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# Study on the inflammasome nlrp3 and blimp-1/nlrp12 after keratinocyte exposure to contact allergens

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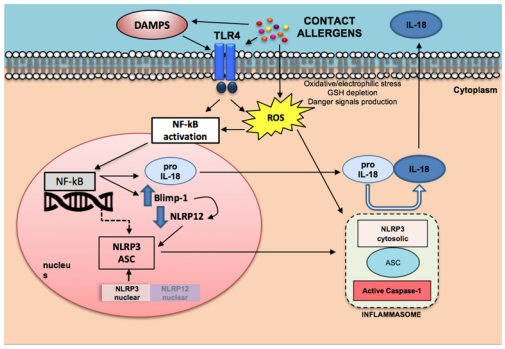
## GRAPHICAL ABSTRACT

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## ABSTRACT

We previously demonstrated that based on their potency, contact allergens differently modulate Blimp-1/NLRP12 expression in human keratinocytes, with the extreme allergen 2,4-dinitrochlorobenzene (DNCB) more rapidly upregulating Blimp-1, leading to downregulation of NLRP12, and to the production of interleukin-18 (IL-18). The purpose of this study was to further investigate the effects of DNCB and para-phenylenediamine (PPD) on the expression of the proteins of the inflammasome, namely NLRP3, ASC and caspase 1 by western blot analysis; to define the intracellular localization and co-localization of NLRP3 and NLPR12 by immunoprecipitation and immunohistochemistry; and to define the role of NF-κB in Blimp-1 induction by pharmacological inhibition. The human keratinocyte cell line NCTC2544 was used for all experiments. Dose and time course experiments were performed to evaluate the effect of the selected contact allergens on the parameters investigated. Results indicate, that consistent with previous finding, DNCB more rapidly (3 h) induces NLRP3, ASC protein expression and caspase-1 activation compared to PPD. Immunoprecipitation studies show the recruitment of ASC to the inflammasome following exposure to both allergens, while high level of NLRP12 and less ASC protein were found associated in control cells. By immunohistochemistry, we found increased NLRP3 expression following exposure to contact allergens, and observed a nuclear co-localization of the two proteins, indicating the NLRP12 likely acts preventing the cytosolic localization of NLRP3 and inflammasome assembly. Finally, contact allergen-induced Blimp-1 mRNA and protein expression can be completely blocked by inhibiting NF-κB activation, confirming the central role of NF-κB in contact allergen-induced keratinocyte activation.

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## 1. Introduction

The NLRP3 (NACHT, LRR and PYD domains-containing protein-3) inflammasome represents the most characterized inflammasone, which is central for the maturation of pro-interleukin 1β and pro-IL-18 (Yang et al., 2019; Tartey and Kanneganti, 2019).

IL-18, a IL-1 family cytokine expressed by several cell types, including dendritic cells and keratinocytes, has been shown to play a key role in various aspects of both innate and adaptive immune responses, including allergic contact dermatitis (Cumberbatch et al., 2001; Antonopoulos et al., 2008; Sanders and Mishra, 2016; Xiao et al., 2018). We previously shown that exposure of the human keratinocyte cell line NCTC2544 or primary human keratinocytes to contact allergens, but not irritants, resulted in a dose dependent induction of IL-18 (Corsini et al., 2009; Galbiati et al., 2011). More recently, we demonstrated a role of Blimp-1 (B lymphocyte-induced maturation protein-1)/NLRP12 in the regulation of contact allergen-induced IL-18 production, with the extreme allergen DNCB more rapidly upregulating Blimp-1, which in turn downregulated NLRP12, resulting in a more rapid production of IL-18 in human keratinocytes (Papale et al., 2014). By silencing experiments, we showed a negative regulation of NLRP12 in contact allergen-induced IL-18 production.

The NLRP3 inflammasome is a multimeric protein complex that acts as a broad sensor of cell homeostasis rupture, serving as a platform for the activation of cysteine protease caspase-1, responsible of the processing and release of IL-1β and IL-18. The NLRP3 inflammasome consists of the NLRP3 scaffold, the apoptotic speck protein containing a caspase recruitment domain adaptor (ASC) and pro-caspase-1. A plethora of stimuli, including endogenous danger-associated molecular patterns (DAMPs) and exogenous pathogen-associated molecular patterns (PAMPs) can activate NLRP3 inflammasome (Yang et al., 2019). Among the DAMPs described to be involved in allergic contact dermatitis, a role of adenosine triphosphate, high-mobility group protein B1 (HMGB1), and hyaluronan can be mentioned (Weber et al., 2010; Galbiati et al., 2014; Nikitovic et al., 2015). Contact allergens use innate immune receptors, like Toll-like receptors TLR2/TLR4 and the NLRP3 inflammasome as well as the induction of oxidative stress to induce skin inflammation, which is critical for successful sensitization and the activation of T cell response (Watanabe et al., 2007; Corsini et al., 2013; Martin, 2017; Corsini et al., 2018).

It is well known that contact allergens vary substantially with regard to the relative potency with which they induce skin sensitization (Kimber et al., 2003). Understanding the mechanisms underlying potency is fundamental for the classification and labeling of skin sensitizers, and to establish thresholds in the induction phase of skin sensitization for the purposes of quantitative risk assessment (Corsini et al., 2018).

The detailed identification of the relevant mechanisms activated by contact allergens of different potency is critical to refine existing, and to develop new in vitro assays for the identification and proper classification and labeling of contact allergens, an important step to replace animal testing (Corsini et al., 2018).

This study aimed to further investigate the effects of two contact allergens of different potency, namely the extreme DNCB and strong PPD, on the expression of the proteins of the NLRP3 inflammasome, to define the intracellular localization and co-localization of NLRP3 and NLPR12, and to define the role of NF-κB in contact allergen-induced Blimp-1 production. DNCB and PPD have been chosen as they represent two contact sensitizers of different potency, namely extreme and strong, widely used to study the sensitization process both in vivo and in vitro. In addition, PPD, the main component of hair dye, is one of the most important contact allergen for humans (Coenraads et al., 2018).

## 2. Materials and methods

### 2.1. Chemicals

The extreme skin sensitizer 2,4-dinitrochlorobenzene (DNCB) (CAS #97-00-7) and the strong sensitizer p-phenylendiamine (PPD) (CAS#106-50-3) were used. DNCB and PPD were obtained from Sigma-Aldrich Co. (St. Louis, Mo, USA) at the highest purity available.

### 2.2. Cells

For all experiments the submerged keratinocyte NCTC 2544 cell line was used (Istituto Zooprofilattico di Brescia, Italy). Cells were seeded at a density of $2.5 \times 10^5$ cells/ml in RPMI 1640 containing 2 mM l-glutamine, 0.1 mg/ml streptomycin, 100 IU/ml penicillin and 0.1% gentamycin, supplemented with 10% heated-inactivated fetal calf serum (media) and cultured at 37 °C in 5% $CO_2$. Cell culture media and all supplements were from Sigma-Aldrich Co. (St. Louis, Mo, USA). For mRNA expression and western blot analysis, cells were seeded in 100 mm Petri dishes (10 ml/Petri). For immunohistochemistry cells were seeded on glass coverlips in a 24-well plate (0.5 ml/well). After o.n. adherence, cells were treated for different times and concentrations as indicated in the legends. DNCB and PPD stock solutions (500x) were prepared in dimethyl sulfoxide (DMSO). DMSO was used as vehicle control in all experiments (0.2% final concentration in culture medium).

### 2.3. Western blot analysis

For western blot analysis, after incubation, culture media was discarded, cells washed with PBS, scraped in 5 ml of cold PBS, transferred in 14 ml polypropylene tubes and centrifuged for 5 min at 1200 rpm at 5 °C. The supernatant was discarded, and cells 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 and 1 µg/mL leupeptin). 100 µl of sample buffer 2X (125 Mm Tris HCl pH 6.8, 4% SDS, 20% glycerol, bromophenol blue, 6% β-mercaptoethanol) were added and samples denatured for 10 min at 100 °C. Protein content was assessed using a commercial kit (Bio-Rad, Hercules, CA, USA). Forty micrograms of proteins were electrophoresed into a 10% sodium dodecyl sulfate (SDS)–polyacrylamide gel under reducing condition. The proteins were then transferred to polyvinylidene difluoride (PVDF) membrane (Amersham, Little Chalfont, UK). Proteins were visualized using mouse monoclonal anti-β actin antibody (clone AC-15, 42 kDa; 1:5000) from Sigma-Aldrich, rabbit polyclonal anti-NLRP12 antibody (95–100 kDa; 1:1000) from Tocris Bioscience (Avonmouth, Bristol, UK), rabbit monoclonal anti-Blimp-1/PRDF1 antibody (clone C14A4, 95–100 kDa; 1:500), rabbit monoclonal anti-NLRP3 antibody (110 kDa; 1:500), rabbit monoclonal anti-TMS1 (ASC) antibody (22 kDa; 1:1000), rabbit monoclonal anti-caspase-1 antibody (the pro-caspase-1: 48 kDa and the activated subunit: 20 kDa) from Cell Signaling Technology (Danvers, MA, USA) as primary antibodies and developed using enhanced chemiluminescence (Bio-Rad). The images of the blots were acquired with the Molecular Imager Gel Doc XR (Bio-Rad). Optical density (OD) of bands was calculated and analyzed by means of the Image Lab version 4.0.1 (Bio-Rad).

### 2.4. Immunoprecipitation

200 µg of cell proteins were incubated in RIA buffer 2X (pH 7.2) (400 mM NaCl, 20 mM EDTA, 20 mM Na2HPO4, 1% Nonidet P-40 and 0.1% SDS) with a polyclonal anti-NLRP12 antibody (95–100 kDa; 1:200) from Tocris Bioscience (Avonmouth, Bristol, UK) overnight at 4 °C under rotation. Protein A/G plus agarose beads (Santa Cruz Biotechnology) were added and incubation was continued for 2 h at 4 °C. Beads were collected by centrifugation and washed in RIA buffer 1X four times. Sample buffer 1X was then added to the samples and boiled for 10 min. Beads were removed by centrifugation, and supernatants loaded onto 10% SDS-PAGE. The proteins were then transferred to PVDF membrane and the immunoprecipitated protein NLRP12 was visualized using rabbit polyclonal anti-NLRP12 antibody (100 kDa;

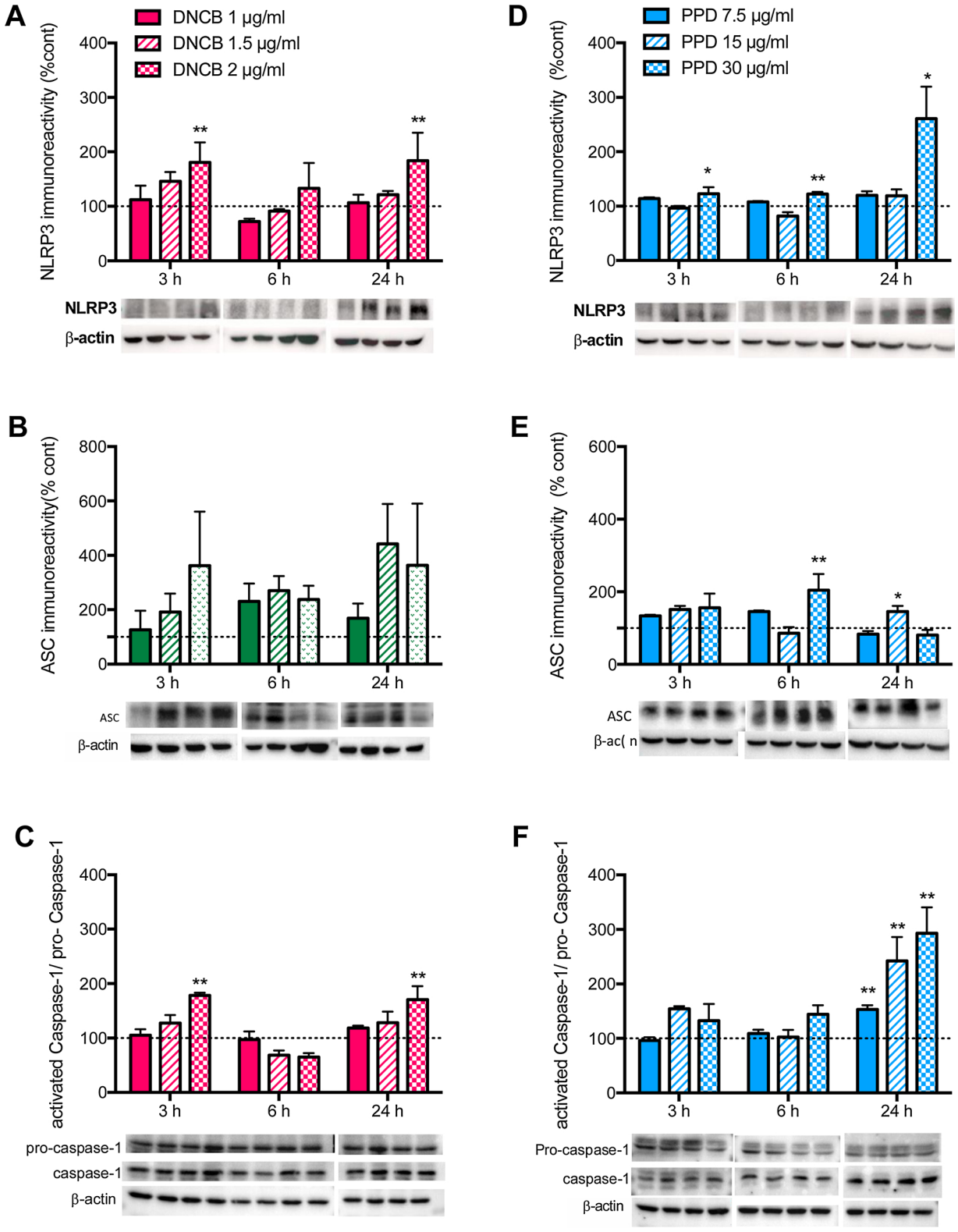


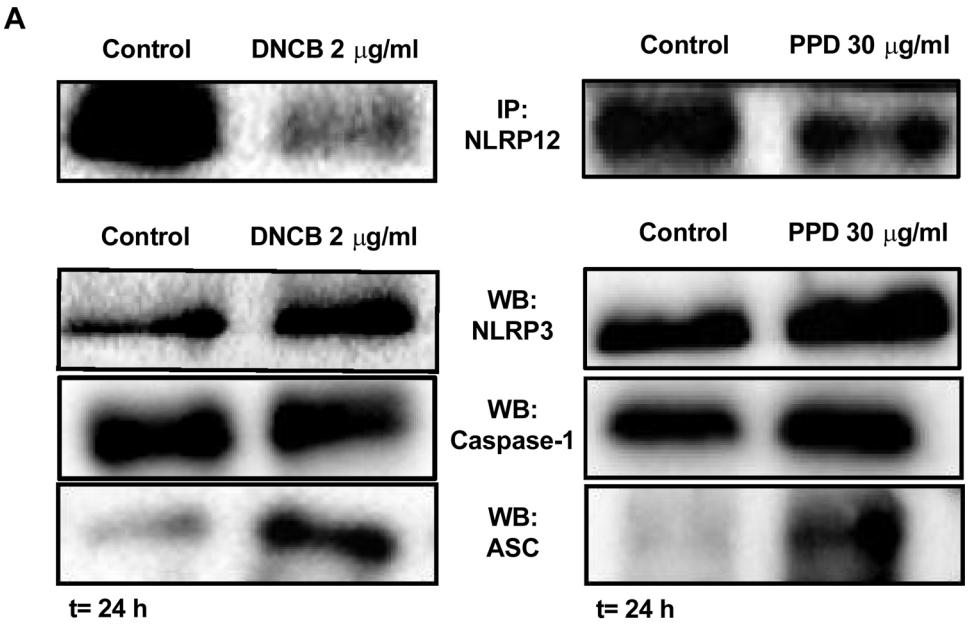

**Fig. 1.** The extreme allergen DNCB more rapidly activate the inflammasome NLRP3.
NCTC2544 cells were treated with increasing concentrations of DNCB (1–2 µg/ml) or PPD (7.5–15 µg/ml) or DMSO as vehicle control (0.2% final concentration) for increasing times (3, 6 and 24 h). Cell lysates were prepared and components of the inflammasome NLRP3 analyzed by western blot analysis. (A; D) Immunoblot of NLRP3 following DNCB (A) or PPD (B) treatment; (B, E) Immunoblot of ASC following DNCB (B) or PPD (E) treatment; (C, F) Immunoblot of the pro-caspase-1 and cleaved caspase-1 in cell lysates following treatment with DNCB (C) or PPD (F). β-actin was also detected as loading control (first lane). Blots were acquired with the Molecular Imager Gel Doc XR, optical density analyzed using the Image Lab version 4.0.1, normalized by β-actin or activated caspase-1/pro-caspase-1, and results expressed as percentage of control (% cont). Data shown are representative of three independent experiments. Statistical analysis was performed by ANOVA followed by Dunnett's multiple comparison test with *$p < 0.05$ and **$p < 0.01$ versus control cells.

1:500), NLRP3, ASC and Caspase-1 are used with the same dilution described in the western blot.

### 2.5. Immunohistochemistry

Following treatment, cells were fixed at RT for 10 min in paraformaldheyde 4% diluted in PBS 0.1 M, pH 7.4. After the incubation in blocking buffer for 45′ minutes at RT, slides were incubated for 3 h at RT with the primary monoclonal antibody rat-anti-human/mouse NLPR3 (R&D Systems, 10 μg/ml) and, after three washing with dilution buffer, with the secondary antibody anti-rat IgG-NL557 (Rhodamine) (R &D Systems, 1:200, 1 h at RT). Then, the incubation with the primary antibody rabbit-anti-human NLRP12 (LSBio, 1:100, 1 h at 37 °C) and the secondary anti-rabbit antibody Alexa Fluor 488 (Molecular Probes, 1:200, 1 h at RT) was performed. Nuclear staining was executed with DAPI 1:50.000 for 5 min at RT and a coverslip was mounted with Mowiol 4-88. Immunostaining was observed by a Nikon Eclipse E600 equipped with a digital camera (DXM1200; Nikon, Tokyo, Japan) and merge pictures obtained with Paint Shop Pro 5.

*Real Time-polymerase chain reaction (RT-PCR).* For total RNA extraction, after treatment culture media was discarded, cells washed with PBS, and scraped in 5 ml of cold PBS, transferred in 14 ml polypropylene tubes and centrifuged for 5 min at 1200 rpm at 5 °C. The supernatant was discarded, and cell pellets lysed with 1 ml of Tri-reagent (Sigma-Aldrich). Total RNA was extracted following supplier's instructions. For the synthesis of cDNA, 2.0 μg of total RNA were retrotranscripted using a high-capacity cDNA reverse transcription kit from Applied Biosystems (Foster City, CA, USA), following the supplier's instructions. For RT-PCR analysis, TaqMan-PCR technology was used. For each PCR, 10 ng of total RNA was used. The 18S ribosomal RNA was used as endogenous reference. Sequences used were for human 18S: Fwd: 5′-CTACCACATCCAAGGAAGCA-3′, Rev: 5′-TTTTTC GTCACTACCTCCCCG-3; and for human Blimp-1: Fwd: 5'-TCGGGTCG TTTACCCCATC-3′, Rev: 5'CACAGCGCTCAGGCCATTA-3′. Quantification of the transcripts was performed by the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).



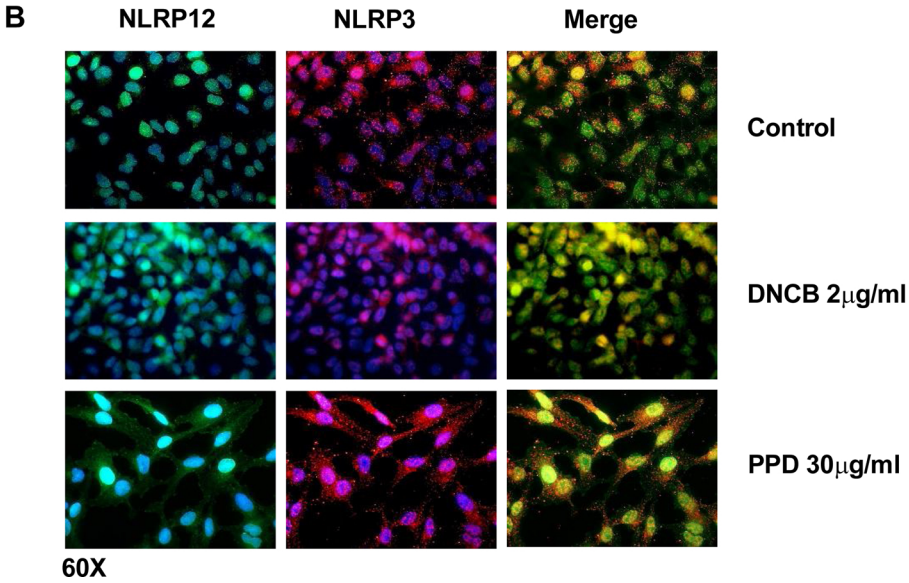




**Fig. 2.** Immunoprecipitation and immunohistochemistry experiments to define NLRP12 and NLPR3 association.
(A) NCTC2544 cells were treated for 24 h with DNCB (2 μg/ml) or PPD (30 μg/ml) or DMSO as vehicle control (Control), immunoprecipitated with anti-NLRP12 (IP), and then immunoblotted (WB) using the indicated antibodies. Results are representative of three independent experiments with similar results. (B) NCTC2544 cells were treated for 24 h with DNCB (2 μg/ml) or PPD (30 μg/ml) or DMSO as vehicle control (Control), fixed and stained with anti-NLRP12 (primary antibody rabbit-anti-human NLRP12 and secondary anti-rabbit antibody Alexa Fluor 488-green conjugated) and anti-NLRP3 (primary antibody rat-anti-human/mouse NLPR3 and secondary antibody anti-rat IgG-NL557 Rhodamine-red conjugated), DAPI was used to counterstain nuclei. Immunostaining was observed by a Nikon Eclipse E600 equipped with a digital camera (DXM1200). Bars: 20 μm.

### 2.6. Statistical analysis

All experiments were repeated at least three times. Data are expressed as mean ± SD. Statistical analysis was performed using InStat software version 7.0 (GraphPad Software, La Jolla, CA, USA). Statistical significance was determined by ANOVA followed by a multiple comparison test or Student's t test as indicated in the legends. Effects were designated significant at $p < 0.05$.

## 3. RESULTS

### 3.1. Effects of allergens of different potency on NLRP3 inflammasome activation

The first aim of this study was to characterize the effect of contact allergen on NLRP3, ASC protein expression and caspase-1 activation. Dose and time course experiments were performed to investigate by western blot analysis the effect of DNCB and PPD on the expression of the constituent proteins of the NLRP3 inflammasome. NCTC2544 were treated for 3, 6, and 24 h with increasing concentrations of DNCB (1, 1.5, 2 μg/ml) and PPD (7.5, 15, 30 μg/ml). The highest concentration used was the CV80 (concentration resulting in a cell viability of 80% compared to DMSO treated cells) as previously established (Papale et al., 2017).

In Fig. 1 results obtained are shown. With a different kinetics depending on potency, contact allergens induce NLRP2 inflammasome proteins and its activation. In details, DNCB induced an earlier and stronger statistically significant induction at 2 μg/ml of NLRP3 (Fig.1 A), ASC (Fig.1B) associated with caspase-1 activation (Fig.1C), compared to PPD. For PPD, statistical significance was achieved at 30 μg/ml at all time points for NLRP3 (Fig.1D), while at 6 and 24 h for ASC (Fig.1E), and only at 24 h for caspase-1 activation (Fig.1 F).

These results support the previous results on IL-18 production, where we found an earlier production with DNCB already statistically significant at 3 h, while for PPD increased IL-18 was observed only after 12 h (Papale et al., 2017).

### 3.2. NLRP3 and NLRP12 intracellular localization

Inflammasomes are believed to form in the cytosol (Brough and

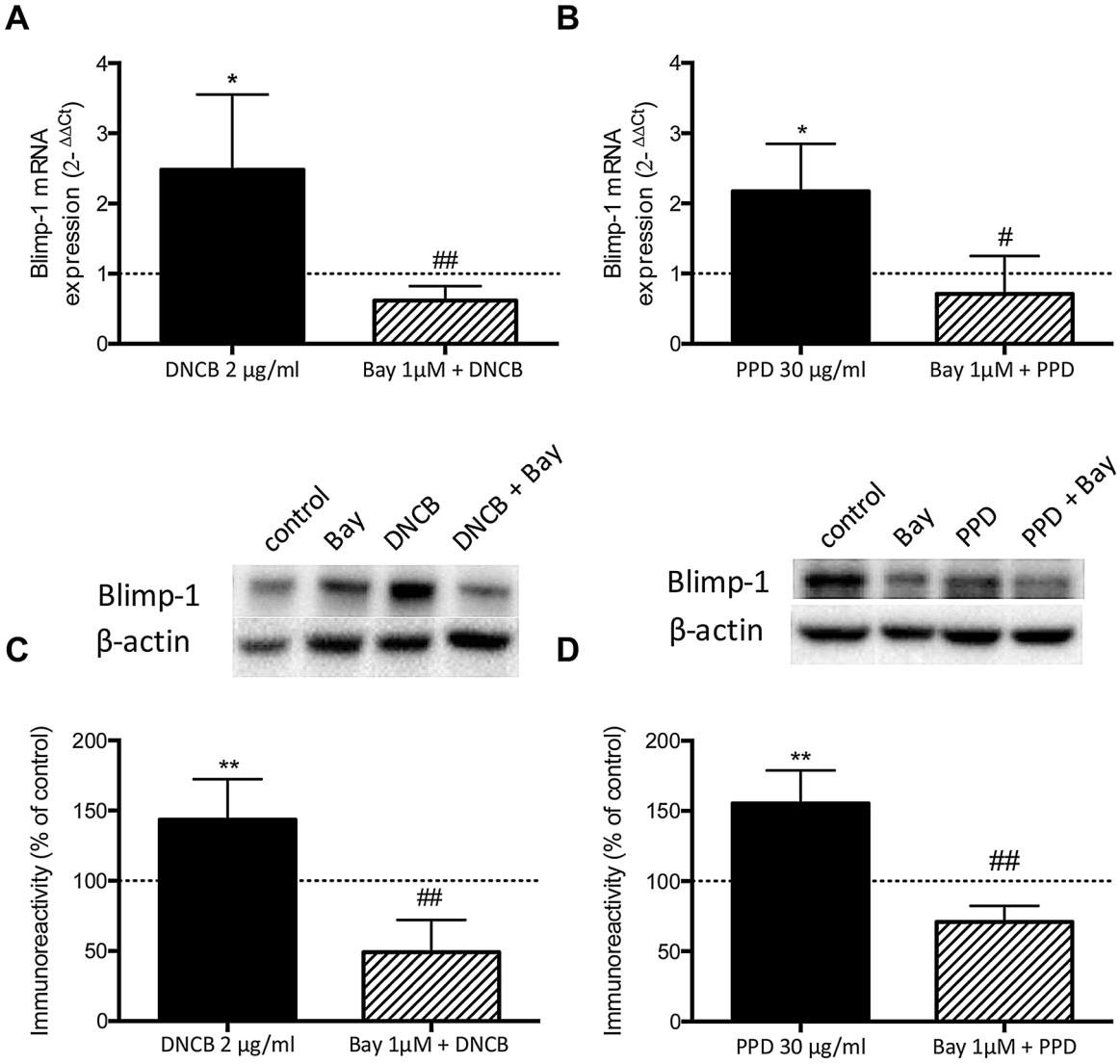



**Fig. 3.** Central role of NF-κB in contact allergen-induced Blimp-1 expression.
NCTC2544 cells were treated for 1 h with the NF-κB inhibitor Bay117085 (1 μM), and then DNCB (2 μg/ml, Panel A and C) or PPD (30 μg/ml, Panel B and D) or DMSO as vehicle control were added for 24 h. Blimp-1 mRNA (A; B) and protein expression (C, D) were measured by RT-PCR and western blot analysis respectively, as described in the Materials and Methods section. Each value represents the mean ± SD, n = 3 independent experiments. Statistical analysis was performed by ANOVA followed by Tukey's multiple comparison test with * $p < 0.05$ and ** $p < 0.01$ vs Control and # $p < 0.05$ and ## $p < 0.01$ vs allergen treated cells.

Rothwell, 2007). We previously demonstrated an inhibitory role of NLRP12 in contact allergen-induced IL-18 production (Papale et al., 2017). The second aim of this study was, therefore, to define the intracellular localization and co-localization of NLRP3 and NLPR12.

Cells were treated for 24 with DNCB 2 μg/ml, PPD 30 μg/ml or DMSO as vehicle control. Results reporting the intracellular localization and co-localization of NLRP3 and NLPR12 are shown in Fig. 2. Time and concentrations were selected based on experiments shown in Fig. 1: at 24 h both DNCB 2 μg/ml and PPD 30 μg/ml induced a statistically significant NLRP3 inflammasome activation. Total cell lysates were immunoprecipitated with a specific antibody against NLRP12 (Fig. 2A). The co-immunoprecipitated complex was then separated by SDS-PAGE, and western blot analysis performed to identify associated inflammasome NLRP3 proteins, namely NLRP3, ASC and caspase-1 (Fig. 2B WB). Consistent with previous results, controls cells have higher NLRP12 expression which was decreased following exposure to allergens (Papale et al., 2017). In control cells, NLRP12 was associated with NLRP3 and caspase-1, with minimal association with ASC. Following treatment with both allergens, ASC was recruited to the complex, thus, going to constitute the inflammasome NLRP3 in its active form. These experiments indicate that NLRP12 is physically associated with NLRP3, and surprisingly, caspase-1 is already part of the complex while ASC recruitment is after allergen-induced activation.

We then investigated their cellular localization, hypothesizing that there could be a different localization, i.e. nuclear vs cytosol, of the two proteins. Cells were treated for 24 h with DNCB 2 μg/ml, PPD 30 μg/ml or DMSO as vehicle control. Immunohistochemistry with specific antibodies was used to visualize the cellular localization of NLRP12 (in green) and NLRP3 (in red) (Fig. 2B). In control cells, NLRP12 is mainly localized in the nucleus. Upon exposure with both allergens, a cytoplasmic localization is also observed. Regarding NLRP3, both a nuclear and cytoplasmatic localization is observed in all groups, with increased fluorescence in allergen-treated cells consistent with western blot results (Fig.1 A, D). Superimposing the two images, a co-localization of NLRP12 and NLRP3 (in yellow), is mainly observed in the nucleus, which could indicate a control system in the cytoplasmic activation of the NLRP3 inflammasome. It would be interesting to evaluate also the location of the other two proteins associated with the NLRP3 inflammasome, namely ASC and caspase-1, which may indicate where the activation takes place.

### 3.3. Role of NF-κB in chemical allergen-induced Blimp-1 production

Last aim of this study was to define the role of NF-κB in Blimp-1 induction, as we previously demonstrated that contact allergen-induced IL-18 production is ROS, NF-κB, and Blimp-1 dependent (Galbiati et al., 2011; Corsini et al., 2013). Therefore, we investigated the involvement of NF-κB in contact allergen-induced Blimp-1. NCTC2544 cells were treated for 1 h with the selective NF-κB inhibitor Bay 117085 (Bay 1 μM), and then DNCB (2 μg/ml) or PPD (30 μg/ml) was added for 24 h. Blimp-1 mRNA and protein expression were evaluated by Real Time-PCR and western blot analysis, respectively. Results are reported in Fig. 3. Both DNCB (Fig. 3A, C) and PPD (Fig. 3B, D) induced Blimp-1 expression were completely prevented by Bay 117085, indicating the centrality of the classical pathway of NF-κB activation in contact allergen-induced IL-18 production in keratinocytes.

## 4. Discussion

Here we described a novel insight in contact allergen-induced NLPR3 inflammasome activation in keratinocytes. We studied possibly differences in the effects of two contact allergens of different potency on the activation of NLRP3 inflammasome, the intracellular localization and co-localization of NLRP3 and NLPR12, and the role of NF-κB in contact allergen-induced Blimp-1 induction. While the involvement of NLRP3 inflammasome in contact allergen-induced IL-18 has been characterized, no detailed studies are available in keratinocytes following in vitro exposure to allergen of different potency. Results indicated that DNCB more rapidly induced NLRP3, ASC protein expression and caspase-1 activation compared to PPD. ASC was recruited to the inflammasome following exposure to both allergens, while high level of NLRP12 and less ASC protein were found associated in control cells. By immunohistochemistry, a nuclear co-localization of the NLRP3 and NLRP12 proteins was observed, indicating that likely NLRP12 acts preventing cytosolic localization of NLRP3 and inflammasome assembly. We propose that part of NLRP3 is sequestered in the nucleus in resting keratinocytes, and becomes available in the cytosol, once keratinocytes are activated in response to contact allergens. This might limit spontaneous activation of caspase-1 in resting cells. Finally, contact allergen-induced Blimp-1 mRNA and protein expression can be completely blocked by inhibiting NF-κB, confirming its central role in contact allergen-induced keratinocyte activation.

In monocytes and macrophages, it is assumed that inflammasomes form in the cytosol. NLRs undergo ATP-dependent oligomerization in response to DAMP recognition, and recruit ASC by PYD-PYD interaction (Duncan et al., 2007). Subsequently, caspase-1 is recruited through the CARD of ASC, which is essential for its activation (Srinivasula et al., 2002). In our study, we observed that in keratinocytes it is ASC that is recruited to the NLRP3 inflammasome, while caspase-1 appears to be already associated with NLRP12 and NLRP3. In monocytes/macrophages, ASC gene ablation or prevention of ASC cytosolic redistribution completely abolished pathogen-induced inflammasome activity (Bryan et al., 2009). Further studies are clearly necessary to evaluate the location of ASC and caspase-1 in keratinocytes, which may help understand the activation process and will provide indication where the activation takes place.

It is interesting to note that the effect of DNCB on the activation of the inflammasome presents a biphasic effect, with activation at 3 and 24 h but not at the 6 h, suggesting the possible involvement of different transduction signals. This is in agreement with the observation made by others that allergens of increasing potency gradually engage more signal transduction pathways and molecules (Albrekt et al., 2014). We are currently investigating the pathways involved. One possible explanation could be that the first wave of activation of the inflammasome induces the maturation of preformed constitutive expressed IL-18, while the second is likely to be associated with a de novo synthesis of IL-18, which can explain the lag phase.

NF-κB is central in inflammatory and immunological reactions, including contact allergy, as many genes encoding immune receptors, cytokines, chemokines, chemokine receptors, and adhesion molecules are under its control (Livak and Schmittgen, 2001; Bell et al., 2003; Kang et al., 2016). In the classical pathway of NF-κB activation, phosphorylation of the inhibitor of NF-κB (IκBα) releases the inhibitor from NF-κB, allowing IκBα degradation and NF-κB activation. Using Bay 117085, an irreversible inhibitor of IκBα phosphorylation, contact allergen-induced Blimp-1 expression could be completely abrogated, indicating that its expression is NF-κB dependent. In keratinocytes, we demonstrated that contact allergen-induced IL-18 production is NF-κB dependent (Galbiati et al., 2011; Corsini et al., 2013), which is consistent with data obtained on Blimp-1 expression and its role in contact allergen-induced IL-18 production (Papale et al., 2017).

## 5. Conclusions

This study demonstrated that contact allergens of different potency activated the NLRP3 inflammasome with a different kinetic, with extreme allergens more rapidly up-regulating inflammasome components and caspase-1 activation. Central to the observed effects is the activation of NF-κB, as its block can prevent all subsequent events from Blimp-1 induction to IL-18 maturation. In addition, our results indicate another layer of complexity in the regulation of IL-18 maturation, where contact allergens affect nuclear to cytosolic redistribution of

NLRP3/NLRP12, and ASC recruitment to the complex.

## Declaration of competing interest

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.

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests.

Valentina Galbiati
Laura Cornaghi
Angela Papale
Elena Donetti
Marina Marinovich
Emanuela Corsini

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