

High-Resolution Transcriptional Profiling of Chemical-Stimulated Dendritic Cells Identifies Immunogenic Contact Allergens, but Not Prohaptens

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Key Words

Contact allergy • Cinnamic aldehyde • Dendritic cells • Exon array • Prohaptens

Abstract

Allergic contact dermatitis is a complex syndrome and knowledge about the in vitro detection of small-molecular-weight compounds, particularly prohaptens, is limited. Therefore, we investigated chemical-induced gene expression changes in human antigen-presenting cells upon stimulation with immunogenic contact allergens, prohaptens and irritants. Monocyte-derived dendritic cells (moDCs) and THP-1 cells were stimulated with the prohaptens cinnamic alcohol (CALC), the haptens cinnamic aldehyde (CALD), an irritant and an obligatory sensitizer in vitro. Whole-genome screening and consecutive PCR analysis of differential gene expression in moDCs stimulated with either CALD or the obligatory sensitizer revealed core-regulation of 11 marker genes which were related to immunological reactions (IL-8, CD1e, CD200R1, PLA2G5, TNFRSF11A), oxidative or metabolic stress responses (AKR1C3, SLC7A11, GCLM) or other processes (DPYLS3, TFPI, TRIM16). In contrast, the prohaptens CALC and the irritant did not change marker gene expression. In THP-1 cells, CALD and the positive control elicited similar expression

changes in only 4 of the previously identified genes (IL-8, TRIM16, CD200R1, GCLM). In conclusion, we provide important insights into the pathophysiological basis of allergic contact dermatitis, identify marker genes suitable for skin hazard assessment and demonstrate that contact-allergenic prohaptens escape in vitro detection if their skin metabolism is not taken into account.

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Introduction

As an interface between the individual and his/her environment, human skin represents the first line of defense against harmful external influences such as small-molecular-weight chemicals. Impairment of skin barrier function is frequently associated with cutaneous inflammation resulting in different clinical phenotypes such as allergic contact dermatitis (ACD) [1, 2]. ACD represents a common inflammatory skin disease with a cumulative prevalence of 15–20% in the general population of western countries. Additionally, ACD as an occupational disease has a major socioeconomic impact and costs up to EUR 3 billion only in Germany [Diepgen T.L., pers. commun.; 3, 4].

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As a T-cell-driven immunological response orchestrated by cutaneous antigen-presenting cells (APCs), ACD can be elicited by a multitude of small-molecular-weight (<1,000 Da) compounds (haptens), particularly metal salts and fragrances [5–7]. While some of these contact allergens are directly immunogenic after binding to proteins, others require bioactivation by metabolizing enzymes (prohaptens). In recent studies, we were able to show that cytochrome P450 (CYP) enzymes expressed in epidermal keratinocytes [8, 9] play a significant role in the activation of prohaptens to highly reactive species which bind to proteins in order to become the nominative antigen [10–12]. Other studies revealed that efflux transport proteins such as the multidrug resistance-related proteins – which are also constitutively expressed in epidermal keratinocytes [8] or monocyte-derived dendritic cells (moDCs) [13] – mediate the active transport of contact allergens in skin cells [14].

Premarketing skin hazard assessment of newly introduced xenobiotic compounds still mainly relies on in vivo animal testing methods such as the murine local lymph node assay [1]. However, a complete ban on in vivo testing of cosmetic and toiletry ingredients for skin-sensitizing properties will come into force within the European Union in 2013 [15]. In addition, new programs to study the toxicity of chemicals and to collect information about the properties of chemical compounds such as the REACH initiative (Registration, Evaluation, Authorization and Restriction of Chemical Substances) will require up to 67 million animals in the near future. Today already about 150,000 animals are needed to test one new chemical which consumes roughly EUR 3 billion for experiments with animals in Europe every year [16]. This increases the demand for alternative approaches to study the toxicity of those compounds including their sensitization potential. Two main goals must be reached by these tests: (1) the recognition of the risk that one compound is a sensitizer and (2) the potential of this risk. In particular the second aspect has led to a focus on the chemical-induced stimulation of moDCs, CD34+ progenitor-derived dendritic cells (DCs) or myeloid/DC lines (THP-1, KG-1, MUTZ 3, U-937). These methods assess phenotypic alterations, cytokine secretion or differential gene regulation of the implemented APCs after incubation with the respective test substance [1].

However, these studies have hitherto not considered the skin-specific CYP-mediated activation of contact-allergenic prohaptens in APC-based in vitro systems. Hence, we employed an enriched skin-like recombinant human CYP cocktail [10] to demonstrate metabolism of

cinnamic derivatives as model compounds for cutaneous bioactivation. Consequently, we employed novel microarray technology to obtain a broader pathophysiological view of the differential effects of haptens, prohaptens and irritants on gene expression in human APCs. We also analyzed whether the marker genes obtained by genomic microarray analysis allowed for the identification of haptens and, particularly, their differentiation from prohaptens and irritants in vitro.

Materials and Methods

Metabolism Studies

Incubation of Cinnamic Alcohol with the Skin-Like CYP Cocktail

Each incubation contained substrate [cinnamic alcohol (CALc) 200 μ M], the previously described skin-like rhCYP cocktail [10], HEPES buffer (50 mM, pH 7.4), $MgCl_2$ (30 mM) and NADPH₂ (1 mM) in a total volume of 500 μ l. The CYP cocktail was prepared by premixing recombinantly expressed CYP 1A1 (3.6 pmol), 1B1 (2.0 pmol), 2B6 (0.035 pmol), 2E1 (11 pmol) and 3A5 (5.6 pmol). The incubations were performed in triplicate and were initialized by addition of NADPH₂ after 3 min of preincubation at 37°C, and terminated after 15, 30 or 60 min by addition of acetonitrile (500 μ l) containing benzyl alcohol (40 μ M) as internal standard. The incubation mixtures were analyzed, after centrifugation (3,000 rpm, 4°C, 10 min), using liquid chromatography-mass spectrometry (LC-MS) as described below. Control experiments were run in the absence of NADPH₂, in the absence of the CYP cocktail and using heat-deactivated CYP enzymes.

Analysis of CYP Cocktail Incubations

The incubation mixtures were analyzed for content of cinnamic aldehyde (CALd) and cinnamic acid (CAcid) using LC-MS. Analyses were performed using electrospray ionization on a Hewlett-Packard 1100 HPLC-MS. The system included a vacuum degasser, a binary pump, an autoinjector, a column thermostat, a diode array detector and a single quadrupole mass spectrometer. The electrospray interface was used with the following spray chamber settings: nebulizer pressure 40 psig (pound-force per square inch gauge), capillary voltage 4,000 V, drying gas temperature 350°C, and drying gas flow rate 10 l/min. For each sample, 10 μ l aliquots were injected onto a HyPURITY C18 column (150 $\times$ 3.0 mm i.d., particle size 3 μ m; Thermo Hypersil-Keystone, Bellafonte, Pa., USA) and eluted with 0.1% acetic acid and 30% acetonitrile in water at a flow rate of 0.40 ml/min and a column temperature of 40°C. The flow was diverted to waste for the first 5 min of each run. Mass spectral analysis was performed in the positive ionization mode with a fragmentor voltage of 70 V. The detector was used in the selected ion monitoring mode and the following ions were monitored: m/z 91 (retention time 3–6 min); m/z 91, 117 (retention time 6–7.4 min) (CALc); m/z 131, 149 (retention time 7.4–9 min) (CAcid), and m/z 115, 133 (retention time 9–15 min) (CALd). Quantifications were carried out using standard curves of CALd and CAcid (dissolved in 30% acetonitrile in water). Benzyl alcohol (20 μ M) was used as internal standard. The detection limits of CALd and CAcid were 0.25 and 0.10 μ M, respectively.

In vitro Cultures of APCs

Monocyte-Derived Dendritic Cells

Human peripheral blood monocytes from a total of 5 healthy donors were separated from purchased single buffy coats (Institut für Transfusionsmedizin, Universitätsklinikum Aachen, Germany) over a Ficoll-Paque gradient (Amersham Pharmacia Biotech, Uppsala, Sweden) and CD2-, CD7-, CD19-, CD56-, CD16- and CD235a-positive leukocytes were depleted using a negative monocyte isolation kit (DynaL Monocyte Negative Isolation Kit, Invitrogen, Oslo, Norway). The remaining monocytes were suspended in complete medium consisting of RPMI-1640 (PAA Laboratories, Pasching, Germany) enriched with 1.5% heat-inactivated autologous plasma and 1.1 ml L-glutamine/100 ml. The cell suspension was plated at a density of 3×10^6 cells per reaction well. In order to induce DC differentiation, the culture medium was supplemented with 800 U/ml GM-CSF and 1,000 U/ml IL-4 (R&D-System, Bühlmann, Basel, Switzerland) and the cells were kept at 37°C and 5% CO₂ for 3 days. After adding 1,600 U/ml GM-CSF and 1,000 U/ml IL-4 to the culture medium on day 4, the resulting DC phenotype was determined 24 h later (day 5) by flow cytometric analysis using saturating concentrations of fluorescein isothiocyanate- or phycoerythrin-conjugated monoclonal antibodies (BD Pharmingen, Erembodegem, Belgium). At this time point, the investigated DCs displayed a phenotype characteristic of immature DCs, i.e. CD1a^{high}, HLA-DR^{intermediate}, CD80^{intermediate}, CD86^{low}, CD83^{negative}, CD14^{negative}.

THP-1 Cells

Human THP-1 monocytes were obtained from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany) and cultured as suspension cells in RPMI-1640 (PAA Laboratories) supplemented with 10% FCS, 100 IU/ml penicillin and 0.1 mg/ml streptomycin at 37°C in a 5% CO₂ humidified incubator. The cells were subcultured at a split ratio of 1:4 and passaged every 3 days.

Stimulation of APCs with Small-Molecular-Weight Compounds

THP-1 cells (3 single experiments) and moDCs (5 single experiments) were incubated with CAIc, CAId, the strong sensitizer 2,4,6-trinitrobenzene sulfonic acid (TNBS) as a positive control, and the model irritant sodium dodecyl sulfate (SDS). The test compounds were individually prepared in complete medium (CAIc 200 µM, CAId 200 µM, SDS 5 µg/ml, TNBS 200 µg/ml) and each test solution was incubated with at least 6×10^6 DCs or THP-1 cells for 30 h, respectively. RNA isolation of the treated cells was performed after 30 h, the RNA samples were harvested (see below) and stored at -80°C prior to further analysis.

RNA Isolation

Total RNA from THP-1 cells and DCs was isolated using the high pure RNA isolation kit (Roche, Mannheim, Germany) according to the manufacturer's instructions, including efficient on-column digestion of DNA with RNase-free DNase I. RNA was quantified by photometric measurement (NanoDrop Technologies, Wilmington, Del., USA) and integrity was proven by using the 2100 Bioanalyzer (Agilent Technologies, Palo Alto, Calif., USA). The obtained amounts of RNA were used for exon array analysis (moDCs) and quantitative real-time PCR (moDCs and THP-1).

Exon Array Analysis of Differential Gene Expression in Stimulated moDCs

The GeneChip Human Exon 1.0 ST array contains approximately 5.5 million probes, forming 1.4 million probe sets that are used to separately interrogate 1 million known and predicted exons [17]. With approximately 4 probes per exon and roughly 40 probes per gene, the Exon 1.0 ST array enables gene level expression analysis, in which multiple probes on different exons are summarized into an expression value of all transcripts from the same gene. It provides a comprehensive coverage of the genome interrogating over 1 million exon clusters, including empirically supported and predicted transcribed sequences, enabling the discovery of previously unidentified novel events.

Two micrograms total RNA from immature moDCs incubated with the respective test substance (see above) were processed using the GeneChip Whole Transcript Sense Target Labeling Assay (Affymetrix, Inc., Santa Clara, Calif., USA). Following the rRNA reduction procedure using the RiboMinus Human/Mouse Transcriptome Isolation Kit (Invitrogen, Carlsbad, Calif., USA) double-stranded cDNA was synthesized with random hexamers tagged with a T7 promoter sequence. The double-stranded cDNA was then used as a template for the subsequent T7 RNA polymerase-mediated cRNA synthesis producing many copies of antisense cRNA. In a second cycle of the first-strand reverse transcription reaction, random hexamers were used to reverse transcribe 10 µg of cRNA from the first cycle to produce single-stranded DNA in the sense orientation with incorporated dUTPs. This single-stranded DNA sample was then treated with a combination of uracil DNA glycosylase and apurinic/apyrimidinic endonuclease 1 that breaks the DNA strand specifically at the unnatural dUTP residues. The fragmented single-stranded DNA was subsequently labeled with recombinant terminal deoxynucleotidyl transferase and the Affymetrix proprietary DNA labeling reagent that is covalently linked to biotin. Hybridization cocktails containing 5.5 µg of fragmented, end-labeled cDNA were prepared and applied to GeneChip Human Exon 1.0 ST arrays. Hybridization was performed in a hybridization oven (45°C) at 60 rpm for 16 h. Using the GeneChip Fluidics Station 450 the hybridized GeneChip Exon 1.0 ST arrays were washed and stained (Fluidics Protocol FS450_0001). Arrays were scanned using the Affymetrix GeneChip Scanner 3000 7G controlled by GeneChip Operating Software version 1.4 to produce CEL intensity files. Data from the exon arrays were processed using the Array Assist Exon 5.5.1 software (Stratagene, La Jolla, Calif., USA) by implementation of the probe logarithmic intensity error algorithm for normalization and the detection-above-background method, to eliminate probes which had a detection intensity comparable to the background of the hybridization signal intensity [9]. Transcripts were considered present in the data set if at least 1 detectable probe set was discovered by the detection-above-background method with a probability of 0.05. Expression values were determined for entire transcripts thereby averaging over multiple probe sets and are scaled logarithmically. Fold changes are reported as an absolute value.

Real-Time Reverse Transcriptase Polymerase Chain Reaction

Purified polyA⁺ RNA was reverse transcribed with the TaqMan Reverse Transcription Reagents kit (Applied Biosystems, Weiterstadt, Germany) with random hexamers as primers. TaqMan experiments were carried out on an ABI PRISM 7000 Se-

quence Detection System (Applied Biosystems) using Assay-on-Demand gene expression products for each investigated marker gene according to the manufacturer's recommendations.

Primer/probe sets for the following genes were obtained from the Applied Biosystems Assays-on-Demand repository: AKR1C2 (Hs00413886_m1), AKR1C3 (Hs00366267_m1), CD200R1 (Hs00373422_m1), CD200R2 (Hs02339410_m1), DPYSL3 (Hs00181665_m1), IL-8 (Hs00174103_m1), TFPI (Hs00196731_m1), CD1e (Hs00229421_m1), TRIM16 (Hs00414879_m1), PLA2G5 (Hs00173472_m1), TNFRSF11A (Hs00187189_m1), CCL4L/CCL4L1/CCL4L2 (Hs00237011_m1), SLC7A11 (Hs00204928_m1), GCLM (Hs00157694_m1) and IFI44L (Hs00199115_m1). At the time of this investigation, a primer/probe set for ME1 was not commercially available. Additionally, preliminary studies revealed that AKR1C4 gene expression in moDCs or THP-1 cells was not reproducibly detectable with the corresponding Assay-on-Demand product (Hs00559542_m1) even after incubation with the very potent sensitizer TNBS (data not shown).

Assay-on-Demand products for HPRT (Hs99999909_m1) and GAPDH (Hs99999905_m1) were used as an internal reference to normalize the target transcripts of THP-1 cells while cyclophilin A (Hs99999904_m1) and GAPDH (Hs99999905_m1) were used as housekeeping genes in DC experiments. The respective rRNA sequences were amplified independently in separate reaction wells in triplicate and real-time PCR efficiencies were determined for each primer/probe set from standard curves generated from serial dilutions of polyA⁺ RNA.

Results

The Prohaptan CALc Is Bioactivated into CALd by a Skin-Like CYP Cocktail

To demonstrate that CALc is a prohaptan converted to CALd by CYP expressed in human skin, we incubated CALc with a previously described panel of CYPs characteristic of human skin [10]. LC-MS analysis detected CALd in all incubations except for the controls. CAcid, which can be formed by further oxidation of CALd, was detected in trace amounts below the limit of quantification in the incubations performed for 30 min and in quantifiable amounts in the 60-min incubations. No metabolites were detected in any of the control incubations.

Exon Array Analysis Identifies Differential Gene Expression Patterns in moDCs after Incubation with Irritant, Prohaptenic and Sensitizing Compounds

In 3 single experiments, immature moDCs of 3 unrelated donors were incubated with the prohaptan CALc, its active metabolite CALd, the nonsensitizing irritant SDS and TNBS, a known contact allergen of very high sensitizing potency that has previously been used as a positive control in similar experiments [18, 19]. Cells were incu-

bated with the respective substance and harvested after 30 h for tRNA isolation. Combined GeneChip Human Exon 1.0 ST array analysis of all 3 experiments revealed that expression of 253 genes was regulated ≥ 3 -fold in moDCs stimulated with TNBS as compared to moDCs incubated with SDS, whereas 492 genes were differentially regulated ≥ 3 -fold in moDCs stimulated with CALd or CALc, respectively (data not shown). To identify potential markers for contact allergen-specific moDC activation, we selected the 50 most regulated genes for each pairwise analysis and found 18 genes to be differentially expressed in moDCs after incubation with both TNBS versus SDS and CALd versus CALc (table 1). Of these candidate genes, 10 were found to be upregulated while 8 revealed down-regulation. Additional calculation of all nonsensitizer (CALc, SDS) versus all sensitizer (CALd, TNBS) data yielded similar results with all 18 genes contained within the 25 most regulated genes (table 1).

RT-PCR Analysis Confirms Significant Regulation of Oxidative Stress-Related and Immunoregulatory Genes in Haptan-Stimulated moDCs and THP-1 Cells

To confirm the differences in gene expression between human APCs stimulated with sensitizers and nonsensitizers, we next used real-time PCR to measure the abundance of transcripts of the microarray-based candidate genes as compared to negative controls, i.e. cell cultures incubated with pure medium.

Considering only genes clearly regulated by both the strong (TNBS) and the moderate (CALd) sensitizer, we detected that stimulation of moDCs of 5 unrelated donors with either substance induced significant differences in expression of 11 genes (fig. 2a). Functional information was obtained through scanning of the EntrezGene (<http://www.ncbi.nlm.nih.gov/omim/>) and OMIM (<http://www.ncbi.nlm.nih.gov/sites/entrez?db=gene>) databases revealing that the included candidate genes related to immunological reactions (IL-8, CD1e, CD200R1, PLA2G5, TNFRSF11A), oxidative or metabolic stress responses (AKR1C3, SLC7A11, GCLM) and other processes (DPYSL3, TFPI) as defined by Gene Ontology terms (table 2). For 1 gene (TRIM16), no assigned Gene Ontology designations could be retrieved.

In 3 independent experiments performed with THP-1 cells, TNBS and CALd elicited similar expression changes in only 4 genes (IL-8, TRIM16, CD200R1, GCLM) while other candidate genes revealed differential regulation after incubation with the extreme sensitizer TNBS, but not after CALd stimulation (AKR1C3, SLC7A11, DPYSL3).

Table 1. Summary of the 50 most regulated genes after incubation of moDCs with TNBS versus SDS and CAld versus CALc

TNBS versus SDS				CAld versus CALc				Sensitizers versus nonsensitizers			
transcript cluster ID	gene symbol	fold change	regulation	transcript cluster ID	gene symbol	fold change	regulation	transcript cluster ID	gene symbol	fold change	regulation
3589458	THBS1	46.41	up	3232979	AKR1C2	49.98	up	3232979	AKR1C2	34.89	up
2731332	IL8	33.63	up	2591421	TFPI	23.93	up	3274758	AKR1C1	22.77	up
3232979	AKR1C2	24.36	up	3363868	RRAS2	17.61	up	2731332	IL8	20.93	up
3233049	AKR1C3	19.80	up	2962820	ME1	17.47	up	3233049	AKR1C3	17.62	up
3692701	CES1	14.99	up	3719020	CCL4L2	16.77	down	2962820	ME1	12.69	up
3846860	SEMA6B	11.27	up	3746845	TRIM16	16.06	up	3746845	TRIM16	11.53	up
2477073	CRIM1	10.38	up	2323847	PLA2G5	15.72	down	2323847	PLA2G5	11.47	down
2962820	ME1	9.22	up	3233049	AKR1C3	15.69	up	2591421	TFPI	11.10	up
3233119	AKR1C4	9.10	up	2688955	CD200R1	14.02	down	2688955	CD200R1	9.78	down
2362230	CD1E	8.78	down	3718977	CCL4	13.95	down	2362230	CD1E	8.64	down
3351675	BLR1	8.76	up	2731332	IL8	13.02	up	3719020	CCL4L2	8.61	down
2548699	CYP1B1	8.63	up	4040932	CCL3	12.76	down	3233119	AKR1C4	7.94	up
2323847	PLA2G5	8.38	down	3589141	SPRED1	12.20	down	4040063	CCL4L2	7.86	down
3696666	NQO1	8.36	up	3266279	SLC18A2	11.91	down	3718977	CCL4	7.85	down
2697863	RBP1	8.34	up	2709132	ETV5	11.33	down	2880292	DPYSL3	7.84	up
3746845	TRIM16	8.28	up	3057955	FGL2	11.31	down	2786322	SLC7A11	7.65	up
3513549	RCBTB2	8.19	up	2997789	GPR141	11.18	down	3692701	CES1	7.54	up
2786322	SLC7A11	7.25	up	3754133	CCL3L1	11.10	down	3791254	TNFRSF11A	7.39	down
2688955	CD200R1	6.82	down	3754070	CCL3L3	11.00	down	3589458	THBS1	7.15	up
3513514	P2RY5	6.82	up	3743306	CLEC10A	10.83	down	3266279	SLC18A2	7.04	down
2571510	IL1B	6.32	up	3507282	FLT1	10.47	down	3718930	CCL4	6.83	down
2921402	SLC16A10	6.31	up	2880292	DPYSL3	10.44	up	2423625	GCLM	6.49	up
3852832	EMR3	6.26	up	3812385	CD226	10.42	down	3507282	FLT1	6.27	down
3823304	CYP4F3	6.02	up	2427981	ADORA3	10.40	down	2688933	CD200R2	6.25	down
3921068	ETS2	6.00	up	3742212	ALOX15	10.00	down	2343473	IFI44L	6.17	down
2880292	DPYSL3	5.90	up	3540068	AKAP5	9.50	down	2477073	CRIM1	6.11	up
3472183	RASAL1	5.88	up	3791254	TNFRSF11A	9.36	down	3057955	FGL2	6.01	down
3791254	TNFRSF11A	5.83	down	2880422	SPINK1	8.77	up	3540068	AKAP5	5.94	down
2423625	GCLM	5.74	up	3251393	DDIT4	8.64	up	3401704	CCND2	5.74	down
2878437	CD14	5.71	up	3409605	MLSTD1	8.63	down	3696666	NQO1	5.71	up
3768412	SLC16A6	5.69	up	2519294	KIAA1946	8.62	up	2997789	GPR141	5.64	down
2343473	IFI44L	5.38	down	2362230	CD1E	8.51	down	3409605	MLSTD1	5.59	down
3279108	NMT2	5.37	up	3105749	ATP6V0D2	8.44	up	3220673	LTB4DH	5.51	up
2591421	TFPI	5.15	up	3401704	CCND2	8.12	down	2362201	CD1C	5.40	down
3791935	SERPINB2	5.05	up	2786322	SLC7A11	8.07	up	4040932	CCL3	5.34	down
3137875	GGH	5.04	up	3978169	TSPYL2	7.90	up	3742212	ALOX15	5.33	down
4016308	BEX1	5.03	up	2688933	CD200R2	7.88	down	3279108	NMT2	5.32	up
3464860	DUSP6	4.97	up	2523855	CTLA4	7.42	down	3718191	CCL8	5.28	down
2688933	CD200R2	4.95	down	3470193	CMKLR1	7.35	down	2348992	VCAM1	5.13	down
2527747	SLC11A1	4.94	up	4013460	CYSLTR1	7.34	down	3743306	CLEC10A	5.08	down
3220673	LTB4DH	4.89	up	2423625	GCLM	7.33	up	3589141	SPRED1	5.07	down
3209384	TMEM2	4.88	up	2494484	NCAPH	7.28	down	3754133	CCL3L1	5.04	down
3061621	TFPI2	4.69	down	3248661	ZNF365	7.13	up	2921402	SLC16A10	4.98	up
3406589	MGST1	4.61	up	3356038	TMEM45B	7.08	down	3754070	CCL3L3	4.84	down
2359664	S100A9	4.59	up	2343473	IFI44L	7.06	down	3137875	GGH	4.64	up
2362201	CD1C	4.46	down	2813866	LOC728506	7.00	down	2427981	ADORA3	4.57	down
3990727	RAB33A	4.42	down	3718191	CCL8	6.96	down	2523855	CTLA4	4.44	down
3719020	CCL4L2	4.42	down	3233119	AKR1C4	6.93	up	2601648	DOCK10	4.41	down
3718977	CCL4	4.41	down	2601648	DOCK10	6.89	down	3258625	GPR120	4.32	down
3275729	IL2RA	4.27	up	2861616	NAIP	6.72	down	2870889	C5orf13	4.22	down

Genes differentially expressed in all 3 analyses are highlighted in italics.

Table 2. Identified marker genes involved in the sensitization phase of allergic contact dermatitis

EntrezGene ID	Gene symbol	Full name	Process (Gene Ontology)
<i>Immunological reactions</i>			
3576	IL-8	interleukin 8	inflammatory response, neutrophil chemotaxis, regulation of cell adhesion
913	CD1E	CD1e molecule	antigen processing and presentation
131450	CD200R1	CD200 receptor 1	interspecies interaction (surface receptor in myeloid lineage cells)
5322	PLA2G5	phospholipase A ₂ , group V	phospholipid metabolic process (leukotriene synthesis)
8792	TNFRSF11A	tumor necrosis factor receptor superfamily 11A	cell-cell signaling (interaction of T-cells and DCs)
<i>Oxidative/metabolic stress response</i>			
8644	AKR1C3	aldo-keto reductase family 1, member C3	oxidation, reduction (conversion of aldehydes and ketones to their corresponding alcohols)
23657	SLC7A11	solute carrier family 7, member 11	amino acid transport (cystine/glutamate transport system)
10626	TRIM16	tripartite motif-containing 16	metal ion binding, dioxin response-associated gene (no Gene Ontology entry)
2730	GCLM	glutamate-cysteine ligase, modifier subunit	cysteine/glutamate metabolic process (first rate-limiting enzyme of glutathione synthesis)
<i>Miscellaneous</i>			
1809	DPYSL3	dihydropyrimidinase-like 3	nervous system development, signal transduction, nucleic acid metabolic process
7035	TFPI	tissue factor pathway inhibitor	blood coagulation, extrinsic pathway

As illustrated by the presented standard deviations, we observed a substantial degree of variation in gene expression patterns between single experiments in both investigated APC types which have been described previously to be inherent in this model of cell culture experiments [20, 21]. However, differential regulation of the identified marker genes remained statistically significant ($p < 0.05$) or even highly significant ($p < 0.005$) as determined by the two-sided Student's *t* test.

Incubation of moDCs and THP-1 cells with either the irritant SDS or the prohaptan CALc did not lead to significant gene expression changes as compared to negative controls (table 1; fig. 2a, b).

Discussion

Despite ongoing efforts, a reliable surrogate for animal-based in vivo assessment of small-molecular-weight compounds has not been established so far. Still, several biological endpoints have been evaluated in APCs stimulated with contact allergens in order to identify skin sensitizers in vitro. Besides the analysis of cytokine secretion [22, 23] or signal transduction pathways [24], chemical-

induced changes in the expression of DC surface markers have been extensively studied by several research groups. However, these test systems are known to preferentially identify strong or even extreme sensitizers whereas weak and moderate sensitizers risk remaining undetected. Even more importantly, these targeted approaches have hitherto only assessed APC responses to fully immunogenic contact allergens while the in vitro effects of pro-haptenic small-molecular-weight compounds have not been investigated so far [25–28]. Therefore, it was the purpose of the present study to provide an exon microarray-based whole-genome analysis of differential gene expression changes in human moDCs treated with an irritant, a prohaptan and its bioactivated metabolite.

To ensure the clinical relevance of our findings, we decided to implement selected cinnamic derivatives as test compounds which have been used to explore sensitization mechanisms in other clinical and laboratory settings [28]. Both CALc and CALd are known to cause ACD after occupational or environmental exposure and have therefore been included in the European patch test standard series (fragrance mix) [29, 30]. Interestingly, CALc is generally regarded as a prohaptan convertible to CALd by cutaneous xenobiotic-metabolizing enzymes such as al-

cohol dehydrogenase or CYPs. However, this assumption has hitherto been based on rather circumstantial evidence implying bioactivation of CALc in ex vivo experiments using human whole-skin specimen [31]. In contrast, we used a set of recombinantly expressed, skin-characteristic CYPs that have recently been shown to activate prohaptens to contact allergenic metabolites in vitro [8, 10]. By means of LC-MS, we were able to directly demonstrate that CALc is a prohapten bioactivated to CALd by a panel of CYP enzymes characteristically expressed in human skin (fig. 1a, b).

Furthermore, we employed genomic array technology to characterize gene expression of chemical-treated moDCs and to identify groups of functionally associated genes relevant in the process of skin sensitization. Although this mode of gene transcript profiling has been chosen before in the context of skin hazard assessment, the microarray used in the current study (GeneChip Human Exon 1.0 ST array) contained approximately 1.4 million probe sets allowing for a substantially higher resolution of gene expression as compared to all previously reported oligonucleotide or targeted microarrays [20, 32–37]. Likewise, Bitton et al. [38] detected a higher correspondence between proteomic and exon array data as compared to that previously observed between proteomic and 3' array data. Robinson and Speed [39] also demonstrated in their studies that the exon/whole-transcript expression array is more sensitive than 3' arrays.

Consequently, this large-scale genomic approach revealed significant (>3-fold) expression changes of more than 250 genes in moDCs incubated with the extreme sensitizer TNBS versus SDS and of almost 500 genes after stimulation with CALd versus CALc. As further RT-PCR confirmation for such a high number of potential marker genes is virtually impossible, we selected the 50 most regulated genes for each pairwise analysis and were able to identify a set of genes differentially regulated by both the very potent and the moderate sensitizer (table 1).

The following RT-PCR experiments confirmed significant gene regulation in moDCs after exposure to TNBS and CALd, but not after incubation with the irritant SDS or the prohapten CALc as compared to negative controls (fig. 2a; table 1). In line with previous investigations, we found relevant alterations in expression of genes known to be associated with antigen processing (CD1e) [35], functional activation (IL-8, CD200R1) [10, 12, 20, 23] and T-cell interaction (TNFRSF11A) [32] of moDCs incubated with contact sensitizers (table 2). In this context, it is noteworthy that TRIM16, a gene encoding for the estrogen-responsive B box protein, was also regulated in hapten-

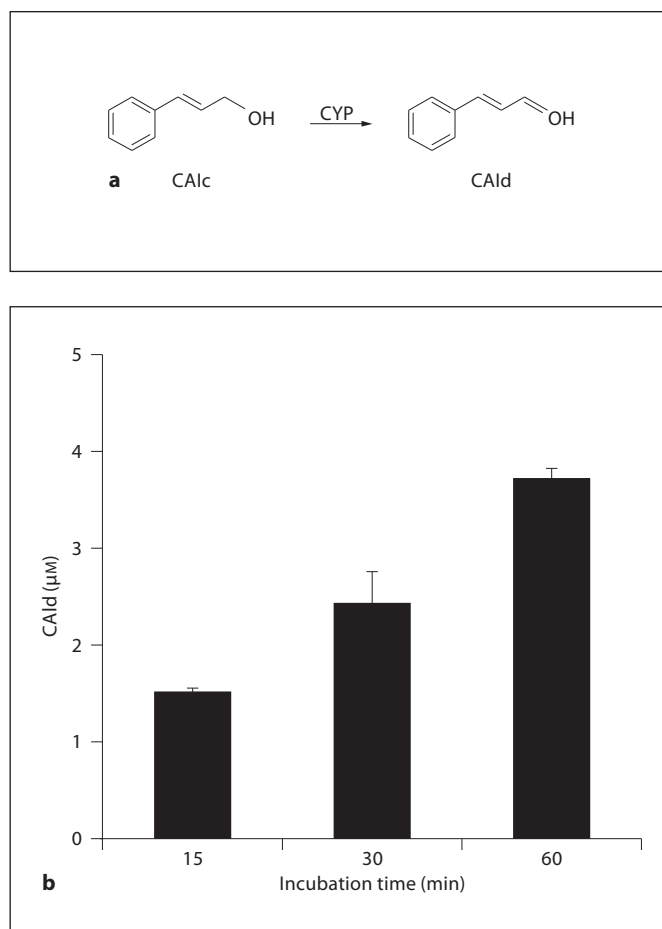
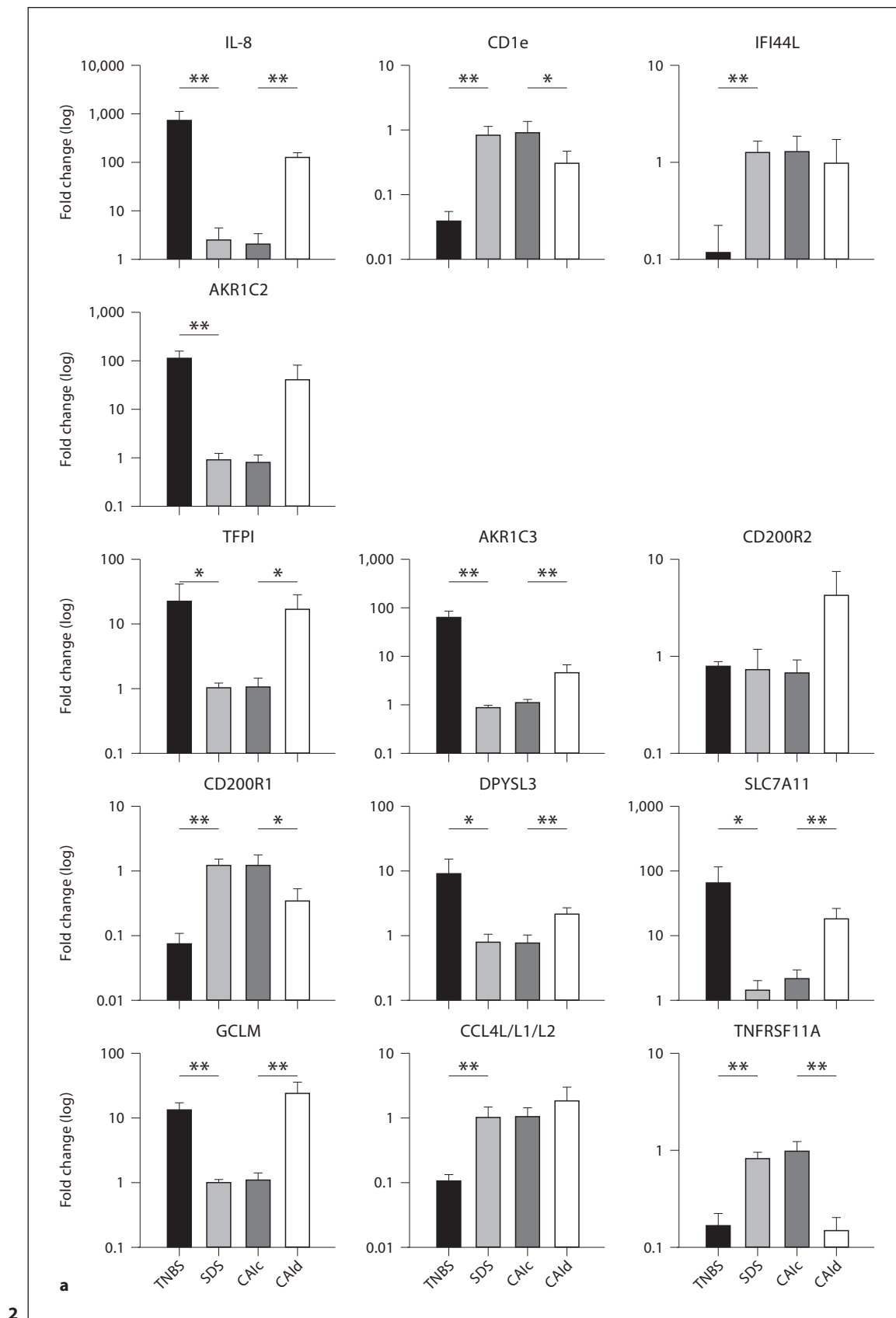


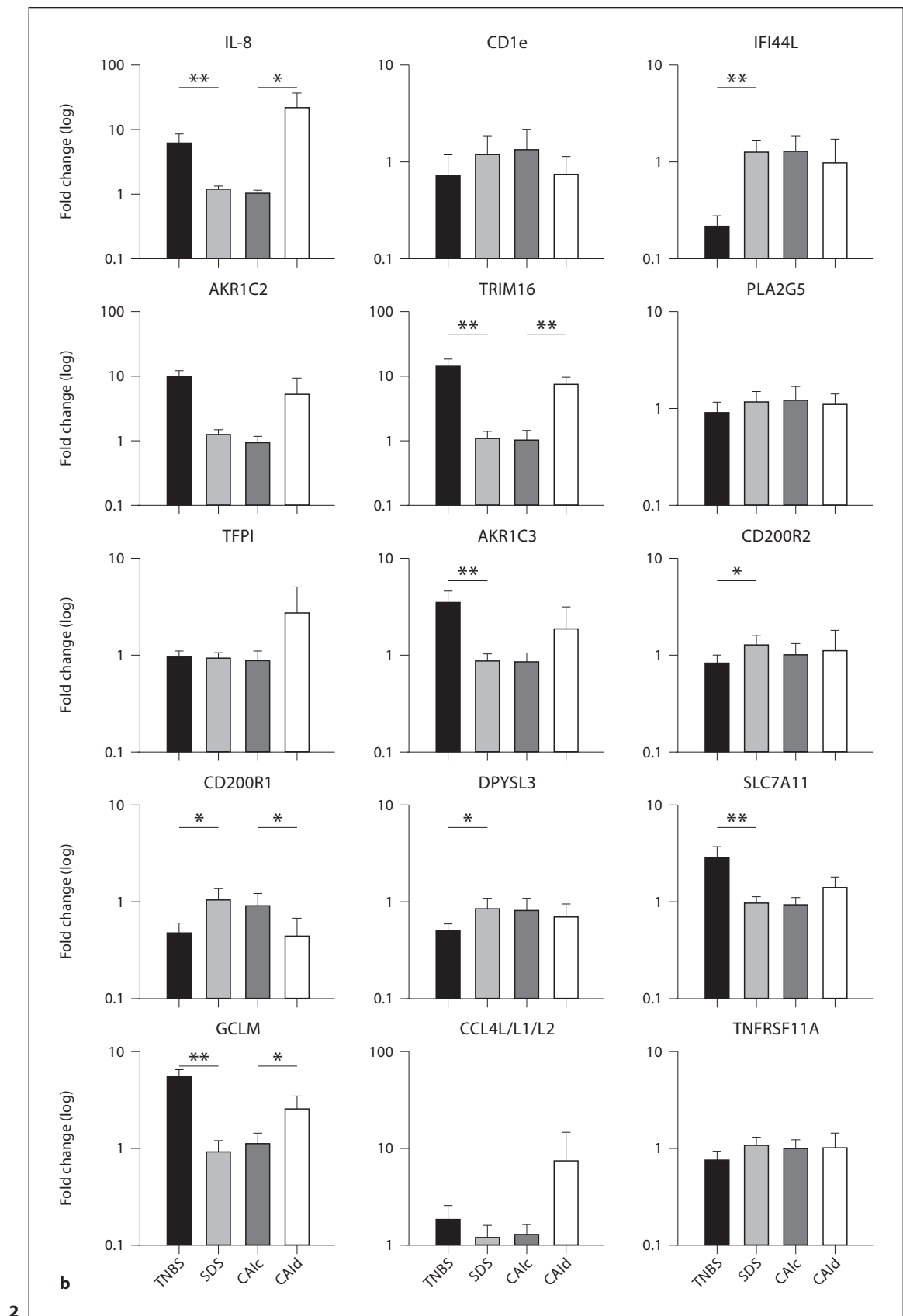
Fig. 1. Chemical structures (a) and detected amounts (b) of CALd in incubations of CALc and the skin-like CYP cocktail. The incubations contained the skin-like cocktail, CALc (200 μM), HEPES buffer (50 mM, pH 7.4), MgCl₂ (30 mM), and NADPH (1 mM) in a total volume of 500 μl. The incubations were terminated after 15, 30, or 60 min and analyzed using LC-MS. The detection limit for CALd was 0.25 μM.

(For figure see next pages.)

Fig. 2. TaqMan real-time PCR analysis of mRNA expression in DCs (a) and THP-1 cells (b) after treatment with TNBS, SDS, CALc or CALd for 30 h. The data shown are mean values ± standard deviation of 5 experiments. The relative RNA levels of each candidate gene are displayed on a log scale and presented as x-fold regulation compared to mRNA expression of the analyzed gene regulation in untreated cells (negative control). Student's t test was used to test the null hypothesis that the respective means of gene expression changes in SDS versus TNBS and CALc versus CALd samples were equal. * p < 0.05; ** p < 0.005.



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stimulated APCs. Besides being involved in keratinocyte differentiation [40] and retinoid metabolism [41], estrogen-responsive B box protein has recently been shown to enhance the secretion of IL-1 β , a cytokine specifically synthesized by APCs upon allergen stimulation [42]. Additionally, the gene encoding for group V phospholipase A₂ (PLA2G5), an enzyme responsible for the endogenous synthesis of arachidonic acid derivatives, was significantly regulated. This suggests a relevant role of these lipid mediators in the pathobiology of contact allergy which supports a recent study demonstrating that topical treatment with a phospholipase A₂ inhibitor markedly improved disease severity in patients with contact dermatitis [43].

Another major result of the present study is transcriptional evidence that oxidative and metabolic stress-associated pathways are involved in contact allergy pathogenesis. In particular, genes related to the homeostasis of glutathione (SLC7A11, GCLM), a tripeptide involved in detoxification of reactive metabolites, were specifically induced by skin sensitizers, but neither the irritant SDS nor the prohaptan CAIc. This is in accordance with recent microarray data revealing that another member of the highly homologous SLC7A family (SLC7A5) was differentially regulated in CD34⁺ progenitor-derived DCs after exposure to sensitizers of various potency (dinitrochlorobenzene, NiSO₄, eugenol, oxazolone). In contrast, this could not be shown after incubation with 2 irritants (SDS, benzalkonium chloride) [34]. Likewise, transcription of AKR1C3, which encodes for a member of the aldo-keto reductase family, was induced in allergen-stimulated moDCs in the present study. Of note, aldo-keto reductases perform oxidoreduction on various substrates and play a central role in the metabolism of endogenous hormones, but also xenobiotics and carcinogens [44]. Accordingly, Ryan et al. [37] found that AKR1C3 and other member genes of the aldo-keto reductases family (AKR1C1/2) were upregulated in peripheral blood monocytes-derived moDCs after incubation with strong (propyl gallate) and extremely strong (dinitrobenzene sulfonic acid) sensitizers, but not with the irritants SDS and benzoic acid [35, 37].

Integrating these data into a pathogenetic model, our findings suggest that, besides immunological reactions, further danger signals consisting of oxidative or metabolic stress responses are required during the sensitization phase of ACD. Intriguingly, we have demonstrated that both signals are only elicited by fully reactive haptens whereas neither the implemented prohaptan CAIc nor the irritant significantly altered marker gene expression. Therefore, future in vitro approaches will have to take

into account that certain small-molecular-weight pro-haptens require cutaneous bioactivation in order to become skin sensitizers [10, 12]. Otherwise, clinically relevant, prohaptenic contact allergens will not be detected by DC-based in vitro assays. As a possible solution, further investigations should envision the implementation of xenobiotic metabolizing enzymes such as recombinant CYP450s in APC-based sensitization assays.

In this regard, larger-scale studies could be facilitated by using human myeloid cell lines that are more readily available and easier to cultivate than precursor-derived DCs. Thus, we used RT-PCR to investigate whether skin sensitizers changed marker gene expression in the human acute monocytic leukemia cell line THP-1. THP-1 cells have previously been shown to be specifically activated by contact sensitizers as determined by enhanced CD86/CD54 surface expression or augmented IL-8 secretion [45, 46]. For the first time, we were able to demonstrate on the transcriptional level that a panel of only 4 genes differentially regulated by chemical-stimulated THP-1 cells (IL-8, TRIM16, CD200R1, GCLM) reliably identified skin sensitizers and therefore represent a promising screening tool in future investigations comprising a broader panel of small-molecular-weight test compounds.

In conclusion, the current study revealed important pathogenetic mechanisms, particularly immunological and oxidative stress-associated responses, implicated in the sensitization phase of ACD. Furthermore, we were able to identify a panel of potential marker genes to be used in animal-free sensitization assays relying on human APCs such as moDCs or THP-1 cells. Finally, we have demonstrated that a contact-allergenic prohaptan escapes APC-based in vitro diagnosis if its skin metabolism is not taken into consideration. Therefore, additional studies are warranted elucidating the diagnostic accuracy of the identified marker genes and, at the same time, integrating APC sensitization assays and cutaneous xenobiotic metabolizing enzymes.

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