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MECHANISTIC UNDERSTANDING OF DENDRITIC CELL ACTIVATION IN SKIN SENSITIZATION: ADDITIONAL EVIDENCES TO SUPPORT POTENCY CLASSIFICATION

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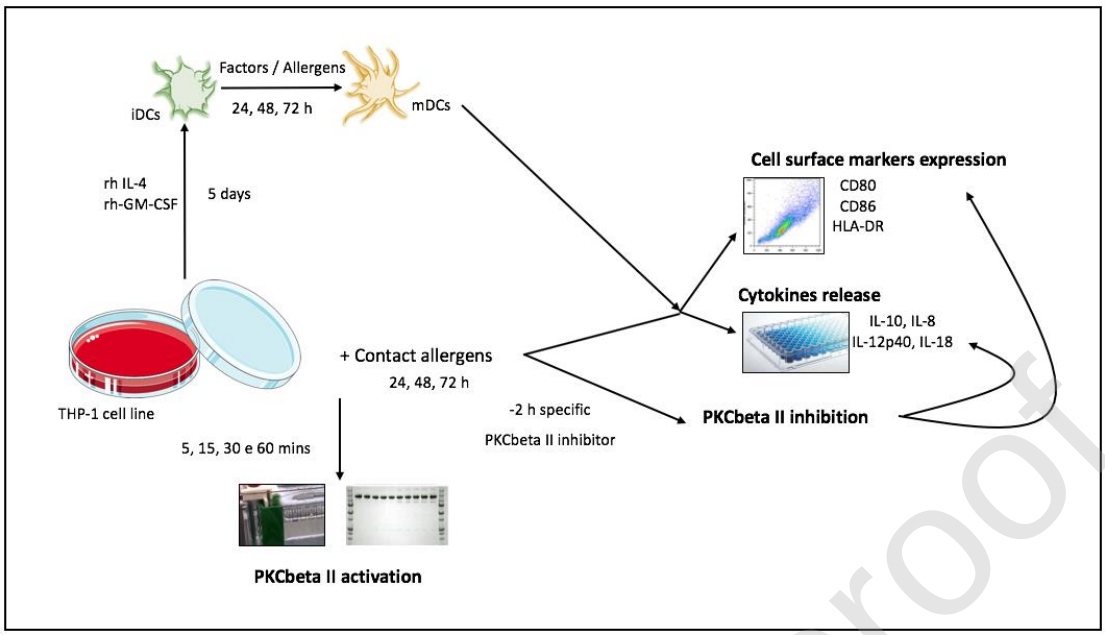
Graphical abstract



Highlights

- The identification of mechanisms influencing allergen potency requires better understanding of molecular events that trigger cell activation
- Allergens of different potency differently modulated cell surface markers expression, with HLA-DR induced only by extreme contact allergens
- A central role is played by PKC beta II in the initiation of chemical allergen-induced DC maturation and activation

Abstract

Allergic contact dermatitis (ACD) is an important occupational and environmental disease caused by topical exposure to chemical allergens. In the EU, it has been calculated that 4% of animals are used in toxicity test for the assessment of skin sensitization (1). To come a complete replacement of animals, evaluation of relative skin sensitization potency is necessary. The identification of mechanisms influencing allergen potency requires a better understanding of molecular events that trigger cell activation. Therefore, (i) the effects of selected allergens on surface markers expression and cytokines release in contact allergen-induced cell activation were assessed, and (ii)

the role of Protein Kinase C (PKC) beta activation in contact allergen-induced cell activation was investigated. The human pro-myelocytic cell line THP-1 was used as experimental model surrogate of dendritic cells. Cells were exposed to select contact allergens of different potency and cell surface marker expression (CD80, CD86, HLA-DR) was determined by flow cytometry analysis. Cytokines production (IL-6, IL-8, IL-10, IL-12p40, IL-18) was evaluated with specific sandwich ELISA. The effective contribution of PKC beta in chemical allergen-induced cell activation was assessed by Western Blot analysis (PKC beta activation) and using a specific PKC beta inhibitor (PKC beta pseudosubstrate). In addition, to investigate if contact allergens are able to induce indeed dendritic cells (DCs) maturation, THP-1 cells were differentiated to immature DC and then exposed to contact allergen of different potency. Overall, our finding provides insights into the process of sensitization and strength of cell activation associated with allergens of different potency. Results obtained suggest that contact allergens of different potency are able to induce a different degree of activation of dendritic cells maturation involved in the process of ACD.

Keywords: in vitro method, potency, contact allergy, skin sensitisation,
PKC beta

Introduction

Allergic contact dermatitis (ACD) is a form of delayed type hypersensitivity caused by small molecular weight chemicals (haptens) and mediate by the T cells (2,3). ACD can have serious impact on the quality of life and represents a common and important environmental and occupational health hazard. The adverse outcome pathways (AOPs) for ACD are reported in the OECD report where mechanisms understanding skin sensitization have been divided into different key events (KEs). The established KEs are: (i) covalent interaction of chemical to cells protein; (ii) activation of inflammatory cytokines and induction of cyto-protective gene pathways with the involvement of keratinocytes (KCs); (iii) induction of inflammatory cytokines and surface markers with the mobilization of the dendritic cells (DCs); (iv) activation, proliferation and polarization of T cells (4).

The evaluation of the contact sensitization potential of chemicals is currently done using the local lymph node assay (LLNA), as described in the OECD guideline 429. In addition, to the hazard identification, the LLNA has proven very useful in assessing the skin sensitizing potency of chemicals, based on the estimation of the concentration of chemical required to induce a stimulation index of three relative to concurrent vehicle-treated controls (i.e., EC3 value). Low EC3 values correlated well with sensitizers known to be potent in human, whereas high EC3 values were usually associated with weakly human sensitizers (5). Driven by the 7th Amendment to the EU Cosmetic Directive, the 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, replacement of the LLNA is become an important issue. The identification of mechanisms influencing the vigor of T cell responses, that can explain the strength of ACD reactions to weak, moderate, strong, and extreme sensitizers (6,7) is a challenge still to be solved and this will require a better understanding of the molecular events that trigger cell activation following exposure to contact allergens. Contact sensitizers have been demonstrated to induce phenotypic and functional changes in DC with the up regulation of surface expression of major histocompatibility complex (MHC) class II molecules, co-stimulatory molecules (e.g. CD80, CD86, CD40 and CCR7) and cytokines, enhancing their antigen-presenting capacity that ultimately could modulate T cell response (8,9). DCs are a heterogeneous population of antigen-presenting cells (APC) that play a central role in the initiation and regulation of adaptive immune responses. It is generally assumed that three signals are required for the activation of naïve T helper cells. Signal 1 or stimulation: the recognition by the T-cell receptor (TCR) of antigenic peptides presented by MHC class II molecules expressed on DCs. Signal 2 or co-stimulation: provided by the triggering of CD28 on the T cell by CD80 and CD86 molecules on the DCs. Signal 3 or polarization: signals delivered from DCs, and other innate immune cells as well as epithelial cells, to T cell that determine its differentiation into various effector phenotypes such as Th1, Th2, Treg (10).

It has been shown that protein kinase C (PKC) activation is necessary and sufficient to drive human CD34+ hematopoietic progenitor cells, peripheral blood monocytes or myeloid leukemic cell lines to DC differentiation (11). PKCs play a key regulatory role in a variety of cellular functions, including cell growth and differentiation, gene

expression, hormone secretion. Different PKC isoforms mediate specific cellular signals required for activation, proliferation, differentiation and survival of immune cells (12,13). Of the classical PKC isoforms, only PKC-βII has been shown to be consistently activated during DCs differentiation-inducing stimuli in normal and leukemic progenitors (11) PKC-βII activation by cytokines (GM-CSF, IL-4 and TNF-α) in DCs was demonstrated to be associated with up-regulation of DCs surface markers (MHC I, MHC II, CD11c, CD40, CD80, CD83 and CD86), the induction of expression of the NF-κB family member c-Rel, and the ability to stimulate allogeneic T cell proliferation (14).

Based on the concept and speculation that the extent of chemical allergen-induced DCs activation, maturation and lifespan drives the quality and magnitude of T cell activation and that PKC-β activation is necessary to drive DCs differentiation and is central to chemical allergen-induced DCs activation, the global aim of this work was to provide a mechanistic point of view able to support *in vitro* potency information, necessary for full replacement of animals in the assessment of the allergenic potential of xenobiotics.

Materials and methods

Chemicals

p-benzoquinone (BZQ), Bandrowski's base (BB), diethyl maleate (DEM), fluorescein isothiocyanate isomer I (FITC), α-hexylcinnamaldehyde (HCA), hydroxycitronellal (HDC), and imidazolidinyl urea (IMZ) were selected as skin sensitizers according to their LLNA potency (15). Chemicals were purchased from Sigma Aldrich and Apollo Scientific (Bandrowski's base) at the highest purity available. BZQ, BB, DEM, HCA, and HDC were dissolved in dimethyl sulfoxide (DMSO) (final concentration of DMSO in culture medium <0.2%), while IMZ was dissolved in Dulbecco's Phosphate Buffered Saline (dPBS). Cell culture media and all supplements were from Sigma Aldrich. The selected contact sensitizers were classified according to their potency on the basis of the EC3 LLNA value data, covering different potencies from extreme to weak (Table 1).

To investigate the role of PKC-β in chemical-induced cell surface markers expression and cytokines release, THP-1 cells were cultured for 2 h in the presence or absence of a selective cell-permeable inhibitor of PKC-β at 5 μM concentration (PKC-β

pseudosubstrate, Tocris, Bristol, UK) in culture medium cell without FCS.

Cells

THP-1. For all experiments, THP-1 cells (Istituto Zooprofilattico di Brescia, Brescia, Italy) were used at a cell density of 10^6 cells/mL in RPMI 1640 containing 2M glutamine, 0.1 mg/mL streptomycin, 100 UI/mL penicillin, 50 μM 2-mercaptoethanol, supplemented with 10% heated-inactivated foetal calf serum (media) and cultured at 37°C in 5% CO_2. For cytotoxicity, cytokines release and surface marker expression 10^6 cells/ml (0.5 ml/well) were seeded in 24-well plates and incubated with increasing concentrations of the selected chemicals or DMSO as vehicle control for 24, 48 and 72h. In dose-response experiments, cells were treated using the CV75 as the highest concentration and with two two-fold dilutions for a total of three concentrations. CV75 represent the concentration resulting in a cell viability of 75% compared to vehicle-treated cells. In time-course experiments, cells were exposed for 24, 48 and 72h to one concentration for each chemical (half of the CV75 to avoid excessive cytotoxicity as cells were cultured up to 72h).

DC differentiation. For DC differentiation, THP-1 cells were treated for 5 days with rhIL-4 (1500 UI/mL – R&D Systems) and rhGM-CSF (1500 UI/mL – ImmunoTools GmbH) to acquire the properties of immature DCs (iDCs) as described by Berges et al. (2005) (16). Mature DCs were then generated from iDCs by addition of rhIL-4 (3000 UI/mL), rhTNF-α (2000 UI/mL – Sigma Aldrich) and ionomycin (200 ng/mL – Sigma Aldrich) or by exposure to the selected contact allergen (BB, DEM and HCA) for 24, 48 and 72h. As markers of DCs differentiation and maturation CD40, CD80, CD86, and HLA-DR (BD, Becton Dickinson and ImmunoTools) were assessed by FACS analysis as described below.

Cell viability

THP-1 cells were treated for 24h with increasing concentrations of the selected chemicals starting from their maximum solubility in appropriate solvents (i.e. DMSO or PBS) and typically with 12 subsequent two-fold dilutions. Cell viability was assessed by propidium iodide (PI) uptake using flow cytometry with the acquisition channel FL-2 (final concentration of PI 0.5 μg/ml). A total of 10.000 cells were acquired. Vehicle treated cells (control) were set as 100% and cell viability of allergen-treated cells

calculated using the cytometer analysis program. The concentration of allergens resulting in 75% of viability (CV75) was then calculated by linear interpolation using InStat software version 7.0 (GraphPad Software, La Jolla, CA, USA).

Cell surface markers expression

Cell surface markers expression was evaluated by FACS analysis. THP-1 cells were treated with the selected chemicals or DMSO as vehicle control. At different times after treatment, cells were centrifuged at 1500 rpm for 5 min, supernatants were collected for cytokines assessment, and cells stained for 30 min with specific FITC/PE-conjugated antibodies against CD40, CD80, CD86 and HLA-DR (BD, Becton Dickinson, and ImmunoTools) or with isotype control antibodies at 4°C following supplier's instructions. The intensity of fluorescence and the percentage of positive cells were analyzed using FACSCalibur flow cytometer, and data were quantified using CellQuest software (Becton Dickinson). Changes in surface markers expression are expressed as stimulation index (SI) calculated on the Geo Mean values (treated cells/vehicles treated cells).

Cytokine production

IL-8, IL-10, IL-18, IL-6 and IL-12p40 release were measured in THP-1 cells supernatants following time-course experiments (24, 48 and 72h). Cell-free supernatants were obtained by centrifugation at 1500 rpm for 5 min and stored at -20°C until measurement. All cytokines were assessed by a specific sandwich ELISA commercially available: IL-8, IL-6, and IL-12p40 from ImmunoTools (Friesoythe, Germany); IL-10 from eBioscence (Affymetrix, San Diego, CA) and IL-18 from MBL (Nagoya, Japan). Results are expressed as stimulation index (SI) of treated cells *versus* cells treated with vehicle.

Western blot analysis

The activation of PKC-βII in cell homogenates was assessed by Western blot analysis. Briefly, cells (3 x 10^6 cells) were treated with selected allergens (BZQ, BB, FITC, DEM, HCA and HDC) for different times: 5, 15, 30 and 60 minutes. Allergens were tested using the CV75. The strong contact allergen FITC was added to the previous list of chemicals tested to cover a broad range of potency, while one of the three weak contact allergen was removed (IMZ). At different times, cells were collected, washed

once with PBS, centrifuged, and lysed in 100 µl of homogenization buffer (50 mM TRIS, 150 mM NaCl, 5 mM EDTA pH 7.5, 0.5% Triton X-100, 50 µM PMSF, 2 µg/ml aprotinin, 1 µg/ml pepstatin, 1 µg/ml leupeptin, and 1X PhosphoStop) and denatured for 10 min at 100°C. The protein content of the cell lysate was measured using a commercial available kit (Bio-Rad). 10 µg of extracted proteins was electrophoresed into a 10% SDS–polyacrylamide gel under reducing conditions. The proteins were then transferred to PVDF membrane (Amersham, Little Chalfont, UK). The proteins were visualized using primary antibodies for Anti-PKC-βII (rabbit monoclonal Ab, dilution 1:40.000, Abcam) and anti-β-actin (mouse monoclonal Ab, dilution 1:1000, BD) and developed using enhanced chemiluminescence (Clarity western ECL blotting substrates Bio-Rad). The image of the blot was acquired with the Molecular Imager Gel Doc XR (BioRad). The optical density of the bands was calculated and analysed by means of the Image Lab program for digital image processing (Version 4.0, Bio-Rad Laboratories).

Data analysis

All experiments were performed at least three times. Statistical analysis was performed using GraphPad InStat version 3.0a for Macintosh (GraphPad Software, San Diego, CA, USA). Dunnet's test was used for multiple comparison analyses of variance. Differences were considered significant at $p \leq 0.05$.

Results

Cell viability

In preliminary experiments, cytotoxicity was established. In Table 1, CV75 results are reported (results are the mean of at least two independent experiments).

Dose-response effects of the selected chemicals on cell surface marker expression following 24 h of treatment

In Fig.1 the effects of the selected chemicals on CD80, CD86 and HLA-DR expression following 24 h of dose-response treatment are reported. Values are expressed as SI (GeoMean). Due to the high auto fluorescence of the FITC, which made it impossible to read at any channels available at the flow cytometer, this compound was discarded for the cell surface markers analysis. BZQ, BB, and HDC were able to induce a

statistically significant increase in the expression of CD86, while CD80 was up-regulated after treatment with BB, DEM, and HCA. Only the extreme contact allergen BB was able to induce a statistically significant increase in HLA-DR expression.

Effects of the selected chemicals on the cell surface marker expression in time course experiments

In Fig.2 the effects of the selected chemicals on CD80, CD86 and HLA-DR expression following 24, 48 and 72 h of treatment are reported. All contact allergens were tested using ½ CV75 values calculated at 24h, as cytotoxicity was expected following longer incubation time. All contact allergens tested induced CD80 and CD86 expression with a different kinetics. In detail, the extreme allergens BB and BZQ induced CD86 and CD80 expression starting from 24h and 48h respectively but maintaining the expression of these markers above control until 72 h. DEM, a moderate allergen, was able to induce CD86 and CD80 expression starting from 48h. More interestingly was the effect of allergens on HLA-DR expression. This surface marker was induced only after treatment with the two extreme contact allergens BB, and BZQ.

Effects of the selected chemicals on cytokines release in time-course experiments

In Fig.3 the effects of the selected chemicals on IL-8, IL-10, IL-12 and IL-18 release following 24, 48 and 72h of treatment are reported. Also IL-6 release was assessed but it resulted always below the limit of detection for all contact allergens (data not reported). All the contact allergens tested were able to induce an increase in IL-8 release, not statistically significant only for BB, confirming the ability of allergens to induce this cytokine in THP-1 (17–19). HCA and IMZ (weak allergens) failed to induce IL-10, IL-18, and IL-12 at all time point. The trend in increased IL-18 release was observed for the extreme allergen BB and the weak allergen HDC, that did not reach a statistically significance due to data dispersion.

Overall, from the analysis of the selected cytokines, a clear picture does not emerge that could help to discriminate allergens based on their *in vivo* potency. The cytokines IL-12 and IL-18, typically associated with Th1 responses, are mainly induced by the two extreme contact allergens tested, respectively BZQ and BB. Also, the weak allergen HDC was able to induce an increase of these two cytokines but, however, it failed to induce the expression of HLA-DR (Signal 1) that is, on the contrary,

statistically significant expressed after exposure to BZQ and BB. Overall, results suggest that extreme allergens are able to fully activate naïve THP-1, used as surrogate of DC.

PKC-βII activation

In Figure 4 western blot analysis and densitometric analysis of PKC-βII activation are reported. Allergens were tested using the CV75. Densitometric analysis shows that all allergens, at concentrations that induce the same level of cell damage, were able to induce PKC-βII activation with potency affecting the time of activation. The extremes contact allergens, respectively BZQ and BB, and the strong FITC were able to activate PKC-βII already at 5 min and the activation persisted until 60 minutes; while DEM (moderate) activated PKC-βII at 15 minutes and the activation decreases more rapidly, after 30 minutes. Results reported clearly indicate that extreme and strong allergens are able to more rapidly activate PKC-βII.

DCs differentiation and maturation

Data presented in Figure 5 indicate a different effect of allergens of different potency on THP-1 activation, with extreme allergens inducing a full maturation of cells, with up-regulation of both co-stimulatory and MHC class II molecules and release of cytokines important for Th1 cell activation (e.g. IL-12/IL-18). To demonstrate that indeed contact allergens induced full DC maturation, THP-1 cells were differentiated to immature DC and then exposed for 24h to three allergens of different potency: BB (extreme), DEM (moderate) and HCA (weak). In Fig. 6, Panel A, the differentiation of cells from THP-1 to immature dendritic cells (iDCs) is reported. THP-1 cells were treated for 5 days according to the protocol described by Berges et al. (16). After five days CD40, CD80, and CD86 were evaluated by flow cytometry and results are reported as % of positive cells. Results showed an increase in cell surface markers expression that indicates THP-1 differentiation. In Fig.6, Panel B, it is reported the maturation of immature DCs induced by a specific cocktail of maturation factors. Cell surface markers CD80, CD86 and HLA-DR were evaluated after 24h of exposure to rhIL-4 (3000 IU/ml), rhTNF-α (3000 U/ml), and ionomycin (200 ng/ml). CD80, CD86, and HLA-DR expression are reported as % of positive cells. As shown in Figure 6, Panel C, both DEM (moderate) and HCA (weak) were able to induce CD80 and CD86

cell surface expression otherwise failed to induce HLA-DR expression. On the contrary, the maturation of iDCs was successfully obtained only after treatment of iDCs with the extreme contact allergen BB as shown by the % of positive cells reported for all the cell surface markers assessed. These data support the hypothesis that only the extreme contact allergens have the capacity to induce a statistically significant increase of ~~a full DCs maturation~~ HLA-DR expression.

Discussion

The main aim of this work was to better understand the mechanism underneath the extent of DC maturation and activation induced by chemical allergens of different potency. Potency refers to the intrinsic property of a sensitizing chemical and is based on the concentration of chemical needed to induce a positive response (20). Allergens are known to vary significantly as much as 10.000-fold in relative sensitization potency with which they can induce skin sensitization. DCs, as APC, play a pivotal role in the initiation and regulation of immune responses, leading to specific T cell activation. During these processes, DCs undergo diverse phenotypical and functional changes such as up-regulated cell surface expression of co-stimulatory molecules and adhesion proteins (i.e. CD86, CD83, CD54, CD40, and MHC II antigens) (21). Maturation of DCs in response to sensitizing agents has been identified as one of the *in vitro* strategies to predict the sensitizing capacity of chemicals (22). Different approaches based on the use of dendritic-like cells cultures have been developed to mimic the maturation process of Langerhans cells. Among them, the THP-1 cell line is one of the most interesting alternatives because of its availability and abundance (23). Using this cell line, we have previously shown 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 (17,24).

Human APCs express three different isoforms of MHC II molecules, denotes as human leukocyte antigen (HLA) -DR, -DQ, and –DP (25). HLA-DR represents a ligand for the T-cell receptor (TCR) and is involved in several immune and autoimmune disorders. Previous studies have indicated that for metal ions, considered as common allergens such as nickel, chromium and cobalt, the availability of surface HLA-DR is needed for T cell stimulation and to induce delayed hypersensitivity reactions (26). It is also well known that 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-c by T-cells(27) . 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 ACD (28). Results presented in this work suggest that allergens of different potency differently modulated cell surface markers expression, with HLA-DR induced only by extreme allergens, which suggest their ability to induce a higher degree of DC maturation. In fact, only the exposure to extreme allergens was sufficient to provide the three signals required for T cell activation: Signal 1 or stimulation by up-regulating HLA-DR. Signal 2 or co-stimulation by up- regulating CD80 and CD86, provided by the triggering of CD28 on the T cell by CD80 and CD86 molecules on the DCs. Signal 3 or polarization by inducing the release of IL-12 or IL-18, signals delivered from DCs to T cell that determine its differentiation Th1 phenotype. Moderate and weak allergens while inducing Signal 2 and in some instances, also Signal 3, failed to induced HLA-DR.

Among the selected allergens, the behavior of the weak allergen HDC was different. It resulted to be more reactive compared to the other weak allergens tested. This behavior may be due to its structure, which shows similarities to citral and limonene, two weak chemical allergens toward which THP-1 cells are particularly reactive. Merckel et al. compared the reactivity of HDC and citral toward glutathione used as a classical model nucleophile in a semi-organic medium and in a microemulsion system: the two weak allergens react with the thiol group of glutathione, in both systems, to form identical adducts (29).

Another important aim of this study was to characterize the role of PKC-β in chemical-allergen induced DC activation. We focused on PKC-βII as the available evidence indicates that PKC-βI and PKC-βII have different functional roles in monocytes: PKC-βI has been associated with the induction of apoptosis (30), while PKC- βII with proliferation and differentiation (11,22). We recently demonstrated a role of PKC-β in chemical allergen-induced CD86 expression and IL-8 release, two markers selectively up-regulated by chemical allergens (31,32), both in the human promyelocytic cell line THP-1 and in primary human DC (33). The ability of the PKC-β pseudosubstrate to completely prevent also chemical allergen-induced CD80 (for BZQ) and HLA-DR expression, as shown by these new results obtained, support the central role of PKC-β also in the initiation of chemical allergen-induced DC maturation and activation.

Overall, our finding provides insights into the process of sensitization and strength of cell activation associated with allergens of different potency. The main aim of this work was to provide a mechanistic understanding able to support *in vitro* potency information, necessary for full replacement of animals in the broader perspective of classification and labeling of potential sensitizers. While the numbers of chemicals tested is limited, and additional compounds should be tested, results obtained support the notion that contact allergens of different potency are able to induce a different degree of activation of dendritic cells maturation with only extreme~~/strong~~ allergens able to induce a statistically significant expression of the cell surface marker HLA-DR, necessary for a full maturation of the dendritic cells.

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships
that could have appeared to influence the work reported in this paper.

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Figures

Fig.1

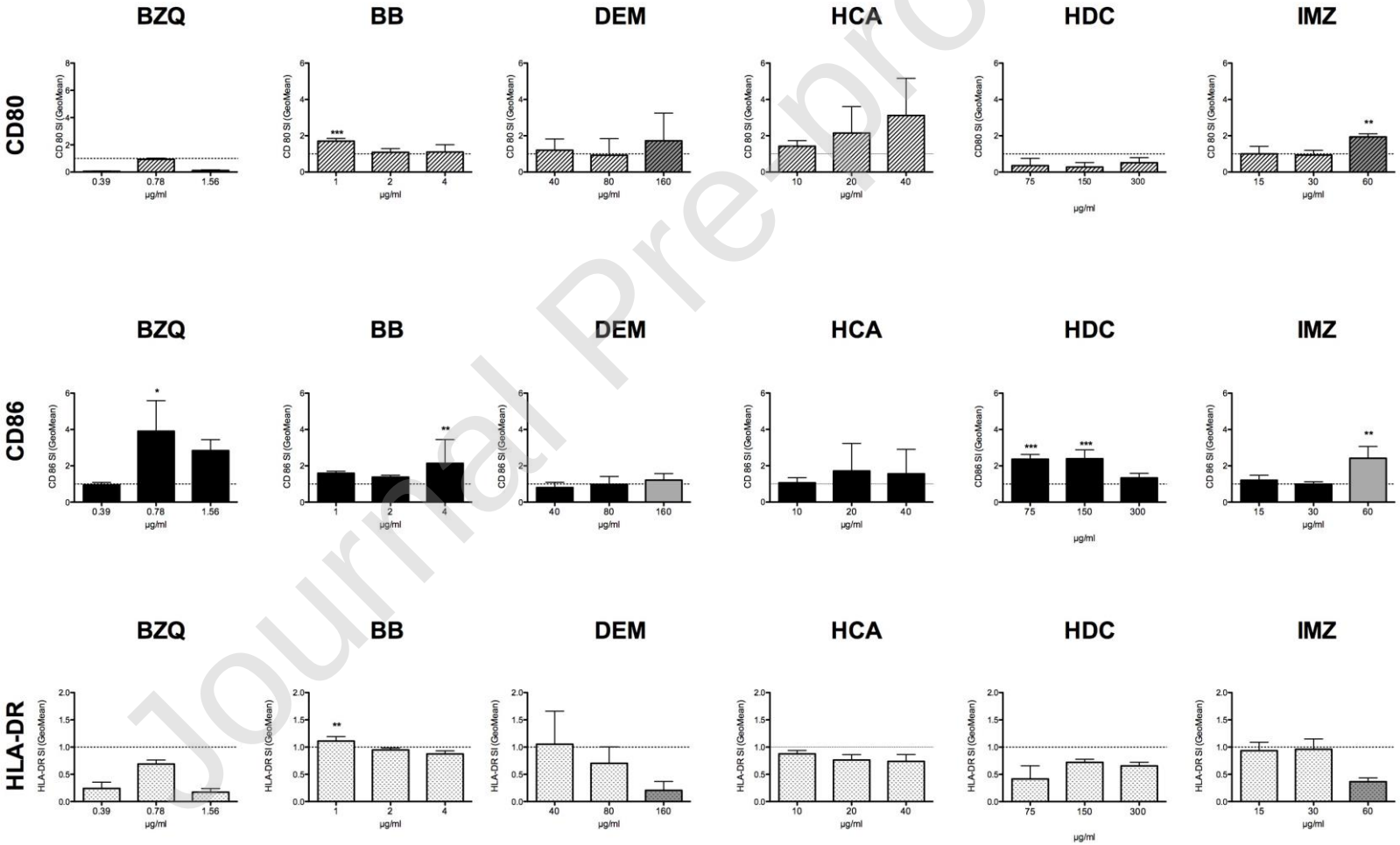


Fig.2

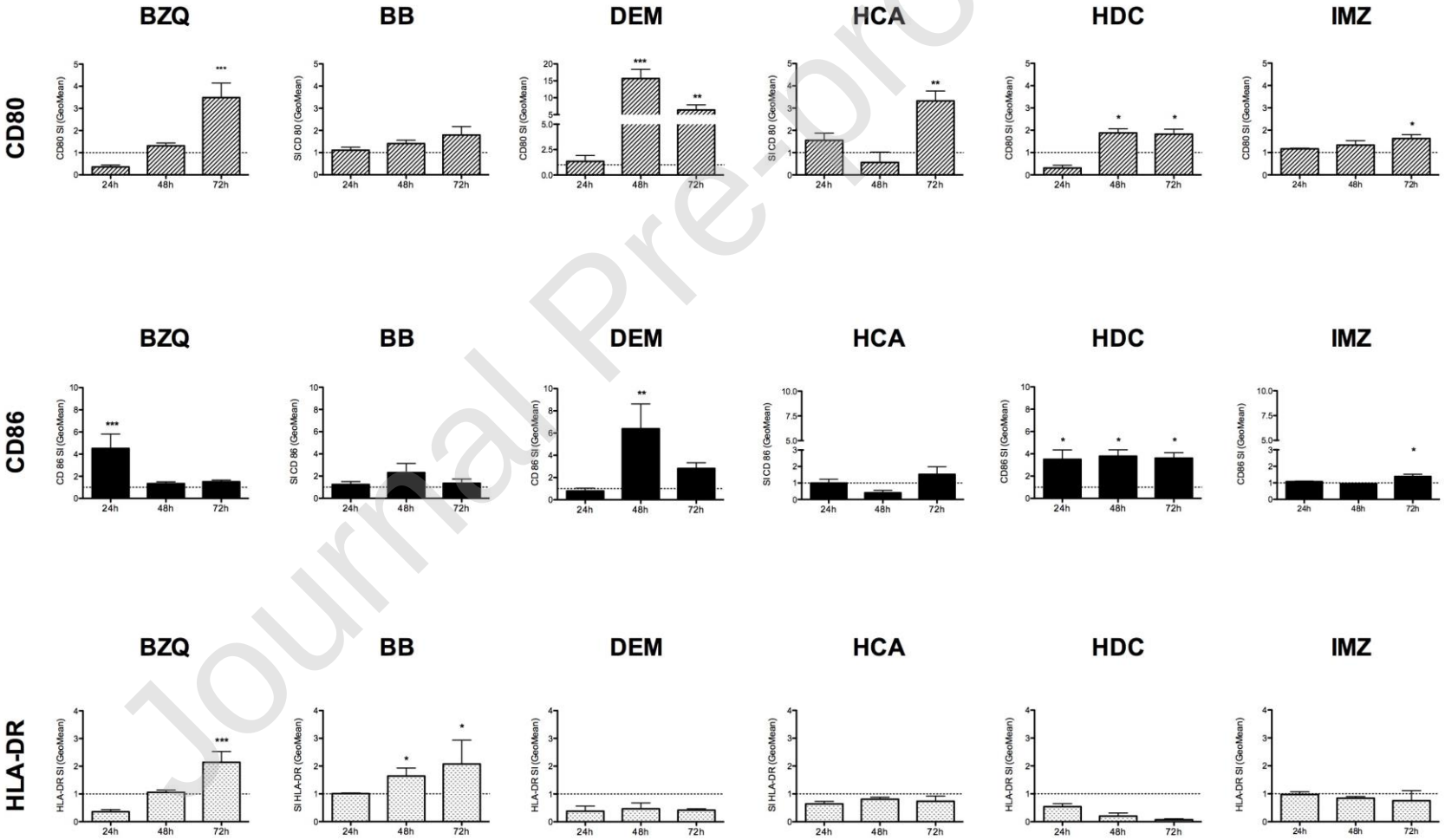


Fig.3

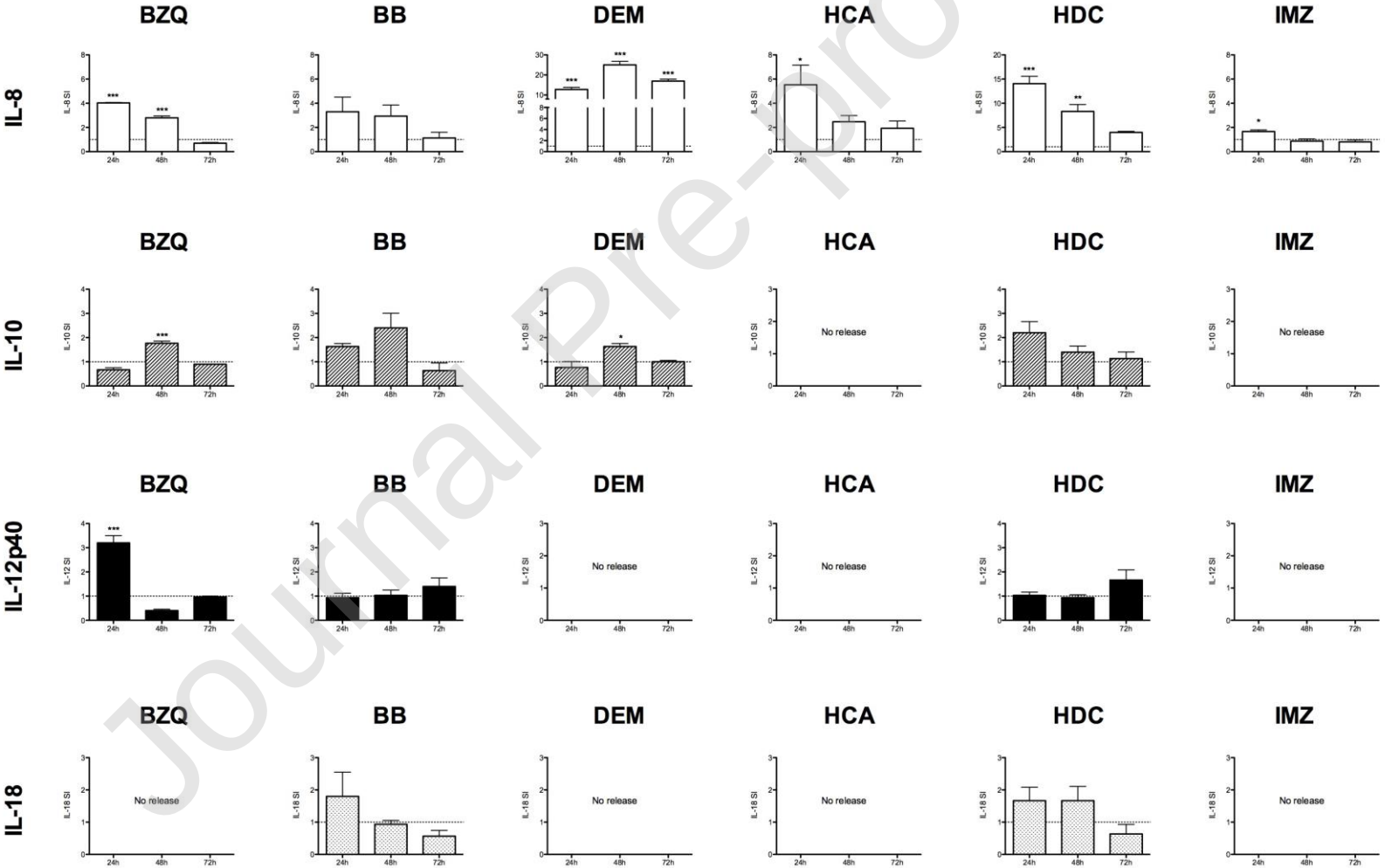


Fig. 4

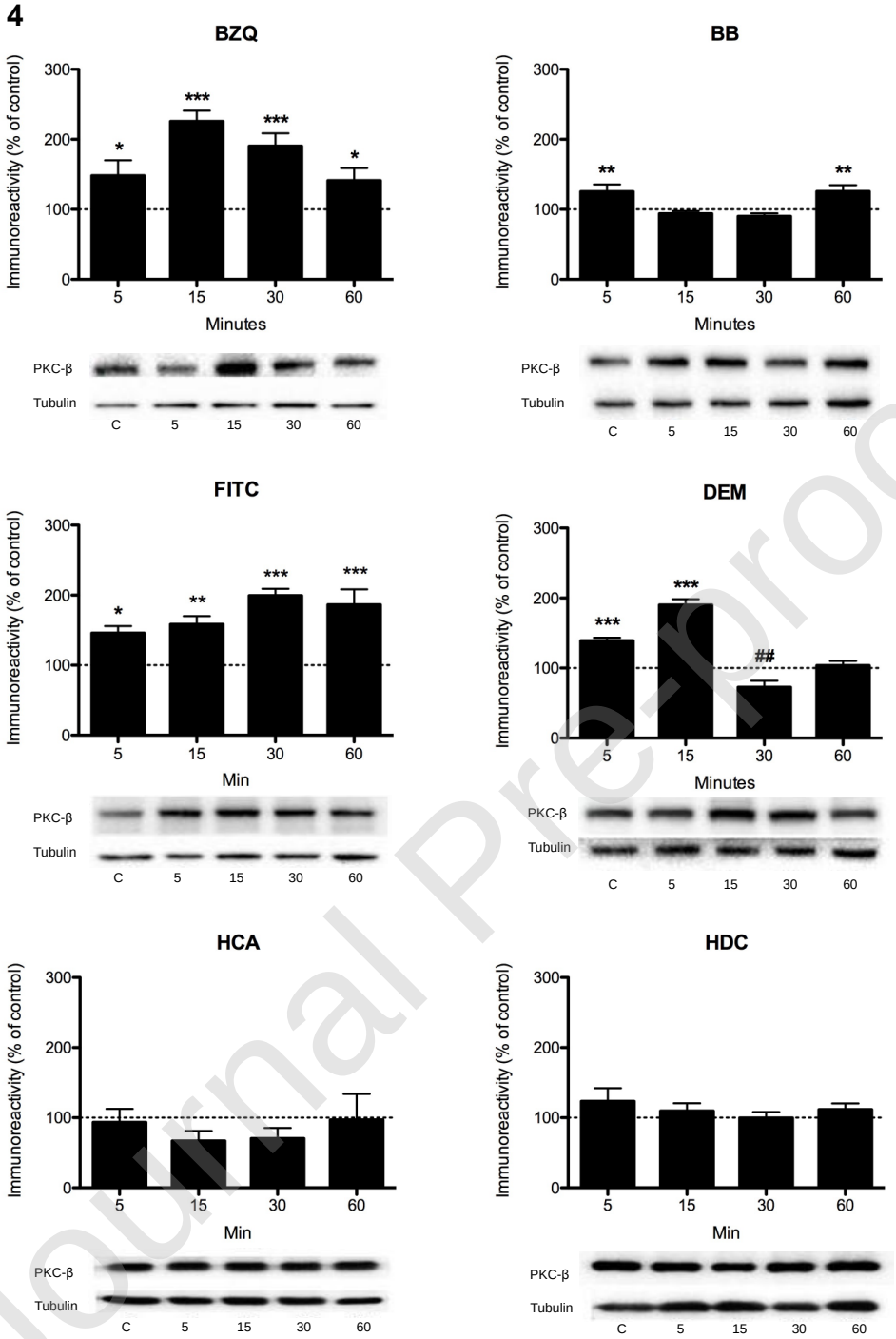


Fig.5

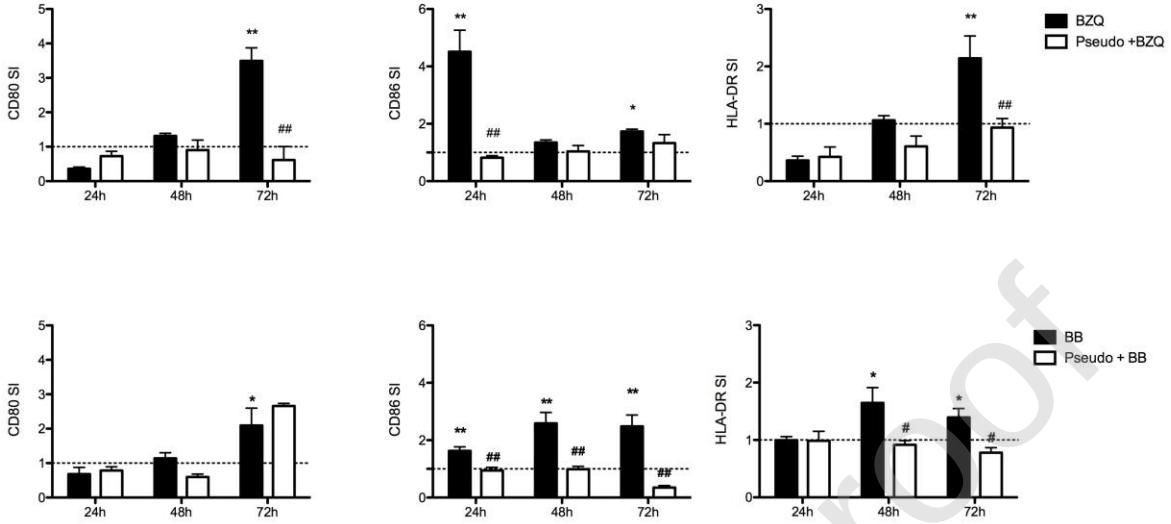
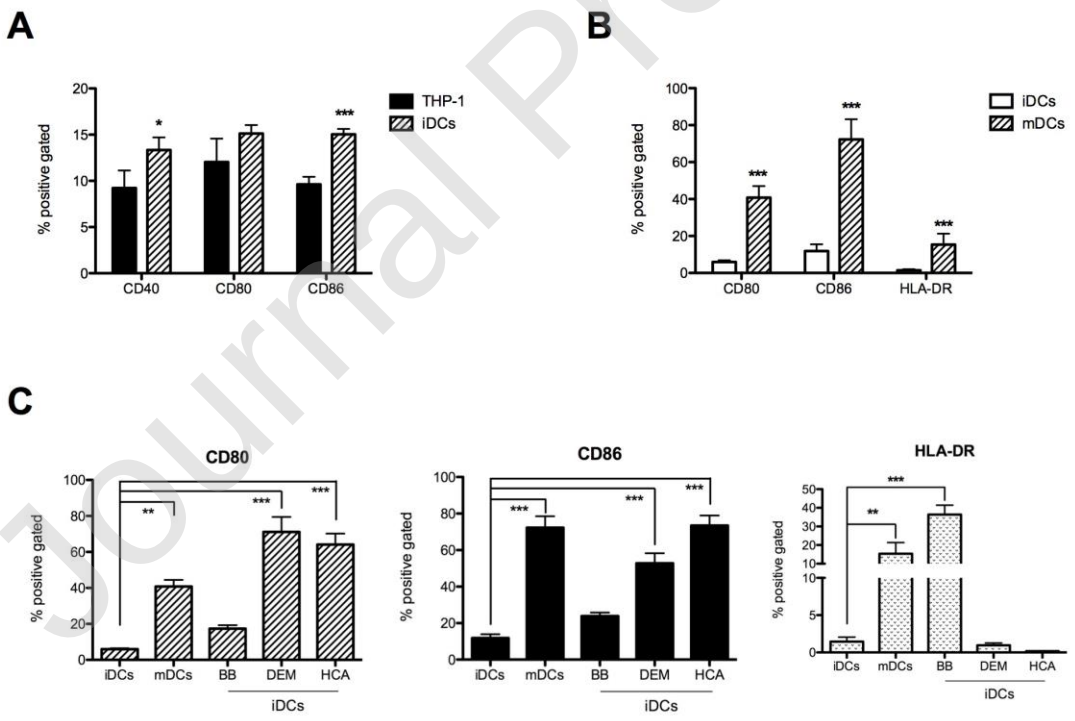


Fig. 6



Figures Legend

Figure 1 – Effects of the selected chemicals on the expression of cell surface marker CD80, CD86 and HLA-DR in dose-response experiments. THP-1 cells were treated for 24 h with increasing concentrations of the selected contact allergens, starting from CV75 and with two 2-fold serial dilutions. Results are expressed as stimulation index (SI Geo Mean). The dotted line reported is set at 1.0 (control). Each value represents the mean ± SD, $n = 3$ independent experiments. Statistical analysis was performed with Dunnett's multiple comparison test, with $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$ vs vehicle treated cells (control). DEM 160 μg/ml and IMZ 60 μg/ml resulted with cell viability less than 75%, despite the CV75 calculation (columns filled in grey colour).

Figure 2 – Effects of the selected chemicals on the expression of cell surface marker CD80, CD86 and HLA-DR in time-course experiments. THP-1 cells were treated for 24, 48 and 72 h with the selected contact allergens. Results are expressed as stimulation index (SI Geo Mean). The dotted line reported is set at 1.0 (control). Each value represents the mean ± SD, $n = 3$. Statistical analysis was performed with Dunnett's multiple comparison test, with $*p < 0.05$, $**p < 0.01$ and $*** p < 0.001$ vs vehicle treated cells (control).

Figure 3 – Effects of the selected chemicals on the cytokines release in time-course experiments. THP-1 cells were treated for 24, 48 and 72 h with the selected contact allergens. Results are expressed as stimulation index (SI). The dotted line reported is set at 1.0 (control). Each value represents the mean ± SD, $n = 3$. Statistical analysis was performed with Dunnett's multiple comparison test, with $*p < 0.05$, $**p < 0.01$ and $*** p < 0.001$ vs vehicle treated cells (control).

Figure 4 –Western blot analysis and densitometric analysis of PKC-βII activation in THP-1 treated with contact allergens of different potency. THP-1 cells were treated for 5, 15, 30 and 60 minutes. Results are expressed as immunoreactivity (% of control). The

dotted line reported is set at 100 (Control). Each value represents the mean ± SD, *n* = 3. Legend: C – control.

Figure 5 – Effective contribution of PKC-β in chemical allergen-induced CD80, CD86 and HLA-DR expression. THP-1 cells were treated for 2 h with a specific PKC-β inhibitor (PKC-β pseudosubstrate) and then BZQ 1.56 μg/ml and BB 2 μg/ml were added for 24, 48 and 72h. Results are expressed as stimulation index (SI). The dotted line on the y-axis at 1.0 of SI represents the threshold above which the chemical is considered positive. Each value represents the mean ± SD, *n=3* independent experiments. Statistical analysis was performed with Dunnett's multiple comparison test, with $^{*,\#}$ $p<0.05$ and $^{**,\#\#}$ $p<0.01$ *vs* vehicle treated cells (*) or *vs* allergens treated cells (#).

Figure 6 – Dendritic cells (DCs) differentiation and maturation starting from THP-1 cells. For DC differentiation, THP-1 cells were treated for 5 days wit rhIL-4 (1500 U/ml) and rhGM-CSF (1500U/ml) to acquire the properties of immature DCs (iDCs – Panel A). Mature DCs (mDCs) were then generated starting from iDCs by addition of rhIL-4 (3000 U/ml), rhGM-CSF (1500U/ml), rhTNF-α (3000 U/ml), and ionomycin (200 ng/ml) or by exposure to contact allergen of different potency for 24h (Panel B and Panel C). CD40, CD80 and CD86 were assessed for the evaluation of cell differentiation (Panel A) while CD80, CD86 and HLA-DR expression were evaluated for the maturation process (Panel B and C). Each value represents the mean ± SD, *n* = 3. Statistical analysis was performed with Dunnett's multiple comparison test, with $*p < 0.05$, $**p < 0.01$ and $*** p < 0.001$ *vs* THP-1 for differentiation experiments (Panel A) or *vs* iDCs for maturation experiments (Panel B and C). For each sample, 10.000 events were acquired and analysis was performed with a calculation of the % of positive gated cells.

Table

Table 1. List of chemicals tested with the *in vitro* CV75 and the *in vivo* classification based on LLNA.

CHEMICAL NAME	CAS #	ACRONYM	CV75 (µg/ml)	LLNA potency category (EC3 % values*)
Benzoquinone	106-51-4	BZQ	1.56	Extreme (0.01)
Bandrowski's base	20048-27-5	BB	4.0	Extreme (0.02)
Fluorescein isothiocyanate isomer I	3326-32-7	FITC	250	Strong (0.14)
Diethyl maleate	141-05-9	DEM	160	Moderate (5.8)
α-hexylcinnamaldehyde	101-86-0	HCA	40	Weak (12)
Hydroxycitronellal	107-75-5	HDC	300	Weak (23)
Imidazolidinyl urea	39236-46-9	IMZ	60	Weak (24)

THP-1 cells were treated for 24h with increasing concentrations of the selected chemicals. Cell viability was assessed by PI staining. CV75 was calculated by linear regression analysis of data. Each value represents the mean of three independent experiments.

* (15)