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NCTC 2544 and IL-18 production: A tool for the identification of contact allergens

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ABSTRACT

Progress in understanding the mechanisms of skin sensitization, provides us with the opportunity to develop in vitro tests as an alternative to in vivo sensitization testing.

Keratinocytes play a key role in all phases of skin sensitization. We have recently identified interleukin-18 (IL-18) production in keratinocyte as a potentially useful endpoint for determination of contact sensitization potential of low molecular weight chemicals. The aim of the present article is to further exploit the performance of the NCTC 2544 assay.

NCTC 2544 is a commercially available skin epithelial-like cell line originating from normal human skin, which possesses a good expression of cytochrome P450-dependent enzymatic activities. Cells were exposed to contact allergens (2-bromo-2-bromomethyl glutaronitrile, cinnamaldehyde, citral, diethylmaleate, dinitrochlorobenzene, glyoxal, 2-mercaptobenzothiazole, nickel sulfate, 4-nitrobenzylbromide, oxazolone, penicillin G, resorcinol, tetramethylthiuram disulfide), to pre-pro-haptens (cinnamyl alcohol, eugenol, isoeugenol, *p*-phenylenediamine), to respiratory allergens (ammonium hexachloroplatinate, diphenylmethane diisocyanate, glutaraldehyde, hexamethylenediisocyanate, maleic anhydride, trimellitic anhydride) and to irritants (benzaldehyde, chlorobenzene, diethylphthalate, hydrobenzoic acid, lactic acid, octanoic acid, phenol, salicylic acid, sodium lauryl sulphate, sulfamic acid). Cell associated IL-18 was evaluated 24 later by ELISA. At not-cytotoxic concentrations (cell viability higher of 80%, as assessed by MTT reduction assay), all contact sensitizers, including pre-pro-haptens, induced a dose-related increase in IL-18, whereas both irritants, with the exception of sulfamic acid, and respiratory allergens failed. A total of 33 chemicals were tested, with an overall accuracy of 97%.

Overall, results obtained indicated that cell-associated IL-18 might provide an in vitro tool for identification and discrimination of contact vs. respiratory allergens and/or irritants.

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1. Introduction

At present, the identification of potential sensitization of chemicals relies on animal models, such as the guinea pig maximization test (Magnusson and Kligman, 1969) and the local lymph node assay (Kimber et al., 1995). However, in the screening of new chemicals, it should be very important both from ethical, safety and economic points of view to have biological markers to discriminate allergy and irritation events without the use of animals. Furthermore, due to the new European policy on chemicals (REACH) and the EU cosmetic directive, in vitro methods are likely to play a major role in the near future. Over the last decade, several in vitro methods have been proposed to identify the potential of chemicals to induce skin sensitization

(reviewed by Corsini and Roggen, 2009; Galbiati et al., 2010), but no accepted in vitro method for the identification of sensitizing chemicals are yet available.

The NCTC 2544 assay, based on the selective induction of IL-18 in human keratinocytes by contact allergens, was developed within the SENS-IT-IV project sponsored by the European Union. The assay proved to be useful in the identification and discrimination of contact allergens from respiratory sensitizers and irritants (Corsini et al., 2009; Galbiati et al., 2011).

Skin sensitization is an immune mediated reaction to low molecular weight exogenous chemicals. Both skin resident cells, such as keratinocytes (KC), Langerhans cells (LC) and mast cells, and immigrating leucocytes, including T lymphocytes and natural killer cells, actively participate in contact allergy (Cavani et al., 2007).

KC were chosen as they play a role in all phases of allergic contact dermatitis, from the early initiation phase with the elaboration of inflammatory cytokines, that plays a role in LC migration, and T-cell trafficking, through the height of the inflammatory phase

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with direct interactions with epidermotrophic T-cells, through the resolution phase of allergic contact dermatitis with the production of anti-inflammatory cytokines and tolerogenic antigen presentation to effector T-cells (Gober and Gaspari, 2008). KC sense haptens, and in turn initiate a program of enhances or de novo expression of inflammatory molecules representing the starting point of primary inflammation.

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). We had focused our attention on IL-18 since this cytokine has been shown to play a key proximal role in the induction of allergic contact sensitization and to favor Th-1 type immune response by enhancing the secretion of pro-inflammatory mediators such as TNF- α , IL-8 and IFN- γ (Okamura et al., 1995; Cumberbatch et al., 2001; Antonopoulos et al., 2008). IL-18 has no apparent role in irritant contact dermatitis (Antonopoulos et al., 2008), indicating that the role of IL-18 in contact hypersensitivity is not simply part of a general requirement for IL-18 in skin inflammation. Furthermore, Hartwig et al. (2008) demonstrated using IL-18-deficient mice that the absence of IL-18 does not affect any of the asthma-specific parameters, including allergen-specific IgE, histological changes of the lungs, infiltrating leukocytes, serum cytokine levels and airway hyperresponsiveness. Human keratinocytes constitutively express IL-18 mRNA and protein (Naik et al., 1999), and works published by Naik et al. (1999) and Van Och et al. (2005) showed the induction of IL-18 following exposure to contact sensitizers.

In the context of the sensitization process, the NCTC 2544 assay was designed to cover the initial phase 'local trauma – proinflammatory cytokine production (danger signals)', necessary for maturation and migration of dendritic cells, and T cells activation. Haptens, acting as danger signals, stress the cells, stimulate the assembly of the inflammasome and NF- κ B activation, resulting in IL-18 neosynthesis and release.

The aim of the present article was to further exploit the performance of the NCTC 2544 assay. Overall, our data suggests that IL-18 represents a promising marker for the screening of potential contact allergens. Its technical simplicity and the good predictivity may make this assay a candidate for rapid validation studies.

2. Materials and methods

2.1. Chemicals

As respiratory allergens the following chemicals were used diphenylmethane diisocyanate (MDI), trimellitic anhydride (TMA), ammonium hexachloroplatinate (HClPt), glutaraldehyde, hexamethylenediisocyanate (HMD), maleic anhydride. As skin sensitizers 2-bromo-2-bromomethyl glutaronitrile, cinnamaldehyde, citral, diethylmaleate, dinitrochlorobenzene (DNCB), glyoxal, 2-mercaptobenzothiazole, nickel sulfate, 4-nitrobenzylbromide, oxazolone, penicillin G, resorcinol, tetramethylthiuram disulfide (TMTD), as pre-prohaptens cinnamyl alcohol, eugenol, isoeugenol, *p*-phenylenediamine (PPD) and, as irritants benzaldehyde, chlorobenzene, diethylphthalate, hydrobenzoic acid, lactic acid, octanoic acid, phenol, salicylic acid (Sal. Ac.), sodium lauryl sulphate (SLS), sulfamic acid were tested. Glycerol was used as negative compound. The SENS-IT-IV program mainly dictated the choice of chemicals. Chemicals have been selected by a dedicated workpackage as relevant and representative of the 'universe' of irritants, respiratory and contact allergens. All reagents were purchased from Sigma (St. Louis, MO, USA) at the highest purity available. All chemicals with the exception of HClPt, glyoxal, nickel sulfate, glycerol, and SLS dissolved in PBS, were dissolved in DMSO (0.1%

final concentration). Cell culture media and all supplements were from Sigma. Solutions were freshly prepared for each experiment. The maximum concentration tested was 1000 μ g/ml.

2.2. Cell culture

For experiments using NCTC 2544 cells (Institute Zooprofilattico di Brescia, Brescia, Italy), cells were cultured in 24-well plate at a cell density of $1.5\text{--}2.5 \times 10^5$ /ml (0.5 ml/well). Cells were treated in 0.5 ml of RPMI 1640 containing 2 mM L-glutamine, 0.1 mg/ml streptomycin, 100 IU/mL penicillin, supplemented with 10% heated-inactivated foetal calf serum (media) and cultured at 37 °C in 5% CO₂ for 24 h.

2.3. Cell viability

Prior to study the effects of the selected chemicals on IL-18 production, their cytotoxicity was assessed by the MTT test (Gerlier and Thomasset, 1986). One hundred microliter of cells ($1.5\text{--}2.5 \times 10^5$ /ml) were seeded in a 96-well plate and treated with increasing concentrations of the test chemical or DMSO as vehicle control (0.2% final concentration). After an incubation period of 24 h, medium was discarded and 100 μ l/well of MTT solution 0.75 mg/ml in culture medium was added. Cells were incubated for 3 h at 37 °C, medium was discarded and cells lysed in 100 μ l/well of a mixture of HCl 1N:isopropanol (1:24). The absorbance of the resulting solutions was read at a wavelength of 595 nm in a microplate reader (Molecular Devices). Eighty percent cell viability (CV80) was calculated for each chemical by linear regression analysis of data.

2.4. Cytokine production

For IL-18 production cells were incubated with increasing concentrations of the selected chemicals, or DMSO as vehicle control (0.2% final concentration). CV80 was the highest concentration tested. PPD 60 μ g/ml was used as positive control. After incubation, culture medium was discarded, monolayers gently washed once with 1 ml of PBS and cells lysed in 0.25 ml of 0.5% Triton X-100 in PBS. Plates were stored at -80 °C until measurement. Intracellular IL-18 content was assessed by specific sandwich ELISA commercially available (MBL, Nagoya, Japan). Results are expressed in pg/mg of total intracellular protein content. The protein content of the cell lysate was determined by the BCA method.

The following PREDICTION MODEL was used: if the fold increases in intracellular IL-18 is ≥ 1.2 and the increase in IL-18 is statistically significant from vehicle treated cells (Dunnett multiple comparisons test) the chemical is classified as contact sensitizer (R43, risk 43: may cause sensitisation by skin contact, as defined in Annex III of European Union Directive 67/548/EEC). If the fold increase in intracellular IL-18 is < 1.2 and there is no statistical significance the chemical is classified as non-contact sensitizer. The positive control is included in each plate. The positive control meets the acceptance if the fold increase in intracellular IL-18 is > 1.4 compared to vehicle treated cells (if the tested chemical is dissolved in PBS or water, the relative control cells for PPD will be the same control cells used for the chemical even if PPD stock solution is dissolved in DMSO. The intracellular IL-18 content of naïve cells or treated with DMSO is very similar). For a given chemical, the same classification must be obtained in at least two out of three independent experiments. The 1.2-fold increase is meant for at least one of the concentrations tested. In Table 1 is reported the effective concentration resulting in 1.2-fold induction in intracellular IL-18 (EC1.2), as assessed by linear regression analysis of data for the allergens tested.

Table 1

Chemicals tested in the NCTC 2544 assay and their in vivo classification.

Chemical	CAS number	In vitro EC1.2 (µg/ml)	LLNA classification
<i>Respiratory allergens</i>			
Ammonium hexachloroplatinate	16919-58-7	Negative	Not available
Diphenylmethane diisocyanate (MDI)	101-68-8	Negative	Strong
Glutaraldehyde	111-30-8	Negative	Strong
Hexamethylenediisocyanate	822-06-0	Negative	Extreme
Maleic anhydride	108-31-6	Negative	Strong
Trimellitic anhydride	552-30-7	Negative	Moderate
<i>Contact allergens</i>			
2,4-Dinitrochlorobenzene	97-00-7	0.43	Extreme
4-Nitrobenzylbromide	100-11-8	1.68	Extreme
Oxazolone	15646-46-5	36	Extreme
2-Bromo-2-bromomethyl glutaronitrile	35691-65-7	0.93	Strong
p-Phenylendiamine	106-50-3	27.2	Strong
2-Mercaptobenzothiazole	149-30-4	36.0	Moderate
Cinnamaldehyde	104-55-2	9.4	Moderate
Diethylmaleate	141-05-9	19.7	Moderate
Glioxal	107-22-2	9.0	Moderate
Isoeugenol	97-54-1	12.8	Moderate
Tetramethylthiuram	137-26-8	1.04	Moderate
Cinnamyl alcohol	104-54-1	103.0	Weak
Citral	5392-40-5	4.45	Weak
Eugenol	97-53-0	344	Weak
Resorcinol	108-46-3	206.0	Weak
Nickel sulfate	7786-81-4	60.0	False negative
<i>Non sensitizers/irritants</i>			
Benzaldehyde	100-52-7	Negative	Not classified
Chlorobenzene	108-90-7	Negative	Not classified
Diethylphthalate	84-66-2	Negative	Not classified
Glycerol	56-81-5	Negative	Not classified
Hydroxybenzoic acid	99-96-7	Negative	Not classified
Lactic acid	50-21-5	Negative	Not classified
Octanoic acid	124-07-2	Negative	Not classified
Phenol	108-95-2	Negative	Not classified
Salicylic acid	69-72-7	Negative	Not classified
Sodium lauryl sulphate	151-21-3	Negative	Not classified
Sulfamic acid	5329-14-6	Positive	Not available

The shading is related to the LLNA potency classification.

2.5. Human skin equivalent cultures

The reconstituted epidermis, EPISKINTM was supplied by Skinethic (Nice, France). It consists of normal adult human derived epidermal keratinocytes, which have been cultured to form a multilayered, highly differentiated model of human epidermis, lacking the dermis. The reconstituted epidermis was cultured at 37 °C in a humidified atmosphere containing 5% CO₂ in air, according to the supplier's specifications. Based on preliminary experiments, skin equivalent cultures were treated with selected chemicals (40 µl), namely DNCB, cinnamaldehyde, TMTD, MDI, TMA, Sal. Ac. and phenol, at a concentration inducing a similar release in IL-1α. All chemicals, except phenol, which was dissolved in assay medium, were dissolved in acetone: olive oil (1:4). Twenty-four hours following treatment the culture medium was used for IL-1α and IL-18 determination. Cytokines were assessed using commercially available ELISA kits (R&D and MBL). Vehicles did not affect IL-1α or IL-18 release, therefore naïve skin equivalent cultures were used as control.

2.6. Data analysis

All experiments were performed at least three times with representative results shown. The data presented are expressed as mean ± SD. Statistical significance was determined by Dunnett's multiple comparison test. Differences were considered significant for $p < 0.05$. For the contingency table the Fisher's exact test was used.

3. Results

3.1. Effects of the selected chemicals on intracellular IL-18 content in NCTC 2544 cells

For each chemical dose response curves were performed (Corsini et al., 2009; Galbiati et al., 2011). In Table 1 the full data set of chemicals tested along with the LLNA classification is presented. The classification of chemicals (i.e. respiratory vs. contact sensitizers vs. non-sensitizers/irritants) is dictated by the SENS-IT-IV project, with the exception of nickel sulfate and sulfamic acid, which were not in the SENS-IT-IV list. For the chemical rated as positive, the EC1.2 values, calculated by linear extrapolation from the values generated, are reported in Table 1. The only chemical that was misclassified was sulfamic acid.

As representative results, the induction of intracellular IL-18 following treatment with two weak allergens resorcinol and citral, with the non sensitizer lactic acid, and two respiratory allergens maleic anhydride and HMD are reported in Fig. 1 together with cell viability data. These chemicals were chosen among the 33 chemical tested for two reasons: (1) sensitivity toward the identification of weak sensitizers, and (2) the lack of relationship between cytotoxicity and IL-18. In Fig. 1A, the effects of resorcinol and lactic acid are shown. For both chemicals the CV80 could not be calculated, as at 1000 µg/ml cell viability was higher than 80% (Fig. 1B). Despite this, only the allergen resorcinol induced a dose-related increase in intracellular IL-18, whereas no changes were observed following treatment with lactic acid. In the case of citral (Fig. 1C and D) a

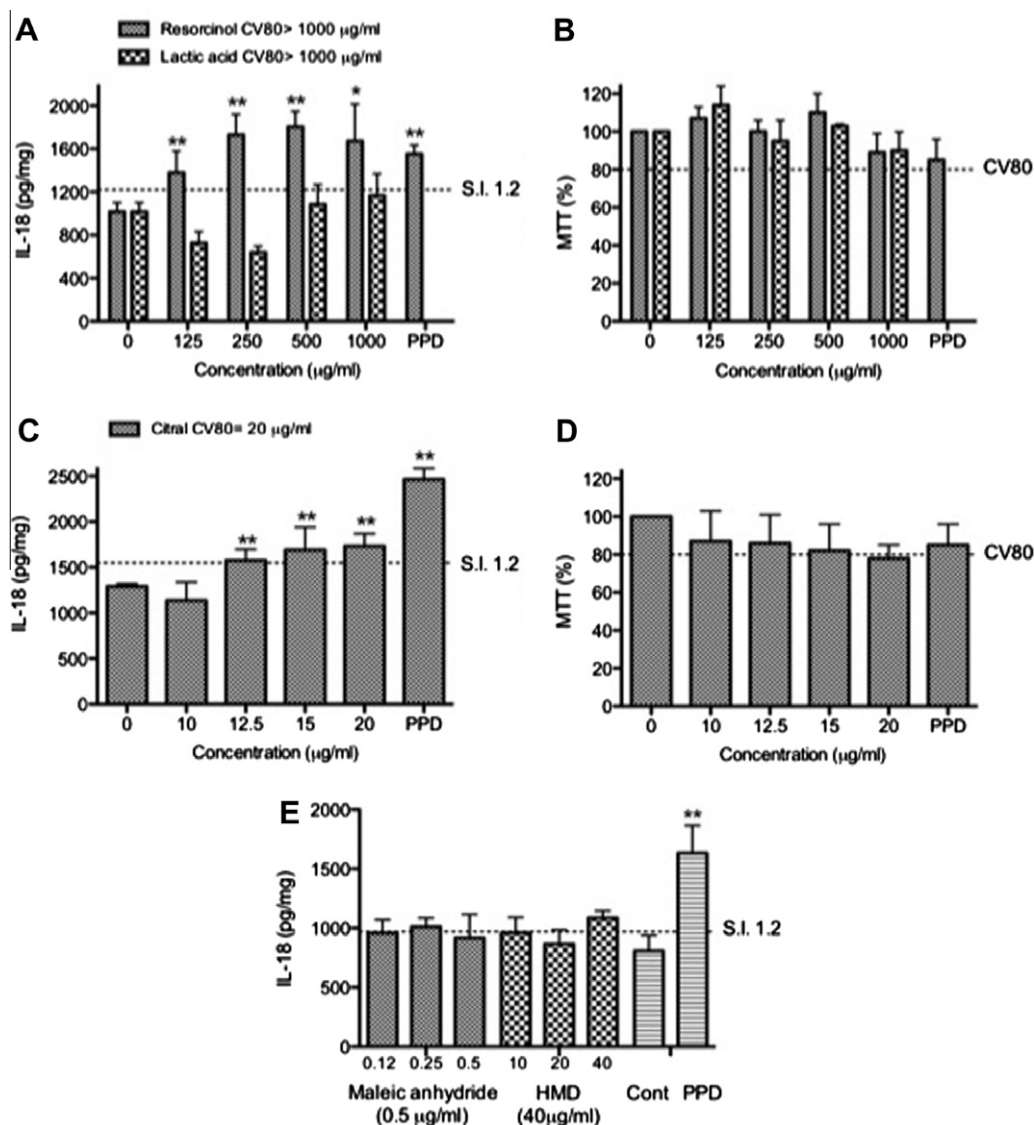


Fig. 1. Dose–response effects of the weak sensitizers resorcinol and citral, and the non sensitizer lactic acid on cell-associated IL-18 and cell viability. NCTC 2544 cells were treated for 24 h with increasing concentrations of resorcinol, citral and lactic acid. PPD (60 μg/ml) was used as positive control. Cells incubated with vehicle were included as a control (0). (A and B) Effect of resorcinol and lactic acid on intracellular IL-18 (A) and cell viability (B). (C and D) Effect of citral on intracellular IL-18 (C) and cell viability (D). Cell viability was assessed by MTT reduction assay. (E) Effect of maleic anhydride and HMD on intracellular IL-18. In parenthesis is reported the CV80. Results are expressed as mean ± SD, $n = 4$. Statistical analysis was performed with Dunnett's multiple comparison test, with * $p < 0.05$ and ** $p < 0.01$ vs. vehicle treated cells (0).

CV80 of 20 μg/ml was calculated, but statistical significant induction of IL-18 could be observed at concentrations above the CV80, indicating that cell damage is not necessary to trigger IL-18 by contact allergens. The two respiratory allergens tested failed, as expected, to induce IL-18 production (Fig. 1E).

3.2. Relationship between EC1.2 and in vivo potency

From inspection of Table 1, no direct correlation between EC1.2 and potency of chemicals is evident at first sight. The Pearson correlation between in vitro EC1.2 (μg/ml) and in vivo LLNA EC3 (%) has a correlation coefficient (r) = 0.4787, with a P value of 0.0711, considered not quite significant. However, particularly low EC1.2 values <2 μg/ml were recorded for many extreme/strong allergens, and, with exception of citral, the EC1.2 for weak sensitizers was in general above 100 μg/ml. In Table 2 the summary results for intracellular IL-18 induction in the NCTC 2544 assay by chemicals of known skin sensitization potency is reported. All contact allergens tested, including the weak ones, were correctly identified.

3.3. Contingency table and false positive

Data relative to contact allergens and non contact sensitizers reported in the contingency table (Table 3) yielded accuracy of 97%, indicating a good predictivity of the assay for the chosen set of reference chemicals. Sensitivity was 100% and specificity 94.1%. The only chemical that was misclassified was sulfamic acid, which is reported to be a strong irritant and was classified in the NCTC 2544 assay as sensitizers. Sulfamic acid induced a dose-related increase in intracellular IL-18 at non-cytotoxic concentrations in 3 out of 3 independent experiments (data not shown). In a textbook (Pohanish, 2012), it is, however, reported that long-term exposure to sulfamic acid may cause dermatitis and skin allergy.

3.4. Critical aspects: cell density, weeks after thawing and the performance of the NCTC 2544 assay

During the refinement of the NCTC 2544 assay two critical aspects emerged: cell density and time after thawing. As shown is

Table 2

Summary results for intracellular IL-18 induction in the NCTC 2544 assay by chemicals of known skin sensitization potency.

Sensitization Class ^a	Number of chemicals tested	IL-18 induction above the 1.2 threshold (positive) ^b	No IL-18 induction (negative)
Extreme	3	3	0
Strong	2	2	0
Moderate	6	6	0
Weak/very weak	4	4	0
None/respiratory	17	1	16
Total sensitizers	15	15	
Total non contact sensitizers	17		
Total compounds tested	32 (+1)		

^a Sensitization class is based on LLNA EC3 values. Nickel sulfate is negative in the LLNA assay and was not included in the table.^b Number of chemicals with significant induction of intracellular IL-18 production.**Table 3**

Contingency table for the NCTC 2544 assay.

		Predicted classification		
		Non contact sensitizer	Contact sensitizer	Total
Chemical classification	Non sensitizers	16	1	17
	Sensitizer	0	16	16
	Total	16	16	26
Sensitivity	100			
Specificity	94.1			
Positive predictivity	94.1			
Negative predictivity	100			
Accuracy	97.0			

Fisher's exact test.

Fig. 2A, $1\text{--}2.5 \times 10^5$ cells/ml represents the optimal cell density (adapted from Galbiati et al., 2011), higher cell density ($>5 \times 10^5$ cells/ml) resulted in a loss of effect. This is also evident comparing the stimulation index: the higher SI was observed at cell density of $1\text{--}2.5 \times 10^5$ cell/ml. A critical point in the performance of the assay is, therefore, the cell density: if cells reach confluence the discriminatory capacity is lost.

The other critical point later identified was the time after thawing appropriate to start with experiments. As reported in the SOP, after thawing from liquid nitrogen, cells should be used for the test after two week of culture, approximately four passages (sensitive-learning.eu/.../SOPs/NCTC_SOP4.doc). However, as clearly shown in **Fig. 2B** (adapted from Galbiati et al., 2011), while this time is appropriate for the strong allergen PPD, it is not optimal for the weak allergen citral, which requires an additional week. On the other hand, consistent results were obtained culturing cells up to five months, longer period have not been tested. Therefore, cells must be used between 3 weeks after thawing, up to five months after thawing.

3.5. Implementation: total IL-18 and IL-18 release in human skin equivalent cultures

In order to develop a high throughput assay, a 96-well plate format assay was developed, in which total IL-18 (intracellular plus secreted) is measured. As previously published (Galbiati et al., 2011), a selective increase in total IL-18 was observed 24 h later in cells treated with contact allergens, whereas no changes were observed following treatment with respiratory allergens and irritants, confirming previous results obtained in a 24-well format assay. Speeding up the assay and reducing cell manipulation (no cell washing steps and no protein determination), the 96-well format assay will increase screening capacity of the NCTC 2544 assay.

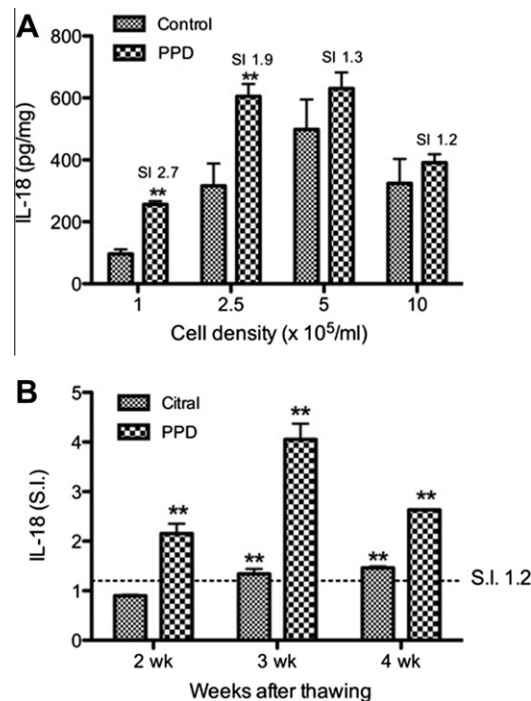


Fig. 2. Effect of cell density and time after thawing on intracellular IL-18. (A) Cell density, NCTC 2544 cells were seeded in 24-well plate at different density ($1\text{--}10 \times 10^5$ cells/ml). After overnight adherence, cells were treated for 24 h with PPD (60 $\mu\text{g/ml}$) or DMSO (0.2% final concentration) as vehicle control. (B) Weeks after thawing, NCTC 2544 were seeded in 24-well plate (2.5×10^5 cells/ml) 2, 3 and 4 weeks after thawing. After adherence, cells were treated for 24 h with citral (20 $\mu\text{g/ml}$), PPD (60 $\mu\text{g/ml}$) or DMSO (0.2% final concentration) as vehicle control. Results are expressed as mean $\pm$ SD, $n = 4$. Statistical analysis was performed with Student's multiple comparison test, with $**p < 0.01$ vs. vehicle treated cells (control). SI, stimulation index.

One limitation of the use of traditional submerged cell culture is chemical solubility and stability in water. To overcome these problems, we conducted preliminary experiments to test the release of IL-18 in commercially available 3D organotypic skin models. We know that the measure of the release of IL-18 in the NCTC 2544 assay did not offer any advantage, since a passive release of this constitutively expressed cytokine could be observed at high concentrations with both allergens and irritants, likely to be due to a cell damage effect (data not shown). Nevertheless, we assessed the release of IL-18 in human skin equivalent cultures hoping to find a cutoff value for determine contact sensitization potential. EpiskinTM was typically exposed for 18–24 h to three contact allergens (DNCEB, TMTD, cinnamaldehyde), two respiratory allergens (MDI, TMA) and two irritants (salicylic acid, phenol). We used concentrations that we know from previous experiments will induce

similar IL-1 α release. As shown in Fig. 3, using 1.5-fold induction of IL-18 compared to naïve cultures as cutoff, a significant increase was observed only after exposure to contact allergens. Additional testing with larger chemical set is required to fully evaluate the utility of this assay.

4. Discussion

Due to their anatomical location and critical role in skin inflammatory and immunological reactions, the use of the KC and skin organotypic culture as a simplified in vitro model to evaluate the potential toxicity of chemicals destined for epicutaneous application is amply justified. In the present work, intracellular IL-18 was investigated after exposure of NCTC 2544 cells to contact allergens, respiratory allergens, and irritants. We showed that exposure of NCTC 2544 cells to contact allergens results in a dose-related induction of intracellular IL-18, whereas exposure to respiratory allergens and irritants fails to induce IL-18 production, indicating the possibility to use IL-18 to specifically identify contact allergens and distinguish them from respiratory allergens and irritants.

As mentioned in the Introduction, IL-18 is mainly associated with Th1-mediated immune responses, but this doesn't exclude its induction or release also in humoral immune responses. As for many chemicals the dichotomy Th1 vs. Th2 is not black and white type of response, we are not excluding to get some positive response for some respiratory allergens. So far, for the limited number of chemicals tested, IL-18 seems to distinguish contact from respiratory allergens. Further chemicals must be tested to confirm this selective induction.

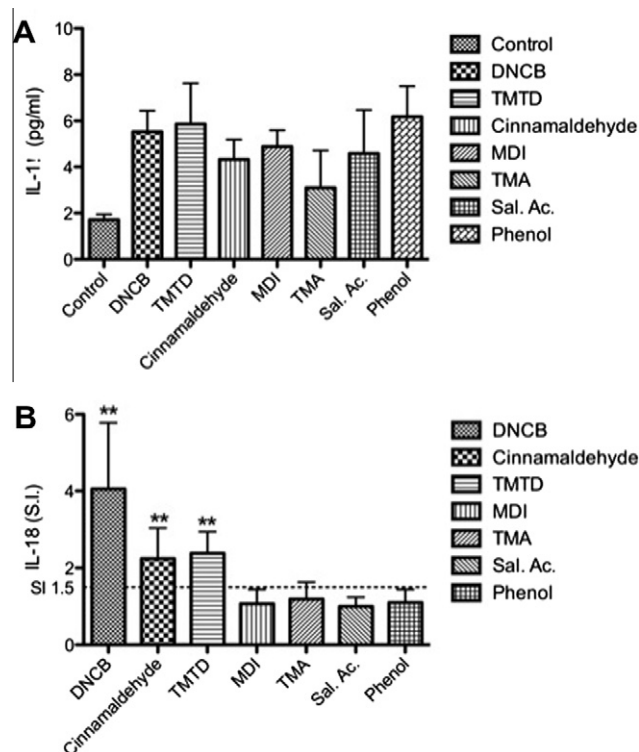


Fig. 3. IL-1 α and IL-18 secretion response to topical treatment with selected chemicals. Chemical (40 μ l) were applied to the apical surface of EpiskinTM tissues for 18–24 h prior to determination of cytokines secretion in the culture medium by ELISA. 1.5-fold induction of IL-18 compared to naïve control is the cutoff for determining contact sensitization potential. Data are expressed as mean $\pm$ SD of 3 independent experiments. Statistical analysis was performed with Dunnett's multiple comparison test, with * p < 0.05 and ** p < 0.01 vs. naïve control.

The only chemical that was misclassified was sulfamic acid, which is considered to be a strong irritant (R35/R36, R35 and R36 stay for R35: causes severe burns, and R36: irritating to eyes, according to the European Union Directive 67/548/EEC). However, its sensitization potential cannot be ruled out, as it has been reported to induce skin allergy (Pohanish, 2012). Nevertheless, in the present evaluation of data, it was classified as false positive.

For the all 33 chemicals tested, the accuracy was 97.0%, indicating a good predictivity of the assay for the chosen set of reference chemicals. All contact allergens, including pre- and pro-haptens and the four weak sensitizers included in the SENS-IT-IV list were correctly classified. Several tests, none validated, are currently available for the identification of skin sensitizers (see Galbiati et al., 2011). Among the most advanced, in term of progress toward validation, are the h-CLAT (human cell line activation test), the MUSST (myeloid U937 skin sensitization test), the KeratinoSensTM and the direct peptide reactivity assay, all currently under pre-validation at ECVAM (Aeby et al., 2010). The overall accuracies of these tests are 85–93% for the h-CLAT (Sakaguchi et al., 2009), 85% for the MUSST, 85.1–93.8% for the KeratinoSensTM depending on the data sets used (Emter et al., 2010), 89% for the direct peptide binding assay (Maxwell et al., 2011). The overall accuracy of NCTC 2544 assay is similar or slightly higher, however, a larger data set is needed to judge whether the NCTC 2544 assay has indeed a better predictivity.

Factors such as compound solubility, chemical reactivity and metabolic activation, which may mask the potential allergenicity of some chemicals, must be considered in the development of in vitro tests. This may be the case of MDI and anhydrides, highly unstable in water. TMA is readily hydrolyzed in water to trimellitic acid and MDI produces inert, solid, insoluble polyurea. The hydrolysis of isocyanate in aqueous solution is rapid, with a half-life of less than 20 s. If submerged cell culture may be unfavorable for many of the respiratory sensitizers, due to chemical instability, we have tested IL-18 production in reconstituted human epidermis, which allows application in organic solvent, i.e. acetone: olive oil, to overcome this problem. Using a 1.5-fold increase in IL-18 release over control as criteria of classification, preliminary results, obtained testing DNCB, cinnamaldehyde, TMTD as contact allergens, phenol, salicylic acid as irritants, TMA and MDI as respiratory allergens, at concentrations inducing a similar IL-1 α release, showed a statistically significant 1.5-fold increase in the release IL-18 following treatment with DNCB, cinnamaldehyde and TMTD, which may support results obtained with submerged keratinocytes. Further studies are clearly necessary, but the use of reconstituted epidermis will overcome the limitation of chemical solubility and stability in water solution, the major drawbacks of the current NCTC 2544 assay.

The lack of metabolic activation may also be a relevant problem in case of pro-haptens. However, NCTC 2544 cells possess both phase I and II metabolic activation capacity (Gelardi et al., 2001), and positive results were indeed obtained with the pro-haptens eugenol, isoeugenol and cinnamyl alcohol. A comparison between the phase I enzyme activities expressed in normal human KC and in several human keratinocyte cell lines, namely HaCaT, SVK14 and NCTC 2544, was established by Cotovio et al. (1997). From that study it appeared that in NCTC 2544 cell line both basal and induced levels of 7-ethoxycoumarin O-deethylase (ECOD) activity (mainly CYP 1A- and CYP 2B-related), are higher than those found in normal. The NCTC 2544 cell line not only compares well with other in vitro models such as reconstructed epidermis and primary cultured KC, but also has even better metabolic properties. The characterization of phase I and phase II metabolism expressed in these cells gives sustained evidence for their suitability to in vitro toxicology studies (Gelardi et al., 2001).

Critical points identified in the performance of the NCTC 2544 assay are the cell density (Corsini et al., 2009; Galbiati et al., 2011) and, presented in the Section 3, the time cells have been cultured before use in experiments. If cells reach confluence at the moment of treatment, the ability to identify contact allergens is lost, therefore a careful check for the optimal cell density using PPD, as reference contact allergen is critical. In our hands, a cell density of $1\text{--}2.5 \times 10^5$ cells/ml gave optimal stimulation. Due to the use of different fetal calf serum, a different cellular doubling time can be expected in the different laboratories. Therefore, we suggest that each laboratory should check for the optimal cell density, using the positive control PPD as a reference compound. Regarding time after thawing, cells should be used between 3 weeks after thawing, up to 5 months after.

The influence of cell density on the discriminatory capacity of the NCTC 2544 assay can be attributed to a decrease in cellular reactivity once cells reach confluence. We have demonstrated that PPD-induced IL-18 production is dependent upon oxidative stress (Galbiati et al., 2011), and it has been demonstrated that the capacity of KC to generate reactive oxygen species decrease as the cells reached confluence (Turner et al., 1998), which offers a logical explanation for the effect observed.

The measurements are to be performed within the described cytotoxic range (>CV80 relative to vehicle treated cells) and cell density (<80% confluence when treatment starts), at a maximal test concentration 1000 µg/ml. We chose not to test chemicals at higher concentrations to avoid false positive response due to unspecific induction of stress response or contaminants in the test chemical preparation. As shown in Table 1, for all skin sensitizers tested the EC1.2 is far below 1000 µg/ml. Furthermore, as detailed in the Results section, cytotoxicity of a compound is not a prerequisite for positive results in the test. Statistically significant increase in intracellular IL-18 occurs at concentration above the CV80.

Among experiments, differences in the absolute value of IL-18 were observed, also in vehicle treated cells. In control cells, historically, the intracellular IL-18 content varied from 300 to 2500 pg/mg. This may be due to differences in the number of cells seeded and adhering to plate wells, to the use of a different cell batch as well as to differences in the IL-18 ELISA and protein assessment performance, which may result in over- or down-estimation of IL-18 content. For this reason, the stimulation index is calculated. As long as cells don't reach confluence and are used after three weeks from thawing, the discriminatory capacity is preserved independently from the absolute value of IL-18.

Regarding to relationship with potency, as outlined in the Results section, there is a general trend for IL-18 induction at lower concentrations for extreme/strong sensitizers whereas higher concentrations are needed in the case of weak sensitizers. The exceptions are oxazolone for which induction occurs at unusually high concentration, which will be under-predicted, and citral, a weak sensitizer with an EC1.2 20–70-fold lower of the other weak sensitizers, which will be over-classified.

Finally, as previously published, it is important to mention that we obtained similar results using other KC cell lines including Ha-CaT and HPKII, as well as primary human KC, confirming the relevance of the proposed model and the possibility to use different source of KC (Corsini et al., 2009; Galbiati et al., 2011).

To summarize, we propose the NCTC 2544 assay as screening, replacement, reduction test for hazard identification and general classification and labeling of skin sensitizers (R43 or No label). Our data suggest that IL-18 production by NCTC 2544 cells may represent a promising in vitro model for the screening of potential contact allergens. The test may also be complementary to the LLNA, allowing for a rapid and easy discrimination of contact sensitizers from respiratory allergens. Results were reproducible, and the method could be transferred to other laboratories, suggesting

the potential use of the test in immunotoxicity testing strategies. The assay is currently under prevalidation in a two-tiered approach project (*Prevalidation of a novel tiered approach to determine the skin sensitizing capacity and potency of chemicals*) sponsored by the Dutch association ZonMw. It is, however, important to stress that due to the complexity of hypersensitivity reactions and the chemical diversity of allergens, a more realistic approach should consider several markers of activation and different cell types, such dendritic like cells activation.

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.

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