

An epidermal equivalent assay for identification and ranking potency of contact sensitizers

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ABSTRACT

The purpose of this study was to explore the possibility of combining the epidermal equivalent (EE) potency assay with the assay which assesses release of interleukin-18 (IL-18) to provide a single test for identification and classification of skin sensitizing chemicals, including chemicals of low water solubility or stability. A protocol was developed using different 3D-epidermal models including in house VUMC model, epiCS® (previously EST1000™), MatTek EpiDerm™ and SkinEthic™ RHE and also the impact of different vehicles (acetone:olive oil 4:1, 1% DMSO, ethanol, water) was investigated. Following topical exposure for 24 h to 17 contact allergens and 13 non-sensitizers a robust increase in IL-18 release was observed only after exposure to contact allergens. A putative prediction model is proposed from data obtained from two laboratories yielding 95% accuracy. Correlating the *in vitro* EE sensitizer potency data, which assesses the chemical concentration which results in 50% cytotoxicity (EE-EC₅₀) with human and animal data showed a superior correlation with human DSA₀₅ (μg/cm²) data (spearman $r = 0.8500$; P value (two-tailed) = 0.0061) compared to LLNA data (Spearman $r = 0.5968$; P value (two-tailed) = 0.0542). DSA₀₅ = induction dose per skin area that produces a positive response in 5% of the tested population. Also a good correlation was observed for release of IL-18 (SI-2) into culture supernatants with human DSA₀₅ data (spearman $r = 0.8333$; P value (two-tailed) = 0.0154). This easily transferable human *in vitro* assay appears to be very promising, but additional testing of a larger chemical set with the different EE models is required to fully evaluate the utility of this assay and to establish a definitive prediction model.

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Introduction

In addition to being able to determine whether or not a chemical is a sensitizer (labelling) it is also equally important to determine the potency of a sensitizer (classification) in order to identify a maximum safe concentration for human exposure (risk assessment). Until now the murine local lymph node assay (LLNA) assay has served the purpose

of both identifying sensitizers and determining their potency. However, in March 2013, a European ban on using animals for this purpose was introduced. Growing political and practical resistance to toxicity testing in animals has driven the development of animal-free methods for screening and prioritized developments for toxicants, including those causing allergic hypersensitivity. Over the last decade, several *in vitro* methods have been proposed to identify the potential of chemicals to induce skin sensitization (Corsini and Roggen, 2009; dos Santos et al., 2009; Galbiati et al., 2010; Luebke, 2012; Martin et al., 2010). A number of these assays are currently under prevalidation by EURL-ECVAM, (European Centre for Validation of Alternative Methods) but no accepted *in vitro* method or integrated strategy for the identification of sensitizing chemicals is yet available.

Skin sensitization is an immune mediated reaction to low molecular weight exogenous chemicals. Both skin resident cells, such as keratinocytes (KC), Langerhans cells (LC), mast cells, and immigrating leukocytes, including T lymphocytes and natural killer cells, actively participate in contact allergy (Cavani et al., 2007). The present study focuses on the role of KC in sensitization and in particular their potential

Abbreviations: CA, cinnamaldehyde; C-OH, cinnamic alcohol; DiSFeB, Dipartimento di Scienze Farmacologiche e Biomolecolari; DNCB, dinitrochlorobenzene; DMF, dimethylformamide; DMSO, dimethylsulfoxide; DSA₀₅ (μg/cm²), induction dose per skin area (DSA) that produces a positive response in 5% of the tested population; EE, epidermal equivalent; EUG, eugenol; HBA, hydroxybenzoic acid; GLC, glycerol; GXL, glyoxal; HBA, hydroxybenzoic acid; IL-18, interleukin-18; LAC, lactic acid; MBT, 2-mercaptobenzothiazole; NBB, 4-nitrobenzyl bromide; PHL, phenol; PPD, p-phenylenediamine; RES, resorcinol; SDS, sodium dodecyl sulphate; VUMC, VU University Medical Centre.

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integration into *in vitro* assays to both identify contact sensitizers (yes/no answer) and also to determine sensitizer potency. KC play a role in all phases of allergic contact dermatitis, from the early initiation phase with penetration through the stratum corneum (bioavailability), the secretion of inflammatory cytokines required for LC migration and T-cell trafficking, the peak of the inflammatory phase with direct interactions with epidermotrophic T-cells, through to the resolution phase of allergic contact dermatitis with the production of anti-inflammatory cytokines and tolerogenic antigen presentation to effector T-cells (Adler et al., 2011; Gober and Gaspari, 2008).

Within the Framework 6 (FP6) European financed project Sens-it-iv 2005–2011, the NCTC2544 IL-18 assay was developed to identify contact sensitizers. The assay was demonstrated to be useful in the identification and discrimination of contact allergens from respiratory sensitizers and irritants (Corsini et al., 2009, 2012; Galbiati et al., 2011). The assay was designed to cover the initial phase of contact sensitization 'local trauma – pro-inflammatory cytokine production (danger signals)', necessary for maturation and migration of dendritic cells, and T-cell activation. IL-18, formerly known as IFN- γ -inducing factor (IGIF), which belongs to the IL-1 cytokine family, is a potent inducer of IFN- γ by activated T-cells (Okamura et al., 1995). IL-18 was chosen as biomarker since this cytokine has been shown to play a key proximal role in the induction of allergic contact sensitization (Antonopoulos et al., 2008; Cumberbatch et al., 2001; Okamura et al., 1995). Human KC constitutively express IL-18 mRNA and protein which is induced following exposure to contact sensitizers (Naik et al., 1999; Van Och et al., 2005).

The second assay developed within the FP6 European financed project Sens-it-iv is the EE potency assay. Reconstructed epidermal models (EE) using primary human keratinocytes are currently used to test whether a chemical has irritant/corrosive properties or not (Fentem et al., 1998; Liebsch et al., 2000; Spielmann et al., 2007). The initial protocol for the potency classification was based on the clinical observation that there is a clear role of irritancy in contact sensitization due to the

irritant property of many allergens (Agner et al., 2002; Basketter et al., 2007; Bonneville et al., 2007; McLelland et al., 1991). Chemicals must cause sufficient local trauma to increase cutaneous cytokine production. The irritant capacity of allergens represents an additional risk factor for sensitization induction (Grabbe et al., 1996). It has been shown that there is a relationship between the strength of the sensitizer and the irritant potential of the chemical (dos Santos et al., 2011; Spiekstra et al., 2009). The EE potency assay on its own is unable to distinguish sensitizers from non-sensitizers.

The purpose of this study was to explore the possibility of combining both assays into a single EE assay. The advantage of the proposed assay would be the possibility to identify in the same test the allergenic potential of a chemical (by the amount of IL-18 release) and its potency (by its irritant potential). The *in vitro* reconstructed 3D epidermis model utilized by the assay allows a chemical exposure that mimics human exposure (topical application), and overcomes all drawbacks of traditional submerged culture, including chemical solubility and stability in culture medium. Furthermore, 3D organotypic epidermis models that possess *in vivo*-like barrier properties and metabolizing capabilities may provide significant benefits over monolayer culture models for the assessment of contact sensitization potential.

Materials and methods

Epidermal equivalents (EE)

epiCS® previously known as EST1000™ (Epidermal Skin Test-1000) (CellSystems, Biotechnology GmbH, Troisdorf, Germany); SkinEthic™ RHE (Reconstructed Human Epidermis) (SkinEthic Laboratories, Lyon, France); EpiDerm™ (MatTek Corp., Ashland, USA and Bratislava, Slovakia) and VU University in house EE (VUMC-EE) were used in this study. EE were maintained according to the suppliers' instructions at 37 °C, 5% CO₂ and 95% relative humidity. After 24 h (1–24 h for EpiDerm™) of equilibration time, EE were exposed to chemicals as described below

Table 1
Tested chemicals and vehicles.

Chemical	CAS #	Category	Vehicle ^a			
			epiCS®	SkinEthic™ RHE	EpiDerm™	VUMC-EE
Oxazolone ^b	15646–46–5	Contact allergen	AOO			AOO
DNCB ^b	97–00–7	Contact allergen	AOO	EtOH/AOO/DMF	AOO/EtOH	DMSO
Formaldehyde ^b	50–00–0	Contact allergen	DMSO			
Isoeugenol ^b	97–54–1	Contact allergen	AOO			DMSO
Phenylacetone aldehyde ^b	122–84–9	Contact allergen	AOO			
Cinnamaldehyde ^b	104–55–2	Contact allergen	AOO		EtOH	DMSO
2-mercaptobenzothiazole ^b	149–30–4	Contact allergen	AOO		EtOH:DMSO 4:1	
α -hexylcinnamaldehyde ^b	101–86–0	Contact allergen	AOO			
Eugenol ^b	97–53–0	Contact allergen	AOO	EtOH/AOO/DMF	EtOH	DMSO
Benzocaine ^b	94–09–7	Contact allergen	AOO			
Citral ^b	5392–40–5	Contact allergen	AOO	EtOH/AOO/DMF		
Cobalt (II) chloride ^b	7646–79–9	Contact allergen	DMSO			
p-Phenylenediamine ^b	122–84–9 epiCS® 122–84–9 EpiDerm™	Contact allergen	AOO		EtOH	
Cinnamic alcohol	104–54–1	Contact allergen			EtOH	
4-Nitro Benzylbromide	100–11–8	Contact allergen			EtOH	
Glyoxal	107–22–2	Contact allergen			Water	
Resorcinol	108–46–3	Contact allergen			Water	
SDS	151–21–3	Irritant	DMSO		Water	
Octanoic acid	124–07–2	Irritant	AOO			
Lactic acid	50–21–5	Irritant	AOO		Water	
Phenol	108–95–2	Irritant	DMSO		Water	
Methyl salicylate	9041–28–5	Irritant	AOO			
Salicylic acid	69–72–7	Irritant	AOO			
Tween 20	9005–64–5	Irritant	AOO			
Chlorobenzene	108–90–7	Irritant	AOO			
Hydroxybenzoic acid (HBA)	99–96–7	Irritant	AOO			
Glycerol	56–81–5	Non-irritant			None	

^a The vehicles used in this study for dissolving chemicals before applying topically to EE. DMSO = 1% DMSO in culture medium; AOO = acetone: olive oil (4:1); EtOH = ethanol; Neat = 100%; Water = distilled H₂O; n.d. = not determined; For details on vehicle and chemical suppliers, see Materials and Methods.

^b Tested coded at DiSFeB and VUMC in the EE potency assay.

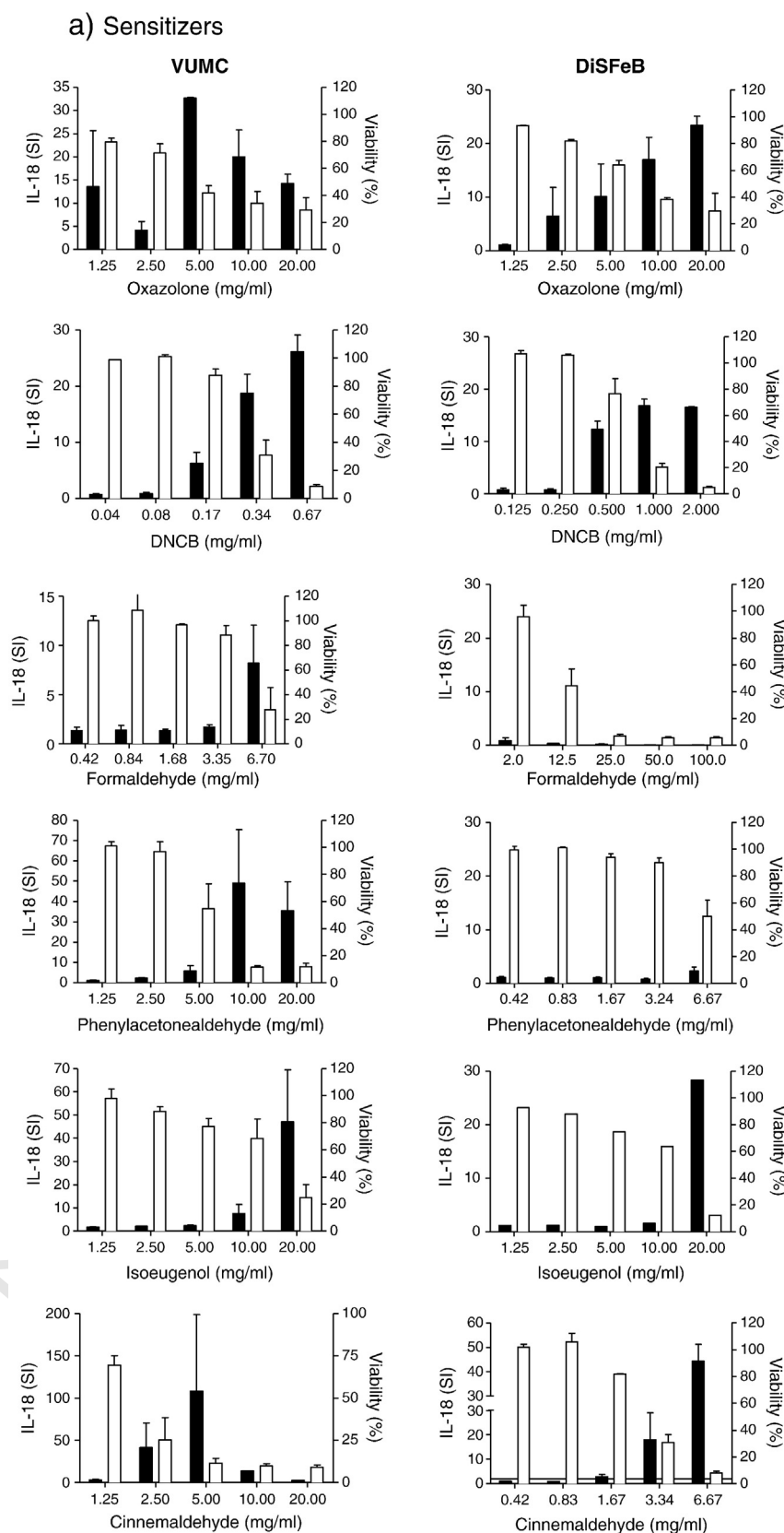


Fig. 1. Dose response effects of the selected sensitizers (A) and non-sensitizers (B) on IL-18 release and cell viability in the epiCS® EE model in VUMC and DiSFeB laboratories. EE were exposed to chemicals as described in Materials and Methods and Table 1. IL-18 was assessed by ELISA and results are expressed as stimulation index (SI) compared to vehicle alone. Cell viability was assessed by MTT reduction assay and is expressed relative to vehicle. Sensitizers were tested independently in duplicate in both laboratories. For non-sensitizers: SDS, lactic acid and methyl salicylate were tested at the VUMC laboratory whilst HBA, phenol, chlorobenzene, octanoic acid, tween 20 and salicylic acid were tested at the DiSFeB laboratory. Filled bars represent the IL-18 release, while open bars the viability. With the exception of isoeugenol in DiSFeB in which only 1 run was performed, results are expressed as average $\pm$ difference, $n = 2$ independent experiments using different batches of EE.

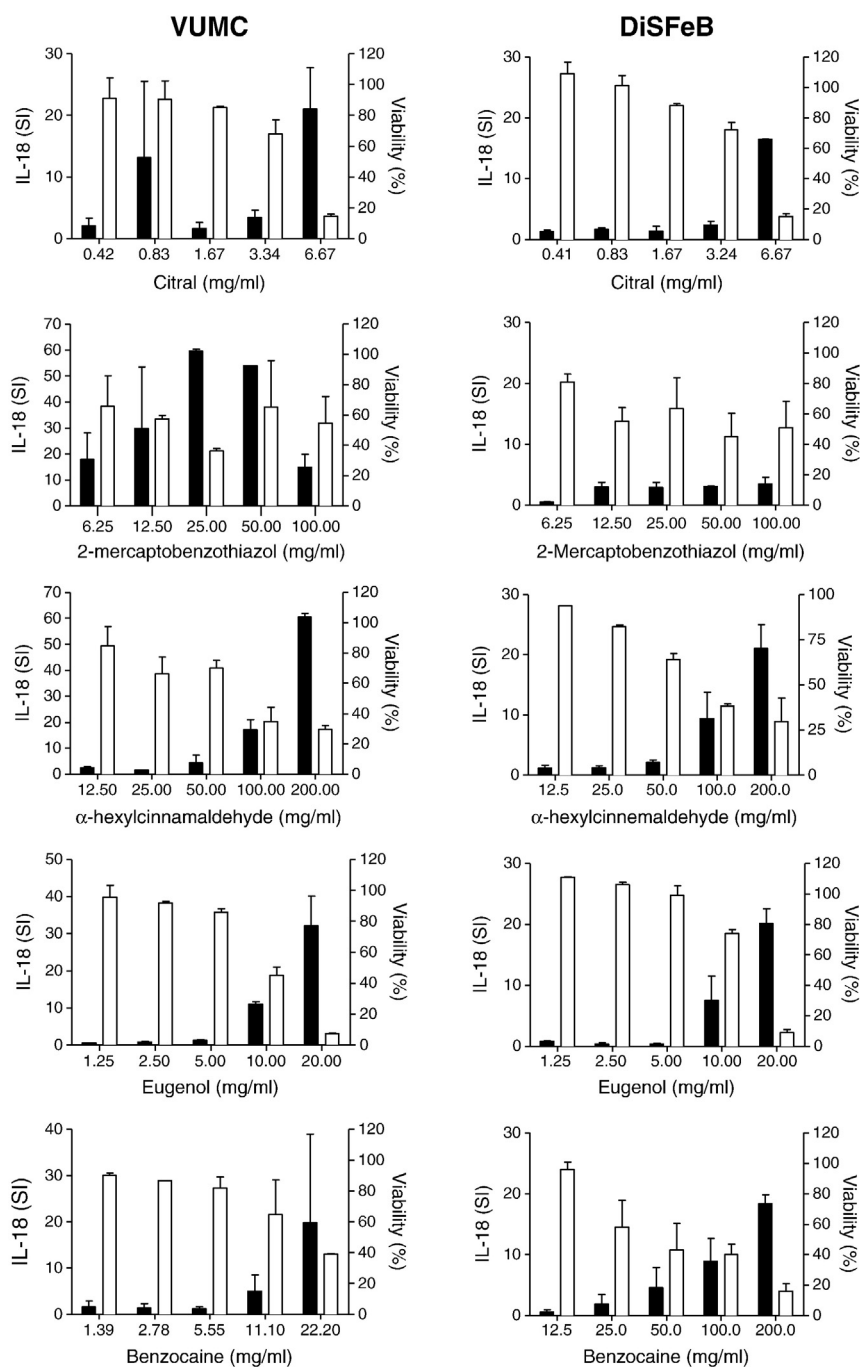


Fig. 1 (continued).

and as described in detail previously (dos Santos et al., 2011; Teunis et al., 2012). All reconstructed epidermal models were derived from normal human keratinocytes. For VUMC-EE, human foreskin was obtained from healthy donors with informed consent. The VU University Medical Centre approved the experiments described in this paper. The study was conducted according to Declaration of Helsinki Principles.

Test chemicals and chemical exposure

A total of 17 contact sensitizers and 10 non-sensitizers were studied (Table 1). At DiSFeB and VUMC, the 13 sensitizers, marked with an asterisk in Table 1, were tested coded in order to further challenge the EE potency assay using the epiCS® model.

At DiSFeB and VUMC where epiCS® was used (and VUMC-EE for limited experiments): (1) EE were allowed to equilibrate for 24 h at 37 °C, 5% CO₂ and then; (2) chemicals were dissolved in either acetone olive oil (AOO 4:1) or 1% DMSO in culture medium. At VUMC and DiSFeB the vehicles and chemicals were purchased from the same supplier: Acetone (Cat. No. 20066.321) from VWR (Radnor, Pennsylvania (VS); olive oil (cat no. 01514) and DMSO (Cat. No. 154938) from Sigma-Aldrich (St. Louis, MO, USA). With the exception of 2-Mercaptobenzothiazole which was purchased from Fisher-Scientific (ACROS Organics; Loughborough, UK), all chemicals were purchased from Sigma-Aldrich (Sigma, Aldrich, SAFC; St Louis, Missouri, USA). Chemicals were >95% pure with the exception of formaldehyde which was 36.5%–38% in H₂O. Isoeugenol was a 98% mixture of the cis and trans form and oxazolone was purified by recrystallization. For experiments, Finn Chamber filter paper discs 7.5

b) Non Sensitizers (including irritants)

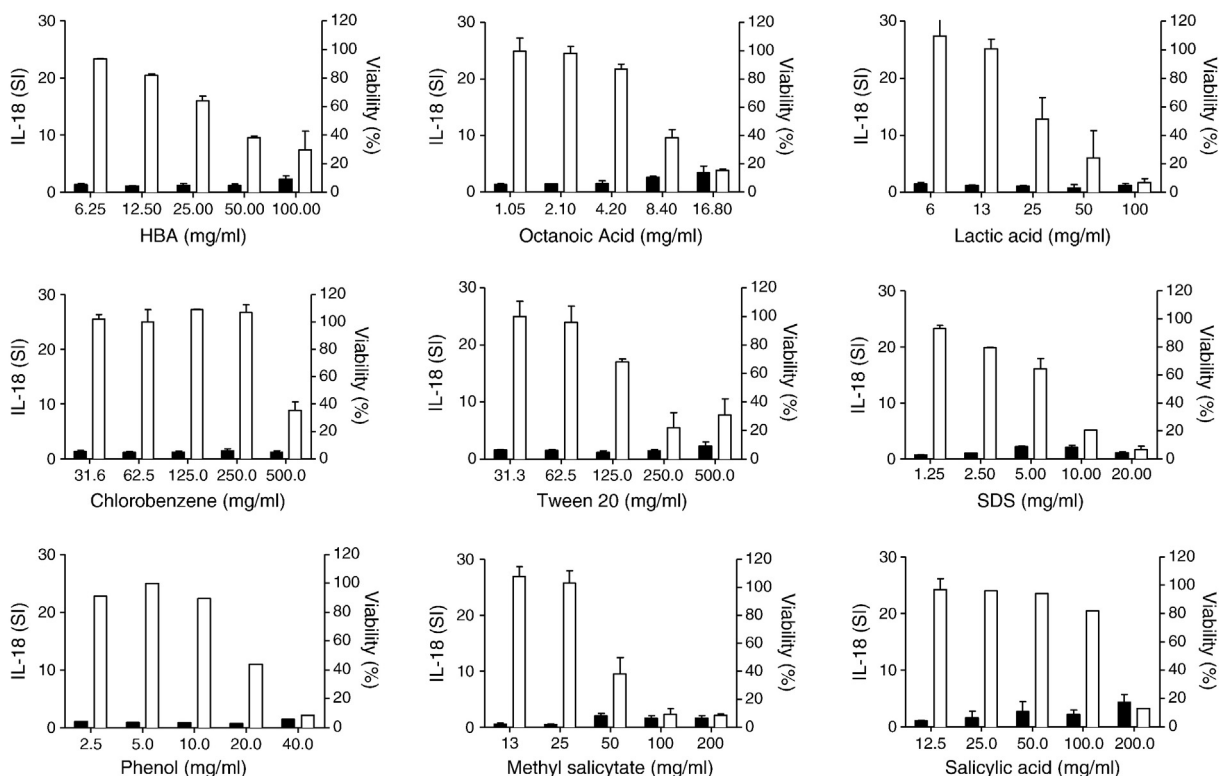


Fig. 1 (continued).

(for epiCS®) or 11 mm (for VUMC-EE) (Epitest LTD Oy, Finland) were impregnated with chemicals or vehicle controls and applied topically to the EE stratum corneum; (3) cultures were incubated with chemicals for 24 h; (4) after chemical exposure, filter paper discs were removed and; (5) metabolic activity was determined immediately by the MTT assay and culture supernatants were harvested and stored at -20°C for IL-18 analysis by ELISA. Chemical exposure and dose finding was performed as described in detail previously (dos Santos et al., 2011; Teunis et al., 2012).

In parallel, at MatTek and MB Research Labs where EpiDerm™ was used, a protocol was developed in which 12 chemicals were tested: (1) Epiderm tissues were allowed to equilibrate for 1–24 h at 37°C , 5% CO_2 and then; (2) test chemicals were directly pipetted to the EE surface, dissolved in either ethanol (25 μl) for lipid soluble compounds or distilled water (100 μl) for water-soluble compounds (Deng et al., 2011). The only exception was ethanol: DMSO 4:1 (25 μl) which was used as vehicle for 10% MBT exposure of EpiDerm™ (Table 1). For MB Research Labs and MatTek Corp., absolute ethanol (200 proof), sterile tissue culture water (ultrapure) and all other chemicals were at least 98% pure ACS grade, and were purchased from Sigma-Aldrich (St. Louis, MO, USA). Sterile DMEM-based tissue culture medium was supplied by MatTek (Ashland, MA, USA); (3) cultures were incubated with chemicals for 24 h; (4) after chemical exposure, the epidermal surface was washed with PBS to remove chemicals and; (5) metabolic activity was determined immediately by the MTT assay and culture supernatants were harvested and stored at -20°C for IL-18 analysis by ELISA.

In addition, a comparison of the effects of different vehicles (AOO, ethanol, water) on 0.15% DNCB, 0.3% citral and 1.0% eugenol induced IL-18 release and cell viability was conducted. This experiment was performed at MB Research in SkinEthic™ RHE EE model, $n = 2$ replicates from a single experiment.

MTT assay and IL-18 ELISA

The MTT assay measures mainly mitochondrial activity, which is representative of cell viability, by quantifying dehydrogenase activity. After a 24 h exposure, test chemicals or filter paper discs impregnated with chemicals were removed from EE or the epidermal surface and then washed with PBS to remove chemicals (the MatTek protocol). The MTT analysis was performed in 24-well plates. Thiazolyl blue tetrazolium bromide (MTT) (Sigma) solution in PBS (0.5 mL of a 5 mg/mL solution) was added to the 24-well plate, EE cultures were placed on top and further incubated for 2–3 h under standard culture conditions. Cultures were then transferred to a new 24 well plate containing 0.3–0.9 mL isopropanol to dissolve the formazine crystals. Plates were incubated overnight, covered with parafilm and protected from the light at room temperature. The absorbance of the formazan dissolved in isopropanol was measured at either 540 or 570 nm and expressed as a percentage relative to the absorbance value of control vehicle.

The amount of IL-18 present in culture supernatants was quantified by a commercially available specific sandwich ELISA according to the supplier's instructions (MBL, Nagoya, Japan). Results were calculated in pg/mL from a standard curve and then converted to a Stimulation Index (SI).

Data analysis

Experiments were performed in duplicate in two of the laboratories (VUMC, DiSFeB) and at least once in each lab by MB Research Labs and MatTek Corp. Different EE batches were used in each experiment. Data are presented as an average of the 2 independent experiments $\pm$ difference (VUMC, DiSFeB), or as average $\pm$ difference of duplicate tissues within a single experiment (EpiDerm™).

Table 2

Prediction models for epiCS®.

Chemical	Prediction model 1 IL18 SI ≥ 2 at $\geq$ EC40		Prediction model 2 IL18 SI ≥ 5 at $\geq$ EC5		Prediction model 3 IL18 SI ≥ 5 at $\geq$ EC5 and $\leq$ EC40	
	VUMC	DiSFeB	VUMC	DiSFeB	VUMC	DiSFeB
Extreme						
oxazolone	4.1 $\pm$ 2.7	10.1 $\pm$ 8.7	32.7 $\pm$ 0.2	23.4 $\pm$ 2.4	32.7 $\pm$ 0.2	23.4 $\pm$ 2.4
DNCB	6.3 $\pm$ 2.9	12.3 $\pm$ 2.29	26.2 $\pm$ 4.2	16.8 $\pm$ 1.8	26.2 $\pm$ 4.2	16.8 $\pm$ 1.8
Strong						
formaldehyde	1.7 $\pm$ 0.3^a	0.35 $\pm$ 0.05	8.2 $\pm$ 5.5	0.9 $\pm$ 0.85^a	8.2 $\pm$ 5.5	0.25 $\pm$ 0.05
Moderate						
Phenylacetonealdehyde	5.8 $\pm$ 3.8	2.3 $\pm$ 1.1	35.6 $\pm$ 20.1	2.3 $\pm$ 1.1^b	35.6 $\pm$ 20.1	(2.3 $\pm$ 1.1 ^b)
Isoeugenol	7.6 $\pm$ 5.4	1.6	47.0 $\pm$ 31.7	28.3	47.0 $\pm$ 31.7	28.3
Cinnamaldehyde	3.2 $\pm$ 1.12	2.9 $\pm$ 1.3	108 $\pm$ 128	44.4 $\pm$ 9.6	108 $\pm$ 128	44.4 $\pm$ 9.6
citral	3.4 $\pm$ 1.7	2.4 $\pm$ 0.9	21.0 $\pm$ 9.5	16.5 $\pm$ 0.1	21.0 $\pm$ 9.5	16.5 $\pm$ 0.1
2-mercaptobenzothiazol	54.0 $\pm$ 0.0	3.0 $\pm$ 1.3	59.7 $\pm$ 0.9	3.5 $\pm$ 1.9^b	59.7 $\pm$ 0.9	(3.5 $\pm$ 1.9 ^b)
Weak						
α -hexylcinnamaldehyde	4.4 $\pm$ 4.3	2.1 $\pm$ 0.59	60.5 $\pm$ 2.0	21.1 $\pm$ 5.6	60.5 $\pm$ 2.0	21.1 $\pm$ 5.6
eugenol	11.1 $\pm$ 0.9	7.5 $\pm$ 5.7	32.1 $\pm$ 11.4	20.2 $\pm$ 3.4	32.1 $\pm$ 11.4	20.2 $\pm$ 3.4
Benzocaine	5.0 $\pm$ 4.9	8.9 $\pm$ 5.4	19.8 $\pm$ 27.2	18.4 $\pm$ 2.0	19.8 $\pm$ 27.2	18.4 $\pm$ 2.0
Non-sensitizers						
SDS	2.2 $\pm$ 0.1		2.2 $\pm$ 0.1		2.2 $\pm$ 0.1	
Octanoic acid		1.5 $\pm$ 0.8		3.4 $\pm$ 1.7		3.4 $\pm$ 1.7
Lactic acid	1.1 $\pm$ 0.3		1.5 $\pm$ 0.3		1.5 $\pm$ 0.3	
Phenol	6.7 $\pm$ 3.1	0.8 $\pm$ 0.0	12.6 $\pm$ 5.6	1.1 $\pm$ 0.1	12.6 $\pm$ 5.6	1.1 $\pm$ 0.1
Methylsalicylate	0.5 $\pm$ 0.2		2.1 $\pm$ 0.5		2.1 $\pm$ 0.5	
Salicylic acid		2.2 $\pm$ 1.1		4.3 $\pm$ 1.9		4.3 $\pm$ 1.9
Tween 20		1.2 $\pm$ 0.5		2.3 $\pm$ 1.0		2.3 $\pm$ 1.0
Chlorobenzene		1.5 $\pm$ 0.3		1.5 $\pm$ 0.3		1.5 $\pm$ 0.3
HBA		1.2 $\pm$ 0.3		2.3 $\pm$ 1.2		2.3 $\pm$ 1.2

Highest SI level of IL-18 release observed within the defined criteria for the different prediction models (see also Fig. 1). For isoeugenol, DiSFeB had data only from 1 run.

False positives and negatives in the prediction models are shown bold underlined. Values obtained from chemicals which do not fulfill the acceptance criteria of the prediction model are shown in brackets.

Prediction model 1: IL-18 SI $2x$ at $\geq$ EC40, VUMC have 1 false negative and 2 false positives. DiSFeB has 2 false negatives and 1 false positive. The maximum IL-18 release (mg/ml) observed in the dose response (see Fig. 1) at a cell viability $\geq$ 40% relative to the vehicle is shown.

Prediction model 2: IL-18 SI $5x$ at $\geq$ EC5, VUMC has in only 1 false positive. DiSFeB has 3 false negatives. The maximum IL-18 release (mg/ml) observed in the dose response (see Fig. 1) at a cell viability $\geq$ 5% relative to the vehicle is shown.

Prediction model 3: IL-18 SI $5x$ at $\geq$ EC5 and $\leq$ 40, VUMC has only 1 false positive and DiSFeB has 1 false negative. The maximum IL-18 release (mg/ml) observed in the dose response (see Fig. 1) at a cell viability $\geq$ 5% and $\leq$ 40% relative to the vehicle is shown.

With the exception of isoeugenol in DiSFeB in which only 1 run was performed, results are expressed as average $\pm$ difference, $n = 2$ independent experiments using different batches of EE.

^a Viability $\geq$ 90%.

^b Viability is $> 50\%$, EC5 and EC40 = 5% and 40% cell viability respectively.

Sensitizer labelling by means of assessing IL-18 release: different prediction models were developed for the epiCS® and EpiDerm™ models. Based on data obtained testing sensitizers and non-sensitizers, for the epiCS® model a cut-off of 5-fold increase of IL-18 release above vehicle control and $\leq$ 40% cell viability was found to be the best for the discrimination of contact allergens, while for EpiDerm™ a cut-off of 1.6-fold increase of IL-18 above vehicle control and between 5 and 50% cell viability (Prediction Model 1.6 $\times$) was found to be optimal to identify allergens.

EE potency assay: the EE-EC₅₀ value is the effective chemical concentration required to reduce metabolic activity (corresponding to cell viability) to 50% of the maximum value. The 100% value for cell viability corresponds to the vehicle control (1% DMSO culture medium or acetone/olive oil 4: 1). EE-EC₅₀ values were obtained by linear regression analysis based on changes in metabolic activity (MTT). Statistical significances were determined by Kruskal–Wallis (nonparametric, one way ANOVA) using GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego, CA, USA). Differences were considered significant for $p < 0.05$.

IL-18 potency assessment: IL-18 SI-2 and SI-5 were obtained by linear regression analysis based on the chemical concentration resulting in a 2 fold or 5 fold release in IL-18. Statistical significances were determined by Kruskal–Wallis (nonparametric, one way anova) using GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego, CA, USA). Differences were considered significant for $p < 0.05$.

Correlations were determined by nonparametric two-tailed correlation Spearman Analyses using GraphPad Software, San Diego, CA, USA.

Results

Dose dependent increase in IL-18 release by sensitizers but not non-sensitizers

A total of 17 sensitizers ranging from extreme, strong, moderate and weak and 10 non-sensitizers were selected for this study and tested using different EE models (Table 1). In the first series of experiments, Cell Systems epiCS® were exposed topically to 13 coded sensitizers and 9 non-sensitizers in a dose dependent manner for 24 h according to the EE potency assay protocol in two laboratories (VUMC, DiSFeB) (Teunis et al., 2012). Culture viability was determined by the MTT assay and IL-18 release into culture supernatants by ELISA. Of the 13 sensitizers and 9 non-sensitizers selected for this study, only 2 chemicals (cobalt II chloride and p-Phenylenediamine (PPD)) had to be excluded due to their interference with the MTT colorimetric assay. For the remaining 11 sensitizers and 9 non-sensitizers, an increase in IL-18 release was generally observed only after exposure to sensitizers but not after exposure to non-sensitizers in both VUMC and DiSFeB laboratories (Figs. 1A and B). Results were further analyzed in order to identify a putative prediction model for identifying sensitizers.

For epiCS®, three prediction models were tested (Table 2). Prediction model 1 ($\geq 40\%$ viability (EC40); ≥ 2 fold increase in IL-18): resulted in VUMC correctly identifying 10/11 sensitizers and DiSFeB correctly identifying 9/11 sensitizers. Only formaldehyde (VUMC and DiSFeB) and isoeugenol (DiSFeB) scored as a false negative in prediction model 1. With regards to the non-sensitizers VUMC correctly identified 2/4 chemicals and DiSFeB correctly identified 5/6 chemicals. Since it was noticed that in general IL-18 release was greater after sensitizer exposure at higher concentrations, prediction model 2 was next tested.

Prediction model 2 ($\geq 5\%$ viability (EC5); ≥ 5 fold increase in IL-18) resulted in the VUMC correctly identifying all sensitizers and DiSFeB correctly identifying 8/11 sensitizers. It should be noted though that the 2/3 false negatives in prediction model 2 in DiSFeB corresponded to dose response curves in which very little cytotoxicity was observed.

EE were still $> 40\%$ viable after exposure to phenylacetone aldehyde and 2-mercaptobenzothiazol. Formaldehyde was the third false negative scored in DiSFeB even though high levels of cytotoxicity were reached. With regards to the non sensitizers VUMC correctly identified 3/4 non sensitizers and DiSFeB correctly identified all 6 non-sensitizers. These results led to the testing of prediction model 3 in which the greater release of IL-18 within a defined cytotoxicity range was tested.

Prediction model 3 ($\geq 5\%$ and $\leq 40\%$ viability (EC5–40); ≥ 5 fold increase in IL-18 release: resulted in exclusion of the 2 false negatives from DiSFeB as critical cytotoxic levels were not reached and therefore all sensitizers with the exception of formaldehyde in DiSFeB were now correctly identified in both laboratories. With regards to non-sensitizers, the results were the same as those obtained in prediction model 2 namely VUMC correctly identified 3/4 non sensitizers and

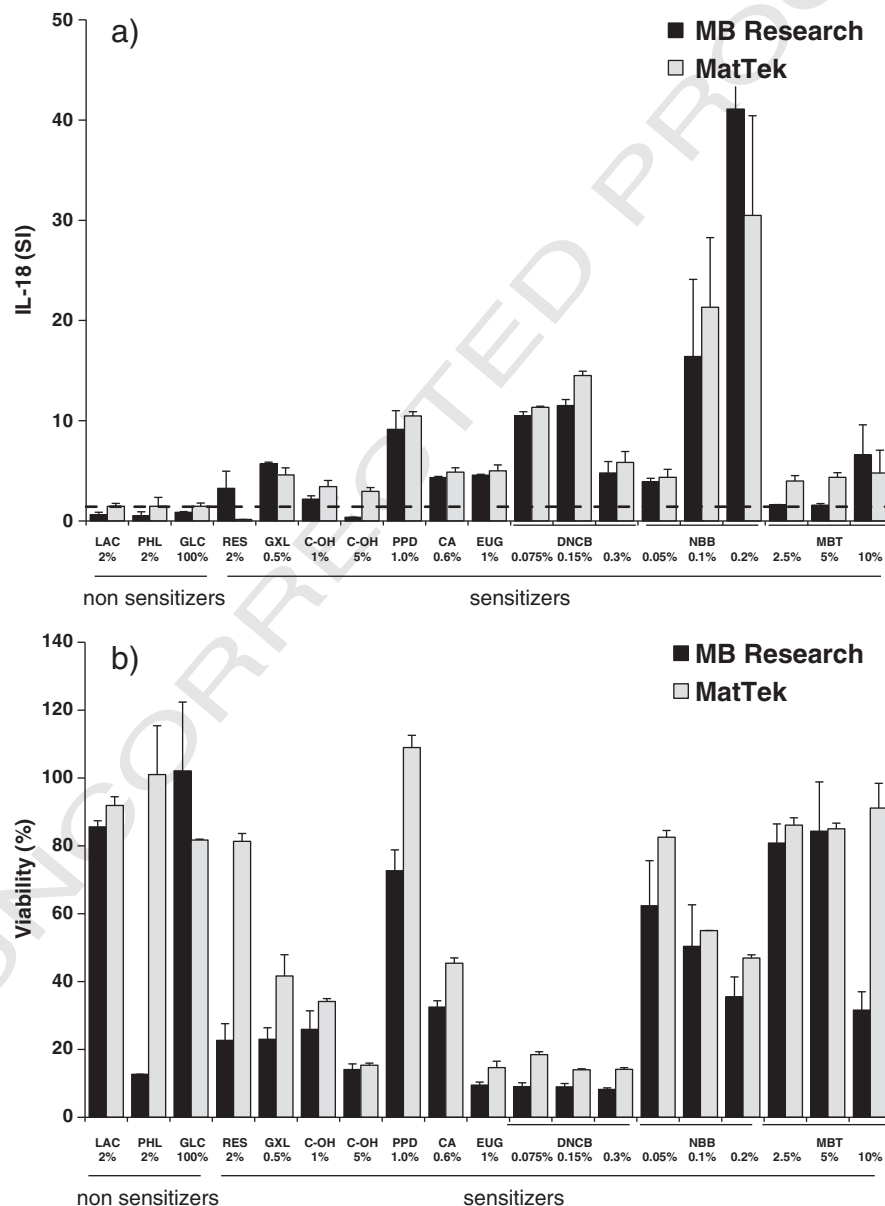


Fig. 2. IL-18 secretion and tissue viability using EpiDerm™. EE were exposed to chemicals as described in Materials and Methods and Table 1. MatTek Corporation and MB Research Labs independently tested IL-18 secretion in duplicate EpiDerm™ tissues. Test chemicals were dissolved in a vehicle of either ethanol or water, with the exception of MBT which was dissolved in 25 μ l of EtOH:DMSO 4:1. After 24 h topical exposure to chemicals, culture supernatant was analyzed by IL-18 ELISA, and an SI was calculated as a fold change above vehicle control (Panel A). An IL-18 SI value of 1.6 or above is the criterion for a positive response is indicated by the dotted line. Tissue viability was determined by MTT assay (Panel B) The figures depict average $\pm$ difference of duplicate EE within a single experiment in each laboratory.

DiSFeB correctly identified all 6 non-sensitizers. Therefore for chemical exposures which fulfilled the criteria for prediction model 3, all but one chemical in each of the laboratories were correctly identified (phenol scored false positive in VUMC, and formaldehyde scored false negative in DiSFeB).

Prediction model 3 using epiCS® was therefore considered the most optimal putative prediction model since it showed 95% accuracy in both VUMC and DiSFeB laboratories. Of note, this prediction model is dependent on the chemical resulting in high cytotoxicity (5–40% viability; 60–95% cytotoxicity) in the EE.

In parallel, at MB Research Labs and MatTek Corp, 9 contact sensitizers (DNCB, NBB, PPD, CA, C-OH, GXL, RES, MBT, and EUG) and 3 non-sensitizers (PHL, LAC, and GLC) were tested using the EpiDerm™ EE model (Fig. 2). Test chemicals were dissolved in a vehicle of either ethanol or water. Ethanol alone caused an IL-18 secretion of 12.5 and 15.1 pg/ml, and water caused secretion of 5.7 and 14.1 pg/ml, respectively for MB Research Labs and MatTek Corp. In support of the above data, sensitizer exposure resulted in a measurable increase of up to 40 fold in IL-18 release over vehicle controls. Using a 'Prediction Model 1.6x' (optimally between 5% and 50% viability (EC50-50); coupled with a ≥ 1.6 fold increase in IL-18 release as indicative of a positive sensitizer response), 9/9 sensitizers tested at MB Research Labs were correctly identified while at MatTek 8/9 sensitizers were correctly identified (RES was a false negative). With regards to the 3 non-sensitizers, at the single chemical concentration tested (LAC 2%, PHL 2% and GLC 100%), no chemical exposure resulted in a ≥ 1.6 -fold increase in IL-18, and therefore all non-sensitizers at these concentrations were correctly predicted as negative.

Taken together our results highlight the advantages of the dose response and demonstrate the ability of the IL-18 endpoint to identify sensitizers in the epiCS® and EpiDerm™ 3D-epidermal models.

Epidermal equivalent EC₅₀ values rank sensitizer potency

Next it was determined whether the EC₅₀ values identified from the 11 coded sensitizers in the dose response experiments, which were used to quantify IL-18 release in the VUMC and DiSFeB laboratories, could be used to rank sensitizer potency. Table 3 shows that both laboratories obtained EC₅₀ values of similar orders of magnitude. Extreme/strong sensitizers classified according to LLNA generally had a lower EC₅₀ value than weak sensitizers. EC₅₀ values for the moderate sensitizers generally ranged between the weak and strong sensitizers (Table 3). When results of both laboratories were combined and a correlation was performed with the LLNA and human DSA₀₅ data, ranking according to the EE potency assay was found to significantly correlate to the ranking obtained by the LLNA (spearman $r = 0.5968$; P value (two-tailed) = 0.0542) and to human DSA₀₅ data (spearman $r = 0.8500$; P value (two-tailed) = 0.0061) (Fig. 3A; Table 3). Of note, correlation between the EE-EC₅₀ data and human DSA₀₅ data was clearly better than between EE-EC₅₀ data and LLNA data.

Next we determined whether the IL-18 SI-2 and SI-5 could also be used to rank sensitizer potency. As can be seen in Figs. 3B and C, the correlation of our *in vitro* human data was again better with human data than with LLNA data. Indeed the correlation SI-2 with human DSA₀₅ data was spearman $r = 0.8333$; P value (two-tailed) = 0.0154 and the correlation SI-5 with human DSA₀₅ data was spearman $r = 0.6267$; P

Table 3
comparison of LLNA_{EC3}, human DSA₀₅ and EE-EC₅₀ values for all sensitizers tested in EE potency assay using VUMC-EE and epiCS®.

Substance	LLNA _{EC3} (%)	Human DSA ₀₅ (μg/cm ²)	EE-EC ₅₀			
			VUMC-EE	epiCS®		
				VUMC	DiSFeB	average
Extreme						
oxazolone	0.001–0.003	–	1.31	1.5 ± 0.2	7.4 ± 1.0	4.45 ± 2.95
dinitrochlorobenzene	0.012–0.047	2.1–5.5	0.13	0.3 ± 0.0	0.7 ± 0.2	0.5 ± 0.2
4-nitrobenzylbromide	0.05	–	0.07			
Strong						
p-phenylenediamine	0.11–2.5	6.9–345	NR	NR	NR	
Formaldehyde	0.28–0.82	89–411		5.7 ± .90	11.8 ± 3.52	8.76 ± 3.05
Phenylacetaldehyde	3	133–938		5.7 ± 1.7	7.7 ± .50	6.7 ± 1.0
Cinnamaldehyde	0.42–3.0	157–1111	1.99	1.9 ± 0.1	2.8 ± 0.4	2.35 ± 0.45
Cobalt chloride	–	172–453		NR	NR	
Moderate						
Citral	2.75–13.2	310–1691		4.4 ± 0.6	4.7 ± 0.2	4.55 ± 0.15
Glyoxal	0.60–1.4	345	12.07			
Isoeugenol	0.92–2.50	775–1333	0.55	15.8 ± 1.3	28.6 ± 16.5	22.2 ± 6.4
2-bromo-2-(bromomethyl) pentanedinitrile	1.3	–	0.76			
2-mercatobenzothiazol	1.7–9.7	1642–2269	No EC ₅₀	16.6 ± 1.4	13.8 ± 4.4	15.2 ± 1.4
Tetramethylthiuram disulfide	5.2–5.92	3832–5388	No EC ₅₀			
Resorcinol	5.5	–	2.64			
Weak						
Eugenol	5.3–18.16	5926	4.46	8.6 ± 2.1	16.4 ± 0.5	12.5 ± 3.9
Cinnamic alcohol	21	625–13122	0.86			
Benzocaine	3.37–22	3831–41667	–	14.8 ± 7.0	48.1 ± 41.5	31.45 ± 16.65
α-hexylcinnamaldehyde	8.6–10.14	–		80.0 ± 15.5	76.9 ± 2.8	78.45 ± 1.55

Table 3 shows all EC₅₀ data obtained until present for the EE potency assay derived from this manuscript and dos Santos et al. (2011).

Human DSA₀₅ (μg/cm²) = induction dose per skin area (DSA) that produces a positive response in 5% of the tested population.

The LLNA_{EC3} values expressed as% and its relative potency classification in parenthesis are reported. According to Basketter et al. (1999), potency classification is based on the mathematical estimation of the concentration of chemical necessary to obtain a threshold positive response (SI = 3); this is termed the EC3 value. Chemicals with an EC3 value (%) > 10 to < 100 are classified as weak, > 1 to < 10 moderate, > 0.1 to < 1 strong, < 0.1 extreme.

For LLNA and human data: – indicates no data available; for EE-EC₅₀: NR = indicates interference with MTT assay therefore no reliable EC₅₀ obtained; No EC₅₀ = due to low solubility of the chemical no EC₅₀ was obtained (dos Santos et al., 2011).

Sensitizers are ranked taking into account ranges obtained from both LLNA and human DSA₀₅ data from the following studies: (Danneman et al., 1983; Friedmann et al., 1983; Gerberick et al., 2005; Griem et al., 2003; ICCVAM, 2011a, 2011b, 2011c; Jordan and King, 1977; Kligman, 1966a, 1966b; Marzulli and Maibach, 1976; Rees et al., 1989; Schneider and Akkan, 2004; Steltenkamp et al., 1980).

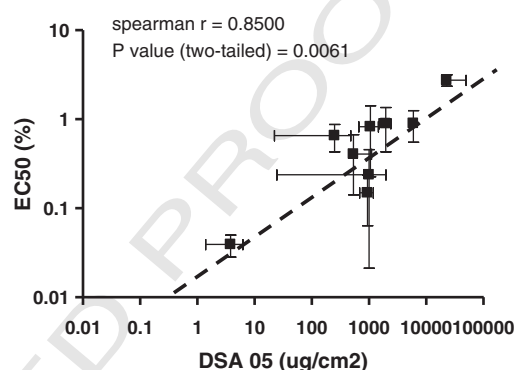
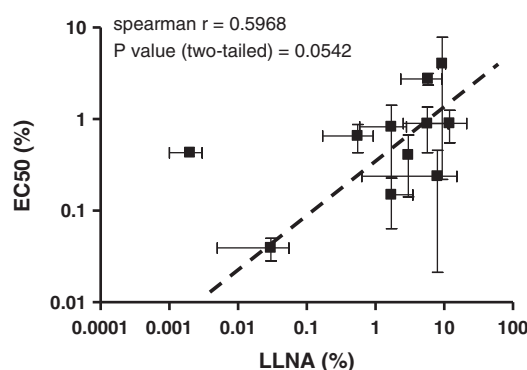
value (two-tailed) = 0.0603 indicating that IL-18 release may supply an additional potency parameter in this assay.

Transferability of EE potency assay to different EE models and effect of different vehicles

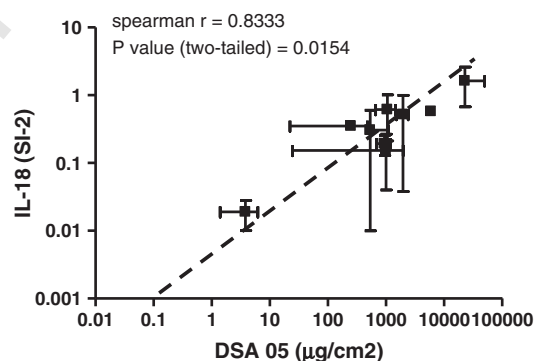
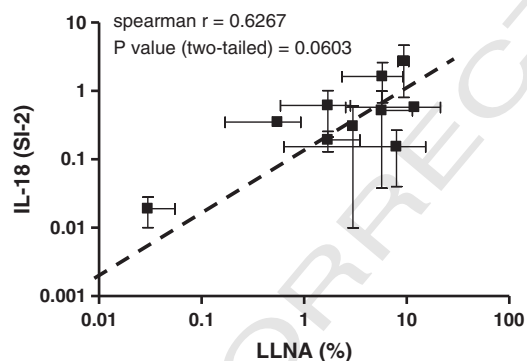
Transferability of EE potency assay to different EE models. In this study, epiCS® was extensively used to further investigate the EE potency assay using coded sensitizers and to determine whether this assay could be combined with an IL-18 readout to identify sensitizers. In order to determine how well the results from one EE type correlate to the results obtained from another EE type, the results from this EE potency study were superimposed onto our previously described results using the

VUMC-EE. The results shown in Fig. 4 indicate that very similar LLNA correlations are obtained for both EE derived from primary human keratinocytes. Oxazolone was a typical outlier when using either EE model. An overall ranking using the combined results of the both EE and 16 chemicals (including oxazolone) was found to significantly correlate to the ranking obtained by the LLNA (spearman $r = 0.52$; P value (two-tailed) = 0.02) and was similar to that obtained by either EE alone (epiCS®: spearman $r = 0.60$; P value (two-tailed) = 0.06; VUMC-EE: spearman $r = 0.58$; P value (two-tailed) = 0.08). This indicates not only that the assay is fully transferable to different EE models but that the results obtained from all different EE may be superimposed onto a single sensitizer ranking correlation graph if identical methodology is used.

a) EE potency assay (EC₅₀)



b) IL-18 release (SI-2)



c) IL-18 release (SI-5)

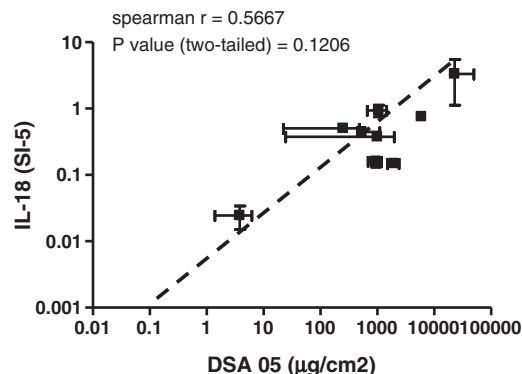
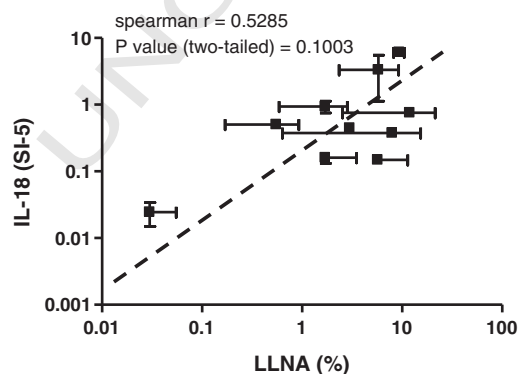


Fig. 3. Correlation of EE assay with LLNA_{EC} and human DSA₀₅. A) Correlation of EE-EC₅₀ data with LLNA_{EC} and human DSA₀₅. B) Correlation of IL-18 SI-2 data with LLNA_{EC} and human DSA₀₅. C) Correlation of IL-18 SI-5 data with LLNA_{EC} and human DSA₀₅. LLNA_{EC} and human DSA data is extracted from Table 3. *In vitro* data is obtained by linear regression analysis based on changes in metabolic activity (MTT) (A) or IL-18 fold release into culture supernatant (B, C). Statistical significances were determined by Kruskal–Wallis (nonparametric, one way ANOVA) using GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego, CA, USA). Differences were considered significant for $p < 0.05$. EE-EC₅₀ data represents the average of the combined data for epiCS® obtained from 2 laboratories (VUMC and DiSeFb) $n = 4 \pm SD$.

The effect of vehicles on different EE viability. Next we compared the effect of three different commonly used vehicles on three different commercially available EE (epiCS®, SkinEthic™ RHE, MatTek EpiDerm™) and one in house EE (VUMC-EE). As can be seen from Fig. 5A, epiCS® and SkinEthic™ RHE produced very similar results after a 24 h exposure to AOO resulting in less than 10% decrease in cell viability and DMSO resulting in approximately 20% decrease in cell viability. For the VUMC-EE less than 10% decrease in cell viability was observed after a 24 h topical exposure to either water, AOO or DMSO. For EpiDerm™ approximately 10% decrease in cell viability was observed after a 24 h topical exposure to either water or AOO. For all EE, the potential cytotoxic effect of the vehicles was within the acceptance criteria of <30% previously proposed for the EE potency assay (Teunis et al., 2012) again supporting the ease of technology transfer of the assay.

Effect of different vehicles and application methods on sensitizer induced IL-18 release. Finally we determined whether different vehicles and application methods influenced the IL-18 readout of the assay. DNCB (150 µg) was topically applied to EpiDerm™ directly in 25 µl of ethanol or 100 µl of AOO (1:20), or by use of a filter disc (25 µl in AOO 4:1). 50 µg of SDS (100 µl in water or 25 µl on a filter paper in water) was applied as reference irritant (Fig. 5B). EpiDerm™ were then incubated for 24 h. At similar cytotoxicity (empty columns), DNCB induced a higher release of IL-18 compared to tissues treated with SDS (SI > 15) as expected independent of the method used for application. However the IL-18 release induced by DNCB was higher if AOO (either directly applied on EE or on filters) was used as vehicle when compared to EtOH. This was later further investigated by MB research using SkinEthic™ RHE with three different sensitizers (DNCB, citral and eugenol) and 3 different vehicles (EtOH, AOO and DMF) (Fig. 5C). In SkinEthic™ RHE, release of IL-18 was similar when either EtOH or AOO was used for vehicle for a given sensitizer. However when DMF was used as vehicle, IL-18 secretion was clearly less. This observation was independent of the degree of cytotoxicity since EtOH and DMF exhibited similar cytotoxicity for each of the sensitizers. AOO appeared to have the least cytotoxic effect on EE when used as vehicle for citral and eugenol. Taken together these results indicate that the choice of vehicle may influence both the amount of IL-18 secretion and the cytotoxicity induced by the chemical together with a vehicle.

Discussion

The goal of this study was to determine whether i) IL-18 released into the culture supernatant of EE models could be used to distinguish sensitizers from non-sensitizers and ii) whether the assay protocol could successfully rank potency of sensitizers. Results obtained in this

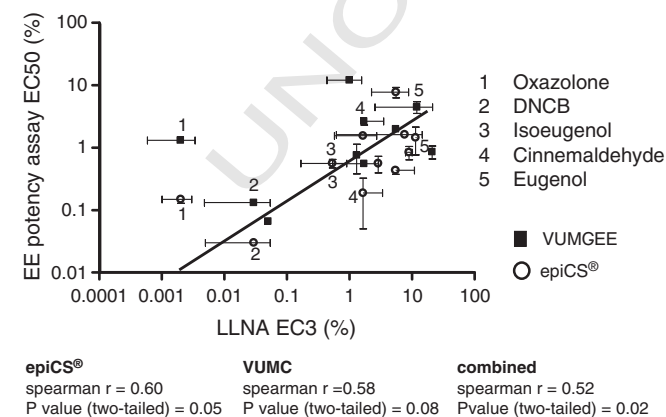


Fig. 4. Correlation EE-EC₅₀ from VUMC-EE and epiCS® with LLNA EC₃ (%). Data obtained from this study using epiCS® are plotted together with previous published data using VUMC-EE (dos Santos et al., 2011) and correlated to LLNA data. Five chemicals are indicated which were tested in both VUMC-EE and epiCS®.

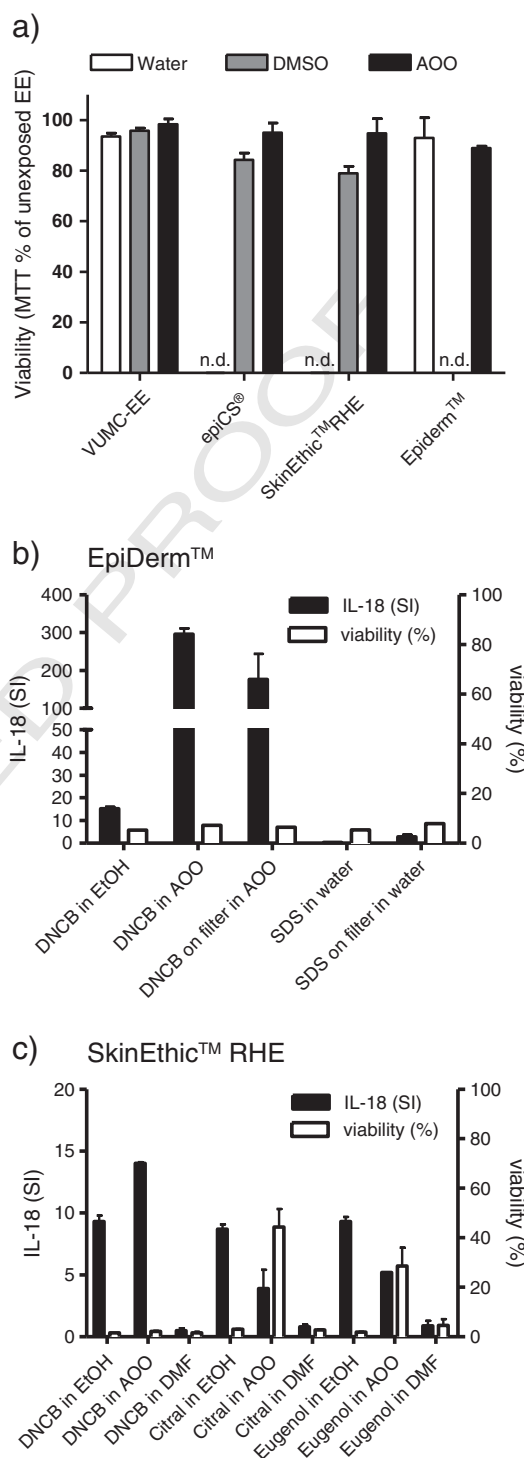


Fig. 5. Effects of different vehicles on cell viability and IL-18 release in different EE models. (A) Effects of water, DMSO and AOO (applied in filter paper disc) on cell viability after 24 h of exposure in different EE models, namely VUMC-EE, epiCS®, SkinEthic™ RHE and EpiDerm™. (VUMC-EE: Water n = 15; DMSO n = 20; AOO n = 17; epiCS®: DMSO n = 14; AOO n = 19; SkinEthic™ RHE: DMSO n = 13; AOO n = 7; mean ± SEM is shown). (B) Comparison of the effects of different vehicles (AOO, ethanol, water) and mode of application (with or without filter paper disc) on DNCB and SDS-induced IL-18 release and cell viability in EpiDerm™ EE model. Results are expressed as average ± difference, n = 2 independent experiments. (C) Comparison of the effects of different vehicles (AOO, ethanol, water, DMF) on 0.15% DNCB, 0.3% citral and 1.0% eugenol induced IL-18 release and cell viability in SkinEthic™ RHE EE model performed at MB Research. Results are expressed as average ± difference, n = 2 replicates from a single experiment.

study indicate that indeed this single *in vitro* human assay may be able to distinguish sensitizers from non-sensitizers as well as rank sensitizers according to potency. The use of reconstituted epidermis overcomes the limitation of chemical solubility and stability in water solution, which are the major limitations of many *in vitro* methods based on the use of submerged cultures. Remarkably consistent results were obtained between the different 3D models, indicating the assays robustness and ease to transfer to other laboratories. It is probable that minor method optimization may be required for different types of commercially available EE and different vehicles.

Due to their anatomical location and critical role in skin inflammatory and immunological reactions, the use of the KC and skin organotypic cultures as a simplified *in vitro* model to evaluate the potential toxicity of chemicals destined for epicutaneous application is amply justified. The proposed method is mechanistically anchored, as IL-18 has been shown to play a key proximal role in the induction of allergic contact sensitization (Antonopoulos et al., 2001; Okamura et al., 1995). The possibility of using IL-18 for the *in vitro* identification of contact allergens is not novel; as we have previously demonstrated in a human keratinocyte cell line (NCTC2544) a selective up-regulation of intracellular IL-18 by contact allergens at cell viability > 80%, whereas irritants and respiratory allergens failed. A sensitivity of 87%, specificity of 95% and an accuracy of 90% was reported (Corsini et al., 2009, 2012; Galbiati et al., 2011). In addition to requiring the analysis of intracellular IL-18 production rather than release of IL-18, the NCTC2544 assay is proving difficult to transfer to different laboratories since it is dependent on critical culture conditions of the NCTC cells (culture time after thawing, seeding density, degree of confluency) and has a cut-off for discriminating sensitizers from non-sensitizers at only 1.2 fold increase in intracellular IL-18 (Corsini et al., 2012). This indicates that considerable cell culture know-how is required to perform the assay and that each laboratory needs to optimize the assay independently. Replacing NCTC2544 cell line with a standardized commercially available EE as described in this manuscript makes the assay extremely transferable and easy to perform for any laboratory with standard cell culture facilities.

In all EE models, we found a robust release of IL-18 only after treatment with contact allergens. Cytotoxicity was essential to observe the release of intracellular IL-18 into culture supernatants. At a similar degree of cytotoxicity only contact allergens, and not irritants, generally induced a dramatic increase in IL-18 release. This is consistent with prior neosynthesis and intracellular accumulation only after sensitizer exposure, and is in line with our previous findings (Corsini et al., 2009). Our observation that cytotoxicity is required indicates that stored intracellular IL-18 is released into the supernatant rather than active secretion of IL-18 and again is in line with previous findings that it is intracellular production rather than secretion, which distinguishes sensitizers from non sensitizers. Increasing the chemical concentration to toxic concentrations enables intracellular protein to be released via membrane porosity rather having to introduce an alternative lysis method as is done in the NCTC assay.

In prediction model 2 a number of false negatives and positives were observed which led to the final proposed prediction model 3. In prediction model 2 ($\geq 5\%$ viability (EC₅); ≥ 5 fold increase in IL-18) 3 false negatives were observed in DiSFeB and 1 false positive in VUMC. Two of the false negatives in DiSFeB corresponded to dose response curves in which very little cytotoxicity was observed. EE were still > 40% viable after exposure to phenylacetone aldehyde and 2-mercaptobenzothiazol. Formaldehyde scored as the third false negative in DiSFeB even though high levels of cytotoxicity were reached. A possible explanation for this may be that too much cytotoxicity may have resulted fixation of the tissue preventing IL-18 release, or in diffusion of formaldehyde into the culture supernatant resulting in denaturation of proteins which in turn may have affected the ELISA. The dose range in the DiSFeB dropped rapidly from $44.5 \pm 17.53\%$ viability to $7.15 \pm 1.55\%$ viability in which the first viability value may have been too high and the next viability value too low. Noticeably, the concentrations of formaldehyde

used by DiSFeB were considerably higher than those used by VUMC. With regards to the non-sensitizers VUMC correctly identified 3/4 non-sensitizers and DiSFeB correctly identified all 6 non-sensitizers. The false positive phenol in the VUMC may possibly be due to extended storage of this chemical resulting in partial oxidation. These results taken together led to the testing of prediction model 3 in which a greater release of IL-18 within a defined cytotoxicity range was tested. Prediction model 3 ($\geq 5\%$ and $\leq 40\%$ viability (EC₅-40); ≥ 5 fold increase in IL-18 release) resulted in exclusion of the 2 false negatives from DiSFeB as critical cytotoxic levels were not reached. For chemical exposures which fulfilled the criteria for prediction model 3, all but one chemical in each of the laboratories were correctly identified (phenol scored false positive in VUMC, and formaldehyde scored false negative in DiSFeB) resulting in 95% accuracy in both VUMC and DiSFeB laboratories. Of note, this prediction model is dependent on the chemical resulting in high cytotoxicity (5–40% viability; 60–95% cytotoxicity) in the EE.

In this study, a previously defined standard operating procedure for the EE potency assay was used (Teunis et al., 2012). The procedure defines how a dose finding is performed for any unknown coded chemical (of unknown molecular mass) in order to obtain an EE-EC₅₀ value. Our results show that by following this procedure not always similar dose responses were obtained between the two laboratories and when difference were observed this generally corresponded with the variation in IL-18 release between the laboratories (e.g. formaldehyde and phenylacetone aldehyde). Minor modifications to the procedure will be expected to remedy this in the future.

Correlation of the EE-EC₅₀ values obtained from the 11 chemicals in two different laboratories showed very good and statistically significant correlation with both LLNA and human DSA₀₅ data. Interestingly the correlation was much better with the human data (spearman $r = 0.8500$; P value of 0.0061) than with mouse LLNA data (spearman $r = 0.5968$; P value of 0.0542) indicating that our human *in vitro* EE assay ranks human sensitizer potency more accurately than the mouse assay. It should be mentioned though that these values may be biased slightly as no human data is available for correlation for the chemical oxazolone which clearly deviates in the LLNA correlation. However, our finding that human *in vitro* assays may prove superior to current animal LLNA in predicting human sensitization is further supported by the findings of Schneider and Akkan (2004) who reported that when comparing human and animal data for 46 substances a correlation of only $r = 0.77$ (HRPIT vs LLNA data) and $r = 0.65$ (HMT vs LLNA data) was obtained (Schneider et al., 2004).

With regards to assessment of sensitizer potency in the NCTC2544 assay, there is a general trend for IL-18 induction at lower concentrations for extreme/strong sensitizers, whereas higher concentrations are required in the case of weak sensitizers (Corsini et al., 2012). In this study we show for the first time that in addition to the EE-EC₅₀, release of IL-18 into culture supernatants of EE may possibly also be used to assess sensitizer potency. Indeed our correlation results of IL-18 SI-2 and SI-5 with human data strongly warrant further testing with extended chemical panels.

Importantly the EE-potency assay is not only transferable between laboratories but also between different types of EE. Results obtained from the LLNA_{EC3}-EE-EC₅₀ correlation in this study using epiCS® could be superimposed onto the correlation graph obtained with VUMC-EE (dos Santos et al., 2011). Even the combined correlation showed a significant spearman $r = 0.52$; P value (two-tailed) = 0.02 with the limited number of chemicals tested including the outlier oxazolone. Further experiments are required to determine whether indeed a single correlation graph can be used in the future independent of the type of EE used to assess sensitizer potency. However it is possible that at least minor differences in exposure times and/or concentrations will need to be optimized among different EE models. For example, EpiDerm™ will be expected to perform slightly differently than epiCS® or SkinEthic™ RHE due to differences in the properties of the

stratum corneum barrier between these models. This is illustrated in the OECD validated skin irritation assay where the treatment time for inducing irritation is 15 min followed by 42 h post-incubation in the EpiSkin model whereas the protocol required optimization for SkinEthic™ RHE (42 min followed by a 42 h post-incubation) and EpiDerm™ (60 min followed by a 42 h post-incubation) (Alepee et al., 2010; Kandarova et al., 2009; Kidd et al., 2007) (see also OECD TG 439; Adopted: 22 July 2010).

In addition to comparing different EE, different vehicles and application procedures were compared. Our preliminary results indicate that the choice of vehicle can greatly influence the result obtained. A lower IL18 secretion was observed when the vehicle ETOH was used rather than AOO. This is an area which clearly requires further investigation using the single protocol developed in this study with epicS® and is also mirrored in the results reported from *in vivo* studies. The broad range of LLNA_{EC3} values and human DSA₀₅ values shown in Table 3 are due in part to the different vehicles used in the different studies. The vehicle effect is thought to differ due to the following two reasons: 1) the vehicle will influence the ability of the test substance to traverse the stratum corneum and reach the viable epidermis and 2) the vehicle will influence the efficiency of Langerhans cell migration from the skin (Basketter et al., 2001; Lea et al., 1999; McGarry, 2007; Wright et al., 2001). The former of these two points is of particular relevance to the EE assay described in this manuscript. In contrast, the means by which the chemical was applied: direct chemical application or filter paper application did not appear to influence the result.

In conclusion, using a single standard operating procedure we have developed a putative prediction model in two different laboratories (VUMC and DiSFeB) using two different types of EE (epiCS® and VUMC-EE). Our results indicate that only minor modifications in the assay methodology and prediction model are required to optimize the assay read-out for the different commercially available EE (eg. SkinEthic™ RHE, EpiDerm™). Factors which may vary between different EE are the optimal duration and method of chemical exposure (direct vs via filter paper disc), the type of vehicle used, the optimal cytotoxicity margin to insure maximum release of IL-18 and the IL-18 induction factor to distinguish a sensitizer from a non sensitizer. Our first results do show that this simple, easily transferable EE assay may enable sensitizers to be distinguished from non-sensitizers (IL-18 release: ELISA) and also rank sensitizers according to potency better than the current LLNA (EC₅₀ value: MTT assay; IL-18 SI-2: ELISA). The assay is easy to implement in any laboratory with standard cell culture and ELISA capabilities.

Conflict of interest

For the past 3 years since the beginning the work, all authors declare not having any financial, personal, or association with any of the individuals or organizations, that have could inappropriately influence the submitted work.

Uncited reference

Mosmann, 1983

Acknowledgments

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