

Discrimination of skin sensitizers from non-sensitizers by interleukin-1 α and interleukin-6 production on cultured human keratinocytes

Daun Jung^a, Jeong-Hwan Che^b, Kyung-Min Lim^c, Young-Jin Chun^d, Yong Heo^{e*} and Seung Hyeok Seok^{a*}

ABSTRACT: *In vitro* testing methods for classifying sensitizers could be valuable alternatives to *in vivo* sensitization testing using animal models, such as the murine local lymph node assay (LLNA) and the guinea pig maximization test (GMT), but there remains a need for *in vitro* methods that are more accurate and simpler to distinguish skin sensitizers from non-sensitizers. Thus, the aim of our study was to establish an *in vitro* assay as a screening tool for detecting skin sensitizers using the human keratinocyte cell line, HaCaT. HaCaT cells were exposed to 16 relevant skin sensitizers and 6 skin non-sensitizers. The highest dose used was the dose causing 75% cell viability (CV75) that we determined by an MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay. The levels of extracellular production of interleukin-1 α (IL-1 α) and IL-6 were measured. The sensitivity of IL-1 α was 63%, specificity was 83% and accuracy was 68%. In the case of IL-6, sensitivity: 69%, specificity: 83% and accuracy: 73%. Thus, this study suggests that measuring extracellular production of pro-inflammatory cytokines IL-1 α and IL-6 by human HaCaT cells may potentially classify skin sensitizers from non-sensitizers. Copyright © 2015 John Wiley & Sons, Ltd.

Additional supporting information may be found in the online version of this article at the publisher's website.

Keywords: cultured human keratinocyte; HaCaT; interleukin-1 α ; interleukin-6; skin sensitizer

Introduction

Most predictive tests for identifying skin sensitizers use animal models, such as the mouse local lymph node assay (LLNA; Kimber *et al.*, 1995) and the guinea pig maximization test (GPMT; Magnusson, 1980). However, *in vivo* assays using animal models are limited in their use, and can be ethically problematic. In addition, legislative changes in the EU, like the Registration Evaluation Authorization and Restriction of Chemicals (REACH) regulation, banned toxicity testing of cosmetic ingredients using animals (Taylor *et al.*, 2011). Thus, the development of novel alternative testing methods to identify the sensitizing potential of chemicals is needed to replace animal experiments.

Currently, human-based reconstructed skin models, such as EpiCS (CellSystemd, Germany), EpidermTM (MatTek, Ashland, MA, USA) and SkinEthicTM RHE (L'Oreal, France), are available for identifying skin irritants (Gibbs *et al.*, 1986; McKim *et al.*, 2012; Saito *et al.*, 2013). Although reconstructed skin models share a more structural resemblance with human skin than monolayer cell culture models (Mehling *et al.*, 2012), there are some economic problems and donor-to-donor variability using human reconstructed skin models. In addition, these models lack immune cells that are essential for recapitulating the sensitization process *in vivo* (Mehling *et al.*, 2012).

Of note, there has been considerable progress in developing alternative *in vitro* skin sensitization models (DPRA, KeratinsensTM, LuSens, MUSST, hCLAT, VitoSens and GARD) (Gerberick *et al.*, 2004; Hooyberghs *et al.*, 2008; Johansson *et al.*, 2011; Nukada *et al.*, 2011; Johansson *et al.*, 2013; Ramirez *et al.*,

2014). Although guidelines for the use of the KeratinoSens (OECD TG 442D) and the Direct Peptide Reactivity Assay (DPRA) (OECD TG 442C) for skin sensitization have been released (February 2015), there remains a need for *in vitro* methods that are more accurate and simpler. Thus, we sought to develop a cell-based assay that can fully replace animal tests currently in use with the utility of currently available non-animal test methods.

*Correspondence to: Seung Hyeok Seok, Associate Professor, Macrophages Laboratory, Department of Microbiology and Immunology, Seoul National University College of Medicine, Seoul 110-799, Republic of Korea.
E-mail: lamseok@snu.ac.kr

Yong Heo, Department of Occupational Health, College of Medical and Public Health Sciences, Catholic University of Daegu, 13-13 Hayang-ro, Gyeongsan-si, Republic of Korea 712-702.
E-mail: yheo@cu.ac.kr

^aDepartment of Microbiology and Immunology, and Institute of Endemic Disease, College of Medicine, Seoul National University, Seoul, 110-799, South Korea

^bBiomedical Research Institute, Seoul National University Hospital, Seoul, 110-744, Republic of Korea

^cCollege of Pharmacology, Ewha Womans University, Seoul 120-808, Republic of Korea

^dChung-Ang University, College of Pharmacy, Seoul 156-756, Republic of Korea

^eDepartment of Occupational Health, College of Natural Sciences, Catholic University of Daegu, Daegu, 712-702, South Korea

Keratinocytes are abundant in the skin and are the first cells that chemicals contact when applied. They are part of the immune system and produce cytokines that play important roles in inflammatory reactions of the skin (Haas *et al.*, 1992). Importantly, keratinocytes produce pro-inflammatory cytokines such as interleukin 1 α (IL-1 α), IL-6, IL-8 and IL-18 (Kupper *et al.*, 1986; Larsen *et al.*, 1989; Grone, 2002). Therefore, several keratinocyte cell lines have been used for skin sensitizer prediction with cytokine levels as a marker (NCTC-2544, HaCaT and HEL-30) (Corsini *et al.*, 2009; Van Och *et al.*, 2005; Son *et al.*, 2013). In the case of the NCTC-2544 assay, selective induction of intracellular IL-18 was used to discriminate sensitizers from contact allergens and irritants (Corsini *et al.*, 2009, 2013; Galbiati *et al.*, 2011). Here we focus on the human keratinocyte cell line, HaCaT, which is easy to culture, displays a transformed phenotype *in vitro* but remains non-tumorigenic, is easy to handle and has less variation than freshly isolated cells (van der Veen *et al.*, 2015). Despite their unlimited growth potential, these cells are similar to normal human keratinocytes (Petra Boukamp *et al.*, 1988).

In this study, we tested a total of 22 chemicals based on their skin sensitizing potential reported in the OECD 429. Because

there were 18 minimum reference substances and four optional reference substances that produced either false-positive or false-negative results in the LLNA, when compared with human results (OECD, 2010). Of the primary cytokines that keratinocytes produce, we focused on quantifying pro-inflammatory cytokine levels of IL-1 α , IL-6, IL-8 and IL-18 by sensitizers. IL-1 α and IL-6 are more appropriate cytokines for this study because high levels of IL-8 or IL-18 secretion were also produced by non-sensitizers. Thus, we aimed to develop an *in vitro* assay as a screening tool for detecting skin sensitizers by evaluating IL-1 α and IL-6 alone or in combination in the human keratinocyte cell line, HaCaT.

Materials and methods

Chemicals

Sixteen relevant skin sensitizers and six skin non-sensitizers were tested (Table 1). All chemicals are OECD TG429 recommended reference substances.

Table 1. Concentration of chemicals that induced 75% cell viability (CV₇₅) of HaCaT cells and their classification

	Chemical	CV ₇₅ ($\mu\text{g mL}^{-1}$)	CAS No ^a	Supplier	Vehicle	LLNA ^b	Human ^c
1	CMI/MI ^d	402.2 $\pm$ 227.6	26172-55-4/2682-20-4	DOW	DMSO	+	+
2	DNCB	4.1 $\pm$ 2.3	97-00-7	Aldrich	DMSO	+	+
3	4-Phenylenediamine	181.2 $\pm$ 25.8	106-50-3	Sigma	DMSO	+	+
4	Cobalt chloride	57.9 $\pm$ 16.8	7791-13-1	Sigma	PBS	+	+
5	Isoeugenol	302.9 $\pm$ 231.3	97-54-1	Aldrich	DMSO	+	+
6	2-Mercaptobenzothiazole	400.0 $\pm$ 16.9	149-30-4	Aldrich	DMSO	+	+
7	Citral	10.0 $\pm$ 16.10	5392-40-5	Aldrich	DMSO	+	+
8	HCA	33.8 $\pm$ 20.1	101-86-0	Aldrich	DMSO	+	+
9	Eugenol	149.5 $\pm$ 22.5	97-53-0	Fluka	DMSO	+	+
10	Phenyl benzoate	315.2 $\pm$ 189.9	93-99-2	Aldrich	DMSO	+	+
11	Cinnamyl alcohol	243.2 $\pm$ 59.8	104-54-1	Aldrich	DMSO	+	+
12	Imidazolidinyl urea	18.0 $\pm$ 11.5	39236-46-9	Aldrich	PBS	+	+
13	Methyl methacrylate	5000.0	119-36-8	Aldrich	DMSO	+	+
14	Chlorobenzene	371.9 $\pm$ 227.3	108-90-7	Sigma-Aldrich	DMSO	-	*
15	Isopropanol	5000.0	67-63-0	Sigma	PBS	-	+
16	Lactic acid	1116.9 $\pm$ 568.9	50-21-5	Aldrich	PBS	-	*
17	Methyl salicylate	193.8 $\pm$ 79.4	119-36-8	Sigma-Aldrich	DMSO	-	-
18	Salicylic acid	805.7 $\pm$ 218.8	69-72-7	Sigma	DMSO	-	-
19	Sodium lauryl sulfate	37.1 $\pm$ 17.8	151-21-3	Sigma	DMSO	+	-
20	Ethylene glycol dimethacrylate	21.3 $\pm$ 14.8	97-90-5	Aldrich	DMSO	+	+
21	Xylene	3451.2 $\pm$ 2263.8	1330-20-7	Sigma-Aldrich	DMSO	+	-
22	Nickel chloride	92.9 $\pm$ 19.8	7718-54-9	Sigma	DW	-	+

Confluent cells were treated for 24 h with different chemicals or DMSO as the vehicle control.

Results are expressed as the means $\pm$ SEM ($\mu\text{g mL}^{-1}$) of the concentration of chemical resulting in 75% cell viability, as assessed by MTT reduction. CV₇₅ was calculated by five-parameter non-regression analysis data.

^aCAS No (Chemical Abstracts Service Number)

^bTesting results using the LLNA (local lymph node assay) and data taken from OECD test guideline (TG429). +; sensitizer, -; non-sensitizer.

^cData taken from OECD test guideline (TG429).

^dCommercially available as Kathon CG, which is a 3:1 mixture of CMI and MI.

DMSO-dimethyl sulfoxide; CMI/MI- 5-Chloro-2-methyl-4-isothiazolin-3-one(CMI)/2-methyl-4-isothiazolin-3-one(MI). DNCB-2,4-dinitrochlorobenzene; HCA- hexyl cinnamyl aldehyde.

* Presumed to be a non-sensitizer in humans based on the fact that no clinical patch test results were located, it is not included as a patch test kit allergen, and no case reports of human sensitization were located.

Cell culture

The human keratinocyte cell line HaCaT was provided by the German Cancer Research Center (Heidelberg, Germany). HaCaT cells were cultured in Dulbecco's modified Eagle medium, supplemented with 10% heated-inactivated fetal bovine serum (FBS; Hyclone, Logan, UT, USA), 1% penicillin–streptomycin (Gibco, Life Technologies, Carlsbad, CA, USA), 1% non-essential amino acids (Gibco, Life Technologies). Cells were cultured at 37 °C in a humidified incubator containing 5% CO₂.

Determination of the 75% cell viability (CV₇₅) for HaCaT cells

Prior to studying the effects of the chemicals on cytokine production, the 75% of cell viability (CV₇₅) was assessed by the MTT assay (Gerlier and Thomasset, 1986). HaCaT cells were seeded in a 96-well plate at a concentration of 1.5×10^4 cells per well. Cells were incubated for 24 h, and the medium was then replaced with fresh medium containing increasing concentrations of test chemicals or DMSO (0.1%) as a vehicle control. After 24-h incubation, 10 μ l of MTT solution (5 mg ml⁻¹) was added to each well. Cells were then further incubated for 4 h at 37 °C, the medium was discarded and cells were lysed in 100 μ l DMSO. The absorbance of the resulting solutions was read at a wavelength of 560 nm in a VICTORTM X3 microplate reader (Perkin-Elmer, Waltham, MA, USA). The CV₇₅ was calculated for each chemical by five-parameter non-linear regression analysis of data.

Cytokine production

To determine IL-1 α and IL-6 production, cells were seeded in 24-well plates at a concentration of 1×10^5 cells ml⁻¹ (500 μ l total

volume/well). After 24 h of incubation, the culture medium was discarded and cells were treated with 0.01, 0.1, 0.5 and $1 \times$ CV₇₅ concentration of each chemical or 0.1% DMSO as vehicle control. After 24 h of incubation, ELISA reagents from a DuoSet ELISA kit (R&D Systems, Minneapolis, MN, USA) were used to measure cytokines in supernatants. The protein content of the cell lysates was measured using the BCA protein assay method (Thermo Scientific, Rockford, IL, USA). The stimulation index (SI) was calculated as follows: SI = cytokine produced in chemical-treated cells / cytokine produced in vehicle control.

Statistical analysis

All experiments were performed at least three times and the data presented are expressed as the mean $\pm$ standard error of the mean (SEM). Differences between the average values of three different duplicated experiments were assessed by Student's *t*-test as indicated in the figure legends. *P* < 0.05 was considered to be statistically significant.

Results

Determination of 75% cell viability (CV₇₅)

Although the OECD TG 429 is a reference for *in vivo* assays using a mouse, it also provides a reference chemical list of human skin sensitizers and non-sensitizers (OECD, 2010). The CV₇₅ for the 16 sensitizers and 6 non-sensitizers are presented in Table 1. The CAS numbers and classification according to the TG429 chemical list are also shown. Methyl methacrylate and isopropanol showed no cytotoxicity at 5000 μ g ml⁻¹, the highest concentration of the MTT assay.

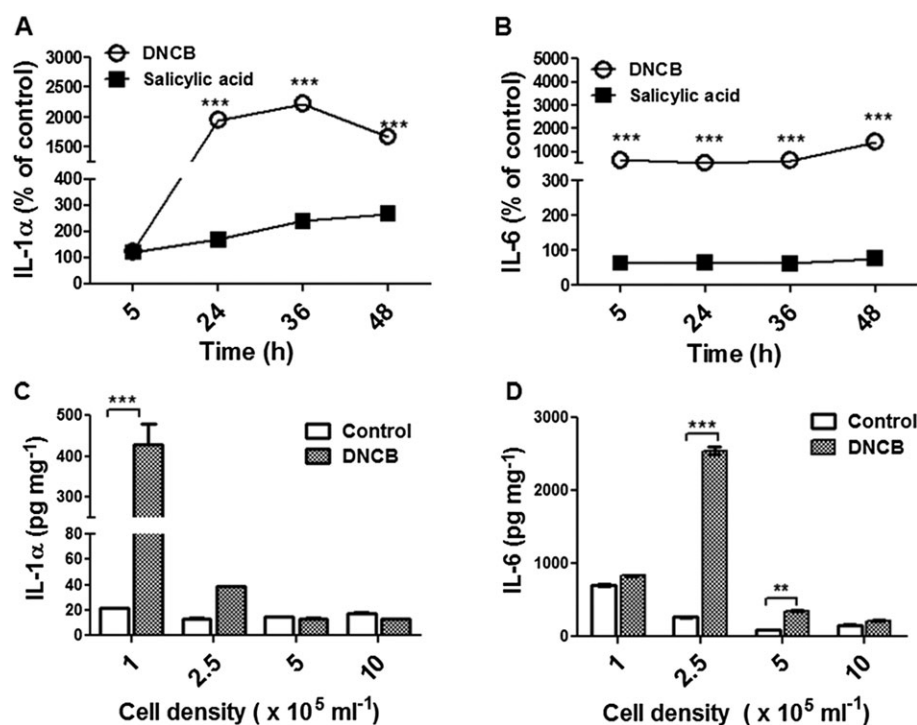


Figure 1. Time course and effect of cell density on extracellular interleukin (IL)-1 α and IL-6 levels. (A–B) Time course. HaCaT cells were treated for different times (5, 24, 36, 48 h) with DNCB (4 μ g ml⁻¹), and salicylic acid (800 μ g ml⁻¹) or vehicle control. (C–D) Effect of cell density: HaCaT cells were seeded in a 24-well plate at different densities (1–10 $\times 10^5$ cells ml⁻¹) and treated with DNCB (4 μ g ml⁻¹) or DMSO as a vehicle control for 24 h. Results are expressed as the means $\pm$ SEM of three duplicated independent experiments.

Effect of the selected chemicals on IL-1 α and IL-6 production in HaCaTs

Preliminary experiments were carried out to determine the best time and cell density to measure extracellular levels of IL-1 α and IL-6. In time course experiments, cells, at 80–90% density, were treated with salicylic acid (800 $\mu\text{g ml}^{-1}$) and DNCB (4 $\mu\text{g ml}^{-1}$) at different time points (5, 24, 30 and 48 h). As shown in Fig. 1A, a statistically significant increase in extracellular IL-1 α was detected in cells treated with DNCB after 24 h of treatment and extracellular IL-6 was detected at 5 h of treatment (Fig. 1B) whereas no changes were observed after treatment with the irritant salicylic acid. Therefore, the incubation time of 24 h was chosen for all subsequent investigations.

The effect of cell density on the response to the sensitizer DNCB was investigated by seeding cells at different densities (1 to 10×10^5 cells ml^{-1} in a 24-well plate). At 24 h post incubation, cells were treated for 24 h with DNCB (4 $\mu\text{g ml}^{-1}$) or DMSO as a vehicle control. As shown in Fig. 1C, 1×10^5 cells ml^{-1} was the optimal cell density for IL-1 α . In the case of IL-6, 2.5×10^5 cells ml^{-1} represents the optimal cell density, whereas a higher cell density ($>5 \times 10^5$ cells ml^{-1}) resulted in a loss of cytokine production. Thus, experiments were conducted using 1.5×10^5 cells ml^{-1} .

Extracellular IL-1 α production after chemical treatment in HaCaTs

We examined extracellular IL-1 α production using HaCaT cells after incubation with the 22 chemicals presented in Table 1. Ten out of

16 sensitizers were categorized as skin sensitizers as a result of a significant increase in IL-1 α production (Table 2). Cobalt chloride, citral, eugenol, MMA, sodium lauryl sulfate and EGDMA failed to be categorized as skin sensitizers (Table 2).

Extracellular IL-6 production after chemical treatment in HaCaTs

Eleven out of 16 sensitizers were categorized as skin sensitizers by a significant increase in IL-6 production (Table 3). However, CMI/MI, cobalt chloride, cinnamyl alcohol, MMA and sodium lauryl sulfate failed to be categorized as skin sensitizers (Table 3). Of the six non-sensitizers, methyl salicylate significantly increased both IL-1 α and IL-6 production in HaCaT cells (Tables 2 and 3)

Classification of tested chemicals as sensitizers or non-sensitizers based on the stimulation index

Chemical classification is determined on the basis of whether the stimulation index (SI) was three or over three in one cytokine production. To assess whether chemicals are skin sensitizers or not, we selected the highest production rates of IL-1 α and IL-6 among the four different treatment doses and the data were graphed in Fig. 2. Non-sensitizers produced an SI under 3 in both IL-1 α and IL-6 production. The accuracy of non-irritants by IL-1 α or IL-6 alone was exactly the same at 83% (5/6) whereas the accuracy of sensitizers was different at 63% (10/16) by IL-1 α and 69% (11/16) by IL-6. However, combination IL-1 α and IL-6 results

Table 2. The relative rate of interleukin-1 α (IL-1 α) production by HaCaT cells at 24 h after the initiation of chemical exposure

Chemicals	IL-1 α production rate (means $\pm$ SEM) ^a			
	0.01 $\times$ CV ₇₅	0.1 $\times$ CV ₇₅	0.5 $\times$ CV ₇₅	1 $\times$ CV ₇₅
CMI/MI	92.8 $\pm$ 24.0	91.8 $\pm$ 28.4	125.1 $\pm$ 14.9	1191.2 $\pm$ 90.9
DNCB	187.6 $\pm$ 31.2	179.7 $\pm$ 38.6	477.3 $\pm$ 459.3	2202.6 $\pm$ 2137.9
4-Phenylenediamine	314.2 $\pm$ 220.1	318.1 $\pm$ 250.1	493.0 $\pm$ 466.7	1298.4 $\pm$ 1655.0
Cobalt chloride	109.5 $\pm$ 28.2	116.6 $\pm$ 49.3	93.4 $\pm$ 16.0	92.2 $\pm$ 12.5
Isoeugenol	109.9 $\pm$ 42.5	100.1 $\pm$ 22.8	343.8 $\pm$ 193.4	104.5 $\pm$ 8.2
2-Mercaptobenzothiazole	91.3 $\pm$ 12.0	132.4 $\pm$ 10.6	135.4 $\pm$ 41.8	422.03 $\pm$ 247.78
Citral	78.7 $\pm$ 15.0	78.0 $\pm$ 6.3	73.1 $\pm$ 11.1	227.3 $\pm$ 143.1
HCA	146.5 $\pm$ 54.4	151.2 $\pm$ 51.3	233.2 $\pm$ 75.6	615.7 $\pm$ 544.7
Eugenol	115 $\pm$ 18.1	108.5 $\pm$ 27.9	112.3 $\pm$ 21.4	112.4 $\pm$ 20.2
Phenyl benzoate	121.6 $\pm$ 21.7	108.6 $\pm$ 22	1342.1 $\pm$ 2039.4	106.4 $\pm$ 1.1
Cinnamyl alcohol	91.7 $\pm$ 15.3	88.4 $\pm$ 39.7	189.7 $\pm$ 61.1	535.3 $\pm$ 277.2
Imidazolidinyl urea	138.8 $\pm$ 59.8	146.5 $\pm$ 46.7	182.0 $\pm$ 52.2	981.5 $\pm$ 904.7
Methyl methacrylate	93.3 $\pm$ 13.2	81.5 $\pm$ 26.6	92.6 $\pm$ 13.1	57.8 $\pm$ 43.2
Chlorobenzene	94.9 $\pm$ 10.7	85.5 $\pm$ 9.6	112.1 $\pm$ 15.3	109.7 $\pm$ 23
Isopropanol	114.2 $\pm$ 26.9	116.1 $\pm$ 25.1	119.2 $\pm$ 22.1	123.7 $\pm$ 25.1
Lactic acid	104.5 $\pm$ 12.1	106.5 $\pm$ 5.4	97.7 $\pm$ 8.0	109.4 $\pm$ 15.2
Methyl salicylate	119.5 $\pm$ 13.2	119.9 $\pm$ 9.3	193.3 $\pm$ 51.9	401.1 $\pm$ 310.9
Salicylic acid	97.2 $\pm$ 24.7	132.2 $\pm$ 33.1	135.2 $\pm$ 36	201.1 $\pm$ 58.2
Sodium lauryl sulfate	117.5 $\pm$ 31.2	118.5 $\pm$ 28	137.5 $\pm$ 41.5	144.2 $\pm$ 57.6
Ethylene glycol dimethacrylate	104.4 $\pm$ 8.3	118.6 $\pm$ 10.3	149.5 $\pm$ 33.4	117.8 $\pm$ 7.0
Xylene	122.8 $\pm$ 16.1	102.2 $\pm$ 18.6	319.5 $\pm$ 236.4	126.1 $\pm$ 15.7
Nickel chloride	105.2 $\pm$ 12.5	93.5 $\pm$ 24.1	100.7 $\pm$ 14.4	107.5 $\pm$ 11.5

CMI/MI- 5-Chloro-2-methyl-4-isothiazolin-3-one(CMI)/2-methyl-4-isothiazolin-3-one(MI); DNCB, 2, 4-dinitrochlorobenzene; HCA, hexyl cinnamyl aldehyde.

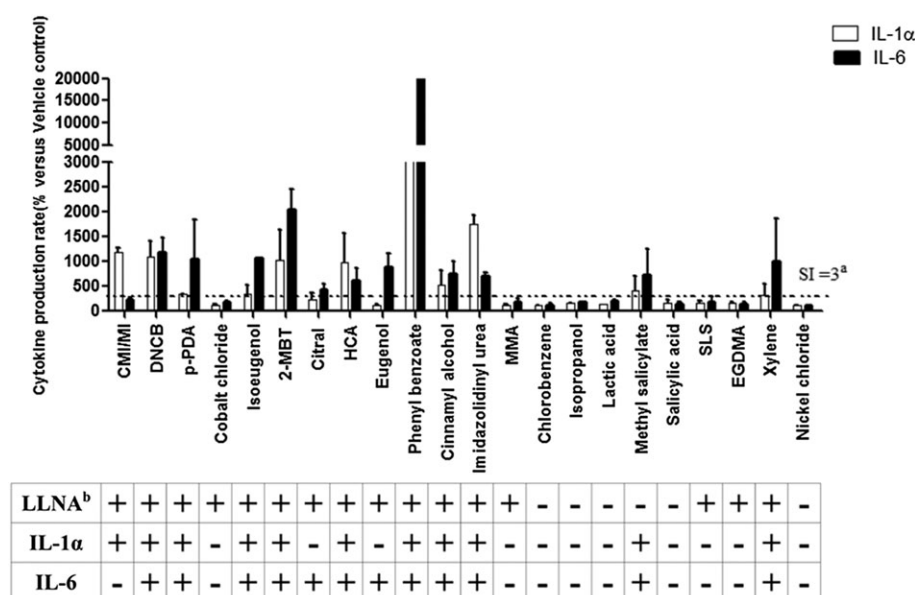
^aThe extracellular IL-1 α production rate, expressed as a percentage relative to control cells, was determined by dividing the amount of IL-1 α (pg ml^{-1}) produced by chemically treated cells by the amount of IL-1 α produced by vehicle control-treated cells and multiplying by 100. Data are expressed as the means $\pm$ SEM of three duplicated independent experiments. Significantly different from the vehicle control; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Vehicle control; 0.1% PBS or 0.1% DMSO.

Table 3. The relative rate of interleukin-6 (IL-6) production by HaCaT cells at 24 h after the initiation of chemical exposure

Chemicals	IL-6 production rate (means $\pm$ SEM) ^a			
	$0.01 \times CV_{75}$	$0.1 \times CV_{75}$	$0.5 \times CV_{75}$	$1 \times CV_{75}$
CMI/MI	88.0 $\pm$ 25.2	87.1 $\pm$ 24.3	109.1 $\pm$ 23.2	228.1 $\pm$ 58.9
DNCB	881.3 $\pm$ 710.5	1142.9 $\pm$ 1027.4	172.4 $\pm$ 56.5	164.3 $\pm$ 78.5
4-Phenylenediamine	789.0 $\pm$ 723.9	325.1 $\pm$ 228.3	401.3 $\pm$ 487.4	537.3 $\pm$ 677.7
Cobalt chloride	90.9 $\pm$ 321.0	106.6 $\pm$ 31.8	151.4 $\pm$ 35.6	157.1 $\pm$ 52.1
Isoeugenol	127.2 $\pm$ 48.4	112.5 $\pm$ 16.4	759.9 $\pm$ 548.4	114.8 $\pm$ 20.5
2-Mercaptobenzothiazole	126.7 $\pm$ 14.4	189.2 $\pm$ 16.4	1444.7 $\pm$ 1259.4	537.81 $\pm$ 3.33
Citral	85.2 $\pm$ 1.5	109.7 $\pm$ 1.4	251.5 $\pm$ 49.1	428.4 $\pm$ 111.0
HCA	505.5 $\pm$ 296.9	263.0 $\pm$ 115.2	152.1 $\pm$ 93.6	161.5 $\pm$ 91.8
Eugenol	115.2 $\pm$ 19.9	114.8 $\pm$ 25.1	362.3 $\pm$ 213.5	633.4 $\pm$ 477.4
Phenyl benzoate	114.9 $\pm$ 20.6	105.1 $\pm$ 18.6	5627.1 $\pm$ 8141.0	106.1 $\pm$ 20.9
Cinnamyl alcohol	101.8 $\pm$ 43.1	124.8 $\pm$ 67.8	354.1 $\pm$ 152.8	746.6 $\pm$ 254.7
Imidazolidinyl urea	1937.4 $\pm$ 1439.6	454.3 $\pm$ 489.3	152.0 $\pm$ 81.1	173.6 $\pm$ 98.9
Methyl methacrylate	61.0 $\pm$ 31.1	105.4 $\pm$ 23.5	117.4 $\pm$ 44.3	117.4 $\pm$ 21.9
Chlorobenzene	126.7 $\pm$ 55.9	123.1 $\pm$ 66.5	109.2 $\pm$ 46.2	113.8 $\pm$ 40.7
Isopropanol	181.2 $\pm$ 26.2	172.3 $\pm$ 58.3	166.1 $\pm$ 36.8	202.1 $\pm$ 70.2
Lactic acid	151.6 $\pm$ 8.5	157.6 $\pm$ 27.6	150.1 $\pm$ 24.1	172.5 $\pm$ 38.9
Methyl salicylate	122.4 $\pm$ 23.2	131.4 $\pm$ 26.4	282.4 $\pm$ 84.4	1030.8 $\pm$ 476.3
Salicylic acid	128.3 $\pm$ 39.8	106.2 $\pm$ 26.3	120.6 $\pm$ 25.4	125.0 $\pm$ 54.3
Sodium lauryl sulfate	108.4 $\pm$ 15.5	104.6 $\pm$ 14.3	179.5 $\pm$ 123.3	182.5 $\pm$ 127.2
Ethylene glycol dimethacrylate	100.0 $\pm$ 8.1	100.3 $\pm$ 3.2	134.2 $\pm$ 54.2	106.0 $\pm$ 15.2
Xylene	111.5 $\pm$ 4.4	109.9 $\pm$ 8.9	992.7 $\pm$ 865.3	136.9 $\pm$ 11.5
Nickel chloride	118.2 $\pm$ 3.5	111.3 $\pm$ 8.7	113.9 $\pm$ 7.7	115.7 $\pm$ 6.0

CMI/MI- 5-Chloro-2-methyl-4-isothiazolin-3-one(CMI)/2-methyl-4-isothiazolin-3-one(MI); DNCB, 2, 4-dinitrochlorobenzene; HCA, hexyl cinnamyl aldehyde.

^aThe extracellular IL-6 production rate, expressed as the percentage relative to control cells, was determined by dividing the amount of IL-6 (pg ml⁻¹) produced by chemically treated cells by the amount of IL-6 produced by vehicle control-treated cells, and then multiplying by 100. Data are expressed as the means $\pm$ SEM of three duplicated independent experiments. Significantly different from the vehicle control; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Vehicle control; 0.1% PBS or 0.1% DMSO.

**Figure 2.** Comparison of cytokine production rates calculated in Tables 2 and 3. The highest cytokine production rate for each chemical is presented.

^aThe small dashed line indicates the stimulation index (SI) 3. Classification: the chemical is classified based on the SI value of our results. ^bPositive (+) ≥ 3 , negative (-) < 3 . CMI/MI, 5-chloro-2-methyl-4-isothiazolin-3-one (CMI)/2-methyl-4-isothiazolin-3-one (MI); DNCB, 2,4-dinitrochlorobenzene; p-PDA, 4-phenylenediamine; 2-MBT, 2-mercaptobenzothiazole; HCA, hexyl cinnamyl aldehyde; MMA, methyl methacrylate; SLS, sodium lauryl sulfate; EGDMA, ethylene glycol dimethacrylate. Results are expressed as the means $\pm$ SEM of three duplicated independent experiments.

showed better accuracy: 75% of sensitizers (12/16), 83% of non-sensitizers (5/6) and 77% overall (17/22) (Tables 4 and 5).

Discussion

Here, we investigated the extracellular production of pro-inflammatory cytokines in the human keratinocyte cell line, HaCaT,

Table 4. Comparison of local lymph node assay (LLNA) results versus classification by the highest relative cytokine production rate

Comparison	Accuracy ^a (%) for		Overall accuracy (%)
	sensitizers	non-sensitizers	
LLNA vs IL-1 α	63(10/16)	83(5/6)	68(15/22)
LLNA vs IL-6	69(11/16)	83(5/6)	73(16/22)
LLNA vs IL-1 α + IL-6 ^b	75(12/16)	83(5/6)	77(17/22)

LLNA, local lymph node assay. ^aAccuracy, the proportion of correct outcomes of a method. Often used interchangeably with concordance. ^bChemical classification determined on the basis of the stimulation index (SI). Sensitizer SI ≥ 3 at least in one cytokine production. Non-sensitizer SI < 3 in both IL-1 α and IL-6 production.

to discriminate skin sensitizers from non-sensitizers. Exposure of the cells to skin sensitizers results in significant production of extracellular IL-1 α and IL-6 compared with non-sensitizers. HaCaT has been widely used for studying skin irritation, skin cancer, genotoxicity, mutagenicity and cytotoxicity (Wilhelm *et al.*, 1994; Little *et al.*, 1996; Merryman, 1999; Wollin and Gorlitz, 2004). In addition, an *in vitro* assay using a gene expression signature in HaCaT cells demonstrates its clinical relevance for a skin sensitization (van der Veen *et al.*, 2015). Of note, KeratinosensTM uses HaCaT carrying an ARE-Nrf2-dependent Luciferase gene that is induced by sensitizers (Johansson *et al.*, 2011). Although a pre-validation study of KeratinosensTM was successful, there was concern that the activation of the ARE/Nrf2 pathway alone may not be sufficient to definitely establish the skin sensitization potential of chemicals. Therefore, a combination of *in vitro* methods might be preferred as alternatives to *in vivo* methods.

Keratinocytes are the major source of cytokines in the epidermis and have been reported to secrete IL-1 α , IL-3, IL-6, IL-8, IL-18, CSF, TNF- α and TGF- β (Ansel *et al.*, 1990; Corsini and Galli, 2000). However, keratinocytes do not normally actively secrete these cytokines; a number of agents mediate keratinocyte cytokine production, including cytokines themselves (Ansel *et al.*, 1990). Thus, several studies have explored the use of these cytokines as a marker of sensitization. For example, IL-18 production was tested using the NCTC 2544 human keratinocyte cell line (Corsini *et al.*, 2009; Teunis *et al.*, 2013). Despite considerable efforts to differentiate sensitizers

Table 5. Classification of the chemical based on the HaCaT IL-1 α and IL-6 assay

Chemical		HaCaT IL-1 α & IL-6 assay prediction			In vivo prediction	
		IL-1 α	IL-6	IL-1 α & IL-6	LLNA ^a	Human ^b
1	CMI/MI	+	-	+	+	+
2	DNCB	+	+	+	+	+
3	4-Phenylenediamine	+	+	+	+	+
4	Cobalt chloride	-	-	-	+	+
5	Isoeugenol	+	+	+	+	+
6	2-Mercaptobenzothiazole	+	+	+	+	+
7	Citral	-	+	+	+	+
8	HCA	+	+	+	+	+
9	Eugenol	-	+	+	+	+
10	Phenyl benzoate	+	+	+	+	+
11	Cinnamyl alcohol	+	+	+	+	+
12	Imidazolidinyl urea	+	+	+	+	+
13	Methyl methacrylate	-	-	-	+	+
14	Chlorobenzene	-	-	-	-	*
15	Isopropanol	-	-	-	-	+
16	Lactic acid	-	-	-	-	*
17	Methyl salicylate	+	+	+	-	-
18	Salicylic acid	-	-	-	-	-
19	Sodium lauryl sulfate	-	-	-	+	-
20	Ethylene glycol dimethacrylate	-	-	-	+	+
21	Xylene	+	+	+	+	-
22	Nickel chloride	-	-	-	-	+

^aTesting results using the LLNA (local lymph node assay) and data taken from OECD test guideline (TG429). +; sensitizer, -; non-sensitizer.

^bData taken from the OECD test guideline (TG429).

* Presumed to be a non-sensitizer in humans based on the fact that no clinical patch test results were located, it is not included as a patch test kit allergen, and no case reports of human sensitization were located. CMI/MI-5-Chloro-2-methyl-4-isothiazolin-3-one(CMI)/2-methyl-4-isothiazolin-3-one(MI). DNCB-2,4-dinitrochlorobenzene; HCA- hexyl cinnamyl aldehyde.

from non-sensitizers, the skin sensitization potential should be validated with several markers because of the complexity of the mechanisms involved. Thus, we have yet to identify and characterize chemicals that accurately assay skin sensitization *in vitro*. In this study, we focused on pro-inflammatory cytokine levels of IL-1 α , IL-6, IL-8 and IL-18 by HaCaT after exposure to sensitizers. Van Och *et al* (2005) previously reported that the ranking of allergens based on the EC3 values derived from IL-18 dose-response relationships in HaCaT cells does not correspond to the *in vivo* classification data from LLNA. In our study, the production levels of IL-18 did not statistically discriminate between sensitizers and non-sensitizers (Supplementary Information Fig. 1A) and skin non-sensitizers induced higher levels of IL-8 than the sensitizers (Supplementary Information Fig. 1B). Hence, we focused on the levels of IL-1 α and IL-6 produced by sensitizers. Twelve out of the 16 sensitizers found in the murine LLNA guideline, OECD TG429, significantly increased either IL-1 α or IL-6 in HaCaT, showing 75% sensitivity (Table 4).

However, both IL-1 α and IL-6 failed to identify cobalt chloride as a sensitizer (Tables 1, 2 and 5). This may be due to the fact that as metal cation, it does not easily pass through the cell membrane. SLS, categorized as a sensitizer by murine LLNA method, also did not change both IL-1 α and IL-6 levels (Tables 1, 2 and 5). However, SLS is also classified as a skin non-sensitizer in the OECD TG429 list. In the case of MMA and EGDMA, they are classified as a weak sensitizer in LLNA (Betts *et al.*, 2006; Loveless *et al.*, 2010). However, in our study, we could not find the change in both IL-1 α and IL-6 levels (Tables 1, 2 and 5). These results showed the limitation of our method with a low predictability on certain metal and weak sensitizers.

IL-1 α is a pleiotropic, potent pro-inflammatory cytokine. Damage to keratinocytes causes the release of IL-1 α , which essentially is a primary event in the skin defence. IL-1 α stimulates the further release of IL-1 α and the production and release of other cytokines such as IL-8, IL-6, GM-CSF, etc. Thus, this cytokine cascades and network leads to rapid generation of the inflammatory response (Corsini and Galli, 2000). IL-6 is also a multifunctional, pleiotropic cytokine involved in the regulation of immune responses, acute-phase responses, hematopoiesis and inflammation. In innate immunity, IL-6 may also influence leukocyte recruitment as the accumulation of neutrophils at sites of infection induces production of acute-phase proteins (Hurst *et al.*, 2001; Akdis *et al.*, 2011). Furthermore, it was reported that various agents such as poly:I:C and PGN can induce IL-6 secretion into the media by HaCaT (Kim *et al.*, 2009). Taken together, when the skin is damaged by sensitizers, neutrophils recruitment occurs by IL-6 secretion and IL-1 α secreted from keratinocytes activates inflammation. Thus, we hypothesize that HaCaT production of these two cytokines may be useful for differentiating skin sensitizers.

As mentioned above, skin sensitizers can induce keratinocytes to produce inflammatory cytokines and test methods including our assay based on this response are considered useful for testing the skin sensitization potential of chemicals. However, as the keratinocyte inflammatory response represents only the second key event of the skin sensitization adverse outcome pathway (AOP), results from our test methods based on the activation of this pathway may not be sufficient when used as a stand-alone method to conclude on the skin sensitization potential of chemicals. Therefore, we should consider combining our data with other *in vitro* assays, e.g. DPRA (OECD, 2015a), KeratinoSens (OECD, 2015b) or h-CLAT (EURL-ECVAM, 2015) addressing other key events of the skin sensitization AOP (OECD, 2012).

In conclusion, the extracellular production of pro-inflammatory cytokines IL-1 α and IL-6 in human HaCaT cells may potentially

classify sensitizers and non-sensitizers as a simple *in vitro* method although additional validation studies should be performed using various contact vs. respiratory allergens and/or irritants and an integrated approach should be studied.

Acknowledgments

This research was supported by grants (13172MFDS987, 11182MFDS575) from the Ministry of Food Drug Safety and the Consortium of Alternative Methods for Safety Evaluation of Cosmetics (CAMSEC).

Conflict of interest disclosure

The authors declare no competing financial interests.

References

- Ansel JC, Perry P, Brown J, Damm D, Phan T, Hart C, Luger TA, Hefeneider S. 1990. Cytokine modulation of keratinocyte cytokines. *J. Invest. Dermatol.* **94**(6 Suppl): 101S–107S.
- Akdis M, Burgler S, Cramer R, Eiwegger T, Fujita H, Gomez E, Klunker S, Meyer N, O'Mahony L, Palomares O, Rhyner C, Ouaked N, Schaffartzik A, Van De Veen W, Zeller S, Zimmermann M, Akdis CA. 2011. Interleukins, from 1 to 37, and interferon- γ : receptors, functions, and roles in diseases. *J. Allergy Clin. Immunol.* **127**(3): 701–21.
- Betts CJ, Dearman RJ, Heylings JR, Kimber I, Basketter DA. 2006. Skin sensitization potency of methyl methacrylate in the local lymph node assay: comparisons with guinea-pig data and human experience. *Contact Dermatitis* **55**: 140–147.
- Corsini E, Galbiati V, Mitjans M, Galli CL, Marinovich M. 2013. NCTC 2544 and IL-18 production: a tool for the identification of contact allergens. *Toxicol. In Vitro* **27**: 1127–1134.
- Corsini E, Galli CL. 2000. Epidermal cytokines in experimental contact dermatitis. *Toxicology* **142**: 203–211.
- Corsini E, Mitjans M, Galbiati V, Lucchi L, Galli CL, Marinovich M. 2009. Use of IL-18 production in a human keratinocyte cell line to discriminate contact sensitizers from irritants and low molecular weight respiratory allergens. *Toxicol. In Vitro* **23**: 789–796.
- EURL-ECVAM. 2015. *Recommendation on the Human Cell Line Activation Test (h-CLAT) for Skin Sensitisation Testing*. 40 pp. <https://eurl-ecvam.jrc.ec.europa.eu/eurl-ecvam-recommendations/eurl-ecvam-recommendation-on-the-human-cell-line-activation-test-h-clat-for-skin-sensitisation-testing>
- Galbiati V, Mitjans M, Lucchi L, Viviani B, Galli CL, Marinovich M, Corsini E. 2011. Further development of the NCTC 2544 IL-18 assay to identify *in vitro* contact allergens. *Toxicol. In Vitro* **25**: 724–723.
- Gerberick GF, Vassallo JD, Bailey RE, Chaney JG, Morrall SW, Lepoittevin JP. 2004. Development of a peptide reactivity assay for screening contact allergens. *Toxicol. Sci.* **81**: 332–343.
- Gerlier D, Thomasset N. 1986. Use of MTT colorimetric assay to measure cell activation. *J. Immunol. Methods*. **94**(1–2):57–63.
- Gibbs S, Corsini E, Spiekstra SW, Galbiati V, Fuchs HW, Degeorge G, Troese M, Hayden P, Deng P, Roggen E. 2013. An epidermal equivalent assay for identification and ranking potency of contact sensitizers. *Toxicol. Appl. Pharmacol.* **272**: 529–541.
- Gron A. 2002. Keratinocytes and cytokines. *Vet. Immunol. Immunopathol.* **88**: 1–12.
- Haas J, Lipkow T, Mohamadzadeh M, Kolde G, Knop J. 1992. Induction of inflammatory cytokines in murine keratinocytes upon *in vivo* stimulation with contact sensitizers and tolerizing analogues. *Exp. Dermatol.* **1**: 76–83.
- Hooyberghs J, Schoeters E, Lambrechts N, Nelissen I, Witters H, Schoeters G, Van Den Heuvel R. 2008. A cell-based *in vitro* alternative to identify skin sensitizers by gene expression. *Toxicol. Appl. Pharmacol.* **231**: 103–111.
- Hurst SM, Wilkinson TS, McLoughlin RM, Jones S, Horiuchi S, Horiuchi S, Yamamoto N, Rose-John S, Fuller GM, Topley N, Jones SA. 2011. IL-6 and its soluble receptor orchestrate a temporal switch in the pattern of leukocyte recruitment seen during acute inflammation. *Immunity*. **14**(6): 705–714.
- Johansson H, Albrekt AS, Borrebaeck CA, Lindstedt M. 2013. The GARD assay for assessment of chemical skin sensitizers. *Toxicol. In Vitro* **27**: 1163–1169.

- Johansson H, Lindstedt M, Albrekt AS, Borrebaeck CA. 2011. A genomic biomarker signature can predict skin sensitizers using a cellbased in vitro alternative to animal tests. *BMC Genomics* **12**: 399.
- Kimber I, Hilton J, Dearman RJ, Gerberick GF, Ryan CA, Basketter DA, Scholes EW, Ladics GS, Loveless SE, House RV. 1995. An international evaluation of the murine local lymph node assay and comparison of modified procedures. *Toxicology* **103**: 63–73.
- Kim H, Han TH, Lee SG. 2009. Anti-inflammatory activity of a water extract of *Acorus calamus* L. leaves on keratinocyte HaCaT cells. *J. Ethnopharmacol.* **122**: 149–156.
- Kupper TS, Ballard DW, Chua AO, McGuire JS, Flood PM, Horowitz MC, Langdon R, Lightfoot L, Gubler U. 1986. Human keratinocytes contain mRNA indistinguishable from monocyte interleukin 1 alpha and beta mRNA. Keratinocyte epidermal cell-derived thymocyte activating factor is identical to interleukin 1. *J. Exp. Med.* **164**: 2095–2100.
- Larsen CG, Anderson AO, Oppenheim JJ, Matsushima K. 1989. Production of interleukin-8 by human dermal fibroblasts and keratinocytes in response to interleukin-1 or tumour necrosis factor. *Immunology* **68**: 31–36.
- Little MC, Gawkrödger DJ, MacNeil S. 1996. Chromium and nickel-induced cytotoxicity in normal and transformed human keratinocytes. An investigation of pharmacological approaches to the prevention of Cr(VI)-induced cytotoxicity. *Br. J. Dermatol.* **134**: 199–207.
- Loveless SE, Api AM, Crevel RW, Debruyne E, Gamer A, Jowsey IR, Kern P, Kimber I, Lea L, Lloyd P, Mehmood Z, Steiling W, Veenstra G, Woolhiser M, Hennes C. 2010. Potency values from the local lymph node assay: application to classification, labelling and risk assessment. *Regul. Toxicol. Pharmacol.* **56**: 54–66.
- Magnusson B. 1980. The identification of contact allergens by animal assay. *Contact Dermatitis* **6**: 46–50.
- McKim JM, Keller DJ, Gorski JR. 2012. An in vitro method for detecting chemical sensitization using human reconstructed skin models and its applicability to cosmetic, pharmaceutical, and medical device safety testing. *Cutan. Ocul. Toxicol.* **31**: 292–305.
- Mehling A, Eriksson T, Eltze T, Kolle S, Ramirez T, Teubner W, van Ravenzwaay B, Landsiedel R. 2012. Non-animal test methods for predicting skin sensitization potentials. *Arch. Toxicol.* **86**: 1273–1295.
- Merryman JL. 1999. Effects of ultraviolet C radiation on cellular proliferation in p53-/-keratinocytes. *J. Environ. Pathol. Toxicol. Oncol.* **18**: 1–9.
- Nukada Y, Ashikaga T, Sakaguchi H, Sono S, Mugita N, Hirota M, Miyazawa M, Ito Y, Sasa H, Nishiyama N. 2011. Predictive performance for human skin sensitizing potential of the human cell line activation test (h-CLAT). *Contact Dermatitis* **65**: 343–53.
- OECD. 2010. OECD guideline for the testing of chemicals: skin sensitization: local lymph node assay. In: *OECD/OCDE (edn)*. pp. 429. Organisation for Economic Co-operation and Development: Paris.
- OECD. 2012. *The Adverse Outcome Pathway for Skin Sensitisation Initiated by Covalent Binding to Proteins. Part 2: Use of the AOP to Develop Chemical Categories and Integrated Assessment and Testing Approaches. Series on Testing and Assessment No. 168.* ENV/JM/MONO(2012)/PART2. Organisation for Economic Co-operation and Development: Paris.
- OECD. 2015a. OECD guidelines for chemical testing no. 442C. In: *Chemico Skin Sensitisation: Direct Peptide Reactivity Assay (DPRA)*. Organisation for Economic Co-operation and Development: Paris. <http://www.oecd.org/env/testguidelines>
- OECD. 2015b. *OECD Guidelines for Chemical Testing No. 442D: In Vitro Skin Sensitisation: ARE-Nrf2 Luciferase Test Method*. Organisation for Economic Co-operation and Development: Paris. <http://www.oecd.org/env/testguidelines>
- Boukamp P, Petrussevska RT, Breitkreutz D, Hornung J, Markham A, Fusenig NE. 1988. Normal Keratinization in a Spontaneously Immortalized Aneuploid Human Keratinocyte Cell Line. *J. Cell Biol.* **106**: 761–771.
- Ramirez T, Mehling A, Kolle SN, Wruck CJ, Teubner W, Eltze T, Aumann A, Urbisch D, van Ravenzwaay B, Landsiedel R. 2014. LuSens: a keratinocyte based ARE reporter gene assay for use in integrated testing strategies for skin sensitization hazard identification. *Toxicol. In Vitro* **28**: 1482–1497.
- Saito K, Nukada Y, Takenouchi O, Miyazawa M, Sakaguchi H, Nishiyama N. 2013. Development of a new in vitro skin sensitization assay (Epidermal Sensitization Assay; EpiSensA) using reconstructed human epidermis. *Toxicol. In Vitro* **27**: 2213–2224.
- Son D, Na Y, Cho WS, Lee BH, Heo Y, Park JH, Seok SH. 2013. Differentiation of skin sensitizers from irritant chemicals by interleukin-1 α and macrophage inflammatory protein-2 in murine keratinocytes. *Toxicol. Lett.* **216**: 65–71.
- Taylor K, Casalegno C, Stengel W. 2011. A critique of the EC's expert (draft) reports on the status of alternatives for cosmetics testing to meet the 2013 deadline. *ALTEX* **28**: 131–48.
- Teunis M, Corsini E, Smits M, Madsen CB, Eltze T, Ezendam J, Galbiati V, Gremmer E, Krul C, Landin A, Landsiedel R, Pieters R, Rasmussen TF, Reinders J, Roggen E, Spiekstra S, Gibbs S. 2013. Transfer of a two-tiered keratinocyte assay: IL-18 production by NCTC2544 to determine the skin sensitizing capacity and epidermal equivalent assay to determine sensitizer potency. *Toxicol. In Vitro* **27**: 1135–1150.
- van der Veen JW, Hodemaekers H, Reus AA, Maas WJ, van Loveren H, Ezendam J. 2015. Human relevance of an in vitro gene signature in HaCaT for skin sensitization. *Toxicol. In Vitro* **29**: 81–84.
- Van Och FM, Van Loveren H, Van Wolfswinkel JC, Machielsen AJ, Vandebriel RJ. 2005. Assessment of potency of allergenic activity of low molecular weight compounds based on IL-1 α and IL-18 production by a murine and human keratinocyte cell line. *Toxicology* **210**: 95–109.
- Wilhelm KP, Samblebe M, Siegers CP. 1994. Quantitative in vitro assessment of N-alkyl sulphate induced cytotoxicity in human keratinocytes (HaCaT). Comparison with in vivo human irritation tests. *Br. J. Dermatol.* **130**: 18–23.
- Wollin KM, Gorlitz BD. 2004. Comparison of genotoxicity of textile dyestuffs in Salmonella mutagenicity assay, in vitro micronucleus assay, and single cell gel/comet assay. *J. Environ. Pathol. Toxicol. Oncol.* **23**: 267–278.

Supporting information

Additional supporting information may be found in the online version of this article at the publisher's web-site.

Supplementary figure 1. The total production level of IL-18 and IL-8. HaCaT cells were seeded in 24-well plate at different densities ($1-10 \times 10^5$ cells/ml) and treated with with skin sensitizers (DNCB, HCA) and non-sensitizers (Lactic acid, Isopropanol) for 24 h. Total (intracellular and extracellular) IL-18 and IL-8 production levels were investigated after exposure to HaCaT cells with skin sensitizers and non-sensitizers. Results are expressed as means $\pm$ SEM of three duplicated independent experiments.

