Research Article

Keratinocytes Improve Prediction of Sensitization Potential and Potency of Chemicals with THP-1 Cells

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Summary

In vitro approaches to address key steps of chemical-induced skin sensitization have been developed, but there is uncertainty how keratinocytes, which play a crucial role not only regarding xenobiotic metabolism but also skin inflammation, impact on a chemical's potential and potency to activate dendritic cells. We investigated these aspects by coculturing THP-1 cells, as surrogate dendritic cells, with HaCaT keratinocytes. We tested our HaCaT/THP-1 model with a set of 14 sensitizers, containing 7 prohaptens, and 10 non-sensitizers. Compared to exposing THP-1 alone, coculturing resulted in up to 3.1-fold enhanced maximal CD86 and/or CD54 upregulation on THP-1, and improved concentration-dependency. All 14 sensitizers were found positive for CD86 and/or CD54 upregulation based on Δ mean fluorescence intensity (MFI) ≥ 10 for CD86 and ΔMFI ≥ 50 for CD54. Only 1 of 10 non-sensitizers was false-positive. Remarkably, coculture with HaCaT keratinocytes improved the rank correlation of the estimated minimum chemical concentrations inducing a positive response *in vitro* with *in vivo* data on sensitization potency, especially for CD54 (Spearman: $r = 0.739$, $p = 0.006$; CD86: $r = 0.571$, $p = 0.041$). These promising data suggest that the coculture model has the potential to support the prediction of sensitization potency based on *in vitro* data.

Keywords: inflammatory microenvironment, skin sensitization potency, cross talk, THP-1, HaCaT keratinocytes

1 Introduction

The hallmarks of chemical sensitizers are their ability to form antigenic haptenated proteins, to induce inflammatory responses in keratinocytes, and to induce maturation of dendritic cells (DC) needed for efficient activation of naïve T cells (OECD, 2012).

DC activation by sensitizers causes the upregulation of co-stimulatory molecules such as CD86 (Linsley et al., 1991), CD54 (Grakoui et al., 1999; Comrie et al., 2015), and other surface molecules (reviewed by Hubo et al., 2013) that are involved in antigen presentation. After interaction with their counterparts on T cells, these surface molecules generate a costimulatory signal (signal 2) that synergizes with the T cell receptor-mediated signal (signal 1) to promote an adaptive immune response.

Keratinocytes exposed to sensitizers release proinflammatory or immunomodulatory cytokines (Gober and Gaspari, 2008; Pasparakis et al., 2014). These factors can trigger DC activation and/or DC mobilization (reviewed by Kaplan et al., 2012). This role of adjacent keratinocytes may therefore have a significant impact on the strength of the chemical-induced DC response. Accordingly, we postulated that keratinocytes impact on the quantitative response of DC to skin sensitizing chemicals.

Quantitative data on sensitization potency of chemicals has long been derived from *in vivo* data obtained with the local lymph node assay (LLNA) in mice. The minimum concentration of a chemical that can induce a sensitization response, i.e. the chemical's sensitization potency, is an essential value in quantitative risk assessment (Mackay et al., 2013). *In vitro* methods intended to reduce or replace animal testing in this area need to be able to predict hazard and also to categorize the potency of sensitizers.

We and others have shown sensitizer-induced CD86 and CD54 upregulation on monocytic THP-1 cells as a model for DC activation (Bocchietto et al., 2007; Goebel et al., 2014; Krutz et al., 2015; Tietze and Blömeke, 2008; Yoshida et al., 2003). However, the impact of the presence of keratinocytes on the activation of THP-1 cells has not yet been fully investigated.

In this study, we used our HaCaT/THP-1 coculture setup (Hennen et al., 2011) to study the impact of keratinocytes on the expression of CD86 and CD54 on THP-1 after treatment with a set of 14 sensitizers and 10 non-sensitizers: We evaluated (1) the magnitude of CD86 and CD54 upregulation in the coculture model in comparison to THP-1 monoculture, (2) the sensitivity, specificity and accuracy of the HaCaT/THP-1 coculture model to

Received June 17, 2016;
Accepted October 18, 2016
Epub October 21, 2016;
https://doi.org/10.14573/altex.1606171

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identify sensitizers and non-sensitizers, and (3) the suitability of calculating the minimum chemical concentrations to induce a positive response to predict sensitization potency subcategories according to the Globally Harmonised System (GHS). Furthermore, we performed a more detailed comparison with continuous data on *in vivo* sensitization potency, and (4) determined the diverse modifications on the chemicals' potency for CD86 and CD54 upregulation mediated by cocultured HaCaT keratinocytes.

2 Materials and methods

2.1 Chemicals

4-Ethoxymethylene-2-phenyl-2-oxazolin-5-one (oxazolone), 2,4-dinitrochlorobenzene, acetaminophen, 3-aminophenol, cinnamic aldehyde, isoeugenol, citral, tetramethylthiuram disulfide, 2-methoxy-4-methylphenol, resorcinol, eugenol, geraniol, cinnamic alcohol, benzalkonium chloride, vanillin, lactic acid, *N,N*-diethyl-3-methylbenzamide and 4-aminoacetanilide were obtained from Sigma Aldrich (Taufkirchen, Germany), whereas N,N'-bis(4-aminophenyl)-2,5-diamino-1,4-quinone-diimine (Bandrowski's base) was purchased from ICN Biomedicals (Aurora, OH, USA). Sodium dodecyl sulfate and dimethyl sulfoxide (DMSO) were purchased from Carl Roth (Karlsruhe, Germany). 4-Acetamidoacetanilide was synthesized as described (Kawakubo et al., 2000). 4-Amino-2-methyl-acetanilide, 4-amino-3-methyl-acetanilide and 2,5-diacetaminotoluene were kind gifts from C. Goebel (P&G, Darmstadt, Germany).

2.2 *In vitro* cell culture

HaCaT keratinocytes, established from the skin from the distant periphery of a melanoma of a 62-year-old male patient (Boukamp et al., 1988), were provided by Prof. Dr. N. E. Fusenig (DKFZ, Heidelberg, Germany) and cultivated in DMEM supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine and 1% antibiotic/antimycotic solution at 37°C, 5% CO_2 in a humidified atmosphere. DMEM, RPMI, L-glutamine and antibiotic/antimycotic solution (10000 U/ml penicillin, 10 mg/ml streptomycin, 25 µg/ml amphotericin B) were obtained from PAA (Cölbe, Germany) or Sigma Aldrich (Taufkirchen, Germany), FBS was purchased from PAA (Cölbe, Germany) or Biochrom (Berlin, Germany). HaCaT cells were grown up to 70% confluence and routinely split (ratio of 1:10 to 1:15) twice per week. Passage numbers 4-16 were used for experiments. THP-1 cells, established from the peripheral blood of a 1-year-old boy with acute monocytic leukemia (Tsuchiya et al., 1980), were obtained from the German Collection of Microorganisms and Cell Cultures GmbH (DSMZ, Braunschweig, Germany) and cultured in RPMI 1640 with 10% FBS, 4 mM L-glutamine, 25 mM 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), 50 µM β-mercaptoethanol and 1% antibiotic-antimycotic solution at 37°C, 5% CO_2 in a humidified atmosphere. Cells were routinely split (ratio of 1:4 to 1:8) twice per week. Passage numbers 4-20 were used for experiments. During this study, both HaCaT and THP-1 cells were tested negative for mycoplasma contamination (Mycoplasma PCR ELISA, Roche Diagnostics, Mannheim, Germany).

2.3 Coculture and chemical treatment

The direct HaCaT/THP-1 coculture was set up as described earlier (Hennen et al., 2011). In brief, HaCaT cells were seeded in 6-well plates (7.5 x 10^4/cm^2) and cultivated for 48 h under standard cell culture conditions at a temperature of 37° and 5% CO_2. THP-1 cells (1.5 x 10^6/3 ml) as well as test chemicals (in 0.2% DMSO or medium as solvent) were added for 24 h. Similar experimental conditions were used for THP-1 monoculture conducted in parallel. Each test chemical was employed in at least three dilutions. A viability decrease of up to 50% with no visible disruption of the confluent HaCaT layer in the presence of the test chemicals was accepted, while samples with a cell viability < 50% were excluded from data analysis.

2.4 Flow cytometry analysis

After exposure to test chemicals, floating THP-1 cells were collected without disruption of the adherent HaCaT monolayer. Analysis of cell surface molecule expression was conducted as described before (Hennen et al., 2011). Briefly, THP-1 cells were stained with fluorescein isothiocyanate (FITC)- or allophycocyanin (APC)-labeled anti-CD86 (clone 2331 [FUN-1]), APC-labeled anti-CD54 (clone HA58) monoclonal antibodies (all mouse IgG1, obtained from BD Pharmingen, Heidelberg, Germany) or corresponding isotype controls. Cells were washed and their cell surface expression of CD86 and CD54 was measured using a FACSCalibur, followed by analysis with CellQuest Pro (BD Biosciences, Heidelberg, Germany). Propidium iodide (PI, 10 µg/ml) was used for determination of cell viability.

Exposure to some chemicals enhanced autofluorescence in the FITC channel to a variable degree in THP-1 cells treated alone or in coculture with HaCaT cells. For this reason, an APC-labeled anti-CD86 antibody was used for THP-1 analysis after exposure to citral, cinnamic aldehyde, 3-aminophenol and acetaminophen. For comparison of results obtained with FITC- and APC-labeled anti-CD86 antibodies, we treated THP-1 cells with various concentrations of 2,4-dinitrochlorobenzene, oxazolone and geraniol for 24 h in the absence and presence of HaCaT cells, then harvested THP-1 cells, and split the samples, separately staining the cells with FITC or APC-labeled anti-CD86, respectively. Then, we correlated ΔMFI values obtained with APC-labeled anti-CD86 to FITC-labeled anti-CD86. Linear regression analysis revealed a conversion factor of 0.28 (n = 42, r = 0.92, r^2 = 0.84). Thus, a ΔMFI of 2.8 obtained using FITC-labeled anti-CD86 was equivalent to a ΔMFI of 10 obtained with APC-labeled anti-CD86. Representative results demonstrating comparability between both anti-CD86 antibody variants are shown in Figure S1 (https://doi.org/10.14573/altex.1606171s).

2.5 Data analysis

PI-positive cells were excluded from analysis of cell surface expression. For data analysis, mean fluorescence intensities (MFI, geometric mean) were first corrected by subtracting MFI of similarly treated isotype-labeled cells (rMFI). Then, the chemical-induced difference in cell surface levels of CD86 or CD54 (ΔMFI) was estimated by subtracting the rMFI of solvent-treated cells from the rMFI of chemical-treated cells.

Tab. 1: Classification of chemicals (sensitizers and non-sensitizers) according to their potential to upregulate CD86 or CD54 on THP-1 cells cocultured with keratinocytes above the cut-offs of ΔMFI of 10 for CD86 or ΔMFI of 50 for CD54

Chemical	LLNA classification	Hapten / prehapten / prohapten	ΔMFI of CD86 ≥ 10	ΔMFI of CD54 ≥ 50
Sensitizers				
oxazolone	extreme	hapten	+	+
Bandrowski's base	extreme	hapten	+	+
2,4-dinitrochlorobenzene	extreme	hapten	+	+
acetaminophen	extreme	prohapten	+	+
3-aminophenol	strong	prohapten	-	+
cinnamic aldehyde	moderate	hapten	+	+
isoeugenol	moderate	prehapten	+	+
citral	moderate	hapten	+	+
tetramethylthiuram disulfide	moderate	hapten	+	+
2-methoxy-4-methylphenol	moderate	prohapten	+	-
resorcinol	moderate	prohapten	+	+
eugenol	weak	prohapten	+	n.a.
geraniol	weak	prohapten	+	+
cinnamic alcohol	weak	prohapten	+	+
Non-sensitizers				
benzalkonium chloride	non-sensitizer[a]		-	+
vanillin	non-sensitizer[b]		-	-
lactic acid	non-sensitizer		-	-
sodium dodecyl sulfate	non-sensitizer[a]		-	-
N,N-diethyl-3-methylbenzamide	non-sensitizer[b]		-	-
4-aminoacetanilide	non-sensitizer		-	-
4-acetamidoacetanilide	non-sensitizer		-	-
4-amino-2-methyl-acetanilide[c]	NA		-	-
4-amino-3-methyl-acetanilide[c]	NA		-	-
2,5-diacetaminotoluene[c]	NA		-	-

[a] false-positive in the LLNA
[b] negative in the LLNA but false-positive in the KeratinoSens + S9 (Natsch and Haupt, 2013)
[c] categorized as non-sensitizer based on available *in vitro* data (Goebel et al., 2014)
n.a., not analyzed; NA, not available

Calculation of the concentration of a chemical needed to reach positivity

The minimum chemical concentration needed to reach a ΔMFI of 2.8 for CD86 (and a ΔMFI of 10 using APC-labeled anti-CD86) or a ΔMFI of 50 for CD54 was calculated via linear interpolation, similar to the LLNA EC3 (Basketter et al., 1999). These estimated concentrations were referred to as ECΔ10 (CD86) and ECΔ50 (CD54).

Statistical analysis

Differences between two groups were determined using Student's *t*-test. A p value < 0.05 was considered to be a statistically significant difference. Spearman's rank correlation coefficients (two-tailed) were estimated for comparison with *in vivo* data using GraphPad Prism (San Diego, CA, USA).

3 Results

3.1 Selection of test chemicals

Chemicals were selected to cover a broad range of sensitizing potencies (Tab. 1). Furthermore, we chose (1) well-established

skin sensitizers, recommended by ECVAM/Cosmetics Europe (Casati et al., 2009), the Sens-it-iv consortium (Rovida et al., 2013) and/or ICCVAM (ICCVAM, 2009), and in addition Bandrowski's base as a known immunogenic auto-oxidation product of para-phenylenediamine (Aeby et al., 2009; Gibson et al., 2015; Krasteva et al., 1996); (2) the prohaptens eugenol, cinnamic alcohol, geraniol and resorcinol, which are commonly recommended for *in vitro* assays (Rovida et al., 2013; Casati et al., 2009), as for those and also 2-methoxy-4-methylphenol, peptide reactivity was clearly enhanced in the presence of peroxidase (Gerberick et al., 2009; Merckel et al., 2013), and for eugenol a clearly enhanced potential for inducing keratinocyte responses in the additional presence of S9 mix as another metabolic system (modified KeratinoSens) has been demonstrated by Natsch and Haupt (2013). Furthermore, we selected (3) chemicals which are commonly accepted as non-sensitizers based on negative LLNA and/or human evidence (Basketter et al., 2014; Aeby et al., 2009; Gerberick et al., 2005). To challenge our coculture model, we additionally included (4) prohaptens (3-aminophenol, resorcinol and acetaminophen) that remained negative with regard to the induction of keratinocyte responses even after exposure in the presence of additional S9 mix (modified KeratinoSens (Natsch and Haupt, 2013)). Here, we included the rare drug allergen acetaminophen as it induces a strong response in the LLNA, which was clearly attenuated in metabolically deficient cytochrome P450 reductase knockout mice (Chipinda et al., 2011). Furthermore, it is a well-established substrate for cytochrome P450 enzymes, forming metabolites with increased reactivity (Patten et al., 1993) and, independently but noteworthy, Goncalo and coworkers found responses comparable to those caused by chemical contact sensitizers after exposure of THP-1 cells to drugs with known potential to induce non-immediate cutaneous adverse drug reactions (Gonçalo et al., 2015). And we further included (5) important skin irritants and molecules that yielded controversial results in other related assays (Basketter and Kimber, 2011; Loveless et al., 1996; Natsch and Haupt, 2013; Willis et al., 1988).

3.2 Enhanced CD86 and CD54 levels on THP-1 cells after treatment with skin sensitizers in coculture

To study the effect of HaCaT keratinocytes on the response of THP-1 cells to sensitizers, we exposed THP-1 cells for 24 h in the absence and presence of HaCaT cells to a set of 14 sensitizers (6 haptens, 1 pre- and 7 prohaptens). It should be pointed out that raw data for Bandrowski's base, 2,4-dinitrochlorobenzene, isoeugenol, 2-methoxy-4-methylphenol, eugenol, and also benzalkonium chloride and sodium dodecyl sulfate were already reported earlier (Hennen et al., 2011) but were included here for refined data analysis.

Representative results are shown in Figure 1 for the sensitizers cinnamic aldehyde, 2,4-dinitrochlorobenzene and 3-aminophenol, and results for 11 additional chemicals are depicted in Figure S2 (https://doi.org/10.14573/altex.1606171s). Due to the impact of HaCaT keratinocytes on the basal levels of CD86 and CD54 on THP-1 cells (Hennen et al., 2011), expression of results as fold of control was not feasible for the comparison of the THP-1 response after exposure alone or in coculture with HaCaT cells. Instead, the net upregulation was calculated (ΔMFI) and used for further analysis.

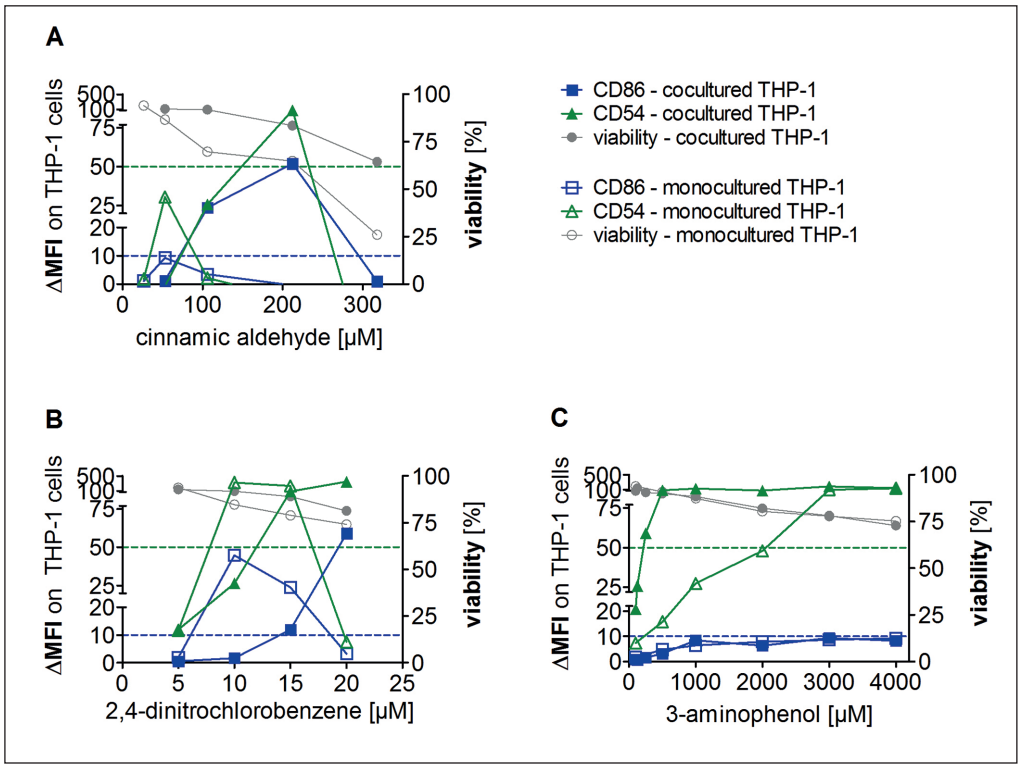

Fig. 1: Impact of HaCaT cells on concentration-dependent upregulation of CD86 and CD54 on THP-1 cells treated with sensitizers

THP-1 cells were treated with cinnamic aldehyde (A), 2,4-dinitrochlorobenzene (B), and 3-aminophenol (C) for 24 h in coculture with HaCaT cells (filled symbols) or alone (open symbols). Expression of CD86 (blue squares) and CD54 (green triangles) on the THP-1 cell surface as well as cell viability (grey dots) were analyzed by flow cytometry. Lines represent thresholds for positivity (blue: ΔMFI of 10 or 2.8 for CD86 and green: ΔMFI of 50 for CD54). Means of at least 3 independent experiments are shown.

Overall, maximal CD86 and/or CD54 upregulation was increased in 10 of 14 sensitizers in coculture compared to the response of THP-1 cells in monoculture (Fig. 2) when considering a modulation of at least 20% as an arbitrary limit for a distinct enhancement or decrease. The increase in upregulation in the presence of keratinocytes over THP-1 cells alone varied between 122 and 310% for CD54, and 132 and 255% for CD86.

Maximal upregulation of CD86 and CD54 was in some cases obtained for different concentrations of sensitizers in the two settings, however the maximal responses were generally observed at comparable viability of THP-1 cells (Fig. 1 and Fig. S2, https://doi.org/10.14573/altex.1606171s). The observed modulation of the maximal CD86 and/or CD54 response was not affected by variable cytotoxicity. However, due to the shift in cytotoxicity, an increased range of concentrations could be tested in coculture, leading to a clearly improved concentration-dependent response.

3.3 Predictive performance of the HaCaT/THP-1 coculture

To examine the predictive performance of the test, the model was also challenged with 10 non-sensitizers, among them well-known skin irritants and chemicals that are known to yield false-positive results *in vivo* and/or *in vitro*. The best overall performance for discrimination of the coculture model between sensitizers and non-sensitizers was achieved when for CD86, a ΔMFI of 10 above the control level, and for CD54, a ΔMFI of 50 were chosen as cut-offs for a positive result.

By means of these criteria, 13/14 and 12/13 sensitizers were positive for CD86 and CD54 upregulation, respectively, as well as 0/10 and 1/10 non-sensitizers (Tab. 1). Among the sensitizers, 3-aminophenol failed to reach the threshold value for CD86 (max. ΔMFI was 8.2 ± 1.6) but was positive with respect to CD54. 2-Methoxy-4-methylphenol did not reach the threshold for CD54 (max. ΔMFI was 15.2 ± 4.5) but reached the cut-off for CD86. None of the non-sensitizers yielded a positive result regarding CD86 upregulation, and only one (benzalkonium chloride) reached a positive result for CD54 upregulation (Tab. 1). Thus, the THP-1/HaCaT coculture can distinguish between sensitizers and non-sensitizers with high specificity, yielding an overall accuracy of 96% if either CD86 or CD54 must exceed the threshold to yield a positive result.

3.4 Prediction of sensitization potency

In order to determine whether the THP-1/HaCaT coculture model could improve *in vitro* potency prediction over the monoculture model, we calculated the concentration of each chemical at the cut-off, i.e. the minimum concentration inducing a positive response (i.e., ECΔ10 for CD86 and ECΔ50 for CD54).

First, we addressed the question whether the calculated ECΔ10 and ECΔ50 values could be used for prediction of GHS potency categories (detailed information on *in vivo* skin sensitization potency of the tested compounds is given in Table S1 (https://doi.org/10.14573/altex.1606171s); the drug allergen acetaminophen was excluded from this analysis as it is not classified as a skin sensitizer). A correct classification of 11/12 (CD86) or 11/11 (CD54) sensitizing chemicals in subcategories 1A and 1B was achieved when applying a threshold of 70 µM regarding ECΔ10 (CD86), or 30 µM for ECΔ50 (CD54), leading to an overall accuracy of 95.5% (CD86) or 95.2% (CD54)

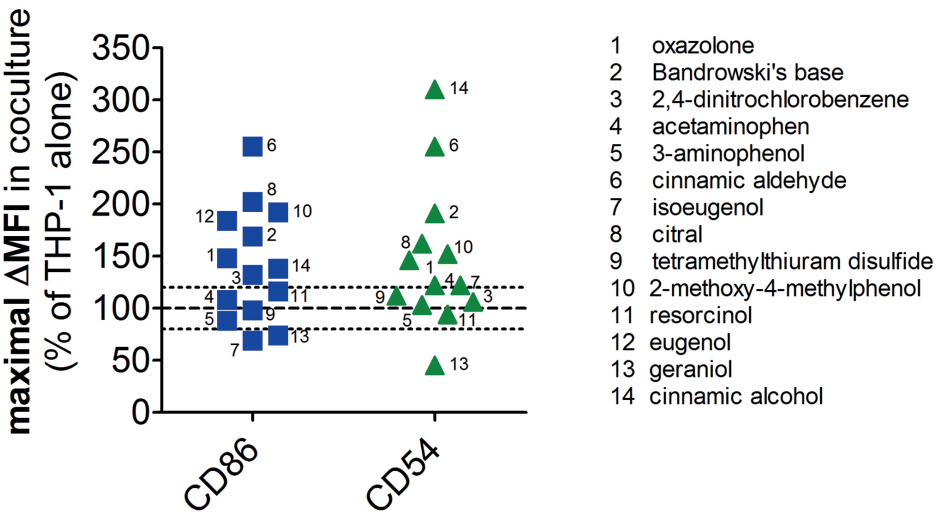

Fig. 2: Modulation of maximal upregulation of CD86 and CD54 on THP-1 cells by coculture with HaCaT keratinocytes
Mean maximal ΔMFI for CD86 and CD54 obtained in HaCaT/THP-1 coculture after treatment with 14 sensitizers, calculated as percent of mean maximal ΔMFI values for CD86 and CD54, respectively, obtained in THP-1 monoculture. Lines represent 100%, i.e., equivalence to results obtained with THP-1 alone, as well as 20% above and below this value.

Tab. 2: Classification of sensitization potency of chemicals according to GHS based on CD86 (ECΔ10) and CD54 (ECΔ50) upregulation in THP-1 cells in the presence of keratinocytes

		CD86				CD54			
		ΔMFI ≥ 10		ΔMFI < 10	total	ΔMFI ≥ 50		ΔMFI < 50	total
		ECΔ10[a] < 70	ECΔ10[a] ≥ 70			ECΔ50[a] < 30	ECΔ50[a] ≥ 30		
Sensitizer	**GHS 1A**	3	0	0	3	3	0	0	3
	GHS 1B	1	8	0	9	0	8	0	8
Non-sensitizer		0	0	10	10	1	0	9	10
Total		4	8	10	22	4	8	9	21

[a] in µM

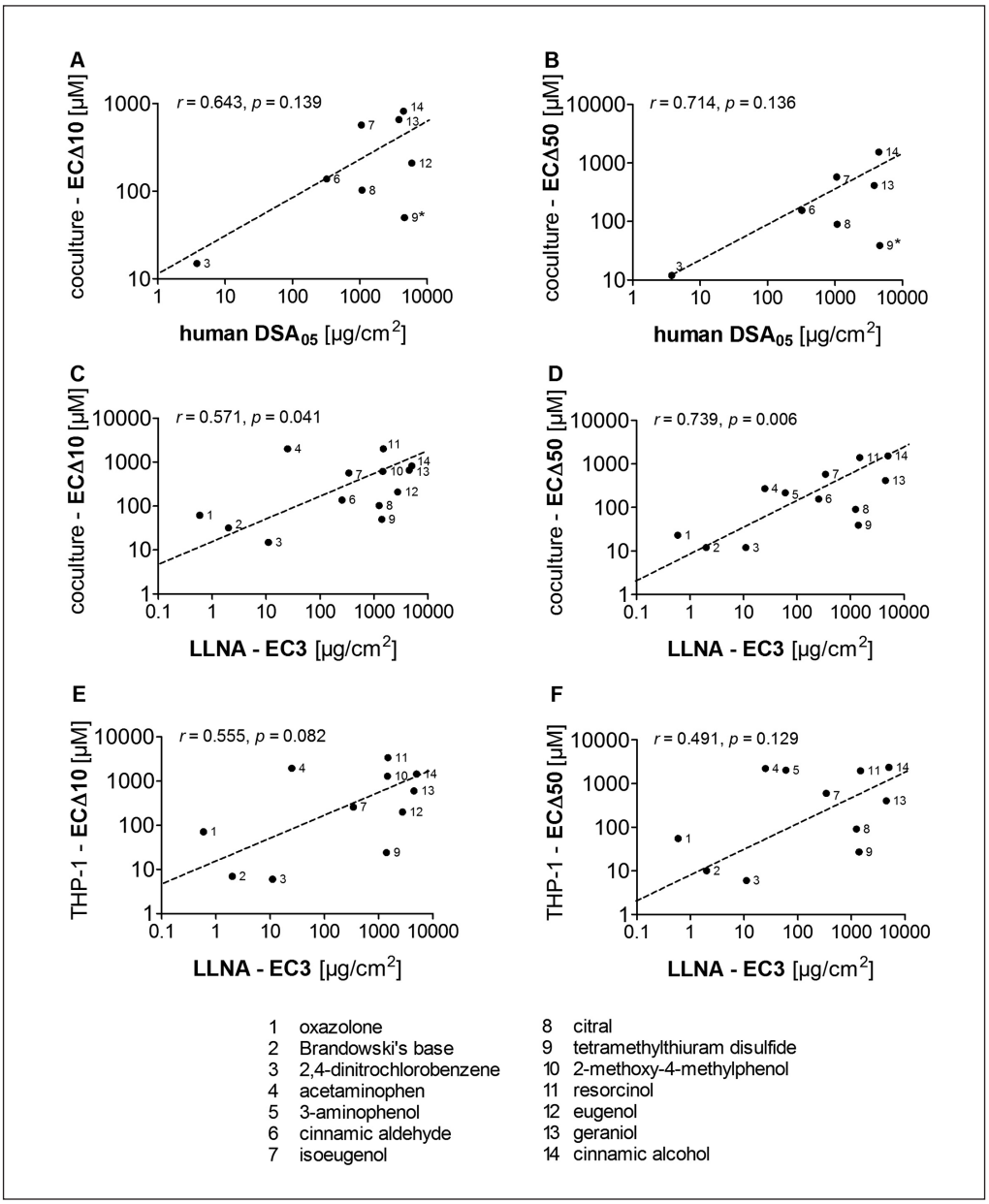

Fig. 3: Correlation analysis of ECΔ10 (CD86) and ECΔ50 (CD54) values obtained in the HaCaT/THP-1 coculture, or in THP-1 monoculture with human DSA_{05} and murine LLNA EC3 values
Correlation of ECΔ10 (CD86) and ECΔ50 (CD54) obtained in HaCaT/THP-1 coculture after treatment with 14 sensitizers with DSA_{05} (A, B) and with EC3 values (C, D). Correlation of ECΔ10 (CD86) and ECΔ50 (CD54) obtained in THP-1 monoculture with EC3 values (E, F). Tetramethylthiuram disulfide was excluded from the line of visual equivalence in panel A and B as indicated by *.

(Tab. 2). Only the moderate sensitizer tetramethylthiuram disulfide would be misclassified into subcategory 1A using CD86.

Next, we questioned how the potencies for CD86 and CD54 upregulation relate to the chemicals' *in vivo* sensitizing potency. We compared ECΔ10 and ECΔ50 values, obtained from coculture of THP-1 cells with keratinocytes, with existing human DSA_{05} values (i.e., the induction dose per skin area (µg/cm^2) that produces a positive response in 5% of the tested population) from ICCVAM (2011). As human DSA_{05} values were not available for each of our compounds, data for only 6 (CD54) or 7 (CD86) chemicals could be compared. We observed that the chemicals with lower DSA_{05} values were often also more potent in CD86 and CD54 upregulation. Excluding the most deviating chemical, i.e., tetramethylthiuram disulfide, from our comparison led to Spearman correlation coefficients r of 0.643 for CD86, and 0.714 for CD54 (Fig. 3A,B).

We further compared ECΔ10 and ECΔ50 values with existing LLNA EC3 values (see Table S1). A positive correlation between the chemicals' potency in the LLNA and the minimum concentration needed for a positive response in our coculture model was found when plotting ECΔ10 (CD86) or ECΔ50 (CD54) against EC3 values (Fig. 3C,D). For comparison, we also evaluated the correlation between values derived from THP-1 cells alone with EC3 values (Fig. 3E,F). With respect to CD86, the correlation between ECΔ10 and EC3 values was only significant in coculture (Spearman $r = 0.571$, $p = 0.041$ for coculture; $r = 0.555$, $p = 0.082$ for THP-1 cells alone), while for CD54, the correlation between ECΔ50 and the EC3 was clearly improved in coculture over monoculture (Spearman $r = 0.739$, $p = 0.006$ for cocultured THP-1 cells; $r = 0.491$, $p = 0.129$ for THP-1 cells alone).

3.5 Comparison of the chemicals' potency to upregulate CD86 and CD54 on THP-1 cells exposed in mono- or coculture

We compared the ECΔ10 (CD86) and ECΔ50 (CD54) values obtained in coculture versus monoculture (Fig. 4). The presence of keratinocytes increased the potency of 6/13 sensitizers (Fig. 4). For these, only between 11% and 72% of the concentrations needed for activation of THP-1 alone were sufficient to yield a positive response regarding either CD86 or CD54 in coculture. In contrast, 4/13 sensitizers (Bandrowski's base, 2,4-dinitrochlorobenzene, isoeugenol and tetramethylthiuram disulfide) only induced a positive reaction at higher concentrations, i.e., up to 457% for CD86 and up to 200% for CD54 (Fig. 4).

Separately focusing on the impact of HaCaT keratinocytes on the modulation of ECΔ10 and ECΔ50 values for haptens or prohaptens revealed that the chemicals for which potency was decreased were mainly haptens, while the majority of the chemicals for which potency was increased are prohaptens (acetaminophen, 3-aminophenol, 2-methoxy-4-methylphenol, resorcinol, cinnamic alcohol) (Fig. 4).

4 Discussion

In vitro tests addressing single key steps (protein reactivity, keratinocyte response, dendritic cell activation) of the adverse outcome pathway (AOP) for skin sensitization have been validated for hazard identification (Ezendam et al., 2016). However, beside hazard, the prediction of skin sensitization potency is crucial for adequate risk assessment. So far, results obtained

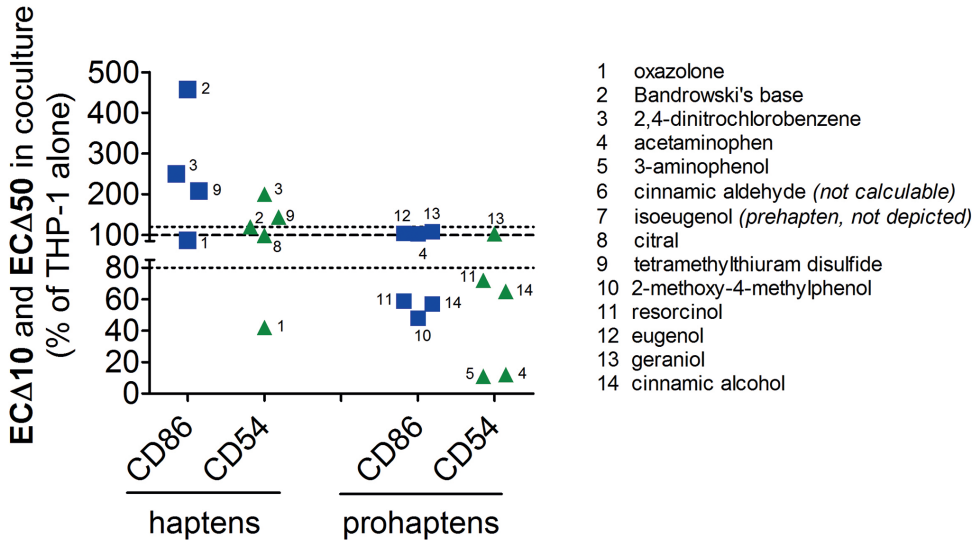

Fig. 4: Modulation of ECΔ10 (CD86) and ECΔ50 (CD54) values by coculture of THP-1 cells with HaCaT keratinocytes
ECΔ10 (CD86) and ECΔ50 (CD54) obtained in HaCaT/THP-1 coculture after treatment with 14 sensitizers, given as percent of ECΔ10 (CD86) and ECΔ50 (CD54), respectively, obtained in THP-1 monoculture. Lines represent 100%, i.e., equivalence to results obtained with THP-1 alone, as well as 20% above and below this value, considered as margin for a distinct modification.

using the available methods show limitations in the demonstration of concentration-dependency and have limited capacities to detect certain prohaptens and for potency prediction (Piroird et al., 2015; Natsch et al., 2015; Nukada et al., 2012; Teunis et al., 2014; Jaworska et al., 2015; Urbisch et al., 2016; Adler et al., 2011).

Keratinocytes may crucially modulate the strength of chemical-induced DC activation by providing xenobiotic metabolism and releasing DAMPs as well as pro-inflammatory cytokines. To explore this aspect, we established a DC-keratinocyte coculture model using THP-1 cells as surrogate DC and HaCaT keratinocytes (Hennen et al., 2011). In line with our working hypothesis, we observed a clearly increased dynamic range after exposure to sensitizers in our HaCaT/THP-1 coculture model compared to THP-1 alone. This was especially evident for CD86 (up to 2.55-fold). The observed increased dynamic range enabled a distinct discrimination between non-sensitizers and sensitizers.

We chose a ΔMFI of 10 as cut-off for a positive CD86 response. This corresponds to an approximately 3-fold upregulation compared to the control level. This threshold correlates with the level of the threshold for positivity used in the LLNA (3-fold), and is much more conservative than with other cell-based tests. For instance, the h-CLAT, based on THP-1 cells alone (Sakaguchi et al., 2009), and the U-Sens, based on U937 cells (Piroird et al., 2015), both use 1.5-fold. Despite this very conservative threshold, 13/14 sensitizers were positive for CD86 upregulation. Consequently, we obtained high sensitivities and specificities for our test chemicals, resulting in an overall accuracy of 96% if either CD86 or CD54 expression must exceed the cut-off to produce a positive result. Only the strong irritant benzalkonium chloride was found to be false-positive regarding CD54.

According to the GHS implemented in the European regulation on classification, labelling and packaging of substances and mixtures (CLP regulation, Regulation (EC) No 1272/2008; EU, 2008), chemical sensitizers are classified in category 1, and further classified in subcategories 1A or 1B depending on their sensitization potency. We tested whether the cut-off concentration in our coculture model could be a suitable parameter to categorize the chemicals into potency classes 1A and 1B. We found that setting thresholds at 70 µM and 30 µM resulted in a good concordance (95.5% for CD86 and 95.2% for CD54). However, a continuous scale might be more helpful as it would allow a more detailed classification, similar to the LLNA.

Indeed, we found a significant correlation between the potency of chemicals to induce CD86 (Spearman $r = 0.571$, $p = 0.041$) and especially CD54 (Spearman $r = 0.739$, $p = 0.006$) upregulation in our coculture with existing *in vivo* sensitization potency values. Comparing this correlation with results obtained with THP-1 alone showed a clear improvement of coculture for the same set of chemicals. Overall, these promising results suggest that the HaCaT/THP-1 coculture model may allow a more fine-scaled prediction of sensitization potency. Consequently, these results suggest that the HaCaT/THP-1 coculture model may be suitable to confirm inconclusive data obtained for pre-/prohaptens and to support combined approaches such as the recently published Bayesian network integrated testing strategy (ITS-3) for the assessment of skin sensitization potency (Jaworska et al., 2015).

Looking in more detail at the correlation of coculture data with *in vivo* potency data, we found that lower chemical concentrations were sufficient to induce a positive response for at least 46% of the tested sensitizers, among them mainly prohaptens, in the coculture model. Although our experiments were not designed to separate between effects of parent compounds and metabolites, the latter are often associated with enhanced protein reactivity and increased potential to generate reactive oxygen species (ROS), and induce cytokine production (Bergström et al., 2007) and further danger molecules (Khan et al., 2007). The latter are all known to support DC activation and enhance the strength of an allergic response (Grabbe et al., 1996). However, our observed correlation with *in vivo* data on sensitization potency did not solely derive from enhanced potency for CD86 and/or CD54 upregulation of certain prohaptens but is equally based on the decreased potency of 4 of 6 haptens in our coculture model. This observation lets us speculate that not only the keratinocytes' capacity for metabolic activation of prohaptens but also the conjugation of haptens plays a role in the modulated chemicals' potency for activation of THP-1 cells in coculture, as the addition of HaCaT to our coculture model also coincides with supplemental targets for reactive molecules and/or conjugation capacities. Interestingly, we also observed a decrease in the concentration-dependent cytotoxicity in coculture for 6 of 14 sensitizers. In line with this, higher concentrations were needed to exceed the thresholds for CD86 and/or CD54 for those chemicals. This indicates a deactivation of these molecules, leading to the assumption that chemical-related danger generation occurs after saturation of conjugation capacities.

In sum, these results suggest that coculture of THP-1 with HaCaT cells modulates the chemicals' DC activation potency, which may improve and support the prediction of the sensitization potency based on *in vitro* data.

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Conflict of interest

The authors declare that there are no conflicts of interest.

Acknowledgements

The authors thank Natalie Lobes and Tim Kinitz for excellent technical assistance. This study was partly supported by the Federal Office of Public Health, Switzerland (11.004203) and by the Program for Women Professors of the Federal Ministry of Education and Research (Germany).

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