

## Role of p38 MAPK in the selective release of IL-8 induced by chemical allergen in naïve THP-1 cells

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Received 14 June 2007; accepted 18 October 2007

Available online 25 October 2007

### Abstract

At present, the assessment of the allergenic potential of chemicals is carried out using animal models. Over the last decade, several in vitro methods mainly using primary dendritic cells have been proposed to identify the potential of chemicals to induce skin sensitization to meet current animal welfare and public opinions. The major limitations of such tests are the donor-to-donor variability, the low levels in the source, and a possible shortage of human sources.

The aim of the present investigation was to establish an in vitro test to identify chemical allergens using the human promyelocytic cell line THP-1 in order to avoid some of these difficulties. We investigated whether the chemokine interleukin-8 or CXCL8 (IL-8) production could provide a methodology for the detection of both respiratory and contact allergens. THP-1 cells were exposed to contact allergens (cinnamaldehyde, dinitrochlorobenzene, nickel sulfate, penicillin G, *p*-phenylenediamine, tetramethylthiuram disulfide), to respiratory allergens (ammonium hexachloroplatinate, diphenylmethane diisocyanate, trimellitic anhydride) and to irritants (salicylic acid, phenol, sodium lauryl sulphate). Following 48 h of incubation, the release of IL-8 was evaluated by sandwich ELISA. IL-8 production was significantly increased after stimulation with all allergens tested, with the exception of trimellitic anhydride, whereas irritants exposure failed to induce IL-8 release. The lack of IL-8 production by trimellitic anhydride can be explained by the rapid hydrolysis of this chemical in water to trimellitic acid, which is not an allergen. In contrast to IL-8 release, CD54 and CD86 expression did not provide a sensitive method failing to correctly identify approximately 30% of the tested compounds. Although CD86 appears to be a more sensitive marker than CD54 when discriminating allergens from irritants neither of these markers provided robust methodology. We also investigated if a common activation pathway in allergen-induced IL-8 production involving p38 mitogen-activated protein kinase could be identified. By Western blot analysis we could indeed demonstrate p38 activation by all chemical allergens tested and, using the selective p38 MAPK inhibitor SB203580, a significant modulation of allergen-induced IL-8 release could be achieved in all cases.

Our data suggests that production of IL-8 by naïve THP-1 cells may represent a promising in vitro model for the screening of potential chemical allergens and activation of p38 MAPK represents a common pathway triggered by allergens.

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**Keywords:** Respiratory allergy; Contact dermatitis; Dendritic cells; Cytokines; In vitro toxicology; Alternative method

### 1. Introduction

Chemical allergy describes adverse health effects, resulting from the stimulation of specific immune response by

chemicals. Hypersensitivity reactions are the result of normally beneficial immune responses acting inappropriately against benign antigens, causing inflammatory reactions and tissue damage. 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.

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Depending on the country, dermatoses comprise from 20% to 70% of all occupational diseases, and between 20% and 90% of these are contact dermatitis (Wahlberg, 1996). While irritant contact dermatitis is a form of 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 that requires previous sensitization by the same chemicals. 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 drugs, cosmetics and other 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. Despite this 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.

Most chemical allergens are less than 1000 daltons and are electrophilic or hydrophylic, 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. Dendritic cells (DCs) are recognized as important antigen presenting cells in adaptive immunity because of their capacity to stimulate naïve lymphocytes (Banchereau et al., 2000). Langerhans cells (LC) are resident immature DCs in the skin capable to take up and process contact allergens. During this process LC differentiate into mature immunostimulatory cells up-regulating the expression of co-stimulatory molecules such as CD80, CD86 and CD40 and adhesion molecules including CD2, CD11a, CD54, CD58 (Quah and O'Neill, 2005). Activated LC move from the epidermis into the dermis,

and into the regional lymphatic system. In the lymph node, LC differentiate into mature dendritic cells and present antigen to specific T lymphocyte using MHC class II molecules to hold the processed antigen in place. Adhesion molecules on both the antigen presenting cell (i.e. CD86) and the T cell (i.e. CD28) ensure appropriate contact and co-stimulation. Following appropriate stimulus, a clone of T cells with the ability to react to the antigen, which caused their expansion, is produced.

According to these observations, some researchers have attempted to obtain purified LC from human and murine sources (see Ryan et al., 2007 for a complete review). However, difficulties found in LC isolation, weak viability, and shortage of human skin makes this alternative not straightforward. Alternatively, monocyte-derived DC from peripheral blood or CD34+ hematopoietic progenitor cells have been frequently used (Aiba et al., 1997; Tuschl et al., 2000; Toebak et al., 2006), despite by the low levels of cells found in the source, donor-to-donor variability, and the high cost.

A recent European centre for the validation of alternative methods (ECVAM) workshop has reviewed the state of the art of the use of dendritic cells and human myeloid cell lines for the predictive identification of skin sensitization hazard (Casati et al., 2005). At present, only a limited number of cell lines such as THP-1, U937, KG-1 or MUTZ-3 have shown promising results. Among these, THP-1, a human monocytic leukemia cell line commercially available, has been proposed by Ashikaga et al. (2002) to identify sensitizers. Recently, Yoshida et al. (2003) reported that naïve THP-1 could respond to sensitizers specifically through augmented expression of co-stimulatory molecules, CD54 and CD86, and considered this as a possible tool to be used as an *in vitro* sensitization test.

Toebak et al. (2006) have recently reported in monocyte-derived DC a selective production of the chemokine IL-8 or CXCL8 by contact allergens, but not contact irritants, next to CD83 and CD86 upregulation. Interestingly, IL-8 is a potent attractant for neutrophils and different subtypes of T cells. IL-8 has an important role in the induction and possibly also in the elicitation of allergic reaction, not only for triggering the influx of leukocytes to the site of inflammation but also for bringing leukocytes within close proximity of maturing DC (Barker et al., 1991).

In this study, we investigated if the production of IL-8, along with CD54 and CD86 expression, increased upon stimulation with chemical allergens in THP-1 cells and, whether IL-8 can be used a sensitive method to identify allergens. THP-1 cells were exposed to a panel of contact and respiratory allergens and irritants. Investigations aimed to characterize if a common signal transduction pathway existed were also conducted. Overall our data suggests that production of IL-8 may represent a promising *in vitro* model for the screening of potential chemical allergens and activation of p38 MAPK represents a common pathway triggered by 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), cinnamaldehyde (Cinn), tetramethylthiuram disulfide (TMTD); nickel sulphate ( $\text{NiSO}_4$ ); *p*-phenylenediamine (PPD); penicillin G (Pen G) and, as irritants sodium lauryl sulphate (SLS); salicylic acid (Sal. Acid) and phenol were used. All reagents were purchased from Sigma (St Louis, MO, USA) at the highest purity available. MDI, TMA, DNCB, Cinn, TMTD, PPD and phenol were dissolved in DMSO (final concentration of DMSO in culture medium 0.1–0.2%), while HClPt, Pen G,  $\text{NiSO}_4$ . Sal Acid and SLS were dissolved in PBS. Cell culture media and all supplements were from Sigma.

### 2.2. Cells

For all experiments, THP-1 cells (Istituto Zooprofilattico di Brescia, Brescia, Italy) were diluted to  $10^6$  cells/ml in RPMI 1640 containing 2 mM L-glutamine, 0.1 mg/mL streptomycin, 100 IU/mL penicillin, 50  $\mu\text{M}$  2-mercaptoethanol, supplemented with 10% heated-inactivated foetal calf serum (media) and cultured at 37 °C in 5%  $\text{CO}_2$ . Prior to study the effects of allergens and irritants on the selected parameters, cytotoxicity of chemicals was performed by the MTT test (Gerlier and Thomasset, 1986). Two hundred microliter of cells 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 48 h, plates were centrifuged; medium aspirated and replaced with 100  $\mu\text{L}$ /well of the MTT solution 0.75 mg/ml in medium. Cells were incubated for 3 h at 37 °C, plates were then centrifuged, medium discarded and cells lysed in 100  $\mu\text{L}$ /well of a mixture of HCl/isopropanol. The absorbance of the resulting solutions was read at a wavelength of 595 nm in a microplate reader (Molecular Devices). Seventy-five percent cell viability ( $\text{CV}_{75}$ ) was calculated for each chemical by linear regression analysis of data.

For IL-8 release or surface markers expression  $0.5 \times 10^6$  cells were seeded in 24 wells plates, while for Western blot analysis  $3 \times 10^6$  cells were used. Cells were incubated for different times with increasing concentrations of the selected chemicals, or DMSO as vehicle control as indicated in the figure legends.

### 2.3. Cytokine production

IL-8 release was measured in cell-free supernatants obtained by centrifugation at 1200 rpm for 5 min and stored at –20 °C until measurement. IL-8 production was assessed by specific sandwich ELISA commercially avail-

able (Immunotools, Friesoythe, Germany). Results are expressed in pg/ml. Limit of detection 15.6 pg/ml.

### 2.4. Flow cytometric analysis of cell surface markers

CD54 and CD86 expression was evaluated by flow cytometric analysis. THP-1 cells were treated with increasing concentrations of the selected chemicals or DMSO as vehicle control. After 24 h, cells were centrifuged, washed once with cold PBS and resuspended in PBS supplemented with 1% FCS and 0.1%  $\text{NaN}_3$ .  $10^5$  cells were stained for 30 minutes with specific FITC-conjugates antibodies against CD54 and CD86 (Dako) or with isotype control antibody at 4 °C (Becton Dickinson) following supplier's instructions. Cells were then washed three times and resuspended in 0.5 ml of PBS supplemented with 1% FCS and 0.1%  $\text{NaN}_3$ . The intensity of fluorescence and the percentage of positive cells were analyzed using a FACSCalibur flow cytometer and data were quantified using CellQuest software (Becton Dickinson). 10,000 viable cells were analyzed for mean fluorescence intensity (MFI) and percentage of positive cells (PC). All experiments were performed in triplicate.

Changes in CD54 and CD86 expression are reported according to Azam et al. (2006) as stimulation index (SI) calculated by the following equation:

$$\text{SI} = \frac{\text{PC}_t \times \text{MFI}_t}{\text{PC}_c \times \text{MFI}_c}$$

$\text{PC}_t$  and  $\text{MFI}_t$  stand for chemical-treated cells, whereas  $\text{PC}_c$  and  $\text{MFI}_c$  for the untreated ones.

Kinetic experiments with a single effective concentration of HClPt (20  $\mu\text{g}/\text{ml}$ ), DNCB (2.5  $\mu\text{g}/\text{ml}$ ) or Sal. Acid (125  $\mu\text{g}/\text{ml}$ ) in front untreated cells were also performed. Cells were treated, collected and analyzed after 3, 6, 24 and 48 h of treatment.

### 2.5. Role of p38 MAPK in IL-8 production and immunodetection of activated p38

In order to evaluate the role of p38 mitogen-activated protein kinase (p38 MAPK) in allergen-induced IL-8 production, the levels of IL-8 was measured in the supernatants of THP-1 cells treated with allergens (48 h) in the presence and absence of SB202190, a highly selective, potent and cell permeable inhibitor of the p38 MAPK. The inhibitor was added to cell culture 1 h prior to the allergens at a final concentration of 50 nM.

Furthermore, Western blot analysis was also performed to study the activation of p38 MAPK. Briefly, cells were washed twice and resuspended to  $1 \times 10^6$  cells/ml in culture medium without FCS. After 1 h incubation at 37 °C to acclimatize cells, allergens were added for 15 min. Cells were then collected, centrifuged and lysed in 100  $\mu\text{L}$  of homogenization buffer (50 mM TRIS, 150 mM NaCl, 5 mM EDTA pH 7.5, 0.5% Triton X-100, 50  $\mu\text{M}$  PMSF, 2  $\mu\text{g}/\text{mL}$  aprotinin, 1  $\mu\text{g}/\text{mL}$  pepstatin and 1  $\mu\text{g}/\text{mL}$

leupeptin) and denatured for 10 min at 100 °C. The protein content of the cell lysate was measured using a commercial kit (Bio-Rad). For the Western blot analysis, 10 µg of extracted proteins were electrophoresed in parallel 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) and non-activated p38 (1:1000) primary antibodies (Sigma) and developed using enhanced chemiluminescence (ECL, Amersham, Little Chalfont, UK).

## 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 or Student'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<sub>75</sub> respect to vehicle treated cells 48 h after treatment was calculated for all chemicals. 500 µg/ml was the highest concentration tested. Results are presented in Table 1. MDI and TMA were the less cytotoxic chemicals according to these results, however it is important to point out that both chemicals are 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. For the majority of chemicals CV<sub>75</sub> represented the highest concentration tested.

Table 1  
Concentration of the chemicals that induced 75% viability (CV<sub>75</sub>)

| Chemical          | µg/ml | µM    | Category             |
|-------------------|-------|-------|----------------------|
| HClPt             | 15    | 34    | Respiratory allergen |
| MDI               | >500  | >2000 | Respiratory allergen |
| TMA               | >500  | >2600 | Respiratory allergen |
| Cinn              | 20    | 151   | Contact allergen     |
| DNCB              | 5     | 25    | Contact allergen     |
| NiSO <sub>4</sub> | 16    | 103   | Contact allergen     |
| Pen G             | >500  | >1342 | Contact allergen     |
| PPD               | 22    | 203   | Contact allergen     |
| TMTD              | 5     | 21    | Contact allergen     |
| Phenol            | 70    | 744   | Irritant             |
| Sal. Acid         | 250   | 1810  | Irritant             |
| SLS               | 30    | 104   | Irritant             |

10<sup>6</sup>/ml cells were treated for 48 h with increasing concentration of the selected chemicals or DMSO as vehicle control (0.2%). Cell viability was assessed by MTT reduction as described in Materials and Methods section. CV<sub>75</sub> was calculated by linear regression analysis of data.

### 3.2. Effect of the selected chemicals on IL-8 release

Preliminary experiments were carried out to determine the best time to measure IL-8 release. For this purpose, cells were treated for different times (24, 48, 72, 96 h) with SDS (30 µg/ml), NiSO<sub>4</sub> (75 µg/ml), DNCB (3 µg/ml) and PPD (100 µg/ml). As shown in Fig. 1, IL-8 was not detected in supernatants of cells treated with vehicle control or SDS, while a time-dependent release was observed for allergens. For both NiSO<sub>4</sub> and PPD an increase in IL-8 release was observed from 24 to 48 h remaining almost constant thereafter. In contrast, maximal levels of cytokine release for DNCB treated cells are detected at 24 h followed then by a decrease and reaching basal levels at 72 h. However, regardless the behavior of the chemical, cytokine levels at 48 h were high enough to be properly detected with all three allergens. Therefore, the incubation time of 48 h was chosen for all other investigations.

THP-1 cells were then treated with increasing concentrations of the contact allergens NiSO<sub>4</sub>, PPD, Pen G, DNCB, Cinn, TMTD, respiratory allergens MDI, TMA, HClPt and with the irritants SDS, Sal. Acid and phenol. With the exception of TMA, all other allergens (both contact and respiratory) induced a dose-related release of IL-8, while all irritants tested failed (Fig. 2). In the case of HClPt, NiSO<sub>4</sub> and TMTD higher IL-8 releases were observed at concentrations higher than CV<sub>75</sub>. The lack of IL-8 production by trimellitic anhydride can be explained by the rapid (seconds) hydrolysis of this chemical in water to trimellitic acid, a non-allergen.

### 3.3. CD54 and CD86 expression after allergen and irritant exposure

THP-1 cells were treated for 24 h with increasing concentrations of chemical respiratory allergens (MDI, TMA and HClPt), contact sensitizers (DNCB, Cinn, TMTD)

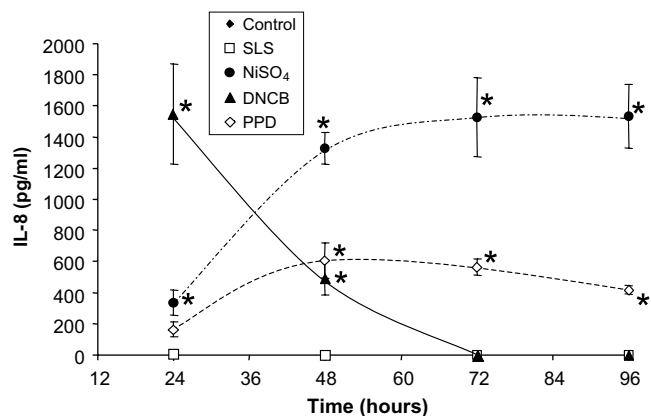


Fig. 1. Time course of IL-8 secretion in THP-1 cells. 10<sup>6</sup>/ml cells were treated for different times (24, 48, 72, 96 h) with SLS (30 µg/ml), NiSO<sub>4</sub> (75 µg/ml), DNCB (3 µg/ml), PPD (100 µg/ml) or vehicle control. Results are expressed as mean ± SD, *n* = 3. Statistical analysis was performed with Dunnett's multiple comparison test, with \**p* < 0.05.

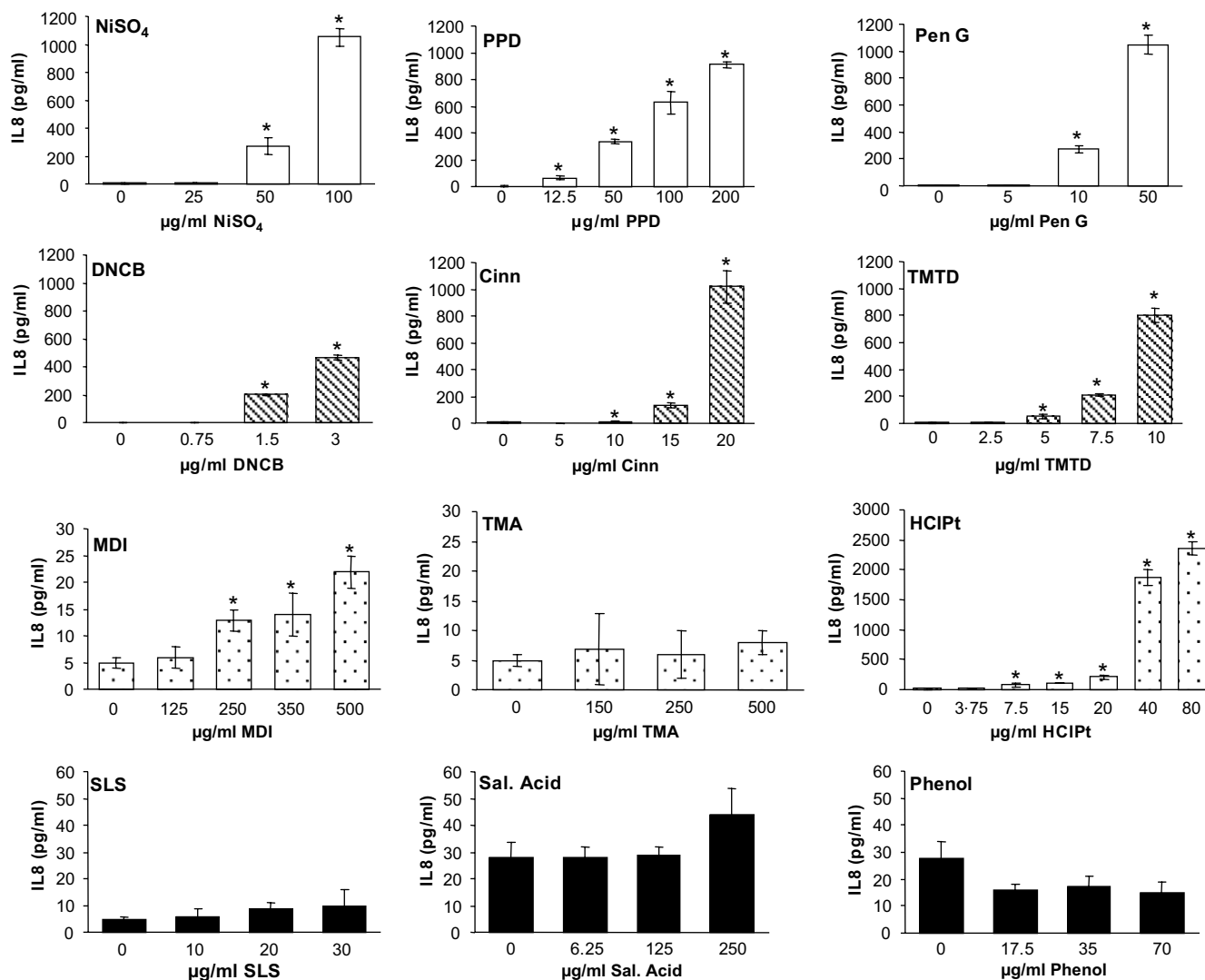


Fig. 2. Effects of respiratory allergens, skin sensitizers and irritants on IL-8 release.  $10^6$ /ml cells were treated for 48 h with increasing concentrations of contact allergens (NiSO<sub>4</sub>, PPD, Pen G, DNCB, Cinn, TMTD), respiratory allergens (MDI, TMA, HClPt) or irritants (SLS, Sal. Acid, Phenol). Vehicle treated cells were included as a control (0 µg/ml). Results are expressed as mean  $\pm$  SD,  $n = 3$ . Statistical analysis was performed with Dunnett's multiple comparison test, with  $*p < 0.05$  vs. vehicle treated cells.

and irritants (SLS, Sal. Acid, Phenol) and then examined by flow cytometry for CD54 and CD86 expression. Among respiratory allergens (Fig. 3a), MDI, TMA and HClPt showed a dose-response increase in the expression of CD54, while only HClPt upregulated in a dose-related manner CD86 expression. Only at the highest concentration tested TMA modestly induced CD54 expression with a SI significantly different from control cells ( $p < 0.01$ ). Contact sensitizers (Fig. 3b) upregulated in a dose dependent-manner especially CD86, with DNCB and Cinn exhibiting the greatest induction. However, when analyzing the behavior of chemical irritants (Fig. 3c), CD54 expression was increased by SLS, and in the case of Phenol treated cells, the SI calculated for all concentrations tested was greater than 2, but no dose-response was found, while none of the irritants tested upregulated CD86.

The expression of CD54 and CD86 induced by the respiratory sensitizer HClPt (20 µg/ml), the skin allergen DNCB (2.5 µg/ml) and the irritant Sal. Acid (125 µg/ml) was evaluated in a time course experiment in order to investigate if a different kinetic exist between respiratory and contact allergens. Fig. 4 reports the SI for CD54 (a) and CD86 (b) at 3, 6 24 and 48 h after treatment. As expected, an increase in the expression of CD54 (a) and CD86 (b) molecules was observed for HClPt and DNCB treated cells, with no significant changes in Sal. Acid treated cells. However, different profiles can be described for the allergens. At 6 h HClPt induced a SI for CD54 of almost 3, which at 48 h increased to approximately 11. In DNCB treated cells, the expression of this surface marker was detected later and at slower rate, with 3 the value of SI calculated at 24 h. By contrast, CD86 was induced to a greater extent in DNCB treated cells in comparison to HClPt treated cells.

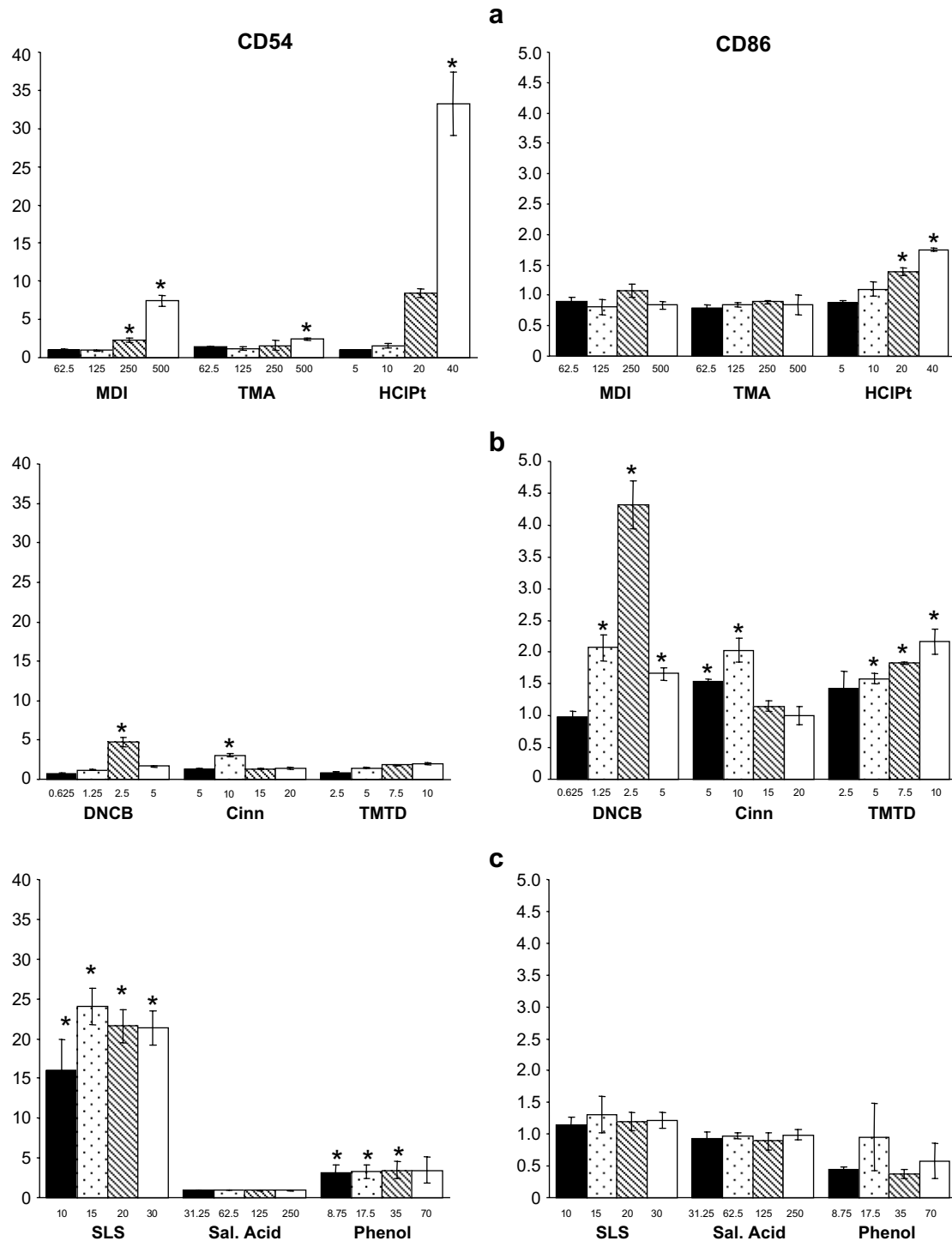


Fig. 3. Dose response effects of respiratory allergens (a), skin sensitizers (b), and irritants (c), on the expression of cell surface markers CD54 and CD86 expressed as stimulation index (SI).  $10^6$ /ml cells were treated for 24 h with increasing concentrations of the selected chemicals. Results are expressed as mean  $\pm$  SD,  $n = 3$ . Statistical analysis was performed with Dunnett's multiple comparison test, with  $*p < 0.05$ .

### 3.4. Role of p38 MAPK in allergen-induced IL-8 production

It is known that nickel can trigger in DC several signal transduction pathways (including p38 MAPK, extracellular signal regulated kinase and nuclear factor- $\kappa$ B) whereas DNCB only activates p38 MAPK (Arrighi et al., 2001; Aiba et al., 2003). We thus investigated if a common acti-

vation pathway in allergen-induced IL-8 production involving p38 mitogen-activated protein kinase could be identified. The activation of p38 MAPK by allergens was assessed by Western blot analysis. Stimulation of the cells by DNCB (2  $\mu$ g/ml), HCIpt (40  $\mu$ g/ml), MDI (500  $\mu$ g/ml), Cinn (20  $\mu$ g/ml), TMTD (10  $\mu$ g/ml) or PPD (100  $\mu$ g/ml) for 15 min induced a statistically significant activation of

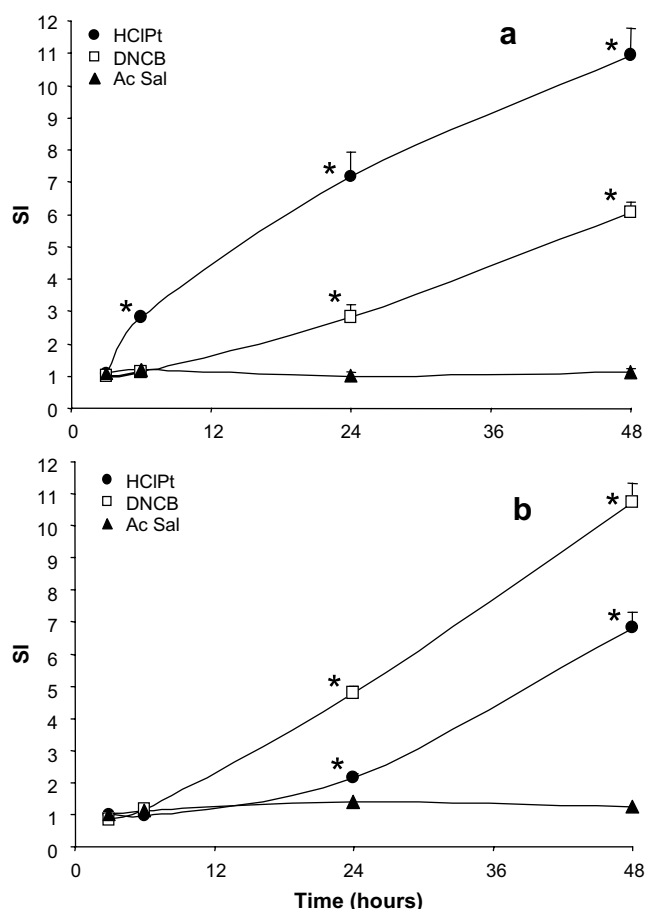


Fig. 4. Time-course expression of CD54 (a) and CD86 (b) following treatment with HClPt (●), DNCB (□) and Salicylic acid (▲). Results are expressed as mean  $\pm$  SD,  $n = 3$ . Statistical analysis was performed with Dunnett's multiple comparison test, with  $*p < 0.05$ . SD smaller than symbols is not shown.

the p38 MAPK as shown by the densitometric analysis of three independent experiments (Fig. 5a and b). The levels of IL-8 were measured in the supernatants of THP-1 cells treated with allergens in the presence or absence of SB202190 (a highly selective inhibitor of the p38 MAPK) in order to evaluate the role of p38 MAPK in allergen-induced IL-8 production. The inhibitor (50 nM) was added to cell culture 1 h prior to the allergens. As shown in Fig. 5c, the release of IL-8 induced by allergens was clearly reduced by the inhibitor. In all cases the decrease was statistically significant. In the case of DNCB, HClPt and MDI the inhibitor totally abolish IL-8 production, indicating that for these allergens IL-8 production is dependent exclusively upon p38 MAPK activation.

#### 4. Discussion

In the present work, next to conventional surface markers upregulation (i.e. CD54, CD86), IL-8 release was investigated after exposure of THP-1 cells to respiratory allergens, contact allergens and irritants. We showed that exposure of THP-1 cells to allergens results in a dose-

related release of IL-8, whereas exposure to irritants fails to induce IL-8 secretion. In contrast, CD54 and CD86 expressions did not provide a sensitive method, failing to identify approximately 30% of the tested compounds. Although CD86 appears to be a more sensitive marker than CD54 neither of these markers provides robust methodology when discriminating allergens from irritants.

Maturation of DC in response to sensitizing agents has been identified as one of the *in vitro* strategies to predict the sensitizing capacity of chemicals (Casati et al., 2005). Different approaches based on the use of dendritic-like cells cultures have been developed to mimic the maturation process of LC. Among them, the THP-1 cell line is one of the most interesting alternatives because of the availability and abundance (Yoshida et al., 2003). Using this cell line, we have studied the maturation of specific markers induced by allergens and non-allergens, to identify selective up regulation by contact and/or respiratory sensitizers. We have also investigated the molecular mechanisms responsible for the regulation of IL-8 release, specifically the activation of the p38 MAPK by sensitizers. Our results suggest that there is a common transduction pathway in which p38 MAPK plays a crucial paper in the activation of IL-8 release, although not exclusive.

Our results are totally in agreement with data obtained in monocyte-derived DCs by Toebak et al. (2006), where IL-8 production was significantly increased after stimulation with allergens tested, and with data obtained by Miyazawa et al. (2007), which suggested a critical role of this cytokine in allergic response together with  $\text{TNF}\alpha$ .

If IL-8 differences can account later in the maturation of Th0 to Th1 or Th2 as well as the effect of released cytokines on cell activation needs further investigation. IL-8 attracts not only cells expressing CXCR1 and CXCR2, like neutrophils and immature DC, but also specific subsets of T cells, including natural killer cells and natural killer T cells (Chuntharapai et al., 1994) and  $\text{CD8}^+$  T cells (Qin et al., 1996), which are all potent interferon- $\gamma$  producing cells. IL-8 most likely plays a pivotal role in the skin during the onset of LC migration from the epidermal layer. Additionally, this cytokine may also indirectly cause T cell accumulation via neutrophil attraction, which in response to IL-8 can release chemotactic mediators attracting T cells (Chertov et al., 1996; Tani et al., 1998). Neutrophils have also been implicated in the pathogenesis of many inflammatory lung diseases, including asthma and IL-8 has been detected in clinical samples from these patients (Pease and Sabroe, 2002).

Kinetic study of IL-8 release have revealed different profiles for DNCB, PPD and  $\text{NiSO}_4$ . While DNCB induced a promptly and high release of IL-8 which disappeared after 48 hours, PPD and  $\text{NiSO}_4$  induced a gradual production of the cytokine that reached the highest values at 24 h and remained stable for 72 h. These results indicate that different cellular pathways may be implicated in the production of the cytokine by chemicals, i.e. p38 MAPK, extracellular signal-related kinases and nuclear factor- $\kappa\text{B}$ . We could

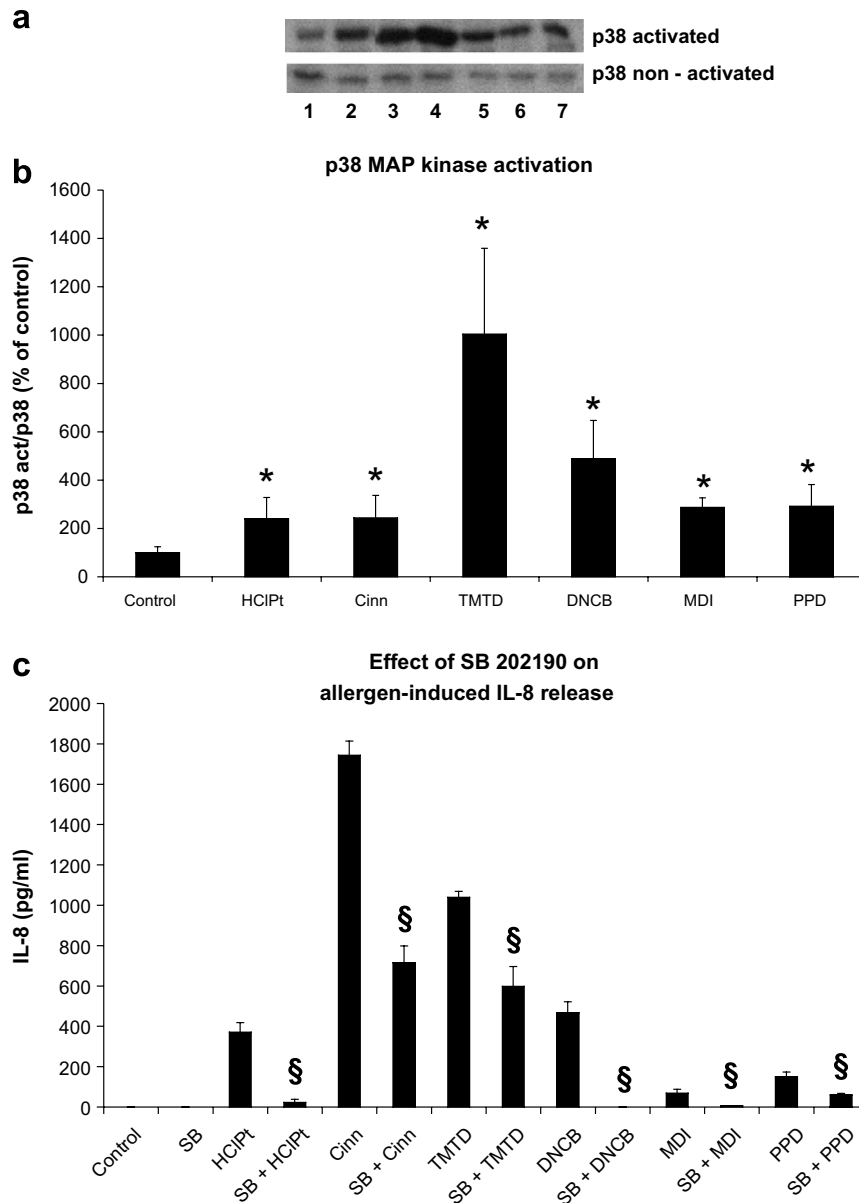


Fig. 5. Role of p38 MAPK in allergen-induced IL-8 production: (a) representative Western blot of activated and non-activated p38 MAPK; (b) relative densitometric analysis of activated vs. non-activated p38 MAPK. Results are expressed as mean  $\pm$  SD of three independent experiments. Statistical analysis was performed with Student's *t*-test, with  $*p < 0.05$  vs. vehicle treated cells. (c) Effect of SB202190 on allergen-induced IL-8 release.  $10^6$ /ml cells were incubated with the different allergens in absence or presence of the selective inhibitor of p38 MAPK SB202190 (50 nM). Results are expressed as mean  $\pm$  SD,  $n = 3$ . Statistical analysis was performed with Student's *t*-test, with  $\$p < 0.05$  vs. cells treated with the respective allergen alone.

indeed demonstrate the role of p38 MAPK activation in allergen-induced IL-8 production. In the case of DNCB, HCIpt and MDI the inhibitor totally abolished IL-8 production, indicating that for these allergens IL-8 production is dependent exclusively upon p38 MAPK activation. The role of p38 MAPK (as one of the activation pathways) in the augmentation of DC surface markers induced by LPS was demonstrated by Ardeshtina et al. (2000) and confirmed by Arrighi et al. (2001). Similarly, Arrighi et al. (2001), Aiba et al. (2003) and Boislevy et al. (2005) have related the activation of p38 MAPK with maturation of DC induced by DNCB and nickel, but in the case of the metal allergen, other signal transduction pathways were also

reported which can explain the highest potential of the chemical. Interestingly, a relationship between IL-8 release and CD86 expression has been suggested for the NiSO<sub>4</sub> (Toebak et al., 2006), as p38 MAPK activation has been related to CD86 expression and IL-8 production (Arrighi et al., 2001).

Variations in the expression of different cell surface markers when exposed to sensitizers and non-sensitizers have been studied in different cell lines. For some contact allergens has been demonstrated an increase of co-stimulatory molecule CD86 expression (Toebak et al., 2006; Yoshida et al., 2003; Azam et al., 2006), while changes in the expression of CD54 were only described by Yoshida

et al. (2003). In this sense, CD54 has been proposed as a good marker to differentiate metal allergens from metal non-allergens because of the stimulation response as described here for HClPt and NiSO<sub>4</sub>. Some authors reported a lack of response of CD54 expression for SLS, even at concentrations where detectable toxicity was induced (Azam et al., 2006), while others described a slightly decrease (Miyazawa et al., 2007). Therefore, CD54 was regarded as a promising tool to distinguish sensitizers from non-sensitizers. We found that both SLS and phenol increased CD54 expression, suggesting that the potential use of CD54 upregulation to discriminate allergens from irritants is questionable. With regards to the CD86 expression, no upregulation was observed after incubation with irritants, while it was upregulated by all contact allergens and by HClPt. Overall, CD86 showed to be a more reliable marker for cell activation than CD54.

In terms of potency, the calculation of concentration of allergen that induced a release of IL-8 of 100 pg/ml by linear regression analysis of data gave the following ranking order: DNCB 0.9 µg/ml, PPD 2.4 µg/ml, TMTD 3.4 µg/ml, HClPt 6.8 µg/ml, Cinn 6.9 µg/ml, Pen G 13.4 µg/ml, NiSO<sub>4</sub> 22.6 µg/ml. If compared with the available in vivo LLNA EC<sub>3</sub> values (DNCB 0.05%, TMTD 5.2%, Cinn 3.0%, Pen G 13.4% and PPD 0.16%), a significant correlation ( $R = 0.924$ ,  $p = 0.0248$ ) was obtained. However, investigations are needed to better address the question of potency.

Other 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 is the case of MDI and TMA, from which very slight or no activation has been found for the different markers investigated. The lack of metabolic activation may be a relevant problem in case of proaptens. At this regards, in preliminary experiments using the proapten cinnamic alcohol, which requires oxidative metabolic activation to the protein-reactive cinnamaldehyde to become sensitizing (Weibel and Hansen, 1989; Basketter, 1992; Smith et al., 2000), negative results were obtained in THP-1 cells. However, we found that co-culture with keratinocytes resulted in a statistically significant release of IL-8 by THP-1 cells, suggesting that co-culture system may solve some of these constraints (data not shown).

In conclusion, IL-8 production by naïve THP-1 cells may represent a promising in vitro model for the screening of potential chemical allergens and p38 MAPK activation represents a common pathway triggered by allergens. On the contrary, the use of the surface markers CD54 and CD86 failed to be as selective. 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, including cytokines and co-stimulatory molecules.

## 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.

## Acknowledgements

This research was supported by the EU grant SENS-IT-IV #018681. Dr. Montserrat Mitjans holded a small fellowship grant BE-2006 from the AGAUR (Generalitat de Catalunya).

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