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Prediction of the contact sensitizing potential of chemicals using analysis of gene expression changes in human THP-1 monocytes

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ABSTRACT

The aim of this study was to find differentially regulated genes in THP-1 monocytic cells exposed to sensitizers and nonsensitizers and to investigate if such genes could be reliable markers for an *in vitro* predictive method for the identification of skin sensitizing chemicals. Changes in expression of 35 genes in the THP-1 cell line following treatment with chemicals of different sensitizing potential (from nonsensitizers to extreme sensitizers) were assessed using real-time PCR. Verification of 13 candidate genes by testing a large number of chemicals (an additional 22 sensitizers and 8 nonsensitizers) revealed that prediction of contact sensitization potential was possible based on evaluation of changes in three genes: *IL8*, *HMOX1* and *PAIMP1*. In total, changes in expression of these genes allowed correct detection of sensitization potential of 21 out of 27 (78%) test sensitizers. The gene expression levels inside potency groups varied and did not allow estimation of sensitization potency of test chemicals. Results of this study indicate that evaluation of changes in expression of proposed biomarkers in THP-1 cells could be a valuable model for preliminary screening of chemicals to discriminate an appreciable majority of sensitizers from nonsensitizers.

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1. Introduction

Allergic contact dermatitis, a delayed-type hypersensitivity reaction induced following skin contact with low molecular weight chemicals, is a common occupational and environmental health problem. Identification of the skin sensitizing potential of chemicals is routinely evaluated using the murine Local Lymph Node Assay (LLNA; OECD 429), which was introduced as an alternative method to guinea pig tests (Buehler, 1965; Magnusson and Kligman, 1969; OECD 406). The growing number of chemicals that require hazard identification together with the increasing interest in animal welfare has prompted multiple efforts to develop new alternative *in vitro* tests capable of identifying skin sensitizers (Casati et al., 2005; Ryan et al., 2005).

A typical approach explored in this line of research is based on analysis of phenotypic changes observed upon contact with tested substances in immune cells cultured *in vitro*. There are many reports showing that dendritic cells (DCs) cultured *in vitro* are especially suitable for development of such *in vitro* tests that are consistent with the pivotal role of Langerhans cells (LCs), immature DCs resident in the skin, in pathogenetic mechanisms underlying contact sensitization. Following the exposure of skin to skin sensitizers, LCs become activated and migrate via afferent lymphatic vessels to the draining nodes undergoing maturation that is characterized by phenotypic and functional changes such as loss of endocytic/phagocytic receptors, up- or downregulation of specific surface antigens (e.g., CD40, CD54, CD80, CD86 or E-cadherin, respectively), cytokines, chemokines and other immune response mediators (e.g., IL-1β and CCR7) (Banchereau et al., 2000; Corsini and Galli, 2000; Kimber et al., 2000; Stępnik and Arkusz, 2003). Similar changes in expression of surface markers and cytokines in LCs in the skin *in vivo* have been reproduced *in vitro* upon contact of LCs with known sensitizers, and this *in vitro* model was proposed as a method for *in vitro* identification of skin sensitizers. Since the isolation of LCs from the epidermis has been shown to be inefficient and cause the spontaneous activation of LCs, *in vitro* generated DCs (derived from $CD34^+$ stem cells (Inaba et al., 1992; Caux et al., 1996) and from peripheral blood monocytes (Sallusto and Lanzavecchia, 1994)) have been devised as a surrogate for LCs. However, DC-based models have certain limitations such as difficulty in access to cell sources, time-consuming and expensive cell culture procedures, and more importantly, significant donor-to-donor variations in cell responsiveness (Aiba et al., 1997; Pichowski et al., 2001; Ryan et al., 2005). Other cell models for use in the detection of skin sensitizing properties of chemicals, such as established

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cell lines of myeloid origin, have also been proposed. Among the most promising cell line-based models for *in vitro* screening of contact sensitizers are THP-1 (Ashikaga et al., 2002, 2006; Miyazawa et al., 2007; Bocchietto et al., 2007; Tietze and Blomeke, 2008), MUTZ-3 (Azam et al., 2006), and U937 cells (Python et al., 2007). The markers of cell activation following contact with skin sensitizing compounds frequently employed in these cell line-based tests include expression of surface antigens CD86/CD80 and CD86/CD54 and expression of IL-1β and IL-8.

Many reports describing *in vitro* cell line-based tests for skin sensitizers employed a limited number of reference compounds to establish a single best phenotypic marker of immune cell activation. There are also reports suggesting that evaluation of expression of multiple genes might provide a better method for detecting sensitizing compounds (Ryan et al., 2004; Gildea et al., 2006; Schoeters et al., 2006, 2007; Cluzel-Tailhardat et al., 2007; Lambrechts et al., 2009). Considering that THP-1 cells have been recognized as a promising model for development of an *in vitro* test (Ashikaga et al., 2002, 2006; Bocchietto et al., 2007; Miyazawa et al., 2007; Tietze and Blomeke, 2008) and show a similar pattern to primary DCs of gene expression following exposure to sensitizing compounds (Lambrechts et al., 2009), we decided to employ this cell line in the two-tier approach experimental protocol utilizing multiple potential genetic markers of cell activation. We also pre-validated this model with multiple skin sensitizers representing different classes of chemicals characterized by different sensitizing potential as determined by the LLNA (Basketter et al., 1995, 1999; Mandervelt et al., 1997; NTP, 1999; Kimber et al., 2003; Gerberick et al., 2005). This approach allows for the development of a testing protocol capable of discriminating between sensitizing and non-sensitizing reference compounds.

2. Materials and methods

2.1. Selection of marker genes

The 35 candidate genes were selected based on available data on sensitizer-induced gene expression changes in DCs (Ryan et al., 2004; Gildea et al., 2006; Schoeters et al., 2006, 2007; Cluzel-Tailhardat et al., 2007). The decision to include a particular gene was based on ranking the genes according to the following criteria: the gene was reported to be significantly regulated on the mRNA level by at least one sensitizer, changes in expression (if there were available data) were detected in the early time point (between 0.5 and 6 h) and the gene had known ontology. In addition to these criteria, priority was given to genes for which changes in expression was detected not only by a DNA array but also by real-time RT-PCR. The experimental protocol was based on a two-tier approach that enabled first for selection of

Table 1
Chemical identity data of test compounds. The compounds represent different classes of chemicals and possess different sensitizing properties from nonsensitizers to extreme sensitizers as determined by the Local Lymph Node Assay (LLNA). IC_{20} values were calculated for every test compound based on the results of the 24-h MTT reduction test for the THP-1 cell line.

	Chemical (abbreviation)	CAS no.	Purity	Solvent	IC_{20} [μM]	LLNA results	
						EC3 [%]	Potency category
Stage 1 experiments							
1	1-Chloro-2,4-dinitrobenzene (DNCB)	97-00-7	≥99.0%	DMSO	7 ± 2	0.05	Extreme [G]
2	2-Mercaptobenzothiazole (2-MBT)	149-30-4	97.0%	DMSO	1000 ± 58	0.16	Moderate [G]
3	p-Phenylene diamine (PPDA)	106-50-3	≥99.0%	DMSO	218 ± 29	1.7	Strong [G]
4	Eugenol (EUG)	97-53-0	99.0%	DMSO	635 ± 64	12.9	Weak [G]
5	Nickel sulfate ($NiSO_4$)	10101-97-0	99.0%	H_2O	312 ± 39	4.8	Metal sensitizer [M]
6	Benzalkonium chloride (BC)	63449-41-2	≥95.0%	H_2O	0.43 ± 0.16[a]	n/a	Nonsensitizer [N]
7	Sodium lauryl sulfate (SDS)	151-21-3	≥98.5%	H_2O	195 ± 6	14	Weak (false positive) [G]
Stage 2 experiments							
8	5-Chloro-2-methyl-4-isothiazolin-3-one (CMI)	26172-55-4	–	cRPMI	572 ± 47	0.009	Extreme [G]
9	Benzo[a]pyrene (BaP)	50-32-8	98.3%	DMSO	>100[b]	0.0009	Extreme [G]
10	Cyanuric chloride (CC)	108-77-0	99.0%	Ethanol	125 ± 29	0.09	Extreme [G]
11	Benzyl bromide (BB)	100-39-0	≥98.0%	DMSO	47 ± 1	0.2	Strong [G]
12	Dimethyl sulfate (DMS)	77-78-1	≥99.0%	DMSO	78 ± 11	0.19	Strong [G]
13	Formaldehyde; formalin (FAD)	50-00-0	36-38%	cRPMI	126 ± 4	0.61	Strong [G]
14	Glutaraldehyde (GA)	111-30-8	72.0%	cRPMI	69 ± 4	0.1	Strong [G]
15	Lauryl gallate (LG)	1166-52-5	≥99.0%	DMSO	4 ± 0.7	0.3	Strong [G]
16	Tolylene-2,4-diisocyanate (TDI)	584-84-9	97.1%	DMSO	>500[b]	0.11	Strong [K]
17	12-Bromo-1-dodecanol (12BD)	3344-77-2	99.8%	Ethanol	44 ± 4	6.9	Moderate [G]
18	3-Aminophenol (3-AP)	591-27-5	99.9%	DMSO	8 ± 2	3.2	Moderate [G]
19	Cinnamic aldehyde (Cinn)	104-55-2	≥93.0%	DMSO	49 ± 6	3,0	Moderate [G]
20	Methyl methanesulfonate (MMS)	66-27-3	99.9%	cRPMI	230 ± 18	8.1	Moderate [G]
21	2,3-Butanedione (2,3-B)	431-03-8	≥99.4%	cRPMI	530 ± 126	11	Weak [G]
22	Aniline (AN)	62-53-3	≥99.5%	DMSO	2,300 ± 300	89	Weak [G]
23	Geraniol (GER)	106-24-1	98.0%	DMSO	1,100 ± 138	26	Weak [G]
24	Pentachlorophenol (PCP)	87-86-5	98.0%	H_2O	31 ± 9	20	Weak [G]
25	1-Bromobutane (1-BB)	109-65-9	99.7%	DMSO	>1000[b]	–	Nonsensitizer [G]
26	1-Butanol (1-B)	71-36-3	≥99.5%	DMSO	>10,000	–	Nonsensitizer [G]
27	Benzocaine (Bzc)	1994-09-07	≥99.0%	DMSO	1600 ± 400	–	Nonsensitizer [G]
28	Glycerol (Glyc)	56-81-5	100%	cRPMI	>10,000	–	Nonsensitizer [G]
29	Isopropanol (Iso)	67-63-0	99.7%	cRPMI	>10,000	–	Nonsensitizer [G]
30	Propylene glycol (PG)	57-55-6	≥99.5%	cRPMI	>10,000	–	Nonsensitizer [G]
31	Sulfanilamide (Sulf)	63-74-1	≥98.0%	DMSO	>10,000	–	Nonsensitizer [G]
32	Potassium dichromate (Cr)	7778-50-9	≥99.5%	H_2O	18 ± 4	0.08	Metal sensitizer [K]Extreme [G]
33	Cobaltous sulfate heptahydrate (Co)	10026-24-1	≥99.0%	H_2O	250 ± 15	n/a	Metal sensitizer [*]
34	Copper(II) chloride dihydrate (Cu)	10125-13-0	99.6%	H_2O	146 ± 32	n/a	Metal sensitizer [B]
35	Mercury(II) chloride (Hg)	7487-94-7	≥99.5%	H_2O	21 ± 1	n/a	Metal sensitizer [B]
36	Ammonium tetrachloroplatinate(II) (TCPA)	13820-41-2	99%	H_2O	114 ± 3	n/a	Metal sensitizer [B]
37	Manganese chloride tetrahydrate (Mn)	13446-34-9	100.2%	H_2O	670 ± 40	n/a	Metal nonsensitizer [B]

(G) – Gerberick et al. (2005); (B) – Basketter et al. (1999); (M) – Mandervelt et al. (1997); (K) – Kimber et al. (2003); (N) – NTP (1999); (*) – Basketter et al. (1999) (LLNA was performed with cobalt chloride).

[a] The concentration given in μg/ml.

[b] Limited solubility.

Table 2
Official symbol and name of test genes as well as primer sequences used for real-time PCR.

Gene symbol	Gene name	Sequence 5′ → 3′ of forward primer	Sequence 5′ → 3′ of reverse primer
IL1B	Interleukin 1 beta	GGTGTTCTCCATGTCCTTTG	GGATCTACACTCTCCAGCTG
IL8	Interleukin 8	GCAGCCTTCCTGATTTCTGC	ACTTCTCCACAACCCTCTGC
CCL2	Chemokine (C–C motif) ligand 2	GATCTCAGTGCAGAGGCTCG	GCTTGTCCAGGTGGTCCATG
CCL4	Chemokine (C–C motif) ligand 4	CCAAACCAAAAGAAGCAAGC	CCTAATACAATAACACGGCAC
CCL23	Chemokine (C–C motif) ligand 23	CTTTGAAACGAACAGCGAGTG	CTTGTGTCCCTTCACCTTG
CXCL1	Chemokine (C–X–C motif) ligand 1	AGGGAATTCACCCCAAGAAC	ACTATGGGGGATGCAGGATT
IL1R2	Interleukin 1 receptor, type II	CGGTTTCGTGGGAGGCATTAC	CGTGTCTCTTCTTCTCCTGGG
IL7R	Interleukin 7 receptor	TGACCTGAGTGTCGTCTA	CATCTCCCCTGAGCTATT
CCR5	Chemokine (C–C motif) receptor 5	GTTTACACCCGATCCACTGG	CATGTGCACAACTCTGACTG
CCR2	Chemokine (C–C motif) receptor 2	AAGCTGAACAGAGAAAGTGGATTGA	AGAACGAGATGTGGACAGCATGT
SLAMF1	Signaling lymphocytic activation molecule family member 1	ATCACTGGAGAACAGTGTCG	GATTCTCCAGATAAAACTTG
CCR7	Chemokine (C–C motif) receptor 7	GCCATCGTCCAGGCTGTCTC	GCTCACTGCTGCTCCTCTGG
CXCR4	Chemokine (C–X–C motif) receptor 4	CTGTTGTCTGAACCCCATCC	ATGAATGTCCACCTCGCTTT
NOTCH3	Notch homolog 3	GCTGGGCATGAAGAACATGG	GTCCACTGACGGCAATCCAC
CREM	cAMP responsive element modulator	GCCTGTTGGGATCGCATTGC	ACAGCCCTGCGGCCATCC
MXD1	MAX dimerization protein 1	GCACGGACTATCTCACAGGTG	GATGCTGGTGCTGGAATAGCC
RGS1	Regulator of G-protein signaling 1	TGCTGAAGTAATGCAATGGTCT	CAAGCCAGCCAGAACTCAAT
NINJ1	Ninjurin 1	ATTACGCCAGCAAGAAGAGC	GGAGATGAGGACCACCAGG
LY75	Lymphocyte antigen 75	GAAGAAGGCGGGCTGTAACC	AGGGGTCATCAGGCTTAGGG
PTGS2	Prostaglandin-endoperoxide synthase 2	GAGATTGTGGAAAAATTGCTTCT	TGGCCCTCGCTTATGATCTGT
TNFAIP2	Tumor necrosis factor, alpha-induced protein 2	CCAGAAGTCTGGCTGAGGTC	AGAAGGAGTGCAGGACTGCC
TNFAIP3	Tumor necrosis factor, alpha-induced protein 3	CCATGGCACAACTCATCTCA	CCATGGGATTCTGGGCGTG
TNFAIP6	Tumor necrosis factor, alpha-induced protein 6	TGTACCACAGAGAAGCACGG	TTTTTCTGGCTGCCTCTAGC
ARHGDIB	Rho GDP dissociation inhibitor (GDI) beta	AAACGCTGCTGGGAGATG	TCCATGGTGATTGGTCCC
AKR1C2	Aldo-keto reductase family 1, member C2	GTAAAGCTCTAGAGGCCGT	CTGGTCGATGGGAATTGCT
AQP3	Aquaporin 3	GACAGAAGGAGCTGGTGTCC	ACATCACCAGGATGAGGGTC
ABCA6	ATP-binding cassette, subfamily A (ABC1), member 6	GATGCATTGGCTCCATCC	GTAAACGTCTGCCACGGG
CLEC10A	C-type lectin domain family 10, member A	ACGGCAGGTGGAATGACGA	CCACCAAAGGCAGCTCAGT
CYP27A1	Cytochrome P450, family 27, subfamily A, polypeptide 1	GCTCAAAGCTGTGCTTAAGG	TCAGAGAAGGCAGTGGGGTC
CYP1A1	Cytochrome P450, family 1, subfamily A, polypeptide 1	GTGGTCTAAGATTCAGCCTG	GGGAAGGCTCCATCAGCATC
PMAIP1	Phorbol-12-myristate-13-acetate-induced protein 1	AGAGCTGGAAGTCGAGTGT	GCACCTTCACATTCCTCTC
HIF1A	Hypoxia inducible factor 1, alpha subunit	GTATTGCACTGCACAGGCCA	TATCCAGGCTGTGTCGACTG
GADD45A	Growth arrest and DNA-damage-inducible, alpha	TCAGCGCACGATCACTGTC	AGGCACAACACCACGTTATC
HMOX1	Heme oxygenase 1	TCCGATGGGTCCTTACACTC	ACACAGTGAGAGGGCTGGAG
CCL3L1	Chemokine (C–C motif) ligand 3-like 1	GACGCAGGCAGCAAAGAGTA	CTGCATGATTGGGAGCAGGT
Control genes			
ACTB	Beta actin	TGACTGACTACCTCATGAAGATCC	CCATCTCTTGCTCGAAGTCC
B2M	Beta-2-microglobulin	TGCTGTCTCCATGTTTGATGTATCT	TCTCTGCTCCCCACCTCTAAGT

the best marker genes based on testing expression of all 35 candidate genes with 7 selected compounds of known sensitizing properties followed by verification of the selected 13 gene markers using an additional 30 compounds (22 sensitizers and 8 nonsensitizers).

2.2. Cell culture

Human peripheral blood monocytes THP-1 (American Type Culture Collection; ATCC#TIB-202) were maintained in cRPMI (RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 10 mM HEPES, 1 mM sodium pyruvate, and 4.5 g/l glucose; Sigma–Aldrich Chemical Company; St. Louis, MO, USA). All tissue culture reagents contained <0.5 EU/ml endotoxin contamination as certified by the manufacturer. Before experiments (after thawing), cells were passaged three times and were cultured no longer than two months. The cells were screened for *Mycoplasma* sp. infection using indicator 3T6 cells (ATCC#CCL-96) and MycoTech Kit (Gibco BRL).

2.3. Chemicals and reagents

Test chemicals were purchased from Sigma–Aldrich Chemical Company (St. Louis, MO, USA), except formalin and isopropanol that were obtained from POCh (Gliwice, Poland). More details (e.g., CAS no., purity and solvent, potency category in LLNA) are given in Table 1. Solutions of liquid chemicals were prepared immediately before use. Stock solutions of the other chemicals were prepared in a suitable solvent, aliquoted and stored at −20 °C. All final solutions of test chemicals were screened for lipopolysaccharide (LPS) contamination using Chromogenic Limulus Amebocyte Lysate QCL-1000 (Lonza). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Sigma–Aldrich Chemical Company (St. Louis, MO, USA).

2.4. Chemical treatment

The study was performed in two stages: in the first stage, the best candidate genes were selected based on testing of five known sensitizers and two skin irritants, and in the second set of experiments, the potential gene markers were verified using a larger number of chemicals (22 sensitizers and 8 nonsensitizers).

In brief, THP-1 cells (1.5×10^6 cells/well/2 ml; a 6-well culture plate) were incubated in cRPMI medium without (solvent; control cells) or with one of the test compounds (treated cells) for 2 h. The test chemicals were used at the concentration that decreased cell viability by 20% (IC_{20}) as determined using the MTT reduction test following a 24-h treatment. Because of solubility limits, it was impossible to achieve sufficient concentrations (IC_{20}) for three selected compounds (B[a]P, TDI and 1-BB). In these three cases, the maximum attainable, but not subtoxic, concentrations were used (100 μM B[a]P, 500 μM TDI and 1 mM 1-BB). For the chemicals that did not show cytotoxic properties, a maximum concentration of 10 mM was used. For chemicals dissolved in DMSO and ethanol, the maximum final concentration of the solvent was 0.5% and 1%, respectively. These concentrations did not induce a cytotoxic effect in the THP-1 cell line. After incubation at 37 °C with 5% CO_2 for 2 h, cells were harvested for quantitative real-time RT-PCR analysis.

2.5. MTT reduction assay for assessment of cytotoxicity

THP-1 cells were plated onto 96-well microplates (2×10^4 cells/well) and incubated in the absence or presence of test compounds for 24 h. Next, the plates were centrifuged to remove supernatants, and the cells were incubated with 100 μl MTT (0.5 mg/ml) for 3 h. After discarding the MTT solution, DMSO (50 μl) was added to each well. The optical density of the solubilized formazan product was determined using a spectrophotometer (with a 550 and 620 nm filter as a reference). Results were expressed as the percent of cell survival (OD of exposed vs. OD of control non-exposed cells). The IC_{20} value (concentration evoking 20% inhibition of growth/viability of cells) for each compound was calculated.

2.6. Analysis of gene expression by real-time RT-PCR

THP-1 cells (1.5×10^6 cells/well/2 ml seeded onto 6-well microplates) were exposed to test compounds for 2 h. Total RNA was isolated with TRI Reagent (Molecular Research Center, Inc., USA) according to the manufacturer's instructions. Extracted total RNA was quantified in nuclease-free water by spectrophotometry. Only samples with high purity (the A260/A280 ratio in the range of 1.8–2.1 in 10 mM

Table 3
Gene expression changes in THP-1 cells exposed to selected chemicals (IC_{20}) for 2 h. Results are presented as the geometrical mean of 2^{-ddCt} values from 5 independent experiments and the geometric standard error (in bold – mean values > 1.7 or <0.6).

Gene symbol	Sensitizers [IC_{20}]					Nonsensitizers [IC_{20}]	
	DNCB [7 μM]	PPDA [218 μM]	2-MBT [1000 μM]	Eugenol [635 μM]	$NiSO_4$ [312 μM]	BC [0.43 μg/ml]	SDS [195 μM]
IL8	**6.2 ± 1.3**	**7.4 ± 1.1**	**16.0 ± 1.8**	**9.5 ± 1.6**	**3.9 ± 1.3**	1.0 ± 1.1	1.2 ± 1.3
PMAIP1	**1.8 ± 1.1**	**2.1 ± 1.1**	**1.8 ± 1.5**	**1.8 ± 1.2**	1.6 ± 1.2	0.9 ± 1.1	1.0 ± 1.2
CXCL1	**4.7 ± 1.2**	**3.8 ± 1.3**	**2.8 ± 1.2**	**3.7 ± 1.4**	1.5 ± 1.6	0.9 ± 1.1	0.8 ± 1.1
HMOX1	**7.6 ± 1.3**	**4.8 ± 1.2**	**3.0 ± 1.2**	**3.1 ± 1.6**	0.8 ± 1.3	0.8 ± 1.3	1.3 ± 1.3
IL7R	**2.4 ± 1.2**	**2.2 ± 1.2**	1.5 ± 1.3	**4.5 ± 1.9**	1.2 ± 1.1	**0.3 ± 1.3**	**0.5 ± 1.3**
IL1B	1.1 ± 1.2	1.4 ± 1.2	**2.8 ± 1.6**	**2.3 ± 1.3**	**2.4 ± 1.4**	0.6 ± 1.3	0.8 ± 1.4
CXCR4	1.7 ± 1.2	1.6 ± 1.0	**2.4 ± 1.4**	**2.3 ± 1.2**	0.7 ± 1.2	**0.3 ± 1.2**	**0.5 ± 1.2**
CCL4	0.8 ± 1.1	0.8 ± 1.2	**5.7 ± 2.4**	1.3 ± 1.7	**3.0 ± 1.7**	**0.4 ± 2.0**	**0.3 ± 2.0**
CCR2	**3.0 ± 1.1**	**2.8 ± 1.1**	0.6 ± 1.4	1.2 ± 1.1	0.8 ± 1.2	1.0 ± 1.1	0.7 ± 1.4
NOTCH3	0.6 ± 1.4	**0.3 ± 1.4**	0.6 ± 1.3	0.6 ± 1.2	0.9 ± 1.2	1.5 ± 1.1	1.1 ± 1.2
IL1R2	1.2 ± 1.1	1.6 ± 1.2	1.2 ± 1.1	**3.4 ± 2.0**	1.6 ± 1.1	0.8 ± 1.3	1.0 ± 1.4
TNFAIP3	1.3 ± 1.1	1.3 ± 1.1	**1.9 ± 1.1**	**1.8 ± 1.2**	**3.1 ± 1.1**	0.9 ± 1.1	1.1 ± 1.2
TNFAIP6	1.2 ± 1.3	1.4 ± 1.2	**2.4 ± 1.4**	**1.9 ± 1.3**	**3.7 ± 1.5**	0.6 ± 1.2	**0.4 ± 1.8**
CCL3L1	0.7 ± 1.1	0.8 ± 1.2	**5.1 ± 1.9**	**1.9 ± 1.5**	**2.2 ± 1.3**	0.9 ± 1.1	0.8 ± 1.1
CCL2	**0.5 ± 1.1**	**0.5 ± 1.2**	1.3 ± 1.2	1.6 ± 1.3	**2.3 ± 1.2**	0.8 ± 1.1	**0.3 ± 1.2**
CCR5	**4.8 ± 1.2**	**5.8 ± 1.2**	1.0 ± 1.3	**4.2 ± 1.5**	0.7 ± 1.1	1.2 ± 1.2	1.5 ± 1.3
SLAMF1	**0.4 ± 1.5**	0.6 ± 1.2	**0.1 ± 1.7**	**0.2 ± 2.1**	0.8 ± 2.2	0.8 ± 2.6	1.5 ± 2.6
MXD1	1.3 ± 1.1	1.2 ± 1.2	1.7 ± 1.1	**4.3 ± 1.2**	1.1 ± 1.1	1.1 ± 1.1	**2.6 ± 1.1**
RGS1	1.0 ± 1.1	**2.0 ± 1.2**	**2.0 ± 1.3**	**2.6 ± 1.25**	1.2 ± 1.4	0.8 ± 1.2	**3.0 ± 1.1**
AKR1C2	**2.5 ± 1.1**	**2.0 ± 1.1**	1.1 ± 1.1	1.7 ± 1.3	0.8 ± 1.3	0.9 ± 1.3	0.7 ± 1.1
CYP1A1	0.7 ± 1.1	1.0 ± 1.2	1.2 ± 1.3	**0.4 ± 1.3**	**0.4 ± 1.4**	**0.4 ± 1.6**	0.6 ± 1.6
CYP27A1	1.5 ± 1.3	0.8 ± 1.2	0.6 ± 1.2	**0.5 ± 1.7**	**0.5 ± 1.3**	**0.5 ± 1.4**	0.6 ± 1.5
ARHGDIB	1.0 ± 1.1	0.8 ± 1.1	0.6 ± 1.3	0.6 ± 1.1	0.6 ± 1.3	0.6 ± 1.4	**0.5 ± 1.3**
AQP3	0.8 ± 1.2	0.6 ± 1.5	0.6 ± 1.4	0.6 ± 1.3	**0.5 ± 1.2**	0.7 ± 1.3	1.0 ± 1.2
PTGS2	0.6 ± 1.1	0.8 ± 1.0	**1.9 ± 1.9**	1.3 ± 1.4	0.7 ± 1.5	0.6 ± 1.2	0.6 ± 1.1
HIF1A	1.2 ± 1.1	1.2 ± 1.1	1.2 ± 1.1	**1.8 ± 1.1**	1.2 ± 1.1	1.0 ± 1.1	1.1 ± 1.2
CCR7	**0.5 ± 1.3**	0.8 ± 1.3	0.6 ± 1.3	1.3 ± 1.2	0.9 ± 1.2	1.0 ± 1.2	1.6 ± 1.1
CCL23	1.3 ± 1.1	1.0 ± 1.1	0.8 ± 1.1	0.6 ± 1.2	0.7 ± 1.1	0.7 ± 1.2	0.7 ± 1.3
ABCA6	0.8 ± 1.1	0.9 ± 1.2	0.8 ± 1.3	0.8 ± 1.2	0.8 ± 1.3	**0.5 ± 1.2**	**0.5 ± 1.4**
CLEC10A	1.1 ± 1.1	1.7 ± 1.2	0.6 ± 1.2	1.0 ± 1.4	0.6 ± 1.3	0.9 ± 1.2	0.7 ± 1.4
CREM	0.7 ± 1.2	1.1 ± 1.1	1.2 ± 1.1	1.6 ± 1.1	1.0 ± 1.1	1.2 ± 1.2	1.0 ± 1.2
NINJ1	1.6 ± 1.1	1.3 ± 1.2	1.2 ± 1.2	1.6 ± 1.3	0.9 ± 1.2	0.8 ± 1.3	1.0 ± 1.3
LY75	1.0 ± 1.1	1.0 ± 1.1	1.0 ± 1.1	1.1 ± 1.1	1.0 ± 1.1	1.0 ± 1.1	1.1 ± 1.1
TNFAIP2	1.5 ± 1.1	1.2 ± 1.1	0.8 ± 1.1	0.9 ± 1.1	1.2 ± 1.2	1.0 ± 1.1	0.8 ± 1.2
GADD45A	1.0 ± 1.2	1.0 ± 1.2	1.0 ± 1.1	1.3 ± 1.1	0.7 ± 1.1	0.8 ± 1.3	1.0 ± 1.2

Tris–HCl; pH 7.5) were used for further analysis. Subsequently, RNA (1 μg) was reverse transcribed into cDNA with random hexamers as primers (RevertAidTM H Minus First Strand cDNA Synthesis Kit; Invitrogen). Real-time analysis was performed in 384-well plates (Roche) using the LightCycler (Roche) as a detection system. Reaction mixtures were plated by the Janus Automated Workstation (Perkin Elmer). Each reaction consisted of 1/25× diluted cDNA, 0.5 μM of each forward and reverse primers, LightCycler 480 Sybr Green I Master (Roche) and nuclease-free water in a total reaction volume of 10 μl. The forward and reverse primer sequences used in the reactions are given in Table 2. A cycle consisted of a denaturation for 10 s at 95 °C, an annealing for 10 s at 60 °C and an elongation at 72 °C for 20 s. The SYBR green fluorescence was detected at the end of each elongation cycle after which a melting curve was assessed to confirm the specificity of the PCR products. The quantification data were then analyzed with the LightCycler software. Results were normalized to the housekeeping genes (*ACTB*, *B2M*; Table 2) as previously described (Vandesompele et al., 2002), and expression was compared to normal levels in control cells using the delta-delta Ct method.

2.7. Statistical analysis

The Ct values for each test gene were normalized using two housekeeping genes (*ATCB* and *B2M*; dCt). The classification of the results was based on distributions of ddCt (dCt for chemical-treated samples adjusted to dCt for solvent-treated samples).

In the first tier, genes were selected assuming a greater than 2-fold upregulation for skin sensitizers and a less than 2-fold upregulation for nonsensitizers. Results for gene expression are shown as geometric mean ± geometric standard error.

In the second tier, sensitization potency from LLNA experiments was taken into account for selection genes for classification of substances. Differences in expression in groups of chemical substances were compared using Generalized Additive Models for Location Scale and Shape (Rigby and Stasinopoulos, 2005). We compared normal and Student's *t*-distribution for error distribution using Akaike Information Criterion (AIC) (Akaike, 1974) and found that for most genes Student's *t*-distribution was better.

Discriminant analysis was used to classify chemical substances into skin sensitizers and nonsensitizers based on expression of selected genes. The density functions of Student's *t*-distribution for expression of each gene in cells exposed to sensitizers and nonsensitizers were calculated using following formula:

$$f(\mathrm{ddCt}) = \frac{\Gamma((\nu+1)/2)}{\sigma\Gamma(\nu/2)\sqrt{(\nu\pi)}}\left(1+\frac{(\mathrm{ddCt}-\mu)}{\sigma^2\nu}\right)^{-((\nu+1)/2)}$$

The classification function (CF) was calculated as the ratio of the density functions of gene expression in two groups. Joint density for each substance for *n* genes was calculated as the product of univariate densities according to the following formula:

$$\mathrm{CF} = \frac{f(\mathrm{ddCt1_S}, \mathrm{ddCt2_S}\ldots)_{\mathrm{gene}\,1} * f(\mathrm{ddCt1_S}, \mathrm{ddCt2_S}\ldots)_{\mathrm{gene}\,2} * f(\mathrm{ddCt1_S}, \mathrm{ddCt2_S}\ldots)_{\mathrm{gene}\,n}}{f(\mathrm{ddCt1_N}, \mathrm{ddCt2_N}\ldots)_{\mathrm{gene}\,1} * f(\mathrm{ddCt1_N}, \mathrm{ddCt2_N}\ldots)_{\mathrm{gene}\,2} * f(\mathrm{ddCt1_N}, \mathrm{ddCt2_N}\ldots)_{\mathrm{gene}\,n}}$$

where $f(\mathrm{ddCt_S})$ and $f(\mathrm{ddCt_N})$ are density functions of Student's *t*-distribution calculated for each gene for sensitizers (S) and nonsensitizers (N), respectively. Classification of chemical substances was based on a cut-off point for CF. The cut-off was selected to minimize false positive results. Sensitizing potential of test chemicals from LLNA experiments was correlated with values of CF to investigate classification of potency. *R* statistical package was used for all statistical analyses.

3. Results

3.1. First screening of candidate genes

Based on available data on sensitizer-induced gene expression changes in both peripheral blood-derived and $CD34^+$-progenitor-derived DCs (Schoeters et al., 2006, 2007), 35 candidate genes

Table 4
Gene expression changes in THP-1 cells exposed to selected chemicals for 2 h. Results are presented as the geometrical mean of 2^{-ddCt} values from 3 independent experiments and the geometric standard error (in bold – mean values > 2). The effects of 0.5% DMSO and 1% ethanol alone on gene expression were taken into account in calculation of relative changes.

Chemical		Gene symbol											
		IL8	*HMOX1*	*PMAIP1*	*IL7R*	*IL1B*	*CXCL1*	*TNFAIP3*	*CCL4*	*NOTCH3*	*TNFAIP6*	*CCR2*	*CXCR4*	*IL1R2*
Sensitizers														
Extreme	CMI [572 μM]	**3.1 ± 1.3**	**5.9 ± 1.2**	**6.8 ± 1.2**	0.5 ± 1.7	0.7 ± 1.5	0.8 ± 1.7	0.9 ± 1.8	1.3 ± 2.0	**4.0 ± 1.1**	0.9 ± 1.7	0.3 ± 1.6	1.2 ± 1.2	1.7 ± 1.2
	BaP [100 μM][a]	0.4 ± 1.2	0.3 ± 1.5	0.4 ± 1.2	0.3 ± 1.2	1.3 ± 1.3	0.6 ± 1.2	1.3 ± 1.3	0.6 ± 1.1	0.5 ± 1.3	0.8 ± 1.5	0.6 ± 1.2	0.5 ± 1.1	0.6 ± 1.2
	CC [125 μM]	**3.8 ± 1.1**	**7.3 ± 1.6**	1.4 ± 1.4	1.6 ± 1.2	1.6 ± 1.2	**3.0 ± 1.0**	1.5 ± 1.1	0.7 ± 1.4	1.2 ± 1.1	1.1 ± 1.5	1.7 ± 1.8	0.9 ± 1.1	1.1 ± 1.1
Strong	BB [47 μM]	**10.7 ± 1.2**	**3.9 ± 2.4**	**7.8 ± 1.2**	0.6 ± 1.4	**2.2 ± 1.0**	0.8 ± 1.2	0.5 ± 1.1	1.2 ± 1.1	0.5 ± 1.9	**2.6 ± 1.6**	0.3 ± 1.4	**2.0 ± 1.1**	**4.3 ± 1.3**
	DMS [78 μM]	1.4 ± 1.0	1.9 ± 1.1	**2.7 ± 1.1**	0.7 ± 1.4	0.9 ± 1.2	0.7 ± 1.2	0.6 ± 1.3	1.2 ± 1.4	1.2 ± 1.4	1.0 ± 1.6	0.7 ± 1.3	1.3 ± 1.1	1.4 ± 1.0
	FAD [126 μM]	1.4 ± 1.1	0.4 ± 2.3	**3.4 ± 1.0**	0.4 ± 1.5	0.7 ± 1.1	0.8 ± 1.3	0.8 ± 1.1	0.4 ± 3.2	0.5 ± 1.5	1.6 ± 1.2	0.2 ± 2.3	1.6 ± 1.1	0.2 ± 2.6
	GA [69 μM]	**3.1 ± 1.3**	0.2 ± 2.5	**5.5 ± 1.2**	0.0 ± 2.3	0.8 ± 1.5	0.5 ± 1.3	0.5 ± 2.0	**2.1 ± 1.7**	0.2 ± 3.3	1.1 ± 1.9	0.2 ± 1.2	1.8 ± 1.1	**2.1 ± 1.7**
	LG [4 μM]	1.6 ± 1.2	1.6 ± 1.1	**3.4 ± 1.4**	1.1 ± 1.2	0.3 ± 1.1	0.6 ± 1.3	1.2 ± 1.0	1.3 ± 1.5	**2.2 ± 1.9**	1.1 ± 1.3	1.2 ± 1.2	1.8 ± 1.3	0.9 ± 1.2
	TDI [500 μM][a]	0.3 ± 1.2	1.4 ± 1.1	0.6 ± 1.3	0.4 ± 1.3	0.5 ± 1.3	0.6 ± 1.1	0.7 ± 1.3	0.5 ± 1.2	1.1 ± 1.1	0.6 ± 1.8	0.6 ± 1.2	0.7 ± 1.1	0.5 ± 1.6
Moderate	12BD [44 μM]	0.5 ± 1.4	1.4 ± 1.1	0.5 ± 1.1	0.5 ± 1.1	0.9 ± 1.0	0.7 ± 1.1	1.3 ± 1.1	0.5 ± 1.0	0.7 ± 1.2	0.7 ± 1.3	0.8 ± 1.1	0.7 ± 1.2	0.5 ± 1.3
	3-AP [8 μM]	**2.3 ± 1.1**	0.9 ± 1.3	1.3 ± 1.1	1.3 ± 1.3	1.3 ± 1.3	1.6 ± 1.1	1.1 ± 1.3	0.9 ± 1.4	0.5 ± 1.1	0.7 ± 1.5	1.4 ± 1.1	1.4 ± 1.1	0.7 ± 1.6
	Cinn [49 μM]	**4.7 ± 1.2**	**30.5 ± 1.7**	**7.1 ± 1.2**	**2.6 ± 1.1**	0.6 ± 1.2	0.9 ± 1.2	1.1 ± 1.1	1.0 ± 1.5	**2.3 ± 1.5**	1.9 ± 1.5	0.2 ± 1.4	1.6 ± 1.1	1.4 ± 1.1
	MMS [230 μM]	1.3 ± 1.1	1.4 ± 1.1	**2.3 ± 1.1**	0.7 ± 1.0	0.9 ± 1.1	1.1 ± 1.1	0.7 ± 1.1	1.2 ± 1.1	0.9 ± 1.1	1.0 ± 1.1	0.8 ± 1.2	1.3 ± 1.1	1.2 ± 1.0
Weak	2.3-B [530 μM]	0.5 ± 1.1	0.4 ± 1.3	1.3 ± 1.1	0.1 ± 1.6	0.4 ± 1.2	0.8 ± 1.0	1.0 ± 1.1	0.8 ± 1.1	0.6 ± 1.3	1.0 ± 1.3	0.5 ± 1.3	1.1 ± 1.1	1.0 ± 1.1
	AN [2.3 mM]	1.1 ± 1.1	1.4 ± 1.2	1.6 ± 1.3	1.5 ± 1.2	0.8 ± 1.1	0.8 ± 1.1	1.0 ± 1.2	1.1 ± 1.3	1.5 ± 1.5	0.8 ± 1.0	0.9 ± 1.2	1.3 ± 1.2	1.7 ± 1.4
	GER [1.1 mM]	**3.9 ± 1.1**	**2.8 ± 1.1**	1.3 ± 1.0	**3.2 ± 1.2**	1.7 ± 1.3	1.9 ± 1.1	1.6 ± 1.4	1.0 ± 1.2	0.4 ± 1.1	1.5 ± 1.3	1.2 ± 1.1	1.9 ± 1.0	0.9 ± 1.6
	PCP [31 μM]	1.2 ± 1.2	1.0 ± 1.1	1.5 ± 1.1	0.9 ± 1.2	1.0 ± 1.2	0.8 ± 1.1	0.9 ± 1.2	1.3 ± 1.3	0.6 ± 1.3	1.1 ± 1.3	0.8 ± 1.2	1.5 ± 1.1	1.0 ± 1.1
Metal	Co [250 μM]	**8.0 ± 1.1**	**2.2 ± 1.2**	**4.6 ± 1.1**	**2.5 ± 1.0**	**5.0 ± 1.3**	**5.5 ± 1.2**	**3.4 ± 1.1**	**11.6 ± 1.5**	0.9 ± 1.2	**7.1 ± 1.3**	0.4 ± 1.2	1.8 ± 1.1	1.7 ± 1.2
	Cr [18 μM]	1.3 ± 1.3	0.7 ± 2.6	**2.5 ± 1.1**	0.8 ± 1.0	0.5 ± 1.5	0.8 ± 1.5	1.2 ± 1.2	1.1 ± 1.6	1.3 ± 1.5	1.3 ± 1.4	0.8 ± 1.0	1.1 ± 1.1	0.8 ± 1.3
	Cu [146 μM]	1.0 ± 1.1	1.0 ± 1.1	**2.5 ± 1.3**	1.2 ± 1.2	0.7 ± 1.0	0.8 ± 1.1	1.0 ± 1.1	1.1 ± 1.1	1.1 ± 1.6	1.2 ± 1.2	1.1 ± 1.2	1.3 ± 1.2	1.0 ± 1.1
	Hg [21 μM]	**3.0 ± 1.3**	**17.8 ± 1.2**	**4.3 ± 1.1**	1.1 ± 1.1	0.4 ± 1.1	1.3 ± 1.1	0.5 ± 1.1	0.4 ± 1.3	1.7 ± 1.3	0.8 ± 1.1	0.6 ± 1.3	1.0 ± 1.1	1.3 ± 1.2
	TCPA [114 μM]	**2.5 ± 1.2**	0.8 ± 1.2	**4.9 ± 1.1**	0.2 ± 1.4	0.7 ± 1.5	0.4 ± 1.4	1.2 ± 1.3	1.2 ± 1.6	**2.1 ± 1.4**	1.8 ± 1.8	0.1 ± 1.1	0.6 ± 1.1	1.3 ± 1.0
Nonsensitizers														
	Mn [670 μM]	1.2 ± 1.3	0.5 ± 1.6	1.5 ± 1.1	1.0 ± 1.1	1.5 ± 1.3	1.1 ± 1.2	1.4 ± 1.2	1.4 ± 1.1	0.7 ± 1.5	1.3 ± 1.2	0.8 ± 1.2	1.4 ± 1.1	0.9 ± 1.2
	1-BB [1 mM][a]	0.4 ± 1.7	1.0 ± 1.2	1.1 ± 1.1	0.5 ± 1.6	0.5 ± 1.5	0.5 ± 1.4	1.2 ± 1.6	0.9 ± 1.1	0.6 ± 2.3	1.1 ± 1.0	0.9 ± 1.7	0.6 ± 1.5	**2.0 ± 1.5**
	1-B [10 mM]	1.1 ± 1.1	0.9 ± 1.1	1.6 ± 1.2	1.2 ± 1.2	0.7 ± 1.0	0.7 ± 1.2	0.8 ± 1.0	1.0 ± 1.1	1.2 ± 1.1	0.9 ± 1.2	0.9 ± 1.1	1.2 ± 1.2	1.3 ± 1.0
	Bzc [1.6 mM]	0.3 ± 1.4	1.4 ± 1.2	0.8 ± 1.2	0.5 ± 1.4	0.4 ± 1.1	0.5 ± 1.2	0.5 ± 1.3	0.3 ± 1.4	0.6 ± 1.1	0.9 ± 1.3	0.4 ± 1.2	1.1 ± 1.1	0.6 ± 1.2
	Glyc [10 mM]	1.0 ± 1.0	0.5 ± 1.8	1.5 ± 1.1	0.7 ± 1.3	0.6 ± 1.2	1.0 ± 1.2	1.0 ± 1.2	0.9 ± 1.2	1.6 ± 1.0	1.2 ± 1.3	1.0 ± 1.1	1.1 ± 1.1	0.8 ± 1.4
	Iso [10 mM]	1.0 ± 1.1	0.7 ± 1.1	1.5 ± 1.0	0.7 ± 1.2	0.7 ± 1.1	0.9 ± 1.3	0.9 ± 1.1	0.9 ± 1.3	1.4 ± 1.2	0.9 ± 1.1	1.0 ± 1.2	1.3 ± 1.0	1.1 ± 1.1
	PG [10 mM]	0.8 ± 1.1	0.9 ± 1.2	1.4 ± 1.2	0.6 ± 1.2	0.7 ± 1.1	0.9 ± 1.1	0.9 ± 1.1	0.9 ± 1.1	0.9 ± 1.5	0.9 ± 1.2	0.8 ± 1.2	1.2 ± 1.1	1.1 ± 1.1
	Sulf [10 mM]	0.5 ± 1.3	1.2 ± 1.2	0.6 ± 1.2	0.9 ± 1.1	0.4 ± 1.2	1.1 ± 1.3	1.1 ± 1.4	0.4 ± 1.1	0.2 ± 1.1	0.9 ± 1.8	1.4 ± 1.2	1.3 ± 1.2	0.4 ± 1.9
Solvents:	0.5% DMSO	0.5 ± 1.1	0.5 ± 1.4	1.8 ± 1.2	0.4 ± 1.2	0.5 ± 1.2	0.3 ± 1.1	0.6 ± 1.2	0.8 ± 1.3	**4.1 ± 1.2**	0.8 ± 1.3	0.3 ± 1.1	0.6 ± 1.1	0.9 ± 1.4
	1% ethanol	0.5 ± 1.1	0.2 ± 2.1	**2.4 ± 1.3**	0.9 ± 1.3	0.3 ± 1.1	0.4 ± 1.2	0.6 ± 1.2	0.6 ± 1.3	**2.3 ± 1.5**	0.9 ± 1.6	0.6 ± 1.5	0.9 ± 1.2	0.8 ± 1.8

[a] Limited solubility of test chemicals.

Table 5
Differences between changes in gene expression in THP-1 cells following the treatment with nonsensitizers and various subgroups of sensitizers representing different sensitizing potency category. Data presented as regression parameter (b), calculated as the difference between mean value of gene expression changes in sensitizing potency subgroups and nonsensitizers, standard error (s.e.) and p-value (p). Generalized Additive Models for Location Scale and Shape with Student's t-distribution for error.

Gene	Sensitizing potency groups	b	s.e.	p
IL8	Nonsensitizers	0.25[a]	0.23	–
	Extreme sensitizers	−1.97	0.42	0.00001
	Strong sensitizers	−1.61	0.36	0.00002
	Moderate sensitizers	−1.86	0.37	0.00000
	Weak sensitizers	−1.23	0.40	0.00249
	Metal sensitizers	−1.45	0.41	0.00065
HMOX1	Nonsensitizers	0.10[a]	0.15	–
	Extreme sensitizers	−2.74	0.29	0.00000
	Strong sensitizers	−0.78	0.24	0.00179
	Moderate sensitizers	−0.55	0.25	0.02819
	Weak sensitizers	−0.57	0.27	0.03428
	Metal sensitizers	−0.53	0.28	0.06258
PMAIP1	Nonsensitizers	−0.23[a]	0.13	–
	Extreme sensitizers	−0.56	0.23	0.01902
	Strong sensitizers	−1.37	0.20	0.00000
	Moderate sensitizers	−0.45	0.20	0.02919
	Weak sensitizers	−0.31	0.22	0.15510
	Metal sensitizers	−1.68	0.23	0.00000
CXCL1	Nonsensitizers	0.29[a]	0.17	–
	Extreme sensitizers	−1.60	0.30	0.00000
	Strong sensitizers	0.16	0.25	0.53580
	Moderate sensitizers	−0.72	0.26	0.00603
	Weak sensitizers	−0.55	0.28	0.05042
	Metal sensitizers	−0.31	0.29	0.29100
IL7R	Nonsensitizers	0.59[a]	0.19	–
	Extreme sensitizers	−0.90	0.34	0.00910
	Strong sensitizers	−0.16	0.29	0.58910
	Moderate sensitizers	−0.73	0.29	0.01354
	Weak sensitizers	−1.39	0.32	0.00004
	Metal sensitizers	−0.61	0.33	0.06822
CXCR4	Nonsensitizers	0.00[a]	0.12	–
	Extreme sensitizers	−0.09	0.21	0.65818
	Strong sensitizers	−0.61	0.18	0.00110
	Moderate sensitizers	−0.27	0.19	0.15767
	Weak sensitizers	−0.70	0.20	0.00074
	Metal sensitizers	−0.18	0.21	0.38971
IL1B	Nonsensitizers	0.61[a]	0.16	
	Extreme sensitizers	−0.80	0.29	0.00709
	Strong sensitizers	−0.39	0.25	0.11467
	Moderate sensitizers	−0.93	0.25	0.00033
	Weak sensitizers	−0.74	0.27	0.00747
	Metal sensitizers	−0.12	0.28	0.67057
TNFAIP3	Nonsensitizers	0.09[a]	0.13	–
	Extreme sensitizers	−0.40	0.23	0.08528
	Strong sensitizers	0.20	0.20	0.30471
	Moderate sensitizers	−0.67	0.20	0.00125
	Weak sensitizers	−0.40	0.22	0.06917
	Metal sensitizers	−0.29	0.23	0.20035

[a] Value b shows the mean value of gene expression changes.

for biomarkers of sensitizing potential were selected. Among the 35 genes chosen for the study, there were genes coding proteins known to be involved in immune response, such cytokines and chemokines (*IL1B*, *IL8*, *CCL2*, *CCL4*, *CXCL1*, *CCL23*, *CCL3L1*) and receptors (*CCR2*, *CCR5*, *CCR7*, *CXCR4*, *IL7R*, *IL1R2*, *SLAMF1*, *LY75*), and other genes involved in apoptosis and stress responses (*HIF1A*, *PMAIP1*, *HMOX1*, *PTGS2*, *TNFAIP3*, *GADD45A*), regulation of transcription/signal transduction (*NOTCH3*, *MXD1*, *RGS1*, *CREM*), cell adhesion/migration (*TNFAIP6*, *CLEC10A*, *ARHGDIB*, *NINJ1*), oxidation reduction (*CYP27A1*, *CYP1A1*, *AKR1C2*), transport (*ABCA6*, *AQP3*) and cell differentiation (*TNFAIP2*). To assess the usefulness of the selected genes, seven chemicals differing in sensitizing properties as determined by LLNA (DNCB, 2-MBT, PPDA, EUG, $NiSO_4$, BC, SDS; Table 1 – stage 1 experiments) were used.

Table 6
Estimated parameters for Student's t-distribution (μ, σ, ν) for nonsensitizers and sensitizers for three selected genes. Parameters estimated using Generalized Additive Models for Location Scale and Shape.

Gene	Nonsensitizers			Sensitizers		
	μ	σ	ν	μ	σ	ν
IL8	0.19	0.58	4.54	−1.39	1.35	4.54
PMAIP1	−0.18	0.56	148.41	−1.12	0.96	148.41
HMOX1	0.11	0.55	3.92	−0.86	1.42	3.92

The expression of all 35 genes was assessed following a 2-h treatment of THP-1 cells with the subtoxic concentration of test chemicals. IC_{20} concentrations of the compounds determined in the 24-h MTT test are given in Table 1. Results of five independent experiments revealed clear expression changes of only a few genes. For the further evaluation, 13 out of 35 genes differently regulated in THP-1 cells by sensitizers and nonsensitizers were selected (first 13 genes marked in bold; Table 3).

The most pronounced expression changes were observed for *IL8*, *PMAIP1*, *CXCL1*, *HMOX1*, *CXCR4* and *IL7R* genes. The significant changes were observed for all or a majority of the sensitizers in each experiment. *IL1B*, *CCL4*, *TNFAIP3*, *TNFAIP6* and *IL1R2* were upregulated mainly by chemicals with mild and weak sensitizing potential and metal sensitizers, whereas expression changes of *CCR2* were affected by haptens with extreme and strong sensitizing properties. Simultaneously, the expression of the above genes was not significantly increased by the nonsensitizers used.

Noteworthy are the results obtained for *NOTCH3*, which was also chosen for further evaluation. Its expression was decreased by all sensitizers to different degrees. Results for *CCL3L1*, *CCL2*, *PTGS2*, *CLEC10A*, *HIF1A*, *CCR7*, *CREM*, *CCR5*, *SLAMF1*, *MXD1*, *RGS1*, *AKR1C2*, and *NINJ1* were ambiguous because their expression levels varied between experiments, similarly to changes in expression of *CCL23*, *LY75*, *TNFAIP2*, *ARHGDIB*, *AQP3*, *ABCA6*, *CYP27A1*, *CYP1A1*, and *GADD45A*.

3.2. Verification of selected genes in THP-1 cells following treatment with a larger number of chemicals

The verification of the chosen 13 genes was done using 30 chemicals belonging to different classes and characterized by different sensitizing potential ranging from nonsensitizers to extreme sensitizers as determined by LLNA (Table 1 – stage 2).

Cytotoxicity of every chemical was assessed in a 24-h MTT reduction test and IC_{20} values were calculated (Table 4). Five out of thirty test compounds (PG, Iso, Glyc, 1-B, Sulf) were not cytotoxic in the 24-h MTT test up to 10 mM, therefore, they were used at a 10 mM concentration. These non-cytotoxic chemicals were proven to be nonsensitizers in LLNA (Gerberick et al., 2005).

Measurements of changes in expression of the 13 selected genes in THP-1 cells following treatment with a larger number of test chemicals proved the usefulness of only 3 genes (*IL8*, *HMOX1* and *PMAIP1*) for distinguishing sensitizers from nonsensitizers (Table 4). The cut-off value of the geometrical mean of 2^{-ddCt} over 2 was adopted.

Almost half of the sensitizers (10 out of 22) could be correctly identified on the basis of results for *IL8* and *HMOX1*. Expression fold change of *IL8*, *HMOX1* or *PMAIP1* in cells exposed to nonsensitizers did not exceed two.

The expression of *IL7R*, *IL1B*, *CXCL1* and *TNFAIP3* was upregulated only by 1–3 out of 22 test sensitizers. No changes in gene expression of *CCR2* and *CXCR4* were found. Although genes such as *NOTCH3*, *CCL4* and *TNFAIP6* were selectively upregulated by some sensitizers, the detected changes were not significant. Results for *IL1R2* were ambiguous, showing upregulation after exposure to some sensitizers and a nonsensitizer.

Table 7
Classification of test chemicals based on the classification function (CF). Chemicals inducing gene expression changes for which the CF is ≥0.05 (5.0E−2) are considered as sensitizers (the concentration used was <IC_{20} and <10 mM; in bold – substances of uncertain classification).

Classification	Chemical	Sensitization category	Value of classification function (CF)				
			IL8	*HMOX1*	*PMAIP1*	Total genes	
Nonsensitizers	BC	Nonsensitizer	5.5E−03	7.9E−02	4.4E−03	1.9E−06	
	PG	Nonsensitizer	8.5E−03	5.4E−02	2.8E−01	1.3E−04	
	Iso	Nonsensitizer	1.7E−02	3.4E−02	2.3E−01	1.3E−04	
	1-BB[a]	Nonsensitizer	8.5E−02	5.3E−02	4.3E−02	1.9E−04	
	SDS	Nonsensitizer	8.4E−02	4.7E−01	1.1E−02	4.3E−04	
	Sulf	Nonsensitizer	4.7E−02	1.4E−01	6.6E−02	4.5E−04	
	2,3-B	Weak sensitizer	1.7E−02	4.2E−01	9.1E−02	6.6E−04	
	PCP	Weak sensitizer	9.3E−02	3.3E−02	3.1E−01	9.7E−04	
	1-B	Nonsensitizer	2.8E−02	3.7E−02	9.8E−01	1.0E−03	
	12BD	moderate sensitizer	7.4E−02	3.4E−01	6.8E−02	1.7E−03	
	Bzc	Nonsensitizer	1.8E−01	4.6E−01	3.2E−02	2.7E−03	
	Glyc	Nonsensitizer	1.4E−02	6.9E−01	3.7E−01	3.5E−03	
	TDI[a]	Strong sensitizer	2.0E−01	3.1E−01	1.3E−01	8.1E−03	
	Mn	**Nonsensitizer**	**1.3E−01**	**2.4E−01**	**4.3E−01**	**1.4E−02**	
	AN	**Weak sensitizer**	**5.1E−02**	**3.9E−01**	**1.1E+00**	**2.3E−02**	
	BaP[a]	**Extreme sensitizer**	**6.3E−02**	**2.0E+00**	**3.4E−01**	**4.2E−02**	≥0.05 ←
Sensitizers	**Cu**	**Metal sensitizer**	**1.5E−02**	**3.3E−02**	**1.8E+02**	**9.3E−02**	
	3-AP	moderate sensitizer	5.4E+01	8.5E−02	9.6E−02	4.4E−01	
	MMS	moderate sensitizer	2.0E−01	3.3E−01	3.1E+01	2.1E+00	
	Cr	Metal sensitizer	3.6E−01	1.2E+01	1.0E+02	4.4E+02	
	DMS	Strong sensitizer	3.5E−01	7.4E+00	4.2E+02	1.1E+03	
	FAD	Strong sensitizer	3.3E−01	6.6E−01	1.1E+04	2.4E+03	
	$NiSO_4$	Metal sensitizer	6.5E+05	3.7E−02	8.2E−01	2.0E+04	
	GER	Weak sensitizer	6.2E+03	1.8E+02	8.1E−02	9.3E+04	
	LG	Strong sensitizer	2.2E+00	1.1E+00	3.9E+04	9.5E+04	
	CC	Extreme sensitizer	4.7E+03	8.8E+03	9.0E−01	3.7E+07	
	TCPA	Metal sensitizer	1.6E+02	4.5E−02	2.1E+07	1.5E+08	
	GA	Strong sensitizer	7.5E+02	2.1E+00	3.0E+08	4.8E+11	
	EUG	Weak sensitizer	1.8E+08	1.2E+03	6.4E+00	1.3E+12	
	Co	Metal sensitizer	1.8E+05	1.8E+01	4.5E+06	1.5E+13	
	Hg	Metal sensitizer	4.9E+02	8.5E+04	1.4E+06	5.9E+13	
	DNCB	Extreme sensitizer	4.5E+07	1.1E+07	2.8E+00	1.4E+15	
	PPDA	Strong sensitizer	3.3E+08	5.9E+05	2.7E+01	5.4E+15	
	2-MBT	moderate sensitizer	7.8E+08	6.0E+03	2.1E+03	9.9E+15	
	CMI	Extreme sensitizer	7.9E+02	9.4E+03	3.6E+10	2.7E+17	
	BB	Strong sensitizer	3.3E+05	2.9E+02	9.4E+11	9.1E+19	
	Cinn	Modetate sensitizer	1.6E+04	1.1E+05	7.9E+10	1.4E+20	

[a] Limit solubility of test chemicals.

3.3. Discriminant analysis and a prediction model of sensitizer detection

Each of the 13 genes selected for the second part of the study was separately assessed for its potential to classify chemicals as skin sensitizers and nonsensitizers. Statistical analysis revealed that only for 8 out of 13 genes (*IL8*, *HMOX1*, *PMAIP1*, *CXCL1*, *IL7R*, *CXCR4*, *IL1B*, *TNFAIP3*) differences in gene expression between sensitizers and nonsensitizers were statistically significant ($p < 0.01$). However, taking into account sensitizing potency groups (chemicals divided into subgroups with the same potency of skin sensitization), only results of IL-8 expression were significantly different from nonsensitizers in all tested subgroups of sensitizers. Also, *HMOX1* and *PMAIP1* were distinguished by significant differences in all sensitizer subgroups except weak sensitizers (*PMAIP1*) or metal sensitizers (*HMOX1*). Unfortunately, regression parameters (*b* value, Table 5) for any of the selected genes did not correlate with the strength of sensitization potential of test chemicals, precluding prediction of sensitization potency of test chemical based on expression of a single gene. There were three genes (*IL8*, *PMAIP1* and *HMOX1*) for which significant differences were observed for at least two extreme, strong and metal sensitizers (Table 5). To classify chemicals into skin sensitizers and nonsensitizers based on combined changes in expression of selected genes, parameters of Student's *t*-distribution of expression of these genes following exposure of cells to sensitizers and nonsensitizers were calculated (Table 6) and used to obtain the density functions. Next, the CF value was calculated for every tested substance, and CF value of 0.05 was found as a cut-off value generating the fewest false positive results. Thus, in the proposed prediction model, the chemicals for which the CF is ≥0.05 (5.0E−2) are considered as sensitizers. However, for additional margin of safety, the substances that induced changes in gene expression resulting in a CF in the range of 0.01–0.1 were considered as uncertain. Using this criteria, the model enables us to predict 20 out of 27 sensitizers from among 37 test chemicals (correct classification of 78% chemicals; sensitivity of 74% for detecting sensitizers) (Table 7). Spearman rank correlation between CF (*in vitro*) and LLNA (*in vivo*) potency rank was equal to 0.74 ($p = 1.9E{-}6$).

4. Discussion

The identification of skin sensitization potential of chemicals for regulatory purposes is performed using exclusively animal tests. Development of reliable and non-expensive *in vitro* predictive models of contact sensitization is desired and a number of such models have been proposed but none of them have yet gained broad acceptance (Ryan et al., 2005). The usefulness of THP-1 cells as a surrogate for DCs in an *in vitro* model has already been demonstrated (Ashikaga et al., 2002, 2006; Bocchietto et al., 2007; Miyazawa et al., 2007; Tietze and Blomeke, 2008). THP-1 cells have been shown to respond to sensitizers by changes in expression of surface antigens (Ashikaga et al., 2006; Sakaguchi et al., 2006; Bocchietto et al., 2007; Miyazawa et al., 2007; Tietze and Blomeke, 2008)

and in gene expression (Hirota and Moro, 2006; Miyazawa et al., 2007). However, it seems to be difficult to identify a single gene whose expression can be correlated to multiple sensitizers and be employed as a marker discriminating sensitizers and nonsensitizers. Recently, there were reports suggesting that one effective way to detect sensitizers in experiments employing DCs (Hooyberghs et al., 2008) and THP-1 (Lambrechts et al., 2009) is parallel observation of changes of expression of several genes followed by discriminant statistical analysis.

The aim of the present study was to identify a set of genes that are strongly associated with activation of THP-1 cells by contact sensitizers of different properties and potency to be employed as a marker for detecting sensitizers. In the first tier of testing, the 35 candidate genes were selected based on available data on sensitizer-induced gene expression changes in DCs (Ryan et al., 2004; Gildea et al., 2006; Schoeters et al., 2006, 2007; Cluzel-Tailhardat et al., 2007), and their expression in THP-1 cells following treatment with seven chemicals with different sensitizing potential were evaluated using real-time RT-PCR. Expression of almost half of the selected genes was significantly changed in THP-1 cells following a 2-h treatment with seven chemicals from different sensitizing potency categories (Table 3). Expression of several genes (*HMOX1*, *CCL4*, *IL1B*, *PMAIP1*, *NINJ1*) was similar whereas expression of certain genes (*HIF1*) differed as compared to the previously observed pattern of gene expression in THP-1 cells after a 24-h treatment with sensitizers (Hirota and Moro, 2006). Following this analysis, we limited the number of evaluated genes from 35 to 13 that met our a priori criteria of at least a 1.7-fold change in expression level following treatment with sensitizers, and we expanded the number of tested compounds to 30, consisting of 22 sensitizers and 8 nonsensitizers (Basketter et al., 1995, 1999; Mandervelt et al., 1997; NTP, 1999; Kimber et al., 2003; Gerberick et al., 2005). This second tier of testing showed that the expression of 11 out of 13 selected genes was upregulated at least 2-fold following exposure to one or more sensitizers but not by nonsensitizers (Table 4). Statistical analysis showed that analysis of the combined expression of 3 out of 13 tested genes (*IL8*, *HMOX1* and *PMAIP1*) was best suited for discrimination between sensitizers and nonsensitizers. Each of these three genes was previously reported to be specifically upregulated by one or more sensitizing compound in at least one cell model *in vitro*. Thus, specific upregulation of *IL8* expression following treatment of THP-1 with sensitizers (10 out of 22 tested) was similar to previously reported sensitizer-mediated upregulation of IL-8 expression in THP-1 and other cell types (Toebak et al., 2006; Coquette et al., 2003; Miyazawa et al., 2007; Nukada et al., 2008; Python et al., 2007). Increased expression of *HMOX1* in THP-1 cells in the present study observed for 7 out of 22 evaluated sensitizers is consistent with reported selective upregulation of *HMOX1* following exposure of THP-1 cells and DCs to strong sensitizers (Hirota and Moro, 2006; Ade et al., 2009). *PMAIP1* expression, which in the present study was increased above the 2-fold threshold level by 13 sensitizers including all 5 sensitizing metals, is consistent with the previous observation that *PMAIP1* was upregulated in $CD34^+$-derived DCs exposed to $NiSO_4$ (Schoeters et al., 2006).

Our approach based on expression levels of three selected genes in exposed THP-1 cells allowed us to correctly identify 74% of sensitizers used (Tables 5 and 6). Among the undetected sensitizers was one compound with moderate (12BD) and three with weak (2,3-B, PCP and AN) sensitization potential and two chemicals with limited solubility (B[a]P and TDI), which negatively affected their bioavailability. Additionally, the sensitizers showed higher cytotoxicity as compared to nonsensitizer compounds. We do not know if this is a random effect of the selection process or represents inherent differences between these two categories of compounds. However, the differences in cytotoxicity could influence the observed differences in gene expression. There are suggestions that there is correlation between cytotoxic effects of chemicals on DCs and their ability to initiate allergic response (Gallucci and Matzinger, 2001).

We have also addressed the question of possible prediction of strength of the sensitization potential of a tested compound. However, the comparison of the gene expression changes between groups of chemicals of different strength of sensitization potential showed that while all the genes were upregulated in THP-1 cells only following sensitizer treatment, the gene expression levels inside potency groups varied and did not correlate with strength of sensitization potential of test chemicals.

In conclusion, the present study revealed that based on the results of gene expression in THP-1 cells following exposure to a tested compound, it is possible to determine if the compound is a sensitizer or nonsensitizer. While this conclusion is supported by data obtained with a limited number of compounds (27 sensitizers and 10 nonsensitizers), it strongly suggests that evaluation of combined changes in expression of selected genes using real-time RT-PCR in THP-1 cells may be a promising *in vitro* model for screening chemicals for their skin sensitization potential.

Conflict of interest

Authors have no conflict of interest.

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