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Comparative DNA microarray analysis of human monocyte derived dendritic cells and MUTZ-3 cells exposed to the moderate skin sensitizer cinnamaldehyde

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ABSTRACT

The number of studies involved in the development of *in vitro* skin sensitization tests has increased since the adoption of the EU 7th amendment to the cosmetics directive proposing to ban animal testing for cosmetic ingredients by 2013. Several studies have recently demonstrated that sensitizers induce a relevant up-regulation of activation markers such as CD86, CD54, IL-8 or IL-1β in human myeloid cell lines (e.g., U937, MUTZ-3, THP-1) or in human peripheral blood monocyte-derived dendritic cells (PBMDCs). The present study aimed at the identification of new dendritic cell activation markers in order to further improve the *in vitro* evaluation of the sensitizing potential of chemicals. We have compared the gene expression profiles of PBMDCs and the human cell line MUTZ-3 after a 24-h exposure to the moderate sensitizer cinnamaldehyde. A list of 80 genes modulated in both cell types was obtained and a set of candidate marker genes was selected for further analysis. Cells were exposed to selected sensitizers and non-sensitizers for 24 h and gene expression was analyzed by quantitative real-time reverse transcriptase-polymerase chain reaction. Results indicated that PIR, TRIM16 and two Nrf2-regulated genes, CES1 and NQO1, are modulated by most sensitizers. Up-regulation of these genes could also be observed in our recently published DC-activation test with U937 cells. Due to their role in DC activation, these new genes may help to further refine the *in vitro* approaches for the screening of the sensitizing properties of a chemical.

Introduction

The cosmetic industry is currently evaluating the sensitizing potential of new chemicals using animal models (e.g., the Murine Local Lymph Node Assay, reviews: Kimber et al., 2002; Basketter et al., 2007; Gerberick et al., 2007a,b). However, the effort in the development of *in vitro* skin sensitization tests has much increased during the last years due to the public interest in animal welfare and to the entry into force of the 7th amendment to the Cosmetics Directive. This latter foresees an animal testing ban for cosmetic ingredients for all human-health related effects by 2009. Furthermore, it introduces a marketing ban on cosmetic products containing ingredients tested on animals by 2009 for all the endpoints except the repeated-dose toxicity endpoints for which the marketing ban deadline is 2013 (Casati et al., 2005; Aeby et al., 2007). As a technical support for the EU directive an integrated research project entitled Sens-it-iv was initiated within the European Sixth Framework Programme. Involving 28 partners from industry, academia or organizations (COLIPA, IVTIP, JRC, VUB) across Europe, Sens-it-iv attempts to develop and validate alternative *in vitro* methods for the risk assessment of potential skin or lung sensitizers (see website: www.sens-it-iv.eu).

A reliable and robust approach to *in vitro* skin sensitization risk assessment of chemicals should involve a battery of tests (see e.g., Jowsey et al., 2006) comprising diverse key sources of information such as *in silico* and structure–activity relationships (SAR) models (see review: Patlewicz et al., 2008), protein reactivity (e.g., the chemical peptide reactivity assay, Gerberick et al., 2007a,b), bioavailability (logP, skin penetration studies), dendritic cell (DC) activation and T-lymphocyte responses. From a biological point of view, DC activation may represent a central part of the test battery due to the pivotal role of DCs in the sensitization phase of allergic contact dermatitis (Banchereau and Steinman, 1998).

Many laboratories including our research group have developed different *in vitro* skin sensitization test systems using human peripheral blood monocyte-derived dendritic cells (PBMDCs) or human myeloid cell lines U937, THP-1 and MUTZ-3 as source of DC-like cells. We already obtained promising results with PBMDCs and U937 cells (Aeby et al., 2004; Python et al., 2007), indicating that the modulation of CD86 expression measured by flow cytometry and IL-1β/IL-8 gene expression analyzed by quantitative real-time reverse transcriptase-polymerase chain reaction (RT-PCR) could discriminate most sensitizers from non-sensitizers. We also demonstrated that AQP3 was

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significantly down-regulated in sensitizer-treated PBMDCs but not in U937 cells. Toebak et al. (2006) reported that IL-8 (CXCL8) production along with CD86 and CD83 expression were promising markers in PBMDCs for discriminating allergens from irritants. IL-8 protein release, measured by ELISA, was also observed in culture supernatants of U937 cells (Python et al., 2007) and THP-1 cells exposed to sensitizers (Mitjans et al., 2008). While CD86 and CD54 were reported as relevant markers for detecting sensitizers in THP-1 cells (Yoshida et al., 2003; Sakaguchi et al., 2007), only CD86 proved to be a reliable marker in MUTZ-3 cells (Azam et al., 2006). Other markers such as HLA-DR, CD1a, CD40, CD54, CD80 or CCR7 were analyzed in PBMDCs and different cell lines in order to define suitable markers for *in vitro* sensitization tests (reviews: Vandebriel et al., 2005; Ryan et al., 2007). Although the research on new DC-activation markers using microarray technologies already started a few years ago (Ryan et al., 2004; Shoeters et al., 2006, 2007; Hirota and Moro, 2006), CD86, CD54 and IL-8 remained the most commonly used DC-activation markers for *in vitro* testing (Tietze and Blömeke, 2008; Sakaguchi et al., 2009; Mitjans et al., 2008; Nukada et al., 2008).

The present study, which is part of the Sens-it-iv project, aimed at identifying new relevant DC-activation markers to detect sensitizing chemicals by comparing PBMDCs and the human cell line MUTZ-3 gene expression profiles after exposure to a sensitizer. Immature PBMDCs have been shown to be suitable cells as surrogate DCs for the *in vitro* screening of sensitizing chemicals (review: Casati et al., 2005). Like THP1 and U937, the MUTZ-3 cell line is considered to be a relevant source of DC-like cells displaying a phenotypic and transcriptional profile close to immature DC after differentiation with cytokines (Masterson et al., 2002; Larsson et al, 2006). In this study, microarray technology was applied to analyze significantly modulated genes in PBMDCs and MUTZ-3 cells after a 24-h exposure to the moderate sensitizer cinnamaldehyde (CIN) at non-toxic concentrations. Subsequently, a comparative analysis of the microarray results was carried out and compared with published data in order to find additional candidate marker genes. A set of promising genes was finally established and evaluated by quantitative real-time RT-PCR in the U937 cell line.

Materials and methods

Human cell line MUTZ-3 and culture medium. MUTZ-3 cells (cat no. ACC 295, Lot no. 8) were purchased from the DSMZ (German National Resource Centre for Biological Material, website www.dsmz.de, Braunschweig, Germany) and cultured using the culture conditions described by Larsson et al. (2006) (use of recombinant human granulocyte–macrophage colony stimulating factor (rh GM-CSF) instead of feeder cell line), in alpha-MEM (Gibco, Lubioscience, Basel, Switzerland), supplemented with 20% (v/v) fetal calf serum (FCS) (heat-inactivated 30 min at 56 °C) (Amimed, BioConcept, Allschwil, Switzerland) and ~400 U/ml rh GM-CSF (Berlex, Bayer, Seattle, US) and 100 U/ml penicillin–100 µg/ml streptomycin (Gibco, Lubioscience, Basel, Switzerland). Cells were maintained in 24-well plates (1 ml/well) (Falcon, Milian, Meyrin, Switzerland) and passaged three times a week at 1×10^5 cells/ml. They were used for the tests after 3 weeks of culture.

Human cell line U937 and culture medium. U937 cells (ATCC cat. no. CRL-1593.2) were ordered in 2001 from the American Type Culture Collection (ATCC, Manassas, USA) and obtained through LGC Promochem (Molsheim Cedex, France). They were cultured according to our recently published protocol (Python et al., 2007). Briefly, U937 cells were cultured in RPMI 1640 medium without phenol red (Sigma, Buchs, Switzerland) supplemented with 2 mM glutamine (Invitrogen, Lubioscience, Basel, Switzerland), 10 mM HEPES (Fluka, Buchs, Switzerland), 1 mM sodium pyruvate (Gibco, Basel, Switzerland), 4.5 g/l glucose (Fluka, Buchs, Switzerland), 100 U/ml penicillin–100 µg/ml streptomycin and 10% FCS (without heat-inactivation). Cells were maintained in flasks and passaged three times a week (twice at 4×10^5 cells/ml and once at 1×10^5 cells/ml before the weekend). Cells were used for the tests after 1 week of culture.

Human monocytes and culture medium. The culture medium was RPMI 1640 without phenol red supplemented with 5% FCS (without heat-inactivation) (Amimed, BioConcept, Allschwil, Switzerland), 2 mM L-glutamine (Biochrom KG, Berlin, Germany), 800 U/ml of rhGM-CSF and 1000 U/ml of IL-4 (Strathmann Biotech GmbH, Hannover, Germany) referred below as complete culture medium. Purification of human monocytes was performed according to the protocol published by Aeby et al. (2004). Briefly, these cells were isolated from fresh buffy coats by sequential density centrifugation on Ficoll-Paque PLUS and Percoll density gradients (Amersham Pharmacia Biotech AB, Uppsala, Sweden) and stored in liquid nitrogen. Human monocyte derived dendritic-like cells (PBMDCs) were generated by thawing the enriched monocytes from a single donor (when used for microarray analysis) or a pool from 4 donors (for confirmation experiments) and growing in 12-well plates (Falcon, Milian, Meyrin, Switzerland) coated with a 2% agarose matrix (Agarose gel, insect cell culture tested, Gibco, Lubioscience, Basel, Switzerland) and cultured up to 5 days in complete culture medium at 37 °C, 5% CO_2 at 3.5×10^6 cells in 2 ml per well. At day 4, the PBMDCs were used for the *in vitro* sensitization test.

Tested chemicals. Sensitizers: cinnamaldehyde (CIN) (98% pure) (Sigma, Buchs, Switzerland), dinitrochlorobenzene (DNCB) (99% pure) (Sigma, Buchs, Switzerland) and trinitrobenzenesulfonic acid (TNBS) (5% (w/v) solution in water) (Sigma, Buchs, Switzerland). TNBS was used as a positive control for PBMDCs. Non-sensitizers: sodium dodecyl sulfate (SDS) (98.5% pure) (Sigma, Buchs, Switzerland) and salicylic acid (SA) (99% pure) (Sigma, Buchs, Switzerland). TNBS and SDS were dissolved in water/medium. CIN, DNCB and SA were dissolved in dimethyl sulfoxide (DMSO) (Sigma, Buchs, Switzerland).

Activation test with the human cell line MUTZ-3. Cells were seeded at 250,000 cells/ml in a 12-well plate (2 ml/well) containing the culture medium previously described. Test chemical was added to the wells and the plates were incubated at 37 °C in a 5% CO_2 humidified incubator. For microarray analysis, triplicate wells were plated for each treatment. After a 24-h exposure to the test chemical, cells were harvested for analysis of CD86 expression by flow cytometry and RNA isolation for microarray analysis and quantitative real-time RT-PCR. The cell viability was measured by flow cytometry with the propidium iodide (PI) (1 µg/ml) staining method or with the fluorescein diacetate (FDA)-staining method (Aeby et al, 2004). PI was from Invitrogen, Lubioscience, Basel, Switzerland. FDA was from Sigma, Buchs, Switzerland. The absolute viability of non-treated MUTZ-3 cells during the test was 75%–85%. When a chemical was dissolved in DMSO, the final in-well DMSO concentration was 0.25% (the highest concentration that does not affect cell viability and gene expression). The same DMSO concentration was used in the relevant negative control.

Activation test with PBMDCs. Four-day cultured PBMDCs were exposed to the test chemical (without well change) for 24 h at 37 °C in a 5% CO_2 humidified incubator and harvested for analysis of CD86 expression by flow cytometry and RNA isolation for microarray analysis and quantitative real-time RT-PCR. The cell viability was measured by flow cytometry with the FDA-staining method. When a chemical was dissolved in DMSO, the final in-well DMSO concentration was 0.5% (the highest concentration that does not affect cell viability and gene expression). The same DMSO concentration was used in the relevant negative control. Three

independent single donor experiments were conducted for microarray analysis.

U937 activation test protocol. The test is based on a recently published protocol (Python et al., 2007) slightly modified to include 100 U/ml penicillin–100 μg/ml streptomycin (Pen/Strep) in the culture medium and 0.1% final in-well DMSO. Briefly, 250,000 cells were seeded in each well of a 12-well plate containing 2 ml of culture medium containing 50 pg/ml IL-4 and Pen/Strep. After 2 to 4 h, test items were added into wells. After incubating for 24 h in a 5% CO_2 humidified incubator, cells were harvested for flow cytometric analysis and RNA isolation for microarray analysis and quantitative real-time RT-PCR. The cell viability was measured by flow cytometry with PI (1 μg/ml) staining method. The absolute viability of non-treated U937 cells during the test was >90%.

Flow cytometry analysis. Anti-CD86-fluorescein isothiocyanate (FITC) (clone 2331 FUN-1) (BD Biosciences, Basel, Switzerland) antibody and its corresponding isotype control were used for labelling. Cells were analyzed on a Coulter Epics XL or FC500 flow cytometer (Beckman Coulter, Nyon, Switzerland). MUTZ-3 cells and PBMDCs were labelled and analyzed as described in Python et al. (2007) and Aeby et al. (2004), respectively.

RNA extraction and reverse transcription. Total RNA was isolated from cell pellets with TRIzol reagent (Invitrogen, Lubioscience, Basel, Switzerland). All reactions took place at 2–8 °C. After centrifugation of the cell suspension at 450 *g* for 5 min, the pellet was resuspended in the TRIzol reagent (400 μl/10^6 cells). 1 μl of LPA (Linear Polyacrylamide, GeneElute LPA, Sigma, Buchs, Switzerland) was added to the sample which was then vortexed for 10 s. After addition of chloroform (80 μl/10^6 cells) (Sigma, Buchs, Switzerland), 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 (Sigma, Buchs, Switzerland) at −20 °C overnight. The pellet was washed with 75% ethanol (Sigma, Buchs, Switzerland), dried and resuspended in 10 μl RNAse-free water. The sample was then aliquoted into 2 tubes. One tube was used for microarray analysis and the other for gene expression by RT-PCR. The RNA was quantified by OD_{260} measurement and adjusted to 50 μg/ml with RNAse-free water. The purity of RNA was assessed by measurement of the $OD_{260/280}$ ratio. 450 ng of purified total RNA was reverse transcribed using random hexamers (50 ng/reaction) with the ThermoScript RT-PCR system (Invitrogen, Lubioscience, Basel, Switzerland) according to the instruction manual.

Quantitative real-time polymerase chain reaction (PCR). TaqMan® probes (TaqMan® Gene Expression Assays, Applied Biosystems, Rotkreuz, Switzerland) were used to perform the gene expression quantification on the LightCycler® (Roche Diagnostics, Rotkreuz, Switzerland) with the kit «Premix Ex Taq Perfect Real-time» (TaKaRa, AxonLab, Le-Mont-sur-Lausanne, Switzerland). The PCR was performed in glass capillaries for 50 cycles. An amplification cycle consisted of a denaturation for 5 s at 95 °C and an annealing/elongation for 30 s at 60 °C. The fluorescence was measured 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 probes used in this study are indicated in Table 1 with their corresponding ID number. As a housekeeping gene for MUTZ-3 cells and PBMDCs, 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) as described in Python et al. (2007). For U937 cells, the 18S ribosomal RNA (18S rRNA) was used as a housekeeping gene (see Table 1). The expression levels of all genes were then normalized to the expression level of 28S or 18S rRNA.

Table 1
Gene probes.

Gene probes	Assay ID
IL-1β	Hs00174097_m1
IL-8	Hs00174103_m1
FCGR2B	Hs00269610_m1
CES1	Hs00275607_m1
NQO1	Hs00168547_m1
CREM	Hs00181804_m1
PIR	Hs00186374_m1
TRIM16	Hs00232396_m1
IFI27	Hs00271467_m1
MX1	Hs00182073_m1
PBEF1	Hs00237184_m1
18S	Hs99999901_s1

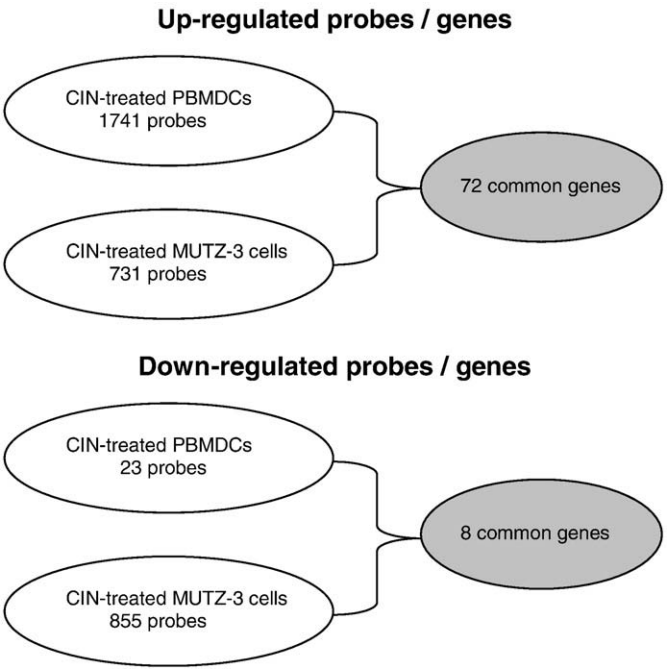

Fig. 1. Distribution of up- and down-regulated probes/genes by CIN in PBMDCs and MUTZ-3 cells. The number of modulated genes shared by both cell types are indicated on a grey background.

Microarray analysis. The microarray analysis was performed by the Microarray Resource Centre (MARC) of Lund University (Sweden), using the GeneChip® Human Genome U133 Plus 2.0 Array containing >54,000 probe sets, including 38,500 human genes (Affymetrix Inc, Santa Clara, USA). The "Significance Analysis of Microarray" (SAM) analysis (Stanford University, USA; Tusher et al., 2001) was performed to detect genes with significant expression changes in CIN-treated PBMDCs (one donor at 2.5 μg/ml CIN and two donors at 5 μg/ml CIN) and MUTZ-3 cells (5 μg/ml CIN). Different statistical tests in SAM analysis had to be used for individual samples (paired *t*-test for PBMDCs) or pooled samples (non parametric Wilcoxon test for MUTZ-3 cells). The statistical significance of the expression was assessed by computing a *q*-value for each gene in the SAM analysis. The high *q*-values (>55%) obtained for PBMDCs are probably due to the donor-to-donor variability. In PBMDCs, the results of SAM analysis were filtered for a fold change (FC) cutoff ≥1.5 and a *q*-value <75% for up-regulated genes. These *q*- and FC cutoff values were chosen according to those obtained for CD86, IL-1β and IL-8 genes. This "standard set" of genes is considered as significantly

Table 2
Biological processes classification of genes significantly up-regulated by CIN in both PBMDCs and MUTZ-3 cells.

Accession no.	Gene name	Gene symbol	FC induced by CIN	
			PBMDCs	MUTZ-3 cells
Apoptosis-related (3)				
NM_006410	HIV-1 Tat interactive protein 2, 30 kDa	HTATIP2	1.66	2.05
NM_003311	Pleckstrin homology-like domain, family A, member 2	PHLDA2	2.14	2.90
NM_014452	Tumor necrosis factor superfamily, member 21	TNFRSF21	2.18	2.44
Cell adhesion-related (3)				
NM_003812	ADAM metallopeptidase domain 23	ADAM23	6.06	2.87
NM_000094	Collagen, type VII, alpha 1	COL7A1	2.03	2.82
NM_177444	PTPRF interacting protein, binding protein 1 (liprin beta 1)	PPFIBP1	1.80	2.54
Cell differentiation-related (1)				
NM_001430	Endothelial PAS domain protein 1	EPAS1	3.09	3.05
Endocytosis-related (1)				
NM_013437	Low density lipoprotein-related 12	LRP12	1.95	2.24
G-protein-related (1)				
NM_005294	G-protein-coupled receptor 21	GPR21	2.27	2.72
Immune response/inflammatory response-related (8)				
NM_006889	CD86 molecule	CD86	1.70	2.47
NM_004001	Fc fragment of IgG, low affinity IIb, receptor (CD32)	FCGR2B	2.04	2.16
NM_201563	Fc fragment of IgG, low affinity IIc, receptor for (CD32)	FCGR2C	2.06	3.04
NM_002032	Ferritin, heavy polypeptide 1	FTH1	1.53	2.90
NM_139010	Hemochromatosis	HFE	2.68	2.60
NM_000576	Interleukin 1, beta	IL1B	1.97	2.06
NM_000584	Interleukin 8	IL8	1.67	2.10
NM_138554	Toll-like receptor 4	TLR4	2.12	2.49
Metabolism-related (4)				
NM_032385	Chromosome 5 open reading frame 4	C5orf4	1.73	2.14
NM_152572	Chromosome 9 open reading frame 98	C9orf98	7.55	4.68
X03674	Glucose-6-phosphate dehydrogenase	G6PD	1.68	2.56
NM_018271	Hypothetical protein FLJ10916 (=threonine synthase-like 2 (*S. cerevisiae*))	THNSL2	3.08	2.40
Protein synthesis/modification-related (2)				
NM_001123	Adenosine kinase	ADK	2.43	2.09
NM_003338	Ubiquitin-conjugating enzyme E2D 1 (UBC4/5 homolog, yeast)	UBE2D1	9.82	2.57
Response to stress-related (1)				
NM_139078	Mitogen-activated protein kinase-activated protein kinase 5	MAPKAPK5	2.07	3.70
Response to toxin-related (2)				
NM_001266	Carboxylesterase 1 (monocyte/macrophage serine esterase 1)	CES1	16.43	5.43
NM_000903	NAD(P)H dehydrogenase, quinone 1	NQO1	2.98	5.98
Response to wounding (1)				
NM_001124	Adrenomedullin	ADM	1.61	2.23
Signal transduction-related (2)				
NM_000922	Phosphodiesterase 3B, cGMP-inhibited	PDE3B	1.82	2.03
NM_016169	Suppressor of fused homolog (*Drosophila*)	SUFU	3.70	2.07
Transcription-related (8)				
NM_182724	CAMP responsive element modulator	CREM	1.50	2.70
NM_003662	Pirin (iron-binding nuclear protein)	PIR	2.22	10.26
NM_018699	PR domain containing 5	PRDM5	3.96	3.99
NM_005901	SMAD family member 2	SMAD2	2.83	4.30
NM_006470/NM_001037330	Tripartite motif-containing 16 /// tripatile motif-containing 16-like	TRIM16/TRIM161I	1.89	2.46
NM_030824	Zinc finger protein 442 /// zinc finger protein 442	ZNF442	3.89	2.06
XM_036218	Zinc finger protein 506	ZNF506	2.01	2.23
NM_016331	Zinc finger protein 639	ZNF639	1.67	3.69
Transport-related (7)				
NM_032189	ATPase, Class VI, type 11A	ATP11A	1.83	2.35
NM_020038	ATP-binding cassette, sub-family C (CFTR/MRP), member 3	ABCC3	2.01	2.42
NM_004171	Solute carrier family 1 (glial high affinity glutamate transporter), member 2	SLC1A2	1.96	2.46
NM_015865	Solute carrier family 14 (urea transporter), member 1 (Kidd blood group)	SLC14A1	5.71	3.53
NM_052885	Solute carrier family 2 (facilitated glucose transporter), member 13	SLC2A13	2.12	2.08
NM_021097	Solute carrier family 8 (sodium/calcium exchanger), member 1	SLC8A1	2.26	2.11
NM_014035	Sorting nexing 24	SNX24	4.51	9.19
Transport-, apoptosis-related (1)				
NM_018844	B-cell receptor-associated protein 29	BCAP29	1.92	2.65
Miscellaneous/unknown (27)				
NM_001353	Aldo-keto reductase family 1, member C1	AKR1C1	2.19	2.81
NM_020919	Amyotrophic lateral sclerosis 2 (juvenile)	ALS2	4.66	2.60
NM_152730	Chromosome 6 open reading frame 170	C6orf170	2.65	3.19
NM_173830	Chromosome 6 open reading frame 182	C6orf182	2.05	2.03
NM_181814	Chromosome 14 open reading frame 29	ABHD12B	10.25	2.49
NM_024764	Chromosome 14 open reading frame 161	C14orf161	1.62	6.22
NM_016441	Cysteine rich transmembrane BMP regulator 11 (chordin-like)	CRIM1	3.68	2.86
NM_030771	Coiled-coil domain containing 34	CCDC34	3.59	2.45
NM_014183	Dynein, light chain, roadblock-type 1	DYNLRB1	1.98	2.03
NM_001978	Erythrocyte membrane protein band 4.9 (dematin)	EPB49	3.57	2.51
NM_017842	Hypothetical protein FLJ20489	FLJ20489	1.71	5.10
AK095081	Hypothetical protein LOC283177	LOC283177	1.56	2.13
BC040669	Hypothetical protein LOC339894	LOC339894	7.61	2.67

Table 2 *(continued)*

Accession no.	Gene name	Gene symbol	FC induced by CIN	
			PBMDCs	MUTZ-3 cells
BM906128	Hypothetical protein MGC14376	MGC14376	1.52	2.99
NM_012211	Integrin, alpha 11	ITGA11	1.66	5.25
NM_025176	KIAA0980 protein	RP4-691N24.1	2.65	2.75
NM_005780	Lipoma HMGIC fusion partner	LHFP	2.12	11.41
NM_001039703	Neuroblastoma breakpoint family, member 10	NBPF10	3.91	2.13
NM_052839	Pannexin 2	PANX2	1.88	2.54
NM_024859	PDZ domain containing, X chromosome	MAGIX	1.69	2.83
XM_290799	Rho GTPase activating protein 23	ARHGAP23	2.06	2.56
BX537948	Sarcoglycan, delta (35 kDa dystrophin-associated glycoprotein)	SGCD	2.70	2.44
BX640843	Similar to NmrA-like family domain containing 1 /// hypothetical protein LOC652465	LOC344887	1.61	7.19
NM_052832	Solute carrier family 26, member 7	SLC26A7	5.39	7.30
NM_004768	Splicing factor, arginine/serine-rich 11	SFRS11	2.38	2.16
NM_014393	Staufen, RNA blinding protein, homolog 2 (*Drosophila*)	STAU2	1.75	3.71
NM_178566	Zinc finger, DHHC-type containing 21	ZDHHC21	2.76	2.66

modulated since they have been previously used as sensitization marker genes in our lab for U937 cells and PBMDCs (Python et al., 2007; Aeby et al., 2004). Significant down-regulated genes were obtained with a FC cutoff ≤ -2.5 (and a q-value <75%). In MUTZ-3 cells, the results of SAM analysis were then filtered for a q-value <5% and a FC cutoff ≥ 2 or ≤ -2 for detecting significant up- or down-regulated genes, respectively. The values of the FC cutoff and q-value were chosen according to those obtained for the "standard set" (CD86, IL-1β and IL-8) in MUTZ-3 cells.

Results

Flow cytometric analysis

Non-toxic concentrations of the moderate sensitizer CIN (2.5 or 5 μg/ml) were applied for 24 h to PBMDCs in three independent experiments (PBMDCs from one single donor per experiment) and to MUTZ-3 cells in a triplicate experiment (three independent wells). As a positive control for PBMDCs, a non-toxic concentration of 2,4,6-trinitrobenzenesulfonic acid (TNBS) (100 μg/ml) was applied. To determine the cytotoxicity of the test compounds, the following viability criterion was used for the PBMDCs and MUTZ-3 cells: a test item concentration is considered as non-toxic when the cell viability reaches ~85% (FDA-staining method) or ~90% (PI-staining method) of the corresponding negative control.

The viability and CD86 expression were analyzed by flow cytometry in MUTZ-3 cells and in PBMDCs from 5 single donors. The measured viability values indicated that for both cell types the used CIN concentrations were not cytotoxic according to our viability criterion and induced an up-regulation of CD86 expression confirming a successful cell activation (triplicate samples from MUTZ-3 cells and PBMDCs from three single donors used for microarray analysis are shown in Table 4). RNA was then isolated from treated cells and shipped to the Microarray Resource Centre (MARC) of Lund University (Sweden). Based on the RNA quality criteria defined by MARC, RNA samples from MUTZ-3 cells and from three single donors were selected for microarray analysis.

Microarray analysis and comparison

Using the criteria described in the "Microarray analysis" section of "Materials and methods", 1741 up-regulated genes and 23 down-regulated genes were detected in PBMDCs, whereas 731 and 855 genes, respectively, were up- and down-regulated in MUTZ-3 cells (Fig. 1). By comparing these results, 72 genes including our standard set of genes (CD86, IL-1β and IL-8, see explanations in "Microarray analysis" section of "Materials and methods") were found to be significantly up-regulated and only 8 genes significantly down-regulated after the 24-h exposure period to CIN in both cell types (Fig. 1). The 80 up- or down-regulated genes were then grouped according to the biological processes defined by the Gene Ontology Consortium (Ashburner et al., 2000) (available on http://source.stanford.edu and http://www.expasy.org). Since a gene may be involved in different biological processes, we decided to assign one of the known biological processes relevant for this study for each gene in 16 different groups (Tables 2 and 3). For example, in the group related to immune/inflammatory response (6th group in Table 2) including CD86, IL-1β and IL-8 genes, we identified 5 additional up-regulated genes (FCGR2B, FCGR2C, FTH1, HFE, TLR4) and two down-regulated genes (IFI27, MX1) (Table 3). Tables 2 and 3 summarize the identified biological pathways. The tested chemical (CIN) has induced the modulation of genes involved in numerous biological pathways both in PBMDCs and MUTZ-3 cells.

Table 3
Biological processes classification of genes significantly down-regulated by CIN in both PBMDCs and MUTZ-3 cells.

Accession no.	Gene name	Gene symbol	FC induced by CIN	
			PBMDCs	MUTZ-3 cells
Immune response-related (2)				
NM_005532	Interferon, alpha-inducible protein 27	IFI27	−6.30	−7.20
NM_002462	Myxovirus (influenza virus) resistance 1, interferon-inducible protein p78 (mouse)	MX1	−3.15	−2.55
Signal transduction-related (1)				
NM_003576	Serine/threonine kinase 24 (STE20 homolog, yeast)	STK24	−5.12	−2.41
Transcription-related (2)				
NM_014515	CCR4–NOT transcription complex, subunit 2	CNOT2	−3.56	−2.24
NM_018456	ELL associated factor 2	EAF2	−8.41	−2.59
Miscellaneous/unknown (3)				
NM_178568	Reticulon 4 receptor-like 1	RTN4RL1	−4.44	−6.18
NM_006820	Interferon-induced protein 44-like	IFI44L	−5.35	−5.78
NM_014583	LIM and cysteine-rich domains 1	LMCD1	−7.56	−6.58

FC: Fold change.

Confirmation of the microarray results through quantitative real-time RT-PCR and flow cytometry

To evaluate the relevance of microarray analyses results and to check the activation status of CIN-exposed cells, the modulation of the expression of IL-1β, IL-8 and AQP3 genes (set of activation markers published by Aeby et al., 2004) was examined by quantitative real-time RT-PCR in the same RNA samples previously analyzed by microarray (Table 4). Similarly, the fold change of CD86 expression was measured by flow cytometry and compared with the fold change obtained by microarray analyses (Table 4). In MUTZ-3 cells, microarray results for theses genes are well supported by the data obtained through quantitative real-time RT-PCR and flow cytometric analyses. In PBMDCs (three single donors), the donor-to-donor variability is reflected in the RT-PCR analysis by the difference in expression for IL-1β, IL-8 and CD86 between donors as well as in the microarray analysis by the *q*-value, indicating a low level of statistical significance in SAM analysis. However, these results indicate that in MUTZ-3 cells as well as in PBMDCs from 2 out of 3 single donors CIN induced a weak to moderate activation of the three DC-activation markers (CD86, IL-1β and IL-8) routinely used in our lab. Although AQP3 gene was found as a significant up-regulated marker in MUTZ-3 cells, in PBMDCs RT-PCR analysis indicated a down-regulation trend of this gene, confirming microarray results (Table 4).

In order to find candidate DC-activation marker genes and to further evaluate our results, six up-regulated genes (CES1, PIR, NQO1, FCGR2B, TRIM16, CREM) and two down-regulated genes (IFI27, MX1) were chosen from the list of 80 up- or down-regulated genes obtained by the comparative microarray analysis (Tables 2 and 3). These genes were selected due to their relevant modulation in PBMDCs and MUTZ-3 cells, to their relation to immune response and/or their link to DC activation reported in the recent literature. CES1 and PIR were chosen because of their strong up-regulation in PBMDCs (16.43-fold up-regulated) or MUTZ-3 cells (10.26-fold up-regulated), respectively. NQO1 is a phase II metabolizing/detoxifying enzyme induced in response to oxidative stress (reviews: Ross et al., 2000; Nioi and Hayes, 2004) and is modulated in PBMDCs (2.98-fold up-regulated) and MUTZ-3 cells (5.98-fold up-regulated). FCGR2B (CD32), involved in the phagocytosis of immune complexes and modulation of antibody production by B-cells, is moderately up-regulated in both cell types (~2-fold up-regulated). TRIM16 was reported as a valuable candidate marker of PBMDCs activation following exposure to the contact allergen dinitrobenzenesulfonic acid (Ryan et al., 2004). PBEF1 was added in the list as an additional gene since Schoeters et al. (2007) reported that this gene as well as CREM could be valuable candidates due to their capacity to predict the sensitizing potential of different classes of chemicals in CD34+ progenitor-derived dendritic cells. However, its expression was significantly up-regulated only in PBMDCs (3.53-fold up-regulated, data not shown). IFI27 and MX1 were also selected as down-regulated candidate genes due to their relation to the immune response. Using the same RNA samples as for the microarray experiments, quantitative real-time RT-PCR experiments confirmed the microarray results for all genes (with the exception of PBEF1 and CREM) in PBMDCs and MUTZ-3 cells. Fig. 2 shows the results obtained for the confirmed up-regulated genes.

Table 4
Comparison of the fold change values (FC) of CD86, IL-1β, IL-8 and AQP3 expression measured by flow cytometry or by quantitative real-time RT-PCR with the mean values obtained by microarray analyses (M) in PBMDCs and MUTZ-3 cells.

Markers	**CIN-treated PBMDCs**					
	RT-PCR (FC)				M (FC)	*q*-value
	Donor 1	Donor 2	Donor 3	Mean		
IL-1β	1.44	4.61	2	**2.68**	**1.97**	64%
IL-8	1.22	4.56	1.13	**2.30**	**1.67**	68%
AQP3	0.76	0.91	0.82	**0.83**	**0.80**	ns
	Cytometry				M (FC)	*q*-value
CD86	1.27	0.87	2.19	**1.44**	**1.70**	72%
Viability	83%	98%	92%	91%		
Markers	**CIN-treated MUTZ-3 cells**					
	RT-PCR (FC)				M (FC)	*q*-value
	Sample 1	Sample 2	Sample 3	Mean		
IL-1β	3.05	3.63	2.09	**2.92**	**2.06**	0%
IL-8	2.02	2.22	1.39	**1.88**	**2.10**	0%
AQP3	1.69	1.49	1.37	**1.52**	**1.65**	0%
	Cytometry				M (FC)	*q*-value
CD86	1.43	1.5	1.49	**1.47**	**2.47**	0%
Viability	94%	95%	95%	95%		

The viability is expressed as the percentage of the respective negative control. (ns = not significant).

Evaluation of the selected candidate DC-activation marker genes in PBMDCs and MUTZ-3 cells by quantitative real-time RT-PCR

The selected candidate DC-activation marker genes (CES1, PIR, NQO1, FCGR2B, TRIM16, IFI27, MX1) were further examined in PBMDCs and MUTZ-3 cells after a 24-h exposure to the sensitizers TNBS, CIN and dinitrochlorobenzene (DNCB) and the non-sensitizers sodium dodecyl sulfate (SDS) and salicylic acid (SA). In order to reduce the donor-to-donor variability observed previously, we used pooled PBMDCs obtained from 4 donors as described by Aeby et al. (2004). Fig. 3 shows the expression of the up-regulated candidate genes measured by quantitative real-time RT-PCR in PBMDCs or in MUTZ-3 cells. Each chemical was tested at the highest non-toxic tested concentration (~90% of viability compared to the negative control), with the exception of TNBS tested at 71% viability in PBMDCs compared to the negative control. CES1 gene expression was very strongly increased in PBMDCs when exposed to TNBS (~47-fold change) and CIN (~12-fold change). It was also strongly induced in DNCB-treated PBMDCs (~6-fold change) and in MUTZ-3 cells exposed to TNBS (~4.5-fold change) and CIN (~6-fold change) (Fig. 3). However, DNCB did not induce CES1 gene expression in MUTZ-3 cells. Among other up-regulated candidate genes, NQO1 and PIR were moderately to strongly up-regulated ($2 < \text{fold change} < 9$) in both cell types after exposure to the sensitizers TNBS and CIN. No relevant modulation of NQO1 and PIR gene expression was detected in DNCB-treated MUTZ-3 cells. Nevertheless, DNCB induced a weak up-regulation (~1.3-fold change) of NQO1 and PIR expression in PBMDCs. No relevant modulation of CES1, NQO1 and PIR was induced by the non-sensitizers SDS and SA in both cell types.

TNBS and CIN induced a moderate increase in TRIM16 expression ($1.9 < \text{fold change} < 3$) in both cells types. However, no modulation of this gene was observed in DNCB, SDS and SA-treated cells. FCGR2B was weakly up-regulated in TNBS- and CIN-treated PBMDCs, but no relevant modulation was found in DNCB-treated PBMDCs and in MUTZ-3 cells exposed to all three sensitizers. MX1 and IFI27, the two selected genes shown to be down-regulated after exposure to CIN (Table 3) were not discriminating sensitizers from non-sensitizers and thus do not represent suitable candidate markers for DC activation by sensitizers (data not shown).

Evaluation of TRIM16, PIR, CES1 and NQO1 genes in U937 cells by quantitative real-time RT-PCR

The TRIM16, PIR, CES1 and NQO1 genes were further examined after a 24-h test chemical exposure in the U937 cell line according to a slightly modified protocol (see "Materials and methods") compared to the original protocol (Python et al., 2007). The gene expression patterns (Fig. 4) obtained in U937 cells were similar to

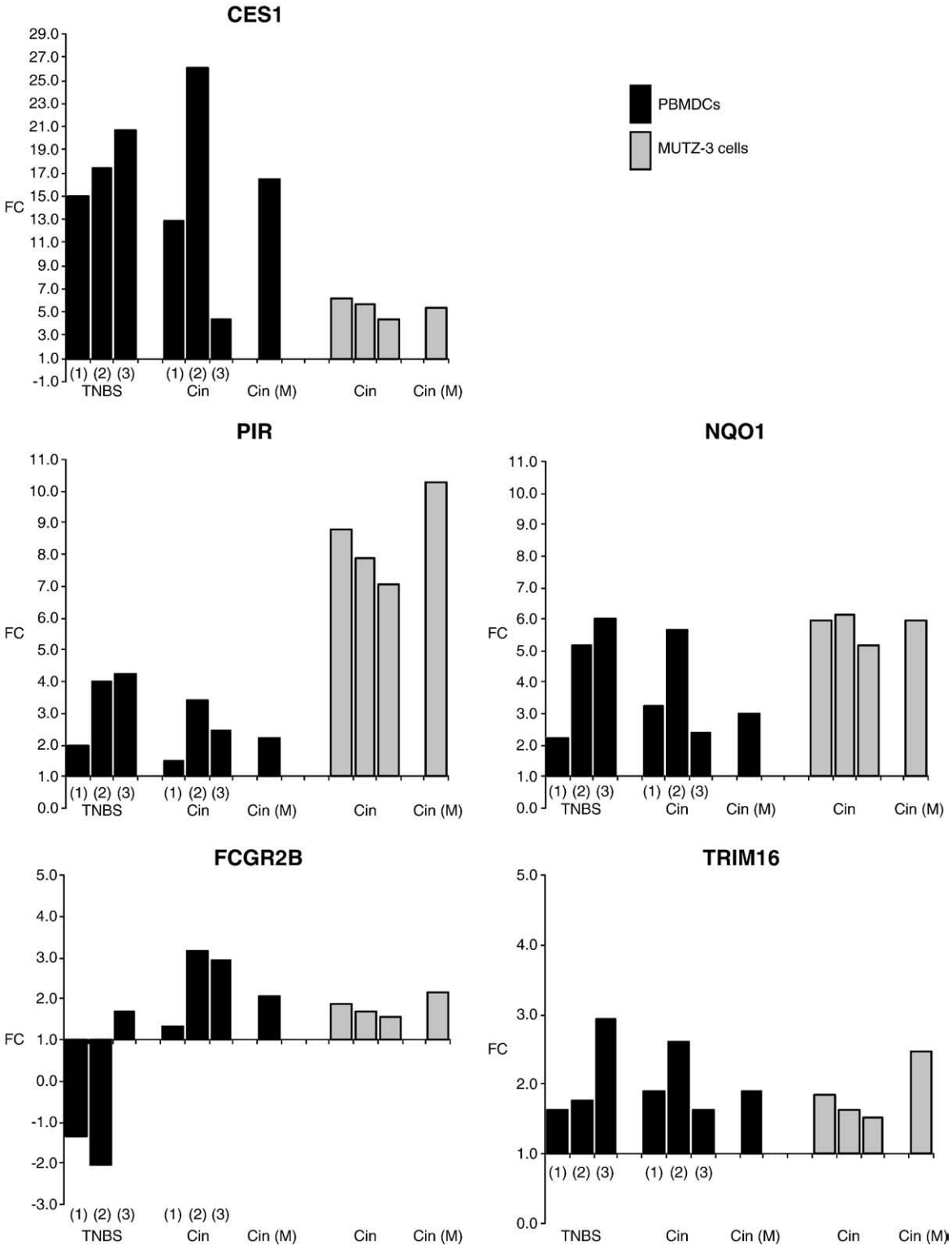

Fig. 2. Comparison of quantitative real-time RT-PCR analysis (X-axis: TNBS, CIN) and microarray analysis (X-axis: CIN (M)) for CES1, PIR, NQO1, FCGR2B and TRIM16 genes after a 24-h exposure to CIN and/or TNBS in PBMDCs obtained from three single donors (X-axis: 1, 2, 3, black bars) and in MUTZ-3 cells in a triplicate experiment (grey bars). Y-axis: fold change (FC) values of selected genes, compared to the negative control. TNBS was used as a positive control in PBMDCs (only analyzed by quantitative real-time RT-PCR).

those obtained in PBMDCs and MUTZ-3 cells (Fig. 3). CIN induced a relevant expression of TRIM16, PIR, CES1 and NQO1 genes in U937 cells, while DNCB induced a weak up-regulation or no change in gene expression. No significant modulations of these genes were observed in U937 cells exposed to non-sensitizers SDS and SA. However, a weak dose response was observed for CES1 in SDS-treated cells.

Discussion

The present study aimed at the identification of new dendritic cell activation markers in order to further improve the *in vitro* evaluation of the sensitizing potential of chemicals. For this project, the human myeloid leukemia cell line MUTZ-3 (Hu et al., 1996; Masterson et al., 2002) was chosen as a source of DC-like cells and its gene expression

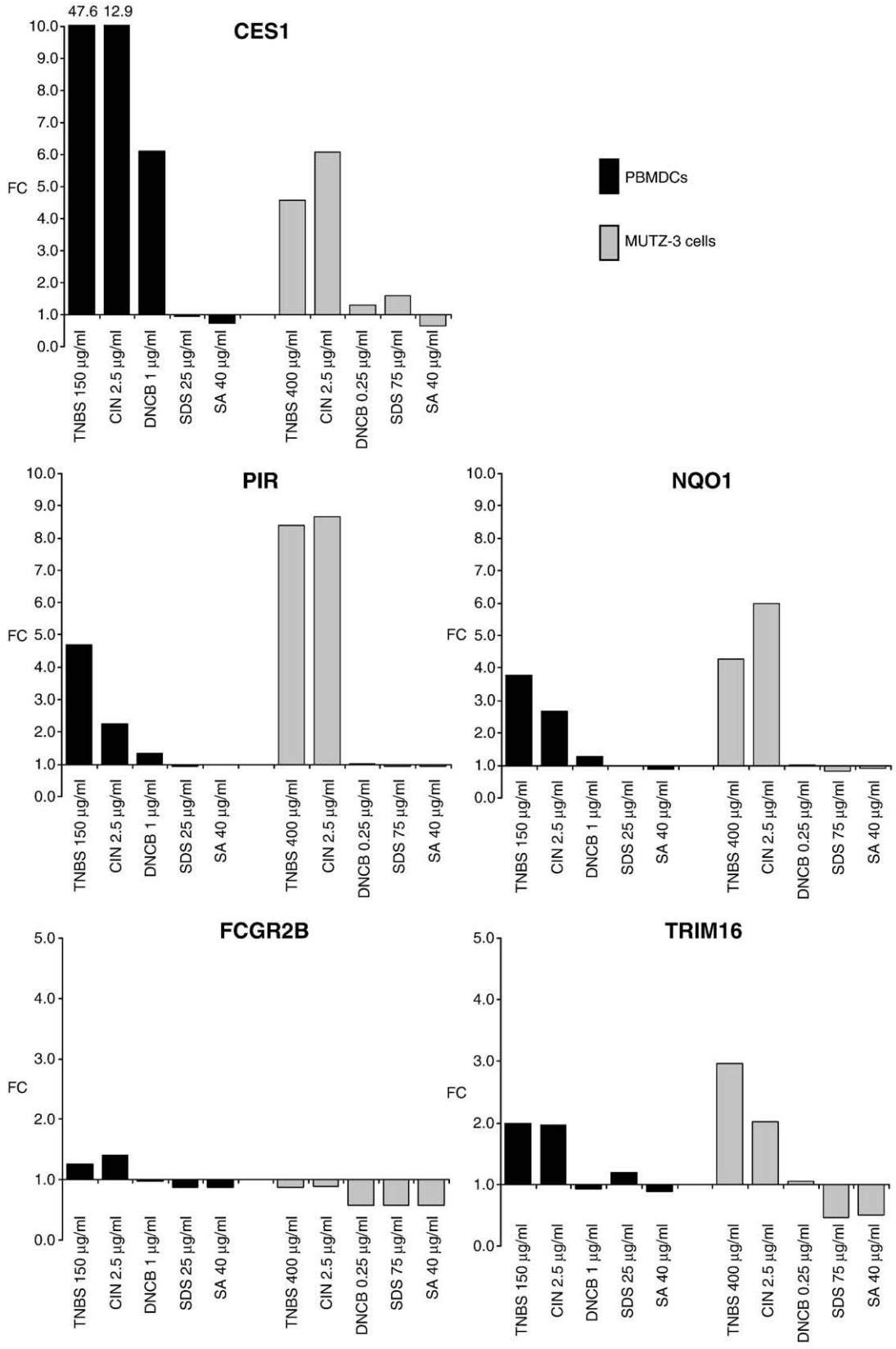

Fig. 3. Quantitative real-time RT-PCR analysis of up-regulated CES1, PIR, NQO1, FCGR2B and TRIM16 genes after a 24-h exposure to the indicated chemicals in PBMDCs obtained from a pool of 4 donors (black bars) and in MUTZ-3 cells (grey bars). Y-axis: fold change (FC) values of selected genes, compared to the negative control.

pattern was compared to the pattern obtained from PBMDCs (Aeby et al. 2004) after exposure to the skin sensitizer CIN. A set of candidate marker genes was selected using microarray analysis and the relevance of the selected genes was further analyzed in both cell types after exposure to a set of test chemicals by quantitative real-time RT-PCR. Based upon relevant up-regulation in MUTZ-3 cells and PMBDCs,

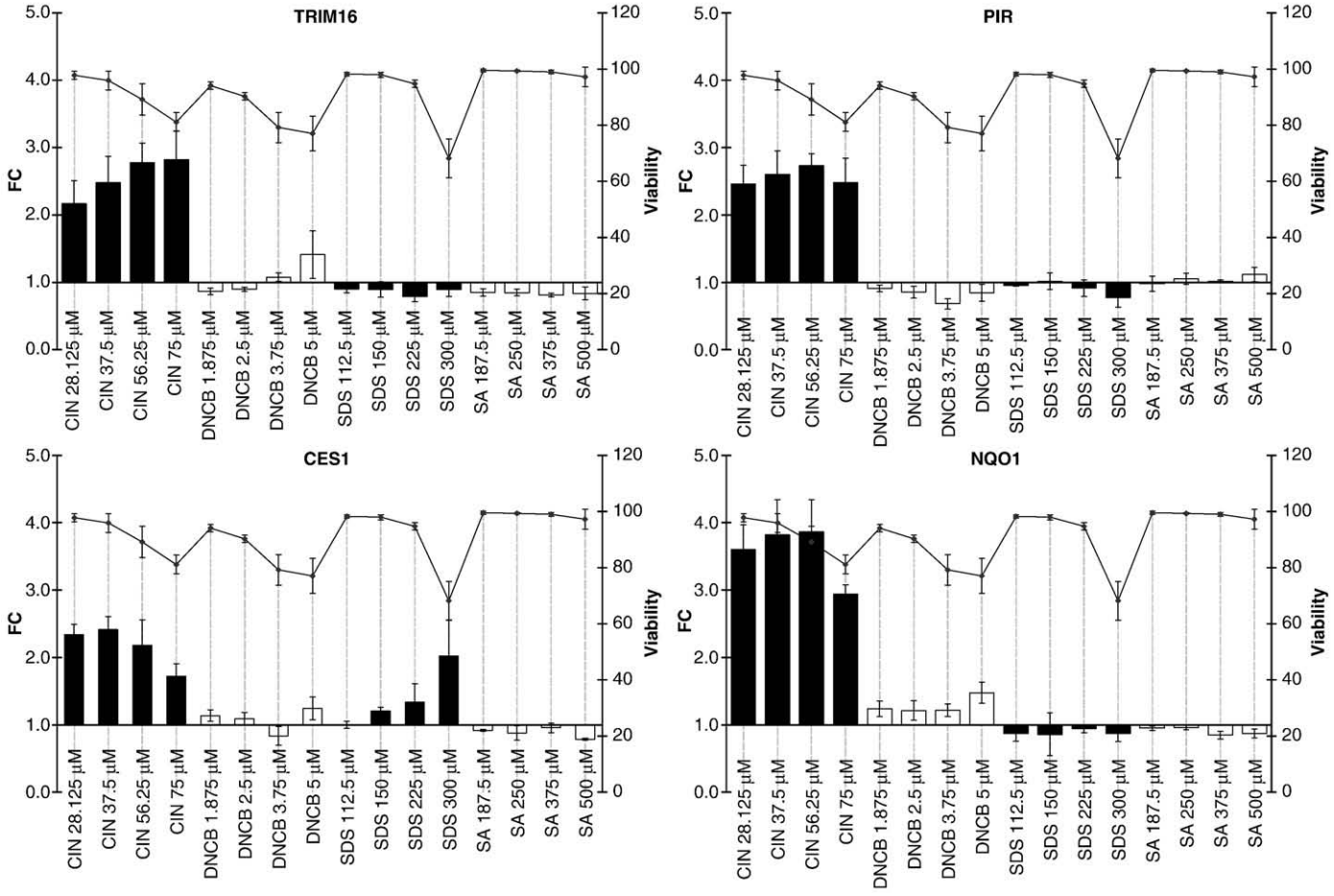

Fig. 4. Quantitative real-time RT-PCR analysis of TRIM16, PIR, CES1 and NQO1 genes after a 24-h exposure to the indicated chemicals in U937 cells. Left Y-axis: fold change (FC) values of selected genes, compared to the negative control. Right Y-axis: percent of viability (black rhombs), compared to the negative control. The mean and the standard deviation of three independent experiments are shown for each chemical.

a set of 4 genes was finally examined using the published U937 activation protocol (Python et al., 2007).

Exposed PBMDCs (three single donors) and MUTZ-3 cells were first analyzed by flow cytometry for CD86 expression and by quantitative real-time RT-PCR for IL-1β, IL-8 and AQP3 gene expression in order to examine their activation status as characterized by Aeby et al. (2004) and to confirm microarray results. Results indicated that CIN was able to induce a weak to moderate up-regulation of CD86 expression and IL-1β/L-8 gene expression in PBMDCs from 2 out of 3 single donors and in the MUTZ-3 cells. However, in PBMDCs obtained from single donors, differences in activation marker expressions (CD86, IL-1β, IL-8) were observed between the three donors (see Table 4). This is best explained by donor-to-donor variability as already observed in other studies (Aeby et al., 2004; Staquet et al., 2004; Hulette et al., 2002; Aiba et al., 1997) and mentioned in the review from Ryan et al. (2005). It was interesting to note that the absolute viability of the non-treated MUTZ-3 cells in the test and in routine cell culture was approximately 80% (data not shown). This relatively low viability value could be due to a DC differentiation of the MUTZ-3 cells in the presence of GM-CSF as already observed under similar conditions by Santegoets et al. (2006).

RNAs isolated from PBMDCs and MUTZ-3 cells exposed to CIN were then examined with the Affymetrix microarray system. The lists of genes obtained from SAM analysis were filtered so that the results included CD86, IL-1β and IL-8 genes which are known DC-activation markers and routinely used in many laboratories (Python et al., 2007; Toebak et al., 2006; Aeby et al., 2004; Aiba et al., 1997). Results indicated that the number of significantly up- or down-regulated genes was almost equally distributed in MUTZ-3 cells. In contrast, PBMDCs displayed a much larger proportion of up-regulated genes compared with down-regulated genes. In order to refine microarray results and to find relevant genes expressed in both cell types, microarray data obtained from PBMDCs were compared with those obtained from MUTZ-3 cells. This approach delivered a short list of 80 genes significantly modulated in both cell types (72 up-regulated and 8 down-regulated genes). The number of overlapping genes between the two cell types is however relatively low. This may be due to the differentiation status of the compared cells: Relatively undifferentiated MUTZ-3 cells were compared with differentiated PBMDCs obtained from different donors. Only 10 genes (including CD86, IL-1β and IL-8) related to immune/inflammatory response were significantly up- or down-regulated in both PBMDCs and MUTZ-3 cells (see Tables 2 and 3). As discussed below, several genes were also reported to be up-regulated in similar microarray studies, supporting the reliability of our microarray results.

For example, in the study from Ryan et al. (2004), TRIM16 was also found to be up-regulated in PBMDCs after a 24-h exposure to dinitrobenzenesulfonic acid. Schoeters et al. (2006) reported CREM and CRIM1 as up-regulated genes in CD34+-progenitor-derived dendritic cells after exposure to nickel sulfate. Hirota and Moro (2006) showed that HTATIP2, FTH1, NQO1, PIR and ABCC3 genes are expressed in THP-1 cells after a treatment with different sensitizers. Recently, Verstraelen et al. (2008) reported that FCGR2B, PIR and NQO1 were significantly up-regulated after exposure to the respiratory sensitizer hexamethylene diisocyanate in THP-1 cells. However, the number of overlaps between our data and microarray data from

the literature is quite limited. This might be due to the differences in PBMDCs isolation, cell types and cell culture procedures influencing the gene expression or the methods and criteria used to analyze the microarray data. Moreover different contact sensitizers are known to induce specific genes related to different biological processes as proposed by Boislève et al. (2004) suggesting that nickel and DNCB induce CCR7 expression on human dendritic cells through different signalling pathways.

A set of 9 genes (CES1, NQO1, CREM, PIR, TRIM16, FCGR2B, IFI27, MX1, PBEF1) selected according to our results and to their relevance in the literature (see above) were used for confirmation of our microarray data and for their evaluation as new candidate marker genes of DC activation. Though PBFE1 was only expressed in PBMDCs (data not shown), it was added to the set of selected genes because Shoeters et al., (2007) reported it as a potential biomarker for skin sensitization in CD34+-progenitor-derived dendritic cells. Quantitative real-time RT-PCR results confirmed our microarray findings for 7 out of 9 selected genes. The confirmed genes (CES1, NQO1, PIR, TRIM16, FCGR2B, IFI27, MX1) were further investigated: Pooled PBMDCs from 4 donors and MUTZ-3 cells were exposed to sensitizers (CIN, DNCB, TNBS) and non-sensitizers (SDS, SA) for 24 h. Cells were then analyzed by flow cytometry for CD86 and viability (data not shown) and by quantitative real-time RT-PCR analysis for gene expression. From the 9-gene set, 4 genes, TRIM16, PIR, CES1 and NQO1, were shown to be promising due to their relevant up-regulation induced by the sensitizers TNBS and CIN in both cell types (see Fig. 3). The expression of these 4 genes was further examined by quantitative real-time RT-PCR in the U937 cells after a 24-h exposure to CIN, DNCB, SDS and SA (see Fig. 4) according to our protocol previously described (Python et al., 2007). Results confirmed with an excellent reproducibility the relevant up-regulation of TRIM16, PIR, CES1 and NQO1 induced by CIN in the U937 cell line. Our results and other published data (see below) indicated that TRIM16, PIR, CES1 and NQO1 although not obviously related to the immune response might be suitable candidate marker genes of DC activation.

TRIM16 expression was moderately induced by CIN in PBMDCs, MUTZ-3 cells and U937 cells. Previous studies identified a role for TRIM16 (EBBP) in a secretion pathway of IL-1β (Munding et al., 2006) and suggested its importance for keratinocytes differentiation (Beer et al., 2002). PIR, CES1 and NQO1 expression were weakly to strongly up-regulated by most sensitizers in PBMDCs and MUTZ-3 cells. Surprisingly, the extreme sensitizer DNCB induced only a weak up-regulation of PIR and NQO1 in PBMDCs and no relevant modulation of PIR, CES1 and NQO1 gene expression in MUTZ-3 cells and U937 cells. The weak or lack of activation of these four genes by DNCB tends to correlate with the observation from Boislève et al. (2004) that DNCB may induce another signalling pathway in DC activation. Another explanation might be that DNCB as an extreme and highly cytotoxic sensitizer has to be tested at very low concentrations; its chemical reactivity at such low concentrations may be directed toward proteins present in the culture medium and thus its DC-activation potential may be reduced or inhibited. From a functional point of view, PIR may be associated with the regulation of transcription (Pang et al., 2004), while CES1 and NQO1 are metabolizing enzymes. NQO1 is a phase II detoxifying enzyme and an antioxidant response element (ARE)-dependent gene regulated by the transcription factor Nrf2 through the Keap1–Nrf2–ARE signaling pathway. This regulatory pathway plays a major role in protecting cell against the toxic effects of oxidative and electrophilic stresses (reviews: Nioi and Hayes, 2004; Dinkova-Kostova et al., 2005, Kwak et al., 2004; Wakabayashi et al., 2004). Interestingly, Natsch and Emter (2008) developed cellular *in vitro* models for predicting sensitizers using Hepa1C1C7 cells and the reporter cell line AREc32 based on the activation of the Keap1–Nrf2–ARE regulatory pathway. Results obtained with NQO1 in our study and the one from Natsch and Emter (2008) indicate that ARE-dependent genes might be useful markers for the detection of DC activation although the role of these genes in sensitization process is yet unclear. In addition, our result confirmed the study from Hirota and Moro (2006) reporting that sensitizers up-regulate NQO1 in sensitizers-treated THP-1 cells and the work from Leon et al. (2007), reporting that NQO1 gene was activated by sensitizers and stress-inducers in two monocytic cell lines (THP-1 and U937). Ade et al. (2009) recently showed that NQO1 and HMOX1 genes are up-regulated in THP-1 cells and dendritic cells after a treatment with contact sensitizers. CES1 encoding a phase I metabolizing enzyme involved in the processing of various xenobiotics and drugs (review: Satoh and Hasokawa, 2006) was also identified with NQO1 as a gene regulated by Nrf2 in the study from Thimmulappa et al. (2002). NQO1 and CES1 are thus both under the control of the Keap1–Nrf2–ARE regulatory pathway. Interestingly, G6PD detected as an up-regulated gene in this study (see Table 2) is also an Nrf2-regulated gene as described by Thimmulappa et al. (2002). Based on these observations, we hypothesize that the activation of these Nrf2-regulated genes is triggered by a detoxification reaction in response to the toxic effects of the tested sensitizers. This might be interpreted as a danger signal required for inducing skin sensitization in the allergic contact dermatitis as previously described in other studies (Aeby et al., 2009; Python et al., 2007; Hulette et al., 2005).

Published data showed that CES1 and NQO1 are Nrf2-regulated genes involved in the Keap1–Nrf2–ARE regulatory pathway. This cellular regulatory pathway plays a central role in response to electrophilic and oxidative stresses (Dinkova-Kostova et al., 2005) and recently, Natsch and Emter (2008) demonstrated that the majority of tested skin sensitizers activate this pathway. Ade et al. (2009) also proposed that this pathway can be activated by sensitizers in dendritic cells and THP-1 cells. This suggests that the oxidative or electrophilic stress via the Keap1–Nrf2–ARE regulatory pathway might play an important role in DC activation and thus in the sensitization phase of allergic contact dermatitis. Interestingly, IL-8 used as a reliable DC-activation marker in our lab in conjunction with CD86 and IL-1β was shown to be up-regulated by the activation of the transcription factor Nrf2 (Zhang et al., 2005). The present study suggests that multiple markers will be needed to reliably detect sensitizers. The four proposed marker genes may not represent the final combination for a reliable detection of the sensitizing potential for chemicals but certainly represent a step toward this goal and may help to further refine the *in vitro* approaches for the screening of the sensitizing properties of a chemical. Other genes from the proposed list (see Tables 2 and 3) should be also considered.

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References

Ade, N., Leon, F., Pallardy, M., Peiffer, J.L., Kerdine-Romer, S., Tissier, M.H., Bonnet, P.A., Fabre, I., Ourlin, J.C., 2009. HMOX1 and NQO1 genes are upregulated in response to contact sensitizers in dendritic cells and THP-1 cell line: role of the Keap1/Nrf2 pathway. Toxicol. Sci. 107 (2), 451–460.

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.

Aeby, P., Python, F., Goebel, C., 2007. Skin sensitization: understanding the *in vivo* situation for the development of reliable *in vitro* test approaches. Altex 24, 3–5 Special Issue.

Aeby, P., Sieber, T., Beck, H., Gerberick, G.F., Goebel, C., 2009. Skin sensitization to p-phenylenediamine: the diverging roles of oxidation and N-acetylation for dendritic cell activation and the immune response. J. Invest. Dermatol. 129 (1), 99–109.

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.

Ashburner, M., Ball, C.A., Blake, J.A., Botstein, D., Butler, H., Cherry, J.M., Davis, A.P., Dolinski, K., Dwight, S.S., Eppig, J.T., 2000. Gene ontology: tool for the unification of biology. Nat. Genet. 25, 25–29.

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., Gerberick, G.F., Kimber, I., 2007. The local lymph node assay: current position in the regulatory classification of skin sensitizing chemicals. Cutan. Ocul. Toxicol. 26 (4), 293–301.

Beer, H.D., Munding, C., Dubois, N., Mamie, C., Hohl, D., Werner, S., 2002. The estrogen-responsive B box protein: a novel regulator of keratinocyte differentiation. J. Biol. Chem. 277 (23), 20740–20749.

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., Aeby, 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.

Dinkova-Kostova, A.T., Holtzclaw, W.D., Kensler, T.W., 2005. The role of Keap1 in cellular protective responses. Chem. Res. Toxicol. 18 (12), 1779–1791.

Gerberick, G.F., Ryan, C.A., Dearman, R.J., Kimber, I., 2007a. Local lymph node assay (LLNA) for detection of sensitization capacity of chemicals. Methods 41 (1), 54–60.

Gerberick, G.F., Vassallo, J.D., Foertsch, L.M., Price, B.B., Chaney, J.G., Lepoittevin, J.P., 2007b. Quantification of chemical peptide reactivity for screening contact allergens: a classification tree model approach. Toxicol. Sci. 97 (2), 417–427.

Hirota, M., Moro, O., 2006. MIP-1β, a novel biomarker for *in vitro* sensitization test using human monocytic cell line. Toxicol. In Vitro 20, 736–742.

Hu, Z.B., Ma, W., Zaborski, M., MacLeod, RAF., 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., 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 (2), 159–166.

Jowsey, I.R., Basketter, D.A., Westmoreland, C., Kimber, I., 2006. A future approach to measuring relative skin sensitising potency: a proposal. J. Appl. Toxicol. 26 (4), 341–350.

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.

Kwak, M.-K., Wakabayashi, N., Kensler, T.W., 2004. Chemoprevention through the Keap1–Nrf2 signaling pathway by phase 2 enzyme inducers. Mutat. Res. 555, 133–148.

Larsson, K., Lindstedt, M., Borrebaek, C.A.K., 2006. Functional and transcriptional profiling of MUTZ-3, a myeloid cell line acting as a model for dendritic cells. Immunology 117, 156–166.

Leon, F., Peiffer, J.L., Tissier, M.H., Bonnet, P.-A., Fabre, I., Ourlin, J.C., 2007. Chemical sensitizers activate stress–response genes HMOX1 and NQO1 in THP-1 and U-937 cells. Toxicol. Lett. 172S, S85–S86 (Poster presentation).

Masterson, J.A., Sombroek, C.C., de Gruijl, T.D., Graus, Y.M.F., van der Vliet, H.J.J., Lougheed, 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 (2), 701–703.

Mitjans, M., Viviani, B., Lucchi, L., Galli, C.L., Marinovich, M., Corsini, E., 2008. Role of p38 MAPK in the selective release of IL-8 induced by chemical allergen in naïve THP-1 cells. Toxicol. In Vitro 22 (2), 386–395.

Munding, C., Keller, M., Niklaus, G., Papin, S., Tschopp, J., Werner, S., Beer, H.D., 2006. The estrogen-responsive B box protein: a novel enhancer of interleukin-1β secretion. Cell Death Differ. 13 (11), 1938–1949.

Natsch, A., Emter, R., 2008. Skin sensitizers induce antioxidant response element dependent genes: application to the *in vitro* testing of the sensitization potential of chemicals. Toxicol. Sci. 102 (1), 110–119.

Nioi, P., Hayes, J.D., 2004. Contribution of NAD(P)H:quinone oxidoreductase 1 to protection against carcinogenesis, and regulation of its gene by the Nrf2 basic-region leucine zipper and the arylhydrocarbon receptor basic helix–loop–helix transcription factors. Mutat. Res. 555, 149–171.

Nukada, Y., Miyazawa, M., Kosaka, N., Ito, Y., Sakaguchi, H., Nishiyama, N., 2008. Production of IL-8 in THP-1 cells following contact allergen stimulation via mitogen-activated protein kinase activation or tumor necrosis factor-α production. J. Toxicol. Sci. 33 (2), 175–185.

Pang, H., Bartlam, M., Zeng, Q., Miyatake, H., Hisano, T., Miki, K., Wong, L.L., Gao, G.F., Rao, Z., 2004. Crystal structure of human pirin: an iron-binding nuclear protein and transcription cofactor. J. Biol. Chem. 279 (2), 1491–1498.

Patlewicz, G., Aptula, A.O., Roberts, D.W., Uriarte, E., 2008. A minireview of available skin sensitization (Q)SARs/expert systems. QSAR Comb. Sci. 27 (1), 60–76.

Python, F., Goebel, C., Aeby, P., 2007. Assessment of the U937 cell line for the detection of contact allergens. Toxicol. Appl. Pharmacol. 220, 113–124.

Ross, D., Kepa, J.K., Winski, S.L., Beall, H.D., Anwar, A., Siegel, D., 2000. NAD(P)H: quinone oxidoreductase 1 (NQO1): chemoprotection, bioactivation, gene regulation and genetic polymorphisms. Chem. Biol. Interact. 129, 77–97.

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 (1), 4–11.

Ryan, C.A., Kimber, I., Basketter, D.A., Pallardy, M., Gildea, L.A., Gerberick, G.F., 2007. Dendritic cells and skin sensitization: biological roles and uses in hazard identification. Toxicol. Appl. Pharmacol. 221 (3), 384–394.

Sakaguchi, H., Miyazawa, M., Yoshida, Y., Ito, Y., Suzuki, H., 2007. Prediction of preservative sensitization potential using surface marker CD86 and/or CD54 expression on human cell line, THP-1. Arch. Dermatol. Res. 298, 427–437.

Sakaguchi, H., Ashikaga, T., Miyazawa, M., Kosaka, N., Ito, Y., Yoneyama, K., Sono, S., Itagaki, H., Toyoda, H., Suzuki, H., 2009. The relationship between CD86/CD54 expression and THP-1 cell viability in an *in vitro* skin sensitization test — human cell line activation test (h-CLAT). Cell Biol. Toxicol. 25 (2), 109–126.

Satoh, T., Hosokawa, M., 2006. Structure, function and regulation of carboxylesterases. Chem. Biol. Interact. 162 (3), 195–211.

Santegoets, S.J., Masterson, A.J., van der Sluis, P.C., Lougheed, S.M., Fluitsma, D.M., van den Eertwegh, A.J., Pinedo, H.M., Scheper, R.J., De Gruijl, T.D., 2006. A $CD34^+$ human cell line model of myeloid dendritic cell differentiation: evidence for a $CD14^+CD11b^+$ Langerhans cell precursor. J. Leukocyte Biol. 80 (6), 1337–1344.

Schoeters, E., Nuijten, J.-M., van den Heuvel, R.L., Nelissen, I., Witters, H., Schoeters, G.E.R., van Tendeloo, V.F., Berneman, Z.N., Verheyen, G.R., 2006. Gene expression signatures in CD34+-progenitor-derived dendritic cells exposed to the chemical contact allergen nickel sulfate. Toxicol. Appl. Pharmacol. 216, 131–149.

Schoeters, E., Verheyen, G.R., Nelissen, I., Van Rompay, A.R., Hooyberghs, J., van den Heuvel, R.L., Witters, H., Schoeters, G.E., van Tendeloo, F., Berneman, Z.N., 2007. Microarray analyses in dendritic cells reveal potential biomarkers for chemical-induced skin sensitization. Mol. Immunol. 44 (12), 3222–3233.

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.

Tietze, C., Blömeke, B., 2008. Sensitization assays: monocyte-derived dendritic cells versus a monocytic cell line (THP-1). J. Toxicol. Environ. Health A 71 (13–14), 965–968.

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.

Tusher, V.G., Tibshirani, R., Chu, G., 2001. Significance analysis of microarrays applied to the ionizing radiation response. Proc. Natl. Acad. Sci. U. S. A. 98 (9), 5116–5121.

Thimmulappa, R.K., Mai, K.H., Srisuma, S., Kensler, T.W., Yamamoto, M., Biswal, S., 2002. Identification of Nrf2-regulated genes induced by the chemopreventive agent sulforaphane by oligonucleotide microarray. Cancer Res. 62 (18), 5196–5203.

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.

Verstraelen, S., Wens, B., Hooyberghs, J., Nelissen, I., Witters, H., Schoeters, G., Cauwenberge, P.V., Heuvel, R.V., 2008. Gene expression profiling of *in vitro* cultured macrophages after exposure to the respiratory sensitizer hexamethylene diisocyanate. Toxicol. In Vitro 22 (4), 1107–1114.

Wakabayashi, N., Dinkova-Kostova, A.T., Holtzclaw, W.D., Kang, M.I., Kobayashi, A., Yamamoto, M., Kensler, T.W., Talalay, P., 2004. Protection against electrophile and oxidant stress by induction of the phase 2 response: fate of cysteines of the Keap1 sensor modified by inducers. Proc. Natl. Acad. Sci. U. S. A. 101 (7), 2040–2045.

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.

Zhang, X., Chen, X., Song, H., Chen, H.-Z., Rovin, B.H., 2005. Activation of the Nrf2/antioxidant response pathway increases IL-8 expression. Eur. J. Immunol. 35, 3258–3267.