



Assessment of the U937 cell line for the detection of contact allergens

François Python ^{a,*}, Carsten Goebel ^b, Pierre Aeby ^a

^a Experimental Product Safety, The Procter and Gamble Co., Wella-Cosmital SA, Marly, Switzerland

^b Human Safety Assessment, The Procter and Gamble Co., Wella Service GmbH, Darmstadt, Germany

Received 3 July 2006; revised 19 December 2006; accepted 26 December 2006

Abstract

The human myeloid cell line U937 was evaluated as an *in vitro* test system to identify contact sensitizers in order to develop alternatives to animal tests for the cosmetic industry. Specific culture conditions (i.e., presence of interleukin-4, IL-4) were applied to obtain a dendritic cell-like phenotype. In the described test protocol, these cells were exposed to test chemicals and then analyzed by flow cytometry for CD86 expression and by quantitative real-time reverse transcriptase-polymerase chain reaction for IL-1 β and IL-8 gene expressions. Eight sensitizers, three non-sensitizers and five oxidative hair dye precursors were examined after 24-, 48- and 72-h exposure times. Test item-specific modulations of the chosen activation markers (CD86, IL-1 β and IL-8) suggest that this U937 activation test could discriminate test items classified as contact sensitizers or non-sensitizers in the local lymph node assay in mice (LLNA). More specifically, a test item can be considered as a potential sensitizer when it significantly induced the upregulation of the expression of at least two markers. Using this approach, we could correctly evaluate the dendritic cell (DC) activation potential for 15 out of 16 tested chemicals. We conclude that the U937 activation test may represent an useful tool in a future *in vitro* test battery for predicting sensitizing properties of chemicals.

© 2007 Elsevier Inc. All rights reserved.

Keywords: CD86; IL-1 β ; IL-8; U937 cell line; *In vitro* sensitization test

Introduction

Allergic contact dermatitis (ACD), a delayed-type hypersensitivity reaction induced following skin contact with allergens of low molecular weight (Lepoittevin et al., 1998; Smith and Hotchkiss, 2001), has become a major public health problem worldwide. During the sensitization phase of ACD, immature dendritic cells (DCs) of the skin (Langerhans cells) pick up and process potential allergens penetrating the skin barrier and become activated. DCs then secrete cytokines such as interleukin-1 β (IL-1 β) (Enk and Katz, 1992; Reutter et al., 1997; Lepoittevin et al., 1998; Pichowski et al., 2000; Aeby et al., 2004) and express co-stimulatory molecules such as CD86 (Lepoittevin et al., 1998; Tuschl et

al., 2000; Aeby et al., 2004). After migration into lymph nodes, mature DCs present the processed allergens to naive T cells. If a T cell recognizes the processed allergen presented on the mature DCs and receives the required secondary signals (e.g., CD86), it becomes activated, proliferates and begins circulating in the body. In case of renewed application of the contact allergens, skin DCs and other antigen-presenting cells will present the allergens directly to specific T cells, which then proliferate and orchestrate an inflammatory response, leading to ACD (elicitation phase) (Banchereau and Steinman, 1998).

The sensitizing potential of new chemicals is usually evaluated in animal tests like the widely used Murine Local Lymph Node Assay (LLNA) (Gerberick et al., 2004; Kimber et al., 2002; Basketter et al., 2002; Dearman et al., 1999). Nevertheless, the increasing interest of the public for reducing animal tests and the industry requirement to respond to the 7th Amendment to the EU Cosmetics Directive are driving the need to develop alternatives to animal tests. Furthermore, an *in vitro* sensitization test system based on the activation of DCs will

* Corresponding author. Rte de Chésalles 21, CH-1723 Marly, Switzerland.
Fax: +41 26 435 26 66.

E-mail address: python.f@pg.com (F. Python).

increase our understanding of the complex *in vivo* mechanism. Playing a key role in the initiation of ACD, DCs were readily proposed and identified as promising candidates for the development of *in vitro* test systems for the detection of potential sensitizers (reviews: Casati et al., 2005; Vandebriel et al., 2005). Recently, our group (Aeby et al., 2004) and others (Ryan et al., 2004; Staquet et al., 2004; Straube et al., 2005; Jugdé et al., 2005; Hulette et al., 2005) reported promising results obtained with human peripheral blood monocyte-derived dendritic-like cells (PBMDs) following exposure to chemical allergens. However, PBMDs major drawbacks are their complex and expensive preparation procedures and their inherent donor-to-donor variability (review: Ryan et al., 2005). As a possible alternative, human myeloid leukemia cell lines represent appropriate candidates as DC surrogate. Several studies demonstrated that the human myeloid cell lines THP-1, KG-1, U937 and MUTZ-3 have the capacity to differentiate towards a DC-like phenotype following cytokine treatment (Koss et al., 1996; Hu et al., 1996; St Louis et al., 1999; Hulette et al., 2001, 2002; Yoshida et al., 2003; Masterson et al., 2002). Ashikaga et al. (2002) evaluated CD86 expression and MHC class II molecule internalization in THP-1 cells after exposure to sensitizers. They showed that some sensitizers induced an increase of CD86 expression after 24-h exposure and an internalization of MHC class II molecules after 2 h. Yoshida et al. (2003) demonstrated that immature THP-1 cells respond to sensitizers by modulating the expression of CD54 and CD86 surface markers. Recently, the MUTZ-3 cell line has also been evaluated as an *in vitro* test system for the screening of contact sensitizers by monitoring the expression of HLA-DR, CD54 and CD86 by flow cytometry (Azam et al., 2006).

In the present study, we evaluated the human myeloid cell line U937 as a source of DC-like cells. We assessed the biological response of this monocytic cell line in an *in vitro* test protocol called the “U937 activation test” based on the measurement of selected activation markers. U937 cells were exposed to 16 test items for 24, 48 and 72 h and the following markers were analyzed: surface expression of CD86 by flow cytometry and IL-1 β and IL-8 mRNA expression by quantitative real-time reverse transcriptase-polymerase chain reaction (RT-PCR) analysis. CD86 and IL-1 β have already been shown to be relevant DC activation markers (Aiba et al., 1997; Enk and Katz, 1992; Reutter et al., 1997; Aeby et al., 2004), whereas the chemokine IL-8 is involved in the inflammation process (reviews: Feghali and Wright, 1997; Rollins, 1997; Scapini et al., 2000). The expression of other genes such as ARHGDI (L γ -GDI), AKR1C3 (HDD2) and NOTCH3 identified by a genomic study (Ryan et al., 2004), or Aquaporin 3 (AQP3), known to be downregulated during the sensitization phase of ACD (de Baey and Lanzavecchia, 2000; Aeby et al., 2004) was also examined by quantitative real-time RT-PCR.

Finally, we have performed a statistical evaluation of the limited *in vitro* dataset obtained in the kinetic studies. Altogether, our results suggest that the described U937 activation test may be a useful tool in an integrated strategy to

evaluate new chemicals for their sensitizing potential, and thus may contribute to reduce the number of animal tests.

Materials and methods

Human cell line and complete culture medium. U937 cells (ATCC cat. no. CRL-1593.2) were ordered in 2001 at the American Type Culture Collection (ATCC, Manassas, USA) and obtained through LGC Promochem (Molsheim Cedex, France). The cells were cultured in RPMI 1640 medium without phenol red (RPMI; Fluka, Buchs, Switzerland) supplemented with 2 mM glutamine (PAA Laboratories GmbH, Coelbe, Germany), 10 mM HEPES (Fluka, Buchs, Switzerland), 1 mM sodium pyruvate (Gibco, Basel, Switzerland), 4.5 g/l glucose (Merck, VWR International SA, Le Lignon, Switzerland), 100 U/ml penicillin–100 μ g/ml streptomycin (PAA Laboratories GmbH, Coelbe, Germany) and 10% fetal calf serum (without heat inactivation) (FCS, Amimed, BioConcept, Allschwil, Switzerland). Cells were passaged for a maximum of 4 weeks.

Modified culture medium. The modified culture medium was the complete culture medium without antibiotics, and containing up to 50 pg/ml IL-4 (cat. no. rhIL-4CC, Strathman Biotech GmbH, Hannover, Germany) to induce the dendritic cell-like phenotype. The in use concentration of IL-4 was adjusted for each lot of cytokine such that IL-4-treated U937 cell population contains between 5 and 12% CD86-positive cells after 48 h.

Tested chemicals. Tested chemicals were dissolved either in water (and then diluted in RPMI basal medium) or in dimethyl sulfoxide (DMSO) when insoluble in water. 2,4,6-Trinitrobenzenesulfonic acid (TNBS) and sodium dodecyl sulfate (SDS) were used as model sensitizer and irritant, respectively. Methyl salicylate (MS) and hexadecyltrimethylammonium bromide (CTAB) were also examined as non-sensitizers. 2,4-Dinitrochlorobenzene (DNCB), oxazolone, eugenol, citral, 2-mercaptobenzothiazole (MBT) and α -hexylcinnamaldehyde (HCA) were tested as sensitizers, and nickel(II) sulfate hexahydrate (NiSO $_4$) as an example of a metal sensitizer. DMSO, sensitizers and non-sensitizers were purchased from Fluka (Buchs, Switzerland), except NiSO $_4$ that was obtained from Applichem GmbH (Darmstadt, Germany). Moreover, five oxidative hair dye precursors of documented purity (Wella production, Hünfeld, Germany) were examined: 4-amino-2-hydroxytoluene (AHT), 3-methyl-4-aminophenol (MAP), 2-amino-5-methylphenol (AMP), hydroxyethyl-*p*-phenylenediamine (HE-PPD) and *p*-toluylendiamine (PTD). TNBS, SDS, CTAB, NiSO $_4$, and HE-PPD were dissolved in water/RPMI. PTD was dissolved in RPMI containing ammonia (final ammonia concentration: 0.00025%) (Merck, Darmstadt, Germany). MS, DNCB, oxazolone, eugenol, citral, MBT, HCA, AHT, MAP and AMP were dissolved in DMSO (final in-well DMSO concentration: 0.25%).

U937 activation test protocol. 250,000 cells were seeded in each well of a 12-well plate (Falcon, Milian, Plan-les-Ouates, Switzerland) containing 2 ml of modified culture medium. After 2 to 4 h at 37 °C with 5% CO $_2$, test items were added into wells. For chemicals dissolved in DMSO, the final DMSO concentration was 0.25%. The negative control (nc) corresponds to the cells treated with the relevant vehicle. Three negative control wells were usually prepared for each time point. After incubating at 37 °C with 5% CO $_2$ for 24, 48 or 72 h (without culture medium change), cells were harvested for flow cytometric and quantitative real-time RT-PCR analyses. The percentage of CD86-positive cells was then measured by flow cytometry and IL-1 β /IL-8 gene expressions analyzed by quantitative RT-PCR with the LightCycler system (Roche Diagnostics GmbH, Rotkreuz, Switzerland).

Labeling and flow cytometric analysis. Fluorescein isothiocyanate (FITC)-conjugated anti-CD86 antibody (mouse IgG1, clone 2331 FUN-1, cat. no. 555657, BD Biosciences, Basel, Switzerland) and the corresponding isotype control (mouse IgG1-FITC, cat. no. 555748, BD Biosciences, Basel, Switzerland) were used. Cell suspensions were diluted 1:1 (400 μ l/400 μ l) in Isoton II solution (Beckman Coulter, Nyon, Switzerland) and incubated 30 min at 4 °C with 8 μ l antibody. After centrifugation (250 $\times$ g, 5 min, 4 °C), cells were resuspended in 800 μ l Isoton II solution at 4 °C and analyzed on a Coulter Epics

XL flow cytometer (Beckman Coulter, Nyon, Switzerland). For each sample, 5000 to 10,000 cells were analyzed. The forward scatter (FS) discriminator was set to channel 25 to exclude debris. Cell viability was determined with the fluorescein diacetate staining (50 ng/ml) (FDA; Fluka, Buchs, Switzerland) method at 4 °C by measuring the percentage of positive cells after 20-min incubation (Aeby et al., 2004). The viability result was expressed as the percentage (%) of the respective negative control (nc, absolute viability of nc is usually 85–90%) and was calculated by the following equation:

$$\text{Viability (\% of nc)} = \frac{\text{Viability \% in treated cells}}{\text{Viability \% in nc}} \times 100$$

Data analysis was performed with the WinMDI 2.8 software (Joseph Trotter, <http://facs.scripps.edu/software.html>). The region R1 and the marker M1 were defined as follows: in the control cell population (without treatment), we selected the homogeneous living cell subpopulation (called region R1) in the side versus forward scatter dotplot (Fig. 1a) and the marker M1 was set to include 0.5% of the isotype control stained cells from R1 (Fig. 1b). Then we selected the homogeneous subpopulation (R1) and the marker M1 to measure the percentage of CD86 and isotype control stained cells for each test item (Figs. 1b and c). The CD86 result was expressed as percent of the respective nc and was calculated by the following equation:

$$\text{CD86 (\% of nc)} = \frac{\text{CD86 pos cells \% in treated cells} - \text{Isotype control pos cells \% in treated cells}}{\text{CD86 pos cells \% in nc} - \text{Isotype control pos cells \% in nc}} \times 100 \text{ (pos cells : positive cells)}$$

Remark. For the test item AMP, the marker M1 was set to 0.5% in the isotype control stained cells from AMP-treated population because strong autofluorescence was observed (>30%).

RNA extraction and reverse transcription. Total RNA was isolated from cell pellets with the TRIzol method (Invitrogen AG, Basel, Switzerland) according to the manufacturer's instructions. Briefly, the cells were pelleted by centrifugation at 450×g for 5 min at 2–8 °C. Subsequent reactions took place at 2–8 °C. The pellet was resuspended in the TRIzol reagent (400 µl/10⁶ cells), homogenized by pipetting vigorously and briefly vortexed. After addition of chloroform (Fluka, Buchs, Switzerland) (80 µl/10⁶ cells) the sample was vortexed, incubated for 5 min and centrifuged at 12,000×g for 30 min. The aqueous phase containing RNA was transferred to a new tube and RNA precipitated with 1 volume isopropanol for at least 45 min (Fluka, Buchs, Switzerland). The pellet was washed with 75% ethanol (Fluka, Buchs, Switzerland) and resuspended in 50 µl TE buffer (Amresco, BioConcept, Allschwil, Switzerland). The RNA was quantified by OD260 measurement and adjusted to 50 µg/ml. The purity of RNA was assessed by measurement of OD260/280 ratio. 450 ng of purified total RNA was reverse transcribed using random hexamers (50 ng/reaction) with the ThermoScript RT-PCR system (Invitrogen AG, Basel, Switzerland), according to the instruction manual.

Quantitative polymerase chain reaction (real-time PCR). The 28S ribosomal RNA (28S rRNA) cDNA was quantified through real-time PCR on the LightCycler with the kit «LightCycler-FastStart DNA Master SYBR Green I» (Roche Diagnostics, Rotkreuz, Switzerland). The IL-1β, IL-8, NOTCH3, HDD2, Ly-GDI and AQP3 cDNAs were quantified with the kit «LightCycler-FastStart DNA Master Hybridization Probes» (Roche Diagnostics, Rotkreuz, Switzerland), using the probes purchased from Applied Biosystems (Rotkreuz, Switzerland) through the website <http://www.appliedbiosystems.com>.

SYBR green real-time PCR. The oligonucleotide primers for 28S rRNA (GenBank accession no.M11167) were 5'-CGGTACACCTGTCAAACGGTAAC-3' and 5'-TTAGAGGCGTTCAGTCATAATCCC-3' (Microsynth GmbH, Balgach, Switzerland). The PCR was performed in glass capillaries for 40 cycles according to the manufacturer's instructions at a final MgCl₂ concentration of 4 mM. A cycle consisted of a denaturation for 15 s at 95 °C, an annealing for 5 s at 65 °C and an elongation at 72 °C for 21 s. The SYBR green fluorescence was detected at the end of each elongation cycle. The specificity of

the amplification products was checked by the melting curve analysis. The quantification data were then analyzed with the LightCycler analysis software using the second derivative maximum method.

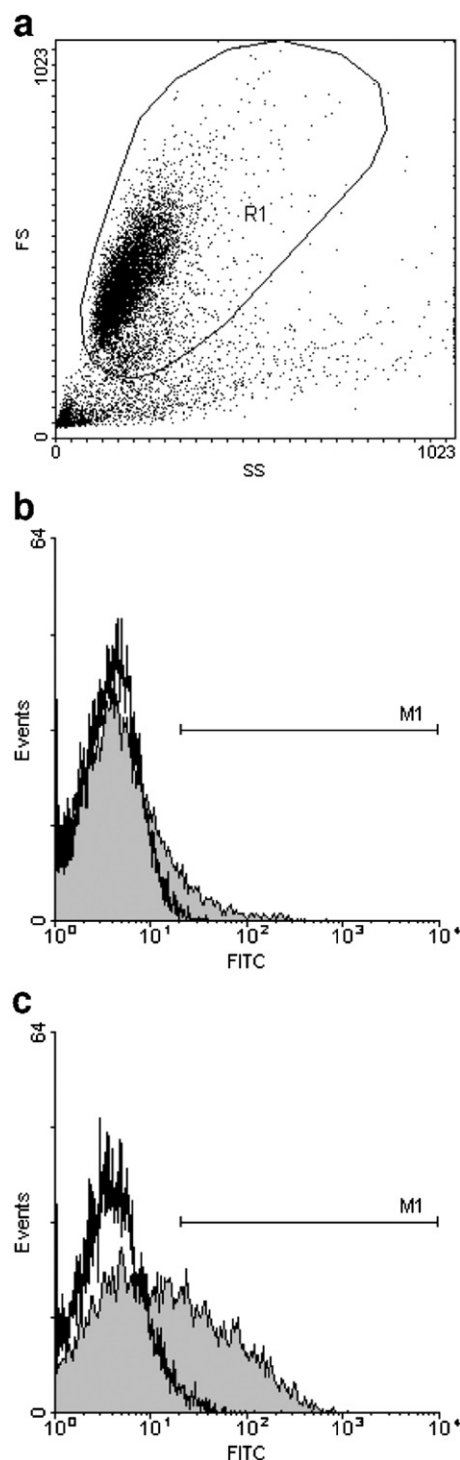


Fig. 1. Cytometric analysis of U937 cells. (a) Linear side scatter (SS) versus linear forward scatter (FS) dotplot of negative control cells (nc). The region R1 defines the gated homogeneous living cell population analyzed in the log FITC histogram. (b) Log FITC histograms of nc labeled with isotype control antibody (black histogram) or with anti-CD86 (grey filled histogram). (c) Log FITC histograms of cells exposed to TNBS labeled with isotype control antibody (black histogram) or with anti-CD86 (filled grey histogram). The percent of CD86 and isotype control positive cells located in the marker M1 were measured for each test item. The results were expressed as the percent of the respective nc.

TaqMan probes real-time PCR. The following TaqMan probes (TaqMan Gene Expression Assays) were used: IL-1 β (Assay ID: Hs00174097_m1), IL-8 (Assay ID: Hs00174103_m1), NOTCH3 (Assay ID: Hs00166432_m1), AKR1C3 (HDD2) (Assay ID: Hs00366267_m1), ARHGDI (Ly-GDI) (Assay ID: Hs00171288_m1) and AQP3 (Assay ID: Hs00185020_m1). The PCR was performed in glass capillaries at a final MgCl₂ concentration of 3 mM for 50 cycles. A cycle consisted of a denaturation for 10 s at 95 °C, an annealing/elongation for 30 s at 60 °C. The fluorescence measurement was detected at the end of each elongation cycle. The quantification data were then analyzed with the LightCycler analysis software using the second derivative maximum method. The expression level of IL-1 β , IL-8, NOTCH3, HDD2 and Ly-GDI cDNAs was finally normalized to the expression level of 28S rRNA cDNA by computing the ratio amount of IL-1 β , IL-8, NOTCH3, HDD2 and Ly-GDI cDNA/amount of 28S rRNA cDNA.

IL-8 ELISA (enzyme-linked immunosorbent assay). Human IL-8 protein secretion was measured using a commercial ELISA kit (BD OptEIA Human IL-8 ELISA Set, cat. no. 555244, BD Biosciences, Basel, Switzerland), according to the manufacturer's recommendations. Samples of cell culture supernatant

were diluted 1:10 and 1:100. The analysis was performed on an Anthos reader 2001 (Anthos Labtec Instruments GmbH, Salzburg, Austria).

Statistical analysis. Student's *t*-test ("Two-sample Assuming Equal Variances" using Microsoft Office Excel) was used to assess the statistical significance of the differences between the mean values of CTAB samples and the mean values of tested chemicals samples.

Results

Dose response study and determination of non-toxic test concentrations at 48 h

Three groups of chemicals were examined in the U937 activation test protocol. The first group (Group 1) was composed of the metal sensitizer nickel(II) sulfate hexahydrate (NiSO₄) and 7 known contact sensitizers (Fig. 2a) identified as

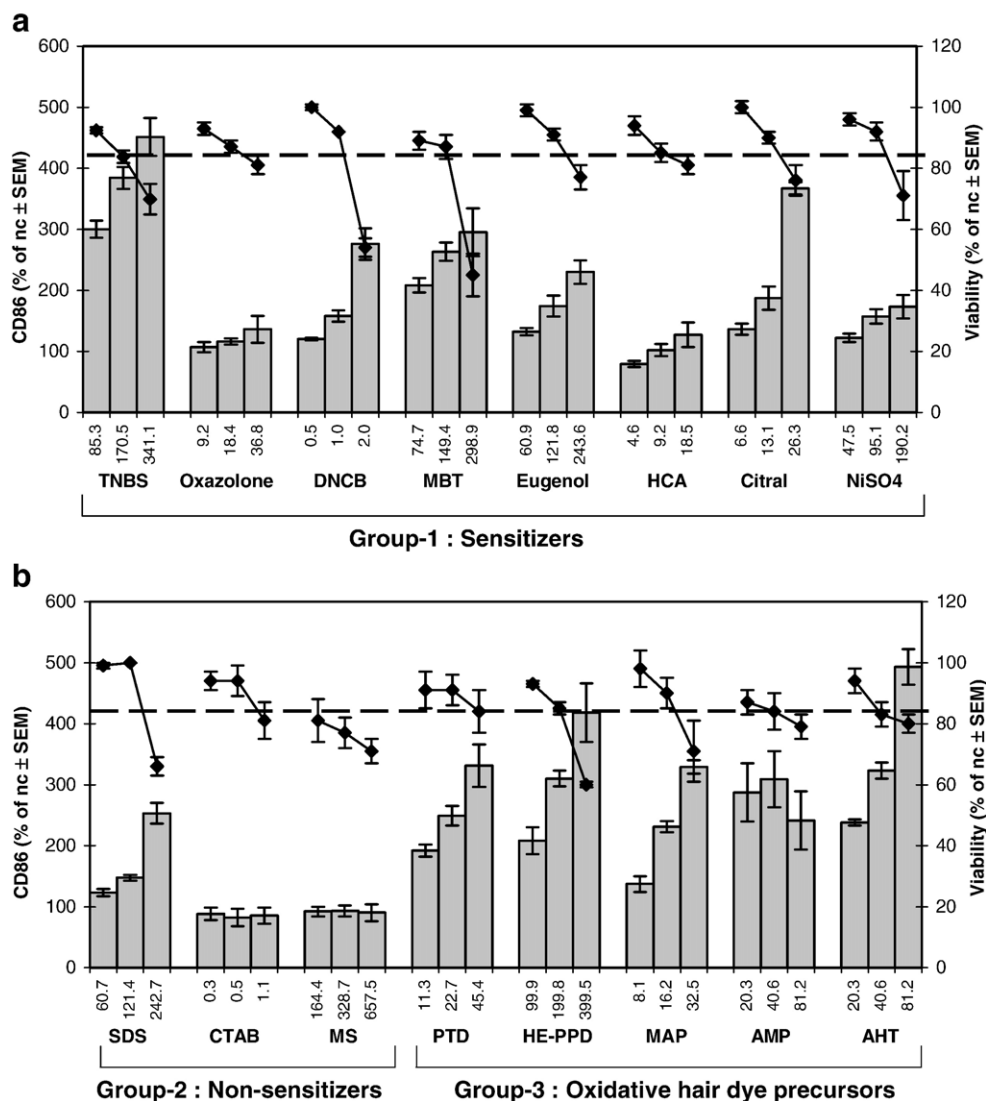


Fig. 2. Dose response study after 48-h exposure to the indicated chemicals. (a) Group 1: sensitizers. (b) Group 2: non-sensitizers and Group 3: oxidative hair dye precursors. X-axis: concentrations of test chemicals (μM). Left Y-axis: percent of CD86-positive cells (grey bars) compared to the negative control (nc). Right Y-axis: percent of viability (FDA incorporation, black rhombs) compared to the nc. The horizontal black dashed line indicates the limit of 85% of nc (viability criterion). The nc corresponds to the cells treated with the relevant vehicle (water/RPMI or 0.25% DMSO). The mean and the standard error of the mean (SEM) of at least three independent experiments are shown for each chemical.

weak to extreme by the LLNA: citral, eugenol, alpha-hexylcinnamaldehyde (HCA), 2-mercaptobenzothiazole (MBT), 2,4,6-trinitrobenzenesulfonic acid (TNBS), 2,4-dinitrochlorobenzene (DNCB) and oxazolone. The second group (Group 2) consisted of 3 standard non-sensitizers (Fig. 2b): sodium dodecyl sulfate (SDS), hexadecyltrimethylammonium bromide (CTAB) and methyl salicylate (MS). The third group (Group 3) contained five oxidative hair dye precursors (Fig. 2b): *p*-toluylenediamine (PTD), hydroxyethyl-*p*-phenylenediamine (HE-PPD), 3-methyl-4-aminophenol (MAP), 2-amino-5-methylphenol (AMP) and 4-amino-2-hydroxytoluene (AHT). Cells were exposed for 48 h to the test items in a dose response experiment and analyzed for viability using fluorescein diacetate (FDA) staining method (see Materials and methods) and CD86 expression (see below). According to our historical data, the following viability criterion was used: a test item concentration is considered as non-toxic when the cell viability reaches at least 85% of the negative control (nc). This 85% limit was set since non-specific effects were observed on CD86 (see below), IL-1 β and IL-8 expressions at higher test item concentrations (data not shown). Three non-toxic to toxic concentrations were examined for each test item (Figs. 2a, b). A clear dose effect on cell viability was observed for each test substance: the two lower concentrations of most chemicals resulted in a cell viability of more than 85% compared to that of the nc (except for MS). The viability decreased below 85% for the third (highest) dose level. The third concentration was thus considered as toxic and unsuitable for use in this *in vitro* test.

CD86 measurements in the dose response study

The 16 tested chemicals showed test item-specific dose effect for CD86 expression (Figs. 2a, b). Most Group 1 sensitizers and most Group 3 oxidative hair dye precursors upregulated CD86 expression in a dose-dependent way. The strong sensitizer TNBS induced a vigorous CD86 response, while the weak sensitizer eugenol induced a weak CD86 response. At cytotoxic concentrations (i.e., <85% viability of nc), most tested chemicals induced an upregulation of CD86 expression. This effect, if observed only at cytotoxic concentrations was considered as unspecific. No CD86 upregulation was detected in cells treated with the irritants MS and CTAB (Group 2), whereas SDS induced an unexpected CD86 upregulation maybe due to its surfactant property. However, for AMP, the strongest CD86 induction was observed at the highest non-cytotoxic concentration tested and no significant change in CD86 expression was found in cells exposed to the extreme sensitizer oxazolone and the moderate sensitizer HCA.

Time-dependent effects on cell viability (kinetic study)

The highest non-toxic concentration as determined in the dose response experiment (Figs. 2a, b) was tested in a kinetic experiment at exposure times of 24, 48 and 72 h. The mean cell viability was generally above 85% of the nc at all tested time points (Table 1). However oxazolone, MBT, HCA and HE-PPD

Table 1

Mean cell viability of each test item concentration in the kinetic study

Chemicals	Tested concentration	Mean viability±SEM (<i>n</i> ≥3)		
		24 h	48 h	72 h
<i>Group 1: sensitizers</i>				
TNBS	170.5 μM	94±1	85±1	86±1
Oxazolone	9.2 μM	84±1	90±1	97±1
DNCB	1 μM	92±4	95±3	99±1
MBT	74.7 μM	91±7	95±3	94±4
Eugenol	121.8 μM	88±4	89±3	100±2
HCA	4.6 μM	87±3	93±1	100±2
Citral	13.1 μM	91±2	93±1	97±1
NiSO ₄	95.1 μM	98±1	99±1	92±1
<i>Group 2: non-sensitizers</i>				
SDS	121.4 μM	100±1	101±1	105±1
CTAB	0.5 μM	98±1	97±1	99±1
MS	328.7 μM	97±11	93±4	97±1
DMSO (vehicle)	0.25%	97±1	96±1	99±1
<i>Group 3: oxidative hair dye precursors</i>				
PTD	22.7 μM	96±1	95±1	102±1
HE-PPD	99.9 μM	96±1	92±2	92±3
MAP	16.2 μM	102±5	101±3	96±3
AHT	40.6 μM	104±10	100±3	91±1
AMP	40.6 μM	103±13	91±2	91±3

were tested at a lower concentration since cytotoxicity occurred at the pre-selected concentration.

Time-dependent effects on CD86 expression (kinetic study)

Group 1 sensitizers induced specific modulations of CD86 expression (Figs. 3a and 4a). The strong sensitizer TNBS upregulated CD86 expression, whereas eugenol and MBT induced a weak to moderate CD86 response, respectively. Only minor or no changes in CD86 expression were measured in cells exposed to oxazolone or HCA (Fig. 4a). Although SDS induced a weak CD86 expression, we observed no significant modulation for the non-sensitizers CTAB and MS (Group 2), as well as for the vehicle dimethyl sulfoxide (DMSO) if compared to cells without treatment (Fig. 5a). In contrast, cells exposed to Group 3 hair dye precursors exhibited relevant exposure time-dependent CD86 modulations (Fig. 6a).

Time-dependent effects on IL-1 β and IL-8 mRNA expression

The quantitative real-time RT-PCR analysis indicated specific modulations of IL-1 β and IL-8 gene expressions for Group 1 sensitizers and Group 3 hair dye precursors (Figs. 3b, c, 4b, c, and 6b, c). Most sensitizers and many hair dye precursors induced a U-shaped expression pattern with a minimum expression at 48 h (Figs. 3b, c, 4b, c, 6b, c). No relevant change of IL-1 β and IL-8 gene expression was detected in cells exposed to the Group 2 non-sensitizers (Figs. 5b, c). However, CTAB and MS induced an upregulation of IL-8 gene expression at the time point 72 h (Fig. 5c). This IL-8 expression induced by an irritant indicates that the 72-h time point might be the duration limit for exposure to chemicals.

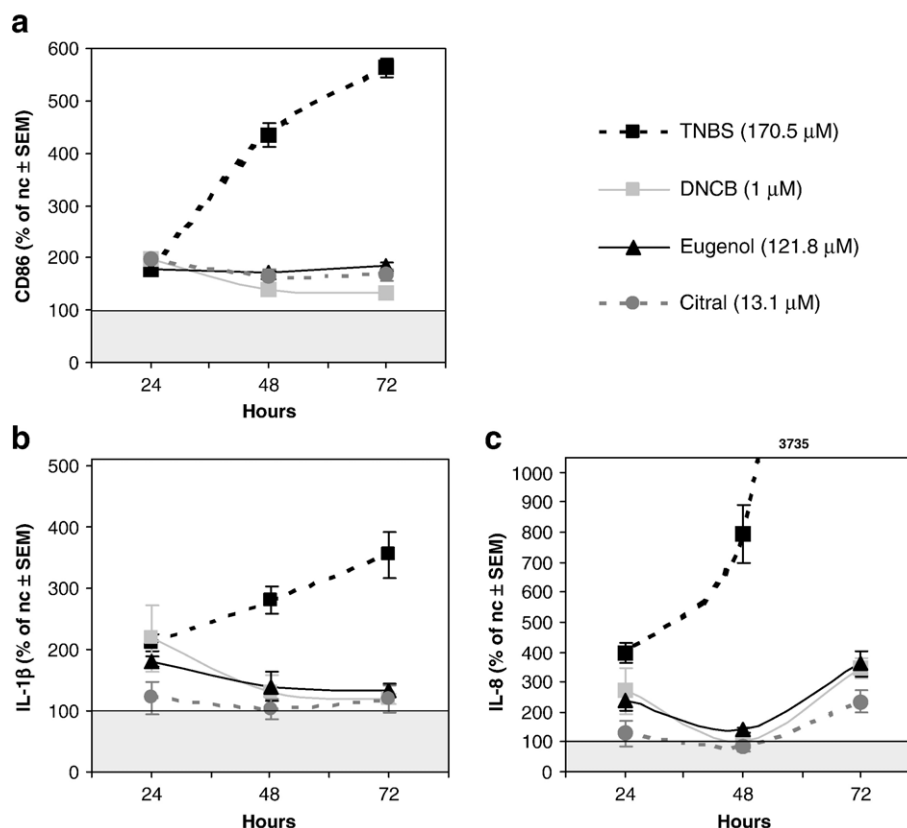


Fig. 3. Time-dependent effect on marker expression in U937 cells after exposure to the indicated Group 1 sensitizers. X-axis: exposure time (hours). Cytometric measurements (a), Y-axis: percent of CD86-positive cells compared to the negative control (nc indicated as 100% by the grey filled area). RT-PCR measurements (b, c), Y-axis: percent of IL-1 β (b) or IL-8 (c) gene expression compared to nc (indicated as 100% by the grey filled area). The nc corresponds to the cells treated with the relevant vehicle (water/RPMI or 0.25% DMSO). The mean and the standard error of the mean (SEM) of at least three independent experiments are shown for each chemical. On top of graph 3c, the number indicates the IL-8 expression level at 72 h for TNBS (3735 $\pm$ 430).

In addition, the cell culture supernatants of treated cells were collected after a 72-h exposure and IL-8 protein measured by ELISA (enzyme-linked immunosorbent assay). This limited set of results revealed no significant increase in the production of IL-8 protein compared to the nc in presence of CTAB but relevant increases in IL-8 concentration after exposure to sensitizers (Fig. 7), suggesting that IL-8 ELISA measurement could be useful to discriminate sensitizers from non-sensitizers after a 72-h exposure time.

Additional markers

Ryan et al. (2004) measured changes in gene expression following exposure to the contact allergen dinitrobenzenesulfonic acid using Affymetrix GeneChip microarrays. From their list of modulated genes, we selected *HDD2*, *Ly-GDI* and *NOTCH3* as putative markers of DC activation and analyzed their expression by quantitative real-time RT-PCR. We also examined the *AQP3* gene expression since Aeby et al. (2004) reported its downregulation in PBMDs exposed to sensitizers. We observed reproducible and test item-specific modulations of *HDD2* expression (a 3 α -hydroxysteroid dehydrogenase from the aldoketoreductase family) during the kinetic studies (Fig. 8). Its expression pattern is similar to that of IL-8. Based on these limited results, *HDD2* mRNA might be a promising sensitizer

marker for the test protocol based on U937 cells. Expression of *Ly-GDI*, a Ras homologous guanosine diphosphate (Rho GDP) dissociation inhibitor beta, could be detected but no significant modulation was measured after exposure to TNBS or SDS. On the other hand, changes in the expression of *NOTCH3* (a receptor for membrane-bound ligands regulating cell-fate determination) and *AQP3* were observed albeit with considerable inter-experiment variability (data not shown). This may be due to very low basal expression levels of both *AQP3* and *NOTCH3* in U937 cells, making these markers unsuitable in this test system.

Statistical analysis of the in vitro results

Table 2 shows the LLNA classification of the tested chemicals and the *P* values calculated with a Student's *t*-test analysis of the *in vitro* data obtained with the U937 activation test. The indicated LLNA data are based upon the available literature or internal data. The indicated *P* values were obtained by computing the statistical significance of the upregulation for CD86, IL-1 β and IL-8 markers at 24, 48 and 72 h for each test item compared to the respective values obtained for the non-sensitizer CTAB. We chose to compare the different test sets against the CTAB values, because it represents a typical non-sensitizer inducing no relevant modulation of the selected

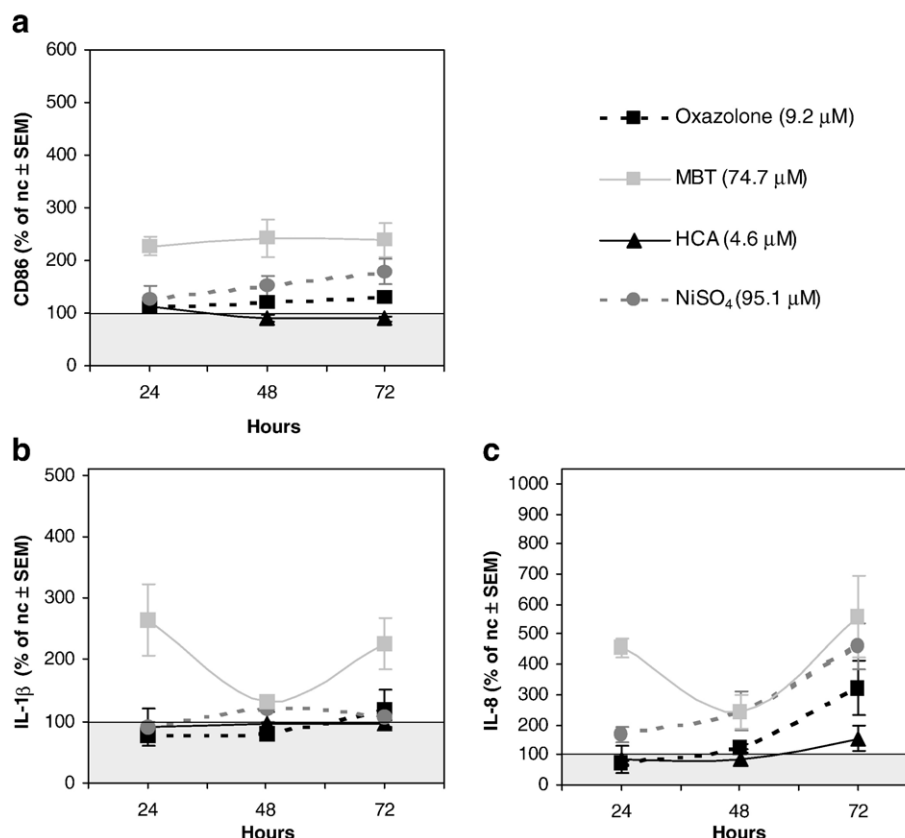


Fig. 4. Time-dependent effect on marker expression in U937 cells after exposure to the indicated Group 1 sensitizers. X-axis: exposure time (hours). Cytometric measurements (a), Y-axis: percent of CD86-positive cells compared to the negative control (nc indicated as 100% by the grey filled area). RT-PCR measurements (b, c), Y-axis: percent of IL-1 β (b) or IL-8 (c) gene expression compared to nc (indicated as 100% by the grey filled area). The nc corresponds to the cells treated with the relevant vehicle (water/RPMI or 0.25% DMSO). The mean and the standard error of the mean (SEM) of at least three independent experiments are shown for each chemical.

markers at 24, 48 and 72 h, with the exception of the upregulation of IL-8 gene expression at 72 h (see Fig. 5c). This upregulation may indicate a limit in the exposure period and therefore 72-h values should be considered carefully. Consequently, *P* values calculated for IL-8 at 72 h are not significant for most tested chemicals.

Discussion

In this study, the biological response of the human monocytic cell line U937 was analyzed in presence of sensitizers and non-sensitizers. Its potential to discriminate sensitizers from non-sensitizers was evaluated and considered for the development of an *in vitro* dendritic-like cell activation test. The effect of eight skin sensitizers, 3 non-sensitizers and 5 oxidative hair dye precursors was investigated. In a first step, dose response experiments were performed to select appropriate, non-toxic test concentrations in order to avoid possible unspecific artifacts caused by cytotoxicity. A test item concentration was considered as non-toxic when the cell viability was above 85% compared to that of the negative control. This 85% limit was set after consideration of the non-specific effects induced on CD86 (see Figs. 2a, b). However, a limited degree of cytotoxicity was considered necessary for optimal DC activa-

tion in order to obtain the equivalent of a danger signal. Such a danger signal is known to be required for the initiation of an immune response by the innate immune system (Gallucci and Matzinger, 2001; Luster, 2002).

In a second step, we investigated the time-dependent modulation of the chosen markers (CD86, IL-1 β and IL-8) in kinetic experiments. Each test item was evaluated for 24, 48 and 72 h at the highest non-toxic concentration defined in the dose response study. Results revealed specific modulations of CD86, IL-1 β and IL-8 for the 16 tested chemicals while cell viability remained $\geq 85\%$ at each time point. Non-sensitizers induced little or no upregulation of CD86, IL-1 β and IL-8, except for the 72-h time point where an IL-8 upregulation was observed with MS and CTAB. In cells exposed to sensitizers, we generally observed an upregulation of the chosen markers with a U-shaped expression pattern for IL-1 β and IL-8 gene expressions with two maxima at 24 and 72 h and a minimum at 48 h. We speculate that this U-shaped gene expression pattern is due to cell division effects, the doubling time of U937 cells being approximately 24 h. After a 24-h exposure period, overall gene expression is probably downregulated during the cell division process and after a lag time (corresponding to the 48-h time point), the respective genes may be re-activated by chemical exposure, resulting in an upregulation of cytokines gene

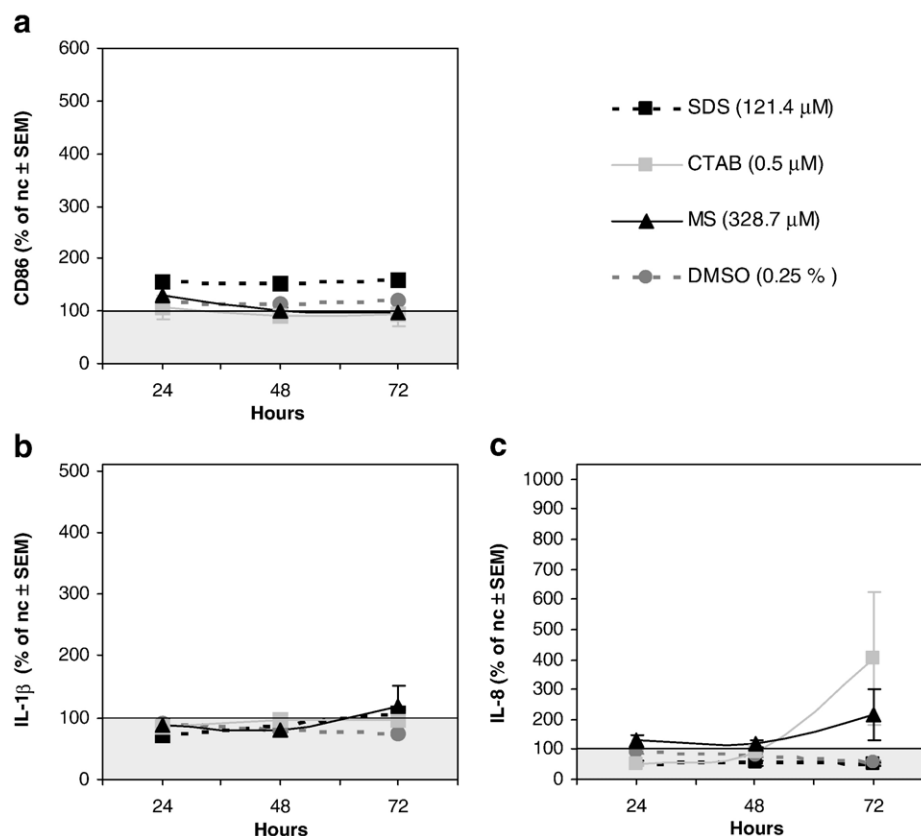


Fig. 5. Time-dependent effect on markers expression in U937 cells after exposure to the indicated Group 2 non-sensitizers. X-axis: exposure time (hours). Cytometric measurements (a), Y-axis: percent of CD86-positive cells compared to the negative control (nc indicated as 100% by the grey filled area). RT-PCR measurements (b, c), Y-axis: percent of IL-1 β (b) or IL-8 (c) gene expression compared to nc (indicated as 100% by the grey filled area). The nc corresponds to the cells treated with the relevant vehicle (water/RPMI or 0.25% DMSO). The mean and the standard error of the mean (SEM) of at least three independent experiments are shown for each chemical.

expression at 72 h. A similar hypothesis has been proposed by Ross et al. (2000) suggesting that the variation in expression of numerous ribosomal genes in human cancer cell lines could be related to the cell doubling time. This observation suggests that 48 h would not be the optimal time point for performing dose effect analyses.

Since the biological response of the U937 cell line showed promising possibilities for differentiating sensitizers from non-sensitizers, the collected *in vitro* data were further evaluated with a Student's *t*-test analysis (see Table 2). Compared to non-sensitizers upregulating only one of the three markers (see Fig. 5), most tested sensitizers significantly induced the upregulation of at least two of the selected markers. Using this criterion (upregulation of at least two markers), the described *in vitro* test could distinguish most sensitizers from non-sensitizers. Six out of 8 sensitizers and all tested non-sensitizers could be correctly classified. The two misclassified sensitizers were citral and HCA. Citral significantly upregulated only one marker (CD86) when compared to CTAB (see Table 2), but also induced a biologically relevant upregulation of a second marker: IL-8 gene expression at 72 h (235% of nc, see Fig. 3c). In the statistical comparison to CTAB, however, this marker could not be detected due to a substantial upregulation of IL-8 by CTAB itself at 72 h (402% of nc, see Fig. 5c). HCA, on the other hand,

was wrongly identified as a non-sensitizer in the *in vitro* test system although the LLNA indicates a moderate sensitizing potency (Table 2). Interestingly, only few positive HCA patch-test reactions were reported in humans by Heydorn et al. (2003). The false negative results observed with HCA may thus be related to the human origin of the U937 cells. Anyway, due to its low water solubility (octanol/water partition coefficient $\text{Log}K_{\text{ow}}$ of 3.770, see Gerberick et al., 2004) HCA might not be readily available to the cells in an aqueous environment. This may represent a general limitation of cell culture based assays for all chemicals with low water solubility. Accordingly, the *in vitro* evaluation of five oxidative hair dye precursors as potential sensitizers reflects the data obtained with the LLNA, since they possess a substantial water solubility (octanol/water partition coefficient $\text{Log}K_{\text{ow}}$ in the range from 0 to 1 for free bases). In addition, Table 2 indicates the concentration at which each chemical induced a relevant effect. It is interesting to note that the test items classified as extreme in the LLNA are inducing significant effects at a very low concentration (oxazolone: 9.2 μM , DNCB: 1 μM).

A direct comparison of *in vivo* (LLNA) and *in vitro* data should however not be attempted due to the absence of a skin barrier in the test system. As shown for HE-PPD in a previous study from our group (Aeby et al., 2004), the presence of a skin

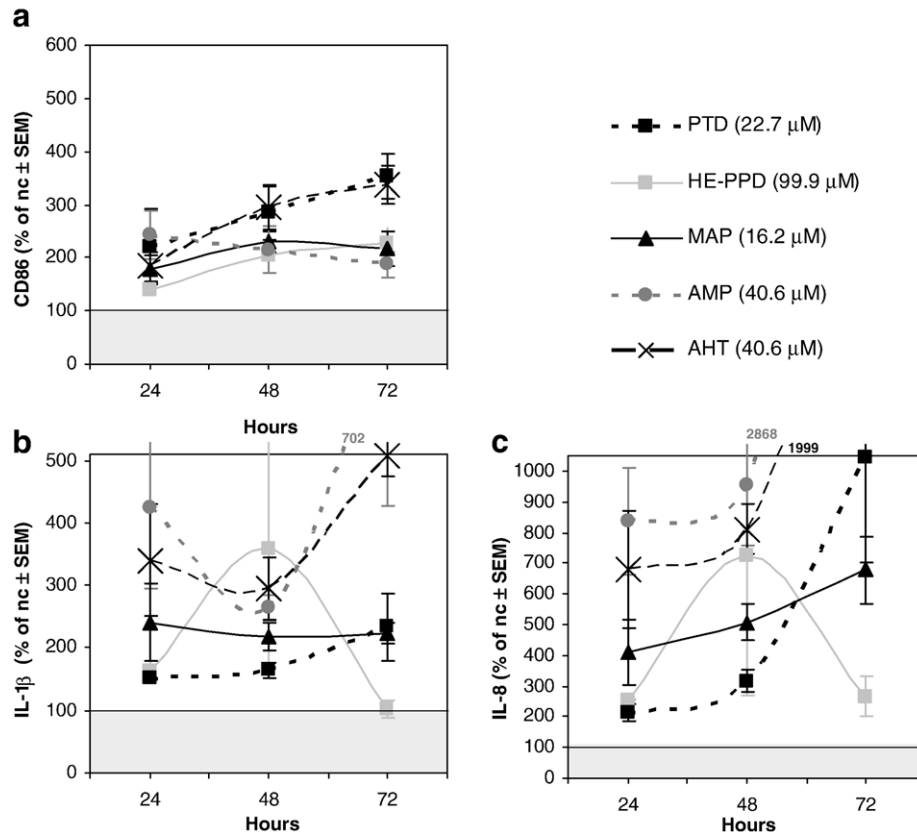


Fig. 6. Time-dependent effect on markers expression in U937 cells to the indicated Group 3 oxidative hair dye precursors. X-axis: exposure time (hours). Cytometric measurements (a), Y-axis: percent of CD86-positive cells compared to the negative control (nc indicated as 100% by the grey filled area). RT-PCR measurements (b, c), Y-axis: percent of IL-1 β (b) or IL-8 (c) gene expression compared to nc (indicated as 100% by the grey filled area). The nc corresponds to the cells treated with the relevant vehicle (water/RPMI or 0.25% DMSO). The mean and the standard error of the mean (SEM) of at least three independent experiments are shown for each chemical. On top of graph b, the number indicates the IL-1 β expression level at 72 h for AMP (702 $\pm$ 274). On top of graph c, the numbers indicate the IL-8 expression level at 72 h for AMP (2868 $\pm$ 1274) and AHT (1999 $\pm$ 260).

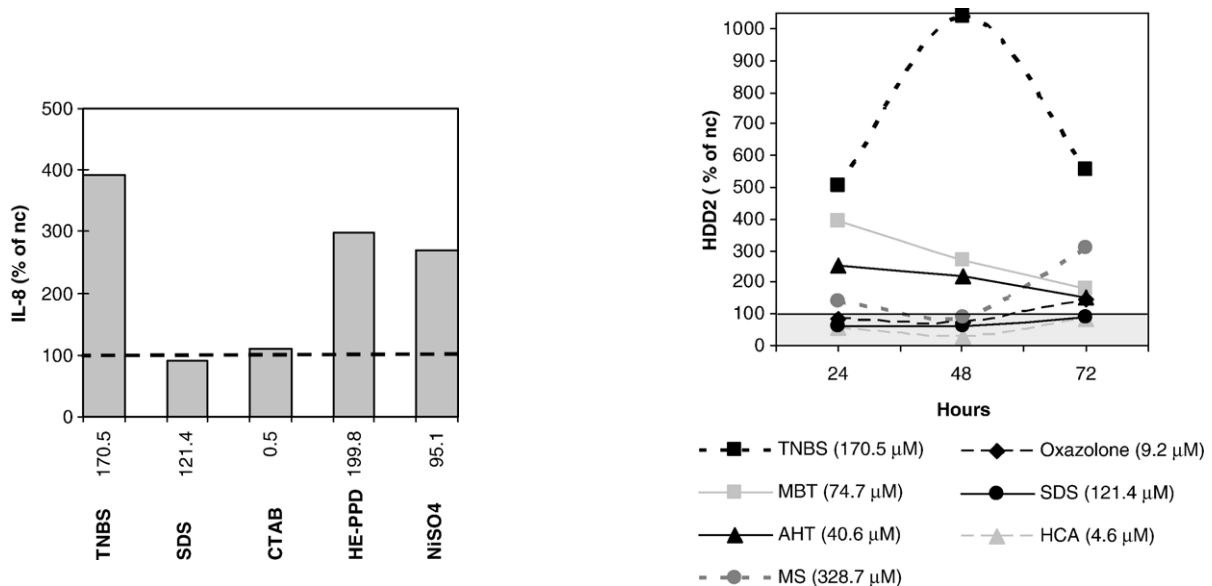


Fig. 7. Detection of IL-8 secretion in U937 cells supernatants after exposure to the indicated test items for 72 h by ELISA assay. X-axis: concentrations of test chemicals (μ M). Y-axis: percent of IL-8 protein expression compared to the negative control (nc indicated as 100% by the black dashed line). The IL-8 concentration range in the nc supernatant was 90–140 pg/ml. The nc corresponds to the cells treated with the relevant vehicle (water/RPMI). The results of a single experiment are shown.

Fig. 8. Time-dependent effect on *HDD2* gene expression in U937 cells after exposure to the indicated test items. X-axis: exposure time (hours). Y-axis: percent of *HDD2* gene expression compared to the negative control (nc indicated as 100% by the grey filled area). The nc corresponds to the cells treated with the relevant vehicle (water/RPMI or 0.25% DMSO). The results of a single experiment are shown.

Table 2
LLNA classification of tested chemicals and statistical significance of markers up-regulation obtained in the U937 activation test

Chemicals	Tested concentration (μM)	LLNA Classification	<i>P</i> value (CD86, <i>n</i> ≥ 3)			<i>P</i> value (IL-1β, <i>n</i> ≥ 3)			<i>P</i> value (IL-8, <i>n</i> ≥ 3)		
			24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h
<i>Group 1: sensitizers</i>											
TNBS	170.5	Strong ^{a, b}	≤ 0.005	≤ 0.005	≤ 0.005	≤ 0.005	≤ 0.005	≤ 0.05	≤ 0.005	≤ 0.005	≤ 0.005
Oxazolone	9.2	Extreme ^{c, d}	ns	≤ 0.05	ns	ns	ns	ns	≤ 0.05	≤ 0.05	ns
DNCB	1	Extreme ^c	≤ 0.05	≤ 0.005	ns	≤ 0.1	ns	ns	≤ 0.05	ns	ns
MBT	74.7	Moderate ^c	≤ 0.05	≤ 0.05	≤ 0.05	≤ 0.05	≤ 0.1	≤ 0.05	≤ 0.005	≤ 0.1	ns
Eugenol	121.8	Weak ^{c, d}	≤ 0.05	≤ 0.005	≤ 0.05	≤ 0.005	ns	≤ 0.1	≤ 0.05	≤ 0.05	ns
HCA	4.6	Moderate ^{c, d}	ns	ns	ns	ns	ns	ns	ns	ns	ns
Citral	13.1	Weak ^c	≤ 0.05	≤ 0.05	≤ 0.05	ns	ns	ns	ns	ns	ns
NiSO ₄	95.1	? ^e	ns	≤ 0.05	≤ 0.1	ns	ns	ns	≤ 0.05	≤ 0.1	ns
<i>Group 2: non sensitizers</i>											
SDS	121.4	? ^e	≤ 0.05	≤ 0.005	≤ 0.05	ns	ns	ns	ns	ns	ns
MS	328.7	— ^d	ns	ns	ns	ns	ns	ns	≤ 0.05	ns	ns
<i>Group 3: oxidative hair dye precursors</i>											
PTD	22.7	Strong ^b	ns	≤ 0.05	≤ 0.05	≤ 0.005	≤ 0.05	≤ 0.1	≤ 0.005	≤ 0.005	ns
HE-PPD	99.9	Strong ^{b, f}	ns	≤ 0.05	≤ 0.05	≤ 0.05	ns	ns	≤ 0.005	ns	ns
MAP	16.2	Moderate ^g	ns	≤ 0.005	≤ 0.05	≤ 0.1	≤ 0.05	≤ 0.005	≤ 0.05	≤ 0.005	ns
AHT	40.6	Strong ^g	≤ 0.1	≤ 0.05	≤ 0.005	≤ 0.05	≤ 0.05	≤ 0.005	≤ 0.05	≤ 0.005	≤ 0.05
AMP	40.6	Moderate ^g	≤ 0.1	≤ 0.05	≤ 0.1	≤ 0.1	≤ 0.005	≤ 0.1	≤ 0.05	≤ 0.05	ns

P values were calculated by comparing the mean value of CTAB samples against the mean values of the tested chemical samples (Student's *t*-test).

ns=Not significant.

? Indicates a false negative for SDS or a false positive for NiSO₄ as reported by the study from Basketter et al. (1994).

^a Data from Smith and Hotchkiss (2001).

^b Data from Aeby et al. (2004).

^c Data from Kimber et al. (2003).

^d Data from Gerberick et al. (2004).

^e Data from Basketter et al. (1994).

^f In LLNAs, HE-PPD was non-sensitizing in AAOO (acetone-olive oil) but a strong sensitizer in DMSO (Aeby et al., 2004).

^g Internal data. LLNA: local lymph node assay.

barrier and the choice of vehicle represent very important parameters when comparing *in vitro* and LLNA results. As an example, HE-PPD was negative in the LLNA when using acetone/aqua/olive oil as vehicle and turned out positive when applied in DMSO, a vehicle known to enhance the bioavailability of many chemicals (Aeby et al., 2004; Ryan et al., 2002). This suggests that a correction factor reflecting the test substance bioavailability should be applied to evaluate *in vitro* sensitization test results for a correct prediction of the skin sensitizing potential of a chemical.

The results obtained in the kinetic study demonstrated the importance of measuring the expression of different markers at different time points. DNCB and NiSO₄ induced a relevant increase of CD86 expression at one time point only, at 24 h or 72 h, respectively (see Figs. 3a and 4a). SDS induced an increase in CD86 expression, but no relevant change was detected in IL-1 β expression (see Figs. 5a, b).

Taken together, these results indicate that in the described test system, a single marker (e.g., CD86, IL-1 β or IL-8) was not sufficient for a reliable evaluation of the DC activation potential of a chemical. In contrast, a combination of multiple markers allowed a more precise analysis. This is further supported by the recent finding that different classes of sensitizers induce different signaling pathways, as suggested by the study from Boislève et al. (2004). The use of multiple markers has also

been recommended in several test protocols recently described. Among the different markers used for the identification of contact allergens in PBMDs, CD86 and/or IL-1 β were generally analyzed (Tuschl and Kovac, 2001; Aeby et al., 2004). Results obtained by Aiba et al. (1997) indicated that the surface expression of CD54, CD86 and of HLA-DR and the production of IL-1 β protein were significantly upregulated in PBMDs after a 48-h exposure to the sensitizers DNCB and NiCl₂. In addition, no significant induction of these markers was detected in PBMDs exposed to the irritants SDS, benzalkonium chloride and ZnCl₂. Coutant et al. (1999) also observed an increase in the surface expression of CD54, CD86, HLA-DR and of CD40 in PBMDs incubated with sensitizers for 48 h. Moreover, recent results with PBMDs indicated that a prolonged incubation time (from 2 to 4 days) increased the number of modulated markers and thus improves the capacity of the test system to discriminate allergens from irritants (Staquet et al., 2004). Our group recently reported that TNBS, HE-PPD and PTD induce a significant upregulation of CD86 and IL-1 β mRNA in PBMDs (Aeby et al., 2004). The results we have now obtained with the U937 cells indicate that many of the described markers are also modulated by exposure to sensitizers. U937 cells may thus represent a convenient alternative to PBMDs. In a similar approach, Sakaguchi et al. (2006) described a test protocol using THP-1 cells, a human

monocyte cell line comparable to U937 as a test system and CD86 and CD54 as activation markers to predict contact allergens.

In our test system, the prolonged (72 h) incubation period may not be optimal for all gene expression markers. For example, the upregulation of IL-8 gene expression at 72 h by CTAB (see Fig. 5c) may indicate the exposure time limit for the test. This limitation could be overcome by measuring the accumulation of IL-8 protein in the culture supernatant over the whole exposure period (IL-8 ELISA, see Fig. 7). Preliminary results indeed indicate that IL-8 protein measurement at 72 h represents an additional promising marker. Although the role of IL-8 has not yet been firmly established during the sensitization process, maturing DCs have been shown to produce large amounts of inflammatory chemokines such as IL-8 after exposure to lipopolysaccharide (Sallusto et al., 1999; Nagorsen et al., 2004). Furthermore, Toebak et al. (2006) observed an increased expression of IL-8 protein (CXCL8) in PBMDs exposed to sensitizers and suggested that IL-8 might be involved in the induction and the elicitation of ACD. In the somewhat different context of using an *in vitro* reconstructed human epidermis model, Coquette et al. (2003) suggested to use the expression and release of IL-8 and IL-1 α for the prediction of skin irritation and/or sensitization. These recent studies and our results suggest that IL-8 plays a specific role in the induction phase of ACD.

In conclusion, the biological evaluation of the U937 cell line in the described DC activation test protocol demonstrated that the analysis of the expression pattern of CD86, IL-1 β and IL-8 allowed a discrimination of sensitizers from non-sensitizers for 15 of the 16 tested chemicals. We found that a combination of at least two markers at more than one time point is needed to establish a reliable evaluation of the DC activation potential of the chemicals tested. The results of the statistical comparison of the tested chemicals to the non-sensitizer CTAB supported our findings since 13/15 were correctly identified with regard to their sensitizing potential.

In a next step, we have further simplified the U937 activation test by measuring CD86 surface expression and cell viability at 24 h and 72 h and by performing IL-8 ELISA tests at 72 h only. Gene expression of IL-1 β and IL-8 at 24 h will only be considered in case of doubtful results. These simplifications should allow to test a large number of compounds to further evaluate the results with adequate statistical tools.

Finally, with regard to the replacement of animal testing for sensitization, one should keep in mind that DC activation is simply a part of the complex chain of events that occurs during the induction phase of an immune response in the skin. Additional parameters i.e. skin penetration, protein binding and eventually metabolic activation need to be considered for an in depth understanding of the relation between *in vitro* DC activation data and *in vivo* results.

Acknowledgments

We are grateful to Sylvie Python for her expert technical assistance in molecular biology and for performing the ELISA

assays and Fabio Protopapa for his expert technical assistance in flow cytometry. We also thank Dr. G. Frank Gerberick and Cindy A. Ryan (Miami Valley Innovation Center, The Procter and Gamble Company, Cincinnati, USA) for their comments on the manuscript. The authors would like to acknowledge the financial support from The European Cosmetic Toiletry and Perfumery Association (COLIPA).

References

- Aeby, P., Wyss, C., Beck, H., Griem, P., Scheffler, H., Goebel, C., 2004. Characterization of the sensitizing potential of chemicals by *in vitro* analysis of dendritic cell activation and skin penetration. *J. Invest. Dermatol.* 122, 1154–1164.
- Aiba, S., Terunuma, A., Manome, H., Tagami, H., 1997. Dendritic cells differently respond to haptens and irritants by their production of cytokines and expression of co-stimulatory molecules. *Eur. J. Immunol.* 27, 3031–3038.
- Ashikaga, T., Hoya, M., Itagaki, H., Katsumura, Y., Aiba, S., 2002. Evaluation of CD86 expression and MHC class II molecule internalization in THP-1 human monocyte cells as predictive endpoints for contact sensitizers. *Toxicol. In Vitro* 16, 711–716.
- Azam, P., Peiffer, J.L., Chamousset, D., Tissier, M.H., Bonnet, P.A., Vian, L., Fabre, I., Ourlin, J.C., 2006. The cytokine-dependent MUTZ-3 cell line as an *in vitro* model for the screening of contact sensitizers. *Toxicol. Appl. Pharmacol.* 212, 14–23.
- Banchereau, J., Steinman, R.M., 1998. Dendritic cells and the control of immunity. *Nature* 392, 245–252.
- Basketter, D.A., Scholes, E.W., Kimber, I., 1994. The performance of the local lymph node assay with chemicals identified as contact allergens in the human maximization test. *Food Chem. Toxicol.* 32, 543–547.
- Basketter, D.A., Evans, P., Fielder, R.J., Gerberick, G.F., Dearman, R.J., Kimber, I., 2002. Local lymph node assay—validation, conduct and use in practice. *Food Chem. Toxicol.* 40, 593–598.
- Boislève, F., Kerdine-Römer, S., Rougier-Larzat, N., Pallardy, M., 2004. Nickel and DNCB induce CCR7 expression on human dendritic cells through different signalling pathways: role of TNF- α and MAPK. *J. Invest. Dermatol.* 123, 494–502.
- Casati, S., Aeb, P., Basketter, D.A., Cavani, A., Gennari, A., Gerberick, G.F., Griem, P., Hartung, T., Kimber, I., Lepoittevin, J.P., Meade, B.J., Pallardy, M., Rougier, N., Rousset, F., Rubinstenn, G., Sallusto, F., Verheyen, G.R., Zuang, V., 2005. Dendritic cells as a tool for the predictive identification of skin sensitization hazard. *ATLA* 33, 47–62.
- Coquette, A., Berna, N., Vandenbosch, A., Rosdy, M., De Wever, B., Poumay, Y., 2003. Analysis of interleukin-1 α (IL-1 α) and interleukin-8 (IL-8) expression and release in *in vitro* reconstructed human epidermis for the prediction of *in vivo* skin irritation and/or sensitization. *Toxicol. In Vitro* 17, 311–321.
- Coutant, K.D., de Brugerolle de Fraissinette, A., Cordier, A., Ulrich, P., 1999. Modulation of the activity of human monocyte-derived dendritic cells by chemical haptens, a metal allergen, and a staphylococcal superantigen. *Toxicol. Sci.* 52, 189–198.
- Dearman, R.J., Basketter, D.A., Kimber, I., 1999. Local lymph node assay: use in hazard and risk assessment. *J. Appl. Toxicol.* 19, 299–306.
- de Baey, A., Lanzavecchia, A., 2000. The role of aquaporins in dendritic cell macropinocytosis. *J. Exp. Med.* 191, 743–747.
- Enk, A.H., Katz, S.I., 1992. Early molecular events in the induction phase of contact sensitivity. *Proc. Natl. Acad. Sci. U. S. A.* 89, 1398–1402.
- Feghali, C.A., Wright, M.T., 1997. Cytokines in acute and chronic inflammation. *Front. Biosci.* 2, d12–d26.
- Gallucci, S., Matzinger, P., 2001. Danger signals: SOS to the immune system. *Curr. Opin. Immunol.* 13, 114–119.
- Gerberick, G.F., Ryan, C.A., Kern, P.S., Dearman, R.J., Kimber, I., Patlewicz, G.Y., Basketter, D.A., 2004. A chemical dataset for evaluation of alternative approaches to skin-sensitization testing. *Contact Dermatitis* 50, 274–288.

- Heydorn, S., Duus Johansen, J., Andersen, K.E., Bruze, M., Svedman, C., White, I.R., Basketter, D.A., Menné, T., 2003. Fragrance allergy in patients with hand eczema—a clinical study. *Contact Dermatitis* 48, 317–323.
- Hu, Z.B., Ma, W., Zaborski, M., MacLeod, R.A.F., Quentmeier, H., Drexler, H.G., 1996. Establishment and characterization of two novel cytokine-responsive acute myeloid and monocytic leukemia cell lines, MUTZ-2 and MUTZ-3. *Leukemia* 10, 1025–1040.
- Hulette, B.C., Rowden, G., Ryan, C.A., Lawson, C.M., Dawes, S.M., Ridder, G.M., Gerberick, G.F., 2001. Cytokine induction of a human acute myelogenous leukemia cell line (KG-1) to a CD1a⁺ dendritic cell phenotype. *Arch. Dermatol. Res.* 293, 147–158.
- Hulette, B.C., Ryan, C.A., Gerberick, G.F., 2002. Elucidating changes in surface marker expression of dendritic cells following chemical allergen treatment. *Toxicol. Appl. Pharmacol.* 182, 226–233.
- Hulette, B.C., Ryan, C.A., Gildea, L.A., Gerberick, G.F., 2005. Relationship of CD86 surface marker expression and cytotoxicity on dendritic cells exposed to chemical allergen. *Toxicol. Appl. Pharmacol.* 209, 159–166.
- Jugdé, F., Boissier, C., Rougier-Larzat, N., Corlu, A., Chesné, C., Semana, G., Heresbach, D., 2005. Regulation by allergens of chemokine receptor expression on in vitro-generated dendritic cells. *Toxicology* 212, 227–238.
- Kimber, I., Dearman, R.J., Basketter, D.A., Ryan, C.A., Gerberick, G.F., 2002. The local lymph node assay: past, present and future. *Contact Dermatitis* 47, 315–328.
- Kimber, I., Basketter, D.A., Butler, M., Gamer, A., Garrigue, J.L., Gerberick, G.F., Newsome, C., Steiling, W., Vohr, H.W., 2003. Classification of contact allergens according to potency: proposals. *Food Chem. Toxicol.* 41, 1799–1809.
- Koss, A., Lucero, G., Kosiner, B., 1996. Granulocyte-colony stimulating factor, granulocyte-macrophage colony stimulating factor and interleukin 4 induce differentiation in the U-937 human monocytic leukemia cell line. *Leuk. Lymphoma* 22, 163–171.
- Lepoittevin, J.P., Basketter, D.A., Goossens, A., Karlberg, A.T., 1998. *Allergic Contact Dermatitis—The Molecular Basis*. Springer-Verlag, Berlin.
- Luster, A.D., 2002. The role of chemokines in linking innate and adaptive immunity. *Curr. Opin. Immunol.* 14, 129–135.
- Masterson, J.A., Sombroek, C.C., de Gruijl, T.D., Graus, Y.M.F., van der Vliet, H.J.J., Loughheed, S.M., van den Eertwegh, A.J.M., Pinedo, H.M., Scheper, R.J., 2002. MUTZ-3, a human cell line model for the cytokine-induced differentiation of dendritic cells from CD34⁺ precursors. *Blood* 100, 701–703.
- Nagorsen, D., Marincola, F.M., Panelli, M.C., 2004. Cytokine and chemokine expression profiles of maturing dendritic cells using multiprotein platform arrays. *Cytokine* 25, 31–35.
- Pichowski, J.S., Cumberbatch, M., Dearman, R.J., Basketter, D.A., Kimber, I., 2000. Investigation of induced changes in interleukin 1 β mRNA expression by cultured human dendritic cells as an in vitro approach to skin sensitization testing. *Toxicol. In Vitro* 14, 351–360.
- Reutter, K., Jaeger, D., Degwert, J., Hoppe, U., 1997. In vitro model for contact sensitization: II. Induction of IL-1 β mRNA in human blood-derived dendritic cells by contact sensitizers. *Toxicol. In Vitro* 11, 619–626.
- Rollins, B.J., 1997. Chemokines. *Blood* 90, 909–928.
- Ross, D.T., Scherf, U., Eisen, M.B., Perou, C.M., Rees, C., Spellman, P., Iyer, V., Jeffrey, S.S., van de Rijn, M., Waltham, M., Pergamenschikov, A., Lee, J.C.F., Lashkari, D., Shalon, D., Myers, T.G., Weinstein, J.N., Botstein, D., Brown, P.O., 2000. Systematic variation in gene expression patterns in human cancer cell lines. *Nat. Genet.* 24, 227–235.
- Ryan, C.A., Cruse, L.W., Skinner, R.A., Dearman, R.J., Kimber, I., Gerberick, G.F., 2002. Examination of a vehicle for use with water soluble materials in the murine local lymph node assay. *Food Chem. Toxicol.* 40, 1719–1725.
- Ryan, C.A., Gildea, L.A., Hulette, B.C., Dearman, R.J., Kimber, I., Gerberick, G.F., 2004. Gene expression changes in peripheral blood-derived dendritic cells following exposure to a contact allergen. *Toxicol. Lett.* 150, 301–316.
- Ryan, C.A., Gerberick, G.F., Gildea, L.A., Hulette, B.C., Betts, C.J., Cumberbatch, M., Dearman, R.J., Kimber, I., 2005. Interactions of contact allergens with dendritic cells: opportunities and challenges for the development of novel approaches to hazard assessment. *Toxicol. Sci.* 88, 4–11.
- Sakaguchi, H., Ashikaga, T., Miyazawa, M., Yoshida, Y., Ito, Y., Yoneyama, K., Hirota, M., Itagaki, H., Toyoda, H., Suzuki, H., 2006. Development of an *in vitro* skin sensitization test using human cell lines; human Cell Line Activation Test (h-CLAT): II. An inter-laboratory study of the h-CLAT. *Toxicol. In Vitro* 20, 774–784.
- Sallusto, F., Palermo, B., Lenig, D., Miettinen, M., Matikainen, S., Julkunen, I., Forster, R., Burgstahler, R., Lipp, M., Lanzavecchia, A., 1999. Distinct patterns and kinetics of chemokine production regulate dendritic cell function. *Eur. J. Immunol.* 29, 1617–1625.
- Scapini, P., Lapinet-Vera, J.A., Gasperini, S., Calzetti, F., Bazzoni, F., Cassatella, M.A., 2000. The neutrophil as a cellular source of chemokines. *Immunol. Rev.* 177, 195–203.
- Smith, C.K., Hotchkiss, S.A.M., 2001. *Allergic Contact Dermatitis—Chemical and Metabolic Mechanisms*. Taylor and Francis, London.
- Staquet, M.J., Sportouch, M., Jacquet, C., Schmitt, D., Guesnet, J., Péguet-Navarro, J., 2004. Moderate skin sensitizers can induce phenotypic changes on in vitro generated dendritic cells. *Toxicol. In Vitro* 18, 493–500.
- St Louis, D.C., Woodcock, J.B., Fransozo, G., Blair, P.J., Carlson, L.M., Murillo, M., Wells, M.R., Williams, A.J., Smoot, D.S., Kaushal, S., Grimes, J.L., Harlan, D.M., Chute, J.P., June, C.H., Siebenlist, U., Lee, K.P., 1999. Evidence for distinct intracellular signaling pathways in CD34⁺ progenitor to dendritic cell differentiation from a human cell line model. *J. Immunol.* 162, 3237–3248.
- Straube, F., Grenet, O., Bruegger, P., Ulrich, P., 2005. Contact allergens and irritants show discrete differences in the activation of human monocyte-derived dendritic cells: consequences for in vitro detection of contact allergens. *Arch. Toxicol.* 79, 37–46.
- Toebak, M.J., Pohlmann, P.R., Sampat-Sardjoepersad, S.C., von Blomberg, B.M.E., Bruynzeel, D.P., Scheper, R.J., Rustemeyer, T., Gibbs, S., 2006. CXCL8 secretion by dendritic cells predicts contact allergens from irritants. *Toxicol. In Vitro* 20, 117–124.
- Tuschl, H., Kovac, R., 2001. Langerhans cells and immature dendritic cells as model systems for screening of skin sensitizers. *Toxicol. In Vitro* 15, 327–331.
- Tuschl, H., Kovac, R., Weber, E., 2000. The expression of surface markers on dendritic cells as indicators for the sensitizing potential of chemicals. *Toxicol. In Vitro* 14, 541–549.
- Vandebriel, R.J., Van Och, F.M., van Loveren, H., 2005. *In vitro* assessment of sensitizing activity of low molecular weight compounds. *Toxicol. Appl. Pharmacol.* 207, S142–S148.
- Yoshida, Y., Sakaguchi, H., Ito, Y., Okuda, M., Suzuki, H., 2003. Evaluation of the skin sensitization potential of chemicals using expression of co-stimulatory molecules, CD54 and CD86, on the naive THP-1 cell line. *Toxicol. In Vitro* 17, 221–228.