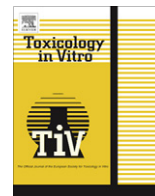




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Toxicology in Vitro

journal homepage: www.elsevier.com/locate/toxinvitFurther development of the NCTC 2544 IL-18 assay to identify *in vitro* contact allergensV. Galbiati^a, M. Mitjans^b, L. Lucchi^a, B. Viviani^a, C.L. Galli^a, M. Marinovich^a, E. Corsini^{a,*}^a Laboratory of Toxicology, Department of Pharmacological Science, Università degli Studi di Milano, Italy^b Dpt. Fisiologia, Facultat de Farmàcia, Universitat de Barcelona, Spain

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ABSTRACT

Several European Union legislations request the use of *in vitro* methods for toxicological evaluations, including sensitization, in order to increase consumer safety but also to reduce the use of animals. The EU project SENS-IT-IV addresses the need of developing predictive *in vitro* tests to assess contact and respiratory hypersensitivity reactions. In this context, we have recently reported the possibility to use IL-18 production in the human keratinocyte cell line NCTC 2544 to discriminate contact sensitizer from irritants and low molecular weight respiratory allergens. The aims of the present study were to further develop this assay in order to optimize experimental conditions; to develop a 96-well plate format to establish a high throughput assay; to test the performance of other available keratinocyte cell lines, and to understand the signal transduction pathway involved in p-phenylenediamine (PPD)-induced IL-18 production.

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. In order to develop a high throughput test, cells seeded in 96-well plate were exposed to contact allergens (2,4-dinitrochlorobenzene, p-phenylenediamine, isoeugenol, cinnamaldehyde, tetramethylthiuram disulfite, resorcinol, cinnamic alcohol and eugenol), irritants (phenol, sodium laurel sulphate, lactic acid and salicylic acid) and respiratory allergens (hexachloroplatinate, diphenylmethane diisocyanate, trimellitic anhydride). A selective increase in total (intracellular plus released) 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. A selective induction of IL-18 was also obtained testing with PPD other keratinocyte cell lines, namely HPKII and HaCaT, with the HPKII showing the highest stimulation index. Regarding the signal transduction pathway, we could demonstrate using selective inhibitors a role for oxidative stress, NF- κ B and p38 MAPK activation in PPD-induced IL-18 production.

In conclusion, results obtained suggest that the production of IL-18 represents a promising endpoint for the screening of potential contact allergens. The assay can be performed in a 96-well plate format, different keratinocyte cell lines can be used, and a role for oxidative stress in contact allergen-induced IL-18 was demonstrated.

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1. Introduction

At present, several animal-based assays are used to assess immunotoxic effects such as immunosuppression and sensitization. Over the last decade, a great deal of progress has been made in the development of alternative *in vitro* test to assess these two realities (Lankveld et al., 2010; Vandebriel et al., 2005; Galbiati

et al., 2010). Animal testing causes increasing ethical problems, and the predictability of results to the human situation is sometimes questionable. Driven by the 7th Amendment to the EU Cosmetic Directive, the new EU policy on chemicals (the REACH system), the update of the European legislation on the protection of animals used in research, and emerging visions and strategies for predicting toxicity, *in vitro* methods are likely to play a major role in the near future. While a number of methods are at various stages of development and use, there are, at present, no cell-based assay capable of distinguish all sensitizer from non-sensitizers in a test panel of chemicals, nor it is possible to rank the sensitizing potencies of the chemicals (Corsini and Roggen, 2009; dos Santos

* Corresponding author. Address: Laboratory of Toxicology, Department of Pharmacological Sciences, Università degli Studi di Milano, Via Balzaretti 9, 20133 Milano, Italy. Tel.: +39 02 50318241; fax: +39 02 50318284.

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

et al., 2009; Galbiati et al. 2010). It is expected that a predictive method to totally replace animal testing will be in the form of a test battery comprising molecular, cell-based, and/or computational methods (Basketter and Kimber, 2009).

Irritant and allergic contact dermatitis is undesired side effects in the development of drugs and cosmetics as well as after contact with environmental or industrial chemicals. Whereas irritant contact dermatitis is a skin inflammation induced by primary contact with chemicals, and is thought not to be mediated by lymphocytes, allergic contact dermatitis represents a lymphocyte-mediated delayed type hypersensitivity reaction, which requires previous sensitization by the same chemicals (Basketter et al., 2008; Nosbaum et al., 2009). 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, 1988; Remaut and Thune, 1992; Paulsen and Andersen, 1993), and various metals including nickel (Picardo et al., 1990) and chromium (Nethercott and Holness, 1989). Keratinocytes (KC) play a role in all phase of allergic dermatitis. They respond to allergen by producing a range of pro-inflammatory cytokines, which are important for dendritic cells maturation and migration to the draining lymph node, where the activation of the specific immune response take place (Vandebriel et al., 2005). Therefore, due to the anatomical location of KC, and their significant role in skin inflammatory and immunological reactions, their use in evaluation of the sensitizing potentials of compounds *in vitro* is amply justified. The *in vivo* observation that IL-1 α expression by mouse KC is specifically increased following the application of contact sensitizer, but not following exposure to a tolerogen or irritant (Enk and Katz, 1992), has been reproduced *in vitro* in the murine KC cell line HEL30 (Corsini et al., 1998; Van Och et al., 2005). Furthermore, it was observed that potency ranking order was similar to that achieved with the LLNA (Van Och et al., 2005). By using human KC, it was demonstrated that allergens, but not irritants or tolerogens, induced IL-12 (Muller et al., 1994; Corsini et al., 2009) and CD40 (Coutant et al., 1999). Recently, a novel assay using the human KC cell line HaCaT containing a reporter construct with a single copy of the ARE-element of the human ARK1C2 gene has been developed, with an overall accuracy of 89.6% (Emter et al., 2010). All these studies indicate that KC may offer the opportunity to identify contact sensitizer.

We recently shown that exposure of the human keratinocyte cell line NCTC 2544 or primary KC to contact allergens, but not to respiratory allergens or irritants, resulted in a dose-related induction of intracellular IL-18 (Corsini et al., 2009) indicating that KC may also offer the opportunity to discriminate contact allergens from respiratory sensitizers and skin irritants. The upregulation of IL-18 by contact allergens confirmed previous results published by van Och et al. (2005) in HaCaT cells.

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). Various cell types, including macrophages and KC, store abundant proIL-18, a biological inactive precursor of IL-18, under normal conditions and increase IL-18 mRNA expression upon stimulation with LPS and/or IFNs (Tsutsui et al., 2004). IL-18 has been shown to play a key proximal role in the induction of allergic contact sensitization, and to favour Th-1 type immune response by enhancing the secretion of pro-inflammatory mediators such as TNF- α , IL-8 and IFN- γ (Antonopoulos et al., 2008; Cumberbatch et al., 2001; Okamura et al., 1995). 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 this cytokine does not affect any of the asthma specific parameters, including allergen-specific IgE, histological changes of the lungs,

infiltrating leukocytes, serum cytokines levels and airway hyper responsiveness.

Together, these studies imply that murine or human KC-based assays can identify contact sensitizers. It is, however, important to stress that the complexity of hypersensitivity reactions and the chemical diversity of allergens probably require any approach for the identification of a chemical allergen to be built on several markers of activation and different cell types, including dendritic cells, like Langerhans cells (Corsini and Roggen, 2009).

The purpose of this work was to further develop the current NCTC 2544 IL-18 assay in order to: (1) to optimize experimental conditions to limit the cell density issue; (2) to develop a 96-well plate format to establish a high throughput assay; (3) to test the performance of other available keratinocyte cell lines; and (4) to understand the signal transduction pathway involved in p-phenylenediamine (PPD)-induced IL-18 production.

2. Materials and methods

2.1. Chemicals

As respiratory allergens the following chemicals were used: ammonium hexachloroplatinate (HClPt), diphenylmethane diisocyanate (MDI), and trimellitic anhydride (TMA). As skin sensitizers 2,4-dinitrochlorobenzene (DNCB), p-phenylenediamine (PPD), isoeugenol, cinnamaldehyde, tetramethylthiuram disulfite (TMTD), resorcinol, cinnamic alcohol and eugenol; and as irritants phenol, sodium laurel sulphate (SLS), lactic acid and salicylic acid (Sal. Ac.) were used. The choice of chemicals was dictated by the SENS-IT-IV program (Newsletter number 02–2007, January, 26, http://www.sens-it-iv.eu/files/newsletter/Sens-it-iv_Newsletter_08.html). Chemicals have been selected by relevant and representative of the ‘universe’ irritants, respiratory and contact allergens. All reagents, including inhibitors, were purchased from Sigma (St Louis, MO, USA) at the highest purity available. MDI, TMA, DNCB, cinnamaldehyde, cinnamic alcohol, resorcinol, TMTD, PPD, salicylic acid, lactic acid and phenol were dissolved in DMSO (final concentration of DMSO in culture medium 0.2%), while HClPt and SLS were dissolved in PBS. Cell culture media and all supplements were from Sigma.

2.2. Cell culture

For experiments using NCTC 2544 cell line (Istituto Zooprofilattico di Brescia, Italy), cells were cultured in 96-well (0.1 ml/well) or 24-well plate (0.5 ml/well) at different density: $1\text{--}10 \times 10^5$ cells/ml. Cells were treated in 0.1–0.5 ml of RPMI 1640 containing 2 mM L-glutamine, 0.1 mg/ml streptomycin, 100 IU/ml penicillin, supplemented with 10% heated-inactivated fetal calf serum (media) and cultured at 37 °C in 5% CO₂.

To study the impact of cell density on the selective production of IL-18 by contact allergens, 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 μ g/ml) or DMSO as vehicle control or after 2 h of adherence, cells were treated for 24 h with PPD (60 μ g/ml), cinnamaldehyde (30 μ g/ml), salicylic acid (500 μ g/ml) or DMSO as vehicle control.

To develop a high throughput assay, NCTC 2544 were seeded at the cell density of $1.5\text{--}2.5 \times 10^5$ cells/ml in 96-well plate and treated after overnight adherence for 24 h with increasing concentration of contact sensitizers (DNCB, PPD, isoeugenol, cinnamaldehyde, TMTD, resorcinol, cinnamic alcohol and eugenol), respiratory allergens (hexachloroplatinate, MDI and TMA) and irritants (phenol, SDS, lactic acid and salicylic acid).

For experiments using HPK II kindly provided by Prof Fusening (Heidelberg University, Germany), and HaCaT cells lines (ATCC, LCG Standards, Sesto San Giovanni, Italy), cells were cultured in 24-well plate at a cell density of 1.5×10^5 cells/ml. NCTC 2544, HPK II and HaCaT cell lines were treated for 24 h with PPD (60 μ g/ml) or DMSO as vehicle control.

To investigate the signal transduction pathway involved in PPD-induced IL-18 production, NCTC 2544 were treated with several inhibitors, namely, the selective inhibitor of protein kinase C α and β 1 isoforms GF109203X (5 μ M), the inhibitor of NF- κ B and anti-oxidant pyrrolidine dithiocarbamate (PDTC 100 μ M), the irreversible inhibitor of I- κ B Bay 11-70-85 (1 μ M), and the inhibitor of p38 MAPK SB203580 (100 nM), for 1 h, and then PPD (60 μ g/ml) or DMSO as vehicle control were added for 24 h. p38 MAPK and NF- κ B activation were assessed by Western blot analysis as described below.

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). A 1.5×10^5 cells/ml (100 μ l/well) were seeded in 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 μ l/well of MTT solution 7.5 mg/ml in culture medium was added. Cells were incubated for 3 h at 37 $^{\circ}$ C, medium was discarded and cells lysed in 100 μ l/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). Eighty percent age cell viability (CV₈₀) was calculated for each chemical by linear regression analysis of data. The CV₈₀ was the highest concentration tested for IL18 production.

2.4. Cytokine production

For the assessment of IL-18 production in cells seeded in 24-well plate after incubation, culture medium was discarded, monolayer gently washed twice 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). 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). Stimulation index (SI) was calculated as follow:

$$SI = \frac{\text{IL-18 in chemical-treated cells}}{\text{IL-18 in vehicle-treated cells}}$$

In the 96-well plate format, total IL-18 production was assessed (both intracellular and released). In this case after incubation, 20 μ l of 3% Triton X-100 (0.5% final concentration) in PBS was added to each well. Plates were then frozen at -80° C, and 100 μ l directly transferred from the 96-well plate to the ELISA plate to assess IL-18 content. In this case, results are expressed as pg/ml.

2.5. Western Blotting

For Western blot analysis of p38 MAPK activation and I- κ B degradation, as measure of NF- κ B activation, NCTC 2544 cells were seeded in 6-well plate at a cell density of 2.5×10^5 cells/ml (2 ml/well). After 24 h of adherence, cells were treated with PPD (60 μ g/ml) or DMSO as vehicle control for different times (5–60 min). After incubation, culture medium was discarded, 1 ml of cold PBS was added, cells were collected by scraping, transferred

in Eppendorf tubes and centrifuge for 5 min at 1200 rpm at 5 $^{\circ}$ C. The supernatant was discarded and cells lysed in 100 μ l of homogenization buffer (50 mM Tris, 150 mM NaCl, 5 mM EDTA pH 7.5, 0.5% Triton X-100, 50 μ M PMSF, 2 μ g/ml aprotinin, 1 μ g/ml pep-

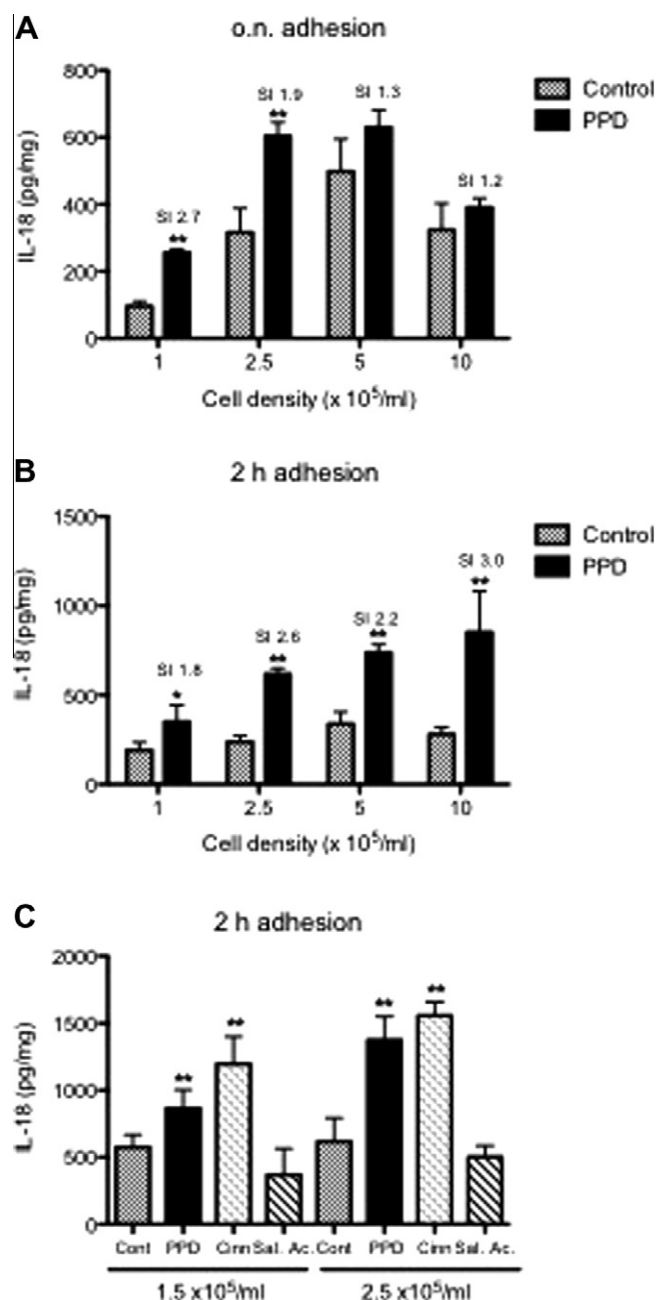


Fig. 1. Effect of cell density and adhesion time on intracellular IL-18. Panel A, over night adhesion. NCTC 2544 were seeded in 24-well plate at different density ($1-10 \times 10^5$ cells/ml). After overnight adherence, cells were treated for 24 h with PPD (60 μ g/ml) or DMSO (0.2% final concentration) as vehicle control. Results are expressed as mean $\pm$ SD, $n = 4$. Panel B, 2 h adhesion. NCTC 2544 were seeded in 24-well plate at different density ($1-10 \times 10^5$ cells/ml). After 2 h adherence, cells were treated for 24 h with PPD (60 μ g/ml) or DMSO (0.2% final concentration) as vehicle control. Results are expressed as mean $\pm$ SD, $n = 4$. Panel C, 2 h adhesion. NCTC 2544 were seeded in 24-well plate at two different density: $1.5-2.5 \times 10^5$ cells/ml. After 2 h of adherence, cells were treated for 24 h with PPD (60 μ g/ml), cinnamaldehyde (30 μ g/ml), salicylic acid (200 μ 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 for panel A, B and Dunnett's multiple comparison test for panel C, with $**p < 0.01$ versus vehicle treated cells (control). SI, stimulation index.

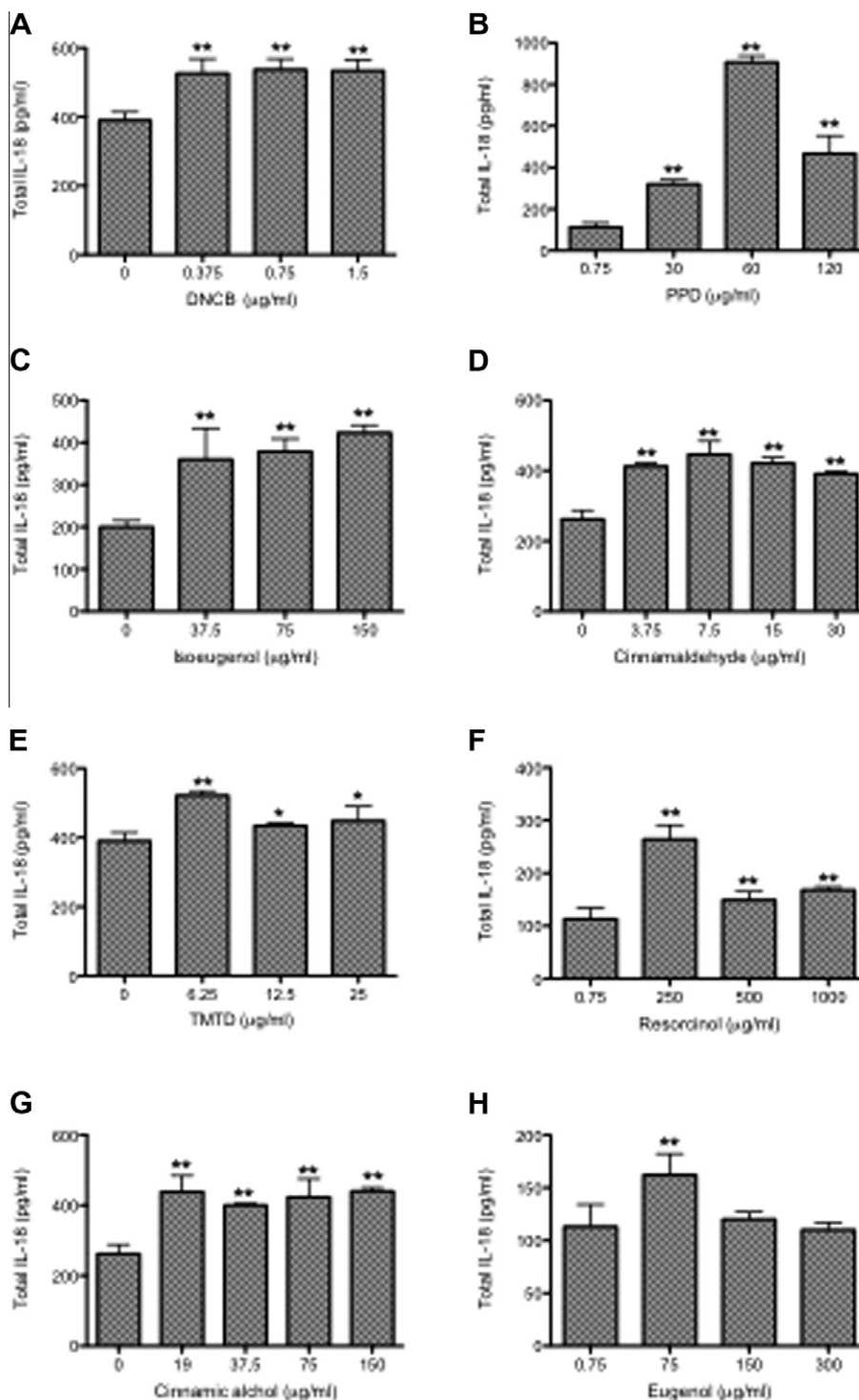


Fig. 2. Effects of contact sensitizers on total IL-18 production. NCTC 2544 seeded in 96-well plate were treated for 24 h with increasing concentration of DNFB (A), PPD (B), isoeugenol (C), cinnamaldehyde (D), TMTD (E), resorcinol (F), cinnamic alcohol (G) and eugenol (H). Cells incubated with vehicle were included as control (0). Results are expressed as mean $\pm$ SD. Statistical analysis was performed with Dunnett's multiple comparison test, with * p < 0.05 and ** p < 0.01 versus vehicle treated cells (0).

statin and 1 μ g/mL leupeptin) and denatured for 10 min at 100 $^{\circ}$ C. The protein content of the cell lysate was measured using a commercial kit (Bio-Rad, Segrate, Italy). For the Western blot analysis, 15 μ g of extracted proteins were electrophoresed into a 15% SDS-polyacrylamide gel under reducing conditions. The proteins were then transferred to PVDF membrane (Amersham, Little Chalfont, UK). The different proteins were visualized using activated p38

(1:1000), non-activated p38 (1:500) I- κ B (1:1000) as primary antibodies (Sigma), and developed using enhanced chemiluminescence (Sigma). The image of the blot was acquired with the Molecular Imager Gel Doc XR (Bio-Rad). The optical density of the bands was calculated and analyzed by means of the Image 1.47 program for digital image processing (Wayne Rasband, Research Service Branch, NIMH, NIH, Bethesda, MD, USA).

2.6. Data analysis

All experiments were repeated at least three times, with representative results shown. Data are expressed as mean $\pm$ standard deviation (SD). Statistical analysis was performed using InStat software version 3.0a (GraphPad Software, La Jolla, CA, USA). Statistical differences were determined using ANOVA followed by a multiple comparison test as indicated in the legends. Effects were designated significant if $p < 0.05$.

3. Results

3.1. Role of cell density and adhesion time on contact allergen-induced intracellular IL-18

The effect of cell density and adhesion time on the response to the contact allergen PPD was investigated seeding cells at different density ($1\text{--}10 \times 10^5$ cells/ml in 24-well plate) and treating them after o.n. or 2 h seeding. As shown in Fig. 1A, $1\text{--}2.5 \times 10^5$ cells/ml represents the optimal cell density, 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. This problem can be overcome treating cells 2 h after seeding, time sufficient to allow cells to adhere. As shown in Fig. 1C, a statistical significant upregulation of intracellular IL-18 was observed at all cell densities tested, including $5\text{--}10 \times 10^5$ cells/ml. Furthermore, as shown in Fig. 1B, seeding cells at two different density ($1.5\text{--}2.5 \times 10^5$ cells/ml), and treating them after 2 h with PPD (60 $\mu\text{g/ml}$), cinnamaldehyde (30 $\mu\text{g/ml}$), salicylic acid (250 $\mu\text{g/ml}$) or DMSO as vehicle control for 24 h, only the contact allergens induced an increase in intracellular IL-18, whereas salicylic acid fails, indicating that the discriminatory capacities are preserved.

3.2. Selective induction of total IL-18 by contact allergens

In order to develop a high throughput assay, a 96-well plate format assay was developed. NCTC 2544 cells were seeded in 96-well plate and treated for 24 h with representative contact allergens, respiratory allergens or skin irritant. The highest concentration tested for each chemical was the CV_{80} . Following treatment the total IL-18 content (intracellular and released) was assessed. NCTC 2544 cells were treated with increasing concentrations of the contact allergens DNCB, PPD, isoeugenol, cinnamaldehyde, TMTD, resorcinol, cinnamic alcohol and eugenol. As shown in Fig. 2, all contact allergens, including the pro-haptens cinnamic alcohol and eugenol, induced a statistical significant increase in total IL-18, confirming results previously obtained in 24-well format assay (Corsini et al., 2009).

We then tested the effect of the respiratory allergens HClPt, MDI, TMA and the irritants phenol, SDS, lactic acid and salicylic acid on total IL-18. None of them was able to increase total IL-18 (Figs. 3 and 4), confirming the selective up-regulation of IL-18 by contact allergens.

3.3. Effects of PPD on cell-associated IL-18 in different human KC

We then investigated if these results were peculiar of this cell line or if similar results could be obtained using other keratinocyte cell lines. At this purpose, two other KC cell lines were used, namely HaCaT and HPK II. As shown in Fig. 5, the treatment with PPD induced an increase in intracellular IL-18 content in all three cell lines tested, with the HPKII showing the highest stimulation

index: 3.4 ± 0.7 vs 1.6 ± 0.3 in NCTC 2544 and 2.1 ± 0.3 in HaCaT cells ($p < 0.05$). These results confirm the possibility to use different keratinocyte models.

3.4. Signal transduction pathway

Finally, we wanted to investigate the signal transduction pathway involved in p-phenylenediamine (PPD)-induced IL-18 production. For this purpose, several selective inhibitors were used:

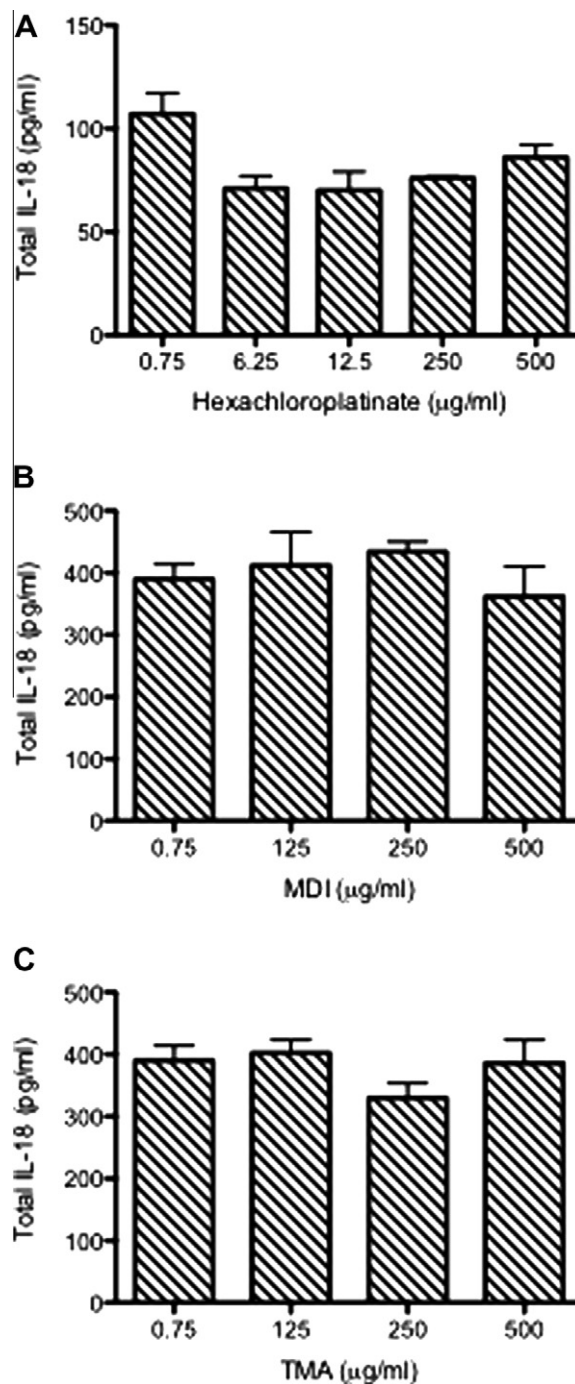


Fig. 3. Effects of respiratory allergens on total IL-18 production. NCTC 2544 seeded in 96-well plate were treated for 24 h with increasing concentration of hexachloroplatinate (A), MDI (B) and TMA (C). Cells incubated with vehicle were included as a control (0). Results are expressed as mean $\pm$ SD. Statistical analysis was performed with Dunnett's multiple comparison test with $*p < 0.05$ and $**p < 0.01$ versus vehicle treated cells (0).

GF109203X (5 μ M) to inhibit protein kinase C, PDTC (100 μ M) and Bay 11-7085 (1 μ M) to inhibit NF- κ B and SB203580 (100 nM) to inhibit p38 MAPK. As show in Fig. 6A, PPD induced intracellular IL-18 was not modulated by GF109203X, excluding the involvement of protein kinase C. On the contrary, PDTC, Bay 11-7085 and SB203580, significantly modulated PPD-induced intracellular IL-18, indicating a role for oxidative stress, NF- κ B and p38 MAPK activation. (Fig. 6B–D). The ability of PPD to activate p38 MAPK and NF- κ B was confirmed by Western blot analysis (Fig. 7). Cells were treated with PPD or DMSO as vehicle control for 5, 15, 30 and 60 min. As shown in Fig. 7, p38 MAPK was clearly activated after 15 min, whereas NF- κ B was activated at later time points.

4. Discussion

In the present manuscript, the ‘NCTC 2544 IL-18 assay’ developed to identify *in vitro* contact allergens, was further characterized. The experimental conditions were optimized to clarify the cell density issue, a high throughput assay was developed, the performance of other available keratinocyte cell lines was tested, and the signal transduction pathways involved in PPD-induced IL-18 production were characterized.

In our previous manuscript (Corsini et al., 2009), and also during technology transfer of the assay to other laboratories, a critical point was identified in the cell density. If cells reach confluence at the moment of treatment, the ability to discriminate contact allergens from skin irritants and respiratory sensitizer is lost. Due to the use of different foetal calf serum, a different cellular doubling time can be expected in the different laboratories. Therefore, as the cells number deeply influences the outcome, each laboratory should carefully check for the optimal cell density, using the positive control PPD as a reference compound. We found that a possible alternative is to treat the cells the same day of seeding after two hours of adherence. Under this condition, the selective upreg-

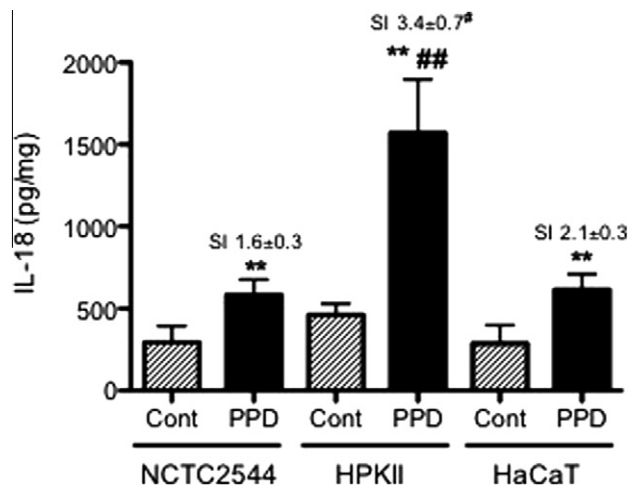


Fig. 5. Effect of PPD on intracellular IL-18 in three keratinocyte cell lines. NCTC 2544, HPKII and HaCaT cells lines were treated for 24 h with PPD (60 μ g/ml) or DMSO (0.2% final concentration) as vehicle control. Results are expressed as mean $\pm$ SD. Statistical analysis was performed with Tukey's multiple comparison test, with ** p < 0.01 versus vehicle treated cells (0). SI, stimulation index. # p < 0.05 and ## p < 0.01 vs NCTC 2544 or HaCaT cells treated with PPD.

ulation of intracellular IL-18 by contact allergens is maintained and the cell density issue is overcome.

Next, in order to further develop the assay and to increase its screening capacity, a 96-well format assay was developed. To speed up the assay and reduce cell manipulation (no cell washing steps and no protein determination), after the treatment, cells were lysed in culture medium directly adding Triton X-100 (final concentration 0.5%). In this case the total IL-18 (intracellular plus released) was assessed. Also under this experimental condition, a selective increase in total IL-18 was observed only following

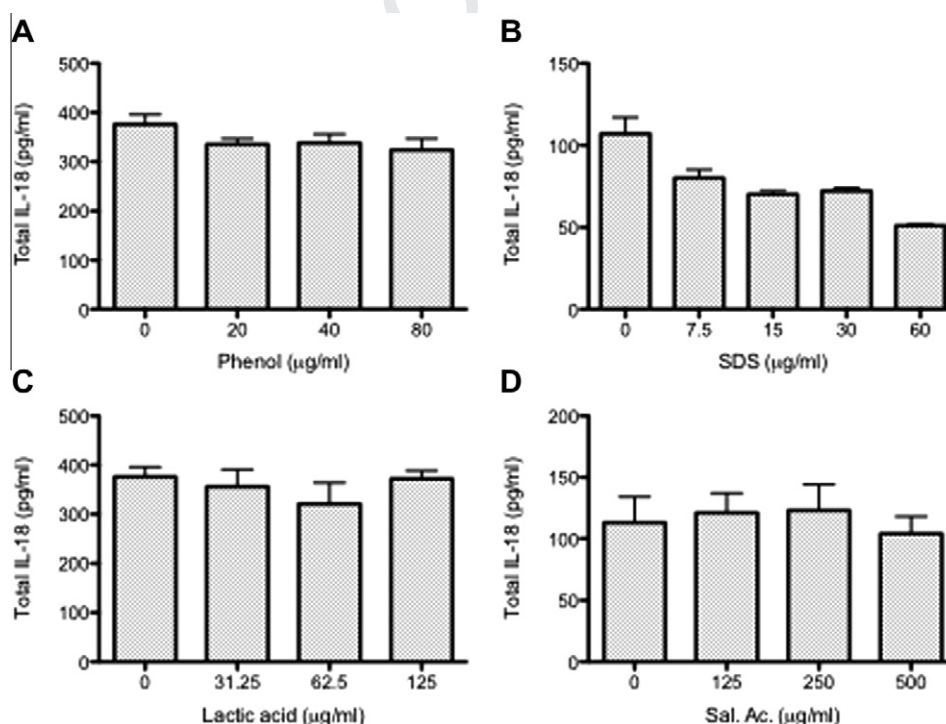


Fig. 4. Effects of irritants on total IL-18 production. NCTC 2544 seeded in 96-well plate were treated for 24 h with increasing concentration of phenol (A), SDS (B), lactic acid (C) and salicylic acid (D, Sal. Ac.). Cells incubated with vehicle were included as a control (0). Results are expressed as mean $\pm$ SD. Statistical analysis was performed with Dunnett's multiple comparison test with * p < 0.05 and ** p < 0.01 versus vehicle treated cells (0).

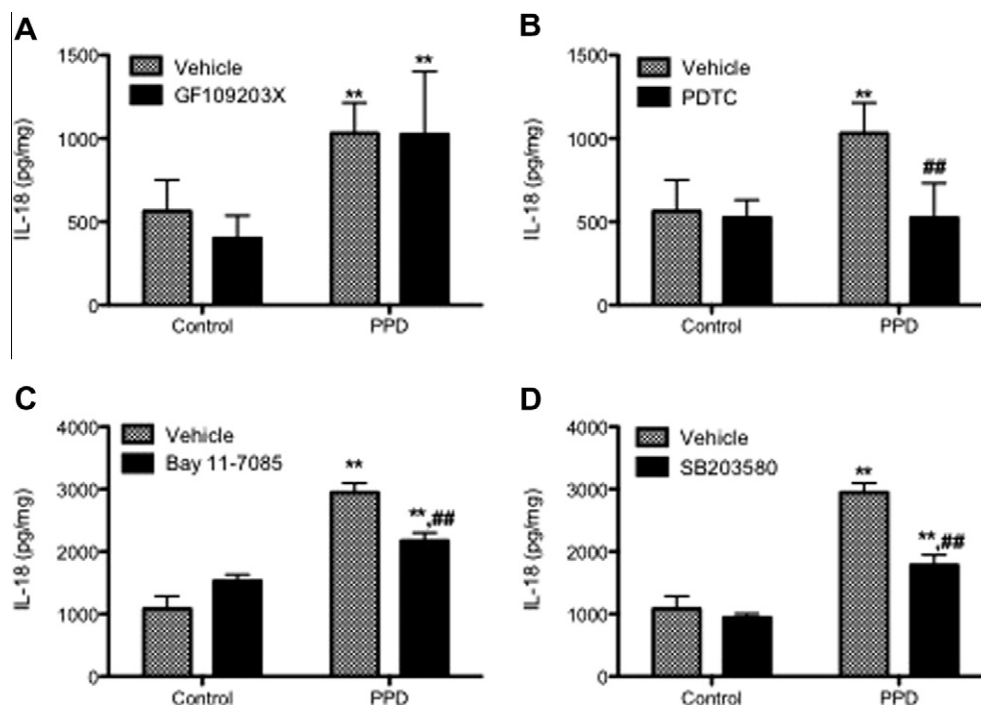


Fig. 6. Role of oxidative stress, p38 MAPK and NF- κ B activation in PPD-induced IL-18 production. NCTC 2544 were treated with GF109203X (5 μ M), PDTC (100 μ M), Bay 11-7085 (1 μ M) and SB203580 (100 nM) for 1 h and after with PPD (60 μ g/ml) or DMSO (0.2% final concentration) as vehicle control for 24 h. Results are expressed as mean $\pm$ SD. Statistical analysis was performed with Tukey's multiple comparison test, with ** p < 0.01 versus vehicle treated cells (0). ## p < 0.01 vs cells treated with PPD alone.

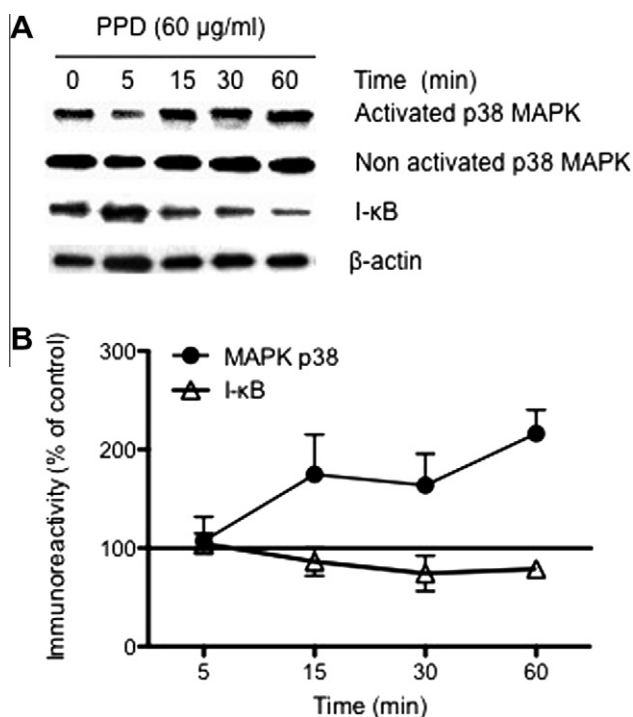


Fig. 7. PPD activated p38 MAPK and NF- κ B in NCTC 2544 cells. Cells were seeded in 6-well plate at a cell density of 2.5×10^5 cells/ml (2 ml/well). After 24 h of adherence, cells were treated with PPD (60 μ g/ml) or DMSO as vehicle control for different times (5–60 min). Panel A, representative Western blots. Panel B, relative densitometric analysis. Each value represents the mean $\pm$ SD of 4 independent experiments.

nol, cinnamaldehyde, tetramethylthiuram disulfite, resorcinol, cinnamic alcohol and eugenol were tested. All, including pre- (i.e. PPD) and pro-haptens (i.e. cinnamic alcohol and eugenol), induced a statistically significant increase in total IL-18. Whereas the respiratory allergens, namely hexachloroplatinate, diphenylmethane diisocyanate, trimellitic anhydride, as well as the irritants phenol, sodium laurel sulphate, lactic acid and salicylic acid did not affect the total IL-18 content. The main limitation of assessing total versus intracellular IL-18 is the loss, in many cases, of a dose–response curve, which may hamper the possibility to use the induction of IL-18 for potency classification. Indeed, as previously shown (Corsini et al., 2009), contact allergens induced a dose-related increase in intracellular IL-18 and by calculation of the concentration of allergen that induced a 1.4 increase in cell-associated IL-18 a significant correlation with the *in vivo* LLNA EC3 values was obtained ($R = 0.754$, $p = 0.0098$). The main advantage of the 96-well format is, of course, time saving, less compound needed and high throughput, which allow a more rapid screening of chemicals for their contact sensitization potential. The lack of a clear dose–response may be due to a different effect, depending on the concentration, of the sensitizer on the release of IL-18 vs the effect on the intracellular IL-18 content, or to a possible interference of the chemicals with the ELISA. Indeed, by adding recombinant IL-18 to cell lysates, as an internal standard, a 20–40% reduction in its recovery was observed in cells treated with resorcinol and eugenol.

The ability of NCTC 2544 to identify contact allergens is not peculiar of this human KC cell lines, as previously demonstrated (Corsini et al., 2009) with primary human KC, similar results were also obtained using other human KC cell lines, i.e. HPKII and HaCaT, further confirming the relevance of the proposed model and the possibility to use different source of KC. Among the three KC cell lines tested, at least for PPD, a higher stimulation index was obtained with HPKII. Further chemicals, however, must be tested to prove the better performance of this cell line.

Finally, we aimed to investigate the signal transduction pathways involved in contact allergen-induced IL-18 production. KC

treatment with contact allergens, whereas both irritants and respiratory allergens failed, indicating the possibility to use total IL-18 to identify contact allergens. A total of eight contact allergens, namely 2,4-dinitrochlorobenzene, p-phenylenediamine, isoeuge-

form an interface between the body and the environment, they are important guardians for the detection of danger signals and the consecutive initiation of an inflammatory response. The transduction of extracellular signals into cellular response is mediated by an array of different kinases, that includes protein kinase C and mitogen activated protein kinases (MAPK). Both kinases have been involved in the induction of allergic contact dermatitis. It has been reported in a mouse model of contact hypersensitivity, that pretreatment with SB202190, a p38 MAPK inhibitor abrogated DNFB-induced contact hypersensitivity, indicating the important role of this kinase (Lisby et al., 1995). The ability of allergens to activate p38 MAPK has been also demonstrated in *in vitro* studies in both keratinocyte cell line A431 (Mehrotra et al., 2007) and dendritic cells (Aiba et al., 2003; Arrighi et al., 2001; Boisleve et al., 2005; Mitjans et al., 2008). Also protein kinase C modulation has been associated with reduction in both irritant and allergic contact dermatitis (Jacobson et al., 1995). Several selective inhibitors were therefore used together with PPD, as reference allergen, to investigate the role of protein kinase C, oxidative stress, NF-B and p38 MAPK in PPD-induced IL-18. In particular, GF109203X a selective inhibitor for the α and β 1 protein kinase C isoforms was used to investigate the role of protein kinase C; PDTC, an inhibitor of NF-B and an anti-oxidant to investigate the role of NF-B activation and oxidative stress; Bay 11-7085 as an irreversible inhibitor I κ B α phosphorylation to assess the role of NF-B activation, and finally, SB 203580 as inhibitor of p38 MAPK activation to investigate the role of MAPK. Results obtained indicated a role for oxidative stress, NF-B and p38 MAPK activation in PDD-induced IL-18 production. No modulation was observed inhibiting protein kinase C activation, suggesting that its activation is not involved in IL-18 production. The results obtained with the inhibitors of NF-B and p38 MAPK are consistent with an inhibition of IL-18 transcription as in 5' upstream region of the human IL-18 gene AP-1, PU.1, NF-B and SP-1 transcription binding sites have been identified (Takeuchi et al., 1999).

Oxidative stress may be the starter point, as it leads to the activation of transcription factors and signalling pathways, including NF-B and p38 MAPK, which leads to the release of cytokines and chemokines (Gloire et al., 2006; Kim and Choi, 2010; Rahman and Biswas, 2004). Reactive oxygen species plays also an important role in the activation of the NLRP3/NALP3 inflammasome (Bryant and Fitzgerald, 2009; Martinon et al., 2009), which is required to direct the proteolytic maturation of inflammatory cytokines such as IL-1 and IL-18 (Martinon, 2010). The ability of contact sensitizers to induce the oxidative stress pathway in KC has been recently confirmed by several authors (Natsch and Emter, 2008; Vandebriel et al., 2010).

In conclusion, total IL-18 production by NCTC 2544, the induction of IL-18 in other keratinocyte cell lines; all give the possibility to identify potential contact allergens *in vitro*. The proposed test may be used as a stand alone assay or as a pre-screen before the LLNA (i.e. the LLNA can be used to confirm a negative finding), or it can be used complementary to other non-animal test (i.e. QSAR, peptide binding assay, etc.), allowing for a rapid and easy discrimination of contact sensitizers from respiratory allergens and skin irritants. Due to the complexity of contact sensitization and the limited number of chemicals tested in the NCTC 2544 assay (only the SENS-IT-IV list has been so far tested), it is, however, likely that a combined analysis of more biomarkers rather than analysis of a single biomarkers will give more satisfactory results. The NCTC 2544 intracellular IL-18 assay has currently entered a prevalidation stage sponsored by SENSTIV and the Dutch Governmental association ZonMW. It is hoped to have a fully validated assay implemented in time to meet the upcoming European regulatory deadlines.

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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