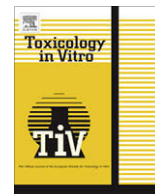




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Use of IL-18 production in a human keratinocyte cell line to discriminate contact sensitizers from irritants and low molecular weight respiratory allergens

Emanuela Corsini^{a,*}, Montserrat Mitjans^b, Valentina Galbiati^a, Laura Lucchi^a, Corrado L. Galli^a, Marina Marinovich^a

^a Laboratory of Toxicology, Department of Pharmacological Sciences, University of Milan, Faculty of Pharmacy, Via Balzaretti 9, 20133 Milan, Italy

^b Departament de Fisiologia, Facultat de Farmàcia, Universitat de Barcelona, Barcelona, Spain

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ABSTRACT

Assessment of allergenic potential of chemicals is performed using animal models, such as the murine local lymph node assay, which does not distinguish between respiratory and contact allergens. 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.

The aim of the present study was to evaluate the possibility to use intracellular interleukin-18 (IL-18) production to assess in vitro the contact sensitization potential of low molecular weight chemicals. The human keratinocyte cell line NCTC2455 was used. Cells were exposed to contact allergens (cinnamaldehyde, dinitrochlorobenzene, glyoxal, isoeugenol, *p*-phenylenediamine, resorcinol, tetramethylthiuram disulfide, 2-mercaptobenzothiazole, 4-nitrobenzylbromide), to proaptens (cinnamyl alcohol, eugenol), to respiratory allergens (diphenylmethane diisocyanate, trimellitic anhydride, ammonium hexachloroplatinate) and to irritants (sodium lauryl sulphate, salicylic acid, phenol). Cell associated IL-18 were evaluated 24 later. At not cytotoxic concentrations (cell viability higher of 75%, as assessed by MTT reduction assay), all contact sensitizers, including proaptens, induced a dose-related increase in IL-18, whereas both irritants and respiratory failed. Similar results were also obtained using primary human keratinocytes. Results were reproducible, and the method could be transferred to another laboratory, suggesting the potential use of the test in immunotoxicity testing strategies. Overall, results obtained indicated that cell-associated IL-18 may provide an in vitro tool for identification and discrimination of contact versus respiratory allergens and/or irritants.

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1. Introduction

Chemical allergy describes adverse health effects, resulting from the stimulation of specific immune response by chemicals. The two most frequent manifestations of chemical-induced allergy are contact hypersensitivity and respiratory sensitization, both of which can have serious impact on the quality of life, and represent a common occupational health problem (Wahlberg, 1996).

Contact hypersensitivity can occur as a result of exposure to a wide variety of chemicals and certain drugs (Nethercott and Holness, 1989), cosmetics (De Groot et al., 1988; Remaut, 1992; Andersen, 1993), and various metals including nickel (Picardo et al., 1990) and chromium (Nethercott and Holness, 1989).

Chemical respiratory allergy, although less common than allergic contact dermatitis, is of considerable significance, not only because asthma resulting from sensitization can be severe, but at times it can also be fatal. A variety of chemicals have been implicated as causing

occupational respiratory allergy. Among these are diisocyanates (i.e. toluene diisocyanate and diphenylmethane diisocyanate), acid anhydrides (i.e. trimellitic anhydride and phthalic anhydride), some reactive dyes, certain chlorinated platinum salts, glutaraldehyde, chloramine-T, plicatic acid and carmine (Corsini and Kimber, 2007).

At present, the identification of potential sensitization of chemicals is carried out using 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 for human use, it should be very important both from safety and economic point of view to have biological markers to discriminate allergy and irritation events that have different impact on human health. Furthermore, due to the new policy on chemicals (REACH), in the European Union, in vitro methods will 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 Vandebriel et al., 2005; Ryan et al., 2007), but no accepted in vitro method for the identification of sensitizing chemicals are yet available.

* Corresponding author. Tel.: +39 02 50318368; fax: +39 02 50318284.

E-mail address: emanuela.corsini@unimi.it (E. Corsini).

Most chemical allergens are less than 1000 daltons and are electrophilic or hydrophilic, acting as haptens and forming a stable link with protein carriers to form a complete allergen. Some of them are not inherently allergenic and must undergo metabolic transformation before participating in an allergic response. 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 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 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).

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 principle, a test system comprised of KC alone may not be useful in establishing allergenic potency as these cells lack antigen presenting capacity. However, in addition to chemical processing LC activation requires the binding of cytokines produced by KC as a result of initial chemical exposure. The irritant capacity of allergens might present an additional risk factor so that irritant allergens may be stronger allergens than non-irritant ones (Grabbe et al., 1996). In that case, the potency of chemicals to induce cutaneous sensitisation may be assessed as a function of KC cytokine expression. Starting from the in vivo observation that in mouse IL-1 α expression by KC was selectively increased after in vivo application of contact sensitizers but not tolerogen or irritant (Enk and Katz, 1992), similar results were reproduced in vitro using the murine KC cell line HEL30 (Corsini et al., 1998). Same results were also obtained by van Och et al. (2005) and, furthermore, the Authors observed that the ranking of potency was similar to the ranking established using the local lymph node assay. Similarly, using human KC it has been demonstrated that allergens but not irritants or tolerogens induced IL-12 (Muller et al., 1994; Corsini et al., 1999). Trinitrobenzene sulphonic acid induced the expression of CD40 on KC, whereas SLS did not (Coutant et al., 1999). All together these studies indicate the possibility to identify contact sensitizers using murine or human KC.

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-12 and IL-18 play important roles in the development of T helper type I (Th1) cells and are synergistic in the induction of IFN- γ by T-cells (Okamura et al., 1995). IL-18 has been demonstrated to favor Th-1 type immune response by enhancing the secretion of pro-inflammatory mediators such as TNF- α , IL-8 and IFN- γ , and to play a key proximal role in the induction of allergic contact dermatitis (Cumberbatch et al., 2001). Human keratinocytes constitutively express IL-18 mRNA and protein (Naik et al., 1999). Antonopoulos et al. (2008) have recently demonstrated that IL-18 is a key proximal mediator of LC migration and contact hypersensitivity, acting upstream of IL-1 β and TNF- α , suggestive of a central role in regulation of cutaneous immune response.

The purpose of this work was therefore to explore the possibility whether IL-18 production in human KC can be used as a sensitive method to identify contact allergens, discriminating them from respiratory allergens and irritants. NCTC 2544 cells were exposed to a panel of contact and respiratory allergens and irritants. Overall, our data suggests that production of intracellular

IL-18 may indeed represent a promising in vitro model for the screening of potential contact allergens.

2. Materials and methods

2.1. Chemicals

As respiratory allergens the following chemicals were used diphenylmethane diisocyanate (MDI); trimellitic anhydride (TMA); ammonium hexachloroplatinate (HClPt). As skin sensitizers 2,4-dinitrochlorobenzene (DNCB), 4-bromobenzylbromide, 2-mercaptobenzothiazole, cinnamaldehyde, cinnamyl alcohol, eugenol, isoeugenol, resorcinol, tetramethylthiuram disulfite (TMTD); p-phenylenediamine (PPD); and, as irritants sodium lauryl sulphate (SLS); salicylic acid, lactic acid, and phenol were used. Glycerol was used as negative compound. The choice of chemicals was dictated by the SENS-IT-IV programme. 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. MDI, TMA, DNCB, 4-bromobenzylbromide, 2-mercaptobenzothiazole, cinnamaldehyde, cinnamyl alcohol, resorcinol, TMTD, PPD and phenol were dissolved in DMSO (final concentration of DMSO in culture medium 0.2%), while HClPt, glyoxal, glycerol, salicylic acid, lactic acid and SLS were dissolved in PBS. Cell culture media and all supplements were from Sigma.

2.2. Cell culture

For experiments using NCTC 2544 cells (Istituto Zooprofilattico di Brescia, Brescia, Italy), cells were cultured in 24-well plate at a cell density of $1.5\text{--}2 \times 10^5/\text{ml}$ (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.

Primary human KC were obtained from Invitrogen (Carlsbad, CA, USA). Cells were seeded at $2.5 \times 10^4/\text{ml}$ in 24-well plate. Cells were grown in serum free supplemented keratinocyte growth medium (Sigma) to 80–90% density prior to treatment. Cells were grown at 37 °C in 5% CO₂. Second and third passages post-cryopreserved seedings were used for experiments.

2.3. Cell viability

Prior to study the effects of allergens and irritants on IL-18 production, cytotoxicity of chemicals was assessed by the MTT test (Gerlier and Thomasset, 1986). About 100 μl of cells ($1.5\text{--}2 \times 10^5/\text{ml}$) 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, 10 μl /well of MTT solution 7.5 mg/ml in culture medium was added. Cells were incubated for 3 h at 37 °C, medium was discarded and cells lysed in 100 μl /well of a mixture of HCl 1 N:isopropanol (1:24). The absorbance of the resulting solutions was read at a wavelength of 595 nm in a microplate reader (Molecular Devices). About 75% cell viability (CV₇₅) was calculated for each chemical by linear regression analysis of data.

2.4. Cytokine production

For IL-18 production cells were incubated for different times with increasing concentrations of the selected chemicals, or DMSO as vehicle control as indicated in the figure legends. 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. Cell lysates were stored at -80°C until measurement. Intracellular IL-18 content was assessed by specific sandwich ELISA commercially available (MBL, Nagoya, Japan). Limit of detection pg/ml. Results are expressed in pg/mg of total intracellular protein content. The protein content of the cell lysate was measured using a commercial kit (Bio-Rad, Segrate, Italy).

2.5. Data analysis

All experiments were performed at least three times, with representative results shown. The data presented are expressed as mean $\pm$ SD. Statistical significance was determined by Dunnett's multiple comparison test or Tukey's *t*-test as reported in the figure legends. Differences were considering significant for $p < 0.05$.

3. Results

3.1. Determination of 75% cell viability

The concentration of chemical resulting in 75% of viability or CV_{75} respect to vehicle treated cells 24 h after treatment was calculated for all chemicals. About 500–1000 $\mu\text{g/ml}$ were the highest concentration tested. Results are presented in Table 1. For the majority of chemicals CV_{75} represented the highest concentration tested. In Table 1 is also reported the category and classification according to the LLNA EC3 values (Basketter et al., 1999; Gerberick et al., 2005). Both weak and extreme sensitizers were used.

3.2. Effect of the selected chemicals on cell-associated IL-18

Preliminary experiments were carried out to determine the best time, cell density and serum concentrations to measure cell-associated IL-18. In time course experiments, cells, at 80–90% density, were treated for different times (5, 24, 30 and 48 h) with salicylic acid (250 $\mu\text{g/ml}$), and PPD (60 $\mu\text{g/ml}$). As shown in Fig. 1 A, statistically significant increase in intracellular IL-18 was detected in cells treated with PPD already after 5 h of treatment, reaching a plateau after 24–30 h, while no changes were observed following treatment with the irritant salicylic acid. Therefore, the incubation time of 24 h was chosen for all other investigations.

The effect of cell density on the response to the contact allergen PPD was investigated seeding cells at different density (1 to 10×10^5 cells/ml in 24-well plate). After over night adherence, cells were treated for 24 h with PPD (60 $\mu\text{g/ml}$) or DMSO as vehicle control. As shown in Fig. 1 B, 1 to 2.5×10^5 cells/ml represents the optimal cell density, higher cell density ($>5 \times 10^5$ cells/ml) resulted in a loss of effect. Experiments were therefore conducted using 1.5 to 2.5×10^5 cells/ml.

To investigate the effect of serum concentrations on contact allergen-induced increase in intracellular IL-18, cells were treated with cinnamaldehyde (60 $\mu\text{g/ml}$), DNCB (0.5 $\mu\text{g/ml}$) and PPD (60 $\mu\text{g/ml}$) or DMSO as vehicle control in the presence of 1 or 10% of FCS. As shown in Table 2, similar results were obtained under the two experimental conditions, with the exemption of cinnamaldehyde, which showed a higher response if cells were cultured in 10% FCS. Therefore, all experiments were conducted using 10% FCS.

3.3. Effect of contact allergens on cell-associated IL-18

NCTC 2544 cells were then treated for 24 h with increasing concentrations of the contact allergens DNCB, TMTD, cinnamaldehyde, cinnamic alcohol, eugenol, isoeugenol, resorcinol, PPD. As shown in Fig. 2, all contact allergens, including the proaptens cinnamic alcohol and eugenol, induced a dose-related increase in intracellular IL-18. Additional contact allergens tested, not reported in Fig. 2, included glyoxal, 4-nitro benzylbromide, 2-mercaptobenzothiazole. Also for these chemicals a statistically significant increase was observed. In particular, the treatment with glyoxal 25 $\mu\text{g/ml}$ the intracellular level of IL-18 was $283 \pm 52^*$ pg/mg, with 50 $\mu\text{g/ml}$ $371 \pm 107^{**}$ (control 132 ± 49); with 4-nitrobenzylbromide 1.25 $\mu\text{g/ml}$ was $368 \pm 15^*$, with 2.5 $\mu\text{g/ml}$ was $442 \pm 14^{**}$ (control 293 ± 13); with 2-mercaptobenzothiazole 62 $\mu\text{g/ml}$ was $817 \pm 57^{**}$ and with 125 $\mu\text{g/ml}$ was $960 \pm 111^{**}$ (control 594 ± 29).

3.4. Effect of respiratory allergens and irritants on cell-associated IL-18

We then tested the effect of the respiratory allergens MDI, TMA, HClPt and the irritants SDS, salicylic acid, lactic acid, and phenol. None of them was able to increase intracellular IL-18 content (Fig. 3), suggesting a selective up-regulation by contact allergens

Table 1
Concentration of the chemicals that induced 75% viability (CV_{75}) and their classification.

Chemical	CV_{75} ($\mu\text{g/ml}$)	Category	LLNA EC3 (%)
HClPt	15	Respiratory allergen	Not available
MDI	>500	Respiratory allergen	0.3 (strong)
TMA	>500	Respiratory allergen	9.2 (moderate)
Cinnamaldehyde	40	Contact allergen	2.8 (moderate)
Cinnamyl alcohol	300	Contact allergen	21 (weak)
DNCB	5	Contact allergen	0.05 (extreme)
Eugenol	225	Contact allergen	12 (weak)
Glyoxal	50	Contact allergen	1.3 (moderate)
Isoeugenol	225	Contact allergen	1.2 (moderate)
PPD	108	Contact allergen	0.22 (strong)
Resorcinol	>1000	Contact allergen	6 (moderate)
TMTD	32	Contact allergen	5.6 (moderate)
2-Mercaptobenzothiazole	125	Contact allergen	5.8 (moderate)
4-Nitrobenzylbromide	2.5	Contact allergen	0.05 (extreme)
Phenol	70	Irritant	Non sensitizer
Lactic acid	>500	Irritant	Non sensitizer
Salicylic acid	250	Irritant	Non sensitizer
SLS	30	Irritant	14.0 (weak, false positive)
Glycerol	>1000	Negative	Non sensitizer

Confluent cells were treated for 24 h with different chemicals or DMSO (0.2% final concentration) as vehicle control. Results are expressed as the concentration of chemical resulting in 75% cell viability, as assessed by MTT reduction. CV_{75} was calculated by linear regression analysis of data.

In the last column, 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 ($\text{SI} = 3$); 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.

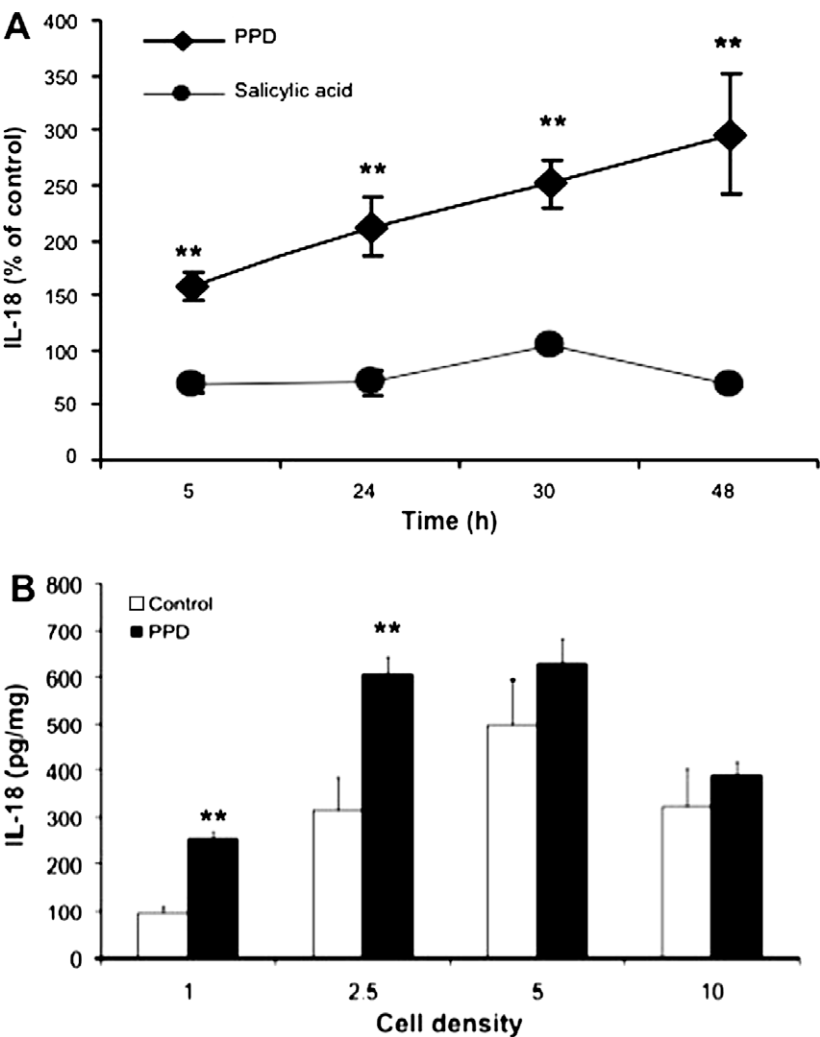


Fig. 1. Time course and effect of cell density on intracellular IL-18. Panel A, time course. NCTC 2544 cells were treated for different times (5, 24, 30, 48 h) with PPD (60 µg/ml), and salicylic acid (250 µg/ml) or vehicle control. Results are expressed as mean ± SD, n = 4. Statistical analysis was performed with Dunnett's multiple comparison test, with ^{***}p < 0.01 versus vehicle treated cells. Panel B, effect of cell density: NTCT 2544 cells were seeded in 24-well plate at different density (1–10 × 10⁵ cells/ml) and treated for 24 h with PPD (60 µg/ml) or DMSO as vehicle control. Results are expressed as mean ± SD, n = 4. Statistical analysis was performed by Tukey's multiple comparison test, with ^{***}p < 0.01 versus vehicle treated cells (control).

Table 2

Effect of FCS on contact allergen-induced intracellular IL-18 production.

Treatment	1% FCS (IL-18% of control)	10% FCS (IL-18% of control)
Cinnamaldehyde 60 µg/ml	584 ± 87	839 ± 148 [§]
DNCB 0.5 µg/ml	146 ± 5	176 ± 29
PPD 60 µg/ml	414 ± 56	358 ± 54

NTCT 2544 cells were treated with three different contact allergens or DMSO (0.2% final concentration) as vehicle control. Twenty-four hours later intracellular IL-18 was evaluated as described in Section 2. Results are expressed as% of control. Each value represents the mean ± SD, n = 4. Statistical analysis was performed by Tukey's multiple comparison test, with [§]p < 0.05 versus cells treated in 1% FCS.

only. We additionally tested glycerol, which also failed to induce IL-18.

3.5. Data reproducibility

We investigated the reproducibility of data by comparing the results obtained in seven independent experiments conducted over a three months period treating NCTC 2544 cells with PPD 60 µg/ml. As shown in Fig. 4, in all experiments PPD induced a sta-

tistically significant increase in intracellular IL-18, with a stimulation index ranging from 1.8 to 3.6.

3.6. Effects of selected chemicals on cell-associated IL-18 in primary human KC

We then asked the question if these results were peculiar of this cell line or if similar results could be obtained using other keratinocyte models. We could demonstrate that similar results were obtained also using primary human KC. As shown in Table 3, while cinnamaldehyde induced a dose-related increase in intracellular IL-18 content, HClPt, SDS and phenol failed, confirming the results obtained with NCTC 2544 cell line, and indicating the possibility to use other keratinocyte models.

4. Discussion

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

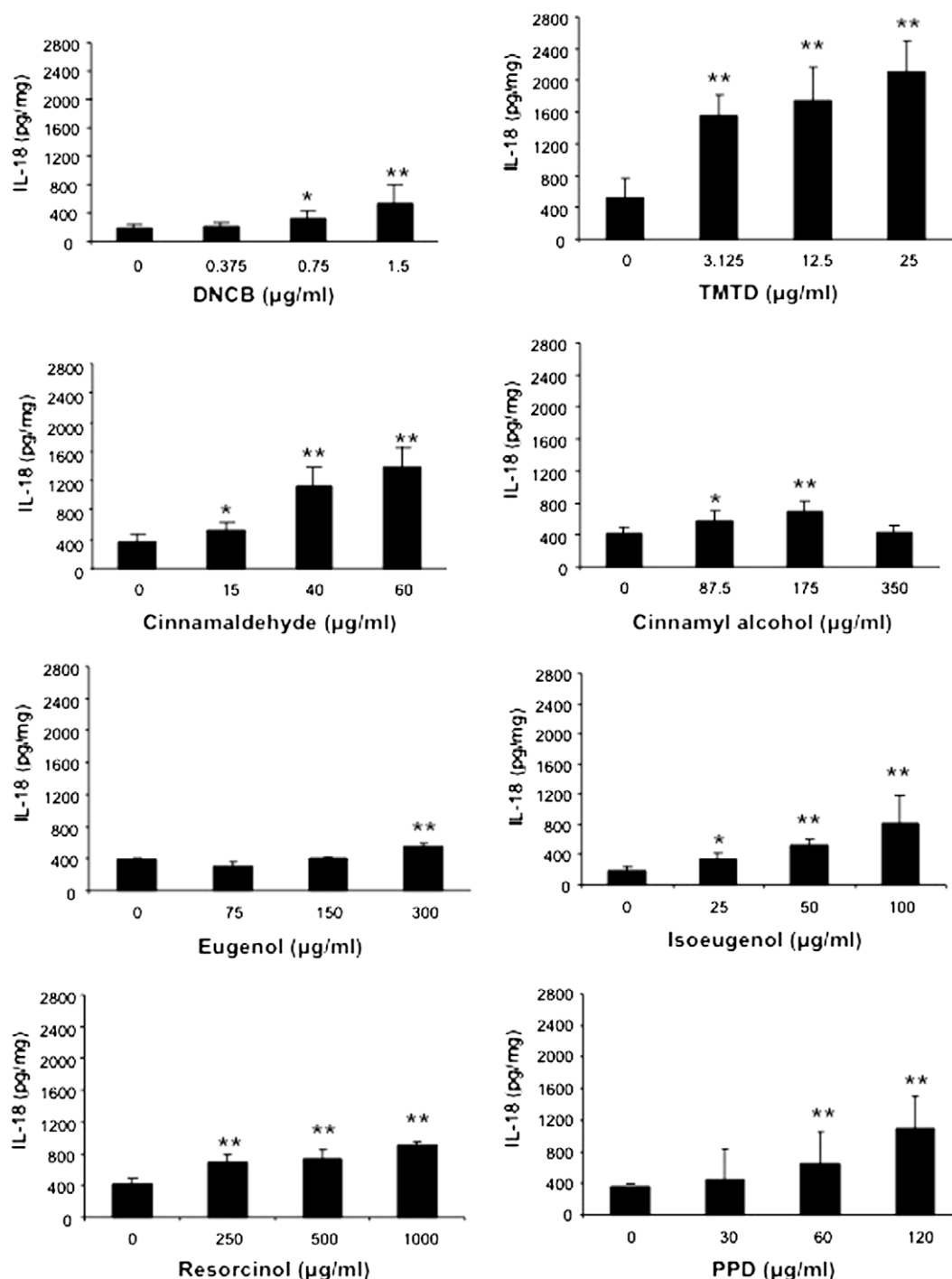


Fig. 2. Dose response effects of contact sensitizers on cell-associated IL-18. NCTC 2544 cells were treated for 24 h with increasing concentrations of DNCB, TMTD, cinnamaldehyde, cinnamyl alcohol, eugenol, isoeugenol, resorcinol and PPD. Cells incubated with vehicle were included as a control (0). Results are expressed as mean $\pm$ SD, $n = 4$. Statistical analysis was performed with Dunnett's multiple comparison test, with * $p < 0.05$ and ** $p < 0.01$ versus vehicle treated cells (0).

to induce IL-18 production, indicating the possibility to use IL-18 it to identify contact allergens.

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 not 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.

We therefore investigated if skin sensitizers could selectively up-regulate this cytokine in human KC. We could indeed demonstrate that contact sensitizers, including proapptens, increase in a dose-related manner intracellular IL-18. In contrast, irritants and

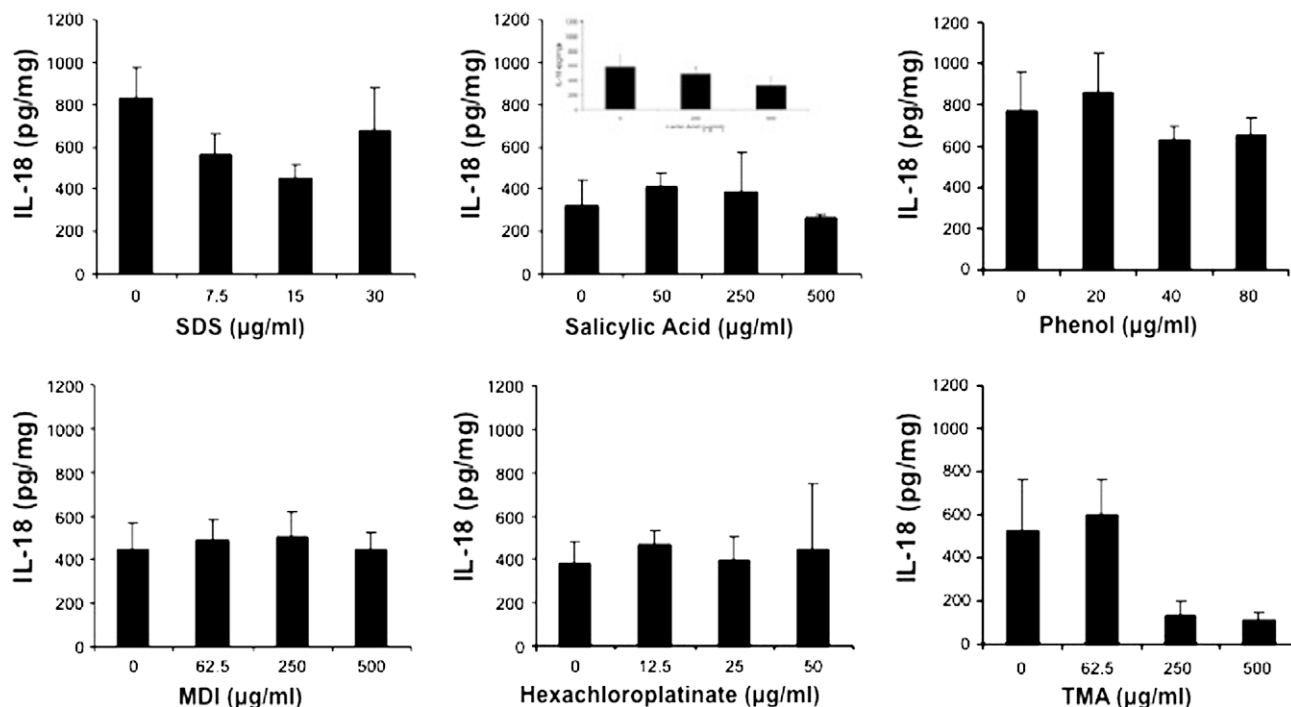


Fig. 3. Dose response effects of irritants and respiratory sensitizers on cell-associated IL-18. NCTC 2544 cells were treated for 24 h with increasing concentrations of SDS, salicylic acid, lactic acid, phenol, MDI, hexachloroplatinate, TMA. Cells incubated with vehicle were included as a control (0). Results are expressed as mean $\pm$ SD, $n = 4$. Statistical analysis was performed with Dunnett's multiple comparison test.

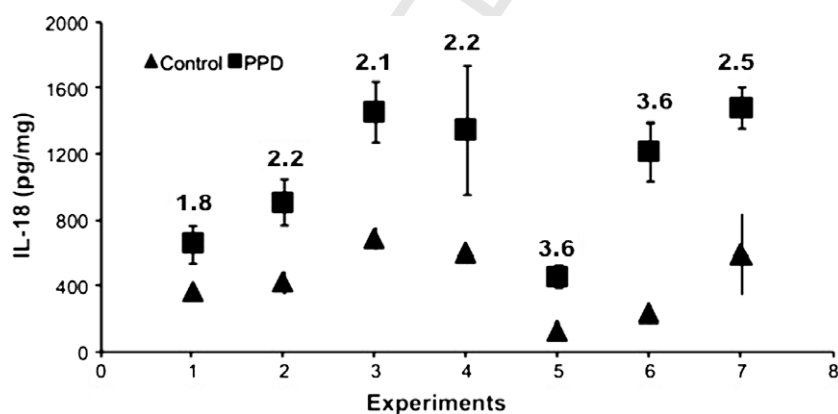


Fig. 4. Reproducibility of data. Results obtained in seven independent experiments performed over a three months period are reported. NCTC 2544 cells were treated for 24 h with PPD (60 μ g/ml) or with DMSO as vehicle control. Intracellular IL-18 was evaluated as described in Section 2. Results are expressed as mean $\pm$ SD, $n = 4$. Statistical analysis was performed with Student's *t*-test, in all experiments PPD induced a statistical significant increase in cell-associated IL-18. The numbers reported in figure represent the stimulation index of the individual experiment.

respiratory allergens failed to up-regulate IL-18. The measure of the release of IL-18 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). Also the evaluation of IL-18 mRNA expression did not provide additional advantage. KC constitutively express high IL-18 mRNA levels, which could not be modulated further following treatment with contact allergens as assessed by real time PCR (data not shown). The latest finding is consistent with data obtained by Naik et al. (1999) in primary human KC treated with DNCB.

Cultures of adult human KC are readily available, even commercially, but differences in metabolism and response to stimuli can exist between cultures derived from different donors (Berghard et al., 1990); therefore, established lines of human KC represent a

good alternative. We obtained similar results using both the NCTC 2544 cell line and primary human KC, confirming the relevance of the proposed model and the possibility to use different source of KC.

Important factors including 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 TMA, highly instable 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 of 20 s. If submerged cell culture may be unfavourable 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

Table 3

Effects of selected chemicals on IL-18 production in primary human keratinocytes.

Chemical (μg/ml)	Intracellular IL-18 (pg/mg)
Control	507 ± 73
HClPt 25	617 ± 110
HClPt 50	482 ± 224
HClPt 100	582 ± 162
Cinnamaldehyde 12.5	1455 ± 367*
Cinnamaldehyde 25	1256 ± 218*
Cinnamaldehyde 50	889 ± 76*
SDS 15	450 ± 13
SDS 30	198 ± 50
Phenol 40	527 ± 134
Phenol 80	438 ± 24

Primary keratinocytes were treated with different concentrations of selected chemicals or DMSO (0.2% final concentration) as vehicle control. Twenty-four hours later intracellular IL-18 content was evaluated as described in Section 2. Results are expressed as pg/mg of total protein content. Each value represents the mean ± SD, $n = 3$. Statistical analysis was performed by Dunnett's multiple comparison test, with $p < 0.05$ versus vehicle-treated control cells.

oil. Preliminary results obtained testing DNCB, phenol, salicylic acid, TMA and MDI showed an increase in IL-18 only following treatment with DNCB, which may support results obtained with submerged keratinocytes.

The lack of metabolic activation may be a relevant problem in case of proaptens. However, NCTC 2544 cells posses both phase I and II metabolic activation capacity (Gelardi et al., 2001), and positive results were indeed obtained with the proaptens eugenol and cinnamic alcohol (Fig. 2). 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 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 have 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).

In terms of potency, the calculation of concentration of allergen that induced a 1.4 increase in cell-associated IL-18 by linear regression analysis of data gave the following values: DNCB (0.5 μg/ml), 4-bromobenzylbromide (2.0 μg/ml), 2-mercaptobenzothiazole (92 μg/ml), cinnamaldehyde (11.0 μg/ml), cinnamyl alcohol (103 μg/ml), eugenol (344 μg/ml), isoeugenol (12.8 μg/ml), glyoxal (9.0 μg/ml), resorcinol (206.0 μg/ml), PPD (31.7 μg/ml), TMTD (1.04 μg/ml). If compared with the available in vivo LLNA EC3 values a significant correlation ($R = 0.754$, $p = 0.0098$) was obtained. Therefore, IL-18 production by KC may also be seen as a possible tool for the prediction of sensitizing strength of a particular chemical. However, investigations are needed to better address the question of potency.

In conclusion, IL-18 production by NCTC 2544 cells, or primary KC, may represent a promising in vitro model for the screening of potential contact allergens and may be useful for ranking chemicals also for their sensitizing potency resulting in a reduction of animal testing. The test may also be complementary to the LLNA, allowing for a rapid and easy discrimination of contact sensitizers from respiratory allergens. Our results warrant further analysis of panels of potential allergens and irritants. 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, including dendritic like cells.

Conflict of Interest statement

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.

5. Uncited reference

Banchereau et al. (1991).

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