

Understanding chemical allergen potency: role of NLRP12 and Blimp-1 in the induction of IL-18 in human keratinocytes

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Abstract Keratinocytes (KCs) play a key role in all phases of skin sensitization. We recently identified interleukin-18 (IL-18) production as useful end point for determination of contact sensitization potential of low molecular weight chemicals. The aim of this study was to identify genes involved in skin sensitizer-induced inflammasome activation and to establish their role in IL-18 production. For gene expression analysis, cells were treated for 6 h with p-phenylenediamine (PPD) as reference contact allergen; total RNA was extracted and examined with a commercially available Inflammasome Polymerase Chain Reaction (PCR) array. Among genes induced, NLRP12 (*Nod-like* receptor P12) was selected for further investigation. NLRP12 promoter region contains Blimp-1 (B-lymphocyte-induced maturation protein-1)/PRDM1 binding site, and from the literature, it is reported that Blimp-1 reduces NLRP12 activity and expression in monocytes/macrophages. Their expression and role in KCs are currently unknown. To confirm NLRP12 expression and to investigate its relationship with Blimp-1, cells were exposed for different times (3, 6 and 24 h) to the extreme sensitizer 2,4-dinitrochlorobenzene (DNCB) and the strong sensitizer PPD. Allergens were able to induce both genes, however, with different kinetic, with DNCB more rapidly upregulating Blimp-1 and inducing IL-18 production, compared to PPD. NLRP12 and Blimp-1 expression appeared to be inversely correlated: Blimp-1 silencing resulted in increased NLRP12 expression and reduced contact allergen-induced IL-18 production. Overall results indicate that contact allergens of different potency differently modulate

Blimp-1/NLRP12 expression, with strong allergen more rapidly downregulating NLRP12, thus more rapidly inducing IL-18 production. Data confirm that also in KCs, NLRP12 has an inhibitory effect on inflammasome activation assessed by IL-18 maturation.

Keywords Allergen potency · Contact allergy · In vitro · IL-18 · Inflammasome

Abbreviations

ACD	Allergic contact dermatitis
AOO	Acetone olive oil
ASC	Apoptosis-associated speck-like protein
Blimp-1	B-lymphocyte-induced maturation protein-1
CARD	Caspase activation and recruitment domain
DAMPs	Damage-associated molecular patterns
DiSFeB	Dipartimento di Scienze Farmacologiche e Biomolecolari
DMSO	Dimethyl sulfoxide
DNCB	2,4-Dinitrochlorobenzene
ELISA	Enzyme-linked Immunosorbent assay
IFN- γ	Interferon- γ
IL-18	Interleukin-18
IL-1 β	Interleukin-1 β
KC	Keratinocytes
LLNA	Local lymph node assay
LLR	Leucine-rich repeat protein
NF- κ B	Nuclear factor- κ B
NLR	NOD-like receptor
NLRP12	Nod-like receptor P12
PAMPs	Pathogen-associated molecular patterns
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PPD	p-Phenylenediamine
PVDF	Polyvinylidene difluoride

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PYD	Pyrin domain
REACH	Registration evaluation authorization and restriction of chemicals
RHE	Reconstituted human epidermis
SDS	Sodium dodecyl sulfate
siRNA	Small interference RNA
TLRs	Toll-like receptors
TNF- α	Tumor necrosis factor- α

Introduction

Allergic contact dermatitis (ACD) is an important occupational and environmental disease caused by topical exposure to low molecular weight chemicals (Thyssen et al. 2007; Peiser et al. 2012). It represents a delayed-type hypersensitivity reaction, which requires previous sensitization by the same chemical (Nosbaum et al. 2005; Cavani et al. 2007; Basketter et al. 2008).

The risk to develop ACD is considered a serious health issue, and the identification of potential sensitizing agents within consumer products is therefore crucial. With the enforcement of the 7th Amendment to the European Cosmetics Directive (76/768/EEC), a ban was introduced on the use of animals. The development of animal alternative methods has become even more urgent due to the REACH (Registration, Evaluation, Authorization and Restriction of Chemicals) regulation, which may demand toxicity tests for chemicals produced or imported in Europe in quantities over 1 ton per year (Grindon 2007; Grindon et al. 2008; Rovida and Hartung 2009).

Understanding how contact allergens promote ACD is important, as the precise roles of each cell type (i.e., KC, skin resident Langerhans and dendritic cells). For the contact allergens studied so far, it appears that they are sensed by Toll-like receptors (TLRs) and the inflammasome (Kaplan et al. 2012). As TLRs evolved to sense microbial pathogens, the inflammation induced by contact allergens is an accident in nature, but it raises the possibility that the activation of these pathways in KC and dendritic cells may be a trait shared by all chemical allergens. The argument is that more potent allergens will cause a stronger innate inflammatory reaction than will less potent allergens, which will impact on the mobilization, migration of skin dendritic cells and their degree of activation that will in turn influence the quality of elicited immune response (Kimber et al. 2011).

In the last decades, an incredible progress has been made in the development of alternative methods to assess immunotoxicity (Galbiati et al. 2014). In this context within the European project *SENS-IT-IV* (LSHB-CT-2005-018681), we have developed a method based on IL-18 production in KCs to discriminate contact allergens from irritants

and respiratory allergens (Corsini et al. 2009; Galbiati and Corsini 2012; Corsini et al. 2013). Sensing of pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) by nucleotide-binding domain, leucine-rich repeat proteins (LLR), can result in the assembly of a multiprotein complex, named inflammasome, which induces the auto-activation of caspase-1 that leads to cleavage and secretion of the proinflammatory cytokines, interleukin-1 β (IL-1 β) and IL-18 (Barton 2008). The inflammasome is typically formed from the induced assembly of caspase-1, a NOD-like receptor (NLR), NOD meaning nucleotide-binding oligomerization domain, and an adapter apoptosis-associated speck-like protein (ASC) (Mariathasan et al. 2006; Martinon and Tschopp 2004). To date the NLRP3 inflammasome is the most studied (Khan and Hay 2015).

When activated by agonists, the NLR components assemble and self-oligomerize in an ATP-dependent way. The effector protein ASC is then recruited, and it assembles with the pyrin domain (PYD). Pro-caspase-1 binds ASC through CARD–CARD (Caspase Activation and Recruitment Domain) associations, which results in auto-proteolysis of pro-caspase-1 into its active form and in the processing of pro-IL-18 into bioactive IL-18.

The aim of this paper was to understand the molecular pathways involved in contact allergen-induced inflammasome activation and IL-18 production, and their possible correlation with allergenic potency.

Materials and methods

Chemicals

As reference skin sensitizers, the extreme skin sensitizer DNCB (CAS#97-00-7) and the strong sensitizer PPD (CAS#106-50-3) were used. All chemicals were purchased from Sigma-Aldrich Co. (St. Louis, Mo, USA) at the highest purity available. For experiments using submerged cell culture, DNCB and PPD were dissolved in dimethyl sulfoxide (DMSO), while acetone/olive oil (AOO, 4:1) was used for experiments using reconstituted human epidermis (RHE). Cell culture media and all supplements were from Sigma-Aldrich Co., while RHE was from MatTek corporation (MatTek In Vitro Life Science Laboratories, Bratislava, Slovakia).

Cell culture

For experiments, using submerged KCs, the human KC cell line NCTC 2544 was used (Istituto Zooprofilattico, Brescia, Italia). Cells were seeded at a density of 2.5×10^5 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 % CO₂. For IL-18 production, cells were seeded in 24-well plates (0.5 ml/well). For NLRP12 and Blimp-1 mRNA expression and Western blotting analysis, cells were seeded in 100-mm Petri dishes (10 ml/Petri).

After overnight adherence, cells were treated for different times 3, 6 and 24 h with DNCB (1.0, 1.5 and 2 µg/ml), PPD (7.5, 15 and 30 µg/ml) or DMSO as vehicle control (0.2 % final concentration in culture medium). Only for DNCB, additional time points were analyzed: 30 min, 1 and 2 h.

Reconstituted human epidermis (RHE)

EpiDerm Skin Model (EPI-200) was used. RHE was maintained according to the supplier's instructions at 37 °C, 5 % CO₂ and 95 % relative humidity. After 24 h of equilibration time, RHE was transfected with siNLRP12 and siBlimp-1 for 48 h (see below). RHE was then exposed to DNCB (1.5 mg/ml) or AOO as vehicle control. Finn Chamber filter paper disks were impregnated with DNCB and AOO and applied topically to the RHE stratum corneum for 24 h (Gibbs et al. 2013). Culture supernatants were harvested for IL-18 analysis by ELISA (enzyme-linked immunosorbent assay).

Cytokine production

For the assessment of IL-18 production in NCTC 2544 cells, monolayers were gently washed twice with 0.5 ml of phosphate-buffered saline (PBS) and lysed in 0.25 ml of 0.5 % Triton X-100 in PBS. Cell lysates were stored at −80 °C until measurement. Intracellular IL-18 content was assessed by specific sandwich ELISA as previously described (Galbiati and Corsini 2012). Results were normalized by cellular protein content. The protein content of the cell lysate was measured using a commercially available kit (Bicinchoninic acid solution and Copper (II) sulfate solution, Sigma-Aldrich Co.). Results are expressed as a stimulation index (SI), calculated as follows:

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

For the assessment of IL-18 release in RHE, after chemical exposure, culture supernatants were harvested and stored at −80 °C for IL-18 analysis by ELISA.

RNA isolation

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).

For total RNA extraction on RHE, RHE was washed with PBS, lysed with 1 ml of Tri-reagent (Sigma-Aldrich Co.), homogenized for 20 s at room temperature, snap-frozen twice, sonicated for 15 s (50 Hz), and finally, centrifuged for 5 min at 15,000 rpm. Total RNA was extracted following supplier's instructions.

Microarray and real-time PCR

To identify genes involved in inflammasome activation, cells were treated for 6 h with PPD 30 µg/ml or DMSO as vehicle control and total RNA extracted.

After retrotranscription, the inflammasome RT² Profiler PCR Array from Qiagen (Hilden, Germany) was used following the supplier's instructions.

For the synthesis of cDNA, 2.0 µg of total RNA was retrotranscribed using a high-capacity cDNA reverse transcription kit from Applied Biosystems (Foster City, CA, USA), following the supplier's instructions. For real-time 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. Quantification of the transcripts was performed by the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001).

Western blotting (WB)

For WB analysis, after established time of 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). Hundred microliters of sample buffer 2X (125 mM Tris HCl pH 6.8, 4 % SDS, 20 % glycerol, bromophenol blue, 6 % β-mercaptoethanol) were added and denatured for 10 min at 100 °C.

The 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) and rabbit monoclonal anti-Blimp-1/PRDI-BF1 antibody (clone C14A4, 95–100 kDa; 1:500) from Cell Signaling Technology (Danvers, MA, USA) as primary antibodies and developed using enhanced chemiluminescence (Bio-Rad). The image of the blots was acquired with the Molecular Imager Gel Doc XR (Bio-Rad). The optical density of the bands was calculated and analyzed by means of the Image Lab version 4.0.1.

Small interference RNA (siRNA)

The effect of inducing RNA interference on NLRP12 and Blimp-1 was performed using commercially available reagents (Qiagen) following manufacturer's instructions.

Briefly, cells were seeded 1.6×10^5 cells/ml in 24-wells plates and after overnight adherence, transfection reagent, siNLRP12 (20 nM final concentration) and siBlimp-1 (40 nM final concentration) were added in RPMI 1640 containing 2 mM L-glutamine, 0.1 mg/ml streptomycin, 100 IU/ml penicillin and 0.1 % gentamycin to promote the silencing (48 h). After 4 h of incubation, fetal calf serum was added (10 % final concentration). As control siRNA, a siRNA sequence that will not cause the specific degradation of any cellular message was used (Qiagen).

RHE was transfected directly in Assay Medium (EPI-100-ASY), supplied by MatTek corporation, with transfection reagent, siNLRP12 (60 nM final concentration) and siBlimp-1 (80 nM final concentration) to promote silencing. As control siRNA, a siRNA sequence that will not cause the specific degradation of any cellular message was used (from Qiagen).

Silencing was conducted following manufacturer's instructions. Forty-eight hours after siRNA transfection, NLRP12 and Blimp-1 contents in whole-cell lysate were assessed by WB using specific antibodies, to confirm the silencing of proteins. Cells were then treated with DNCB (2 µg/ml) and PPD (15 µg/ml), or DMSO as vehicle control, while RHE was treated with DNCB (1.5 mg/ml), or AOO as vehicle control for 24 h to assess IL-18 production.

Data analysis

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

Table 1 Genes overexpressed by PPD treatment in NCTC 2544 cells

Genes	Fold change $2^{-\Delta(\Delta CT)}$
CARD18	5.31
CCL7	10.57
NLRP12	15.35
NLRX1	23.10
CCL5	30.70

Cells were treated for 6 h with PPD (30 µg/ml). Inflammasomes RT² Profiler PCR Array was used to identify modulated genes. *CARD18* caspase recruitment domain family, member 18, *CCL5* chemokine (C–C motif) ligand 5, *CCL7* chemokine (C–C motif) ligand 7, *NLRP12* nod-like receptor family, pyrin domain containing 12, *NLRX1* NLR family member X1

Results

PCR array

To identify genes selectively upregulated by contact allergens and correlated with the activation of the inflammasome a commercially available inflammasome RT² Profiler PCR array was used. Cells were treated for 6 h with PPD (30 µg/ml) or with DMSO as vehicle control. In Table 1, the more activated genes identified in this preliminary screen are reported. To confirm these findings, cells were treated for 3, 6, 24 h with PPD (30 µg/ml) or DMSO as vehicle control. The expression of CARD18, NLRP12, NLRX1 and CCL5 was confirmed by real-time PCR (Fig. 1), while the upregulation of CCL7 was not confirmed. Among them, NLRP12 was chosen and its role in contact allergen-induced KCs activation was investigated. Its specific role in KCs is currently unknown.

Effects of contact allergens on NLRP12 and Blimp-1 expression

NLRP12/Monarch1 is an intracellular protein consisting of an N-terminal PYD, a central NACHT (NOD domain) and a C-terminal leucine-rich repeat (LRR) region (Tuncer et al. 2014). The full-length human NLRP12 cDNA encodes for 1062-aa protein with an estimated molecular weight of approximately 120 kDa (Ye et al. 2008). Human NLRP12 is expressed predominantly in cells of myeloid lineage, including neutrophils, eosinophils, monocytes, macrophages and immature dendritic cells, and its expression is downregulated in response to pathogens, pathogens products and inflammatory cytokines, indicative of an inhibitory role in innate immune activation (Wu et al. 2013; Shami

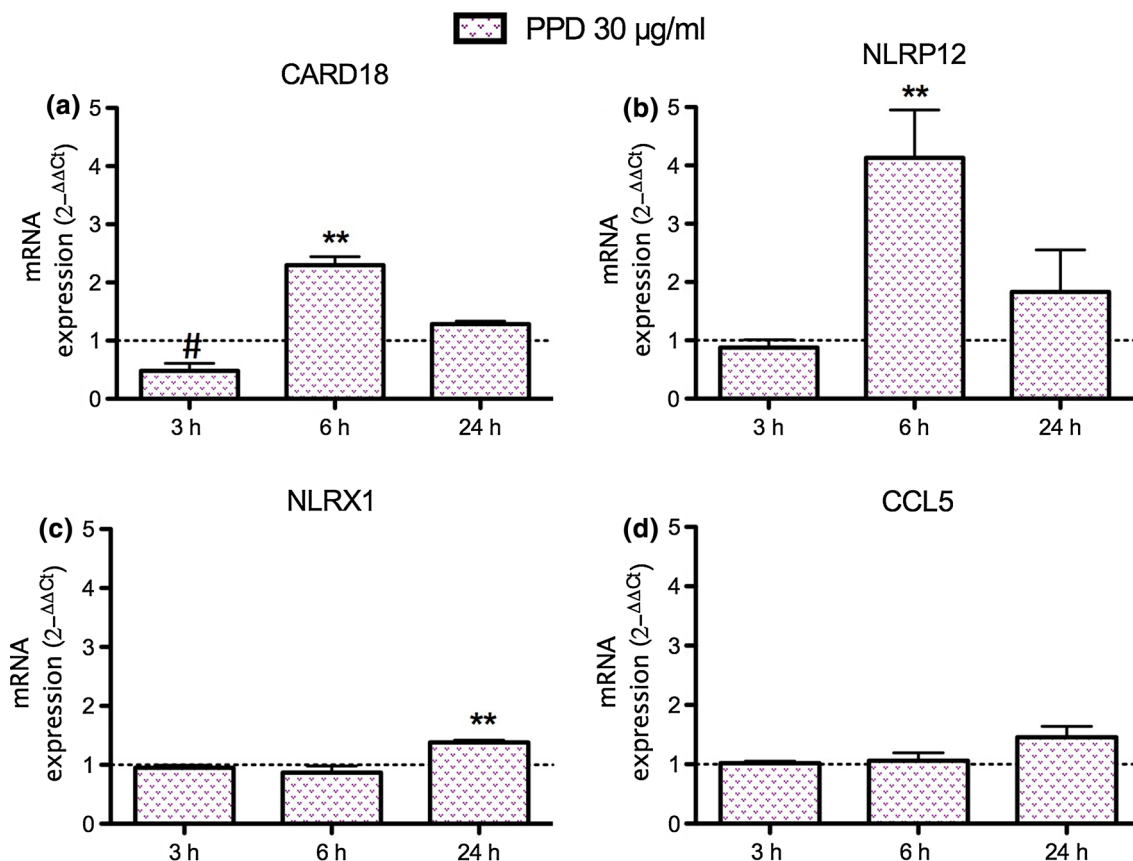


Fig. 1 Induction of the selected genes confirmed by real-time PCR. NCTC 2544 cells were treated for 3, 6, 24 h with PPD 30 μg/ml, or DMSO as vehicle control. To confirm the expression of the identified genes, real-time PCR was used, as described in “[Materials and meth-](#)

[ods](#)” section. Each value represents the mean ± SEM of $n = 3$ independent experiments. Statistical analysis was performed with analysis of variance followed by Dunnett’s test, with $**p < 0.01$ and $#p < 0.05$ versus vehicle-treated cells at the different time points

et al. 2001; Wang et al. 2002; Lich et al. 2007). Moreover, Lord et al. (2009) found that the promoter of NLRP12 contains a Blimp-1/PRDM1 binding site. It has been shown that Blimp-1 reduces NLRP12 activity and expression in myeloid cells during cell differentiation (Martins et al. 2006; Chang et al. 2000).

To investigate the effects of contact allergens on NLRP12 and Blimp-1 expression in KCs, dose–response and time-course experiments were performed. Cells were treated with the extreme sensitizer DNCB (LLNA EC₃ = 0.05 %) and the strong allergen PPD (LLNA EC₃ = 0.11 %) at concentrations selected from previously published data (Corsini et al. 2009).

As NLRP12 and Blimp-1 are reported to be inversely related (Lord et al. 2009), data are presented as ratio Blimp-1/NLRP12. An increased Blimp-1/NLRP12 ratio should be indicative of increased inflammasome activation.

As shown in Fig. 2, both allergens modulated NLRP12 and Blimp-1 gene expression with a different kinetic. In the first series of experiments, cells were treated with

increasing concentration of DNCB (1–2 μg/ml) and PPD (7.5–30 μg/ml) for 3, 6 and 24 h. PPD showed statistically significant changes in Blimp-1/NLRP12 ratio at 6 h (Fig. 2b), while no changes were observed with DNCB (Fig. 2a). This prompted us to investigate the effect of DNCB at earlier time points. Cells were, then, treated for 30 min, 1 and 2 h. As shown in the inset of Fig. 2a, DNCB induced a very early activation of Blimp-1, suggesting that extreme allergens may more rapidly activate the inflammasome.

In a similar way, as shown in Fig. 3, both allergens modulated NLRP12 and Blimp-1 protein expression. In particular, the extreme allergen DNCB modulated the expression already at 30 min (Fig. 3a), while the strong allergen PPD modulated protein expression after 24 h of treatment (Fig. 3b). In both cases, contact allergens induced an increase of Blimp-1 protein expression and a decrease of NLRP12 protein expression, where the extreme allergen more rapidly modulated their expression (Fig. 3a).

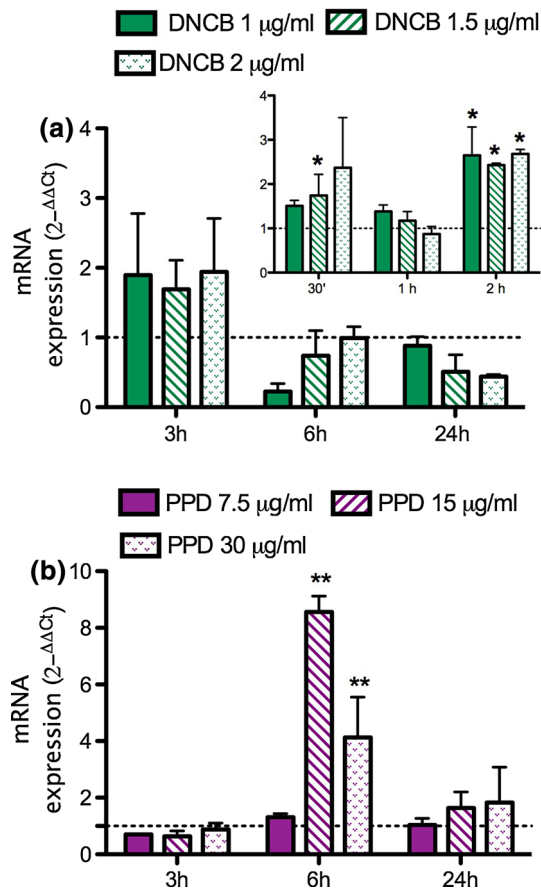


Fig. 2 Effects of contact allergens on NLRP12 and Blimp-1 gene expression. NCTC 2544 cells were treated for different times with DNCB 1–2 µg/ml (a) and PPD 7.5–30 µg/ml (b) or DMSO as vehicle control. After treatments, NLRP12 and Blimp-1 mRNA expression was evaluated by real-time PCR, as described in “Materials and methods” section. Results are expressed as Blimp-1/NLRP12 ratio. Each value represents the mean $\pm$ SEM of $n = 3$ independent experiments. Statistical analysis was performed with analysis of variance followed by Dunnett’s test, with $*p < 0.05$, $**p < 0.01$ versus vehicle-treated cells at the different time points

As a consequence of an increase of Blimp-1 protein expression and a decrease of NLRP12 protein expression, a different kinetic of IL-18 production was observed, with DNCB inducing a more rapid (3 h) production of IL-18 (Fig. 3c) compared to PPD (Fig. 3d).

Role of NLRP12 and Blimp-1 in allergen-induced IL-18

NLRP12 is one of the most elusive members of the NLR family. In monocytes/macrophages, its expression is associated with the shutdown of the inflammatory response

Fig. 3 Effects of contact allergens on NLRP12 and Blimp-1 protein expression. NCTC 2544 cells were treated for different times with DNCB 1–2 µg/ml (a) and PPD 7.5–30 µg/ml (b) or DMSO as vehicle control. After treatment, NLRP12 and Blimp-1 protein expression was assessed by Western blot analysis. Representative Western blots are reported at 30 min and 6 h for DNCB (3a) and 24 h for PPD (3b). At 3, 6, 12 and 24 h IL-18 production was assessed by ELISA (c, d). Results are expressed as mean $\pm$ SEM of $n = 3$ independent experiments. Statistical analysis was performed with analysis of variance followed by Dunnett’s test, with $*p < 0.05$, $**p < 0.01$ and $#p < 0.05$ versus vehicle-treated cells at the different time points

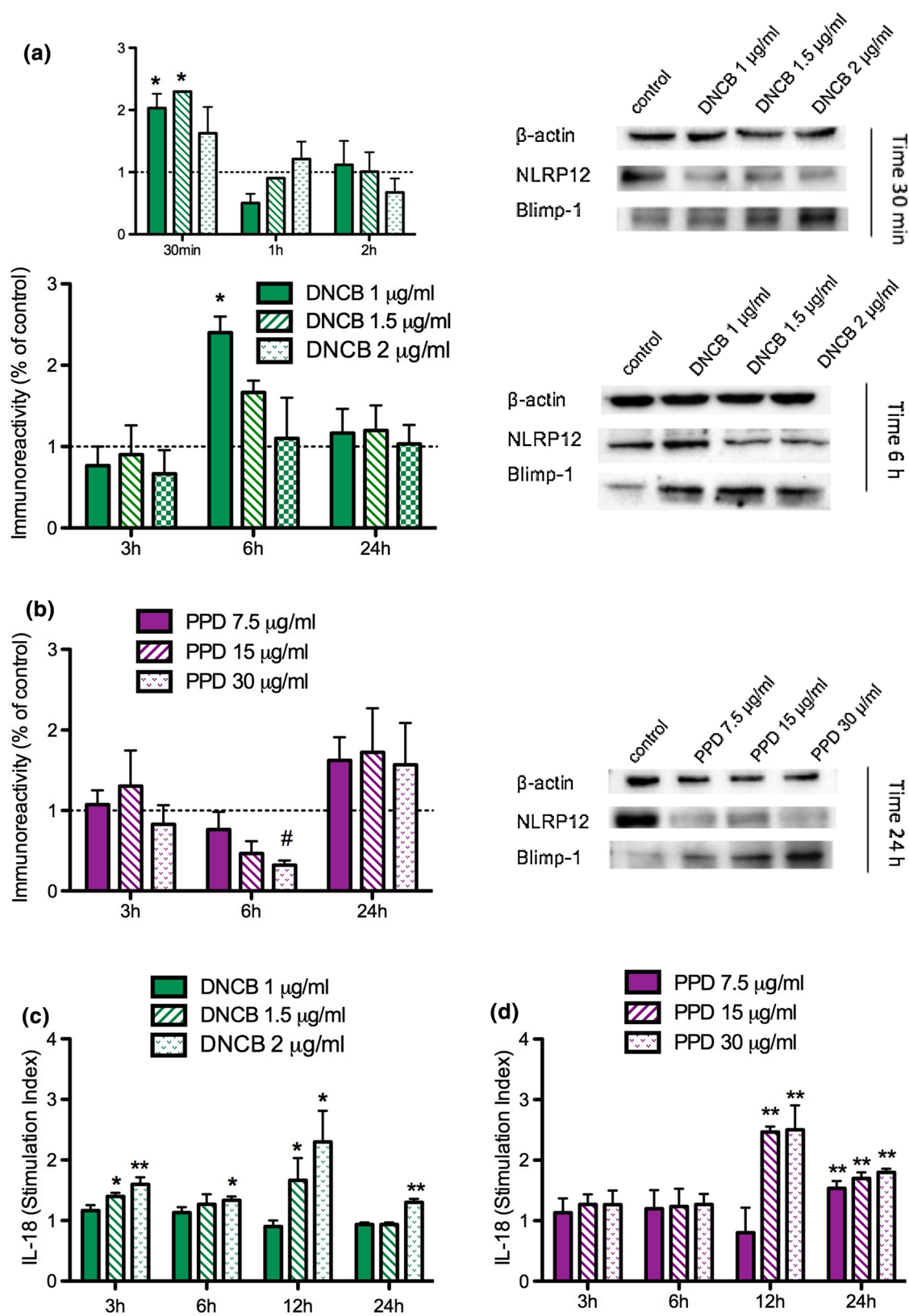
(Arthur et al. 2010). To investigate its role in IL-18 production, the effects of inducing RNA interference on NLRP12 and Blimp-1 were assessed.

NCTC 2544 cells were transfected with NLRP12 and Blimp-1 siRNA for 48 h. After transfection, cells were treated for 24 h with DNCB 2 µg/ml or PPD 15 µg/ml and intracellular IL-18 production assessed by ELISA.

As shown in Fig. 4, NLRP12 silencing, despite its reduced expression both at mRNA and protein level (Fig. 4a, b), did not result in increased allergen-induced IL-18 production (Fig. 4e, f), while Blimp-1 silencing was associated with a significant induction of NLRP12 mRNA and protein expression (Fig. 4c, d), which resulted in a significant reduction in contact allergen-induced IL-18 (Fig. 4e, f). These data are suggestive of an inhibitory role of NLRP12 in inflammasome activation, consistent with data reported in immune cells (Lord et al. 2009). To strengthen these results, an experiment was set up upregulating Blimp-1 expression by treating cells with DNCB 1 µg/ml for 6 h (Fig. 3a). Cells were then extensively washed with culture media to remove DNCB and then exposed to PPD 30 µg/ml or DMSO as vehicle control for 18 h. As shown in Fig. 5, a statistically significant increase was observed in cells pulse DNCB compared to cells exposed to DMSO 6 h + PPD 18 h, further supporting our hypothesis.

Similarly, to confirm the silencing results obtained in the NCTC 2544 cell line, a more realistic model was used. Commercially available RHE was used. RHE was transfected with NLRP12 and Blimp-1 siRNA for 48 h. After transfection, RHE was treated for 24 h with DNCB 1.5 mg/ml or with AOO as vehicle control. The amount of IL-18 present in culture supernatants was quantified by ELISA.

As shown in Fig. 6, Blimp-1 silencing was associated with the induction of NLRP12 (Fig. 6b), as observed in the NCTC 2544 cells. Under this experimental condition, a statistically significant reduction in DNCB-induced IL-18 release was observed in RHE in which Blimp-1 was



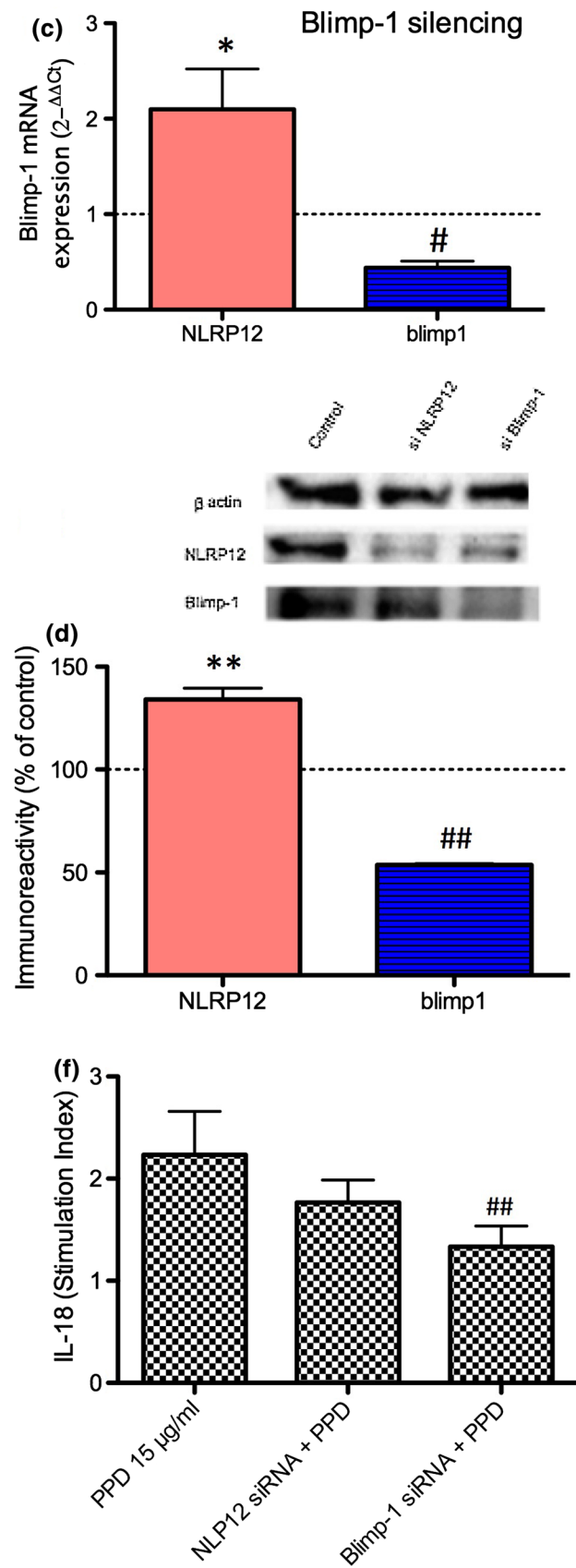
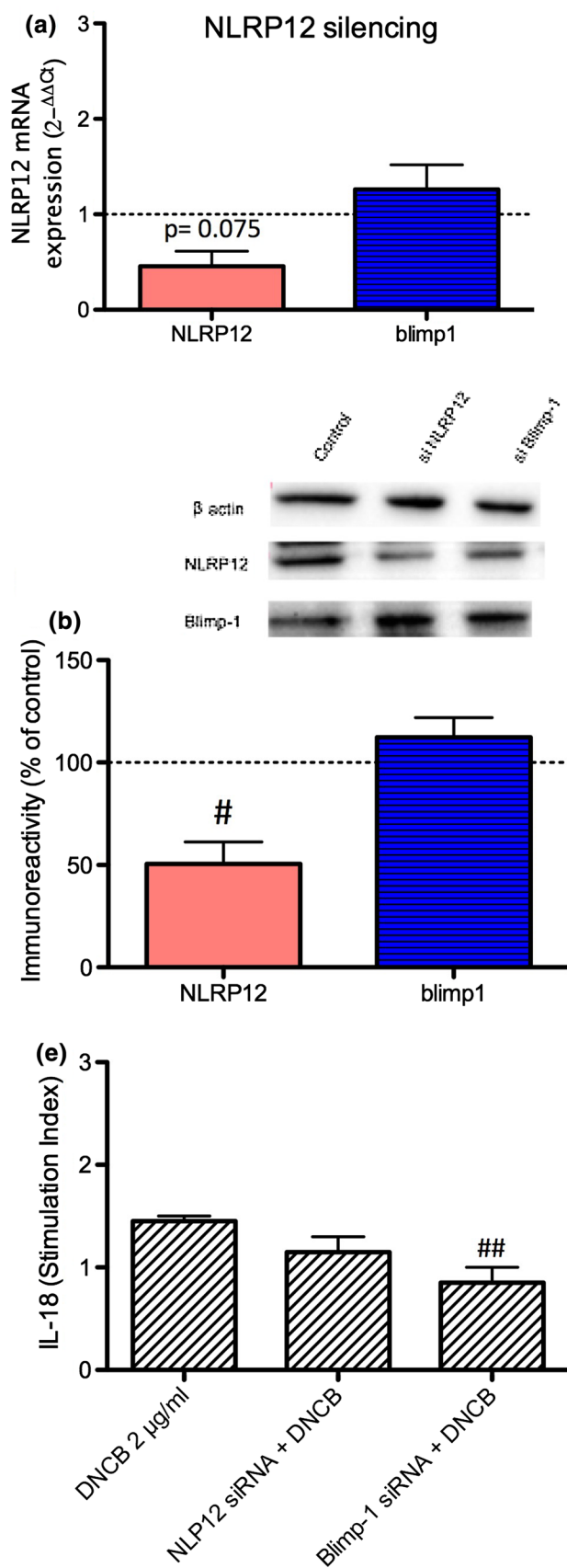


Fig. 4 Effects of NLRP12 and Blimp-1 silencing on contact allergen-induced IL-18 production. NCTC 2544 cells were transfected with NLRP12 and Blimp-1 siRNA for 48 h. After silencing, gene expression was assessed by real-time PCR (a, c), while protein expression was assessed by Western blot analysis (b, d) with representative Western blots shown. After silencing, cells were treated with DNCB (2 µg/ml) or PPD (15 µg/ml) for 24 h and then IL-18 production was assessed by ELISA (e, f). Results are expressed as mean ± SEM of $n = 3$ independent experiments. Statistical analysis was performed with analysis of variance followed by Dunnett's test, with $*p < 0.05$, $**p < 0.01$, $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ versus vehicle (transfection reagent)-treated cells. Results are expressed as mean ± SEM of $n = 3$ independent experiments. Statistical analysis was performed with analysis of variance followed by Dunnett's test, with $^{\#\#}p < 0.01$ versus DNCB or PPD alone (e, f)

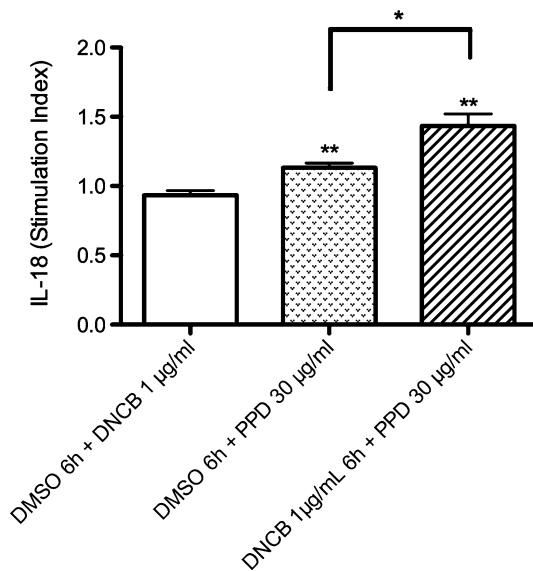


Fig. 5 Effects of Blimp-1 induction on DNCB-induced IL-18 production. NCTC 2544 cells were treated with DNCB 1 µg/ml or DMSO as vehicle control for 6 h and then with DNCB 1 µg/ml or PPD 30 µg/ml for 18 h, and IL-18 production was assessed by ELISA. Results are expressed as mean ± SEM of $n = 3$ independent experiments. Statistical analysis was performed with analysis of variance followed by Dunnett's test, with $**p < 0.01$ versus vehicle-treated cells and $*p < 0.05$ versus vehicle + PPD 30 µg/ml-treated cells

silenced (Fig. 6c), confirming results obtained in the KCs cell line. Also in this case, NLRP12 silencing failed to upregulate DNCB-induced IL-18 production.

Discussion

The aim of this study was to better understand the molecular mechanism involved in contact allergen-induced inflammasome activation and IL-18 production. Using the inflammasome RT² Profiler PCR array, among the genes selectively upregulated by PPD, NLRP12 was identified.

We could then demonstrate that DNCB and PPD differently affected Blimp-1/NLRP12 expression. Interestingly, the extreme allergen DNCB more rapidly downregulated NLRP12 expression and more rapidly induced IL-18 production. As demonstrated in monocytes/macrophages (Williams et al. 2005) also in KCs, NLRP12 and Blimp-1 expression is inversely correlated.

IL-18 is a cytokine that plays a key proximal role in the induction of allergic contact sensitization and favors Th-1-type immune response by enhancing the secretion of pro-inflammatory mediators such as tumor necrosis factor- α (TNF- α) and interferon- γ (Okamura et al. 1995; Cumberbatch et al. 2001; Antonopoulos et al. 2008). Human KCs constitutively express IL-18 mRNA and protein, and it has been shown its induction following exposure to contact sensitizers (Corsini et al. 2009; Galbiati and Corsini 2012; Naik et al. 1999).

NLRP12, in contrast to other NLR proteins that promote inflammation, acts as an attenuator of inflammatory responses (Ye et al. 2008), and it has been characterized in monocytes/macrophages as a negative regulator of both canonical and non-canonical nuclear factor- κ B (NF- κ B) signaling (Lich et al. 2007). NLRP12 was also found to attenuate the development of contact hypersensitivity (Arthur et al. 2010). More recently, mutations that result in a truncated form of NLRP12 were linked to hereditary periodic fevers that manifest with recurrent fevers, joint pain and skin urticaria (Jéru et al. 2008).

Lord et al. (2009) found that reducing NLRP12 expression with small interference RNA in myeloid/monocyte cells, an increase in NF- κ B activation and cytokine expression in response to TLR2/TLR4 agonists (TNF α) was observed, suggesting that NLRP12 is a negative regulator of inflammation. The NLRP12 promoter contains a Blimp-1/PRDM1 binding site. It has been shown that Blimp-1 reduces NLRP12 activity and expression (Williams et al. 2005). The expression of NLRP12 and Blimp-1 appears to be inversely correlated. It has been proposed that the transcriptional silencing of NLRP12 is essential for the development of a proper immune response (Struwe and Warren 2010). NLRP12 gene silencing is mediated by Blimp-1 during both innate immune activation and cellular differentiation (Lord et al. 2009).

We speculate that the ability of different allergens to induce IL-18 may be linked to their ability to modulate NLRP12 and Blimp-1 gene and protein expression differently, depending on their potency. Indeed different kinetics were observed, with the extreme allergen DNCB modulating Blimp-1/NLRP12 gene and protein expression more rapidly, compared to the strong allergen PPD.

SiRNA is widely used to silence the expression of specific genes to assess the individual contribution of these genes to an assortment of cellular phenotypes. In order to

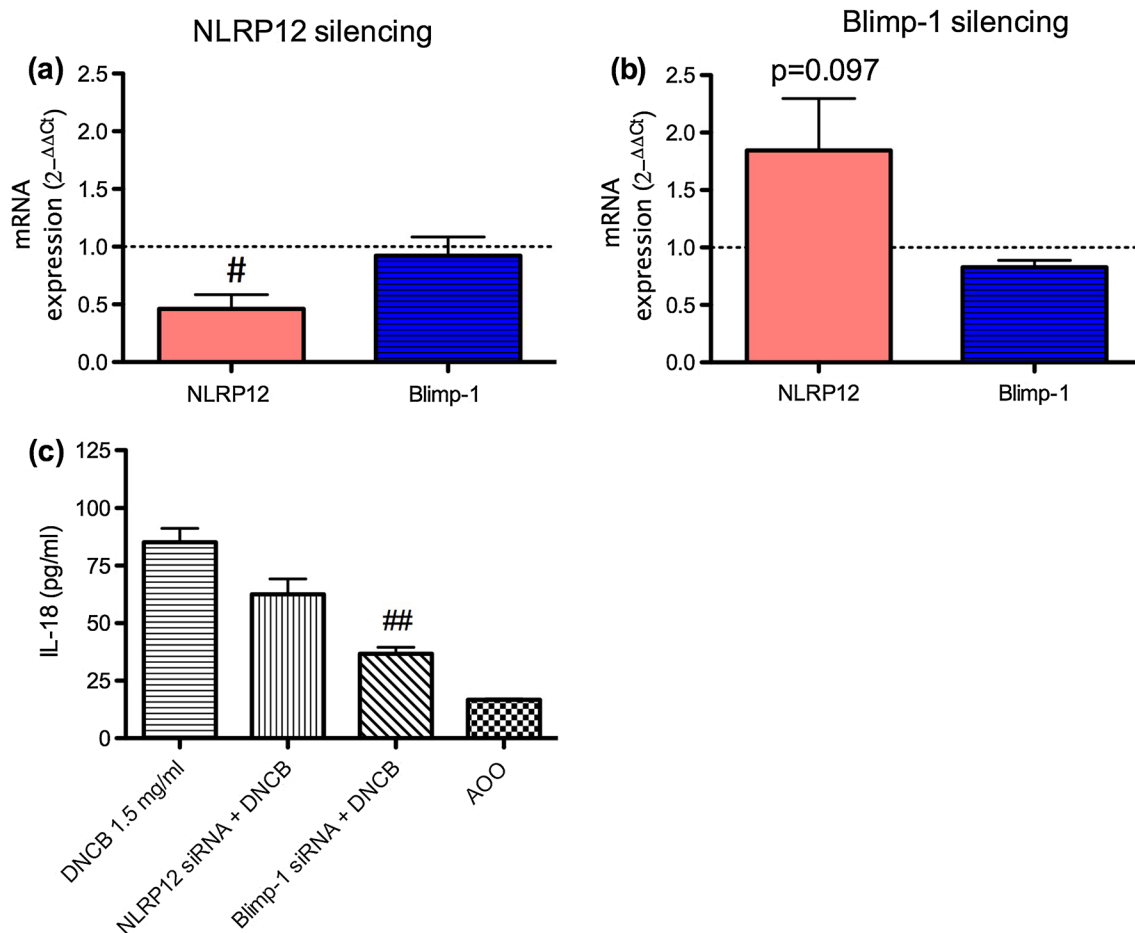


Fig. 6 Effect of NLRP12 and Blimp-1 silencing in reconstructed human epidermis. RHE was transfected with NLRP12 and Blimp-1 siRNA for 48 h. After silencing, gene expression was assessed by real-time PCR (a, b). After silencing, RHE was exposed to DNCB (1.5 mg/ml) or AOO as vehicle control for 24 h, and IL-18 release was assessed by ELISA (c). Results are expressed as mean $\pm$ SEM

of $n = 3$ independent experiments. Statistical analysis was performed with analysis of variance followed by Dunnett's test, with $^{\#}p < 0.05$ versus vehicle (transfection reagent)-treated cells (a, b). Results are expressed as mean $\pm$ SEM of $n = 3$ independent experiments. Statistical analysis was performed with analysis of variance followed by Dunnett's test, with $^{##}p < 0.01$ versus DNCB alone (c)

understand the role of NLRP12 and Blimp-1 in allergen-induced IL-18 production, silencing experiments were performed. Silencing Blimp-1, the gene and protein of NLRP12 was significantly increased, supporting the role of Blimp-1 as repressor of NLRP12 expression (Williams et al. 2005). The following scenario can be imagined (Fig. 7): following exposure to contact allergens, Blimp-1 expression significantly increase to downregulate NLRP12 expression, which in turn facilitates the activation of the inflammasome and IL-18 maturation (Fig. 7b). On the contrary, we speculate that NLRP12 is present before immune stimulation and it acts as a negative regulator of inflammation. This event could prevent the inflammasome activation and consequently IL-18 production (Fig. 7a). The inhibitory role of NLRP12 in IL-18 production was evident after Blimp-1 silencing, which resulted in NLRP12

overexpression and in an almost complete abrogation of contact allergen-induced IL-18 production. Equally, inducing Blimp-1 by DNCB, it was evident a statistical increase in IL-18 (Fig. 5), supporting the role of NLRP12 as negative regulator of inflammation. Surprisingly, NLRP12 downregulating did not result in increased contact allergen-induced IL-18 production, suggesting that in our experimental conditions, the degree of reduction was not sufficient to significantly affect inflammasome activation and IL-18 production induced the strong allergens used.

In conclusion, we demonstrated the role of NLRP12 and Blimp-1 in contact allergen-induced IL-18 production in human KCs. Current investigations are focused on the effects of weak allergens and other allergens of different potency on Blimp-1/NLRP12 expression to support our working hypothesis that allergens with different in vivo

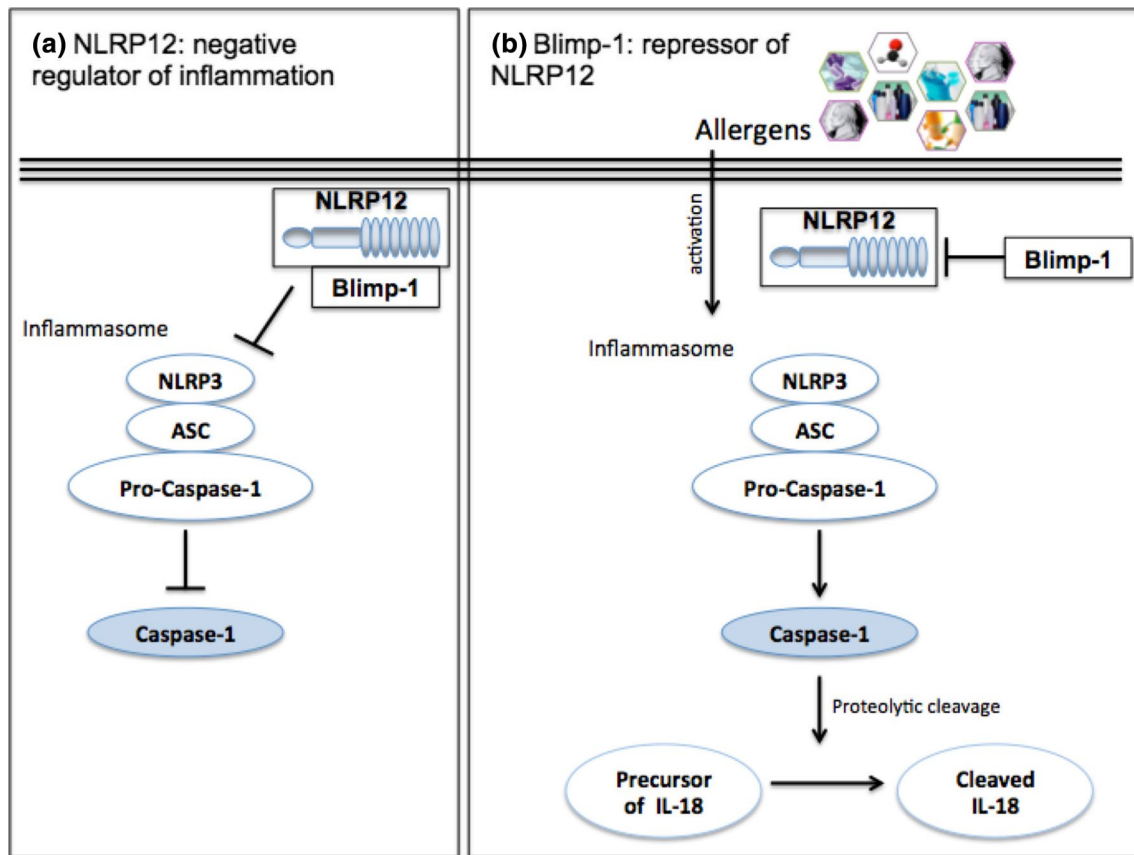


Fig. 7 NLRP12 and Blimp-1 role in inflammasome activation. NLRP12 is supposed to act as a negative regulator of inflammation before immune stimulation (**a**), but during infection its expression is reduced to allow for a full inflammatory response. In this con-

test, Blimp-1 plays a pivotal role (**b**): It is a repressor of NLRP12. NLRP12 inhibition leads to inflammasome activation and the activated caspase-1 drives the proteolytic cleavage of pro-IL-18 in IL-18

potency can differently modulate their expression resulting in a different degree of IL-18 maturation, which will affect the vigor of contact hypersensitivity.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

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