agent preventing toxic levels of oxygen radicals and that is often used in cell culture media.

The DMEM medium for cultivation of MDA-AREbn2 was completed under sterile conditions by the addition of 10 % FCS, 1x Pen/Strep and 1x Non-essential amino acids.

3.1.3 ANTIBODIES

The antibodies used within this study are listed in Table 3-4.

Table 3-4: Used antibodies.

Antibody	Clone	Supplier
FITC Mouse anti-human CD86	2331 (FUN-1)	BD Pharmingen™ (San Jose, USA)
Goat Anti-Mouse IgG1:HRP	Polyclonal	AbD Serotec (Puchheim, Germany)
Purified Mouse Anti-Human CD54	HA58	BD Pharmingen™ (San Jose, USA)
Purified Mouse Anti-Human CD86	2331 (FUN-1)	BD Pharmingen™ (San Jose, USA)
Purified Mouse IgG1, κ Isotype Control	Clone MOPC-21	BD Pharmingen™ (San Jose, USA)

3.1.4 KITS

All kits used in the experiments are listed in Table 3-5.

Table 3-5: Used kits.

Kit	Supplier
EZ4U Cell Proliferation Assay	Biomedica (Oxford, UK)
MycoAlert™ mycoplasma detection kit	Lonza (Basel, Switzerland)
Nano-Glo® Dual-Luciferase® Reporter Assay System	Promega (Fitchburg, USA)
TMB Peroxidase EIA Substrate Kit	Bio-Rad (Hercules, USA)

3.1.5 CHEMICALS AND SOLUTIONS

The used chemicals and solutions are listed in Table 3-6.

Table 3-6: Used chemicals and solutions.

Chemical/Solution	Supplier
2-Mercaptobenzothiazole	Sigma Aldrich (Saint Louis, USA)
2-Mercaptoethanol	Sigma Aldrich (Saint Louis, USA)
Acetone	Sigma Aldrich (Saint Louis, USA)
Acetonitrile	Avantor/J. T. Baker (Deventer, Netherlands)
Ammonium acetate	Sigma Aldrich (Saint Louis, USA)
Calcein AM solution	Sigma Aldrich (Saint Louis, USA)
Certipur® buffer solution pH 4.00	Merck Millipore (Billerica, USA)
Certipur® buffer solution pH 7.00	Merck Millipore (Billerica, USA)
Cinnamic aldehyde	Sigma Aldrich (Saint Louis, USA)
Cobalt chloride ($CoCl_2$)	Alfa Aesar (Ward Hill, USA)

Cohn fraction	Sigma Aldrich (Saint Louis, USA)
Crystal violet	VWR (Radnor, USA)
Diethyl phthalate	Sigma Aldrich (Saint Louis, USA)
Dimethyl sulfoxide	Merck (Kenilworth, USA)
Disodium phosphate (Na_2HPO_4)	Sigma Aldrich (Saint Louis, USA)
DPBS (1x) without Ca^{2+} and Mg^{2+}	Lonza (Basel, Switzerland)
DPBS (1x), no calcium, no magnesium	Gibco Life Technologies (Paisley, UK)
Ethanol	VWR (Radnor, USA)
Hydrochloric acid (HCl)	Merck (Kenilworth, USA)
Isopropanol	Avantor/J. T. Baker (Deventer, Netherlands)
Methanol	Sigma Aldrich (Saint Louis, USA)
Methyl methacrylate	Sigma Aldrich (Saint Louis, USA)
Monopotassium phosphate (KH_2PO_4)	Sigma Aldrich (Saint Louis, USA)
Sodium dihydrogen phosphate (NaH_2PO_4)	Sigma Aldrich (Saint Louis, USA)
Sodium hydroxide (NaOH) pellets	Merck (Kenilworth, USA)
Sodium hydroxide (NaOH) 1 mol/L	Merck (Kenilworth, USA)
Natrium chloride (NaCl)	Sigma Aldrich (Saint Louis, USA)
Paraformaldehyde 97 %	Alfa Aesar (Ward Hill, USA)
p-Benzoquinone	Sigma Aldrich (Saint Louis, USA)
Phthalic anhydride	Sigma Aldrich (Saint Louis, USA)
Potassium chloride (KCl)	VWR (Radnor, USA)
p-Phenylendiamine	Alfa Aesar (Ward Hill, USA)
Sulfuric acid (H_2SO_4)	Sigma Aldrich (Saint Louis, USA)
Trypan blue solution 0.4 %	Sigma Aldrich (Saint Louis, USA)
1-Chloro-2,4-dinitrobenzene	VWR (Radnor, USA)

Preparation of paraformaldehyde solution

To obtain a 2 % paraformaldehyde solution for fixation of CD86/CD54 stained THP-1 cells 200 mg PFA were dissolved in 1 mL ultrapure water and 5 µL 1 mol/L NaOH at 70 °C in the waterbath. Upon cooling to room temperature 9 mL DPBS were added to a total volume of 10 mL. The solution was either prepared freshly or aliquots were stored in the freezer at -20 °C.

Preparation of staining buffer 1 (DPBS with 0.1 % FCS)

Staining buffer 1 for the blocking procedure included in the ELISA protocol (see 4.5.2) consists of 1x DPBS w/o Mg^{2+} and Ca^{2+} supplemented with 0.1 % FCS. In a falcon tube 50 µL FCS were added to 50 mL DPBS.

Preparation of staining buffer 2 (DPBS with 0.1 % FCS and 0.01 % Cohn fraction)

For the preparation of staining buffer 25 mg Cohn fraction were dissolved in 50 mL 1x DPBS. The 10 % solution was diluted 1:100 in DPBS to obtain a 0.1 % solution, which was frozen in 1 mL and 2 mL aliquots. The aliquots were thawed on demand and were further diluted 1:10 in DPBS. For 50 mL staining buffer 5 mL of 0.1 % Cohn fraction in DPBS were added to 45 mL DPBS and 50 µL FCS to a final concentration of 0.01 % Cohn fraction.

3.1.6 EQUIPMENT

All equipment needed for the experiments is listed in Table 3-7.

Table 3-7: Used equipment.

Equipment	Manufacturer
450 nm Filter	Perkin Elmer (Waltham, USA)
490 nm Excitation Filter	Perkin Elmer (Waltham, USA)
520 nm Emission Filter	Perkin Elmer (Waltham, USA)
550 nm Filter	Perkin Elmer (Waltham, USA)
650 nm Filter	Perkin Elmer (Waltham, USA)
-80°C freezer Igloo	Telstar technologies (Terrassa, Spain)
Analytical balance A200S	Sartorious (Göttingen, Germany)
Centrifuge 5702 (Rotor A-4-38)	Eppendorf (Hamburg, Deutschland)
Centrifuge 5810R (Rotor F45-30-11)	Eppendorf (Hamburg, Deutschland)
CO2-Incubator New Brunswick-Galaxy 170R	Eppendorf (Hamburg, Deutschland)
Drying oven FD115	Binder (Tuttlingen, Germany)
HPLC Column Luna 3u C18(2) 100A, 100 x 2 mm	Thermo Scientific (Waltham, USA)
HPLC-System Thermo U3000	Thermo Scientific (Waltham, USA)
Incubator Shaker Laborshaker THL 500	Gerhardt (Königswinter, Germany)
Laminar flow Biosafe 7-130	Ehret GmbH (Mahlberg, Germany)
Magnetic stirrer with heating plate RCT	Ika (Staufen, Germany)
Microscope Eclipse TS1000	Nikon (Tokyo, Japan)
Multichannel pipette Xplorer 50-1200 µL	Eppendorf (Hamburg, Deutschland)
MultilabelCounter 1420 Victor3™	Perkin Elmer (Waltham, USA)
Nitrogen Dewar GT11	Air Liquide (Paris, France)
pH-meter Lab 860	Schott (Mainz, Germany)
Pipetboy acu	Integra Biosciences (Zizers, Switzerland)
Pipetman L Multichannel P12x200	Gilson (Middleton, USA)
Pipetman L Multichannel P8x20	Gilson (Middleton, USA)
Pipetman L Multichannel P8x300	Gilson (Middleton, USA)
Pipetman P10	Gilson (Middleton, USA)
Pipetman P100	Gilson (Middleton, USA)
Pipetman P1000	Gilson (Middleton, USA)
Pipetman P200	Gilson (Middleton, USA)

Pipetman Ultra U8x300	Gilson (Middleton, USA)
Pipette controller, Accurpette	VWR (Radnor, USA)
Rotary evaporator hei UAP precision	Heidolph (Schwabach, Germany)
Rotary evaporator Laborota 4003 control	Heidolph (Schwabach, Germany)
Ultrapure water system Reference A+	Millipore (Billerica, USA)
Varioklav 400E (autoclave)	HP Medizintechnik (Oberschleißheim, Germany)
Vortex Genius 3	Ika (Staufen, Germany)
Water bath – 1002	GFL (Burgwedel, Germany)
XS205 DualRange Analytical Balance	Mettler Toledo (Greifensee, Switzerland)

3.1.7 DISPOSABLES

The used disposable items are listed in Table 3-8.

Table 3-8: Used disposables.

Disposables	Supplier
1 mL Eppendorf tubes	Eppendorf (Hamburg, Deutschland)
1.8 mL cryo tube vials, sterile	Nunc (Roskilde, Denmark)
10 mL pipette, sterile	Sterilin (Newport, UK)
15 mL polypropylene falcons, sterile	Sterilin (Newport, UK)
2 mL Eppendorf tubes	Eppendorf (Hamburg, Deutschland)
2 mL pipette, sterile	Sterilin (Newport, UK)
2 mL vials, clear glass	Merz (Raleigh, USA)
20 mL vials, clear glass	Merz (Raleigh, USA)
25 mL pipette, sterile	Sterilin (Newport, UK)
50 mL polypropylene falcons, sterile	Sterilin (Newport, UK)
75 cm^2 cell culture flasks	VWR (Radnor, USA)
96 well flat bottom plates	VWR (Radnor, USA)
96 well flat bottom plates (tissue culture treated), sterile	VWR (Radnor, USA)
96 well V-bottom plates, sterile	VWR (Radnor, USA)
Divided reagent reservoirs, 3-wells	VWR (Radnor, USA)
Pipette tips 200 µL	VWR (Radnor, USA)
Pipette tips Diamond Tip 0.1-20 µL	Gilson (Middleton, USA)
Pipette tips epT.I.P. 50-1250 µL	Eppendorf (Hamburg, Deutschland)
Reagent reservoirs	VWR (Radnor, USA)
Reagent reservoirs, sterile	VWR (Radnor, USA)
Weighing dishes	VWR (Radnor, USA)

3.2 BASIC CELL CULTURE WORK

3.2.1 CULTIVATION OF CELLS

All used cell lines were cultivated at 37 °C and 5 % CO_2 in the CO_2 incubator.

3.2.1.1 THP-1

THP-1 cells were cultivated in suspension in RPMI 1640 medium (Gibco #42401-018) supplemented with FCS, GlutaMax, Pen/Strep and 2-mercaptoethanol in 75 cm^2 (10-20 mL cell suspension) or 175 cm^2 (40-50 mL cell suspension) culture flasks. The cells were passaged every two to four days at different rates depending on their usage and cell density, mostly at a 1:2, 1:4 or 1:10 ratio. Passages were made by transferring the respective volume of the old cell suspension to a new culture flask with a sterile pipette and adding fresh medium to obtain the original volume. The medium was tempered to 37 °C previously. The cell density during cultivation was always kept between $2*10^5$ and 10^6 cells/mL (see Figure 3-1).

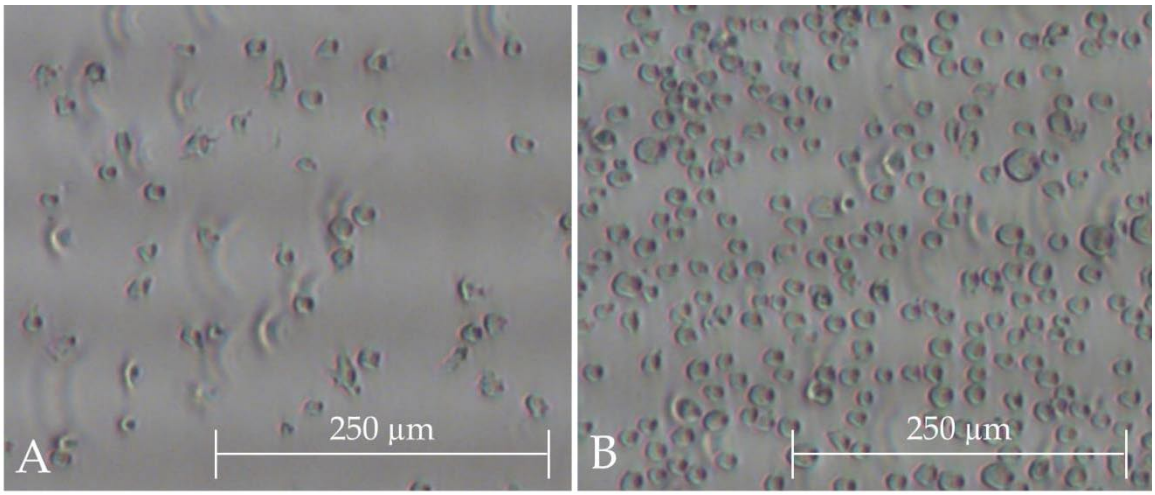

Figure 3-1: THP-1 cells at low and high density of $2.5*10^5$ (A) and $9*10^5$ cells/mL (B). Magnification factor = 100x.

If a defined cell count was needed, the cell suspension was transferred to sterile centrifuge tubes and centrifuged for 5 min at 1200 rpm (225 g). The medium was discarded and the cell pellet resuspended in a previously determined volume of medium, which was calculated as described by the following equation.

$$\text{resuspension volume [mL]} = \frac{\text{centrifuged volume [mL]} * \text{cell count} * 10^4}{\text{desired cell concentration [cells/mL]}}$$

3.2.1.2 U937

U937 cells were cultivated in suspension in RPMI 1640 medium (Gibco #42401-018) supplemented with FCS, GlutaMax and Pen/Strep in 75 cm^2 (10-20 mL cell suspension) culture flasks. The flasks were filled with 10 or 20 mL cell suspension. The cells were passaged every three to four days at different rates depending on their usage and cell density, mostly at a 1:2, 1:4 or 1:10 ratio. Passages were made by transferring the respective volume of the old cell suspension to a new culture flask with a sterile pipette and adding fresh medium to obtain the original volume. The medium was tempered to 37 °C previously. If a defined cell count was needed, the cell suspension was transferred to sterile centrifuge tubes and centrifuged for 5 min at 1200 rpm (225 g). The medium was discarded and the cell pellet resuspended in a previously determined volume of medium, which was calculated as described above.

3.2.1.3 MDA-AREBN2

MDA-AREbn2 cells were cultivated in complete DMEM high glucose medium supplemented with FCS, 1x NEAA and 1x Pen/Strep in 75 cm^2 culture flasks. The adherently growing cells were passaged (1:3, 1:5 or 1:10) twice or three times a week thereby preventing growth over 80 % confluence (see Figure 3-2).

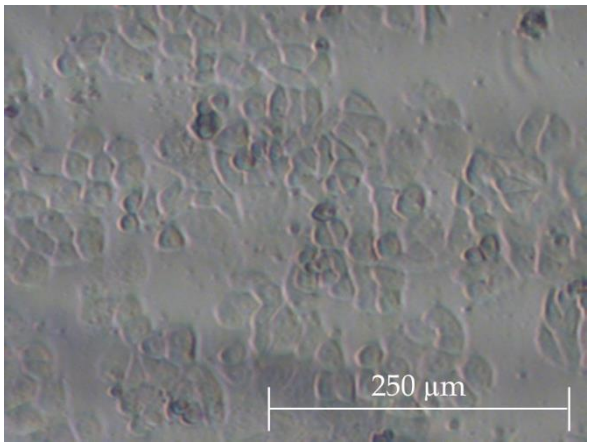

Figure 3-2: MDA-AREbn2 cells at ca. 80 % confluence. Magnification factor = 100x.

Therefore, the medium was discarded and the cells were rinsed carefully with 10 mL tempered, sterile DPBS. After discarding the PBS the cells were incubated with 1 mL trypsin-EDTA solution for 10-15 min. The detachment was checked under the microscope. If the cells were detached, 4 mL medium were added to inhibit trypsin and the respective volume of cells was transferred to a new culture flask, which had been prepared with 20 mL tempered medium.

3.2.2 DETERMINATION OF THE CELL COUNT

The cell count of cell suspensions was determined using a Neubauer counting chamber. First the cover glass was fixed on top of the counting chamber with 96% ethanol. Then, a few microliters of cell suspension were transferred to the two chambers with a pipette, 4 squares were counted and a mean cell count was calculated. Since one quarter of the Neubauer chamber is 1 x 1 cm, the volume is 0.1 µL. To obtain the cell concentration [cells/mL] the following equation is used

$$\text{cells/mL} = \text{mean cell count} * 10^4.$$

3.2.3 TRYPAN BLUE EXCLUSION ASSAY

The trypan blue exclusion assay is an easy and quick way to determine cell viability. Living cells are capable to exclude the azo dye whereas the disrupted cell membrane of dead cells allows the dye to pass, resulting in a bright blue stain of dead cells. The incubation time should not exceed 2-3 min, as trypan blue is slightly cytotoxic resulting in a drop of viability over time (Schmitz 2011). 5 µL trypan blue were transferred to an Eppendorf tube and mixed with 45 µL cell suspension with a pipette, to obtain a 1:10 ratio. Cells were counted using a Neubauer chamber and cell viability was calculated using the following equation.

$$\text{Viability [\%]} = \frac{\text{unstained cells}}{\text{total cell count}} * 100$$

3.2.4 THAWING OF CELLS

For thawing of cells stored in liquid nitrogen, 9 mL medium were transferred to a 75 cm^2 culture flask and equilibrated in the CO_2 incubator at 37 °C for 10-15 min. The vial containing the cell suspension was thawed quickly for approximately 3 min in the laminar flow workbench in a beaker filled with water at 37 °C. The temperature was monitored with a thermometer. To avoid contamination the vial was not completely dipped into the water. The cells were then transferred from the vial to a sterile centrifuge tube using a pipette and 9 mL tempered medium were added slowly. To remove the freezing medium, which contains DMSO, the cells were centrifuged for 7 min at 125 g and the supernatant was removed with a pipette. The pellet was dissolved in 1 mL tempered medium and transferred to the prepared culture flask which was then placed in the CO_2-incubator at 37 °C.

3.2.5 GENERATION OF A MASTER CELL BANK

After their delivery the vials containing the cells were immediately transferred to liquid nitrogen for storage until their further usage. The cells were thawed quickly as described above (see 3.2.4). After incubation over night the first passage was made at a 1:2 rate, by splitting the cultured volume in half and adding 5 mL medium to both flasks, which were incubated for another 3 days at 37 °C and 5 % CO_2 to allow the majority of the cells to reach the log phase. The cells were passaged at a 1:10 ratio and cultured in 75cm² tissue culture flasks for another three days. Labelled cryovials were stored in the freezer and freezing medium was prepared by the addition of 15 % highly pure DMSO to complete RPMI 1640. Cell suspension from 3 culture flasks were pooled in a centrifuge tube and spun down for 10 min at 125 g. The appropriate resuspension volume to achieve the desired cell count of $1*10^6$ cells/mL was calculated. The cell pellet was resuspended in complete RPMI 1640 (1/3 of the calculated volume) and freezing medium was slowly added (2/3 of the calculated volume), resulting in a final DMSO concentration of 10 %. As DMSO is slightly cytotoxic, the cell suspension was aliquoted to 1.5 mL as quickly as possible. To assure that the cells were frozen slowly a freezing box was used. The box was filled with 250 mL isopropyl alcohol which allows the temperature to drop 1 °C per minute. The box containing the vials was stored at -80 °C over night, before being placed in liquid nitrogen.

24 h after cryopreservation, one vial was thawed and cultivated to test the quality of the frozen cells.

3.2.6 MYCOPLASMA TEST

Besides cross-contamination the contamination with mycoplasma is one of the major issues in cell culture and can have various undesired effects on the cultivated cells (Drexler and Uphoff 2002). Since mycoplasma are resistent to most of the commonly used antibiotics, the cultured cells were tested for mycoplasma contamination using MycoAlert mycoplasma detection kit. The kit uses the activity of mycoplasma enzymes, which are not present in eukaryotic cells. Through lysis of the mycoplasma the enzymes are able to react with the MycoAlert substrate and convert ADP to ATP. ATP levels can be measured through conversion into a light signal by luciferase contained in the MycoAlert reagent.

600 µL of buffer were added to the substrate and the reagent and both were equilibrated to room temperature. In the meantime 1 mL of the cell suspension to be tested was spun down for 5 min at 200 g. 100 µL of the cleared supernatant were mixed with 100 µL MycoAlert

reagent and luminescence was measured after 5 min incubation at room temperature (Read A). Then 100 µL of substrate were added and the samples were incubated for 10 min. The luminescence was measured again (Read B) and the ratio was calculated by dividing Read B by Read A to determine if mycoplasma are present. Interpretation of the data was done as shown in Table 3-9.

Table 3-9: Interpretation of the mycoplasma test data according to manufacturer instruction.

Ratio	Interpretation
< 0.9	Negative for mycoplasma
0.9-1.2	Quarantine cells & retest in 24 h
> 1.2	Mycoplasma contamination

3.3 TESTING SUBSTANCES

3.3.1 SELECTION OF TEST CHEMICALS

Nine test chemicals were chosen in order to cover known non-sensitizers and sensitizers with different potency to induce ACD. The chemicals listed in Table 3-10 were selected for use in the h-CLAT after the determination of their cytotoxicity by means of EZ4U assay.

Table 3-10: Chemicals selected for cytotoxicity and h-CLAT.

Substance	Abbreviation	Classification in literature (LLNA)[1]	Reference
2-Mercaptobenzothiazole	2-MBT	++	Bauch et al. 2012
Phthalic anhydride	PA	+++	Bauch et al. 2012
p-Phenylendiamine	p-PD	+++	Santos et al. 2009
Diethylphthalate	DEP	-	Santos et al. 2009
Methylmethacrylate	MMA	+	Borak et al. 2011
p-Benzoquinone	p-BQ	++++	Bauch et al. 2012
Cinnamic aldehyde	CA	++	Santos et al. 2009
Cobalt chloride	$CoCl_2$	++	Bauch et al. 2012

Dimethyl sulfoxide (DMSO) was used as vehicle. Although it acts as an irritant, it is considered a non-sensitizer (Yoshida et al. 2003a) and is therefore a suitable vehicle.

[1] Extreme sensitizer ++++
Strong sensitizer +++
Moderate sensitizer ++
Weak sensitizer +
Non-sensitizer -

Stock solutions of all chemicals were prepared by dissolving or diluting the respective amount of substance in DMSO (see Table 3-11). For the biological assays the substances were further diluted in DMSO and complete RPMI1640 medium, resulting in the desired concentrations and a final DMSO concentration of 0.1 %.

Table 3-11: Preparation of 1 mol/L stock solutions of the test chemicals.

Substance	M [g/mol]	Added substance	DMSO [µL]
2-MBT	167.24	0.1658 g	991
PA	148.12	0.1467 g	990
p-PD	108.14	0.0202 g	188
DEP	222.24	0.2231 g	1004
MMA	100.12	0.0961 g	960
p-BQ	108.10	0.1100 g	1018
CA	132.16	126 µL	874
DNCB	202.55	0.2025 g	1000

As 1 mol/L cobalt chloride was not soluble in DMSO, it was directly dissolved in complete RPMI1640 medium containing 0.2 % DMSO to obtain a solution of 1 mmol/L. Therefore, 5 mg cobalt chloride were added to 38.5 mL medium and 77 µL DMSO and the solution was stored at 4 °C.

For the cytotoxicity assay and the h-CLAT the same stock solutions were used, but the final dilutions in RPMI1640 were prepared freshly for every assay.

3.3.2 EZ4U CYTOTOXICITY ASSAY

The EZ4U (easy for you) Cell Proliferation Assay is a method for distinguishing between living and dead cells and can be used for example for the determination of the cytotoxicity of substances. The assay is based on the fact that living cells are able to reduce non- or slightly colored tetrazolium salts to formazan derivativs of intensely red color in their mitochondria. As mitochondria become inactive a few min after cell death, the metabolization of the tetrazolium salt substrate correlates with the living cell count, which can be measured photometrically at 450 or 492 nm.

For the construction of a cytotoxicity curve the substances were diluted according to Table 3-12. The substances were first solved in DMSO to obtain 1 mol/L stock solutions which were then further diluted in DMSO to maintain a constant DMSO concentration of 0.2 %. These 1000x stock solutions were diluted 1:500 in medium resulting in 2x concentrated dilutions (containing 0.2 % DMSO). The final DMSO concentration in the wells did not exceed 0.1 %.

Table 3-12: Dilution of the test substances for the EZ4U assay. The test substances were first diluted in DMSO to obtain a 1000x stock, then the dilutions in medium were prepared resulting in 2x concentrated dilutions. As the cells and test substances were mixed 1:2 in the wells, the final concentrations were 1x (0.01-1 mmol/L).

	1 mol/L Stock [µL]	DMSO [µL]	1000x conc. [mmol/L]	Substance [µL]	Medium [µL]	2x conc. [mmol/L]	1x conc. [mmol/L]
D1		-	1000	2 D1	998	2	1
D2	30	70	300	2 D2	998	0.6	0.3
D3	10	90	100	2 D3	998	0.2	0.1
D4	3	97	30	2 D4	998	0.06	0.03
D5	10	990	10	2 D5	998	0.02	0.01

The cell count and the viability of the cells were determined by trypan blue in a Neubauer counting chamber. Therefore, the cell suspension was centrifuged for 5 min at 225 g (1200 rpm) and the pellet was resuspended in a defined volume of RMPI1640 medium without phenol red to adjust the cell count to $1*10^5$ cells/mL. White medium was used, because of the interference of phenol red with the red color of the formazan derivatives. 100 µL cell suspension were seeded in a 96 well plate in triplicate and 100 µL of the respective dilution of the test substances were added. Medium containing 0.2 % DMSO was added to the cells as blank. The cells were incubated with the test substances for 24 h at 37 °C and 5 % CO_2. Based on the stimulation time used in the h-CLAT, an incubation time of 24 h was chosen instead of the 72 h commonly used for EZ4U.

For staining with the EZ4U kit one flask of substrate was dissolved in 2.5 mL activator both of which should be used at room temperature and tempered at 37 °C prior to addition to the cells. After adding 20 µL of the staining solution to each well the cells were incubated for 3.5 h at 37 °C and 5 % CO_2 to allow them to metabolize the tetrazolium salts. Absorbance was then measured photometrically at 450 nm and at 690 nm. The reference measured at 690 nm is used to subtract impurities on the 96 well plate, which would alter the actual absorbance. Medium containing 0.2 % DMSO was measured as blank for uncolored test substances. For substances with intrinsic color the dilution series of the substances without cells were measured as blanks. All blanks were subtracted from the measured values of the samples afterwards.

DETERMINATION OF IC50 VALUES

To define a suitable concentration range for each test substance for the h-CLAT, the concentration at which approximately 50 % of the THP-1 cells are viable (IC50) after 24 h incubation with the test substance was determined for each substance. The % survival was calculated for each concentration in relation to the cells that had been treated with the vehicle control and were therefore defined as 100 % survival according to the following equation:

$$\text{Survival [\%]} = \frac{\text{absorbance}_{\text{sample}}}{\text{absorbance}_{\text{vehicle control}}} * 100 \,.$$

The % survival were the blotted against the applied concentrations (see Figure 3-3) and the IC50 value was assessed graphically.

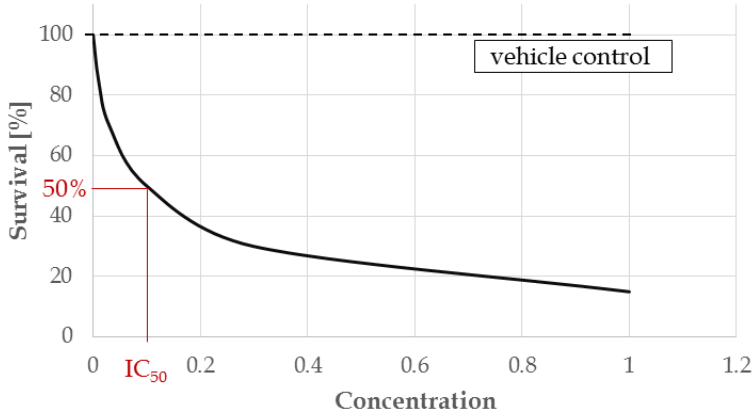

Figure 3-3: Example for the determination of the IC50 values of the test substances. The concentration at which 50 % of the cells are viable is defined as IC50.

3.3.3 HAZARD EVALUATION BY MEANS OF DPRA

The principle of the direct peptide reactivity assay is described in 2.3.2.1.

Dose-response curves were measured for diethyl phthalate (DEP), p-phenylendiamine (p-PD), p-benzoquinone (p-BQ) and cinnamic aldehyde (CA). Lysine (Lys) as well as cysteine (Cys) peptides were incubated with the test substances for 24 h in the dark at RT and the depletion of the peptides was measured using HPLC. The used peptides are specified in

Table 3-13.

Table 3-13: Peptides and buffers used for DPRA.

Lys	**Peptide**	Ac-RFAAKAA-COOH
	MW [g/mol]	820.92
	Buffer Lys	100 mmol/L ammonium acetate buffer (pH 10.2) + 20 % ACN
	c [mmol/L]	0.658 in buffer Lys
Cys	**Peptide**	Ac-RFAACAA-COOH
	MW [g/mol]	795.87
	Buffer Cys	100 mmol/L phosphate buffer (pH 7.5) + 20 % ACN
	c [mmol/L]	0.686 in buffer Cys

Stock solutions of the substances were prepared in ACN (100 mmol/L p-BQ, 13.5 mmol/L CA, 22.2 mmol/L DEP and 10 mmol/L p-PD) and the test substances were mixed with the peptides at different ratios (see Table 3-14) to obtain a concentration range.

Table 3-14: Ratios of peptide to test substance used for the measurement of the dose-response curves.

Lys:p-BQ	Lys:CA/DEP/p-PD	Cys:p-BQ	Cys:CA/DEP/p-PD
1:0	1:0	1:0	1:0
1:0.5	1:1	1:0.05	1:1
1:1	1:2.5	1:0.1	1:2.5
1:2.5	1:10	1:0.25	1:10
1:10	1:25	1:0.5	1:25
1:25	1:50		1:50

50 µL of the respective substance-peptide mixtures were injected into the HPLC system and the samples were eluted (at a column temperature of 50 °C) using a gradient, which is described in Figure 3-4.

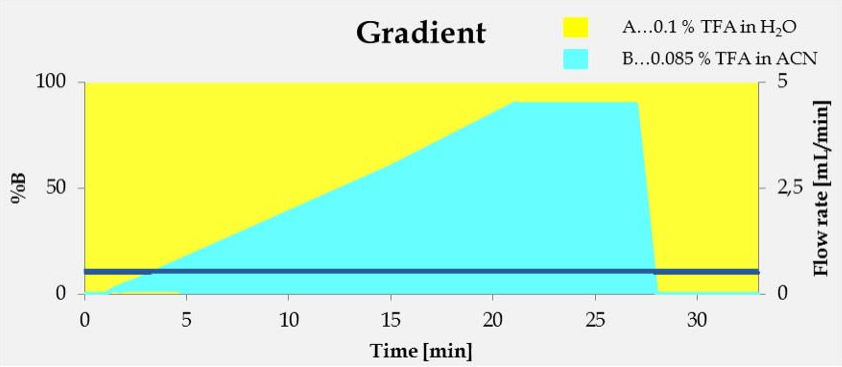

Figure 3-4: Gradient used for eluation. Eluent A consists of 0.1 % trifluoroacetic acid in water, eluent B consists of 0.085 % trifluoroacetic acid in acetonitrile.

For detection a wavelength detector was used at 220 nm.

The peak areas were integrated and the % peptide depletion was calculated using the following equation:

$$\text{Peptide depletion [\%]} = \left(1 - \frac{\text{Peptide peak are in replicate}}{\text{Mean peptide peak area in referece control}}\right) * 100.$$

The HPLC measurements were carried out by Daniela Neubert (OFI, Vienna).

3.3.4 HAZARD EVALUATION BY MEANS OF ARE

3.3.4.1 ESTABLISHMENT OF ARE

MDA-AREbn2 cells were cultivated as described in 3.1.2. The cell line carries a stably integrated construct containing nanoluciferase (NanoLuc) as reporter gene following 4 AREb sequences as well as a constitutively expressed firefly luciferase (Luc). ARE sequences play a role in the Nrf2-antioxidant response pathway which is shown in 2.3.2.2. 100 µL of the cell suspension are seeded per well of a 96 well plate at a density of 10^4 cells/well. Dilutions of the test substances are prepared in 1x PBS. After 24 h incubation at 37 °C and 5 % CO_2 the DMEM medium is removed from the adherent cells. 100 µL of the test substances are added to the wells and the plates are incubated for 6 h at 37 °C. In the meantime the reagents for the luciferase assay (Nano-Glo® Dual-Luciferase® Reporter Assay System) are equilibrated at room temperature. After incubation the test substances are removed and the cells are washed with 40 µL PBS. First viability is measured by adding Luc substrate (ONE-Glo™ EX). The substrate is dissolved in 10 mL luciferase buffer. According to the manufacturer the same volume used for incubation (100 µL) should be added to each well. Due to high costs the volume was reduced (shown below). The cells are incubated with luciferase substrate for at least 3 min at 300-600 rpm on a shaker and the solution is stored at -20 °C. The luminescence caused by Luc is measured using a photometer. Then, NanoLuc substrate (NanoDLR™ Stop&Glo) is prepared by mixing 1:100 substrate and NanoLuc buffer. The same volume as for firefly luciferase is added to each well and the plates are incubated for 10 min at 600-900 rpm on a shaker. The NanoLuc substrate solution contains not only substrate, but inhibitor of firefly luciferase as well. This way the luminescence caused by the two different luciferases does not interfere. The NanoLuc luminescence, which determines sensitization, is measured using a photometer. An overview of ARE assay is shown in Figure 3-5.

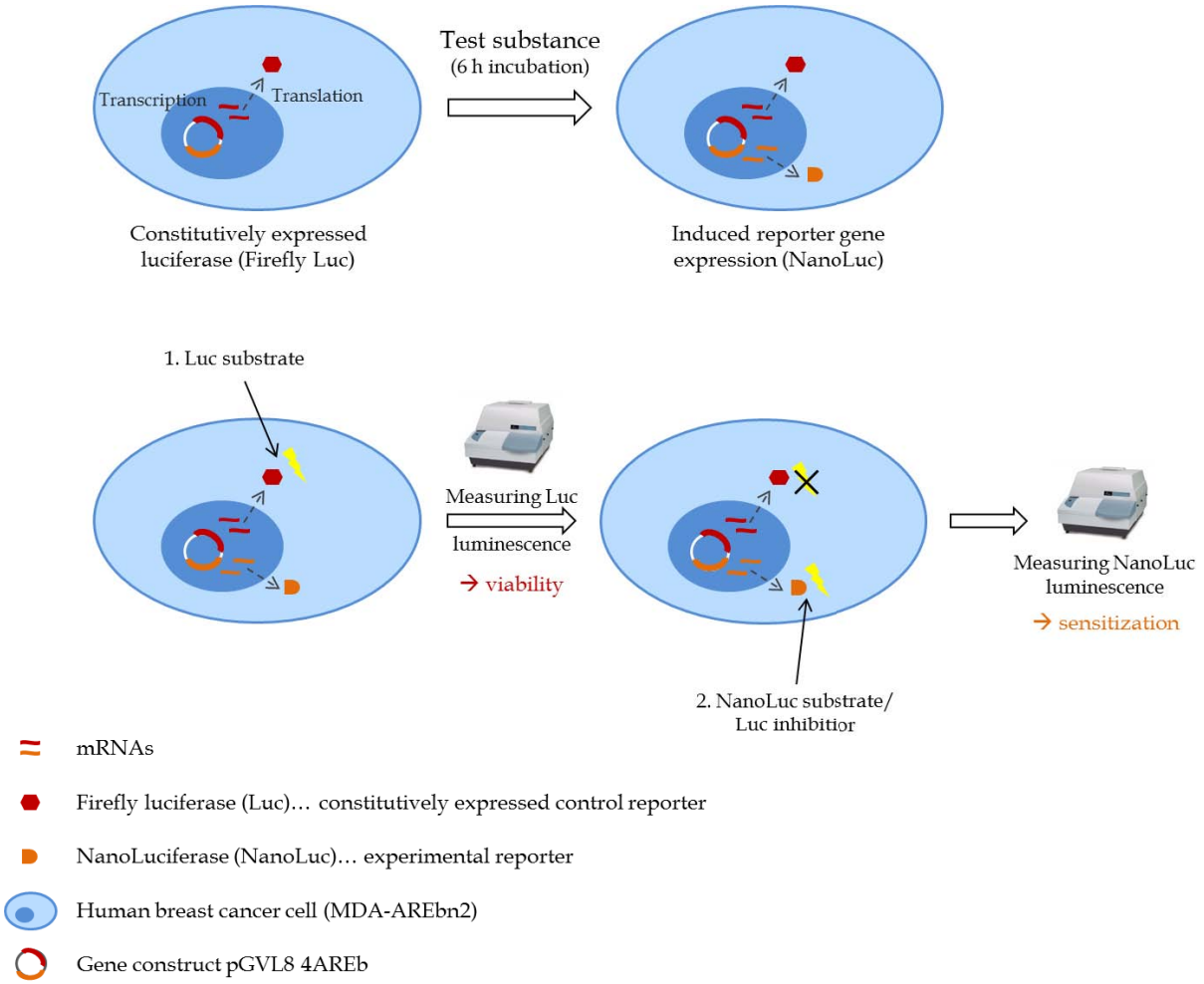

Figure 3-5: Schematic overview of an ARE assay. 10^4 MDA-AREbn2 cells are seeded into a 96 well plate and pre-cultured for 24 h to allow the cells to adhere to the surface of the wells. For stimulation the medium is discarded and the respective dilutions of the test substances in PBS are added. After 6 h incubation at 37 °C the substrate for the Firefly luciferase (Luc) which serves as constitutively expressed control reporter is added. The plate is placed on the shaker at 600 rpm for at least 3 min and the luminescence is measured. Then the substrate for the experimental reporter luciferase (NanoLuc) which contains a Luc inhibitor is mixed freshly and added to the wells. After incubation for at least 10 min on the shaker at 900 rpm the luminescence is measured again. Through the Luc inhibitor added to the NanoLuc buffer just the NanoLuc luminescence is measured and the Luc signal is excluded.

3.3.4.2 OPTIMIZATION OF ARE

According to the manufacturer (Promega Nano-Glo® Dual-Luciferase® Reporter Assay System Quick Protocol) the same volume of luciferase substrate solution should be added, that is used for incubation. Since adding 100 µL per well would mean using a whole kit for one plate, an experiment was conducted to reduce volume and consequently costs. ARE protocol (see 3.3.4) was followed, except for the added GloMix volumes. MDA-AREbn2 cells were stimulated with a concentration range of p-BQ dissolved in PBS with 0.1 % DMSO. The dilutions are shown in Table 3-15.

Table 3-15: Dilution of p-BQ for ARE.

p-BQ [µmol/L]	100 µmol/L p-BQ Stock [µL]	PBS + 0.1 % DMSO [µL]	V_{ges} [mL]
1	10	990	3
2.5	25	975	
5	50	950	

After washing the cells 1x with PBS different volumes of luciferase and NanoLuc substrate were added and the luminescences were measured and compared.

3.3.5 DEVELOPMENT OF A CELL-BASED ELISA FOR DETECTING SKIN SENSITIZERS

3.3.5.1 TESTING FITC-CONJUGATED ANTIBODY

Since in the original protocol FACS detection with FITC-conjugated antibodies is used, anti-CD86-FITC was tested first. It was checked, whether the photometer was sensitive enough to detect the FITC signal. Therefore, THP-1 cells were adjusted to $2*10^6$ cells/mL and 500 µL of the cell suspension were seeded in a 24 well plate. The cells were then stimulated with 500 µL of 90 µg/mL $CoCl_2$ (resulting in a final concentration of 45 µg/mL) which was chosen in order to get a clearly detectable signal ($EC_{1,5}$ = 29 µg/mL; Bauch et al. 2012). After 24 h incubation at 37 °C and 5 % CO_2 the cells were transferred to Eppendorf tubes and stained for CD86 with monoclonal FITC mouse anti-human CD86 antibody on ice. First the stimulated THP-1 cells were washed twice with cold 1x PBS, incubated with 1 mL staining buffer 1 (1x PBS with 0.1 % FCS) for 5 min and afterwards with 600 µL staining buffer 2 (staining buffer 1 with 0.01 % Cohn fraction) for 15 min in order to block Fc binding sites. 180 µL of the cell suspension were then transferred to new Eppendorf tubes, spun down and resuspended in 50 µL antibody working solution (1:7 anti-CD86 in 1x PBS). After 30 min incubation at 4 °C in the dark, unbound antibodies were removed by two washing steps with staining buffer 1. The cell pellet was finally resuspended in 200 µL staining buffer 1 and 100 µL were transferred to a 96 well plate. Fluorescence was measured by means of a photometer with a 485 nm excitation filter and a 535 nm emission filter.

The experiment was repeated using 10 mmol/L and 20 mmol/L p-benzoquinone for stimulation instead of $CoCl_2$. The cells were incubated with the chemical for 48 h, apart from that the protocol above was not altered.

3.3.5.2 TESTING ENZYME-CONJUGATED ANTIBODY

According to the manufacturer the anti-mouse:HRP conjugate can be applied in dilutions ranging from 1:4000 to 1:8000. In order to determine a suitable dilution, THP-1 cells were stained using different concentrations of the secondary antibody. Prior to the staining process the cells were stimulated with 50, 100 and 200 µmol/L p-BQ respectively in a 24 well plate and incubated at 37 °C and 5 % CO_2 for 24 h. 180 µL cell suspension were then transferred to 96 well V-bottom plate and spun down for 5 min at 200 g and 4 °C. The cells were washed once with cooled 1x PBS by removing the supernatant with a multichannel pipette and afterwards resuspending the pellet. All centrifugation and incubation steps were done at 4 °C and in the dark (for incubation the plate was placed in the cooled centrifuge). The cells were first incubated with staining buffer 1 (1x PBS containing 0.1 % FCS) for 5 min and then the unspecific binding sites were blocked by a 15 minute incubation step with staining buffer 2 (1x PBS containing 0.1 % FCS and 0.01 % Cohn fraction). Following the 30 min incubation with 50 µL 1:7 anti-CD86, the cells were washed twice with 1x PBS. Then, they were stained with anti-mouse:HRP as secondary antibody for 1 hour. The cells were stained in double measurement with 50 µL antibody working solution. The antibody dilutions 1:4000 and 1:8000 were tested. After another two washing steps with PBS the stained cells were resuspended in 150 µL TMB solution (1:10 H_2O_2 in substrate solution) and incubated at RT until the color change occurred.

3.3.5.3 OPTIMIZATION OF THE WASHING PROCEDURE

Due to the low reproducibility of the assay when using a pipette to remove the supernatant from pelleted cells, the plates should be inverted to discard the supernatant instead. Therefore, a more compact cell pellet was needed. The first approach was to test how robust the cells are towards centrifugation at a higher g-force than the originally used 200 g. 100 µL per well of a $2*10^6$ cells/mL THP-1 cell suspension were seeded in three 96 well plates, which were then centrifuged for 10 min at 400 g, 600 g and 800 g. The condition of the cells was then examined under the microscope through trypan blue staining and visual control of the cell shape.

Cell loss during washing steps

Since unbound antibodies have to be removed, washing steps are included in the staining protocol. For the ELISA four different washing procedures were tested for their efficiency. 100 µL per well of a $2*10^6$ cells/mL cell suspension were seeded in three 96 well plates and spun down at 500 g. The cell pellets were resuspended in 50 µL 1:8000 anti-mouse:HRP in

1x PBS to mimic the staining step. As just the washing procedure for the secondary antibody needed to be tested, the cells were not stained for CD86 and also the anti-mouse:HRP incubation step was skipped. After addition of the secondary antibody all wells were subjected to two or four washing steps using the different strategies (see Table 3-16).

Table 3-16: Washing scheme for testing cell loss with different methods.

Wells 1-5	Washed 2x with pipette	Method 1
Wells 6-10	Washed 4x with pipette	
Wells 11-15	Washed 2x by inverting & pouring out supernatant	Method 2
Wells 16-20	Washed 4x by inverting & pouring out supernatant	

The inverted plates were blotted onto tissue paper to remove fluid drops. As a control, five wells were not washed at all, but also centrifuged. After finishing the washing steps finally 150 µL TMB solution were added to each well. Washing using a multichannel pipette will be referred to as method 1, washing through discarding the supernatant and blotting the plate onto tissue paper will be referred to as method 2.

As the cell loss needed to be determined for the actual number of eleven washing steps included in the ELISA protocol another experiment using washing method 2 was conducted. 100 µL cell suspension were seeded into a 96 well plate at a concentration of approximately $2*10^6$ cells/mL. The actual cell count was then determined for each of the wells individually using a Neubauer chamber. The cells were washed with 1x PBS eleven times using method 2 and the cell counts before and after washing were compared.

3.3.5.4 TESTING THE CHOSEN ANTIBODIES AND OPTIMIZED WASHING PROCEDURE

All changes in the assay setup, which were chosen according to the previously conducted tests were tested combined in a single experiment. $1*10^6$ THP-1 cells/mL were stimulated with 90 µg/mL $CoCl_2$ in a 24 well plate. The cells were incubated at 37 °C and 5 % CO_2 for 24 h (see 3.3.5.1) before staining of the surface marker CD54 in a V-bottom 96 well plate. First the cells were washed with PBS, then the unspecific binding sites were blocked by incubation with staining buffer 1 (5 min) and staining buffer 2 (15 min). The cells were then incubated with 50 µL anti-CD54 primary antibody per well which had been diluted 1:16 in PBS. The primary antibodies were then stained with 50 µL 1:8000 anti-mouse:HRP conjugated detection antibodies and the binding was visualized by adding 150 µL TMB substrate solution (1:10 solution A to solution B). 50 µL 1 mol/L sulphuric acid was added to stop the reaction and the

cells were removed by centrifugation. 100 µL of the supernatant was transferred to a flat-bottom 96 well plate and the absorbance was measured at 450 nm.

Additionally, cells were incubated in complete RPMI1640, while medium containing 0.1 % DMSO was used as vehicle control.

To test CD86 as well, $1*10^6$ THP-1 cells/mL were stimulated with a concentration range of p-BQ. The range was based on the determined IC50 value of 100 µmol/L, which was used as highest concentration. Additionally, 25 and 50 µmol/L were chosen. The cells were incubated at 37 °C and 5 % CO_2 for 24 h. Then, they were transferred to a 96 well plate and washed with PBS. After blocking the unspecific binding sites with staining buffer 1 and 2, the cells were incubated with 50 µL 1:14 diluted anti-CD86 antibody. After incubation, the plate was washed and incubated with the 1:8000 diluted HRP-conjugated secondary antibody. After addition of 150 µL TMB substrate solution the reaction was stopped with 1 mol/L sulfuric acid. 100 µL of the supernatant was transferred to a flat-bottom 96 well plate and the absorbance was measured at 450 nm.

The obtained results were compared with data from Bauch et al. 2012. Therefore, the EC1.5 value was calculated using linear interpolation between the lowest (25 µmol/L) and the highest concentration (100 µmol/L). Interpolation was done using the following equation

$$x = x_0 + \frac{\left(f_{(x)} - f_0\right) * (x_1 - x_0)}{f_1 - f_0}$$

with x being the concentration used for stimulation and f(x) the resulting enhancement in marker expression (see Table 3-17). The x-fold increase in expression was calculated by dividing the measured absorption of the sample by the absorption of the blank.

Table 3-17: Calculation of EC1.5 of p-BQ.

x0	25 µmol/L
x = EC1.5	?
x_1	100 µmol/L
f_0	1.07-fold increase
f(x)	1.50-fold increase
f_1	2.83-fold increase

To directly compare the results with LLNA data especially concerning sensitivity, the EC3 [%] needs to be calculated. Therefore, linear interpolation was used as well, like mentioned above.

The calculated value was then converted to [g/L] using the molar mass of p-BQ of 108.1 g/mol and divided by the assumed density of 1 kg/L of the medium the cells were stimulated in.

3.3.5.5 ANTIBODY TITRATION

Since an increased antibody concentration could improve the sensitivity of the assay, but would also cause higher background the chosen primary antibody dilutions should maximize sensitivity while minimizing the unspecific background. The optimal dilution of the primary antibodies anti-CD86, anti-CD54 and the isotype control should be determined with regards to sensitivity as well as cost reduction. The stimulation of the cells was performed as described by Bauch et al. 2012. For stimulation of the THP-1 cells two different concentrations of p-BQ (25 and 100 µmol/L) and $CoCl_2$ (25 and 100 µg/mL) were used (see Table 3-18).

Table 3-18: Dilution of p-BQ and $CoCl_2$ used for stimulation during primary antibody titration.

p-BQ					
c [µmol/L] 1x	**c [µmol/L] 2x**	**Substance**	**Medium [µL]**	**Medium + 0.2 % DMSO [µL]**	**V_{ges} [mL]**
2000		2 µL 1 mol/L stock	998	-	1
25	50	50 µL 2 mmol/L stock	-	1950	2
100	200	200 µL 2 mmol/L stock	-	1800	2
$CoCl_2$					
c [µg/mL] 1x	**c [µg/mL] 2x**	**Substance**		**Medium + 0.2 % DMSO [µL]**	**V_{ges} [mL]**
400		20 mg $CoCl_2$		50	50
25	50	250 µL 400 µg/mL stock		1750	2
100	200	1000 µL 400 µg/mL stock		1000	2

The established ELISA protocol (see 3.3.6.3) was performed using different primary antibody dilutions. The p-BQ/$CoCl_2$ stimulated cells and the blanks (BL) were stained with anti-CD86 and anti-CD54 primary antibodies as well as the isotype control anti-IgG1 in a V-bottom 96 well plate. A dilution range from 1:10 to 1:200 was used to stain for the cell surface markers CD86 and CD54 (see Table 3-19). The binding was visualized using HRP-labelled anti-mouse secondary antibody and TMB substrate solution. The color change was measured at 450 nm using a photometer.

Table 3-19: ELISA scheme used for primary antibody titration in a 96 well plate.

		p-BQ						$CoCl_2$					
		25 µmol/L			100 µmol/L			25 µg/mL			100 µg/mL		
		1	2	3	4	5	6	7	8	9	10	11	12
1:10	A	1	2	3	1	2	3	1	2	3	1	2	3
1:20	B	1	2	3	1	2	3	1	2	3	1	2	3
1:50	C	1	2	3	1	2	3	1	2	3	1	2	3
1:100	D	1	2	3	1	2	3	1	2	3	1	2	3
1:200	E	1	2	3	1	2	3	1	2	3	1	2	3
Blanks	F	BL 1:10	BL 1:10	BL 1:10	BL 1:20	BL 1:20	BL 1:20	BL 1:50	BL 1:50	BL 1:50	BL 1:100	BL 1:100	BL 1:100
	G	BL 1:200	BL 1:200	BL 1:200									
	H												

3.3.5.6 NORMALIZATION OF THE SIGNAL TO THE CELL COUNT

A major difference between the application of the established ELISA and the detection via FACS is the influence the cell count has on the measured signal. Each cell is measured individually by means of FACS, which is why the cell count that is lost during antibody staining has no relevance. The photometrical measurement of the ELISA results in a total signal of each well, which makes the cell count a very important factor. Calcein AM should be used to normalize the obtained signal to the cell count in each well. This non-fluorescent dye is taken up by the cells. The acetomethoxy group is cleaved off by intracellular esterases, causing green fluorescence. Since only live cells possess active esterases calcein staining also provides a tool for the discrimination between dead and live cells.

A fluorescence curve was measured for calcein stained THP-1 cells. Therefore, the cell count was adjusted to 10^5 and $9*10^5$ cells/mL and 100 µL of each cell suspension were seeded into a 96 well plate. Then, 10 µL of calcein AM solution were added to each well and the plate was immediately placed inside the photometer. A well containing $9*10^5$ cells/mL without calcein was used as negative control. The absorption was measured every 2 min using a 490 nm excitation and a 520 nm emission filter until saturation was reached.

For integrating calcein staining in the ELISA protocol, a calibration curve is needed for the determination of the cell count. The calibration curve was measured by Micheal Leitner (OFI Vienna) by incubating a 1:2 dilution series of THP-1 cells starting with a concentration of $7.7*10^5$ cells/mL with 10 µL calcein and measuring the fluorescence using a photometer.

3.3.6 HAZARD EVALUATION BY MEANS OF H-CLAT

3.3.6.1 H-CLAT

THP-1 cells were cultivated in complete RPMI1640 medium with HEPES, which was additionally supplemented as described in 3.1.2. The sensitizing potential of chemicals was assessed through incubation of THP-1 cells with the test substances and subsequent measurement of the expression of the cell surface marker molecules CD86 and CD54. Suitable doses of the test substances for evaluation of the augmentation of CD86 and CD54 expression were set according to the results of the cytotoxicity tests (see 4.1). Based on the IC50 values that had been calculated by means of the EZ4U assay, the application concentrations of each substance were chosen in order to cover the sub-lethal as well as the toxic range. The chemicals were dissolved in DMSO to obtain 1 mol/L stock solutions.

THP-1 cells were prepared through seeding a defined cell count of $1*10^5$ or $2*10^5$ cells/mL into a culture flask. The cells were pre-cultured for 48 or 72 h to ensure a good expression level of both surface markers (Ashikaga et al. 2006). The cell count was then adjusted to $2*10^6$ cells/mL by centrifugation for 5 min at 1200 rpm (228 g) and followed by resuspension in a defined volume of tempered medium. The viability of the cells was determined by staining with trypan blue and cell numbers were determined using a Neubauer counting chamber. 500 µL per well of the cell suspension were then seeded into a 24 well plate and 500 µL of the respective dilutions of the test chemicals were added. Complete RPMI1640 medium containing 0.2 % DMSO was added as vehicle control. After incubation for 24 h at 37 °C and 5 % CO_2 the cells were stained for the cell surface marker molecules CD86 and CD54.

3.3.6.2 FLOW CYTOMETRY

DI Thomas Mohr (Science Consult – DI Thomas Mohr KG) conducted all flow cytometric measurements. Staining of stimulated cells (see 3.3.6.1) with fluorescence-labelled antibodies and measurement of the fluorescence signal using flow cytometry was done as described by Bauch et al. 2012. After incubation of the cells with the test substances (which was done in duplicates), they were transferred to 1.5 mL Eppendorf tubes and centrifuged at 200 g and 4 °C for 5 min. After 2 washing steps with 1 mL staining buffer (PBS with 0.1 % BSA) the cells were resuspended in 600 µL staining buffer with 0.01 % Cohn fraction. Following 15 min incubation, 180 µL of the cells were transferred to a 96 well plate. Each Eppendorf tube was split into 3 wells. The plates were centrifuged at 200 g and 4 °C for 5 min and the pellet was resuspended in 50 µL antibody working solution. The antibody dilutions (1:7 for anti-CD86-FITC (#555657,

BD Pharmingen), 1:16 for anti-CD54-FITC (#F7143, DAKO) and 1:16 for anti-IgG1-FITC isotype control (#X092701, DAKO)) were performed in PBS. The cells were incubated with the respective antibody solutions for 30 min in the dark. Afterwards the cells were washed with staining buffer and resuspended in 200 µL staining buffer. Then the cells were incubated for 5 min with 5 µL of 50 µg/mL PI solution. Fluorescence levels of the FITC-conjugated antibodies and PI were measured via flow cytometry and the RFI were calculated using the following equation

$$\text{RFI}\,[\%] = \frac{\text{MFI of chemical treated cells} - \text{MFI of chemical trated isotype crontrol cells}}{\text{MFI of vehicle treated cells} - \text{MFI of vehicle treated isotype control cells}}.$$

3.3.6.3 ELISA

The cells were incubated with the test substances for 24 h (see 3.3.6.1) and subsequently the expression of CD86 and CD54 was detected by means of an ELISA. If one well (which equals 1 mL) per substance in the 24 well plate did not yield enough volume for the staining process, more wells were prepared and pooled in centrifuge tubes after incubation.

For staining of the surface molecules 180 µL of the stimulated THP-1 cells were seeded in a 96 well V-bottom plate in triplicates. The cells were centrifuged for 5 min at 500 g. Since viable cells were stained, all centrifugation steps were done at 4 °C. The supernatant was discarded by inverting the plate and blotting it briefly onto tissue paper. All following washing steps included in the staining procedure were done in the same way. After centrifugation the cells were washed with cold 1x DPBS without Ca^{2+} and Mg^{2+} once. The cell pellets were then resuspended in 200 µL staining buffer 1 (DPBS with 0.1 % FCS (v/v)) and incubated for 5 min inside the centrifuge. For all incubation steps including staining buffer or antibody solutions which need to be cooled to 4 °C and incubated in the dark, the 96 well plates were placed inside the centrifuge. The cells were then centrifuged, the supernatant was discarded as previously described and the pellets were resuspended in 200 µL staining buffer 2 (DPBS with 0.1 % FCS (v/v) and 0.01 % Cohn fraction (w/v)). After 15 min incubation the cells were spun down again, resuspended in 50 µL primary antibody working solution and incubated for 30 min. Monoclonal mouse anti-human CD86 antibody (#555655, BD Pharmingen) and mouse anti-human CD54 antibody (#555510, BD Pharmingen) were used to stain for CD86 and CD54 surface markers. To detect undesired unspecific binding, a mouse IgG1 isotype control (#555746, BD Pharmingen) was used in the same dilutions as the primary antibodies (see Table 3-20). After two washing steps with 100 µL cooled DPBS the cells were incubated with the anti-mouse:HRP detection antibody for 1 hour. The cells were washed three times with 200 µL

DPBS to remove unbound antibodies. To be able to photometrically detect the bound antibodies, 150 µL TMB substrate solution (1:10 H_2O_2 in TMB solution) were added to each well. The 2 solutions have to be mixed freshly before each staining in a centrifuge tube and protected from light exposure by wrapping the tube in aluminium foil. The peroxidase conjugated to the secondary antibody activates the 3, 3', 5, 5'-Tetramethylbenzidine upon substrate addition which then turns the solution bright blue. The following 15-30 min incubation step was the only one done at room temperature and without light protection. The reaction was stopped by adding 50 µL 1 M sulphuric acid, which makes the solution turn yellow. The cells were removed by pelleting through a 5 min centrifugation step at 500 g. 100 µL supernatant of each well were then transferred to a 96 well flat bottom plate and the absorption at 450 nm was measured by a photometer.

Table 3-20: Antibody dilution for one 96 well plate.

Antibody	Dilution	V_{ges} [µL]	$V_{antibody}$ [µL]	V_{PBS} [µL]
α-CD86	1:100	5000	50	4050
α-CD54	1:200	5000	25	4075
α-IgG1	1:100	5000	50	4050
	1:200	5000	25	4075
α-mouse:HRP	1:8000	8000	1	7999

4. RESULTS

4.1 EZ4U CYTOTOXICITY ASSAY

Dose-response cytotoxicity curves were constructed for all test chemicals to determine their IC50 values for THP-1. According to the calculated IC50 values the concentration ranges used for h-CLAT were chosen.

For the determination of the IC50 values by means of the EZ4U assay, THP-1 cells were incubated with the chemicals for 24 h. After staining the cells using EZ4U kit, the absorption was measured at 450 nm (see 3.3.2). The measured optical density was plotted against the used concentrations. The resulting dose-response curves of the test chemicals are shown in Figure 4-1.

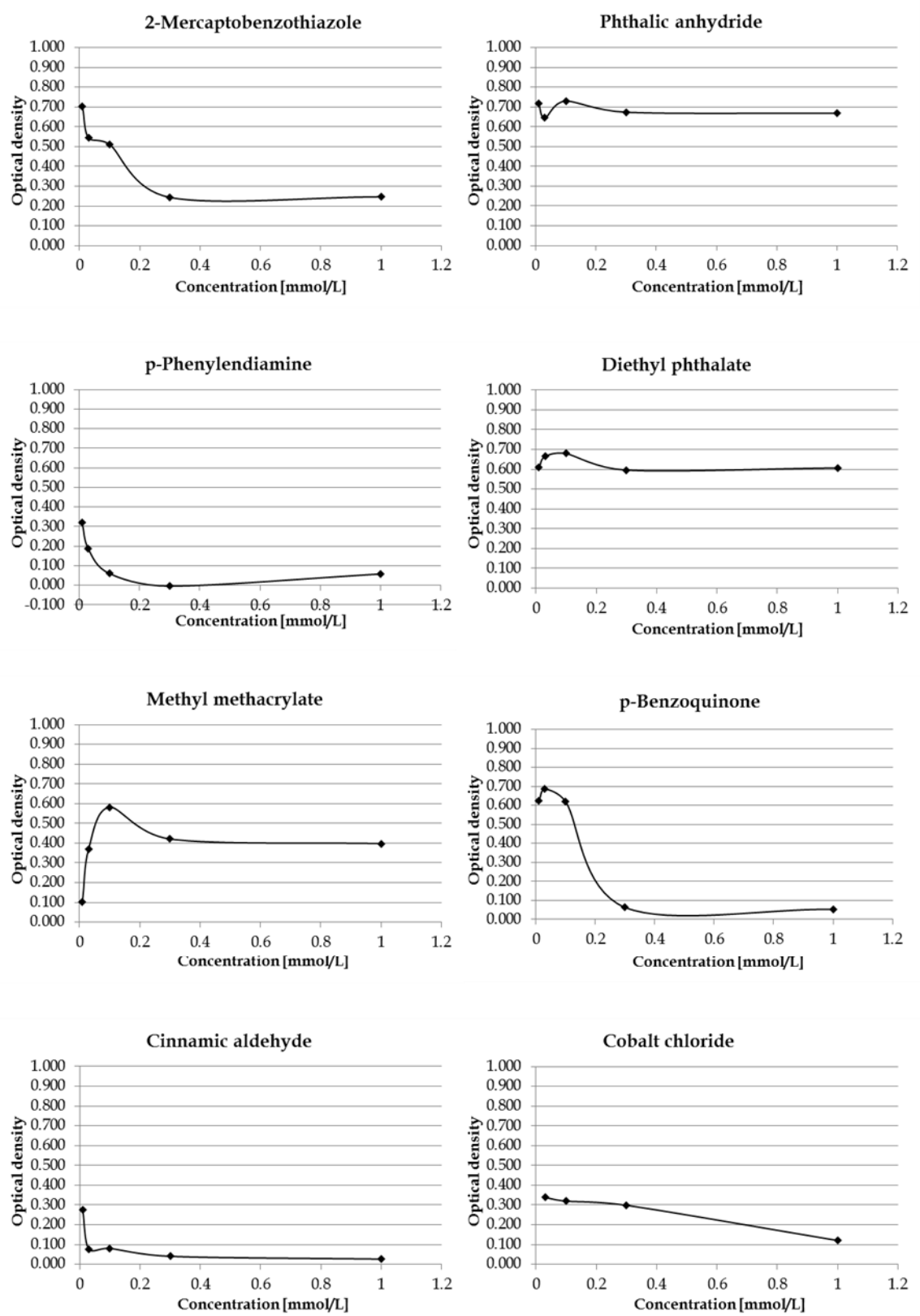

Figure 4-1: Cytotoxicity curves of the selected test substances.

THP-1 cells incubated with phthalic anhydride, diethyl phthalate and methyl methacrylate showed constant viability and were hence considered non-toxic in the tested concentrations. The other test substances however showed a correlation between cell viability and the used concentrations.

The IC50 values of the toxic test substances were estimated graphically, with respect to the vehicle control (cells incubated with medium containing 0.1 % DMSO) which was considered to represent 100 % cell survival. A scheme of IC50 determination is shown in Figure 3-3.

The estimated IC50 values of all test chemicals for 24 h incubation with THP-1 are listed in Table 4-1. They were used for choosing a concentration range for the h-CLAT.

Table 4-1: Values determined for the test substances.

Substance	Non-toxic	IC50 [µmol/L]
2-Mercaptobenzothiazole		170
Phthalic anhydride	x	-
p-Phenylendiamine		30
Diethyl phthalate	x	-
Methyl methacrylate	x	-
p-Benzoquinone		100
Cinnamic aldehyde		50
Cobalt chloride		230

4.2 HAZARD EVALUATION BY MEANS OF DPRA

Since a stand-alone assay like the developed ELISA is not suited for the determination of skin sensitizers, also DPRA and ARE were established.

4.2.1 DOSE-RESPONSE CURVES OF SELECTED CHEMICALS MEASURED BY MEANS OF DPRA

The chemicals p-BQ, DEP, p-PD and CA were incubated with lysine and cysteine peptides at different ratios for 24 h. The measured peptide depletions were plotted against the concentrations of the test chemicals (Figure 4-2 and Figure 4-3).

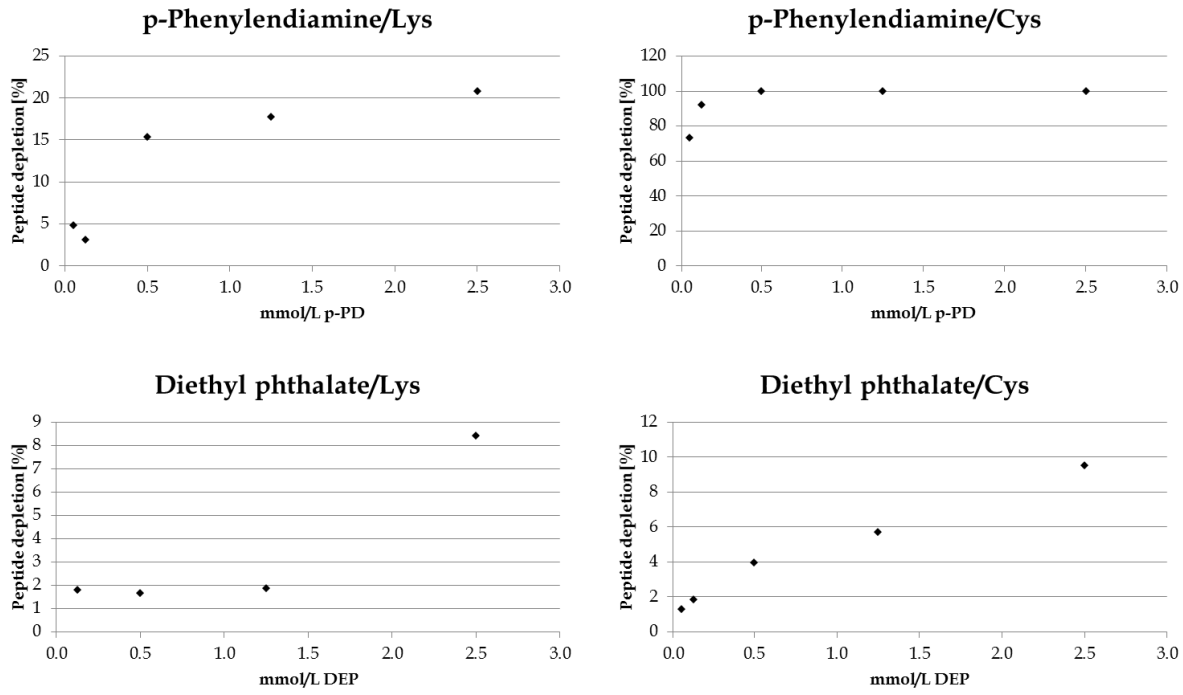

Figure 4-2: Depletion of cysteine peptides after 24 h incubation with different chemicals measured by means of DPRA. For all tested substances the concentration correlates with the peptide depletion.

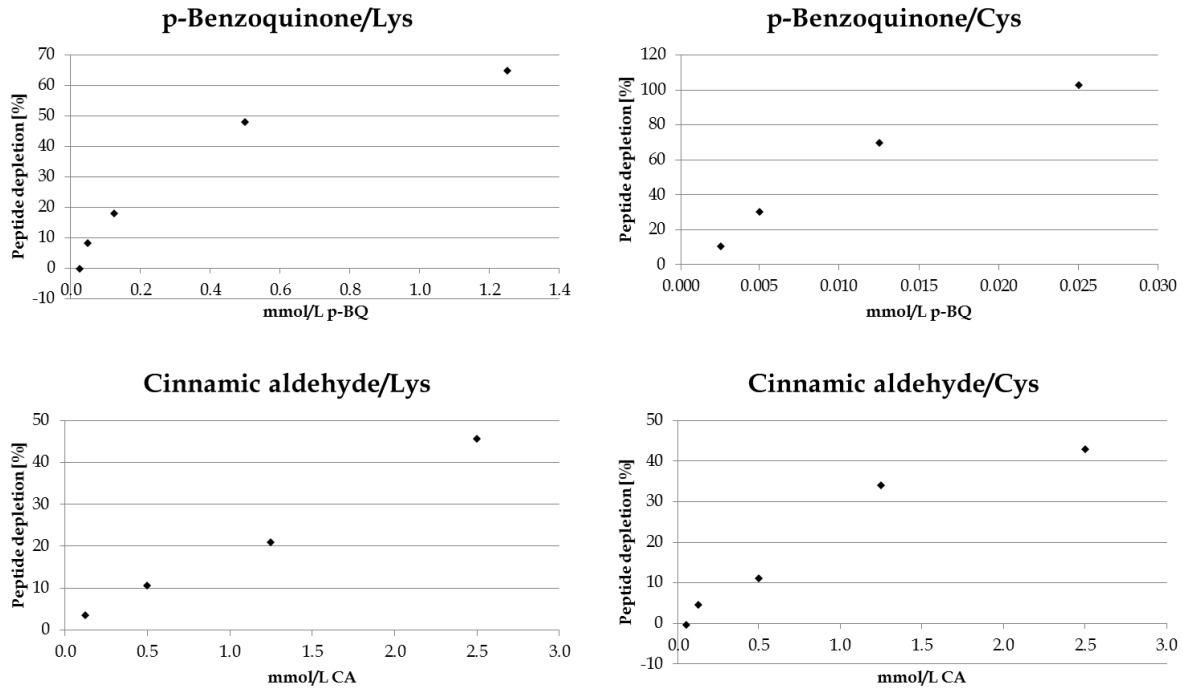

Figure 4-3: Depletion of lysine peptides after 24 h incubation with different chemicals measured by means of DPRA. For all tested substances the concentration correlates with the peptide depletion.

Although the dose-response curves are non-linear (Figure 4-2 and Figure 4-3), a clear correlation between chemical concentration and peptide depletion can be observed. For the extreme/strong sensitizers (p-BQ and p-PD), but not the moderate (CA) and non-sensitizer (DEP) cysteine was much more sensitive and showed stronger binding than the lysine peptide (see Table 4-2).

Table 4-2: Measuring the sensitization potential of p-BQ, p-PD, DEP and CA using DPRA. The % depletion values indicating that the substances are sensitizers are marked in grey.

	Lysine				Cysteine			
	Ratio	**Peptide [mmol/L]**	**Subst. [mmol/L]**	**Depl. [%]**	**Ratio**	**Peptide [mmol/L]**	**Subst. [mmol/L]**	**Depl. [%]**
p-BQ	1:0	0.049	0.000	0.00	1:0	0.040	0.000	0.00
	1:0.5	0.049	0.025	-0.19	1:0.05	0.036	0.003	10.57
	1:1	0.045	0.050	8.26	1:0.1	0.028	0.050	30.15
	1:2.5	0.040	0.125	18.05	1:0.25	0.012	0.013	69.74
	1:10	0.025	0.500	48.06	1:0.5	0.000	0.025	100.00
	1:25	0.017	1.250	64.96				
p-PD	1:0	0.052	0.000	0.00	1:0	0.051	0.000	0.00
	1:1	0.049	0.050	4.90	1:1	0.014	0.050	73.19
	1:2.5	0.050	0.125	3.18	1:2.5	0.004	0.125	92.39
	1:10	0.044	0.500	15.42	1:10	0.000	0.500	100.00
	1:25	0.042	1.250	17.75	1:25	0.000	1.250	100.00
	1:50	0.041	2.500	20.81	1:50	0.000	2.500	100.00
DEP	1:0	0.048	0.000	0.00	1:0	0.053	0.000	0.00
	1:1	0.046	0.050	3.74	1:1	0.052	0.050	1.30
	1:2.5	0.047	0.125	1.80	1:2.5	0.052	0.125	1.84
	1:10	0.047	0.500	1.68	1:10	0.051	0.500	3.98
	1:25	0.047	1.250	1.89	1:25	0.050	1.250	5.70
	1:50	0.044	2.500	8.42	1:50	0.048	2.500	9.55
CA	1:0	0.046	0.000	0.00	1:0	0.052	0.000	0.00
	1:1	0.044	0.050	4.64	1:1	0.052	0.050	0.30
	1:2.5	0.045	0.125	3.54	1:2.5	0.050	0.125	4.54
	1:10	0.041	0.500	10.68	1:10	0.046	0.500	11.15
	1:25	0.037	1.250	20.86	1:25	0.034	1.250	34.07
	1:50	0.025	2.500	45.59	1:50	0.030	2.500	42.87

According to the threshold defined by the OECD 2014 (see 2.3.2.1) for the identification of sensitizers (mean % depletion ≤ 6.38) p-benzoquinone, p-phenylendiamine (extreme/strong sensitizers) and cinnamic aldehyde (moderate sensitizer) were identified correctly. For diethyl phthalate the highest concentrations gave false negative results since DEP is considered a non-sensitizer (see 3.3.1). However, the threshold obtained from the OECD guideline was

defined for 1:50 lysine and 1:10 cysteine with test substance concentrations of 100 mmol/L and thus are not directly comparable with the results above.

4.3 HAZARD EVALUATION BY MEANS OF ARE

Within this work, ARE assay was established at OFI. Also a reduction of costs by reducing the used volume of luciferase substrate solution (see 3.3.4.2) could be accomplished.

ARE was conducted using a concentration range of 1, 2.5 and 5 mmol/L p-BQ for stimulation. For the luciferase assay different volumes of 10-40 µL of the two substrate solutions per well were added and the luminescence was measured using a photometer. The comparison of the measured NanoLuc expressions is shown in Figure 4-4.

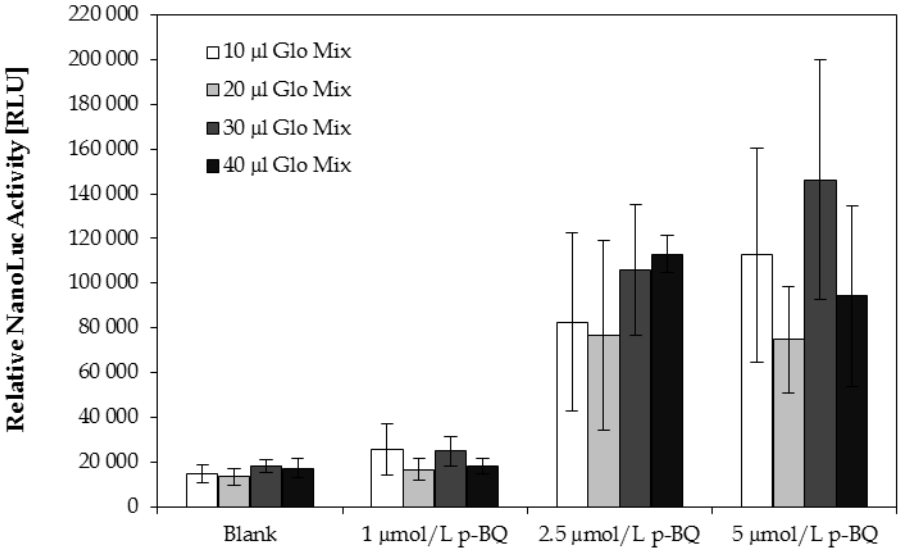

Figure 4-4: Comparison of NanoLuc reporter gene expression using a concentration range of p-BQ and different volumes of NanoLuc substrate.

NanoLuc serves as reporter gene, its expression caused by the activation of ARE sequences when sensitizing substances are present. Although the standard deviations indicated by error bars are quite high, an increase in NanoLuc expression with increasing p-BQ concentration can clearly be observed. Since Figure 4-4 shows no significant difference in the measured luminescence signals, the different used volumes of luciferase substrate solution (10, 20, 30 and 40 µL) all seem to work well. Therefore, for all further experiments 10 µL luciferase substrate solution was used.

Testing substances and measuring dose-response curves using ARE assay is subject to another master thesis. Also the high variability of the results should be investigated in order to improve the accuracy of the assay.

4.4 DEVELOPMENT OF A CELL-BASED ELISA FOR DETECTING SKIN SENSITIZERS

4.4.1 TESTING FITC-CONJUGATED ANTIBODY

Neither the first nor the second experiment using FITC-conjugated antibodies resulted in any detectable signal, which implies that the photometers sensitivity is not sufficient to detect any signal of FITC-stained CD86/54 surface markers. Therefore, a different setup was selected. Purified anti-CD86 and anti-CD54 were chosen as primary antibodies. Since the primary antibodies were not available as enzyme-conjugates, a HRP-conjugated anti-mouse antibody in combination with a TMB substrate kit was selected as secondary antibody.

4.4.2 TESTING ENZYME-CONJUGATED ANTIBODY

To determine a suitable anti-mouse:HRP dilution, THP-1 cells which had been stimulated using the sensitizer p-BQ were stained with anti-CD86 primary antibody and two different dilutions of the anti-mouse:HRP secondary antibody.

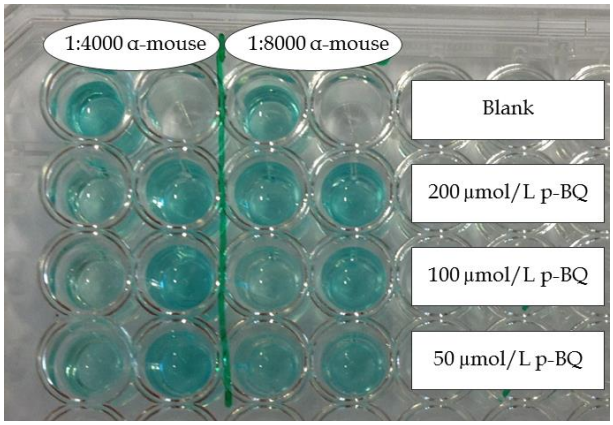

Figure 4-5: Comparison of different secondary antibody (anti-mouse:HRP) dilutions. THP-1 cells stimulated with different concentrations of p-benzoquinone (200, 100 and 50 µmol/L) were stained for the surface marker molecule CD86 with purified anti-CD86 antibody and two different dilutions of anti-mouse:HRP as secondary antibody. The staining gave no reproducible results and indicates, that an optimization of the washing procedure is necessary.

As shown in Figure 4-5, all wells including the blanks were brightly coloured. This indicates that despite the washing steps there was still unbound secondary antibody left in the wells. Therefore the experiment is not analysable regarding the different concentrations of p-BQ. Nevertheless 1:8000 dilution was chosen to be used in the next experiment.

Figure 4-5 also indicates that the washing steps using the multichannel pipette are not sufficient, as there remain unbound antibodies in the wells. This is due to the fact that necessarily a rest of supernatant cannot be removed, if the cell pellet is not touched with the pipette tip (which would lead to the loss of cells). An additional problem arising from the irregularity between the wells caused by the multichannel pipette is also a differing loss of cells in each well. Therefore, the washing procedure had to be improved, which was achieved by changing a few different parameters.

4.4.3 OPTIMIZATION OF THE WASHING PROCEDURE

To eliminate the background caused by remnants of the secondary antibody, the washing procedure was optimized especially regarding centrifugation and the following removal of the supernatant.

4.4.3.1 DEFINING CENTRIFUGATION PARAMETERS

THP-1 cells were centrifuged at different g forces to obtain a more compact cell pellet and the results were examined under the microscope. The cells, which had been centrifuged at 400 g showed a good viability, whereas there were many dead and burst cells and much cell debris in the wells of the plates that had been exposed to 600 and 800 g. Thus 500 g instead of the originally used 200 g were chosen for centrifugation steps. The time for each centrifugation step (5 min) as well as the temperature of 4 °C were retained.

4.4.3.2 DETERMINATION OF CELL LOSS DURING WASHING STEPS

As all of the four different washing procedures worked well and were able to remove the unbound anti-mouse:HRP antibodies from the wells (Figure 4-6, none of the washed wells are coloured), the loss of cells caused by the individual strategies was compared. Therefore, the cells in five wells washed through method 1 and in five wells washed through method 2 were counted in a Neubauer chamber and the results were then compared with the previously determined cell count of $2.1*10^6$ cells/mL of the seeded cell suspension.

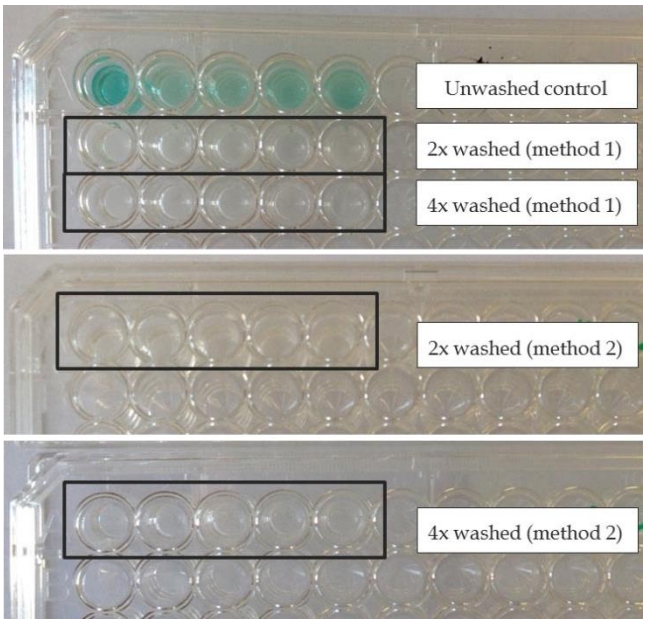

Figure 4-6: Result of the washing procedure optimization experiment. All washing procedures were sufficient to remove the unbound anti-mouse:HRP antibodies from the wells, as none of the washed wells show any colour. In contrast the unwashed control-wells are coloured brightly.

Compared with the wells washed through method 1, which show a standard deviation of $6.9*10^5$ cells/mL and a coefficient of variation of 39.4 %, method 2 causes a much lower standard deviation of $1.8*10^5$ cells/mL and coefficient of variation of 8.4 % (see Figure 4-7).

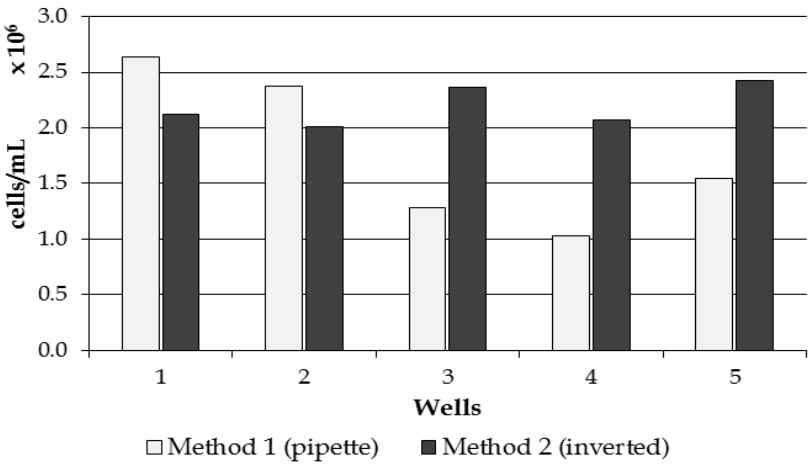

Figure 4-7: Comparison of washing method 1 and 2 regarding the cell count before and after washing. The variation in cell count between the wells washed with the multichannel pipette is much higher, than between the wells washed through discarding the supernatant by inversion.

Considering the centrifugation- and cell loss-experiments the parameters of the washing procedure for the ELISA were defined as follows. The plates were washed with 1x PBS centrifuged for 5 min at 500 g, followed by adjacent inversion and blotting onto tissue paper in order to discard the supernatant.

Another experiment was conducted involving the actual number of eleven washing steps (using method 2) included in the ELISA protocol. The cell counts before and after washing were compared (see Figure 4-8).

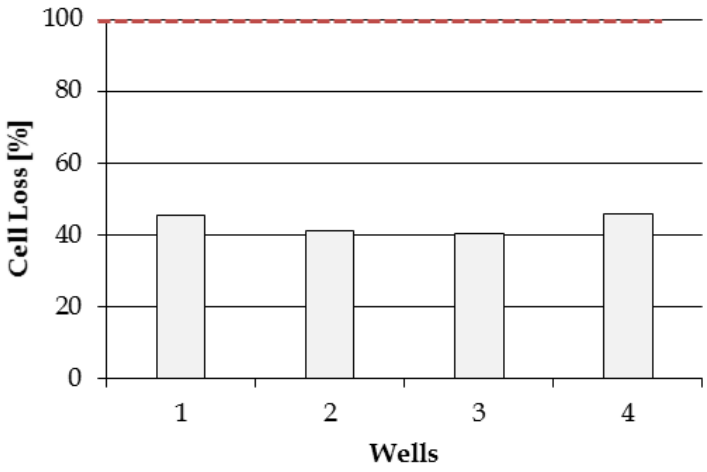

Figure 4-8: Cell loss [%] of THP-1 cells washed eleven times. In all four wells the loss of cells through washing varies between 40 and 45 % of the initial cell count, which confirms the good reproducibility of the procedure.

All wells showed a constant cell loss of 40 to 45 %, resulting in a variability of around 6 %. Despite the quite high quantity of cells washed out, the washing method was considered adequate because of the low variability.

4.4.4 VERIFICATION OF THE DEVELOPED PROTOCOL

The newly developed ELISA protocol was tested using $CoCl_2$ stimulated THP-1 cells. The surface marker CD54 was stained using anti-CD54 with a dilution of 1:16 since titration of the primary antibody had not been done yet. Anti-CD54 primary antibodies were then stained with anti-mouse:HRP conjugated detection antibody (1:8000).

The $CoCl_2$ concentration of 90 µg/mL was chosen according to the EC1.5 value of 45 µg/mL $CoCl_2$ (which had been determined by h-CLAT) found in the literature (Bauch et al. 2012). To obtain a clearly detectable signal 2x EC1.5 was used for stimulation. Medium was used as blank, while medium containing 0.1 % DMSO was tested as vehicle control to verify that DMSO does not cause an upregulation of surface marker expression.

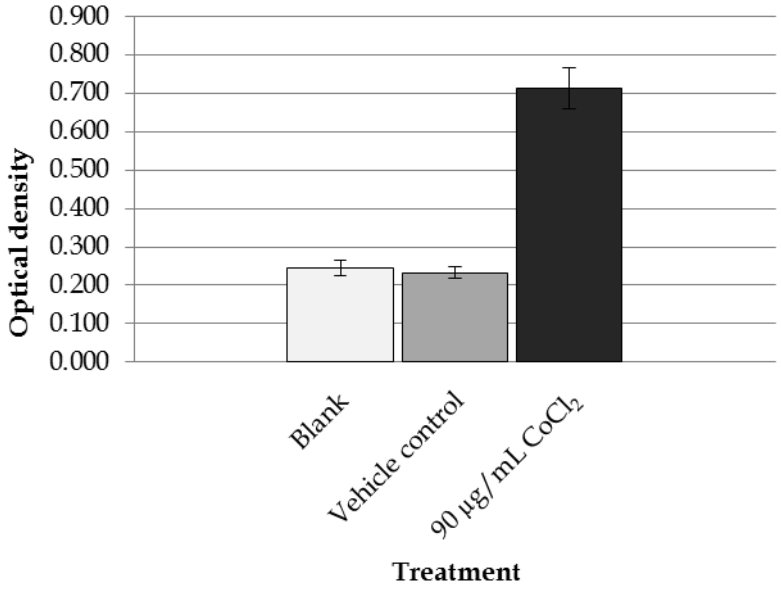

Figure 4-9: Staining of $CoCl_2$ stimulated THP-1 cells with anti-CD54 (1:16). THP-1 cells were stimulated with $CoCl_2$ for 24 h. Then, the optimized washing procedure and chosen antibodies (1:16 dilution for mouse anti-human CD54 (Bauch et al. 2012) and 1:8000 dilution for anti-mouse:HRP) were tested.

The cells that had been incubated with complete RPMI1640 medium containing 0.1 % DMSO showed the same CD54 marker expression as cells incubated with medium alone (see Figure 4-9). This confirms the assumption, that DMSO has no influence on the expression of the sensitization marker CD54 (since it is classified as a non-sensitizer).

The measurement not only identified the sensitizer $CoCl_2$ correctly, CD54 expression for $CoCl_2$ stimulated THP-1 cells also corresponds well to the EC1.5 value obtained from the literature. Bauch and co-workers have shown that 45 µg/mL $CoCl_2$ leads to a 1.5-fold upregulation of CD54 expression (Bauch et al. 2012). Figure 4-9 shows that stimulation with 90 µg/mL led to a 2.9-fold increase of CD54 marker expression. This finding also indicates that both values are within the linear section of the dose-response curve.

Since only CD54 antibody had been analysed, the established ELISA was further tested for its suitability to measure the dose-response curve of p-BQ stimulated CD86 expression in THP-1 cells. According to Bauch et al. p-BQ causes enhanced CD86 expression. In order to compare EC1.5 and EC3 values with data obtained from literature, the cells were stimulated with different concentrations that were chosen according to the IC50 value that had previously been determined using EZ4U assay (see 4.1). The IC50 value of 100 µmol/L p-BQ was used as

highest concentration. To reduce costs, the surface marker CD86 was stained with 1:14 diluted anti-CD86 antibody instead of the 1:7 dilution recommended by the manufacturer. As in the previous experiment, 1:8000 diluted anti-mouse:HRP secondary antibody and TMB substrate solution were used. The dose-response curve of p-BQ stimulated THP-1 cells is shown in Figure 4-10.

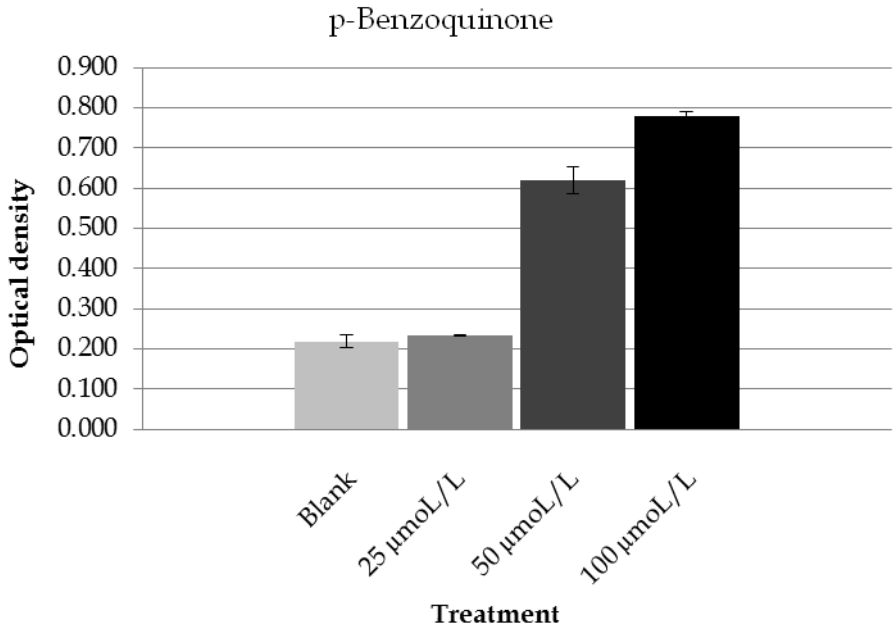

Figure 4-10: p-BQ stimulated THP-1 cells stained with anti-CD86 (1:14). THP-1 cells were stimulated with 25, 50 and 100 µmol/L p-BQ for 24 h. Expression of the surface marker CD86 was then measured using anti-CD86 antibody (1:14).

Figure 4-10 shows that p-BQ induces a concentration dependent increase in CD86 expression. Whereas 25 µmol/L p-BQ shows no upregulation of CD86 compared to the blank, 50 µmol/L caused a 2.8-fold increase in CD86 surface expression. Stimulation with 100 µmol/L p-BQ resulted in a 3.6-fold increased signal. Since 100 µmol/L p-BQ equals the determined IC50, it can be assumed that only approximately half of the cells were viable compared to the blank, which explains the lower increase in signal intensity.

THP-1 cells stimulated with different concentrations of isopropyl alcohol (IPA) ranging from 100-1000 µmol/L were used as negative control. IPA does not cause enhancement of CD86 expression as it is considered a non-sensitizer (Bauch et al. 2012). The negative control is shown in Figure 4-11.

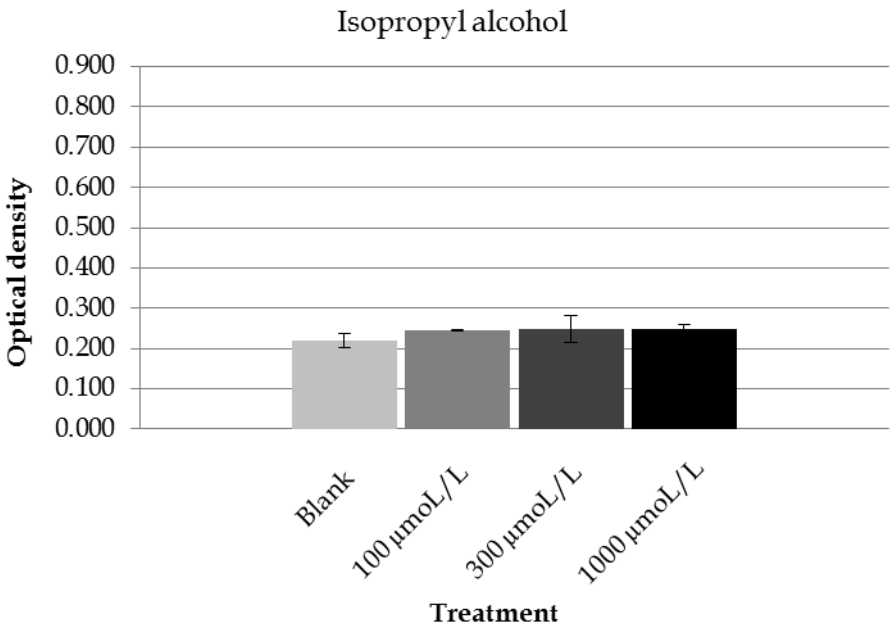

Figure 4-11: IPA stimulated THP-1 cells used as negative control. As expected, no increased CD86 expression could be observed at any applied IPA concentration.

In Table 4-3 and the results of surface marker staining of $CoCl_2$ and p-BQ stimulated THP-1 cells are compared to LLNA and h-CLAT data obtained by Bauch et al. 2012.

Table 4-3: Comparison of the ELISA results with LLNA and h-CLAT data for $CoCl_2$ obtained from literature.

$CoCl_2$

LLNA (Bauch et al. 2012)		**h-CLAT CD54** (Bauch et al. 2012)		**h-CLAT+ELISA CD54**		
	EC3 [%]		EC1.5 [µg/mL]		EC2.9 [µg/mL]	EC2.9 [%]
+ (moderate sensitizer)	4.8	+	45	+	90	0.09

Since only one concentration rather than a concentration range of $CoCl_2$ was used for stimulation, linear interpolation for the calculation of the EC1.5 was not possible. Therefore, the increase in marker expression was calculated in respect to the blank value. 90 µg/mL $CoCl_2$ caused a 2.9-fold increase of CD54 expression. Compared to the LLNA, the ELISA shows improved sensitivity, which is demonstrated by converting the EC2.9 to %. The values cannot be compared directly because of the missing interpolation, but they give an impression of the increased sensitivity.

Table 4-4 shows the comparison of CD86 marker expression of p-BQ stimulated cells measured using ELISA with data from literature.

Table 4-4: Comparison of the ELISA results with LLNA and h-CLAT data for p-BQ obtained from literature.

p-Benzoquinone						
LLNA (Bauch et al. 2012)		**h-CLAT CD86** (Bauch et al. 2012)		**h-CLAT+ELISA CD86**		
	EC3 [%]		EC1.5 [µmol/L]		EC1.5 [µmol/L]	EC3 [%]
+ (extreme sensitizer)	0.0099	+	36	+	38	0.0009

Since for p-BQ a concentration range was used, an EC1.5 value of 38 µmol/L could be calculated by linear interpolation, which corresponds well to the value given by Bauch et al. An EC3 of 83 µmol/L was calculated as well via linear interpolation. To compare it directly to LLNA data, it was converted to %. Benzoquinone shows about 10-fold increase in sensitivity, which is even higher than for $CoCl_2$.

Although the developed ELISA protocol has been shown to give good results, a high amount of the primary antibodies is needed, resulting in high costs. Thus the protocol was further optimized by titration of anti-CD86 and anti-CD54 primary antibodies.

4.4.5 PRIMARY ANTIBODY DILUTIONS

THP-1 cells were stimulated with two different concentrations of $CoCl_2$ and p-BQ respectively. While the lower concentrations of 25 µg/mL $CoCl_2$ and 25 µmol/L p-BQ were expected not to cause enhanced surface marker expression, the higher concentrations of 100 µg/mL $CoCl_2$ and 100 µmol/L p-BQ should show a clearly detectable upregulation without exceeding the determined IC50 values for both substances (see 4.1). $CoCl_2$ as well as p-BQ have been shown to upregulate both CD86 and CD54 (Bauch et al. 2012). The results of staining stimulated cells for CD86 expression are shown in Figure 4-12 and Figure 4-13, titration of CD54 is shown in Figure 4-14 and Figure 4-15. Additionally $CoCl_2$ stimulated THP-1 cells were stained with an isotype control for IgG1 to analyse unspecific Fc receptor binding.

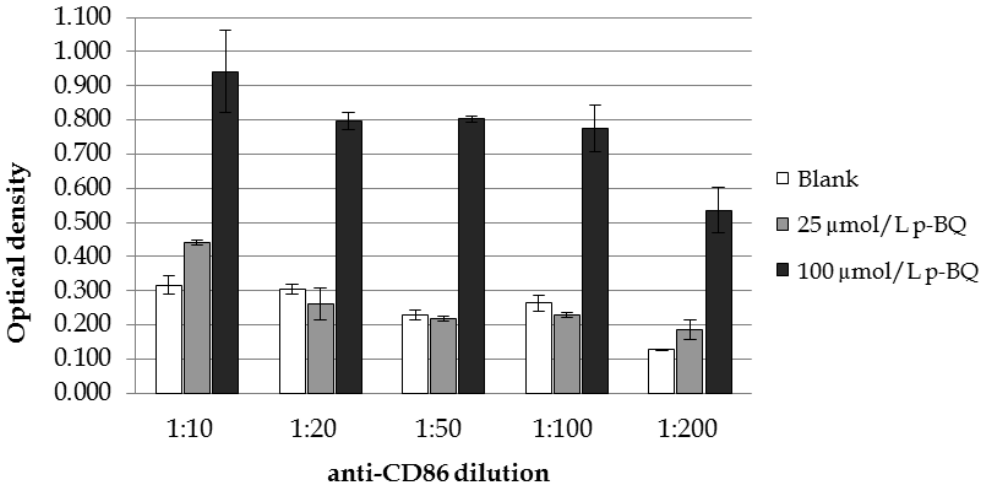

Figure 4-12: Titration of anti-CD86 primary antibody with p-BQ stimulated cells. Unstimulated cells stained with 1:200 diluted anti-CD86 primary antibody show the lowest signal meaning the lowest background, nevertheless all measured signals are quite low. Therefore 1:100 was considered the most suitable dilution.

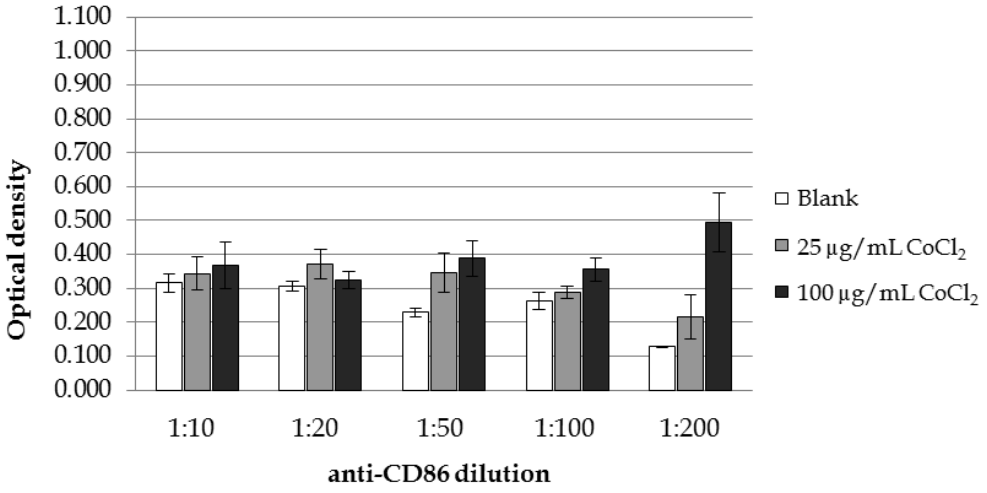

Figure 4-13: Titration of anti-CD86 primary antibody with $CoCl_2$ stimulated cells. Enhanced expression of CD86 is only detectable on cells stained with 1:200 diluted anti-CD86 antibody.

Figure 4-12 clearly shows that although the overall signal decreases, the unspecific background decreases as well at higher dilutions. Lower background at the higher dilutions can also be observed in Figure 4-13. This implies better sensitivity at higher dilutions of the anti-CD86 antibody. Especially the enhanced CD86 expression resulting from stimulation with 100 µg/mL $CoCl_2$ is only detectable when the background is considerably decreased at 1:200 diluted anti-CD86. A similar upregulation of CD86 expression was detected with a treatment up to 100 µmol/L p-BQ. Although the staining with 1:200 diluted anti-CD86 shows the lowest background, also the signals of the stimulated cells are low compared to the other dilutions.

1:100 was therefore considered the most suitable dilution for the anti-CD86 antibody and was included in the ELISA protocol.

Figure 4-14 and Figure 4-15 show that (similar to the anti-CD86 staining) the background resulting from unspecific antibody binding decreases at higher anti-CD54 dilutions. Thus, higher dilutions are preferable. 1:100 and 1:200 diluted anti-CD54 work equally well concerning background and signal intensity. Since CD54 shows a higher constitutive basic level expression (resulting in a higher blank value) than CD86 and 1:200 still yields sufficiently high signal intensity, 1:200 was considered the most suitable dilution for anti-CD54 antibody. Furthermore, diluting the antibody 1:200 reduces costs by half, compared to the 1:100 dilution.

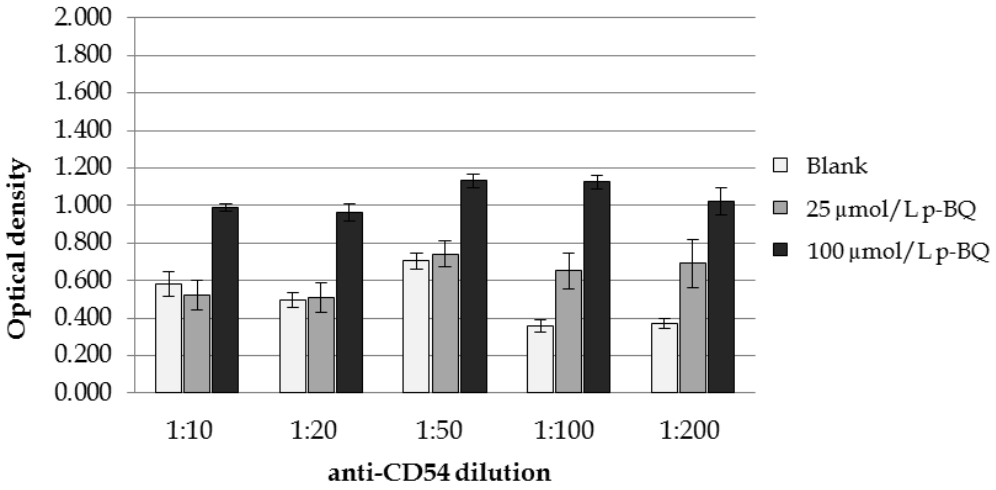

Figure 4-14: Titration of anti-CD54 primary antibody with p-BQ stimulated cells. The highest dilutions for anti-CD54 primary antibody show the lowest blank value meaning the lowest background, therefore 1:100 and 1:200 were considered the most suitable dilutions.

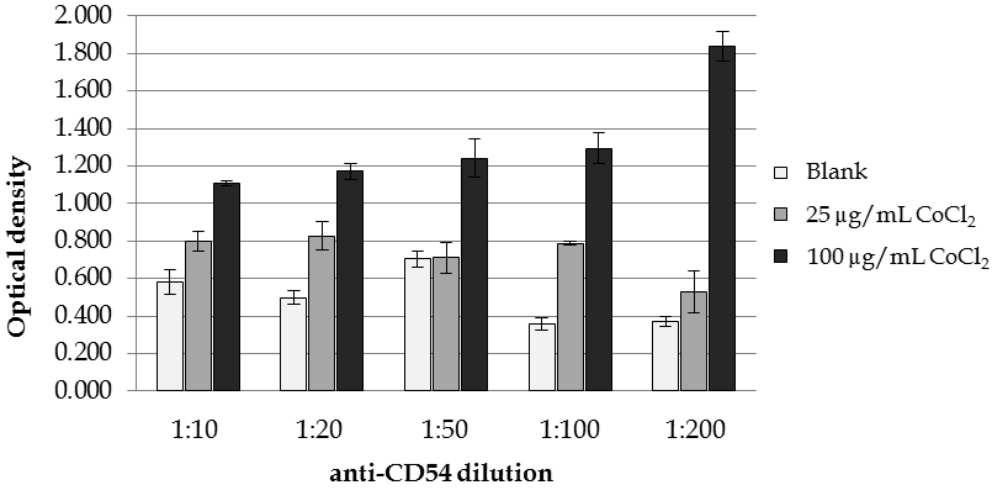

Figure 4-15: Titration of anti-CD54 primary antibody with $CoCl_2$ stimulated cells. Staining with 1:100 and 1:200 diluted anti-CD54 antibody shows the lowest background, therefore the higher dilutions are preferable.

Although Bauch et al., 2012 reported no enhanced CD54 expression upon p-BQ stimulation, 100 µmol/L p-BQ causes clear upregulation in the titration experiment as well as in flow cytometry experiments (see 4.5.1). This is also confirmed by others, e.g. dos Santos et al. 2009.

A κ isotype control was titrated as well, to analyse unspecific Fc receptor binding. Signal background results from undesired, non-specific binding of the antibodies to Fc receptors via their Fc fragments. The same dilutions as for anti-CD86 and anti-CD54 antibodies were used for the anti-IgG1 antibody. As isotype control anti-IgG1 exhibits the same Fc fragment as anti-CD86 and anti-CD54, but lacks antigen binding sites. The 1:50, 1:100 and 1:200 dilutions of the isotype control show reduced unspecific binding compared to the other tested dilutions (see Figure 4-16), hence the chosen antibody dilutions for anti-CD86 and anti-CD54 are substantiated.

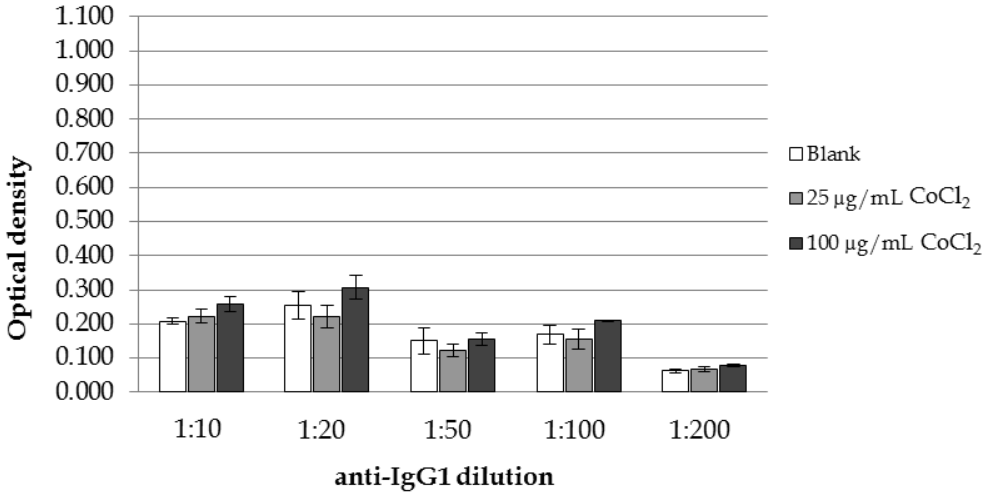

Figure 4-16: Titration of anti-mouse IgG1, κ isotype control. The 1:50, 1:100 and 1:200 diluted isotype controls show reduced Fc receptor binding compared to the lower dilutions. Thereby the 1:100 and 1:200 dilutions for the tested anti-CD86 and anti-CD54 primary antibodies are confirmed as the most suitable ones.

4.4.6 NORMALIZATION OF THE SIGNAL TO THE CELL COUNT

During flow cytrometric measurement the signal of each cell is detected individually. Since for the established combination of h-CLAT and cell-based ELISA photometrical measurement is the detection method of choice, the overall absorption of each well is measured. Therefore, a constant cell count and equal cell loss is of crucial importance to obtain a reliable signal. Previous tests have shown an acceptable variability of approximately 6 % regarding cell loss (see 4.4.3.2). Nevertheless, staining of the cells with calcein AM should be performed to link the measured signal to the actual cell count in the respective well. Therefore cells were adjusted to $9*10^5$ cells/mL and $1*10^5$ cells/mL and 100 µL/well were seeded into a 96 well plate. The cells were incubated with calcein for 150 min and fluorescence curves were measured which are shown in Figure 4-17. The fluorescence of THP-1 cells without calcein were measured as negative control (see Figure 4-18).

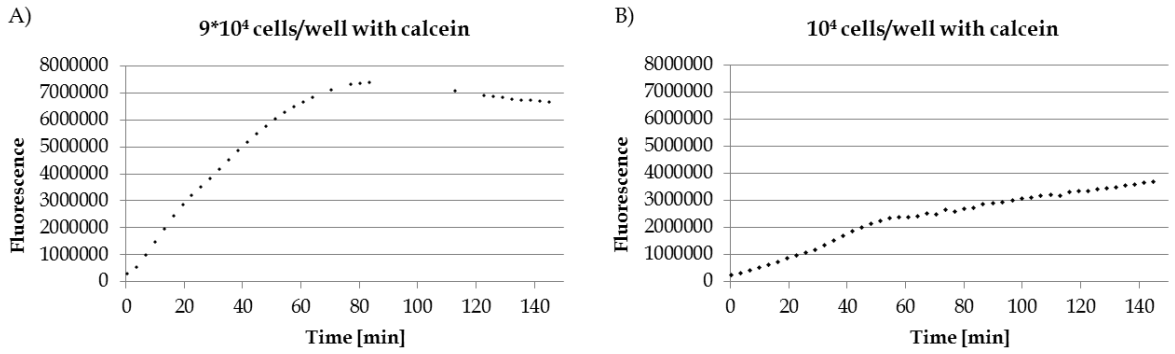

Figure 4-17: A) Fluorescence curve of 9*10⁴ THP-1 cells/well stained with calcein. B) 1*10⁴ THP-1 cells/well stained with calcein.

Figure 4-17 shows that 9*10⁴ cells/well (A) cause a nearly doubled fluorescence signal compared to 10⁵ cells/mL (B), which indicates that the cell count correlates with the measured fluorescence signal. After ca. 120 min of incubation, saturation was reached and the fluorescence signal stayed constant, which can be observed best at the highest cell density. The negative control shown in Figure 4-18 (cells without calcein staining) exhibits no fluorescence at all.

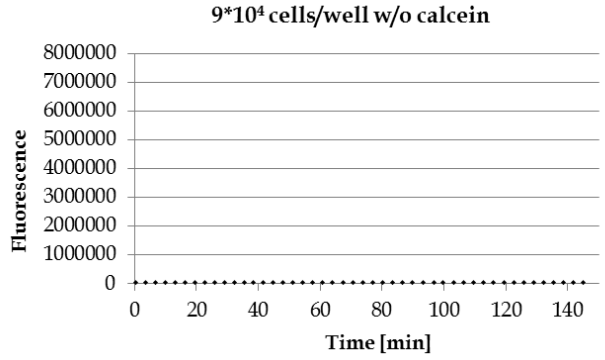

Figure 4-18: Absorption curve of the negative control (9*10⁵ THP-1 cells/mL without calcein).

For the determination of the cell count during the ELISA, a calibration curve of calcein is necessary. The measurement of a calibration curve was performed by Michael Leitner (OFI Vienna) by incubating a 1:2 dilution series of THP-1 cells starting with a concentration of 7.7*10⁵ cells/mL with calcein. The calibration curve is shown in Figure 4-19.

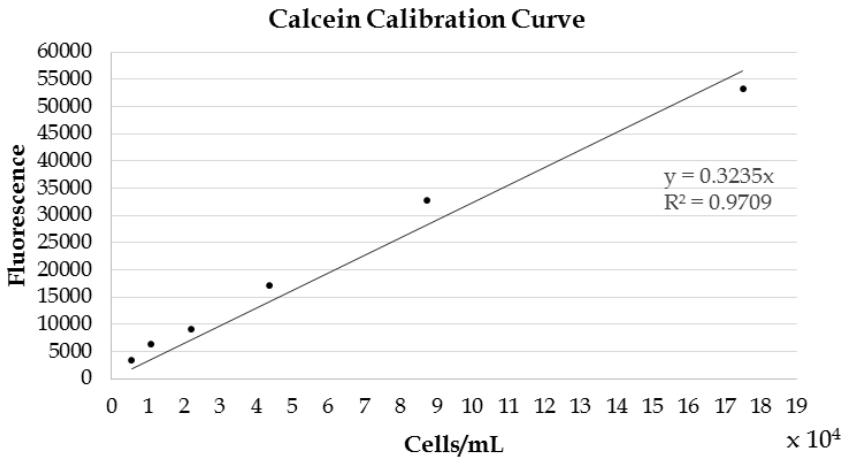

Figure 4-19: Calibration curve of THP-1 cells incubated with calcein. The calibration curve should be used for the determination of the cell count after performing the established ELISA.

Calcein staining of THP-1 cells worked well, nevertheless the calibration curve shows non-linearity already at the highest measured cell count of about $1.7*10^5$ cells/mL. Therefore, the cell density of about 10^6 cells/mL (used for h-CLAT) is not within the linear range of the calibration curve and calcein is not suitable for the determination of the exact cell count.

4.5 HAZARD EVALUATION BY MEANS OF H-CLAT

4.5.1 DOSE-RESPONSE CURVES OF SELECTED CHEMICALS MEASURED BY MEANS OF FLOW CYTOMETRY

THP-1 cells stimulated with different concentrations of p-BQ and $CoCl_2$ as described by Bauch et al. 2012 were stained with anti-CD86-FITC (diluted 1:7) or anti-CD54-FITC (diluted 1:16). Viability of the cells was monitored via PI staining. Marker expression as well as viability was determined by measuring the fluorescence signals using a flow cytometer. Dose-response curves for both test substances are shown in Figure 4-20 (p-BQ) and Figure 4-21 ($CoCl_2$). Unlike described in the original protocol, single measurements were conducted instead of double determinations. The viability was measured four times, from which a standard deviation could be calculated.

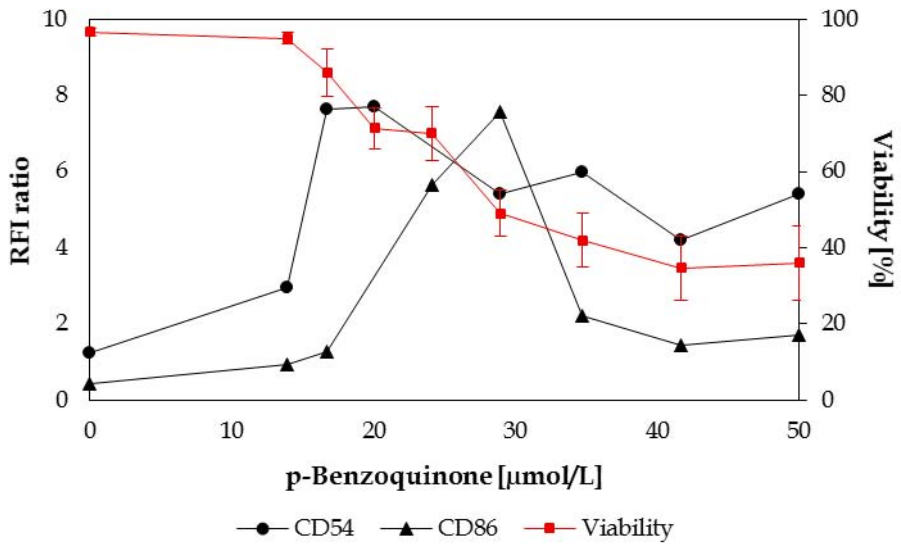

Figure 4-20: Surface marker expression of p-BQ stimulated THP-1 cells. Expression of both CD86 and CD54 is enhanced upon incubation with the extreme sensitizer p-BQ. The viability decreases with increasing p-BQ concentration.

The dose-response curves of CD86 and CD54 measured for p-BQ stimulated THP-1 cells show that expression of both surface markers is enhanced at increasing concentration. CD86 expression peaks at approximately 30 mmol/L p-BQ while expression of CD54 is highest at approximately 20 mmol/L before decreasing again. The apparent decrease in marker expression can be explained by the decrease of cell viability caused by toxicity of p-BQ.

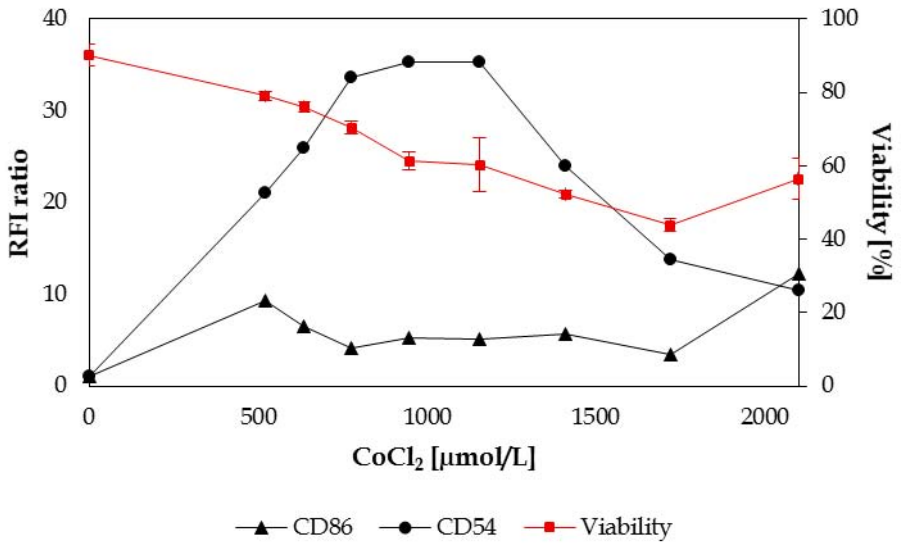

Figure 4-21: Surface marker expression of $CoCl_2$ stimulated THP-1 cells. While CD86 expression stays the same, CD54 expression is enhanced by $CoCl_2$. The measured signal begins to decrease, when approximately half of the cells are dead.

Figure 4-21 shows that CD54 expression of $CoCl_2$ stimulated cells increases until the applied concentration reaches approximately the IC50 of the test substance at 1200 µmol/L. THP-1 cells do not seem to induce CD86 expression upon $CoCl_2$ stimulation.

Data from h-CLAT/ELISA experiments was compared to the results from the h-CLAT/flow cytometry experiment as well as data from literature. An overview is given in Table 4-5.

Table 4-5: Comparison of h-CLAT/ ELISA and h-CLAT/flow cytometry results with data from literature. + = enhanced expression, - = no enhanced expression, n.d. = no data

	$CoCl_2$		p-BQ	
Source	**CD86**	**CD54**	**CD86**	**CD54**
h-CLAT/ELISA	~	+	+	+
h-CLAT/FACS	-	+	+	+
Bauch et al. 2012	+	+	+	-
Nukada et al. 2013	-	+	+	+
dos Santos et al. 2009	n.d.	n.d.	+	+

Whereas FACS detected no enhanced expression for CD86 upon $CoCl_2$ stimulation, the cell-based ELISA gave no clearly interpretable result, since higher CD86 expression could only be observed at considerable reduced unspecific background at the highest tested anti-CD86 antibody dilution of 1:200. Bauch et al. 2012 reported an increase in CD86 expression, while Nukada et al. 2013 detected no augmented expression. CD54 expression after incubation with $CoCl_2$ was found to be upregulated by ELISA, flow cytometry and all included literature sources. Upon stimulation with the extreme sensitizer p-BQ the expression of both CD86 and CD54 is significantly enhanced. However, Bauch et al. 2012 reported an increase of CD86 only.

4.5.2 ESTABLISHED ELISA PROTOCOL

- Centrifuge is switched on to allow it to cool down to 4 °C.
- Preparation of antibody working solutions by dilution in 1x DPBS:

Table 4-6: Antibody working solutions.

Antibody	Dilution
anti-CD86	1:100
anti-CD54	1:200
anti-IgG1	1:100
	1:200
anti-mouse:HRP	1:8000

Until usage the solutions are stored at 4 °C in the fridge. Antibody solutions are prepared for a few wells more as reserve.

- All wells containing cells incubated with the same substance and concentration are pooled, if there have been prepared more than one well in the 24 well plate.
- Transfer of 180 µL triplets of the stimulated cells for each substance and concentration to a 96 well V-bottom plate.
- Transfer of six times 180 µL of the blank to the same 96 well V-bottom plate.
- The samples to be stained for CD86 and CD54 are placed on one plate, the same samples to be stained with the isotype control are placed on a corresponding plate, thus eliminating the need to balance the plates before every centrifugation.
- Plates containing the viable cells, 1x DPBS for washing, staining buffers 1 and 2, antibody and TMB solutions are always stored cooled on ice or in the fridge.
- Cells are pelleted by centrifugation for 5 min at 500 g and 4 °C.
- Transfer of 80 µL of the supernatant of each well to a new 96 well plate. Upon IL tests the samples are pooled in vials and frozen at -20 °C.
- Removal of the rest of the supernatant by inverting the plate and pouring the medium out. The plates are then blotted onto tissue paper to remove drops.
- Plates are washed once with 100 µL cold 1x DPBS.
- Washing procedure: After centrifugation the supernatant is removed by inverting the plate, pouring the liquid out and drying the plate by blotting it shortly onto tissue paper. The pellet is resuspended in cold 1x DPBS w/o Ca^{2+} and Mg^{2+} by filling all wells with an automatic multichannel pipette in dispenser mode and afterwards resuspending all triplets with the same pipette tips.

- Resuspension of the cell pellets in 200 µL cold staining buffer 1 (1x DPBS w/o Ca^{2+} and Mg^{2+} containing 0.1 % FCS).
- Incubation in staining buffer 1 for 5 min inside the centrifuge (in the dark at 4 °C).
- Centrifugation for 5 min at 500 g and 4 °C.
- Removal of supernatant as described above.
- Resuspension of the cell pellets in 200 µL staining buffer 2 (1x DPBS w/o Ca^{2+} and Mg^{2+} containing 0.1 % FCS and 0.01 % Cohn fraction).
- Incubation in staining buffer 2 for 15 min inside the centrifuge for blocking of unspecific and Fc receptor binding.
- Centrifugation for 5 min at 500 g and 4 °C.
- Removal of supernatant.
- Resuspension of the cell pellets in 50 µL primary antibody working solution (1:100-anti-CD86, 1:200 anti-CD54, 1:100 anti-IgG1 or 1:200 anti-IgG1). If small volumes of antibody solution are used, divided reagent reservoirs are used.
- Incubation for 30 min inside the closed centrifuge.
- Centrifugation for 5 min at 500 g and 4 °C.
- Removal of supernatant.
- Plates are washed twice with 100 µL cold 1x DPBS.
- Resuspension of the cell pellets in 50 µL secondary antibody working solution (1:8000 anti-mouse:HRP).
- Incubation for 1 hour inside the closed centrifuge.
- Preparation of the TMB substrate solution: addition of 1:10 H_2O_2 to TMB solution. The substrate solution is prepared shortly before the end of the incubation with the secondary antibody and stored cooled and protected from light exposure. TMB solution is prepared for a few wells more in reserve.
- Centrifugation for 5 min at 500 g and 4 °C.
- Removal of supernatant.
- Plates are washed three times with 200 µL cold 1x DPBS.
- Resuspension of the cell pellets in 150 µL TMB substrate solution.
- Incubation at room temperature for 15-30 min (until bright blue colour appears).
- Addition of 50 µL 1 M sulphuric acid to each well to stop the reaction.
- Pelleting of the cells by centrifugation for 5 min at 500 g and 4 °C.

- Transfer of 100 µL of the supernatant of each well to a 96 well flat bottom plate. The isotype controls and blanks are measured on the same plate as the respective samples.
- Measurement of absorbance at 450 nm in the photometer.

A schematic setup of the established ELISA protocol is shown in Figure 4-22.

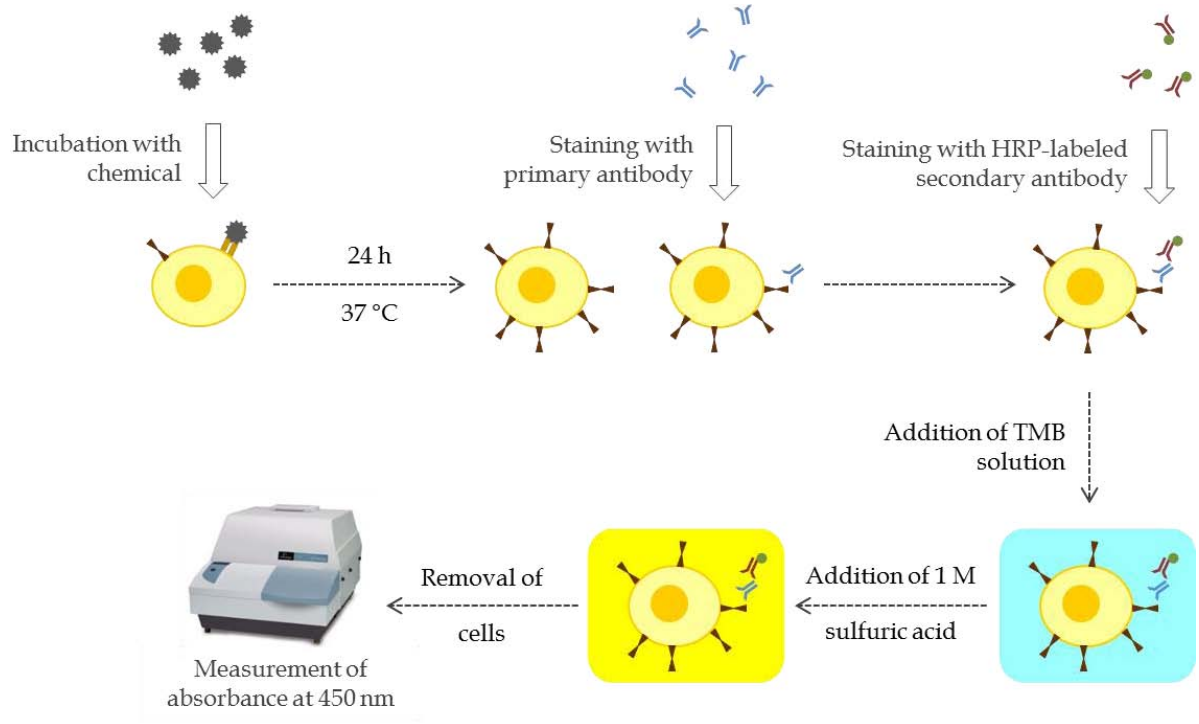

Figure 4-22: Setup of h-CLAT assay including an ELISA for detection. 1*10^6 THP-1 cells/mL are incubated with the respective test substances in a 24 well plate for 24 h at 37 °C and 5 % CO_2. The augmentation of the expression of the cell surface molecules CD86 and CD54 in response to sensitizing chemicals is subsequently detected through antibody staining. After staining of CD86 and CD54 with mouse anti-human CD86/CD54 and staining with mouse IgG1 as isotype control, the cells are stained for the primary antibodies with an anti-mouse IgG1 HRP conjugated secondary antibody. HRP activity is detected by addition of TMB solution yielding a blue colour that turns yellow upon addition of sulphuric acid. The absorption is then photometrically measured at 450 nm.

Figure 4-23: Key for Figure 4-22.

5. DISCUSSION

Allergic contact dermatitis is one of the most prevalent skin conditions affecting up to 20 % of the population (Karlberg et al. 2008; Martin et al. 2011). With more than 3000 known contact allergens present in the environment, but also in consumer products like personal care products it is a great regulatory issue to identify potentially hazardous ingredients (Divkovic et al. 2005; Martin et al. 2011). Especially in the last few years there has been increasing need to establish reliable *in chemico* and *in vitro* assays to replace animal tests like the LLNA, which had been the model of choice for hazard evaluation of chemicals until its EU wide ban in 2013.

Thus, aim of this thesis was the development of an ELISA-based *in vitro* assay measuring the cellular response to allergen treatments. Measuring cellular response is a promising approach since dendritic cell activation represents a key step in skin sensitization (Yoshida et al. 2003b). Examples for the successful exploitation of the cellular response of DC surrogate cell lines to allergen exposure are the h-CLAT (THP-1) developed for Shiseido and Kao (Ashikaga et al. 2006) and the MUSST (U937). Since THP-1 cells have been found to predict sensitization better (Ashikaga et al. 2006), h-CLAT was preferred over MUSST and was used as basis for the development of a cell-based ELISA. The h-CLAT links the sensitization potential of a substance to the increased expression of the surface markers CD86 and/or CD54 on THP-1 cells upon exposure to allergens. For the developed cell-based ELISA, THP-1 cells are stimulated through incubation with the test substance following the h-CLAT protocol published by Bauch et al. 2012. The concentration ranges of the test substances are chosen according to the IC50 values of the substances, determined by EZ4U cytotoxicity assay. Unlike the h-CLAT, which uses fluorescent-labelled antibodies for the detection of marker expression by flow cytometry, the developed ELISA is much more simplified regarding the required equipment. As the photometers sensitivity is not sufficient for detection of staining with FITC-conjugated antibodies like in the original protocol, enzyme-linked detection was used. Since the antibodies used by Bauch et al. are not available as enzyme-conjugates, purified anti-CD86, anti-CD54 and anti-IgG1 were used in combination with a HRP-labelled anti-mouse secondary antibody. TMB solution is used as substrate for the peroxidase. The resulting color reaction can be measured photometrically, which eliminates the need for an expensive flow cytometer. This makes the assay feasible for basically every laboratory with standard cell culture equipment. The handling and especially the evaluation of the results is easier than in the

standard h-CLAT protocol were a gating strategy is needed. Another advantage is the use of 96-well plates, which allows higher throughput compared to flow cytometric measurements.

A major challenge concerning the ELISA was the impact the cell count has on the measured signal. Since the analysed markers are attached to the cell surface and photometrical measurement determines the overall signal of each well, the seeded cell count as well as the loss of cells (e.g. through the washing procedure) crucially influences the obtained signal. During flow cytometric measurement the cell count has no influence, since each cell is measured individually. The problem was addressed by optimizing the washing steps, to ensure an equal loss of cells in all wells. This includes centrifugation at higher g forces as usually used in cell culture experiments. Examination of the centrifuged cells under the microscope showed no increase in dead cells. A variability of 6 % could be achieved, with 40 – 45 % of the cells being lost on average. In order to further improve reproducibility, staining with calcein-AM was tested, which should be used to normalize the measured absorption to the actual cell count for every single measurement. Unfortunately, the cell density of 10^6 cells/mL used for h-CLAT exceeds the linear section of the calibration curve. Therefore, calcein staining is not suitable for the normalization of the signal to the cell count.

The chosen antibody combination of anti-CD86/-CD54 with HPR-labelled anti-mouse antibody and TMB substrate solution was tested using the optimized washing procedure. Two experiments were conducted to test the antibody combination and washing procedure. Staining of THP-1 cells stimulated with $CoCl_2$ as well as p-BQ showed satisfying results. The ELISA results of staining THP-1 cells stimulated with $CoCl_2$ for CD54 were compared to FACS data from Bauch et al. (Bauch et al. 2012). While Bauch et al. reported an EC1.5 value of 45 µg/mL $CoCl_2$, the ELISA was able to identify the sensitizer correctly and showed a 2.9-fold increase of CD54 expression upon exposure to 90 µg/mL $CoCl_2$. An EC1.5 could not be calculated by linear interpolation, since only one concentration of $CoCl_2$ was used for stimulation. Nevertheless, the results are comparable which suggests that both methods are equally sensitive. This was confirmed by staining THP-1 cells stimulated with p-BQ for CD86. The used concentrations were 100 µmol/L p-BQ (the IC50 for p-BQ, which had been determined by EZ4U cytotoxicity assay) as well as 0.25 x IC50 and 0.5 x IC50. The lowest concentration of 25 µmol/L did not cause enhanced CD86 expression, whereas 50 and 100 µmol/L gave a clearly detectable signal. An EC1.5 value of 38 µmol/L p-BQ was calculated for CD86 via linear interpolation between the highest and the lowest concentration. This ELISA result again correlates with data from (Bauch et al. 2012b), who reported an EC1.5 of

36 µmol/L. Comparing both results to LLNA data demonstrated that the sensitivity of h-CLAT was higher than in the animal assay, which is also reported by Nukada et al. 2011.

Since in the original h-CLAT protocol very high primary antibody concentrations are used, a titration experiment was conducted. Different antibody dilutions were used to stain THP-1 cells stimulated with $CoCl_2$ and p-BQ. Thereby the ideal dilutions yielding sufficiently high signal while minimizing the background were determined for anti-CD86 and anti-CD54. Compared to the recommended dilutions (1:7 for anti-CD86 and 1:16 for CD54) the background as well as the costs could be lowered considerably by increasing the dilutions to 1:100 for anti-CD86 and 1:200 for CD54.

The data from h-CLAT/ELISA measurements was compared to data from h-CLAT/FACS experiments using $CoCl_2$ and p-BQ for stimulation as well as data from literature. Generally the ELISA results correlate well with literature and flow cytometry data. The ELISA was not only able to predict both sensitizers correctly, but also the detected marker expressions coincide with the findings from the flow cytometry experiments as well as data obtained from dos Santos et al. 2009 and Nukada et al. 2013. Bauch et al. 2012 also identified both substances as sensitizers, but their results in marker expression differ a little. This may be due to the fact, that expression of surface markers on THP-1 cells is quite sensitive to culture conditions (Ashikaga et al. 2006). This may also be the reason, why the determination of the cell viability is more variable. This is observed, when different literature data and experiments from this work are compared. While the IC50 for $CoCl_2$ found in the EZ4U cytotoxicity assay (230 µmol/L) and through PI staining in the h-CLAT/flow cytometry experiment (200 µmol/L) are similar, the results for p-BQ differ considerably. The IC50 of 100 µmol/L which was determined using EZ4U assay is comparable to the value reported by Sakaguchi et al. 2009 (IC50 = 80 µmol/L), but is more than two times higher than the IC50 of 35 µmol/L determined by PI staining.

In the original protocol published by Ashikaga et al. 2008, an inter-laboratory reproducibility study found varying cytotoxicity data, too. Also the marker expressions differ between the different laboratories, although most substances were predicted correctly. This may be due to the unstable features and variance of the used leukaemia cell line THP-1 and is also strongly influenced by culture conditions. It was observed that the time the cells are pre-cultured before conducting h-CLAT assay as well as the time the cells have been maintained in culture have a strong impact on marker expression levels. Cells pre-cultured for 24 h rather than 48 or 72 h and cells at higher passages were found to exhibit lower expression levels (Ashikaga et al.

2006; An et al. 2009). Although h-CLAT has good predictive capacity which is comparable to LLNA (Nukada et al. 2011), the assay seems to be quite error prone because of the cell lines instability (An et al. 2009). The low reproducibility was also observed in the experiments performed during the course of this thesis. A troubleshooting guide was therefore assembled, which addresses mistakes and gives solutions (see Table 7-1, appendix). Nevertheless, the h-CLAT/ELISA assay yielded reasonable sensitivity and the results are comparable to literature data.

The major goal of developing *in vitro* alternatives to animal assays is sparing the animals while yielding equal or even better performance. Skin sensitization involves complex processes, thus h-CLAT and the developed cell-based ELISA as stand-alone assay would not be sufficient to obtain a reliable prediction of the sensitization potential of a substance. Instead multiple assays integrated into a test battery, each testing a different key step of the sensitization phase is more suitable. The measurement of multiple markers allows more precise prediction, since different classes of skin sensitizers cause response through diverse signalling pathways and mechanisms. The developed cell-based ELISA could be integrated in a test battery combining various assays, e.g. the DPRA and the ARE. DPRA targets the haptenation of self-proteins, which is the first step in skin sensitization, after the allergens have passed the skin barrier. The ARE targets the second step in sensitization by measuring the level of keratinocyte activation through allergen exposure using reporter luciferases. The advantage of this simple assay is the simultaneous measurement of cell viability and sensitization through the utilization of two different luciferases. The cell-based ELISA is testing the response of THP-1 cells to incubation with allergens, which resembles dendritic cell response, another crucial step in skin sensitization. Such a battery of three distinct assays could be sufficient for the discrimination between sensitizers and non-sensitizers as well as the prediction of the sensitization potential of chemicals. Another possibility would be the additional measurement of interleukin expression (e.g. IL-β1 or IL-8), since they have been shown to play a role in the response of keratinocytes and dendritic cells to allergens. Interleukins accumulate in the culture supernatant, which makes the measurement of their concentration from supernatant samples very convenient.

So far, DPRA as well as ARE have been established at the OFI. Testing substances with ARE remains subject to another thesis. Also the further investigation of test substances (e.g. by measurement of dose-response curves) using h-CLAT/ELISA will be done in another thesis. The results need to be validated against flow cytometry data and thresholds (like fluorescence

intensity thresholds for FACS measurement) have to be defined. Categorization into sensitizers and non-sensitizers is the first milestone, but also classification of the sensitization potential of substances needs to be established.

Furthermore, data obtained by DPRA, ARE and h-CLAT/ELISA should be compared, when more experiments have been performed. The three methods could then be integrated in a test battery with a better prediction than the single tests.

6. LITERATURE

Al-Otaibi, Sultan T., and Hatem Ali M. Alqahtani. 2015. 'Management of Contact Dermatitis'. *Journal of Dermatology & Dermatologic Surgery*, February. doi:10.1016/j.jdds.2015.01.001.

An, Susun, Seoyoung Kim, Yong Huh, Tae Ryong Lee, Han-Kon Kim, Kui-Lea Park, and Hee Chul Eun. 2009. 'Expression of Surface Markers on the Human Monocytic Leukaemia Cell Line, THP-1, as Indicators for the Sensitizing Potential of Chemicals'. *Contact Dermatitis* 60 (4): 185–92. doi:10.1111/j.1600-0536.2009.01528.x.

Ashikaga, T., M. Hoya, H. Itagaki, Y. Katsumura, and S. Aiba. 2002. 'Evaluation of CD86 Expression and MHC Class II Molecule Internalization in THP-1 Human Monocyte Cells as Predictive Endpoints for Contact Sensitizers'. *Toxicology in Vitro: An International Journal Published in Association with BIBRA* 16 (6): 711–16.

Ashikaga, T., Y. Yoshida, M. Hirota, K. Yoneyama, H. Itagaki, H. Sakaguchi, M. Miyazawa, Y. Ito, H. Suzuki, and H. Toyoda. 2006. 'Development of an in Vitro Skin Sensitization Test Using Human Cell Lines: The Human Cell Line Activation Test (H-CLAT). I. Optimization of the H-CLAT Protocol'. *Toxicology in Vitro: An International Journal Published in Association with BIBRA* 20 (5): 767–73. doi:10.1016/j.tiv.2005.10.012.

ATCC. 2015a. 'THP-1 ATCC ® TIB-202™'. Accessed August 25. http://www.lgcstandards-atcc.org/Products/All/TIB-202.aspx.

———. 2015b. 'U-937 ATCC ® CRL-1593.2™'. Accessed August 25. http://www.lgcstandards-atcc.org/Products/All/CRL-1593.2.aspx.

Bauch, Caroline, Susanne N. Kolle, Tzutzuy Ramirez, Tobias Eltze, Eric Fabian, Annette Mehling, Wera Teubner, Bennard van Ravenzwaay, and Robert Landsiedel. 2012. 'Putting the Parts Together: Combining in Vitro Methods to Test for Skin Sensitizing Potentials'. *Regulatory Toxicology and Pharmacology: RTP* 63 (3): 489–504. doi:10.1016/j.yrtph.2012.05.013.

Bock, M., A. Schmidt, T. Bruckner, and T.L. Diepgen. 2003. 'Occupational Skin Disease in the Construction Industry'. *British Journal of Dermatology* 149 (6): 1165–71. doi:10.1111/j.1365-2133.2003.05748.x.

Borak, Jonathan, Cheryl Fields, Larry S. Andrews, and Mark A. Pemberton. 2011. 'Methyl Methacrylate and Respiratory Sensitization: A Critical Review'. *Critical Reviews in Toxicology* 41 (3): 230–68. doi:10.3109/10408444.2010.532768.

Bordel-Gómez, Ma T., A. Miranda-Romero, and J. Castrodeza-Sanz. 2010. '[Epidemiology of contact dermatitis: prevalence of sensitization to different allergens and associated factors]'. *Actas Dermo-Sifiliográficas* 101 (1): 59–75.

Cohen, David E., and Noushin Heidary. 2004. 'Treatment of Irritant and Allergic Contact Dermatitis'. *Dermatologic Therapy* 17 (4): 334–40. doi:10.1111/j.1396-0296.2004.04031.x.

Divkovic, Maja, Camilla K. Pease, G. Frank Gerberick, and David A. Basketter. 2005. 'Hapten-Protein Binding: From Theory to Practical Application in the in Vitro Prediction of Skin Sensitization'. *Contact Dermatitis* 53 (4): 189–200. doi:10.1111/j.0105-1873.2005.00683.x.

dos Santos, G. Galvão, J. Reinders, K. Ouwehand, T. Rustemeyer, R. J. Scheper, and S. Gibbs. 2009. 'Progress on the Development of Human in Vitro Dendritic Cell Based Assays for Assessment of the Sensitizing Potential of a Compound'. *Toxicology and Applied Pharmacology* 236 (3): 372–82. doi:10.1016/j.taap.2009.02.004.

Drexler, Hans G., and Cord C. Uphoff. 2002. 'Mycoplasma Contamination of Cell Cultures: Incidence, Sources, Effects, Detection, Elimination, Prevention'. *Cytotechnology* 39 (75 – 90, 2002).

Ettenberger-Bornberg, Gabriele, Nicole Glück, Elisabeth Riegel, and Thomas Czerny. 2015. 'Cell Based Test Systems for the Antioxidant Response Pathway'. In *9. Forschungsforum Der Österreichischen Fachhochschulen*.

European Parliament, and Council of the European Union. 2011. 'Regulation (EU) No 1169/2011 of the European Parliament and of the Council of 25 October 2011 on the Provision of Food Information to Consumers, Amending Regulations (EC) No 1924/2006 and (EC) No 1925/2006 of the European Parliament and of the Council, and Repealing Commission Directive 87/250/EEC, Council Directive 90/496/EEC, Commission Directive 1999/10/EC, Directive 2000/13/EC of the European Parliament and of the Council, Commission Directives 2002/67/EC and 2008/5/EC and Commission Regulation (EC) No 608/2004 Text with EEA Relevance'.

Goebel, Carsten, Pierre Aeby, Nadège Ade, Nathalie Alépée, Aynur Aptula, Daisuke Araki, Eric Dufour, et al. 2012. 'Guiding Principles for the Implementation of Non-Animal Safety Assessment Approaches for Cosmetics: Skin Sensitisation'. *Regulatory Toxicology and Pharmacology* 63 (1): 40–52. doi:10.1016/j.yrtph.2012.02.007.

Honda, Tetsuya, Gyohei Egawa, Stephan Grabbe, and Kenji Kabashima. 2013. 'Update of Immune Events in the Murine Contact Hypersensitivity Model: Toward the Understanding of Allergic Contact Dermatitis'. *The Journal of Investigative Dermatology* 133 (2): 303–15. doi:10.1038/jid.2012.284.

Jurado-Palomo, Jesus, Alvaro Moreno-Ancillo, Irina Diana, Carmen Panizo, and Ivan Cervigon. 2011. 'Epidemiology of Contact Dermatitis'. In *Contact Dermatitis*, edited by Young Suck Ro. InTech. http://www.intechopen.com/books/contact-dermatitis/epidemiology-of-contact-dermatitis.

Karlberg, Ann-Therese, Moa Andresen Bergström, Anna Börje, Kristina Luthman, and J. Lars G. Nilsson. 2008. 'Allergic Contact Dermatitis––Formation, Structural Requirements, and Reactivity of Skin Sensitizers'. *Chemical Research in Toxicology* 21 (1): 53–69. doi:10.1021/tx7002239.

Martin, S. F., P. R. Esser, F. C. Weber, T. Jakob, M. A. Freudenberg, M. Schmidt, and M. Goebeler. 2011. 'Mechanisms of Chemical-Induced Innate Immunity in Allergic Contact Dermatitis: Contact Allergen-Induced Innate Immunity'. *Allergy* 66 (9): 1152–63. doi:10.1111/j.1398-9995.2011.02652.x.

Merk, Hans F., Jens M. Baron, Mark M. Neis, Daniela Höller Obrigkeit, and Ann-Therese Karlberg. 2007. 'Skin: Major Target Organ of Allergic Reactions to Small Molecular Weight Compounds'. *Toxicology and Applied Pharmacology* 224 (3): 313–17. doi:10.1016/j.taap.2007.01.004.

Metz, Martin, and Marcus Maurer. 2009. 'Innate Immunity and Allergy in the Skin'. *Current Opinion in Immunology* 21 (6): 687–93. doi:10.1016/j.coi.2009.09.009.

Nukada, Yuko, Takao Ashikaga, Hitoshi Sakaguchi, Sakiko Sono, Nanae Mugita, Morihiko Hirota, Masaaki Miyazawa, Yuichi Ito, Hitoshi Sasa, and Naohiro Nishiyama. 2011. 'Predictive Performance for Human Skin Sensitizing Potential of the Human Cell Line Activation Test (H-CLAT)'. *Contact Dermatitis* 65 (6): 343–53. doi:10.1111/j.1600-0536.2011.01952.x.

Nukada, Yuko, Masaaki Miyazawa, Saitou Kazutoshi, Hitoshi Sakaguchi, and Naohiro Nishiyama. 2013. 'Data Integration of Non-Animal Tests for the Development of a Test Battery to Predict the Skin Sensitizing Potential and Potency of Chemicals'. *Toxicology in Vitro: An International Journal Published in Association with BIBRA* 27 (2): 609–18. doi:10.1016/j.tiv.2012.11.006.

OECD. 2014. 'OECD Guideline for the Testing of Chemicals - Draft Proposal for a New Test Guideline: In Chemico Skin Sensitisation: Direct Peptide Reactivity Assay (DPRA)'.

Saint-Mezard, P., M. Krasteva, C. Chavagnac, S. Bosset, H. Akiba, J. Kehren, J. Kanitakis, D. Kaiserlian, J. F. Nicolas, and F. Berard. 2003. 'Afferent and Efferent Phases of Allergic

Contact Dermatitis (ACD) Can Be Induced after a Single Skin Contact with Haptens: Evidence Using a Mouse Model of Primary ACD'. *The Journal of Investigative Dermatology* 120 (4): 641–47. doi:10.1046/j.1523-1747.2003.12093.x.

Sakaguchi, Hitoshi, Takao Ashikaga, Masaaki Miyazawa, Nanae Kosaka, Yuichi Ito, Katsurako Yoneyama, Sakiko Sono, Hiroshi Itagaki, Hidekazu Toyoda, and Hiroyuki Suzuki. 2009. 'The Relationship between CD86/CD54 Expression and THP-1 Cell Viability in an in Vitro Skin Sensitization Test – Human Cell Line Activation Test (H-CLAT)'. *Cell Biology and Toxicology* 25 (2): 109–26. doi:10.1007/s10565-008-9059-9.

Santos, G. Galvão dos, J. Reinders, K. Ouwehand, T. Rustemeyer, R. J. Scheper, and S. Gibbs. 2009. 'Progress on the Development of Human in Vitro Dendritic Cell Based Assays for Assessment of the Sensitizing Potential of a Compound'. *Toxicology and Applied Pharmacology* 236 (3): 372–82. doi:10.1016/j.taap.2009.02.004.

Schmitz, Sabine. 2011. *Der Experimentator: Zellkultur*. 3. Aufl. Der Experimentator. Heidelberg: Spektrum Akad. Verl.

Smith Pease, C. K., D. A. Basketter, and G. Y. Patlewicz. 2003. 'Contact Allergy: The Role of Skin Chemistry and Metabolism'. *Clinical and Experimental Dermatology* 28 (2): 177–83.

Sundström, C., and K. Nilsson. 1976. 'Establishment and Characterization of a Human Histiocytic Lymphoma Cell Line (U-937)'. *International Journal of Cancer. Journal International Du Cancer* 17 (5): 565–77.

Thyssen, Jacob Pontoppidan, Allan Linneberg, Torkil Menné, and Jeanne Duus Johansen. 2007. 'The Epidemiology of Contact Allergy in the General Population – Prevalence and Main Findings'. *Contact Dermatitis* 57 (5): 287–99. doi:10.1111/j.1600-0536.2007.01220.x.

Tsuchiya, S., M. Yamabe, Y. Yamaguchi, Y. Kobayashi, T. Konno, and K. Tada. 1980. 'Establishment and Characterization of a Human Acute Monocytic Leukemia Cell Line (THP-1)'. *International Journal of Cancer. Journal International Du Cancer* 26 (2): 171–76.

Yoshida, Y., H. Sakaguchi, Y. Ito, M. Okuda, and H. Suzuki. 2003. 'Evaluation of the Skin Sensitization Potential of Chemicals Using Expression of Co-Stimulatory Molecules, CD54 and CD86, on the Naive THP-1 Cell Line'. *Toxicology in Vitro* 17 (2): 221–28. doi:10.1016/S0887-2333(03)00006-7.

7. APPENDIX

ELISA-TROUBLESHOOTING GUIDE

Table 7-1: Troubleshooting Guide for the ELISA-based sensitization assay.

Problem	Possible cause	Prevention/Solution
High background	Viability of cells: dead cells can lead to increased unspecific binding	Check viability of the cells prior to stimulation
	Contamination (i.e. with antibody) in the reagent reservoirs or solutions	Do not bring used pipette tips in contact with the reagent reservoirs, use new solutions (staining buffer, PBS)
	Incorrect blocking procedure	Check staining buffers and incubation time
	Insufficient washing	Check washing procedure (number of washing steps may be increased)
	Incubation times too long	Do not extend incubation times from the protocol
No signal	All reagents added (and in correct order)?	Tick finished steps in the protocol
	Wrong antibody dilution	Check dilution of antibodies
	Old reagents	Check expiration date of reagents (especially antibodies and TMB kit)
No difference between different concentrations of known sensitizers	Wrong dilution of test substances used for stimulation	Check dilutions of test substances
	Age of cells: decrease of marker expression	Do not passage cells too often (ca. 30x)
Higher signal for lower concentrations of test substances than for higher concentrated samples	Cytotoxicity of substances used for stimulation	Check IC50 values of substances
	Wrong dilution of test substances used for stimulation	Check dilutions of test substances (also where the samples are on the plate)
High blank values	Contamination of blanks	Avoid contact of used tips with reagent reservoirs und do net reuse reagent reservoirs
	Incorrect washing procedure (remains of unbound antibodies)	Wash properly (see protocol)
	Precultivation of cells	Cells express less after 24 h of precultivation compared to 48

No difference between known sensitizers and blanks (uniformly blue plate)	Age of cells: after too many passages the surface marker expression decreases	Do not passage cells too often (ca. 30x)
	Contamination of blanks	Avoid contact of used tips with reagent reservoirs and use new reservoirs for each staining for the antibodies and TMB solution (residual HRP turns solution unspecifically blue)
	Incorrect washing procedure (was all unbound antibody washed away?)	Wash properly (see protocol)
No difference between known sensitizers and blanks (uniformly blue plate)	Wrong concentrations of test substances	Check used concentrations/dilution of the test substances used for stimulation
	TMB substrate solution mixed too early	Prepare solution freshly and protect from light
	HRP-contaminated solutions	Use fresh solutions
High variation between triplets	Contamination of some of the wells	Do not contaminate reagent reservoir with used tips Use the same tips just for the resuspension of triplets
	Improper supernatant removal (after pouring out the supernatant following centrifugation, sometimes fluid remains in the wells)	Check all wells after pouring out the supernatant (is any liquid left?)

GROWTH CURVES OF THE CELL LINES THP-1 AND U937

Growth curves of THP-1 and U937 were measured to determine their growth rate and doubling time to be able to assess when cells enter the log phase, e.g. for the establishment of a master cell bank consisting of cell population in the log phase. Cells were seeded into 75 cm² tissue culture flasks at a density of $1*10^5$ cells/mL and incubated for three days. Samples were drawn and cell number as well as viability was determined by a trypan blue exclusion assay as described. For the construction of a growth curve and the calculation of the growth rate μ and the doubling time g, the cell count was plotted against the incubation time (see Figure 7-1). The growth rate μ and the doubling time g were calculated based on the equations below.

$$N(t) = N_0 * e^{\mu * t} \qquad \mu = \frac{\ln(N) - \ln(N_0)}{t - t_0} \qquad g = \frac{\ln(2) * \Delta t}{\ln(N) - \ln(N_0)} = \frac{\ln(2)}{\mu}$$

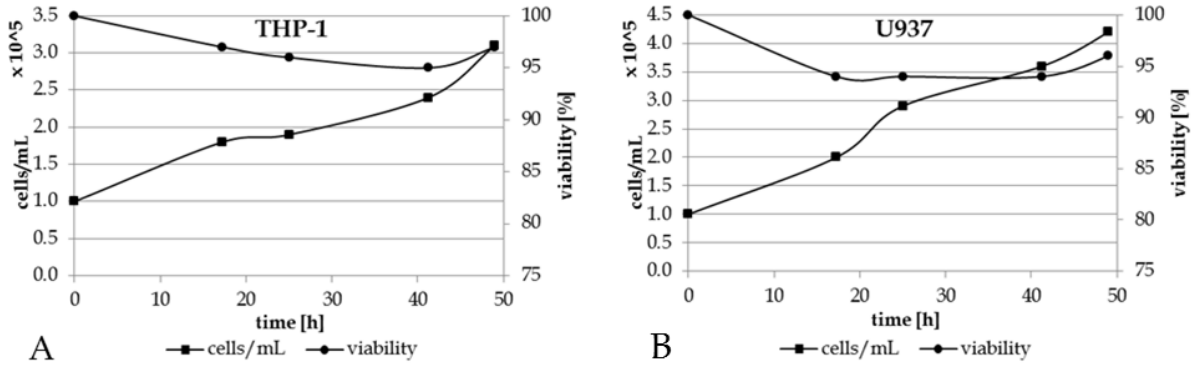

Figure 7-1: Growth characteristics of the cell lines THP-1 and U937. The cell density and viability of THP-1 (A) and U937 (B) were determined and plotted against the incubation time.

The average population doubling time calculated for THP-1 was 27 h, the average population doubling time of U937 was 20 h. Both cell lines showed slower growth rates over time (data not shown), since the nutrient level decreases with increasing cell density. Also the viability trended to decrease during the course of cultivation, if no new media was added after 2 days.

It was observed that THP-1 cells cultured at very high densities ($1*10^6$ cells/mL or higher) were not provided with enough nutrients to grow properly, which caused their differentiation towards an adherent, macrophage-like phenotype. Therefore the cell density range of $2*10^5$-$8*10^5$ cells/mL provided by the supplier (ATCC 2015a) was adhered to.