Corinna Tietze

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The impact of ascaridol on human monocyte-derived dendritic cells and on the monocytic leukemia cell line THP-1

Betreuende: Univ.-Prof. Dr. rer. nat. Brunhilde Blömeke
Berichterstattende: Univ.-Prof. Dr. rer. nat. Brunhilde Blömeke
Univ.-Prof. Dr. Dr. h.c. mult. Paul Müller

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II Abbreviations

AB	antibiotic/antimycotic solution
ACD	allergic contact dermatitis
APC	allophycoerythrin
BSA	bovine serum albumine
CBA	Cytometric Bead Assay
CCR2	CC chemokine receptor 2
CD	cluster of differentiation
CD184	CXC motive chemokine receptor 4 (CXCR4)
CD209	receptor c-type lectin (DC-SIGN)
CD54	intercellular adhesion molecule-1 (ICAM-1)
DC	dendritic cell(s)
DMSO	dimethylsulfoxide
DNCB	2,4-dinitrochlorobenzene
ELISA	Enzyme Linked Immunosorbent Assay
FACS	flow cytometry associated cell sorter
FBS	foetal bovine serum
FITC	fluorescein-5-isocyanat
GM-CSF	granulocyte/macrophage-colony stimulating factor
h-CLAT	human Cell Line Activation Test
HEPES	4-(2-hydroxylethyl)-1-piperazin-ethylsulfonic acid
HLADR	human leukocyte antigen DR-1
i. a.	inter alia
IFN	interferon
IL	interleukin
LLNA	local lymph node assay
LPS	lipopolysaccharide
MAPK	mitogen-activated protein kinase
MCP-1	monocyte-chemotactic protein-1
MFI	mean fluorescence intensity
MHC	major histocompartibility complex
MoDC	monocyte-derived dendritic cell(s)
MOPS	3-(n-morpholino)-propane-sulfonic acid
NFkappaB	nuclear factor-kappaB

PBMC	peripheral blood-derived mononuclear cells
PBS	phosphat buffered saline
PE	phycoerythrine
PE-Cy5	phycoerythrine-cyano dye 5 tandem
PerCyp	peridin-chlorophyll-a protein
PI	propidium iodide
PPD	para-phenylendiamine
PVDF membrane	polyvinylidene fluoride membrane
REACH	registration, evaluation, authorization of chemicals
RFI	relative fluorescence intensity
rMFI	relative mean fluorescence intensity
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	standard error of mean
SLS	sodium lauryl sulfate
TBS	tris buffered saline
TBST	tris buffered saline with Tween 20
TBSTT	tris buffered saline with Tween 20 & Triton X
TCR	T-cell-receptor
Th1	T helper cell type 1
Th2	T helper cell type 2
THP-1	human acute monocytic leukemia cell line-1
TNBS	2,4,6-trinitrobenzene sulfonic acid
TNFalpha	tumor necrosis factor alpha
TTO	tea tree oil

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1. Introduction

The skin is continuously challenged by environmental antigens that may evoke protective or detrimental immune responses. A variety of small chemicals including pharmaceutical, industrial and metallic compounds can act as general denoted haptens which penetrate the skin and become sensitizing entity by binding to epidermal proteins. These hapten-carrier complexes are immunogenic and can elicit the development of allergic contact dermatitis (ACD), one of the most frequent allergic skin diseases (Kimber *et al.*, 2002). The medical treatment for ACD is limited - in fact only acute symptoms can be cured and for secondary prevention of the disease a lifelong avoidance of the allergen(s) is necessary. In order to improve cure probability the identification of potent contact allergens and the better understanding of their molecular mechanisms of action are essential.

It has been demonstrated that dendritic cells, which play a crucial role in the immunity, can be activated by certain chemicals and therefore, have become a promising tool for detection of contact allergens (Kimber *et al.*, 2001; Casati *et al.*, 2005). Moreover, several cell lines, which are suggested as surrogates for dendritic cells, are currently explored for their potential to predict sensitizers. However, the greatest weight in this type of approaches is given to the discrimination of skin sensitizers from those chemicals which are non-sensitizing. It has been shown that skin sensitizing chemicals cover not only a broad spectrum in terms of their chemistry, but also present a very wide range of potencies. Studies for the detection of subtle modifications induced by weak haptens, including fragrances, dyes and preservatives, which represent the majority of haptens relevant to human ACD, become necessary. Hence, further investigations into whether promising *in vitro* sensitization assays with dendritic cells or dendritic cell surrogates are sensitive enough even to predict presumably weak allergens like ascaridol, a substance which is associated with tea tree oil contact dermatitis, are needed.

1.1 Ascaridol

Ascaridol (1,4-epodioxy-p-menth-2-ene) is an asymmetric monoterpene endoperoxide, naturally occurring as a major constituent (60 – 80 %) in the volatile oil of American wormseed fruit (*Chenopodium ambrosioides*), and in lesser amounts in the leaves of the boldo tree (*Peumus boldus)* (Johnson and Croteau, 1984) as well as in leaves of *Artemisia molinieri* (Masotti *et al.*, 2003). Ascaridol belongs to the terpenoides, which are typically composed of isoprene units. The chemical structure of ascaridol is shown in figure 1.

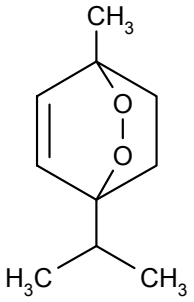

Figure 1: Chemical structure of ascaridol.

Infusions of *Chenopodium ambrosioides* have been used for centuries in America as a popular remedy against intestinal worm infections and therefore its main component ascaridol has been presumed to be a potent anthelmintic (Smillie and Pessoa, 1924). Admittedly, MacDonald and co-workers showed that almost 90 % of the nematocidal activity of *Chenopodium ambrosioides* infusions was due to a hydrophilic component different from ascaridol (MacDonald *et al.*, 2004). However, Okuyama *et al.* found that ascaridol, when given to mice at a dose of 100 mg/kg body weight produced hypothermia, and decreased locomotory activity (Okuyama *et al.*, 1993). Effereth and colleagues found first hints that ascaridol may be an interesting novel candidate drug for cancer treatment. They analyzed its activity against different tumor cell lines *in vitro* and found that ascaridol exerts antineoplastic activity (Efferth *et al.*, 2002).

Intriguingly, ascaridol has been found in old tea tree (*Melaleuca alternifolia*) oil (Pirker *et al.*, 2003). Tea tree oil (TTO) is generally composed of terpene hydrocarbons, mainly monoterpenes, sesquiterpenes, and their associated alcohols. Brophy and co-workers, who analyzed over 800 TTO samples, reported approximately 100 components (Brophy *et al.*, 1989). Among other ingredients alpha-terpinene was identified with approximately 10 %. Allergic reactions due to TTO have been reported frequently in the past 15 years (Aberer, 2008). Although a range of components has been suggested to be responsible, the most definitive work indicates that TTO allergy is caused mainly by oxidation products that occur in aged or

improperly stored oil (Hausen *et al.*, 1999). Degradation products of photo-oxidized commercial TTO are three times as sensitizing as the non-oxidized newly opened fresh TTO (Hausen *et al.*, 1999). In Australia Rutherford and co-workers retrospectively reviewed patch test data over a 4.5-year period and identified 41 cases of positive reactions to oxidized TTO out of 2320 people patch-tested, giving a prevalence of 1.8 % (Rutherford *et al.*, 2007). The TTO ingredient alpha-terpinene can be oxidized to ascaridol in the presence of air and light (Esser *et al.*, 1994). Schenck synthesized ascaridol from alpha-terpinene through photo-oxidation first in 1943 (Esser *et al.*, 1994). Johnson and Croteau (1984) showed the biosynthetic pathway of ascaridol in *Chenopodium ambrosioides*: alpha-terpinene leads to ascaridol with a single enzyme, an iodide peroxidase. The observation that freshly distilled TTO rarely contains the offending sensitizers, while on the contrary patients who had used oxidized TTO showed strong irritant reactions on controls and extreme allergic reactions on those being sensitized to the oil (Hausen *et al.*, 1999), indicates that ascaridol may be one of the allergens in TTO. Both Lippert *et al.* (Lippert *et al.*, 2000) and Pirker *et al.* (2003) patch tested TTO-sensitized patients with ascaridol and almost all patients showed a positive reaction: all 10 patients in Pirker's study and 7 of 8 patients in Lippert's study. Furthermore, Hausen and colleagues used ascaridol to perform epicutaneous tests and 15 out of 20 TTO-sensitized patients reacted positively at the 72 h reading (Hausen, 2004). These clinical data strongly indicate that ascaridol exhibits immuno-modulating properties or even a sensitizing potential, but this has not been further explored yet.

1.2 Allergic contact dermatitis

TTO use is increasing, with considerable interest in it being bactericidal in nature, although it may be bacteriostatic in lower concentrations (Carson *et al.*, 2006). It is found in many commercially available skin and hair care products. In general, plant-constituents are regarded as critical allergens by dermatologists (Aberer, 2008). Virtually all alternative remedies can cause hypersensitivity reactions. The mechanisms which are required for acquisition of skin sensitization and subsequent elicitation of allergic reactions in skin are complex and dependent on highly orchestrated molecular and cellular interactions. Allergic contact dermatitis (ACD) is a T cell-mediated inflammatory reaction occurring at the site of challenge with a contact allergen in sensitized individuals. It is characterized by redness, papules, and vesicles, followed by scaling and dry skin (Krasteva *et al.*, 1999). The pathophysiology of ACD consists of two distinct phases. Firstly, the so-called sensitization phase arises, which comprises the first contact of skin with the hapten leading to the generation of antigen-specific T cells in the lymph nodes. In detail, haptens accessing the stratum corneum are taken up by cutaneous dendritic cells including Langerhans cells and dermal dendritic cells (see figure 2, (1)). The hapten uptake induces activation and further maturation of dendritic cells to profes-

sional antigen-presenting cells, which migrate through the afferent lymphatic vessels to the draining lymph nodes (figure 2 (2)). In the lymph node dendritic cells present haptenated peptides to naive $CD8^+$ and $CD4^+$ T cell which clonally expand and differentiate into hapten-specific effector cells and hapten-specific memory T cells (3). Activated T cells recirculate via the blood and migrate into tissues including the skin (4). Secondly, the elicitation phase develops, if the susceptible individual is exposed to the same hapten again (5), which leads to the activation of hapten-specific T lymphocytes in the dermis (6) and triggers the inflammatory process responsible for the cutaneous lesions (Kimber *et al.*, 1998; Kimber *et al.*, 2002; Kimber and Dearman, 2002; Cumberbatch *et al.*, 2004).

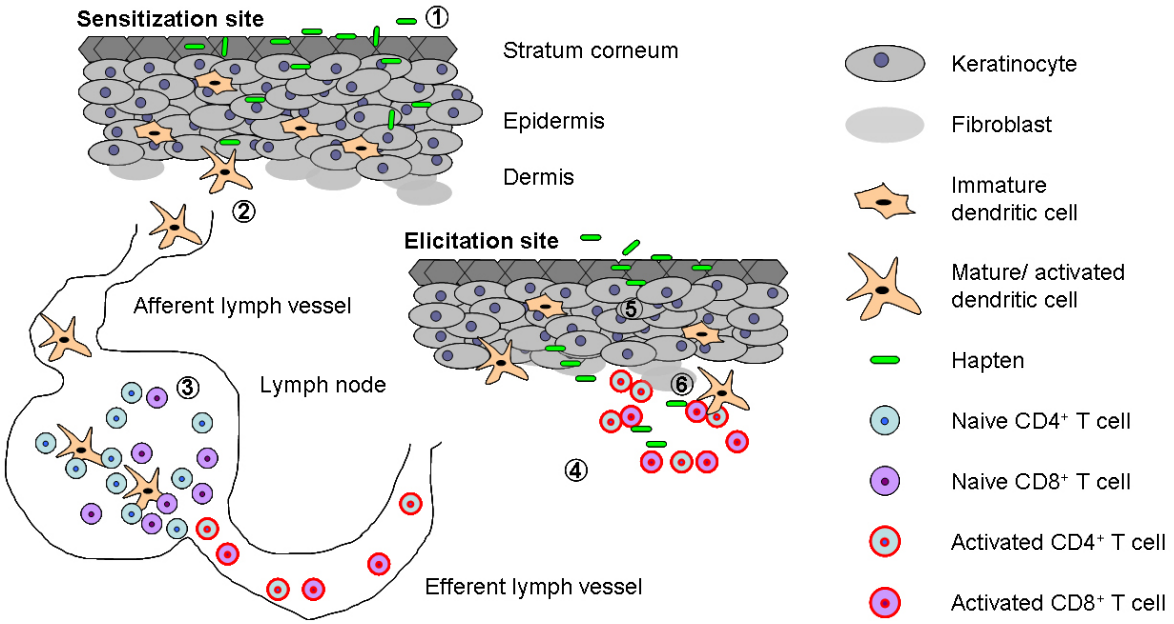

Figure 2: Pathophysiology of allergic contact dermatitis.

Figure 2 shows a general scheme of the pathophysiology of allergic contact dermatitis which is an adaptive immune response toward chemicals penetrating the skin. Allergic contact dermatitis progresses in two phases. During the initial sensitization phase, the host is immunized to the hapten. After re-exposure to the hapten, a more rapid and potent secondary immune response is mounted. The second, elicitation or effector phase, manifests in allergic contact dermatitis. Numbers refer to explanation in the text above.

The initial step in the development of ACD is decided by the participitation of dendritic cells. Their actions as sentinels of the immune system and antigen presenting cells are essential. Therefore, dendritic cells are one of the key players in the development of ACD, and their reaction to environmental antigens should take centre stage for identification of sensitization potentials.

1.3 Dendritic cells - key players in the induction phase of ACD

In the following the action of dendritic cells (DC) in the induction phase of ACD, hence the reaction of DC to environmental antigens or haptens is surveyed. DC form an epidermal cellular network with their dendrites which allows the capture of haptens that have penetrated the skin barrier. Although haptens do not have dedicated cellular receptors to interact with, as it is known for pathogens (e.g. toll-like receptor mediated recognition), they diffusely distribute into the skin due to their lipophilic nature and bind to a great number of extracellular and cell-membrane-associated proteins as a consequence of their chemical reactivity (Divkovic *et al.*, 2005). Unless haptens are per se already protein-reactive due to their electrophilic properties, they may be chemically or metabolically activated prior to or upon cutaneous absorption. Subsequently, they must bind to skin protein(s) to form a macromolecular immunogen. These chemicals which must be activated by abiotic means (e.g. UV light, autoxidation) are denoted as pre-haptens, while chemicals activated by enzymatic conversions are called pro-haptens (Aptula *et al.*, 2007).
After antigen detection, its processing and presentation occur followed by the migration of DC to lymphoid tissues. During these processes DC change phenotypically and become mature DC. Differences in the maturation stadium are reflected by a heterogeneous expression of several cell surface molecules called cluster of differentiation (CD).

1.3.1 Phenotypical changes of dendritic cells during maturation

Initially, antigens are bound to presentation molecules of DC such as major histocompatibility complex (MHC). While the MHC I pathway is required for presentation of endogenous antigens resulting most often from viral infections (Rodriguez *et al.*, 1999), the MHC II pathway is essential to present extracellular soluble antigens taken up by pinocytosis or receptor mediated endocytosis (Banchereau and Steinman, 1998). Antigen fragments are loaded onto the human leukocyte antigens (HLA) such as HLA-DR-1 (HLADR) molecule in so-called MHC class II compartments (Andersson *et al.*, 1998). Haptens are usually bound to MHC II molecules (Tuschl *et al.*, 2000), which are presented to $CD4^+$ T cells (Nijman *et al.*, 1995). MHC I complexes are displayed to $CD8^+$ T cells (Rodriguez *et al.*, 1999). However, it has become clear that both $CD4^+$ and $CD8^+$ T cells can act as effector cells in skin inflammatory reactions (Saint-Mezard et al., 2004). The precise contribution of these cells in human ACD is still not known (Girolomoni *et al.*, 2001). In addition, the stimulation of T cells by DC involves, beside T-cell-receptor (TCR) recognition of peptide-MHC complexes, further signalling events of co-stimulatory molecules such as CD86 and CD80. Their principal mode of action is by binding to CD28 on T cells (Freeman et al., 1991; Azuma et al., 1993; Lenschow et al., 1996). The different kinetics and expression patterns of CD86 and CD80 have led to

the hypothesis that CD86 upon binding of CD28 provides co-stimulatory signals needed for the activation of naive T cells within draining lymphoid tissues, whereas CD80 plays a major role in amplifying and regulating T cell activity at peripheral inflammatory sites (Sansom *et al.*, 2003). A consequence of such antigen-specific T cell - DC interactions is the formation of the so-called immunological synapse, comprising a central cluster of TCR-peptide-MHC complexes and CD28-CD86 interactions surrounded by rings of engaged accessory molecules (Grakoui *et al.*, 1999).

Moreover, mature DC in contrast to immature DC augment the expression of various surface molecules such as integrins (van Helden *et al.*, 2006) and adhesion molecules like intercellular adhesion molecule-1 (ICAM-1, CD54) (Aiba *et al.*, 1997; Coutant *et al.*, 1999a). CD54 triggers the accumulation, and thereby increases the effective concentration of immune receptors, as well as MHC molecules in particular at the contact area to T cells (Lebedeva *et al.*, 2005). Additionally, the expression of several lectins, e.g. the receptor C-type lectin (DC-SIGN, CD209), are influenced. CD209 acts as an antigen capture receptor as well as a cell adhesion molecule or rolling receptor that allows leukocyte trans-endothelial migration for passing lymphoid endothelium (Neumann *et al.*, 2008). Geijtenbeek and colleagues predicted that CD209 also enables T cell receptor engagement via stabilization of the DC-T cell contact zone (Geijtenbeek *et al.*, 2000). Furthermore, it is shown that environmental antigens can augment CD40 expression on DC (Vidalain *et al.*, 2000; Staquet *et al.*, 2004; Coulter *et al.*, 2007). The ligation of DC expressed CD40 by its ligand CD154 on T cells enhances maturation of the DC and leads to production of interleukin-12 (Cella *et al.*, 1996). In addition, the analysis of DC maturation disclosed that mature DC express CD83 (Zhou and Tedder, 1996; Aiba and Tagami, 1999).

However, the signal transduction pathways involved in DC maturation are poorly characterized. A few reports describe the activation of mitogen-activated protein kinase (MAPK) pathways in the process of human DC maturation. Ardeshna *et al.* (2000) showed that during exposure of immature DC to lipopolysaccharide (LPS) the p38 MAPK is responsible for up-regulation of CD86, CD80 and CD83. Furthermore, Arrighi and co-workers have reported that in DC 2,4-dinitrofluorobenzene (DNCB) and nickel sulfate induce p38 MAPK phosphorylation and that p38 MAPK inhibition suppress the augmentation of CD86 (Arrighi *et al.*, 2001). Finally, Aiba *et al.* confirmed, using DNCB and nickel chloride, that haptens stimulate DC via MAPK pathways. Especially DNCB preferentially stimulated p38 (Aiba *et al.*, 2003).

Further on, there are some reports disclosing that also nuclear factor (NF)kappaB are involved in the signal transduction of DC maturation (Ade *et al.*, 2007).

1.3.2 Functional changes of dendritic cells during maturation

Immediately after antigen contact, cytokines like interleukin (IL)-1beta are expressed followed by secretion of IL-6 and IL-8, typically proinflammatory mediators which act as chemoattractants and activators for leukocytes in the affected environment (Baggiolini and Dahinden, 1994; Baggiolini *et al.*, 1994; Cumberbatch *et al.*, 1996). The pleiotropic inflammatory cytokine tumor necrosis factor (TNF)alpha is an important mediator for migration of DC to draining lymph nodes and is necessary for an optimal contact sensitization (Cumberbatch and Kimber, 1995; Flint *et al.*, 1998). Other secreted cytokines like IL-12 and IL-10 play a role in T cell polarization. In lymph nodes DC have the potential to induce a polarized T helper cell type 1 (Th1) or T helper cell type 2 (Th2) response (Bellinghausen *et al.*, 1999; Moser and Murphy, 2000). It is further known that IL-12 or IL-12p75, which consists of the two subunits p40 and p35 (Trinchieri *et al.*, 2003), induces the development of Th1 cells (Moser and Murphy, 2000), while IL-10 represses Th1 and seems to polarize Th2 response (Iwasaki and Kelsall, 1999). To specify, IL-12p75 directs T cell polarization towards Th1 (Macatonia *et al.*, 1995), whereas IL-12p40 effects the T cell polarization differently. On the one hand IL-12p40 as a homodimer may act as an antagonist of IL-12p75, and on the other hand as heterodimers containing different polypeptides, they could influence the nature (strength, effector class, duration, etc.) of the immune response to ensure that it is appropriate to the site and the antigen involved (Abdi, 2002). In general, DC produce low levels of IL-12 and high levels of IL-12p40 after activation (Brombacher *et al.*, 2003). In addition, IL-4, the major Th2-inducing cytokine, is hard to detect in human DC as they produce only a modicum of this cytokine *in vitro* (Rissoan *et al.*, 1999). However, the proinflammatory cytokines and the expression of specific chemokines by inflammatory tissues, endothelial venules or lymphoid organs (Springer, 1994) and their interaction with appropriate receptors expressed by DC, orchestrate the mobilization and movement of these cells (Kimber *et al.*, 2000). For instance, DC precursors typically express CC chemokine receptor 2 (CCR2) and CXC motive chemokine receptor 4 (CXCR4, CD184) (Rustemeyer *et al.*, 2003; Cruz *et al.*, 2005).

In summary, the type of antigen and its concentration as well as the cytokine microenvironment, in which immature DC take up antigen, critically influence the DC phenotype. Figure 3 shows the progression of immature to mature DC due to antigen uptake. Further on, the phenotype then influences whether a Th1 or Th2 response occurs. ACD is usually the result of an exaggerated immune response sustained by Th1 cell (Cavani *et al.*, 2003). Interestingly, Th2 cells were found in human biopsies of ACD resulting from chronic and repeated stimulations with the antigen (Cavani *et al.*, 1998). It is pointed out that the modality how haptens influence DC phenotype will decide on whether a skin sensitization will be elicited or not.

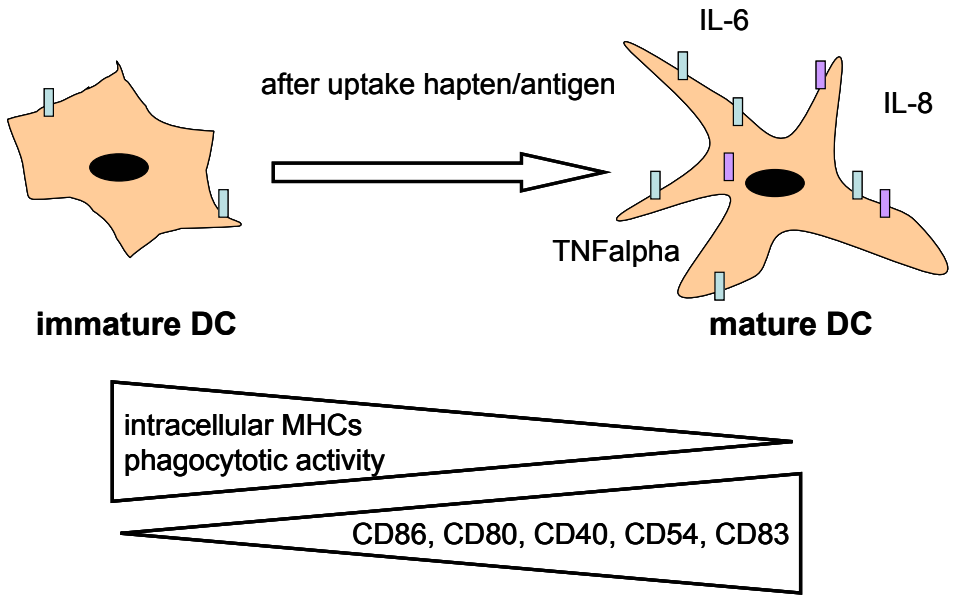

Figure 3: Features that can change during DC maturation.

Immature DC are unable to stimulate T cells, but are well equipped to capture antigens, through several ways like phagocytosis. Antigens are able to induce maturation of DC, whereupon the phenotype of DC changes: antigen-capturing devices disappear and T cell stimulatory functions increase. Mature DC can therefore be characterized by augmented expressions of various surface molecules like CD86, CD80, HLADR, CD54, CD40 or CD83. In addition the secretion of different cytokines such as interleukin (IL)-6, IL-8 or tumor necrosis factor (TNF)alpha can be determined.

1.4 Studying of sensitization potential of chemicals

In vivo predictive tests are currently available to identify sensitization potential of chemicals. The guinea pig maximization test (Magnusson and Kligman, 1969), the occluded patch test of Buehler (Buehler, 1985), the mouse ear swelling test (Gad *et al.*, 1986) and the murine local lymph node assay (Kimber *et al.*, 1989) have been used as standard procedures for many years. However, recent regulatory changes have placed a major emphasis on *in vitro* safety testing and alternative models. In regard to skin sensitization tests, DC as key players in the initial process of skin sensitization have been considered in the development of *in vitro* alternatives. Hence, DC derived from $CD34^+$ haematopoietic progenitor cells (De Smedt *et al.*, 2001) and those derived from human donor-derived peripheral blood $CD14^+$ monocytes (Coutant *et al.*, 1999a; Aiba *et al.*, 2003) were consulted. Recently, several studies demonstrated that human myeloid cell lines such as THP-1, U937 and MUTZ-3 are good surrogates for DC (Ashikaga *et al.*, 2002; Azam *et al.*, 2006; Python *et al.*, 2007).

1.5 Monocyte-derived dendritic cells

Blood monocytes are one of the most commonly used precursor cells for generating human DC *in vitro*. In the presence of the cytokines granulocyte/macrophage-colony stimulating factor (GM-CSF) and IL-4 they can be generated to DC after six days of culture (Sallusto and Lanzavecchia, 1994; Bender *et al.*, 1996; Romani *et al.*, 1996) showing little or no proliferate expansion. These *in vitro* generated so-called human monocyte-derived DC (MoDC) are similar to Langerhans cells (Steinbach *et al.*, 1998; Aiba *et al.*, 2000) and are known to respond to various chemicals. Some chemicals such as DNCB, but not irritants such as sodium dodecyl sulfate, caused an alteration of CD86, CD80, HLADR, CD40, CD83, or CD54 surface expression (Aiba *et al.*, 1997; Coutant *et al.*, 1999a). These phenotypic changes are accompanied by cytokine production such as IL-6, TNFalpha, IL-8 and IL-1beta (Aiba *et al.*, 1997; Coutant *et al.*, 1999a; Tuschl and Kovac, 2001). Furthermore, MoDC can stimulate naive T cells to effector cells for hypersensitivity reactions (Cella *et al.*, 1997b).

1.6 The monocytic cell line THP-1 as DC surrogate

Recently, several studies pointed out that a variety of skin sensitizers significantly induce CD54, CD86, CD40 and HLADR expression on the human monocytic leukaemia cell line THP-1, while non-sensitizers do not (Ashikaga *et al.*, 2002; Yoshida *et al.*, 2003; Miyazawa *et al.*, 2007). In addition, THP-1 cells have a high capacity to induce IL-8 and TNFalpha release following antigen treatment. Hence, it was concluded that the features of THP-1 cells after contact with strong allergens reflect *in vitro* allergen-induced DC activation during skin sensitization, and that therefore the establishment of these cells as an *in vitro* skin sensitization test is promising. Due to these results, the human Cell Line Activation Test (h-CLAT) has been developed and optimized (Ashikaga *et al.*, 2006; Sakaguchi *et al.*, 2006). The experimental conditions of the currently established h-CLAT protocol recommend the use of THP-1 cells at growth-sated initial cell concentration and set the appropriate sample treatment time to 24 h.

Recently, similar to what was explored for DC, Miyazawa *et al.* found that the inhibition of p38 MAPK caused a selective inhibition of CD86, CD54 and/or CD40 expression following treatment with DNCB or nickel sulfate in THP-1 cells (Miyazawa *et al.*, 2008a). Thus, it can be assumed that p38 MAPK is involved in the chemical-induced activation of THP-1 cells.

1.7 Aim of this study

Due to the continuously changing environmental conditions, it is necessary to regularly monitor and update the spectrum of contact allergens that elicit contact dermatitis. In particular, due to the widely spread use of 'natural' ingredients in cosmetic and alternative medicine products, allergic contact dermatitis to a number of substances derived from plants was reported recently (Brasch, 2007; Aberer, 2008). Therefore, the prediction of the sensitization potential of chemicals and naturally occurring substances is important. In the context of developing non-animal alternative methods, the use of sensitive *in vitro* assays is needed. Several working groups demonstrated that *in vitro* generated DC and DC surrogates can distinguish between strong allergens and irritants by eliciting different phenotypic and functional cell responses. The nuances of sensitization potential which can be strong, moderate, weak, or mild are difficult to determine and still need more investigations. Thus, it should be evaluated whether *in vitro* test systems are approaches which are sensitive enough to detect even weak allergens and therefore, might be available for assessment of relative potency for the purposes of categorizing chemical allergens. The aim of this study was to explore the immune modulating impact of a presumably weak and naturally occuring allergen in *in vitro* sensitization models. For this purpose, ascaridol, predicted as one of the possible causes for TTO contact dermatitis, was studied and its effects on antigen presenting cells were characterized. As suggested *in vitro* DC models, on the one hand *in vitro* generated DC from primary blood monocytes and on the other hand a continuous monocytic cell line THP-1 were used to explore the sensitization potential of ascaridol.

2. Material

2.1 Cell culture

RPMI 1640 with L-glutamine	PAA, Coelbe, Germany
antibiotic/antimycotic solution (AB):	PAA, Coelbe, Germany
penicillin 10.000 U/ml	
streptomycin sulphate 10 mg/ml	
amphotericin B 25 µg/ml	

2.1.1 THP-1 cell culture

THP-1	'Deutsche Sammlung von Mikroorganismen und Zellkulturen' (DSMZ) GmbH, Braunschweig, Germany
L-glutamine 200 mM	PAA, Coelbe, Germany
foetal bovine serum (FBS) F7524	Sigma, Taufkirchen, Germany
β-mercaptoethanol	Roth, Karlsruhe, Germany
4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES)	Calbiochem, Darmstadt, Germany

phosphate buffered saline (PBS) (10 x):

Na_2HPO_4 x 2 H_20	10 mM	Roth, Karlsruhe, Germany
KH_2PO_4	1.8 mM	Sigma, Karlsruhe, Germany
NaCl	137 mM	Roth, Karlsruhe, Germany
KCl	2.7 mM	Roth, Karlsruhe, Germany

pH 7.4
ad 1 l aqua deion.

2.1.2 Monocyte-derived dendritic cell (MoDC) generation and cell culture

buffy coat	kindly provided by blood bank of University Hospital Aachen, Germany
foetal bovine serum Gold (FBS Gold)	PAA, Coelbe, Germany
interleukin-4 (IL-4)	Peprotech, Hamburg, Germany
granulocyte/macrophage-colony stimulating factor (GM-CSF)	Peprotech, Hamburg, Germany
Ficoll-Paque™ PLUS	GE Healthcare, Uppsala, Sweden

PBS	PAA, Coelbe, Germany
bovine serum albumin (BSA), endotoxin free	PAA, Coelbe, Germany
monocyte negative isolation kit	Dynal® Biotech, Hamburg, Germany

2.2 Chemicals and reagents

ascaridol (purity ≥ 99.8 %)	extraction from American worm seed by Dr. M. Neubauer and Dr. R. Mayer, Institute of Pharmacology, University of Bonn, Germany
dimethylsulfoxide (DMSO) (purity ≥ 99.5 %)	Roth, Karlsruhe, Germany
2,4-dinitrochlorbenzene (DNCB) (purity ≥ 99 %)	Sigma, Taufkirchen, Germany
fluorescein isothiocyanate-dextran FD-40	Sigma, Taufkirchen, Germany
lipopolysaccharide, E.Coli (LPS)	Sigma, Taufkirchen, Germany
polymyxin B sulfate	Sigma, Taufkirchen, Germany

2.3 Flow cytometry analysis

Table 1: Overview of all used fluorochrome-labeled antibodies.

antibody	format	clone	isotype
CD1a	FITC	HI 149	mouse IgG_1, kappa
CD11c	PE	B-ly6	mouse IgG_1, kappa
CD184 (CXCR4)	PE-Cy5	12G5	mouse IgG_{2a}, kappa
CD3	FITC	UCHT1	mouse IgG_1, kappa
CD40	FITC	5C3	mouse IgG_1, kappa
CD45/CD14	FITC/PE	2D1, МΦР9	mouse IgG_1/IgG_{2a}
CD54 (ICAM-1)	APC/PE	HA58	mouse IgG_1, kappa
CD80	FITC/PE	L307.4	mouse IgG_1, kappa
CD86	FITC/PE	2331 (FUN-1)	mouse IgG_1, kappa
HLADR	FITC	G46-6 (L243)	mouse IgG_{2a}, kappa
CD86/CD209/CD83	CD86 PE	FUN-1	mouse IgG_1, kappa
	CD209 PerCyp-Cy5.5	DCN146	mouse IgG_{2b}, kappa
	CD83 APC	HB15e	mouse IgG_1, kappa

Except CD45/CD14, which were purchased from R&D Systems (Bad Nauheim, Germany), all antibodies including corresponding isotype controls were acquired from Becton Dickinson, (Heidelberg, Germany). Propidium iodide was purchased from Sigma (Taufkirchen, Germany). Further chemicals and solution used for flow cytometry like PBS, FBS, or BSA were purchased from PAA (Coelbe, Germany).

2.4 Cytokine measurement

IL-12p40 ELISA kit, Quantikine	R&D Systems, Bad Nauheim, Germany
Human IL-8 ELISA kit II	BD OptEIA™, Heidelberg, Germany
Human TNF ELISA kit II	BD OptEIA™, Heidelberg, Germany
Cytometric Bead Array (CBA)	Becton Dickinson, Heidelberg, Germany

2.5 Cell lysis

lysis buffer complete lysis-M, EDTA-free	Roche Diagnostics, Mannheim, German
Protease Inhibitor Cocktail Tablets, complete Mini, EDTA-free	Roche Diagnostics, Mannheim, Germany
Bradford Reagent	Sigma, Taufkirchen, Germany

2.6 SDS-PAGE

XCell Novex SureLock™ Mini-Cell	Invitrogen, Karlsruhe, Germany
NuPAGE® Novex Bis Tris gradient gel	Invitrogen, Karlsruhe, Germany
NuPAGE® Sample Reducing Agent (10 x)	Invitrogen, Karlsruhe, Germany
NuPAGE® LDS Sample Buffer (4 x)	Invitrogen, Karlsruhe, Germany
PageRuler™, Prestained Protein Ladder	Fermentas, St.Leon-Rot, Germany
MagicMark™ XP Western Protein Standard	Invitrogen, Karlsruhe, Germany
3-(n-morpholino)-propane-sulfonic acid (MOPS) buffer (20 x)	Invitrogen, Karlsruhe, Germany
Ponceau-S	Sigma, Taufkirchen, Germany

2.7 Western Blot

Mini Trans-Blot cell	Bio-Rad, Munich, Germany
PVDF membrane	Amersham Hybond-P, Buckinghamshire, UK
Blotting Grade Blocker Non-Fat Dry Milk	Bio-Rad, Munich, Germany

phospho-p38 MAP kinase antibody		Cell Signaling Technology®, New England Biolabs GmbH, Frankfurt a. M., Germany
p38 MAP kinase antibody		Cell Signaling Technology®, New England Biolabs GmbH, Frankfurt a. M., Germany
horseradish peroxidase (HRP)-linked bovine anti-rabbit IgG		Santa Cruz Biotechnology Inc. Heidelberg, Germany

transfer buffer (20 x):

Tris	24.22 g	ICN Biomedical Inc., Aurora, USA
glycine	150.14 g	Biorad, Munich, Germany
ad 1 l with aqua deion. (storage at 4 °C)		

transfer buffer ready to use (1 x):

transfer buffer (20 x)	50 ml	
methanol	100 ml	Roth, Karlsruhe, Germany
ad 1 l with aqua deion.		

tris buffered saline (TBS) (10 x):

Tris - HCl pH 8	100 mM	ICN Biomedical Inc., Aurora, USA
NaCl	1500 mM	Roth, Karlsruhe, Germany

TBS with Tween 20 (TBST):

1 x TBS	
1 % (v/v) Tween 20	Fluka, Steinheim, Germany

TBS with Tween 20 and Triton X (TBSTT):

1 x TBST	
1 % (v/v) Triton X 100	Roth, Karlsruhe, Germany

2.8 Consumable materials

Consumable materials were purchased from Sarstedt (Nuembrecht, Germany), Applied Biosystems (Darmstadt, Germany), BD Biosciences (Heidelberg, Germany), and Roche Diagnostics (Mannheim, Germany) if not otherwise specified.

2.9 Equipment

centrifuge	Eppendorf Centrifuge 5417R (Hamburg, Germany)
centrifuge	Thermo Fisher Scientific Heraeus Multifuge 1 S-R (Karlsruhe, Germany)
clean bench	Thermo Fisher Scientific Heraeus Hera Safe (Karlsruhe, Germany)
flow cytometer	Becton Dickinson FACSCalibur (Heidelberg, Germany)
incubator	Thermo Fisher Scientific Heraeus cell 240 (Karlsruhe, Germany)
microscope	Carl Zeiss Axiovert 40 CFL (Offenbach,Germany)
power supply	Biometra PowerPack P25 (Goettingen, Germany)
spectrophotometer	Bio-Tek Synergy HT (Bad Friedrichshall, Germany)
shaker	Buehler SM 25 (Hechingen, Germany)
thermomixer	Eppendorf thermomixer compact (Hamburg, Germany)
ultrasonicator, homogenizer	Ultraschallprozessor UP 50H dr hielscher GmbH (Teltow, Berlin, Germany)
vortexer	Labnet VX100 (Windsor, Berkshire, United Kingdom)

3. Methods

3.1 THP-1 cell culture and cryopreservation

THP-1 cells were cultured in RPMI 1640 medium supplemented with L-glutamine (final concentration 4 mM), 10 % FBS, 1 % antibiotic/antimycotic solution (AB) (10 U/ml penicillin, 100 µg/ml streptomycin, 0.25 µg/ml amphotericin B), 50 µM β-mercaptoethanol and 25 mM HEPES as further described (Yoshida *et al.*, 2003). Cells were maintained under standard culture conditions at a temperature of 37 °C in 5 % humidified CO_2. Cell densities of 0.1 till 0.5 cells/ml were applied for subculture. Passage numbers 4 to 12 were used for experiments.

For cryopreservation, THP-1 cells (10×10^6 /ml) were frozen in FBS containing 10 % dimethylsulfoxide (DMSO) in cryogenic vials and placed in an isopropanol freezing container over night at -80 °C. Finally, for long-term storage frozen cells were preserved in liquid nitrogen.

3.2 MoDC generation and cell culture

Human peripheral blood-derived mononuclear cells (PBMC) were isolated from buffy coats of healthy donors by Ficoll-Paque density gradient centrifugation and then washed three times with phosphate buffered saline (PBS). Afterwards, PBMC (6×10^6 cells/ml) were cultured for 60 min in RPMI 1640 supplemented with 10 % FBS at 37 °C in order to allow monocyte to adhere on the plastic bottom of Petri dishes as described (Hausser *et al.*, 1997). Non-adherent cells were aspirated and the monocyte enriched fraction was further purified using a monocyte negative isolation kit according to manufacturer's instructions. Briefly, the enriched monocyte fraction was incubated with blocking reagent (containing gamma globulin for blocking unwanted bindings of the antibodies) and a mixture of monoclonal antibodies against the non-monocytes. Through adding Dynabead®, which binds to the non-monocytes, the monocytes were gained by removing the bead-bound cells with a magnet. The monocyte fraction was finally cultured in six-well plates (3×10^6 cells/well) with RPMI 1640 medium containing 10 % FBS, 1 % AB including 800 U/ml granulocyte/macrophage-colony stimulating factor (GM-CSF) and 1000 U/ml interleukin (IL)-4 for six days. One third of the medium was replaced every second day by fresh culture medium containing 1600 U/ml GM-CSF and 1000 U/ml IL-4. The differentiation of monocytes to MoDC was monitored by measuring the diminution of the monocyte marker CD14 via flow cytometry. Only cells, which down-regulated CD14 by more than 50 % between culture day 2 and 4, were used for experiments. The resulting cell preparations on culture day six were consistently ≥ 97 % $CD11c^+$; ≥ 80 % $CD1a^+$; ≤ 20 % $CD14^+$ and less than 2 % $CD3^+$. The cells were characteristically dendritic in

appearance and non-adherent in the culture dish. These immature MoDC were resuspended as 1 x 10^6 cells/ml in freshly supplemented culture media and used for experiments.

3.3 Chemical treatment

Both MoDC and THP-1 cells were seeded 1 x 10^6 cells/ml and exposed selectively to 2,4-dinitrochlorobenzene (DNCB, 5 µM), lipopolysaccharide (LPS, 0.1 µg/ml), polymyxin B (10 µg/ml) or various concentrations of ascaridol (10 - 120 µg/ml). Ascaridol, LPS and polymyxin B were solubilised in medium. DNCB was dissolved in dimethylsulfoxide (DMSO). The final concentration of DMSO in culture media was less than 0.1 %, and therefore 0.1 % DMSO was used as a vehicle control for DNCB.

3.4 Assessment of cell viability

Cell viability was assessed flow cytometrically using propidium iodide (PI). PI is a non-permeant dye that penetrates membranes of dying/dead cells. It intercalates into the major groove of the DNA and produces a highly fluorescent adduct so that non-viable cells can be identified by positive red fluorescence (Bedner *et al.*, 1999). In order to study cell viability, cells (1 x 10^6 cells/ml) were incubated with different concentrations of ascaridol for 24 h. Further on, cells were collected,washed twice with PBS and then stained with PI at a final concentration of 1 µg/ml. Apoptotic and necrotic cells were determined via flow cytometry within 5 min after PI addition. Total counting events for living cells were 10.000. The percentage of cell viability was calculated.

3.5 Immunophenotypic analysis

After chemical treatment of MoDC or THP-1 cells for 24 h or 96 h as described above (cf. 3.3), cells were collected, washed twice, resuspended in PBS and incubated with fluorochrome-conjugated mouse antibodies or respective isotype-matched controls at the ratio of 1:10 for 20 min at room temperature. After a washing step to remove excess antibodies samples were resuspended in PBS and analyzed using a FACSCalibur. Cells were electronically gated according to their light-scatter properties to exclude cell debris. As dead cells can introduce analytical errors arising from (1) non-specific uptake of fluorescent probes leading to erroneous percentages of positive labelled cells, (2) increased autofluorescence, and (3) altered antigen expression, they were excluded from analysis via PI staining as described in 3.4. Total counting events for living cells were 5000 for MoDC and 10.000 for THP-1. Both the percentage of stained cells and the (geometric) mean fluorescence intensity (MFI) of stained cells were determined by using the Cell Quest Pro™ software (BD Biosciences, Hei-

delberg, Germany). The relative MFI (rMFI) was calculated by subtracting the MFI of the corresponding isotype control.

In order to compare the results from h-CLAT assay (cf. 1.6) in this study with published data, the relative fluorescence intensity (RFI) was also calculated as described in (Sakaguchi *et al.*, 2007). An overview of all used equations for FACS analysis is provided in table 2.

Table 2: Formula used for FACS analysis.

relative Mean Fluorescence Intensity (rMFI)	$rMFI = MFI_{chemical-treated\ cells} - MFI_{corresponding\ isotype\ control}$
Relative Fluorescence Intensity (RFI)	$RFI = \frac{MFI_{chemical-treated\ cells} - MFI_{corresponding\ isotype\ control}}{MFI_{vehicle\ control} - MFI_{corresponding\ isotype\ control}} \cdot 100$

3.6 Antigen uptake assay

MoDC (1 x 10^6 cells/ml) were incubated in the presence or absence of 10 µg/ml ascaridol for 24 h and 96 h. Subsequently, after collecting and washing, cells were resuspended in 0.2 % BSA-PBS and incubated with FITC-labelled dextran (m_r 40. 000) at a final concentration of 1 mg/ml for 60 min at 4 and 37 °C. At 4 °C, cell metabolism is inhibited and MoDC are incapable of capturing antigens. The background of FITC-labelled dextran uptake can be determined. The incubation at 37 °C affords condition for antigen uptake. After thorough washing of cells with cold 0.2 % BSA-PBS, fluorescence was measured by flow cytometry and analysis of the dextran uptake was performed with Cell Quest Pro™ software. Difference between the mean fluorescence intensity (MFI) of samples incubated with FITC-labelled dextran at 37 °C and MFI of samples maintained at 4 °C was calculated.

3.7 Cytokine detection

Concentrations of the cytokines IL-1beta, IL-6, IL-10, tumor necrosis factor(TNF)alpha and IL-12p75 secreted by MoDC in cell culture supernatants were evaluated with the BD™ Cytometric Bead Array (CBA) human inflammatory kit in accordance with the manufacturer's protocol. In addition, culture supernatants were also analyzed via Enzyme-linked Immunosorbent Assay (ELISA) for IL-12p40 and IL-8. THP-1 culture supernatants were analyzed via ELISA for IL-8 and TNFalpha, according to the manufacturer's instructions. Therefore,

treated cells were collected, centrifuged (245 x g, 10 min for MoDC and 300 x g, 5 min for THP-1 cells) and supernatants were immediately stored at -80 °C for subsequent analysis.

3.8 Preparation of cell lysates

THP-1 cells (1 x 10^6 cells/ml) were cultured in 25 cm^2 flasks and incubated with ascaridol as described in 3.3 for 24 h. After collecting and centrifuging cells with ice cold PBS and washing them twice, cells were lysed with lysis buffer complete lysis-M EDTA-free containing protease inhibitors and sonicated 4 x 8 s on ice. Clear lysates were prepared by centrifugation at 20.000 x g for 10 min at 4 °C. Protein content was determined by using Bradford Reagent according to the manufacturer's instructions.

3.9 SDS-PAGE

Separation of proteins on the basis of their molecular weights was performed by discontinuous sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using pre-cast gradient Bis Tris SDS-PAGE gels 4 - 12 %. Dissolved protein (50 µg) was mixed with sample buffer at a ratio of 3:1 (NuPAGE® LDS Sample Buffer (4 x)) including reducing agent (NuPAGE® Reducing Agent (10 x)), heated at 70 °C for 10 min, and loaded onto the gel. Voltage was constantly at 200 V.

3.10 Western blot

Following discontinuous SDS-PAGE, the proteins were electrophoretically transferred to a polyvinylidene fluoride (PVDF) membrane at a constant electric current of 400 mA for 2 h by using a wet blot system. After transfer, membranes were stained with Ponceau-S according to the manufacturer's instructions to ensure equal protein loading and document transfer efficiency. Subsequently, membranes were blocked with 5 % non-fat dry milk in TBST for 60 min at ambient temperature. Further on, membranes were incubated with the primary antibody over night by gently shaking at 4 °C in 5 % BSA-TBST (1:1000 dilution). After washing membranes three times in TBSTT and once in TBST, the blots were incubated for 60 min with the appropriate peroxidase-conjugated secondary antibody. Membranes were washed four times in TBST, once in TBS, and developed with SuperSignal® West Pico Chemiluminescent Substrate according to the manufacturer´s instructions. Antibody binding to protein was analyzed by using the Lumi-Imager from Roche Diagnostics (Mannheim, Germany) and the software LumiAnalyst.

3.11 Statistical analysis

All data are presented as means ± standard error of mean (SEM). Differences between two groups were evaluated by a paired two tailed Wilcoxon test, using the SPSS® version 15.0 software (Chicago, USA). P-values of $p \leq 0.05$ (*) and $p \leq 0.005$ (**) were considered as statistically significant.

4. Results

4.1 Preliminary study on cell viability

In order to exclude unspecific effects of ascaridol, a dose-response study was performed by analyzing cell viability. Therefore, monocyte-derived dendritic cells (MoDC) were exposed to various concentrations of ascaridol (1 - 50 µg/ml) for 24 h and analyzed after propidium iodide (PI) staining. In general, a tested concentration of chemicals was considered as non-cytotoxic, when at least 75 % of cells were viable (Toebak *et al.*, 2006). In this way it was observed that concentrations of 1, 5 and 10 µg/ml ascaridol yielded no effects on the cell viability (85 ± 5 %), while higher concentrations reduced cell viability down to less than 75 % (see figure 4; n = 3). A concentration of 20 µg/ml ascaridol resulted in a cell viability of approximately 60 % (n = 3). Thus, 10 µg/ml ascaridol was regarded as the maximum non-cytotoxic dose, and was chosen for further experiments.

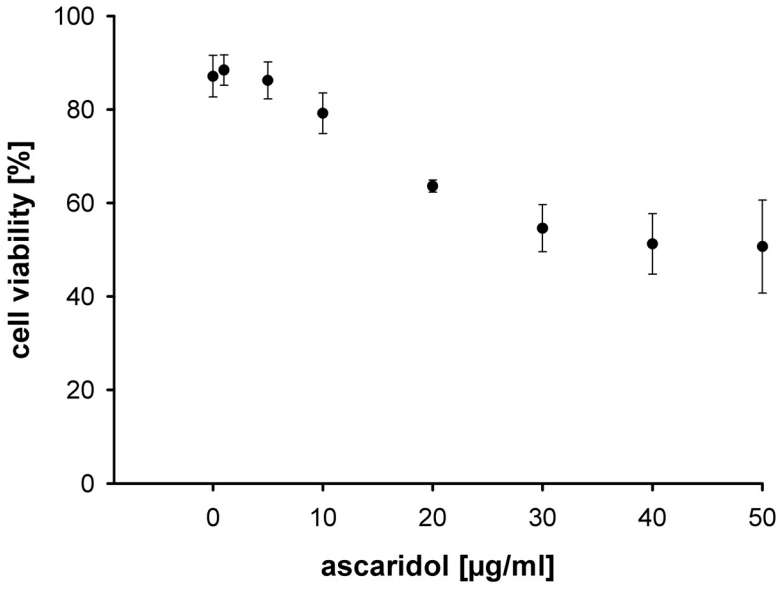

Figure 4: Effects of ascaridol on the viability of MoDC.

MoDC were incubated with different concentrations of ascaridol (1 - 50 µg/ml) for 24 h. Cells were stained with propidium iodide and the percentage of dead/dying cells was determined by flow cytometry. The cell viability was calculated. Values shown are the mean ± SEM of three independent experiments, with $p > 0.05$.

In case of THP-1, cells were exposed to 10 - 160 µg/ml ascaridol and the cell viability was also determined via PI flow cytometry analysis. As shown in figure 5, concentrations between 10 and 120 µg/ml ascaridol did not impact cell viability, which was 75 - 100 %. A concentration of 140 µg/ml ascaridol decreased cell viability down to 70 ± 8 %, and 160 µg/ml ascaridol resulted in a cell viability of approximately 59 ± 14 %. Therefore, the highest non-cytotoxic concentration for further experiments was set to 120 µg/ml ascaridol, for which 74 ± 7 % cell viability was obtained (n = 9).

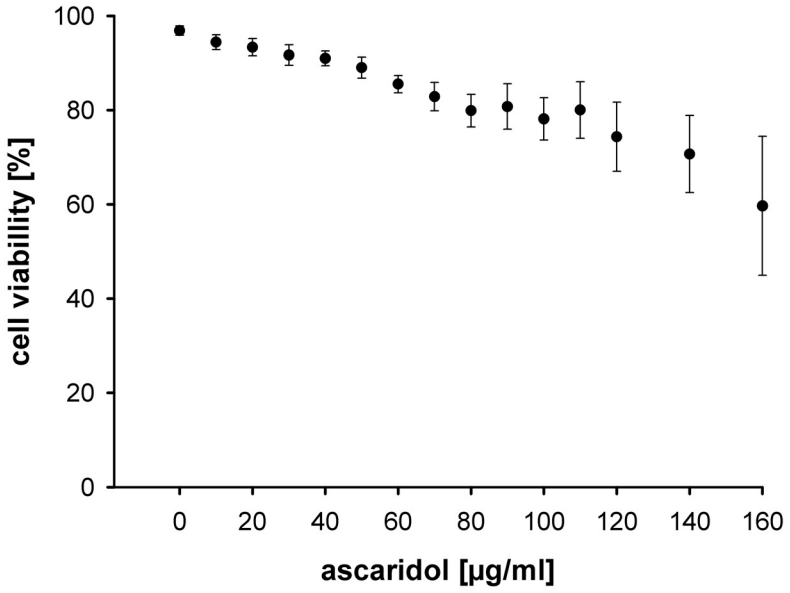

Figure 5: Effects of ascaridol on the viability of THP-1 cells.

THP-1 cells were incubated with different concentrations of ascaridol (10 - 160 µg/ml) for 24 h. Cells were stained with propidium iodide and the percentage of dead/dying cells was determined by flow cytometry. The cell viability was calculated. Values shown are the mean ± SEM of nine independent experiments, with $p > 0.05$.

4.2 Impact of ascaridol on CD86, CD80, HLADR, and CD83 surface expression of MoDC

MoDC are known to respond to a variety of chemical allergens by up-regulation of several co-stimulatory molecules. It has been postulated that ascertainment of this effect might provide the basis for an *in vitro* approach for the identification of skin sensitizing chemicals (Hulette *et al.*, 2005). Therefore, MoDC from nine healthy donors were exposed to either medium alone or 10 µg/ml ascaridol for 24 h and 96 h. Lipopolysaccharide (LPS, 0.1 µg/ml) was used as a positive control for activation in order to assess the dynamic range of expression (Gosset *et al.*, 2003). CD86, CD80, HLADR and CD83 surface expression was analyzed via flow cytometry. Figure 6 shows data from one representative experiment for each exposure point in time.

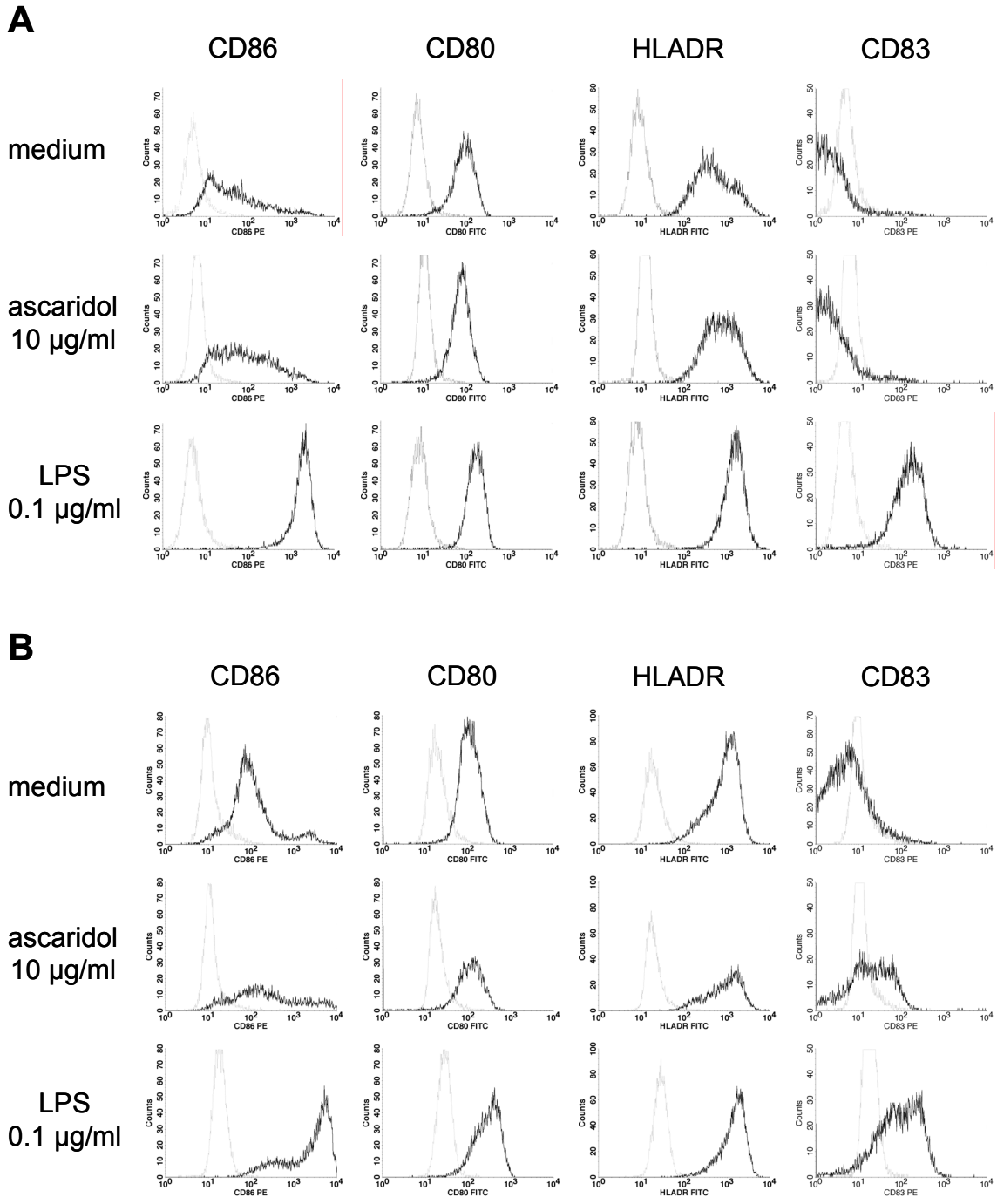

Figure 6: Representative histograms of CD86, CD80, HLADR, and CD83 surface expressions on MoDC after treatment with ascaridol for 24 and 96 h.

MoDC were treated with medium alone (negative control), 10 µg/ml ascaridol or 0.1 µg/ml LPS (positive control) for 24 h (panel A) and 96 h (panel B). Surface expression of CD86, CD80, HLADR, and CD83 were analyzed via flow cytometry. Black opened histograms indicate stained cells, grey histograms corresponding isotype controls. Histograms of one representative experiment for each incubation time are shown.

Figure 7 gives an overview of the obtained results. After 24 h incubation LPS consistently induced the expression of CD86, CD80, HLADR as well as CD83 in all experiments, whereas ascaridol treatment showed a different pattern. Compared to non-treated MoDC, ascaridol induced a significant increase of the relative mean fluorescence intensity (rMFI) for CD86 in six of nine healthy donors after 24 h. The rMFI-values for ascaridol varied between 83 and 280 and for non-stimulated cells between 54 and 255 depending on the donor (figure 7A). The CD86 expressing cell population increased only slightly after ascaridol treatment compared to the control (6 - 14 %). These six CD86 responsive donors were further analyzed for surface expression of CD80, HLADR and CD83.

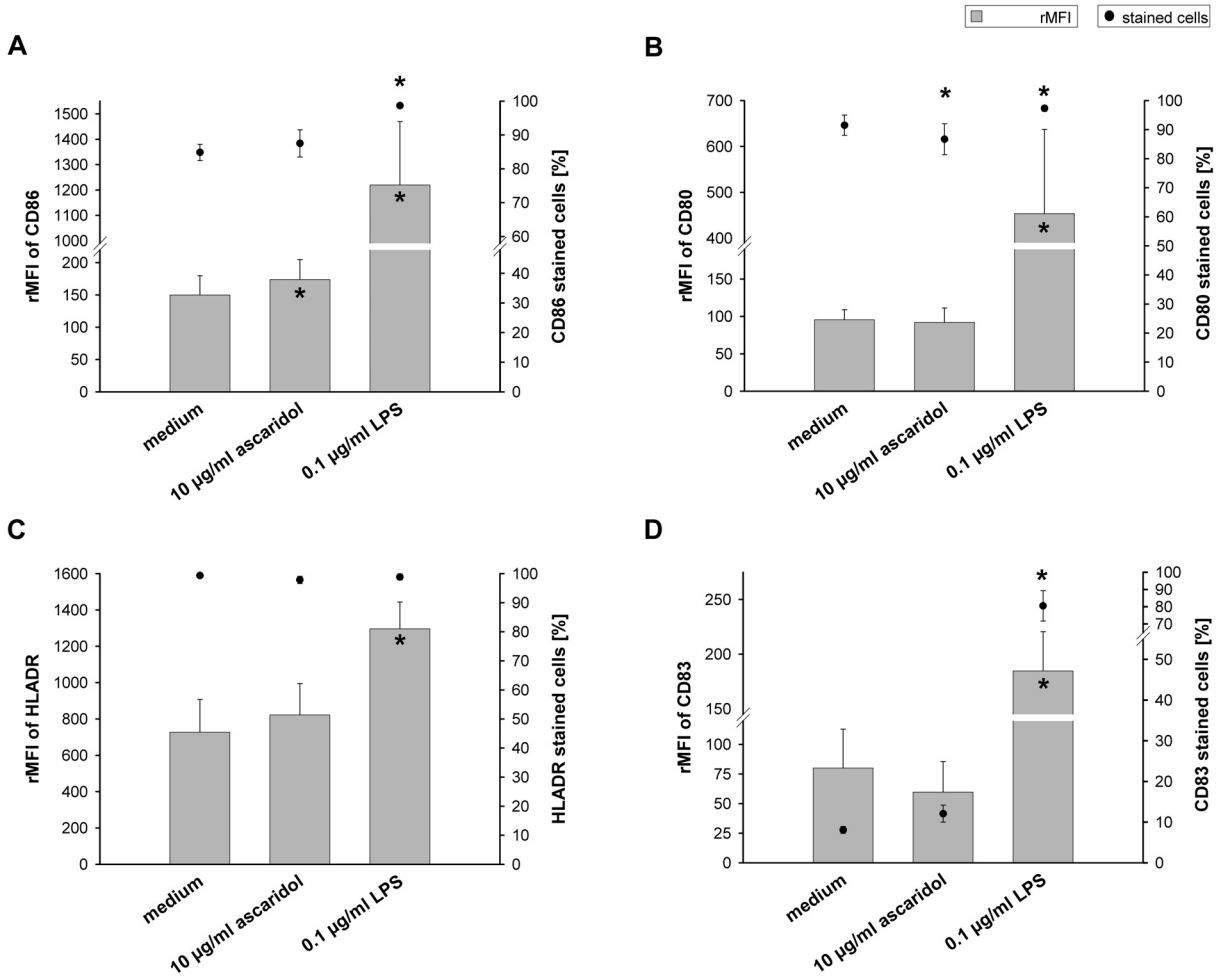

Figure 7: Surface expression analysis of MoDC treated with ascaridol for 24 h.

Surface expression of CD86 (panel A), CD80 (panel B), HLADR (panel C), and CD83 (panel D) was determined as relative mean fluorescence intensity (rMFI) via flow cytometry after treatment with 10 µg/ml ascaridol, 0.1 µg/ml LPS (positive control) or medium alone for 24 h. Bars represent the rMFI mean value ± SEM of six independent experiments for each panel. Scatters represent the mean values ± SEM of the stained cell population in percent (%). Statistical significances between chemical-treated cells and vehicle control cells are quoted as asterixes. Asterixes placed within bars indicate statistical significance for rMFI values, while asterixes above scatters indicate statistical significance of mean values for percentage of positive stained cells. $P \leq 0.05$ (*).

Figure 7B shows that no changes of CD80 rMFI after 24 h ascaridol stimulation was found, even the percentage of CD80 expressing cells was decreased significantly. A moderate rise of the rMFI values for HLADR was determined (figure 7C). Four of six donors showed an increased rMFI for HLADR after ascaridol treatment with values between 395 and 1535 compared to 278 and 610 in the absence of ascaridol. In addition, the percentages of CD83 stained cells increased from 3 to 10 % after ascaridol exposure in three of six donors, while the rMFIs were not enhanced, rather decreased (figure 7D).

Figure 8 represents the obtained results of surface expression analysis on MoDC via flow cytometry after 96 h of stimulation.

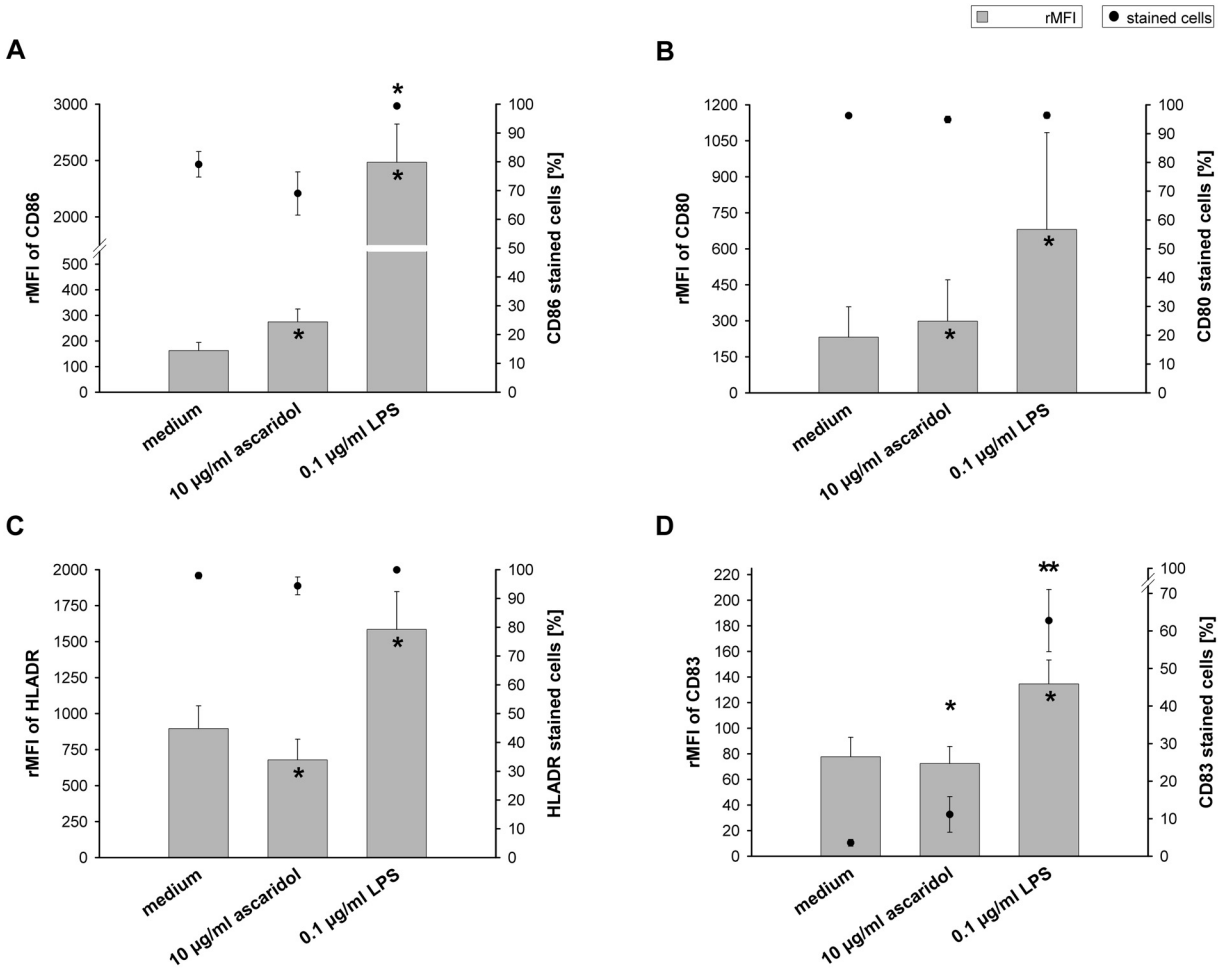

Figure 8: Surface expression analysis of MoDC treated with ascaridol for 96 h.

Surface expression of CD86 (panel A), CD80 (panel B), HLADR (panel C), and CD83 (panel D) was determined as relative mean fluorescence intensity (rMFI) via flow cytometry after treatment with 10 µg/ml ascaridol, 0.1 µg/ml LPS (positive control) or medium alone for 96 h. Bars represent the rMFI mean value ± SEM of nine independent experiments for each panel. Scatters represent the mean values ± SEM of the stained cell population in percent (%). Statistical significances between chemical-treated cells and vehicle control cells are quoted as asterixes. Asterixes placed within bars indicate statistical significance for rMFI values, while asterixes above scatters indicate statistical significance of mean values for percentage of positive stained cells. $P \leq 0.05$ (*) and $p \leq 0.005$ (**).

After 96 h incubation with ascaridol the expression of CD86 on the surface of MoDC was still significantly augmented in all independent experiments with rMFI values ranging from 122 to 541 compared to 93 and 464 for non-stimulated cells (see figure 8A). The percentage of CD86 expressing cells was lower after ascaridol treatment in comparison to medium alone. For CD80 expression a moderate increase of the rMFI values (93 - 180 in treated compared to 56 - 128 in non-treated cells) was observed after 96 h by almost equal percentages of stained cells (figure 8B). The rMFI value of HLADR after ascaridol stimulation was significantly decreased after 96 h. rMFI values from 227 to 1412 were calculated, while for non-stimulated cells HLADR rMFI values ranging from 388 to 1719 were found (figure 8C). Even the HLADR stained cell population of ascaridol-treated MoDC of two donors was reduced to approximately 10 and 30 % compared to medium-control. In case of CD83, six of nine experiments yielded a significant increase in the percentage of CD83 stained cells after exposure to ascaridol from 5 to 37 %, although differences in rMFI could not be observed (see figure 8D).

4.2.1 Exclusion of endotoxin contamination

During the process of MoDC generation, recombinant proteins like GM-CSF or IL-4 were added to the culture media as described in section 2.1.2. These proteins were obtained from bacterial systems or protein extracts of ectoparasites. In order to exclude that LPS contaminations due to these proteins are responsible for the observed effects of ascaridol on MoDC, in some experiments polymyxin B was added to experimental approaches. Polymyxin B is a well known and potent antibiotic that binds and neutralizes bacterial endotoxin (LPS) (Cuellar *et al.*, 2004). Figure 9 shows that polymyxin B itself did not alter the surface expression of CD86, CD80 and HLADR on MoDC neither after 24 h (panel A) nor after 96 h (panel B). LPS alone increased the CD86, CD80 and HLADR expression at each investigated point in time, whereas the co-incubation of LPS with polymyxin B resulted in expression levels of all determined surface markers similar to those which were obtained for polymyxin B alone. In contrast, polymyxin B did not prevent the ascaridol-mediated increased CD86 and HLADR expression after 24 h (figure 9A). Further on, after 96 h (figure 9B) the co-incubation of ascaridol and polymyxin B resulted in enhanced CD86 and CD80 expression and decreased HLADR expression compared to only polymyxin B-treated cells. Thus, similar results for the treatment with ascaridol alone and the co-incubation of ascaridol with polymyxin B were obtained. Therefore, endotoxin contamination could be excluded.

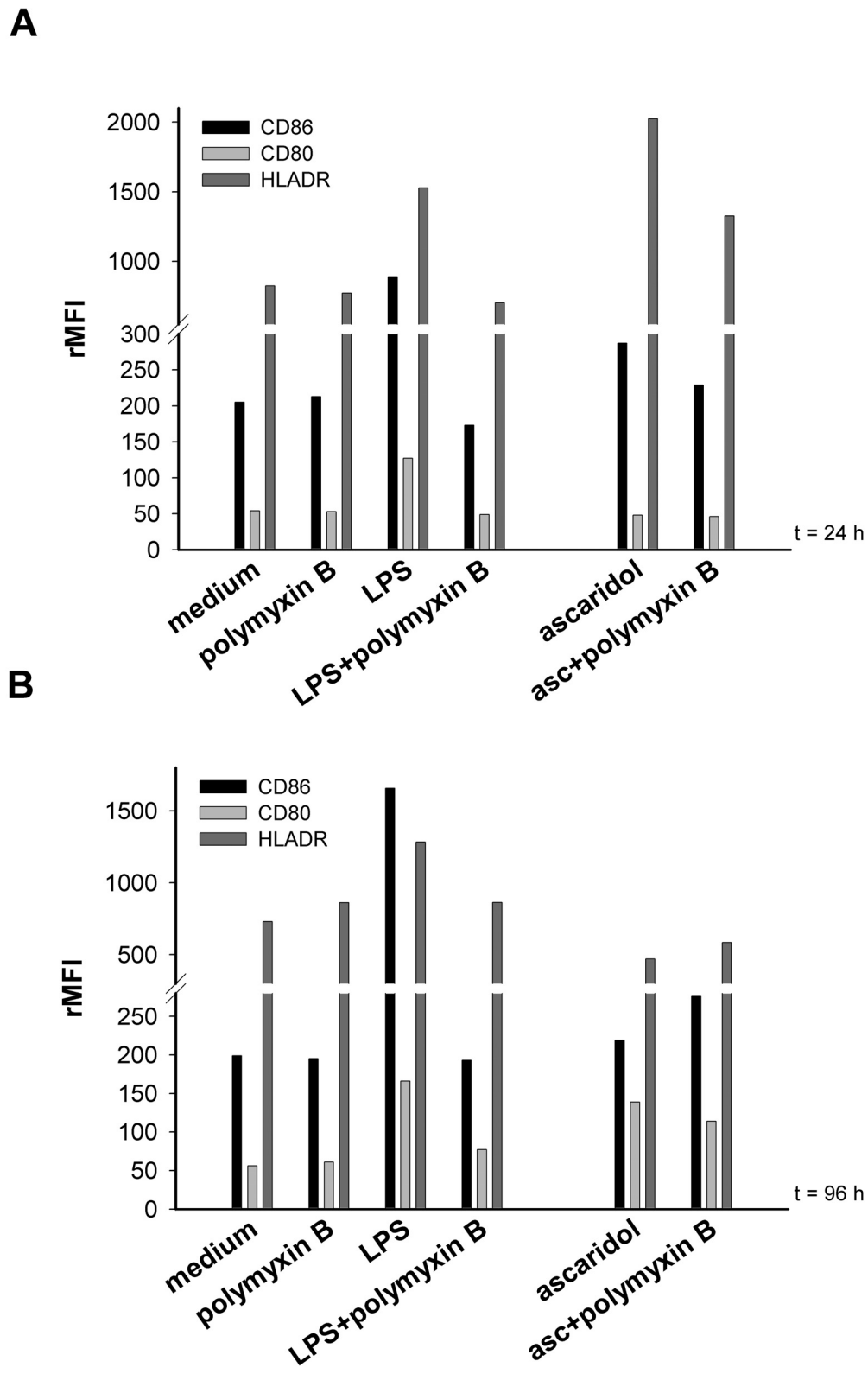

Figure 9: Co-incubation of ascaridol- or LPS-treated MoDC with polymyxin B for 24 and 96 h.

Surface expression of CD86, HLADR and CD80 of MoDC cultured in medium alone or in the presence of either 10 µg/ml polymyxin B or 10 µg/ml ascaridol (asc) or both for 24 h (panel A) or 96 h (panel B) was determined via flow cytometry. MoDC were also treated with 0.1 µg/ml LPS or LPS with polymyxin B as positive control. One representative experiment of two is shown for each incubation period.

4.3 Impact of ascaridol on CD54 and CD40 surface expression of MoDC

It has been found that some contact allergens are able to increase the expression of CD54 (Aiba *et al.*, 1997; Tuschl *et al.*, 2000) and CD40 (Coulter *et al.*, 2007). Therefore, we investigated both markers on MoDC after treatment with ascaridol for 24 h and 96 h. We only studied MoDC of donors who showed a clear response to LPS (i.e. augmented CD86, CD80 and HLADR expression) and an increased expression of CD86 after 24 h ascaridol stimulation. Ultimately, for CD54, MoDC from eight healthy donors were analyzed via flow cytometry. Figure 10 presents the results after 24 h (panel A) and 96 h (panel B) incubation period.

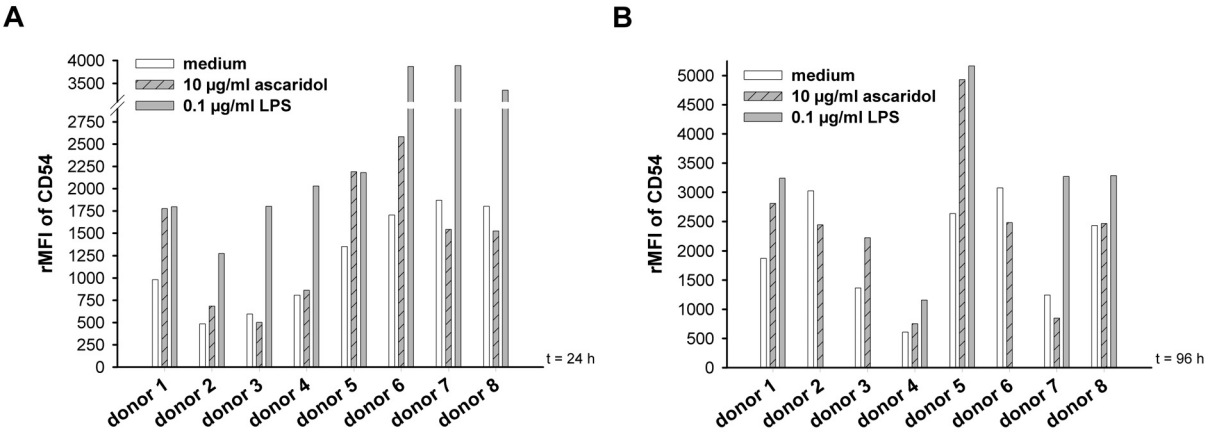

Figure 10: CD54 surface expression of ascaridol-treated MoDC.

CD54 surface expression of MoDC cultured in medium alone or in the presence of 10 µg/ml ascaridol was determined via flow cytometry. As positive control MoDC were also treated with 0.1 µg/ml LPS as indicated. Panel A shows results after 24 h incubation and panel B after 96 h. White bars show results of non-treated cells (medium), grey pattern bars display results for ascaridol-treated MoDC, while grey bars show results of LPS-treated cells. Values of relative mean fluorescence intensity (rMFI) of eight independent experiments are given.

While LPS augmented CD54 expression after 24 h in all investigated cases, the response after 96 h of LPS treatment was not determined for all experiments due to limited cell availability. However, those measured showed clear augmented CD54 expression as expected. Exposure with ascaridol revealed no clear pattern. After 24 h treatment five of eight donors showed an increase of CD54 expression - four explicitly (donor 1, 2, 5, 6), one rather moderately (donor 4). Ascaridol exposure for 96 h resulted in augmented CD54 expression on MoDC from four donors (donor 1, 3, 4, 5), but not the identical donors as named above. By comparing CD54 expression after 24 h with CD54 expression after 96 h, three of eight donors showed augmented CD54 at both point in time (donor 1, 4, 5), while in two experiments an increased CD54 expression was detected only after 24 h, but not after 96 h (donor 2, 6). One donor (donor 3) displayed an augmented CD54 expression after 96 h, but not after 24 h.

Overall, independent from treatment, all analyzed cells expressed CD54 and therefore no differences in the percentages of CD54 stained cells were observed (data not shown).

Figure 11 presents the obtained results after 24 h (panel A) and 96 h (panel B) incubation period in case of CD40 expression. LPS increased the CD40 expression on MoDC of all studied donors at each measured time except donor 5 at 24 h. Ascaridol did not increase the surface expression of CD40 after 24 h, in contrast MoDC from five of six donors showed a clear decrease of CD40 expression (donor 1, 3, 4, 5, 6). After 96 h ascaridol treatment of MoDC an increase of CD40 expression was determined in all donors studied.

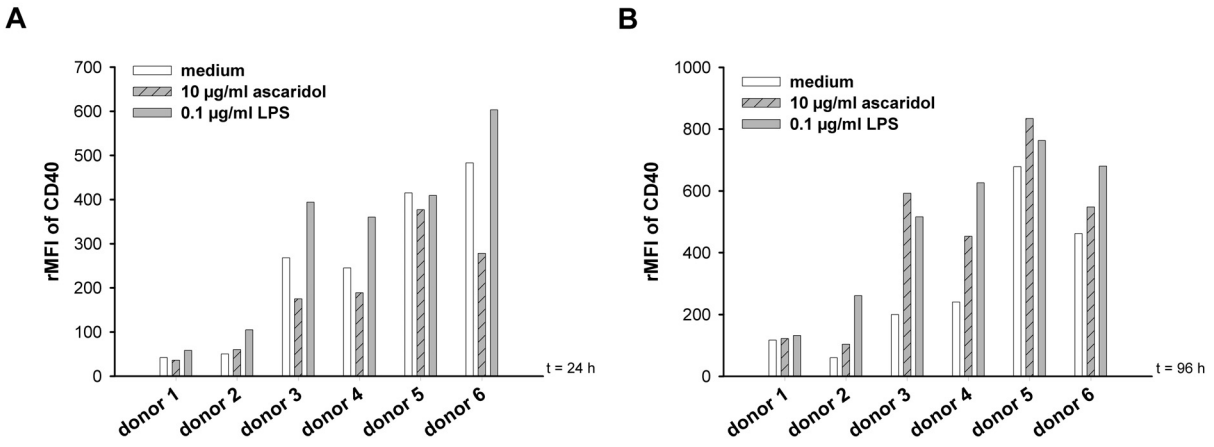

Figure 11: CD40 surface expression of ascaridol-treated MoDC.

CD40 surface expression of MoDC cultured in medium alone or in the presence of 10 µg/ml ascaridol was determined via flow cytometry. As positive control MoDC were also treated with 0.1 µg/ml LPS. Panel A shows the results after 24 h incubation and panel B after 96 h. White bars show results of non-treated cells (medium), grey pattern bars display results for ascaridol-treated MoDC, while grey bars show results of LPS-treated cells. Values of relative mean fluorescence intensity (rMFI) of six independent experiments are given.

4.4 Impact of ascaridol on CD209 surface expression of MoDC

In another series of experiments MoDC were analyzed to their CD209 (DC-SIGN) expression after ascaridol treatment. CD209, which mediates both DC trafficking (through binding to ICAM-2) and the DC - T cell interactions during the generation of an immune response (through binding to ICAM-3), is abundantly expressed by DC both *in vitro* and *in vivo* (Bleijs *et al.*, 2001). In agreement, CD209 was consistently expressed on all tested MoDC independently from treatment at both investigated points in time. Therefore, only differences in CD209 rMFI were further analyzed. In addition, CD209 expression was only analyzed when ascaridol and LPS had augmented CD86 or CD83 expression as shown above (cf. 4.2). Figure 12 shows that after 24 h MoDC from five of six donors reduced the expression of CD209 in response to ascaridol treatment (donor 1, 3, 4, 5, 6), whereas after 96 h the surface ex-

pression of CD209 was increased clearly on MoDC from all investigated donors. LPS which binds to CD209 (Zhang *et al.*, 2006) and thereby can provoke its internalisation, reduced the CD209 expression (Relloso *et al.*, 2002). Within the present study it was found that after 24 h incubation with LPS MoDC from four donors down-regulated CD209 as expected (donor 3, 4, 5, 6), while two others did not (donor 1, 2). The same result was determined after 96 h. In four experiments LPS decreased CD209 expression (donor 1, 2, 4, 5), while in two approaches an augmented CD209 expression was found (donor 3, 6), although a down-regulation was observed after 24 h of stimulation.

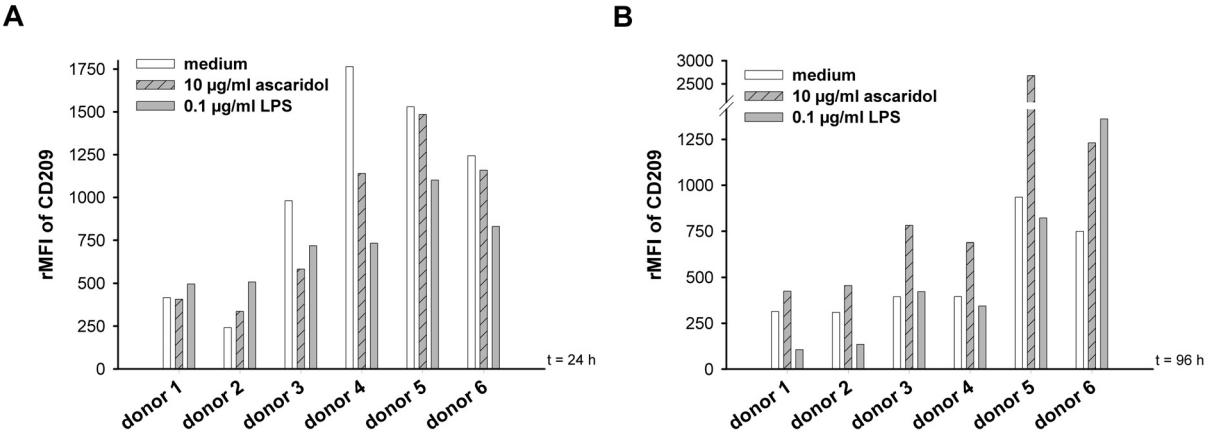

Figure 12: CD209 surface expression of ascaridol-treated MoDC.

CD209 surface expression of MoDC cultured in medium alone or in the presence of 10 µg/ml ascaridol was determined via flow cytometry. As positive control MoDC were also treated with 0.1 µg/ml LPS. Panel A shows results after 24 h incubation and panel B after 96 h. White bars show results of non-treated cells (medium), grey pattern bars display results for ascaridol-treated MoDC, while grey bars show results of LPS-treated cells. Values of relative mean fluorescence intensity (rMFI) of six independent experiments are given.

4.5 Impact of ascaridol on CD184 surface expression of MoDC

Chemokines are involved in the control of DC trafficking, which is critical for the initiation of an adaptive immune response. Therefore, the effect of ascaridol on the surface expression of the chemokine receptor CD184 (CXCR4) was investigated. Due to the high expression in white blood cells and marked sequence similarity to IL-8 receptor, Loetscher and co-workers suggested that CD184 may function in the activation of inflammatory cells (Loetscher *et al.*, 1994). Moreover, its ligand CXCL12 has been related to the constitutive basal trafficking of DC (Sozzani *et al.*, 2000). Figure 13 shows representative histograms of the obtained results. We found that the basal expression of CD184 on MoDC was low. After ascaridol treatment no changes of CD184 expression were observed - neither after 24 h nor 96 h of incubation. For LPS an increase of CD184 was determined at both studied times.

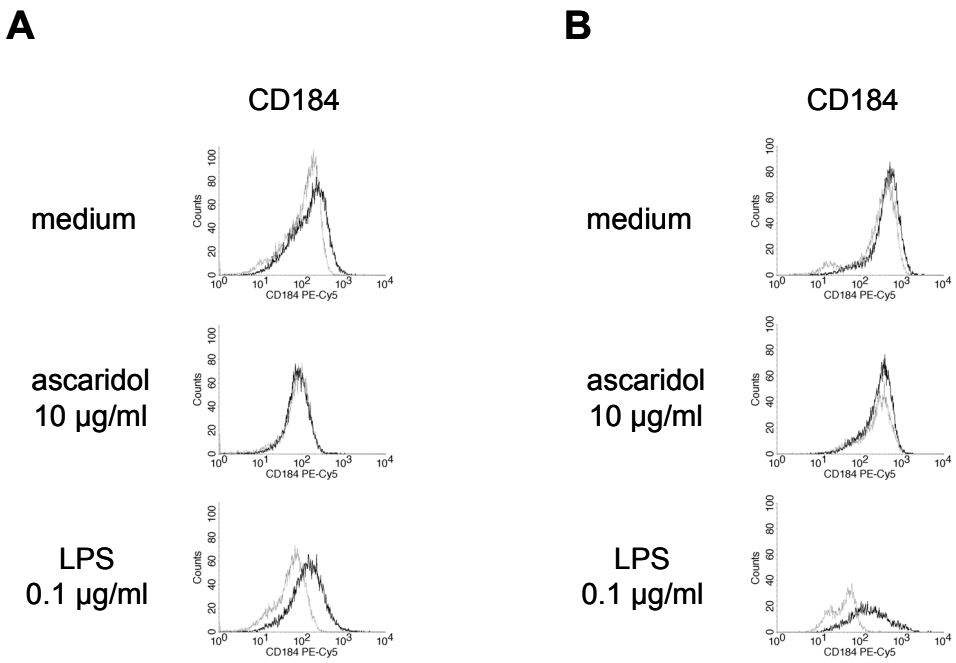

Figure 13: Representative histograms of CD184 surface expression on MoDC after treatment with ascaridol for 24 and 96 h.

MoDC were treated with medium alone (negative control), 10 µg/ml ascaridol or 0.1 µg/ml LPS (positive control) for 24 h (panel A) and 96 h (panel B). Surface expression of CD184 was analyzed via flow cytometry. Black opened histograms indicate CD184 stained cells, while grey histograms display the corresponding isotype controls. Histograms of one representative of three independent experiments for each incubation period are shown.

4.6 Ascaridol affects phagocytotic property of MoDC

Immature DC take up antigens efficiently, but lose this ability on maturation (Sallusto *et al.*, 1995). Therefore, antigen uptake of DC treated with ascaridol in comparison to DC cultured only in media was determined by flow cytometry. The differences between the mean fluorescence intensity (MFI) of samples incubated with FITC-labelled dextran at 37 °C and the MFI of samples maintained at 4 °C were calculated. Only experiments, which showed a clear CD86 and/or CD83 augmentation due to ascaridol treatment, were analyzed for FITC-labelled-dextran uptake. As depicted in figure 14 an increased FITC-labelled dextran uptake was measured for ascaridol-treated MoDC after 24 h (n = 3). An average ascent of 55 % was found compared to non-treated cells. However, after 96 h the dextran uptake of ascaridol-treated MoDC was decreased by 30 % compared to medium-treated cells (n = 5).

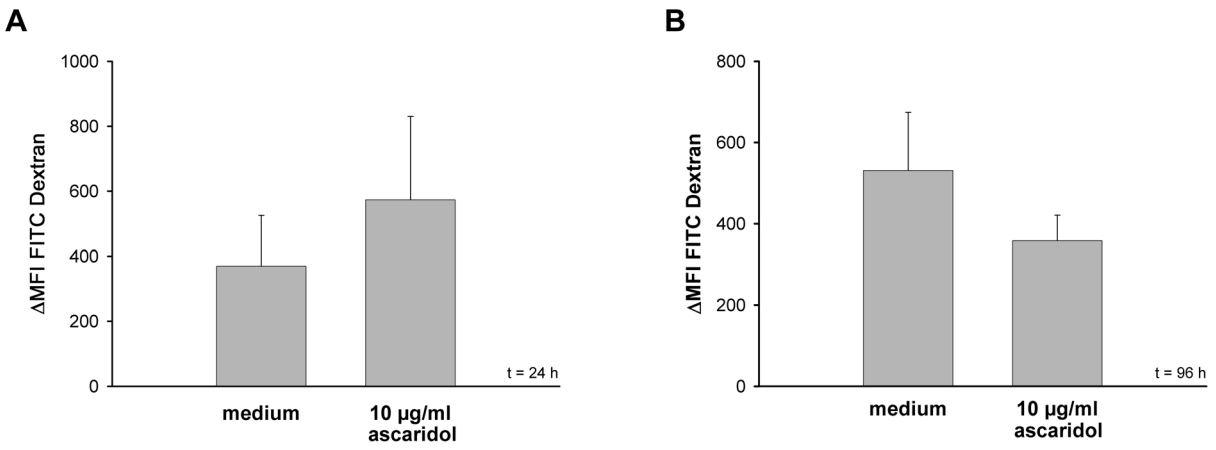

Figure 14: Antigen uptake of MoDC after ascaridol treatment for 24 and 96 h.

MoDC were cultured in medium alone or in the presence of 10 µg/ml ascaridol for 24 h (panel A) or 96 h (panel B). After this treatment cells were further incubated with FITC-labelled dextran at 4 °C or 37 °C for 60 min. The mean fluorescence intensity (MFI) of cells that took up FITC-labelled dextran was determined by flow cytometry and the difference between the MFI of parallel samples maintained at different temperatures was calculated. Panel A shows the mean value ± SEM of three independent experiments and panel B gives the results of five independent experiments. $P > 0.05$.

4.7 Cytokine release in cell culture supernatants of MoDC after treatment with ascaridol

A critical event during the development of cutaneous immune responses provoked by contact allergens is the mobilisation of dendritic cells. A successful induction of migration requires cytokine signals, and therefore the cytokine production of interleukin (IL)-8, IL-1beta, tumor necrosis factor (TNF)alpha and IL-6 was studied in cell culture supernatants of MoDC after 24 h stimulation with ascaridol. Further on, LPS was used as a positive control. After LPS treatment increased levels of all determined cytokines were measured in all investigated cell culture supernatants (data not shown). As figure 15 shows, after 24 h exposure with ascaridol an increased production of IL-8 was found. While for MoDC incubated with medium alone an average IL-8 production of 3485 pg/ml was determined, ascaridol increased the IL-8 concentration up to 6378 pg/ml.

In addition, we found that ascaridol enhanced the release of TNFalpha (figure 16). While in supernatants of untreated MoDC 5 pg/ml TNFalpha was determined in average, for ascaridol-treated MoDC 12.6 pg/ml was measured. The concentration of IL-6 was increased by ascaridol between 1.5 and 2.6-fold induction in three of six donors studied (figure 16). Furthermore, we determined in three of six donors an ascaridol-induced IL-1beta production with 1.2 to 2.5-fold increase compared to analysis of supernatants from non-treated cells (figure 16).

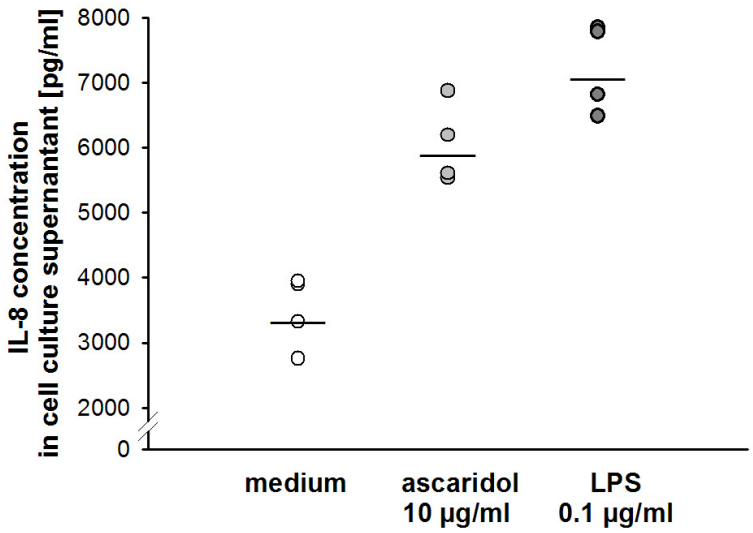

Figure 15: Concentration of IL-8 in cell culture supernatants of MoDC after treatment with ascaridol in comparison to medium-treated cells.

Cell culture supernatants of MoDC showing an augmented CD86 expression by ascaridol were analyzed via ELISA for interleukin (IL)-8 after 24 h incubation. Cytokine production in pg/ml is shown for ascaridol-treated as grey circles, for medium-treated cells as white circles and for LPS-treated as dark-grey circles. The mean value of four independent experiments is shown as a horizontal mark. P > 0.05.

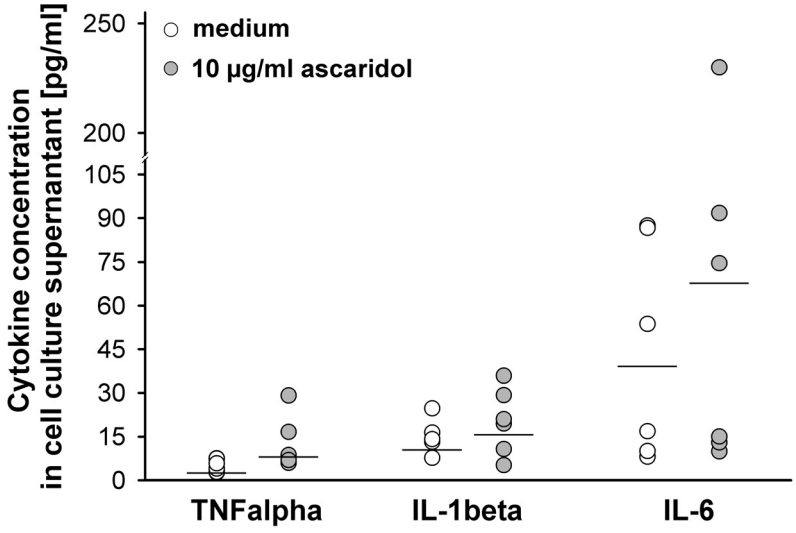

Figure 16: Concentration of TNFalpha, IL-1beta and IL-6 in cell culture supernatants of MoDC after treatment with ascaridol in comparison to medium-treated cells.

Cell culture supernatants of MoDC showing an augmented CD86 expression by ascaridol were analyzed via CBA for tumor necrosis factor (TNF)alpha, interleukin (IL)-1beta and IL-6 after 24 h incubation. Cytokine production in pg/ml is shown for ascaridol-treated as grey circles and for medium-treated cells as white circles. The mean value of six independent experiments is shown as a horizontal mark. P > 0.05.

Cytokines such as IL-12p75, IL-12p40 and IL-10 have been implicated in the polarization of T cells (Snijders *et al.*, 1998). In this context, IL-12 production by DC is considered to be critical for T helper cell type 1 development, while IL-10 may play a role in the T helper cell type 2 system. Therefore, it was studied whether ascaridol stimulation of MoDC influence the concentration of IL-12p75, IL-12p40 or IL-10. As figure 17 shows, no enhanced concentration of IL-12p75 or IL-12p40 was detected after ascaridol treatment. Furthermore, the IL-10 level was unaffected by ascaridol.

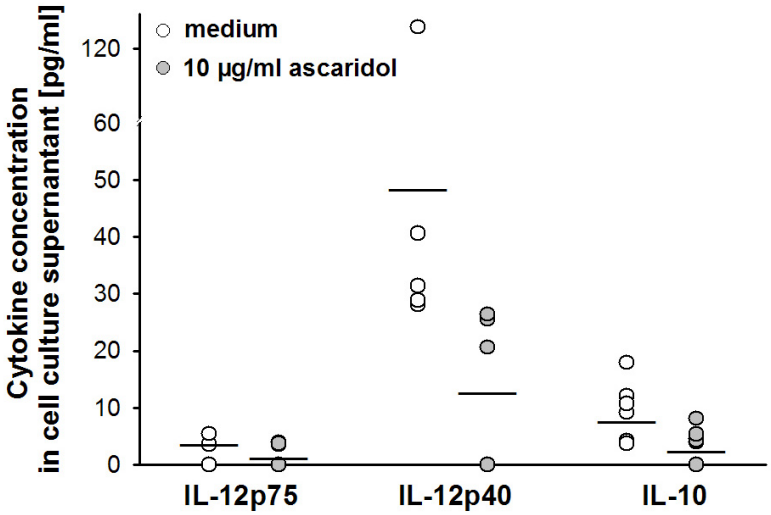

Figure 17: Concentration of IL-12p75, IL-12p40 and IL-10 in cell culture supernatants of MoDC after treatment with ascaridol in comparison to medium-treated cells.

Cell culture supernatants of MoDC showing an augmented CD86 expression by ascaridol were analyzed via CBA for T helper cell polarization involved cytokines like interleukin (IL)-12p75 and IL-10 after 24 h incubation. In addition the IL-12p40 concentration was measured by ELISA. The cytokine production in pg/ml is shown for ascaridol-treated cells as grey circles and for medium-treated cells as white circles. The mean value of six independent experiments is shown as a horizontal mark. $P > 0.05$.

4.8 Impact of ascaridol on CD86, CD54, CD40, and HLADR surface expression of THP-1 cells

Miyazawa and colleagues previously showed that THP-1 cells can discriminate between allergens and irritants by different expression of surface markers following chemical exposure (Miyazawa *et al.*, 2007). In order to investigate the allergenic potential of ascaridol THP-1 cells were exposed with several concentrations of ascaridol for 24 h and subsequently CD86, CD54, CD40, and HLADR surface expression were analyzed via flow cytometry. One representative experiment is shown in figure 18.

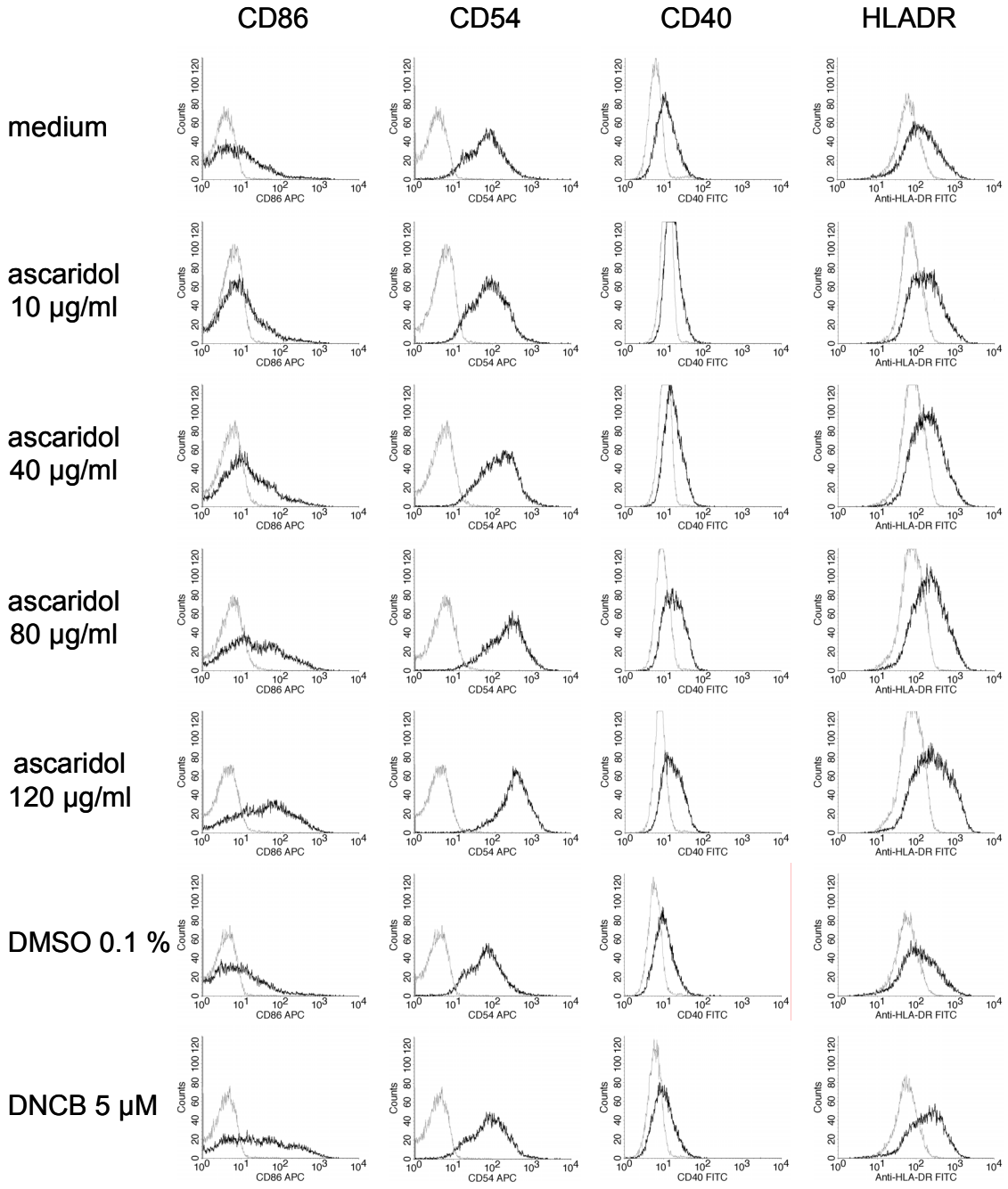

Figure 18: Representative histograms of CD86, CD54, CD40 and HLADR surface expressions on THP-1 cells after treatment with different concentrations of ascaridol for 24 h.

THP-1 cells were exposed to 10, 40, 80 and 120 µg/ml ascaridol or medium alone for 24 h. In addition, the response for 5 µM DNCB served as a positive control and its vehicle control DMSO was analyzed. The surface expression of CD86, CD54, CD40, and HLADR was determined via flow cytometry. Black opened histograms indicate stained cells; grey histograms show corresponding isotype controls. Histograms of one representative experiment are shown.

A total of eight independent experiments were analyzed for CD86, CD54, and CD40 expression and four independent experiments for HLADR expression. The well known sensitizer 2,4-dinitrochlorobenzene (DNCB, 5 µM), investigated for THP-1 cells by Sakaguchi (2006), was used as a positive control. As figure 19 shows, except of the only moderately augmented CD40 expression, the rMFI of all determined surface markers was clearly increased by DNCB compared to its vehicle control DMSO as expected.

Ascaridol-treated cells showed a dose-dependent augmented CD86 surface expression as shown in figure 19A. Ascaridol concentrations of 80 and 120 µg/ml augmented the rMFI value of CD86 significantly up to 61, whereas the CD86 rMFI value of untreated cells was 34 in average (n = 6). Likewise, the percentages of $CD86^+$ cells were increased from approximately 16 % up to 38 %.

CD54 expression was also augmented in a dose-dependent manner by ascaridol as shown in figure 19B. The rMFI value of CD54 was induced significantly up to 120 after treatment with 80 µg/ml ascaridol and 120 µg/ml ascardiol enhanced the rMFI value up to 162 compared to the averaged rMFI value of 89 for untreated cells (n = 6). The percentage of CD54 expressing cells was also increased. Already 10 µg/ml ascaridol augmented the CD54 expressing cells up to 85 % in average, while the percentage of CD54 expressing cells after medium treatment was 76 %. Higher doses of ascaridol increased the $CD54^+$ cell population up to 89 % in average.

For CD40 expression, the rMFI values between ascaridol-treated and non-treated cells were similar. The percentages of cells expressing CD40 were augmented after ascaridol treatment (see figure 19C, n = 6). In average 37 % of non-treated cells expressed CD40, whereas after treatment with 10 and 40 µg/ml ascaridol an increase of 15 % more $CD40^+$ cells was determined. Incubation with either 80 or 120 µg/ml ascaridol augmented the percentage of $CD40^+$ cells up to approximately 60 %.

Furthermore, ascaridol induced a dose-dependent augmentation of HLADR expression indicated by an increase of $HLADR^+$ cells and augmented rMFI values as represented in figure 19D (n = 4). While for untreated cells about 30 % $HLADR^+$ cells with an average rMFI value of 498 were found, for 10 µg/ml and 40 µg/ml ascaridol-treated cells 39 % $HLADR^+$ cells with an average rMFI of 576 were determined. Compared to untreated cells a further increase up to 43 % of $HLADR^+$ cells with an average rMFI value of 626 for 80 µg/ml ascaridol and 47 % of $HLADR^+$ cells with an average rMFI value of 648 for 120 µg/ml ascaridol was found.

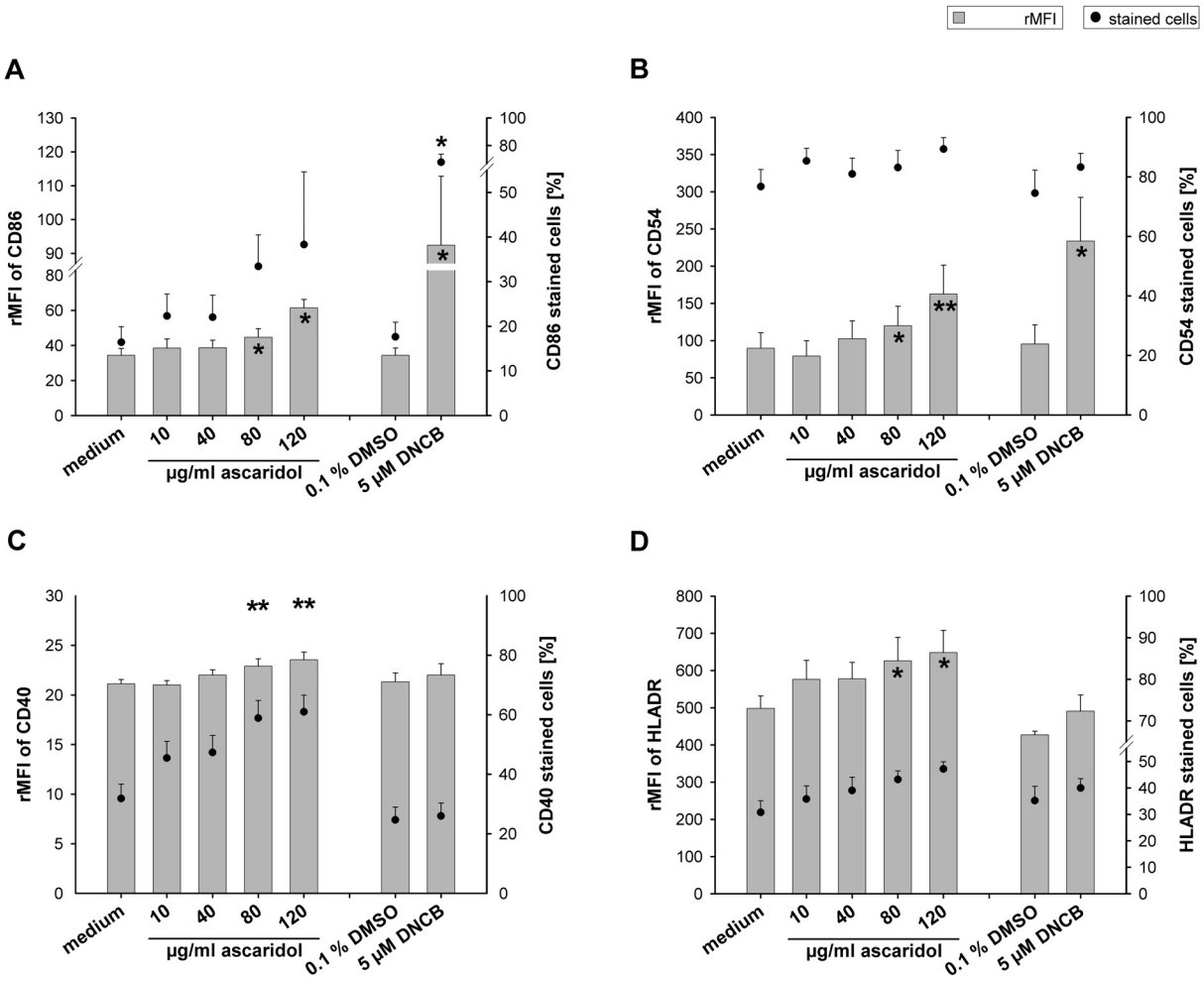

Figure 19: Surface expression analysis of THP-1 cells after treatment with different ascaridol concentrations for 24 h.

THP-1 cells were exposed to 10, 40, 80 and 120 µg/ml ascaridol or medium alone for 24 h. In addition, the response for 5 µM DNCB served as a positive control and its vehicle control DMSO was analyzed. Surface expression of CD86 (panel A), CD54 (panel B), CD40 (panel C) and HLADR (panel D) was determined via flow cytometry. Bars show the mean value of the relative mean fluorescence intensity (rMFI) ± SEM of eight independent experiments for CD86, CD54 and CD40 analysis and four for HLADR. Furthermore, the percentage of stained cells is given. Black scatters display the mean of stained cells in % + SEM.
Asterixes placed within the bars indicate statistical significance for rMFI values, while asterixes above the scatters indicate statistical significance of mean values of the percentage of stained cell population between chemical-treated cells and vehicle control cells.
$P \leq 0.05$ (*) and $p \leq 0.005$ (**).

4.9 Analysis of THP-1 cell response to ascaridol using proposed criteria of the human cell line activation test

The human cell line activation test (h-CLAT) suggested by Ashikaga *et al.* (2006) proposes a significant induction of the CD86 and/or CD54 expression on THP-1 cells for the classification of sensitizers. Sakaguchi *et al.* (2007) recommended that substances enhancing the relative fluorescence intensity (RFI) values for CD86 over 150 and /or CD54 over 200 should

be declared as sensitizers. Thus, analogous to h-CLAT examination RFI values were also calculated from rMFI values, presented in the previous chapter 4.8. Table 3 shows the obtained results. For DNCB as a well known sensitizer averaged RFI values of 292 for CD86 and 340 for CD54 were determined. Thus, obtained RFI values would categorize DNCB as a sensitizer according to criteria requested by Sakaguchi (Sakaguchi *et al.*, 2007). Ascaridol fulfilled the criteria for CD86 at the concentration of 120 µg/ml with an averaged RFI value of 202. For CD54 expression an averaged RFI value of 198 was determined.

Table 3: Representation of CD86 and CD54 surface expression on THP-1 cells after ascaridol or DNCB treatment as RFI values analogous to Sakaguchi *et al.* (2007).

THP-1 cells were exposed to 10, 40, 80 and 120 µg/ml ascaridol or 5 µM DNCB for 24 h. Cells cultured in medium are used as negative control for ascaridol-treated cells; 0.1 % DMSO served as negative control for DNCB-treated cells. Analogous to h-CLAT examination for THP-1 relative fluorescence intensity (RFI) was calculated from relative mean fluorescence intensity values of THP-1 which are presented in section 4.8. Mean RFI values ± SEM of eight independent experiments for each surface marker are shown.

	CD86	**CD54**
10 µg/ml ascaridol	108 ± 25	110 ± 21
40 µg/ml ascaridol	115 ± 18	118 ± 32
80 µg/ml ascaridol	136 ± 31	144 ± 55
120 µg/ml ascaridol	202 ± 32	198 ± 96
5 µM DNCB	292 ± 121	340 ± 124

4.10 Impact of ascaridol on CD184 and CCR2 surface expression of THP-1 cells

The recruitment of leukocytes to inflamed tissues involves chemotactic molecules that attract and activate leukocytes. CD184 is expressed on a variety of cell types, including monocytes (Wang *et al.*, 2006). According to Loetscher (1994) CD184 may fullfil a function in the activation of inflammatory cells. In addition, CCR2, the receptor for monocyte-chemotactic protein-1 (MCP-1), is expressed on monocytes. MCP-1 is a member of the chemokine family that mediates leukocyte chemotaxis. The potent and specific activation of monocytes by MCP-1 may mediate the monocytic infiltration of inflammatory tissues (Charo *et al.*, 1994).

Therefore, it was investigated whether ascaridol treatment influenced the CD184 or the CCR2 surface expression on THP-1 cells after 24 h of incubation. Representative results for both surface markers are depicted in figure 20.

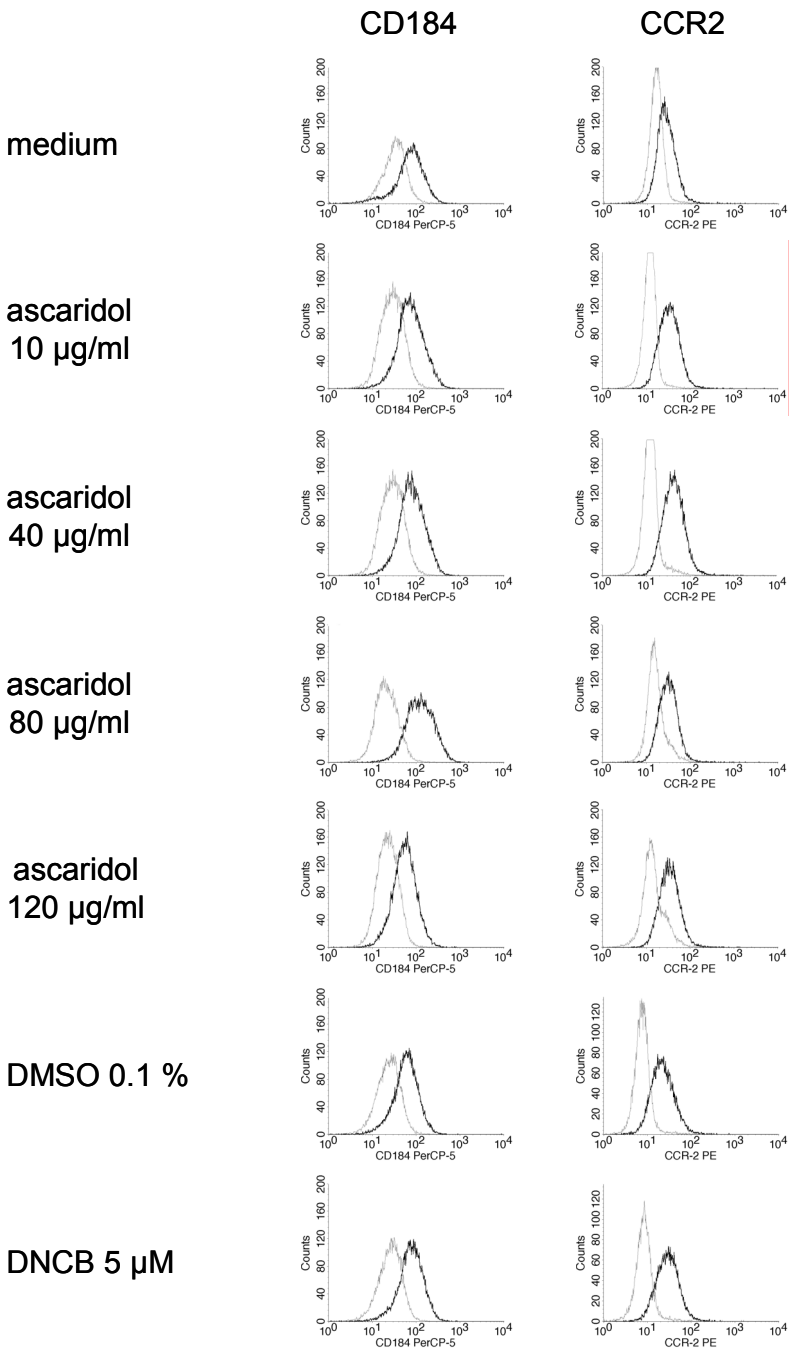

Figure 20: Representative histograms of CD184 and CCR2 surface expression on THP-1 cells after treatment with different concentrations of ascaridol for 24 h.

THP-1 cells were exposed to 10, 40, 80 and 120 µg/ml ascaridol or medium alone for 24 h. In addition, the response for 5 µM DNCB served as positive control and its vehicle control DMSO was analyzed. Surface expression of CD184 and CCR2 was analyzed via flow cytometry. Black opened histograms indicate stained cells; grey histograms corresponding isotype controls. Histograms of one representative experiment are shown.

As depicted in figure 21A, a slight increase of the CD184 expression was found for ascaridol. For non-treated cells an averaged rMFI value of 170 was determined, while the rMFI value of 80 µg/ml ascaridol-treated cells was up to 180. The highest tested concentration of ascaridol (120 µg/ml) revealed a clear down-regulation of CD184. Both the CD184 rMFI value and the percentage of CD184 stained cells were decreased compared to non-treated cells. DNCB induced an increase of CD184 expression with an averaged CD184 rMFI value of 174 compared to its vehicle control (rMFI = 166), while the percentages of $CD184^+$ cells were similar.

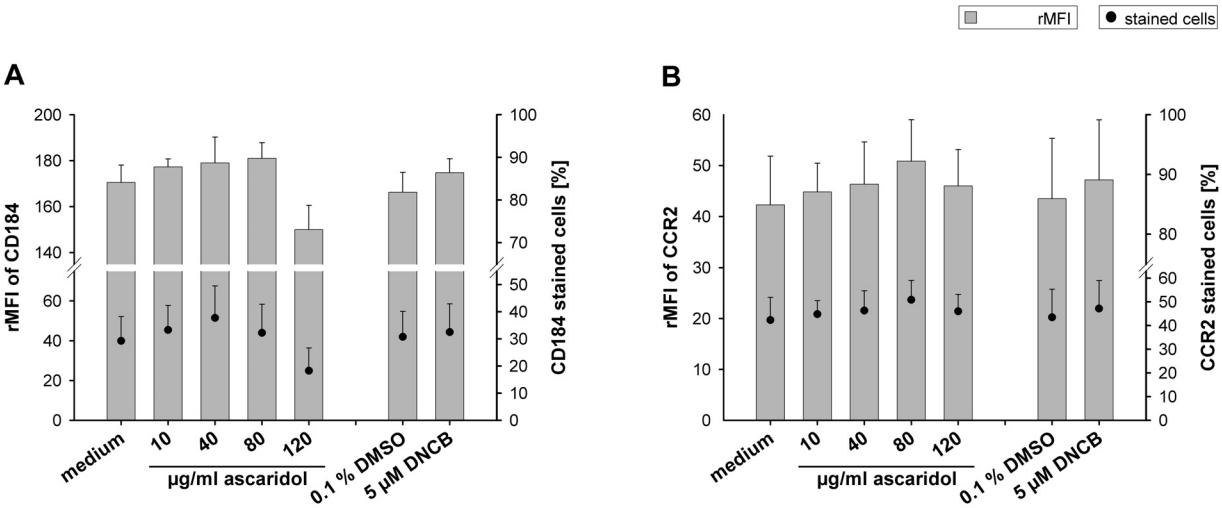

Figure 21: CD184 and CCR2 surface expression analysis of THP-1 cells after treatment with different concentrations of ascaridol for 24 h.

THP-1 cells were exposed to 10, 40, 80 and 120 µg/ml ascaridol or medium alone for 24 h. In addition, the response for 5 µM DNCB served as positive control and its vehicle control DMSO was analyzed. Surface expression of CD184 (panel A) and CCR2 (panel B) was determined via flow cytometry. Bars represent the relative mean fluorescence intensity (rMFI) mean value ± SEM of four independent experiments for CD184 and six for CCR2. Scatters represent the mean values + SEM of the stained cell population in percent (%).
$P > 0.05$.

Figure 21B shows the results for analysis of CCR2 surface expression. Similar percentages of $CCR2^+$ cells of about 45 - 50 % in average were found for all tested approaches. However, a slight increase of CCR2 rMFI was observed for all tested ascaridol doses compared to untreated cells, culminated with value of 44 for 80 µg/ml ascaridol vs. 30 of non-treated cells. Both lower ascaridol concentrations 10 and 40 µg/ml yielded similar rMFI (35 in average). An averaged rMFI value of 40 was determined for 120 µg/ml ascaridol.

4.11 Impact of ascaridol on cytokine secretion of THP-1 cells

Miyazawa and colleagues showed that different allergens induced a change of IL-8 and TNFalpha production of THP-1 cells (Miyazawa *et al.*, 2007). Therefore, the concentrations of both proteins were determined in cell culture supernatants after treatment with different concentrations of ascaridol.

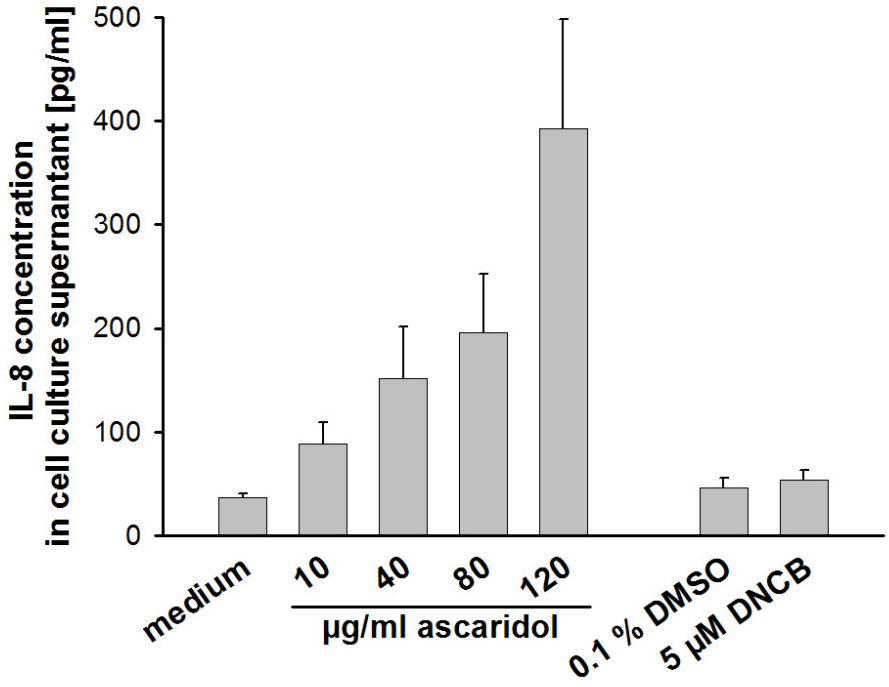

Figure 22: Concentration of IL-8 in cell culture supernatants of THP-1 after treatment with ascaridol in comparison to medium-treated cells.

Cell culture supernatants of THP-1 cells, which had been incubated with different concentrations of ascaridol (10, 40, 80 and 120 µg/ml), medium alone, 5 µM DNCB as positive control or its vehicle control DMSO for 24 h, were analyzed by ELISA for interleukin (IL)-8 production. Bars indicate the mean value ± SEM in pg/ml of four independent experiments. $P > 0.05$.

As shown in figure 22, ascaridol increased the production of IL-8 in a dose-dependent manner. While a basal production of 37 ± 3 pg/ml IL-8 was measured for untreated THP-1 cells, 10 µg/ml ascaridol increased the production up to 89 ± 20 pg/ml ($n = 4$; $p = 0.07$). The highest tested concentration of ascaridol yielded 393 ± 105 pg/ml IL-8 in average ($n = 4$; $p = 0.07$).

TNFalpha production was not detected in cell culture supernatants neither after DNCB nor after ascaridol treatment (data not shown).

4.12 Impact of ascaridol on p38 mitogen-activated protein kinase activation in THP-1 cells

It has previously been found that DNCB as well as nickel sulfate can induce CD86 expression on THP-1 cells via p38 mitogen-activated protein kinase (MAPK) phosphorylation (Miyazawa *et al.*, 2008a). Therefore, it was investigated via Western Blot analysis whether ascaridol, which induced CD86 expression on THP-1 cells (see figure 19), affects the activation of p38 MAPK.

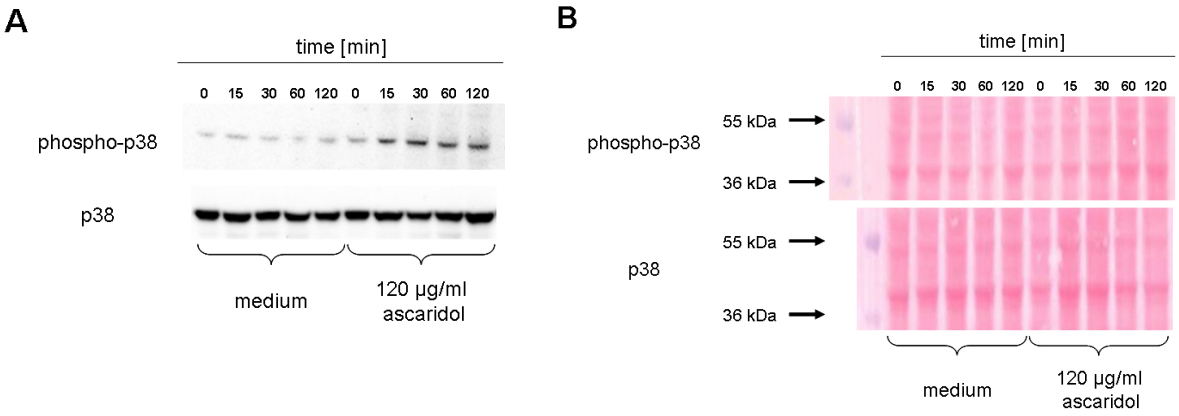

Figure 23: Impact of ascaridol on the phosphorylation of p38 MAPK in THP-1 cells.

THP-1 cells were treated with either medium alone or 120 µg/ml ascaridol for 0, 15, 30, 60 and 120 min. After collecting the cells, the expression of phospho-p38 MAPK and total p38 MAPK were analyzed by Western Blot (panel A). Ponceau-S staining was used as loading control (panel B). Similar results were obtained in three independent experiments.

As depicted in figure 23, in comparison to untreated THP-1 cells, ascaridol (120 µg/ml) induced the phosphorylation of p38 MAPK within 15 min of treatment prolonging up to 2 h.

5. Discussion

The continuous increase of commercialized compounds with legal requirements for toxicity testing and the demand to reduce animal experiments in view of legislative initiatives, such as REACH (registration, evaluation, authorization of chemicals) as well as the 7th Amendment to the Cosmetics Directive (2003/15/EC), have forced the development of *in vitro* models for the prediction of sensitizing potential of new substances. The current *in vitro* models mainly make use of cells involved in the sensitization phase, such as dendritic cells (DC). Hence, *in vitro* generated DC from human blood monocytes or various cell lines including the human acute monocytic leukemia cell line-1 (THP-1) as potential DC surrogate are suggested as promising tools for skin sensitization prediction (Tuschl and Kovac, 2001; Yoshida *et al.*, 2003). However, it is clear that contact sensitizers vary substantially with regard to the relative potency with which they are able to induce skin sensitization. Thus, whether *in vitro* test systems are approaches that are sensitive enough to detect even weak sensitizers, and thus, might be available for assessment of relative potency for the purpose of categorizing chemical allergens, must be evaluated. Therefore, the immune modulating impact of a presumably weak sensitizer was explored. The response to ascaridol, a naturally occurring substance which is predicted as one of the possible causes for tea tree oil contact dermatitis, was studied. Investigations focusing on the sensitizing potential of this compound are missing, therefore, we studied whether ascaridol has the property to influence both monocyte-derived dendritic cells (MoDC) and THP-1 cells, recently described as human Cell Line Activation Test (h-CLAT).

5.1 Response of MoDC to ascaridol

5.1.1 Ascaridol influenced the surface expression of co-stimulatory molecules on MoDC

Cells obtained by culture of peripheral blood monocytes with granulocyte/macrophage-colony stimulating factor (GM-CSF) and interleukin (IL)-4 were shown to resemble immature dendritic cells (Schuler *et al.*, 1995; Bender *et al.*, 1996; Romani *et al.*, 1996; Ebner *et al.*, 1998). During maturation they achieve skills in antigen uptake and processing and are able to activate T cells. These cells are well suited to substitute Langerhans cells, and therefore they promise to be a useful test system for the identification and discrimination of sensitizing and irritating chemicals. Several investigators discriminate chemical allergens and irritants by measuring surface expression markers like CD86, CD80, CD40, CD54, and HLADR which were expressed higher in the presence of sensitizers (Coutant *et al.*, 1999a; Hulette *et al.*,

2002). Within the present study the highest non-cytotoxic concentration of ascaridol in the *in vitro* MoDC test system was evaluated with 10 µg/ml and a significant increase of CD86 expression was found after 24 and 96 h of stimulation. CD86 is a molecule which provides an important co-stimulatory signal necessary for T cell activation and survival by binding CD28 (Azuma *et al.*, 1993). Several groups received similar CD86 up-regulation for various chemical allergens like 2,4-dinitrochlorobenzene (DNCB), 2,4,6-trinitrobenzene sulfonic acid (TNBS), nickel salts, as well as cobalt, and chrome (III) chloride (Aiba *et al.*, 1997; Tuschl *et al.*, 2000; Straube *et al.*, 2005; Toebak *et al.*, 2006). Hulette and colleagues found that DC treated with the highest non-cytotoxic concentrations of several chemical allergens (e. g. dinitrofluorobenzene acid and methylisothiazolinone) display marginal up-regulation of CD86 (Hulette *et al.*, 2002) and therefore, other activation markers are necessary to investigate. Based on their results, additional changes such as major histocompatibility complex (MHC)-peptides class II like HLADR, whose stable expression on the surface of mature DC is also crucial for T cell priming (Moser and Murphy, 2000), was explored. Thus, for ascaridol it was varified that HLADR was up-regulated during the early activation phase in MoDC but not at a later time (96 h). This time-dependent regulation was initially observed in mice by Aiba and Katz (Aiba and Katz, 1990). However, increased expression of MHC class II molecules is generally regarded as an early activation marker of human myeloid DC *in vitro* (Hellman and Eriksson, 2007). Additionally, CD54, an adhesion molecule, which i. a. triggers the accumulation of MHC molecules in particular at the contact area to T cells (Lebedeva *et al.*, 2005), was not augmented constantly by ascaridol. Only for half of the donors studied an increase was determined after 24 and 96 h. Commonly, it is assumed so far that strong allergens induced the up-regulation of CD86, HLADR, and CD54 on DC (i. e. MoDC and $DC34^+$ precursor-derived DC, respectively), whereas weaker allergens (citronellal, coumarin) only changed the CD86 expression after 24 h exposure (Aiba *et al.*, 1997; Coutant *et al.*, 1999a; Rougier *et al.*, 2000; Straube *et al.*, 2005). Thus, ascaridol induced an increased CD86 expression accompanied by a moderate HLADR and/or donor dependently CD54 augmentation after 24 h, which directs to the assumption that ascaridol could be a weak sensitizer. This is consistent with the observation that ascaridol induced the CD80 up-regulation only after long term incubation (96 h), but not after 24 h. Besides CD86, CD80 provides another co-stimulatory signal for the T cell receptor and thereby T cell activation. Comparable with the surface expression pattern found for ascaridol after 24 h, the fragrance coumarin with weak sensitizing effects was shown to increase CD86 expression after 48 h treatment in MoDC but not CD80, whic was rather decreased (Jugde *et al.*, 2005).

However, the response pattern of DC to chemical stimulations is not uniform rather variable depending on the properties of each chemical (Manome *et al.*, 1999). For instance, both nickel chloride and DNCB induced similar magnitudes of the up-regulation of CD86, whereas

the expressions of CD54 and HLADR were more strongly increased after nickel chloride compared to DNCB treatment (Aiba and Tagami, 1999; Coutant *et al.*, 1999a), although nickel salts are identified as moderate allergens in guinea pig maximization test (Arimura *et al.*, 1998). Furthermore, the well known strong immunogen para-phenylendiamine (PPD) was demonstrated to up-regulate only CD40 and HLADR, but not CD86 (Coulter *et al.*, 2007). Additionally, there are many reports of donor-to-donor variability in the responses to allergen exposure including differences in cell surface marker expression (Aiba *et al.*, 1997), mRNA expression (Pichowski *et al.*, 2000; Straube *et al.*, 2005), and cytokine production (Aiba *et al.*, 1997). Thus, well known sensitizers induced responses in DC that differ in quality and quantity and sometimes are inconsistent with their estimated potency via animal tests, the use of MoDC *in vitro* test for classification of sensitization potential (whether a chemical is a strong or a weak sensitizer) seems to be more complicated. The classification of sensitization potential must be derived very carefully from how many surface expression markers are influenced and how strong the augmentation is.

5.1.2 Ascaridol influenced the surface expression of markers which enhance MoDC maturation

The ligation of DC expressed CD40 by T cell CD154 leads to further maturation of the dendritic cell and production of IL-12, a cytokine that promotes T helper cell type (Th) 1 immune responses (Cella *et al.*, 1996). Hence, the change of CD40 expression on MoDC was determined for ascaridol. Similar to the CD80 expression pattern, ascaridol pushed the CD40 expression after long time stimulation predominantly. Five of six studied donors up-regulated CD40 after 96 h incubation. Several investigators demonstrated that strong allergens like DNCB, sulfamethoxazole, or 4-aminophenol and even moderate antigens like nickel sulfate and chlorpromazine up-regulated CD40 on MoDC after 24 or 48 h incubation (Coutant *et al.*, 1999a; Sanderson *et al.*, 2007). Besides, Coutant and co-workers studied early phase activation events due to contact sensitizers with a murine epidermal cell assay by determination of possible modulation of CD40 expression among others. After *in vitro* treatment with the strong contact sensitizer TNBS, Langerhans cells demonstrated a rapid up-regulation of CD40, whereas no change of CD40 expression was observed after treatment with the mild or weak contact sensitizer, citral and citronellal, respectively (Coutant *et al.*, 1999b). These results encourage to evaluate ascaridol as a rather weak sensitizer which elicited CD40 augmentation predominantly after long term incubation.

There are two major phases in the life of DC: an immature stage characterized by a high efficiency in taking up and processing antigens associated with high expression of some molecules involved in antigen uptake such as CD209, which promotes internalization of antigens

(Steinman, 2000; Geijtenbeek *et al.*, 2002; Giordano *et al.*, 2003); and a mature stage in which the antigen uptake capacity is lost. For ascaridol-treated MoDC an increased capacity of taking up antigens was observed when compared to untreated MoDC, while some maturation markers were predominantly inhibited (CD80, CD40) after 24 h. In contrast, after long term incubation (96 h) the capacity to take up antigens got lost and several co-stimulatory molecules were augmented donor dependently. Moreover, ascaridol influenced also the expression of the adhesion receptor CD83. CD83 is one of the most characteristic cell surface markers for fully matured dendritic cells (Prechtel and Steinkasserer, 2007). The membrane-bound CD83 has immune stimulatory capacity, activates T cells and is important for the generation of thymocytes. The loss of CD83 led to a strong reduction of the DC - T cell stimulatory capacity, suggesting that CD83 acts as an essential enhancer during T cell activation (Kruse *et al.*, 2000; Hirano *et al.*, 2006; Prechtel *et al.*, 2006). For ascaridol-treated MoDC only in some experiments an increased percentage of $CD83^+$ cells was found after 24 h, but the effect became significant after long term stimulation (96 h), thus conforming to other above described immuno-modulating influences of ascaridol (e.g. augmented CD80 and CD40 expression as well as the reduced capacity to take up antigens).

The present study illustrates that ascaridol influenced the surface expression of different markers on MoDC, but particularly after long term incubation (96 h vs. 24 h). This observation is not unique for ascaridol. Staquet and co-workers found that a 2-day treatment with the representative allergens DNCB (strong) and nickel sulfate (moderate) induced significant changes of most surface marker expressions (e.g. CD86, HLADR, CD40, CD54, and CD83) while other chemicals such as balm of Peru (strong), kathon (moderate), cinnamic aldehyde (mild) or the irritant sodium lauryl sulfate (SLS) had no significant effect. In contrast, the 4-day treatment with sensitizers substantially improved the results. Indeed, the number of modified expression markers was significantly increased in comparison with the irritant SLS (Staquet *et al.*, 2004). Consistent with their observations the present study indicated that the screening of mild or moderate sensitizers requires both the consideration of many maturation markers and a prolonged time of incubation with the chemicals.

5.1.3 Ascaridol influenced the surface expression of CD209 on MoDC

CD209, which has been considered as a DC-specific marker due to its high expression restricted to DC, is shown to be involved in the antigen recognition of bacterial pathogens provoking their uptake by internalization which leads to decreased expression of CD209 after stimulation (Giordano *et al.*, 2003; Zhang *et al.*, 2006; Zhou *et al.*, 2006). Interestingly, after 24 h of ascaridol treatment, a decreased expression of CD209 was determined. However, after 96 h CD209 is clearly up-regulated by ascaridol. Scarce information is available concerning direct effects of contact sensitizers on CD209. One possibility could be that the

chemical induced secretion of mediators such as tumor necrosis factor (TNF)alpha or interleukin (IL)-1beta amplifies the increase of CD209 surface expression as it was shown for other surface proteins involved in dendritic maturation (Cella *et al.*, 1997a; Berthier-Vergnes *et al.*, 2005). Another possibility is that the augmented CD209 expression is caused as a response of the altered expression of other dendritic maturation markers as it is known for MHC class II - induced maturation (Lokshin *et al.*, 2002). It is also possible that ascaridol directly effects signalling pathways leading to CD209 expression and thereby induces the stabilization of DC-T cell contact as it can be conceived for CD209 augmentation (Steinman, 2000; Geijtenbeek *et al.*, 2002). Furthermore, it can also be assumed that the augmented CD209 expression increases the probability to capture antigens/haptens (Giordano *et al.*, 2003; Neumann *et al.*, 2008). This study clearly indicates that the participation of CD209 in chemical-induced DC maturation should be further explored with other well known allergens.

5.1.4 Ascaridol elicited cytokine release in MoDC

5.1.4.1 The impact of ascaridol on pro-inflammatory cytokines

Cytokines play an important role in the induction of immune reactions by providing further important signals for the activation of naive T cells. Only fully mature DC induce immunity, while semi-mature DC, which also present co-stimulatory molecules but do not secrete pro-inflammatory cytokines, fail. Thus, the decisive immunogenic signal seems to be the release of pro-inflammatory cytokines from the DC to amplify and direct the type of immune response (Lutz and Schuler, 2002). Dendritic cells are known to produce specific cytokine profiles after activation *in vitro* (de Saint-Vis *et al.*, 1998; Lore *et al.*, 1998). IL-1beta, IL-6 and TNFalpha have been shown to participate in DC activation during sensitization both in mouse (Cumberbatch and Kimber, 1992; Enk and Katz, 1992; Enk *et al.*, 1993) and human (Aiba and Tagami, 1999; Pichowski *et al.*, 2000; De Smedt *et al.*, 2001). In response to ascaridol enhanced levels of IL-1beta, IL-6 and TNFalpha after 24 h were found. Consistent with our results, Aiba and co-workers reported that an augmented CD86 expression induced by well known allergens DNCB and nickel chloride was accompanied by an increased secretion of IL-1beta, IL-6 or TNFalpha in MoDC after 24 h (Aiba and Tagami, 1999). Admittedly, the ascaridol-induced production of these mediators was found only in some donors. Indeed, others observed an explicit significance of TNFalpha secretion only after nickel chloride or mangan chloride exposure, but TNFalpha response induced by other tested chemicals including DNCB was occasional (Manome *et al.*, 1999). Furthermore, the chemokine IL-8 was induced by ascaridol in all tested donors. IL-8 is a potent attractant for neutrophiles and different subsets of T cells (Chuntharapai *et al.*, 1994). More recent data propose that during *in vitro* activation of human myeloid DC with bacterial allergens such as lipopolysaccharide (LPS), the

production of IL-8 among others is the earliest sign of activation preceding MHC class II, CD40, CD80, and CD86 expression (Hellman and Eriksson, 2007). Interestingly, *in vitro* reconstructed human epidermis exhibits a sharp maximum of IL-8 release after exposure to several sensitizers (e.g. DNCB, 2.4-dinitrofluorobenzene, TNBS, oxazolone), whereas in the presence of the tested irritants the inverse cytokine release profile was observed (Coquette *et al.*, 2003). Thus, IL-8 secretion may also play a pivotal role in sensitization by chemicals and could be used as a marker for discrimination of irritants and even weak sensitizers such as ascaridol. Indeed, Toebak and co-workers demonstrated that the IL-8 production was significantly increased in MoDC after stimulation with different tested allergens, e.g. DNCB and nickel sulfate, whereas exposure with irritants led to decreased IL-8 production (Toebak *et al.*, 2006).

5.1.4.2 The impact of ascaridol on T cell polarization signals

Generally, the production of the immuno-stimulatory form of IL-12, the IL-12p75 heterodimer consisting of p35 and p40 subunits, is required to induce Th1 responses, whereas secretion of IL-10 favours Th2 responses and in some cases regulatory T cell generation (Moser and Murphy, 2000). IL-12p75 activates STAT4 at sites of Th1 mediated inflammation (Frucht *et al.*, 2000). Inversely, IL-10 indirectly favours Th2 development partly by inhibition of IL-12 production (Moore *et al.*, 1993; Moser and Murphy, 2000). In addition the homodimer IL-12p40 might act as a natural antagonist of IL-12p75 (Abdi, 2002). *In vitro* only a few sensitizers are known to induce the IL-12 or IL-10 secretion of DC (Coutant *et al.*, 1999a; Aiba *et al.*, 2000). In agreement with these observations the DC generated within this study failed to produce IL-12p75 or IL-12p40 upon stimulation with ascaridol for 24 h and no increased concentration of IL-10 was found, either. Other investigators using different stimuli observed that IL-12 is produced most abundantly by DC that are beginning to respond to DC activators, and further on DC reduce this capacity as maturation proceeds (Kalinski *et al.*, 1998; Ebner *et al.*, 2001). This may explain partly the obtained results for ascaridol. Moreover, the optimal induction of IL-12p75 heterodimer seems to require additional signals, which may be provided by interferon(IFN)-gamma, CD40 cross-linking or LPS (Hilkens *et al.*, 1997; Snijders *et al.*, 1998). Interestingly, Holschbach demonstrated by using the same methods that substantial amounts of IL-12p75 were found after addition of LPS to various concentrations of ascaridol in some donors (Holschbach, 2007). Hence, the observation by Holschbach and the absence of IL-12p40 or IL-10 production after ascaridol stimulation within the present study indicate that ascaridol may elicited a Th1 response.

Taken together, within the present study, it was demonstrated for the first time that ascaridol is able to activate MoDC *in vitro*. Hence, a combination of early matured DC, which are strong producers of cytokines, and late matured DC, which have high expression of co-

stimulatory molecules, could prove beneficial in the attempt to initiate *in vitro* and *in vivo* cell-mediated immune responses. Co-stimulatory signals, namely the interactions between CD28 of T cells and their ligands expressed on primed DC (CD86 and CD80) accompanied by augmented CD40 expression - as it was found for late-matured, ascaridol-primed DC - have been shown to exert a clear-cut effect on modulating the Th1 and/or the Th2 development (Gause *et al.*, 1997; Maggi, 1998). Especially, the stimulation of CD40 on the DC by CD40 ligand on the T cell results in promotion of IL-12 production by the DC and Th1 shift (Onoe *et al.*, 2007). Therefore, it can be conjectured that ascaridol-primed DC maybe support a Th1 immune response, although at early times no secretion of IL-12 was found. However, it is important to prove whether ascaridol-treated DC are able to prime naive $CD45RA^+$ T cells and whether these primed T cells show IFN-gamma (Th1) or IL-4 (Th2) secretion.

5.2 Response of ascaridol in THP-1 cells

5.2.1 Ascaridol influenced the surface expression of THP-1 cells

Ashikaga and co-workers explored the usefulness of a human monocyte cell line in the development of *in vitro* models for predictive testing of contact sensitizers (Ashikaga *et al.*, 2002). Based on the finding of several investigators that chemicals induced altered surface expression such as CD86 on *in vitro* generated dendritic cells, Ashikaga *et al.* used THP-1 cells as a replacement for DC and demonstrated that well known allergens like DNCB and PPD enhance CD86 expression, while irritants such as sodium dodecly sulfate (SDS) and methylsalicylate did not. Furthermore, HLADR and CD54 expression were augmented after DNCB treatment (Yoshida *et al.*, 2003). Moreover, Miyazawa and co-workers investigated a series of chemicals, including known allergens like eugenol and hydroquinone, and non-allergens such as methyl paraben, in order to determine their potential to increase CD86, CD54 and CD40 expression on THP-1 cells and found that all tested allergens caused significant increased changes, while non-allergens did not (Miyazawa *et al.*, 2007). For ascaridol a dose-dependent increase of CD86, CD54, CD40 and HLADR was found on THP-1 cells after 24 h. The highest doses tested for ascaridol (80 and 120 µg/ml) yielded significant up-regulations of all four analyzed markers. Hence, ascaridol clearly induced the activation of THP-1 cells.

However, when THP-1 cells were treated with various chemicals, sensitizers primarily augmented the expression of CD54 and/or CD86 (Yoshida *et al.*, 2003; Sakaguchi *et al.*, 2007; Sakaguchi *et al.*, 2008). From these results, it was considered that measuring CD54 and CD86 expression on naive THP-1 cells could be utilized as an *in vitro* method for predicting sensitization which was termed human Cell Line Activation Test (h-CLAT). Moreover, after testing various known sensitizers (e.g. methylisothiazolinone, formaldehyde) in comparison

to non-sensitizers (e.g. methylparaben) Sakaguchi *et al.* (2007) suggested two threshold concentrations which may be used to categorize skin sensitization potency like the murine local lymph node assay (LLNA) estimated concentration that yield a three-fold stimulation (EC3) value. These corresponding values are the estimated concentration which gives a relative fluorescence intensity (RFI) of 150 for CD86 and a RFI of 200 for CD54. According to Sakaguchi *et al.* substances enhancing RFI values for CD86 over 150 and/or CD54 over 200 should be declared as sensitizers. Further on, they conducted an inter-laboratory study and found that this criteria resulted in an accuracy of 93 %, which confirms appropriate cut-off criteria for h-CLAT (Sakaguchi *et al.*, 2008). Following these criteria, only the ascaridol-enhanced CD86 expression (120 µg/ml ascaridol) can be regarded as significant in the present study, whereas the CD54 response missed the limited barrier tersely (evaluated RFI value of 198 ± 96 vs. 200). Regarding basal expression of CD54 on THP-1 cells, Miyazawa and colleagues determined that CD54 is expressed at low levels - even lower than CD86 and CD40 basal levels (Miyazawa *et al.*, 2007). In contrast, within the present study a distinctly higher basal level for CD54 was found. In average, 70 % of non-treated THP-1 cells expressed CD54 with an averaged relative mean fluorescence intensity (rMFI), which was 5-fold higher in comparison to rMFI values of CD86 and CD40. Due to the high background level, the changes of CD54 expression induced by ascaridol were established in a smaller response window. Therefore, further investigations are needed whether the proposed Sakaguchi's criterion for CD54 are applicable in our circumstances, and even concerning weak sensitizers in general.

However, ascaridol achieved one criterion in the prediction model, as it was also observed for metal allergens like nickel sulfate and cobalt sulfate, which lack to respond to CD86 on THP-1 cells and only augment CD54 expression (Yoshida *et al.*, 2003), hence ascaridol would be categorized as a sensitizer.

The use of THP-1 cells in respect to discriminating the potency of different allergens affords some problems. Allergens which showed different potencies in guinea pig maximization test, caused similar responses in THP-1 cells. For instance, both nickel sulfate, which is classified as moderate sensitizer, and cobalt sulfate, identified as an extreme sensitizer, induced similar CD86 expression on THP-1 cells (Ashikaga *et al.*, 2002). Moreover, hydroxycitronella, considered as a weak allergen in LLNA (Basketter *et al.*, 1994), strongly activated THP-1 cells for CD86, CD54, CD40 and IL-8 (Miyazawa *et al.*, 2007). Overall, the magnitude of TNFalpha and IL-8 production varied depending on the allergen and was also independent from the classification of allergens determined by LLNA (Miyazawa *et al.*, 2007). Thus, these observations indicate that the potency of an allergen cannot be deduced from THP-1 responses, but nevertheless the use of these cells to screen sensitizers is very promising. In-

deed, using THP-1 cells the sensitizing potential of ascaridol initially indicated by the use of MoDC is confirmed within the present study.

5.2.2 Ascaridol elicited cytokine release in THP-1 cells

Beside the induced expression of several markers, also the up-regulation of cytokine production has been shown upon maturation in THP-1 cells. TNFalpha and IL-8 secretion were markedly induced by DNCB and nickel sulfate in a dose-dependent manner in THP-1 cells (Miyazawa *et al.*, 2007). For ascaridol a dose-dependent production of IL-8 was determined. This is consistent with prior studies indicating that enhanced IL-8 release accompanying increased surface expression is at least necessary to discriminate and classify irritants and sensitizing agents (Coquette *et al.*, 2003; Toebak *et al.*, 2006). TNFalpha production induced by ascaridol was not found (data not shown). Just recently, Miyazawa and colleagues explored that the TNFalpha production induced by DNCB or nickel sulfate peaked at maximum within 6 and 12 h of incubation and then decreased (Miyazawa *et al.*, 2008b). Therefore, the determination of this cytokine secretion, especially in the case of ascaridol, should be further investigated at earlier times. Regarding molecular signalling, Miyazawa (2008) found that blocking p38 mitogen-activated protein kinase (MAPK) pathway selectively suppressed DNCB-induced TNFalpha release, whereas nickel sulfate-induced TNFalpha secretion was suppressed by inhibition of the extracellular-signal regulated kinase pathway (Miyazawa *et al.*, 2008a). Their data indicate that the augmentation of TNFalpha and co-stimulatory molecules expression is regulated by distinct MAPK pathways, depending on the stimuli. The present study shows that ascaridol also induced the phosphorylation of p38 MAPK in THP-1 cells. Based on the observed lack of TNFalpha release after 24 h and assuming that no TNFalpha production can be determined at earlier times, it can be conceived that maybe one of the several TNFalpha signalling pathways are blocked by ascaridol, but this aspect needs further investigations. Interestingly, it was shown that IL-8 expression in monocytes was driven, in part by nuclear factor (NF-)kappaB signal cascade (Gray and Pestka, 2007), hence the participation of NFkappaB pathway in ascaridol sensitization signalling needs further exploration and may provide information about signalling mechanisms induced by ascaridol.

5.3 Ascaridol - the sensitizer

By using two different *in vitro* test systems, ascaridol was classified as a sensitizer with probably weak potency within the present study. Personal care products containing oxidized tea tree oil are mainly applied onto the skin, so that the ingredients can penetrate and may be adsorbed by the body. Therefore, they can be considered as the most likely source for exposition with ascaridol. The oxidative conversion of alpha-terpinene, one compound of tea

tree oil, yields the epoxide ascaridol (Johnson and Croteau, 1984; Esser *et al.*, 1994). Thus, the examined immuno-modulatory impacts of ascaridol on DC comply with the observation that an increasing range of chemicals appears to be indirectly capable to cause skin sensitization as a result of their capacity to undergo air oxidation (Basketter *et al.*, 2002). For instance, it is concluded that auto-oxidation of the weakly allergenic linalyl acetate leads to formation of allergenic oxidation products (Skold *et al.*, 2008). Linalool, present in lavender oil, auto-oxidizes upon air exposure forming allergenic oxidation products (Skold *et al.*, 2002). Furthermore, oxidation products originating from e.g. P450-mediated oxidative metabolism provide a hint that skin sensitization reactions are caused not only by auto-oxidation but also by the metabolism of terpenes in skin tissues, as it was explored for linalool oxidation products (Meesters *et al.*, 2007). There are enzymes being capable of generating ascaridol from alpha-terpinene (Johnson and Croteau, 1984), thus it might be presumed that bacteria on the skin or metabolic competent skin cells themselves convert alpha-terpinene to ascaridol by oxidative metabolism. Subsequently, ascaridol may react with nucleophilic sites of skin proteins to form haptens, as it was shown for other epoxides (Bergstrom *et al.*, 2006), and thereby may elicit an allergic reaction. Although our data indicate that ascaridol is a weak sensitizer, it has to be pointed out that the sensitization potential can be potentiated by contemperaneous exposure with other compounds and that in real life chemical exposure usually involves several substances simultaneously, and therefore combinatory, perhaps additive effects should be considered. Due to the fact that TTO is a mixture of components, there is notionally the probability that any of its ingredients might have sensitizing potential like ascaridol. It can be conceived that ascaridol with its weak sensitization potential as it was confirmed within this study, may act as an enhancer in this context. The combined effect of several allergens and allergens with non-allergens are less characterized. Nilsson and coworkers demonstrated by using the allergen carvon that non-allergenic compounds with a structure not resembling haptens can reduce the sensitizing effect of haptens (Nilsson *et al.*, 2004). Whether the simultaneous stimulation of several allergens results in additive or reductive sensitizing effects is not known so far and therefore, further investigations regarding this issue are needed. Nevertheless, to determine whether a chemical is a sensitizer or not can successfully be explored on purified DC *in vitro* as it was performed for ascaridol with the MoDC *in vitro* system and the THP-1 h-CLAT assay.

5.4 Chemokine receptors as markers for sensitization prediction

Chemokines are important mediators of immune-mediated skin diseases such as allergic contact dermatitis. It is established that migration of DC from sites of inflammation to the lymph nodes is related to a balance in both chemokine receptor expression and chemokine production. CD184 (CXCR4) is constitutively expressed on human DC (Sallusto *et al.*, 1999).

More recent data indicate that CD184 engagement is important not only for maturation but also survival of DC. Consistently, the dinitrobenzene sulfonic acid-induced *in vitro* proliferation of previously sensitized lymph node cells was enhanced by CD184 ligand and suppressed by CD184 antagonist (Kabashima *et al.*, 2007). Only few attempts were done to evaluate the potential of this marker to discriminate between sensitizers and irritants. Rustemeyer and colleagues evaluated the applicability of CD184 on MoDC for a panel of sensitizing methacrylate congeners and suggested that CD184 was most sensitive in discriminating between the contact sensitizers and irritants (Rustemeyer *et al.*, 2003). However, within the present study a very slight basal surface expression was found on MoDC, and in contrast to LPS, ascaridol did not induce an up-regulation at both measured times (24 h and 96 h). Interestingly, an augmented expression of CD184 induced by ascaridol and DNCB were determined on THP-1 cells. Thus, the relevance of CD184 augmentation induced by different chemicals needs further examinations.

In addition, the changed expression of CCR2 on THP-1 was investigated. CCR2 is a chemokine receptor expressed on monocytes which recognizes the ligand monocyte chemoattractant protein-1 (MCP-1) (Berkhout *et al.*, 1997). When MCP-1 binds to CCR2, it induces a rapid increase in intracellular calcium concentration, increased expression of cellular adhesion molecules, cellular degranulation, and the promotion of leukocyte migration (Dawson *et al.*, 2003). It may also be involved in the regulation of the immune system through an effect on T cell polarization (Johnson *et al.*, 2004). CCR2 knock out mice show defects in macrophage recruitment and in production of Th1 cytokines, such as IFN-gamma (Boring *et al.*, 1997). Within this study a CCR2 up-regulation on THP-1 by DNCB and ascaridol was determined; even ascaridol increased the CCR2 expression in a dose-dependent manner. Concerning mechanisms, CCR2 up-regulation is associated with p38 MAPK phosphorylation in THP-1 cells (Ko *et al.*, 2007), thus p38 MAPK might be involved as well in ascaridol-induced CCR2 expression particularly with regard to the observed ascaridol-mediated activation of p38 MAPK. Hence, the response of CCR2 to ascaridol encourages to perform further investigations utilizing broad panels of contact allergens and irritants to explore whether chemokine receptors can be a tool to discriminate between sensitizers and irritants on THP-1 cells.

5.5 *In vitro* sensitization assays: MoDC versus THP-1 cells

The development of *in vitro* methods for predicting the sensitizing potential of new chemicals is of major importance for the reduction of animal testing and the maintenance of product safety. Several approaches, with different levels of complexity, have been described. Some of the most promising assays are based on the use of dendritic-like cell cultures in order to reconstitute the maturation process of Langerhans cells after exposure to sensitizers, by

monitoring activation markers. In the present study, the property of monocyte-derived dendritic cells (MoDC) and the monocytic cell line THP-1 (h-CLAT) to discriminate between skin sensitizers and non-sensitizers was used to investigate a presumably weak allergen, ascaridol. Both test systems use phenotypical alterations as predictive endpoints for contact sensitizers. For both test systems ascaridol induced changes in the phenotypical surface expressions. Whereas for h-CLAT system the determination of the surface markers CD86 and CD54 is recommended for the discrimination between irritants and strong to moderate allergens (Sakaguchi *et al.*, 2007), for MoDC the changed expression of CD86, CD54 and HLADR was suggested to be useful (Tuschl *et al.*, 2000). For both test systems the incubation of 24 h was proposed. In the case of ascaridol the analysis of these features would be sufficient in order to determine its sensitizing potential effectively, although the analysis of MoDC at late maturation yielded more information about the characterization of ascaridol's immuno-modulating impact (e.g. T cell polarization). Overall, ascaridol elicited an augmented CD86 expression in both *in vitro* test systems accompanied with other altered surface expression of several activation markers. While CD54 expression was increased in dose-dependent manner on THP-1 cells after ascaridol treatment, it failed to gain the minimum requirement according to Sakaguchi *et al.* (2007). In MoDC, several responses to CD54 augmentation were found. The observed donor variability in the response of MoDC to ascaridol presumably originates in the individual differences in immune reactivity. On the one hand this finding highlights the use of MoDC which better converge with the *in vivo* observed response to chemicals, but on the other hand it complicates a useful and simple analysis with respect to drawbacks in reproducibility and standardization. Thus, chemicals induced responses differing in quality and quantity, the classification whether a chemical is a strong or a weak sensitizer seems to be complicated and must deduce very carefully on how many surface expression markers are influenced and how strong the augmentation is. Admittedly, the classification can be achieved by including chemicals with proved potency in experimental series and comparing the responses, although the experimental approach still struggles with donor variability. However, consistent with Staquet *et al.* (2004) the present study demonstrated that both the consideration of many activation markers and a prolonged time of incubation substantially improve the identification of weak or mild allergens. Furthermore, the combination of measuring alteration of surface expression molecules and determination of cytokines in cell culture supernatants provides the minimum requirement to avoid false positive as well as false negative results.

However, the observed response of THP-1 cells to ascaridol enhanced the use of this DC surrogate for simple, short-term *in vitro* sensitization testing. The nonexistence of a standardized protocol for generating DC-like cells and the inherent variability of immature DC obtained from human donors support the use of THP-1 cells as a good alternative. Although

THP-1 cells may have a relatively heterogeneous composition in terms of the differentiation stage of its cellular components (Bocchietto *et al.*, 2007), compared to DC they have the advantage of being easy to obtain, being less expensive and much easier to be cultured, thus providing sufficient numbers of cells so that the test design is not limited. It is important that test protocols using cell lines are robust enough so that the impact of any lot-to-lot variability on the performance of the assay is minimized. It is also necessary to consider the use of methods to assess different batches of cells (with respect to both the growth characteristics and performance within the assay) prior to their use. The present study shows that although a different basal level of CD54 on THP-1 cells was found in comparison to published data, the impact of DNCB was reproducible.

While MoDC seem to be more sensitive, and therefore may be more precise to distinguish between mild, moderate, and strong sensitizers, only in THP-1 cells a dose-response analysis for ascaridol was possible due to the strict intolerance of MoDC to higher concentrations of ascaridol. Although MoDC of some donors were exposed to higher ascaridol concentration (15, 20 and 40 µg/ml), only controversial results were obtained (data not shown). Only few viable cells were found and due to the possibility that dead or dying cells can yield false positive or false negative responses (Hulette *et al.*, 2002), further investigations were quitted. Hence, the present study emphasizes the investigation of sensitization potential at sub-cytotoxic concentrations, although it has been suggested that cytotoxicity is needed to gain successful immune responses (Hulette *et al.*, 2005; Sakaguchi *et al.*, 2007). Within the present study responses for ascaridol in both THP-1 cells and MoDC were found at sub-cytotoxic concentrations (dead cells between 10 - 25 %). This is consistent with the results of Hulette and colleagues (2005) and Miyazawa and co-workers (2007), who reported that up-regulation of CD86 expression in MoDC and THP-1 cells, respectively, was observed when cells were exposed to allergens at concentrations inducing 10 - 15 % cytotoxicity. Furthermore, skin irritants were able to activate MoDC in concentrations that were cytotoxic and yielded more than 50 % of dead MoDC (Straube *et al.*, 2005). Therefore, a DC-based assay for contact allergens should be performed at sub-cytotoxic concentrations. This subject is still controversially discussed, hence, even for strong contact allergens tested, the effective concentrations were very close to cytotoxic concentrations. Up to the present it is not clear whether chemicals themselves can modulate phenotypical changes in immune cells, or whether the alterations are caused due to their toxicity inducing danger signals. The *in vivo* effect of skin irritations, which may appear as cytotoxicity *in vitro*, is closely linked to the immunogenic potency of chemicals (Straube *et al.*, 2005). Contact allergens show a higher *in vivo* sensitizing potential if they cause strong irritation simultaneously. Conversely, weaker contact sensitizers have a lower irritating potential (Ulrich *et al.*, 2001). Therefore, in MoDC

and THP-1 cells, a close association between cell activation and cell toxicity might exist and be allergen specific.

The main shortcomings of both experimental approaches are the necessity to include a metabolizing system for chemical prohaptens which need activation by abiotic or biotic means to become immunogenic. Hence, the described *in vitro* methods have their endpoint specific events that occur during skin sensitization, there is a concern that the relative loss of biological complexity in these assays (when compared with the holistic *in vivo* process) may result in reduced predictive accuracy and a compromised ability to determine potency. It is necessary to explore the extent of the metabolic activity of DC and THP-1 and whether this is sufficient for the routine identification of prohaptens. However, there are approaches integrating these aspects such as co-culture experiments including the participation of e.g. keratinocytes which may be involved in the proteinization process of prohaptens (Vandebriel *et al.*, 2005; Bergstrom *et al.*, 2007; Schreiner *et al.*, 2007), but these need further investigations.

In conclusion, it is clear that the use of cell lines as surrogate DC in the development of *in vitro* test methods offers advantages compared with primary cultures of DC from peripheral blood. Within the present study, the h-CLAT assay was useful as short-term assay as suggested by Yoshida *et al.* (2006), while the MoDC assay yielded more information about immuno-modulating impact at long term incubation as specified before. However, both test systems classified ascaridol as sensitizer. Thus, the direct *in vivo* verfication of this observation by current chosen methods to assess skin sensitizing potency such as the murine LLNA is required imperatively.

It is clear that accurate characterisation of skin sensitization hazards will require the effective integration of various sources of information. Consequently, the combination of chemical assays with *in vitro* techniques may provide a useful surrogate to animal testing for skin sensitization, but there remain significant challenges to overcome for potency assessment. More comprehensive investigations are needed to confirm the robustness and resilience of *in vitro* methods and hence, to expand their acceptance. Due to the continuously changing environmental conditions, it is necessary to regularly monitor and update the spectrum of sensitizers that elicit contact dermatitis. Therefore, both debated *in vitro* test systems will become indispensable tools. The use of cosmetics and medical formulations, which are applied directly onto the skin, is increasingly widespread and due to this intensive exposure, even ingredients with rather moderate sensitizing potential need to be considered. In this context the presumable weak allergen ascaridol was clearly identified as sensitizers in both *in vitro* systems MoDC and THP-1 cells by screening its immuno-modulating effects.

6. Summary

The skin is continuously challenged by environmental antigens that may penetrate and elicit a skin sensitization, which can develop into allergic contact dermatitis. Medical treatment for allergic contact dermatitis is limited - in fact only acute symptoms can be cured and for secondary prevention of the disease a lifelong avoidance of the allergen(s) is necessary. Therefore, the screening of the sensitization potential of substance used in commercially available products is indispensable to prevent such diseases. Hence, risk assessment is deduced from data obtained by murine local lymph node assay predominantly, but there exists a need to develop methods capable of providing the same information that do not require the use of animals in view of legislative initiatives such as REACH (registration, evaluation, authorization of chemicals) as well as the 7th Amendment to the Cosmetics Directive (2003/15/EC). Therefore, a number of promising *in silico* and *in vitro* approaches are being developed to address this need. *In vitro* test systems using the response of dendritic cells, which are the key player in the elicitation process of contact dermatitis, are established, but, although these novel methods for hazard identification might find application in the context of screening, it is not clear whether these approaches are useful for the purposes of risk assessment and risk management to predict allergic potency. Therefore, it was investigated whether on the one hand *in vitro* generated dendritic cells from primary blood monocytes (MoDC) and on the other hand a continuous monocytic cell line, the THP-1 cells, suggested as dendritic cell surrogate, react to a presumably weak allergen. Ascaridol, predicted as one of the possible causes for tea tree oil contact dermatitis, was studied and its effects in these two *in vitro* skin sensitization models were explored. Thus, the surface expression of CD86, HLADR, CD54, and CD40, which are known as activation markers in both *in vitro* models, were measured via flow cytometry. For MoDC, an augmented CD86 and HLADR surface expression in comparison to untreated cells were determined after 24 h exposure with ascaridol. An increased CD54 and CD40 surface expression were found only in some donors. After long term incubation of 96 h, ascaridol-treated MoDC still up-regulated CD86 and additionally an augmented CD40 expression was measured in all studied donors. An enhanced CD54 expression was determined for 50 percentage of all investigated donors. Furthermore, CD80, CD83 and CD209 protein expression were up-regulated in MoDC after 96 h of ascaridol incubation. In addition, it was determined that after 24 h ascaridol-treated MoDC showed an increased capacity to uptake antigens, whereas after 96 h this capacity got lost and antigen-capturing devices were reduced in comparison to non-treated MoDC. Moreover, the cytokine release of ascaridol-treated MoDC were measured after 24 h. Tumor necrosis factor (TNF)alpha, interleukin (IL)-1beta and IL-6 secretion were determined in some donors. Furthermore, IL-8 release was clearly increased after 24 h ascaridol treatment. Secretion of cytokines such as IL-

12 and IL-10, which play a role in T cell polarization, were not found. By the same token, THP-1 cells were analyzed after ascaridol treatment for several activation markers. We found a similar response pattern as measured in MoDC. Ascaridol induced CD86 expression as well as CD54 after 24 h incubation. Additionally, the impact of ascaridol on phosphorylation of p38 mitogen-activated protein kinase, which had been shown to be involved in increased expression of activation markers like CD86 by others, were studied via Western blot analysis. A phosphorylation of p38 was determined after 15 min of ascaridol stimulation. Moreover, an augmented CD40 and HLADR surface expression were measured in a dose-response manner after 24 h ascaridol treatment. Also similar to MoDC an enhanced IL-8 secretion after ascaridol stimulation was observed in THP-1 cells. Hence, for the first time it was shown that ascaridol has immuno-modulating effects. The obtained data from both *in vitro* systems, MoDC and THP-1 cells, identified ascaridol as a sensitizer. Although for both systems there remain significant challenges to overcome for potency assessment, ascaridol is presumed to be a weak sensitizer probably. Interestingly, ascaridol treatment of THP-1 cells resulted also in an increased augmentation of CD184 and CCR2, two chemokine receptors expressed on monocyte. Therefore, these data encouraged the exploration of chemokine receptors as tools in skin sensitization prediction. Consequently, the combination of chemical assays with *in vitro* techniques may provide a useful surrogate to animal testing for skin sensitization. Due to the continuously changing environmental conditions, it is necessary to regularly monitor and update the spectrum of sensitizers that elicit contact dermatitis. Therefore, both debated *in vitro* test systems will become indispensable tools.

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9. Declaration

Hereby, I declare that the presented thesis *The impact of ascaridol on human monocyte-derived dendritic cells and on the monocytic leukemia cell line THP-1* is my own work and that it has not been submitted to any other university for any degree or examination.

Hiermit erkläre ich, dass die vorliegende Arbeit *The impact of ascaridol on human monocyte-derived dendritic cells and on the monocytic leukemia cell line THP-1* selbständig von mir verfasst wurde und bisher weder im ganzen noch in Teilen in dieser Fakultät oder einer anderen akademische Institution eingereicht worden ist.

Corinna Tietze

10. Curriculum Vitae

name	Corinna Tietze
address	Heinrich-Luebke-Str. 32; 54296 Trier
date of birth	11.02.1981
place of birth	Leipzig
nationality	German
School	
1987-1992	37. Polytechnische Oberschule, Leipzig
1992-1999	Neues Nikolai Gymnasium Leipzig university-entrance diploma
University	
1999-2004	University of Leipzig academic studies of biochemistry
2004	diploma thesis: Dimerisation studies of the hY1- and hY5-receptors
since 2005	Universität Trier Faculty of Geography/Geosciences Department Ecotoxicology/Toxicology doctoral thesis: The impact of ascaridol on human monocyte-derived dendritic cells and on the monocytic leukemia cell line THP-1

11. Summary in German

Täglich ist unsere Haut verschiedensten Umweltreizen ausgesetzt. Substanzen mit kleinem Molekulargewicht u. a. aus kosmetischen Produkten, Arzneimitteln, Kleidung und anderen Gegenständen des alltäglichen Umganges stammend, können durch die Haut in den Körper eindringen und Hautkrankheiten hervorrufen. Die Kontaktallergie ist dafür das bekannteste Beispiel. Sie äußert sich in Entzündungsreaktionen wie Rötungen, Ödeme, Bläschen, Schuppen, Knötchen oder Hautabschälungen. Eine Heilung für Patienten gibt es bislang nicht. Die Vermeidung der entsprechenden Allergene ist bisher die erfolgreichste Strategie, um das Auslösen dieser Krankheit zu verringern. Daher ist das Erkennen von Kontaktallergie auslösenden Substanzen entscheidend für einen gesicherten Verbraucherschutz. Bislang werden die Hautsensibilisierungspotenziale von chemischen Stoffen durch Tierversuche wie dem lokalen Lymphknoten-Test (*local lymph node assay* = LLNA) in der Maus oder dem Meerschweinchen-Maximierungstest validiert. Im Zuge der neuen europäischen Chemikalienverordnung REACH und der Europäischen Direktive vom 15. März 2003 gibt es große Bestrebungen *in vitro* Systeme zu entwickeln, aus denen die gleiche Information abgeleitet, aber die Anzahl der Tierversuche verringert werden kann. Die dendritische Zelle, welche die durch die Haut eindringenden Substanzen zu erkennen vermag, nimmt bei der Entwicklung einer Kontaktdermatitis eine Schlüsselposition ein. Die Reaktion der dendritischen Zelle auf eine Substanz entscheidet, ob sich eine Kontaktdermatitis bildet oder nicht. Daher basieren einige *in vitro* Systeme zur Validierung von Sensibilisierungspotenzialen auf *in vitro* generierten dendritischen Zellen. Es wird zum Beispiel der Einsatz von aus Blutmonozyten abgeleitete dendritische Zellen, so genannte MoDC, oder adäquate Ersatzzellen wie die Monozytenzelllinie THP-1 untersucht. Beide Zellmodelle können bekannte, stark sensibilisierende Chemikalien von Reizstoffen unterscheiden. Aber ob diese *in vitro* Modelle über eine ausreichende Sensitivität verfügen, die das Erfassen von schwachen und milden Sensibilisierungspotentialen von Allergenen ermöglicht, bedarf weiterer Forschung. Daher wurden beide Systeme herangezogen, um ein schwaches Allergen zu charakterisieren. Die Reaktion auf Ascaridol, eine Substanz, die im Zusammenhang mit Teebaumöl-Kontaktdermatitis diskutiert wird, wurde im Folgenden analysiert. Dazu wurde einerseits die Ausprägung von Oberflächenproteinen bestimmt, die eine Aktivierung der dendritischen Zelle anzeigen, und andererseits die Ausschüttung von pro-inflammatorischen Mediatoren gemessen. Eine 24-stündige Exposition von MoDC mit Ascaridol resultierte in einer erhöhten Expression von kostimulierenden Molekülen wie CD86 und HLADR. Des Weiteren wurden im Vergleich zu unbehandelten Zellen in einigen Spendern erhöhte Konzentrationen von Tumornekrosefaktor (TNF)alpha, Interleukin (IL)-1beta sowie IL-6 gefunden. Für alle untersuchte Spender wurde auch eine höhere IL-8 Konzentration gemessen. Es konnten keine erhöhten Konzentrationen

der Zytokine IL-12p75, IL-12p40 und IL-10, welche eine tragende Rolle in der T Zellpolarisierung spielen, nachgewiesen werden. Im Weiteren wurde zur Untersuchung der späten Reifung der dendritischen Zellen eine 96-stündige Inkubation mit Ascaridol durchgeführt. Die gesteigerte CD86 Expression wurde wiedergefunden, begleitet von einer verstärkten Expression von CD80, CD40,CD209 und CD83. Die Hälfte aller untersuchten Spender zeigte sowohl nach 24 als auch nach 96 Stunden eine erhöhte CD54 Expression. Demnach konnte also bereits nach einer 24-stündigen Stimulation mit Ascaridol eine Aktivierung der MoDC festgestellt werden. Einer längeren Inkubation mit Ascaridol folgte ein verstärkter immunmodulatorischer Einfluss, der sich in der gesteigerten Expression von weiteren Aktivierungsmarkern widerspiegelte.

Die Stimulation von THP-1 Zellen mit Ascaridol resultierte in einer Dosis-abhängigen erhöhten Expression von CD86 and CD54. Des Weiteren wurde auch eine erhöhte Expression von CD40 und HLADR gefunden. Ebenfalls wurde im Vergleich zu unbehandelten Zellen eine höhere Konzentration von IL-8 in Zellkulturüberständen gemessen. Damit kann Ascaridol auch in THP-1 Zellen eine Aktivierung auslösen und verglichen mit den von Sakaguchi *et al.* (2007) etablierten Kriterien für THP-1 Zellen als Testsystem zur Erkennung von Chemikalien mit sensibilisierenden Eigenschaften, als Sensibilisierer eingestuft werden. Da die Reaktion auf Ascaridol in beiden *in vitro* Systemen nicht zur vollständigen Aktivierung der jeweiligen Zellen führte, sollte Ascaridol als schwacher Sensibilisierer klassifiziert werden. Die vorliegende Studie zeigt auf, dass beide *in vitro* Modelle auch schwache Sensibilisierer erkennen können und ermutigt daher die Erforschung dieser Modelle als mögliche Testsysteme zur Vorsortierung von Substanzen mit Sensibilisierungspotential bei der Erfassung von neuen Chemikalien.

12. Publications

Tietze C and Bloemeke B (2008) Sensitization assays: Monocyte-derived dendritic cells versus a monocytic cell line (THP-1); *J Toxicol Environ Health Part A*, **71**, 965-968