Activation of the IL-1β-Processing Inflammasome Is Involved in Contact Hypersensitivity

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The inflammasome is a cytosolic protein complex regulating the activation of caspase-1, which cleaves the pro-inflammatory cytokines IL-1β and IL-18 into their active form. The inflammasome is composed of a NACHT-, LRR- and pyrin (NALP) family member that acts as a sensor for danger signals and the adaptor protein apoptosis-associated speck-like protein containing a CARD domain (ASC), which allows the recruitment of caspase-1 in the complex. In the skin, exposure to contact sensitizers (CS) such as trinitro-chlorobenzene causes an immune response called contact hypersensitivity (CHS) or eczema. In this delayed-type hypersensitivity response, efficient priming of the adaptive immunity depends on the concomitant activation of the innate immune system, including IL-1β/IL-18 activation in the skin. To determine if the inflammasome contributes to CHS, we have analyzed its capacity to react to CS *in vitro* and *in vivo*. We show here that key components of the inflammasome are present in human keratinocytes and that CS like trinitro-chlorobenzene induce caspase-1/ASC dependent IL-1β and IL-18 processing and secretion. We also show that ASC- and NALP3-deficient mice display an impaired response to CS. These findings suggest that CS act as danger signals that activate the inflammasome in the skin, and reveal a new role of NALP3 and ASC as regulators of innate immunity in CHS.

INTRODUCTION

The skin protects us from our environment and is therefore constantly subjected to microbial, chemical, and physical insults that ultimately result in the generation of non-self antigens. Accordingly, the skin is endowed with the capacity to trigger immune responses (Murphy *et al.*, 2000). Sensitizing chemicals that penetrate the skin surface induce a delayed-type hypersensitivity reaction called contact hyper-sensitivity (CHS) (Grabbe and Schwarz, 1998). There are few T cells in normal skin and antigen presentation occurs mostly in draining lymph nodes. Uptake, transport, and presentation of antigens is achieved primarily by specialized skin cells called Langerhans cells (LCs) (Grabbe and Schwarz, 1998). Efficient LC migration and antigen presentation as well as the later recruitment of primed lymphocytes to the skin is dependent on the concomitant presence of pro-inflammatory stimuli (like IL-1β) induced by the contact sensitizers (CS) themselves (Grabbe *et al.*, 1996; Griffiths *et al.*, 2005). However, how sensitizing chemicals induce activation of these cytokines is still unknown.

IL-1β and IL-18 mediate acute inflammatory responses and provide a link between innate and adaptive immunity in CHS (Shornick *et al.*, 1996; Wang *et al.*, 2002). Both cytokines are produced as inactive cytoplasmic precursors, which are processed into their active forms by caspase-1 (Cerretti *et al.*, 1992; Thornberry *et al.*, 1992; Ghayur *et al.*, 1997; Dinarello, 1998). Interestingly, a functional caspase-1 was found to be necessary for LC migration and optimal CHS in mice (Antonopoulos *et al.*, 2001). The molecular mechanisms controlling caspase-1 activity were totally unknown until very recent studies demonstrated that it relied on a cytosolic multiprotein complex composed of a NACHT-, LRR-, and pyrin domain (NALP) family member, the protein apoptosis-associated speck-like protein containing a CARD (ASC) and caspase-1 (Srinivasula *et al.*, 2002; Agostini *et al.*, 2004; Martinon and Tschopp, 2005). As this complex regulates pro-inflammatory cytokine activation, it was given the name inflammasome (Martinon and Tschopp, 2004).

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Abbreviations: ASC, apoptosis-associated speck-like protein containing a CARD domain; CHS, contact hypersensitivity; CS, contact sensitizers; DAMPs, danger associated molecular patterns; LC, Langerhans cell; NALP, ACHT- (Neuronal apoptosis inhibitory protein, CIITA, HET-E, TP1), LRR- and pyrin domain; PAMPs, pathogen associated molecular patterns; PK, primary keratinocytes; TNCB, trinitrochlorobenzene

The role of the 14 human NALP proteins identified is still poorly understood. NALPs form a subfamily of the NACHT-LRR family that includes nucleotide oligomerisation domain (NOD) proteins and is reminiscent of Toll-like receptors (Martinon and Tschopp, 2005). Interestingly, the latter are key sensors of the innate immune system that detect pathogen-associated molecular patterns (Akira and Takeda, 2004). Recent studies using mice deficient in NALP3 or ASC show that NALPs may sense bacterial components and uric acid crystals. Upon exposure to these ''danger signals'', they induce ASC-mediated caspase-1 recruitment and activation, thereby triggering an inflammatory response (Kanneganti et al., 2006; Mariathasan et al., 2006; Martinon et al., 2006). Hence, NALPs complete the NOD and toll-like receptors (TLR) repertoire of danger sensors.

Mutations in the gene encoding NALP3 are associated with severe auto-inflammatory disorders characterized by recurrent fevers owing to excessive IL-1β secretion that are alleviated by IL-1 receptor antagonists (Hawkins et al., 2003; Agostini et al., 2004; Hoffman et al., 2004). This underlines the central role of the inflammasome in IL-1β regulation and the necessity to tightly control its activity. As patients affected by these diseases have inflammatory skin lesions, we hypothesized that an active inflammasome is present in the skin and that it may be responsible for CS-mediated IL-1β activation. We show here that the inflammasome is indeed present and can be activated in keratinocytes. Moreover, ASC- and NALP3-deficient mice display an impaired CHS to trinitro-chlorobenzene (TNCB) and dinitro-1-fluorobenzene (DNFB), thus identifying the NALP3 inflammasome as a key regulator of innate immunity in contact hypersensitivity. Hence, pro-inflammatory signals necessary for the priming of T cells are provided, at least in part, by danger sensors of the NALP family.

RESULTS

TNCB, SDS, and UV induce caspase-dependent IL-1β secretion by keratinocytes

Evidence suggests that several cytokines implicated in CHS are produced by resident cells within the skin (Grabbe et al., 1996; Griffiths et al., 2005). In particular, LC and keratinocytes can secrete IL-1β and IL-18 upon exposure to CS (Enk et al., 1993; Naik et al., 1999) and these cytokines are crucial for LC migration to the lymph nodes (Cumberbatch et al., 2002). We decided to determine if keratinocytes, which form the major cell population in the skin, could react to danger signals by activating and secreting IL-1β. In macrophages, IL-1β secretion follows two sequential events: (i) upregulation of pro-IL-1β through NF-κB-mediated transcriptional activation (Martinon and Tschopp, 2005) and (ii) inflammasome/caspase-1-mediated processing of pro-IL-1β (Dinarello, 1998; Martinon et al., 2002). We found that exposure of primary keratinocytes to purified flagellin (activating TLR5), MALP-2 (activating TLR6), or TNFα, which all activate NF-κB indeed resulted in an increase of the expression level of pro-IL-1β (Figure 1a and data not shown). The promoter of pro-IL-1β is known to contain an NF-κB binding site (Hiscott et al., 1993), and other potent NF-κB activators are therefore likely
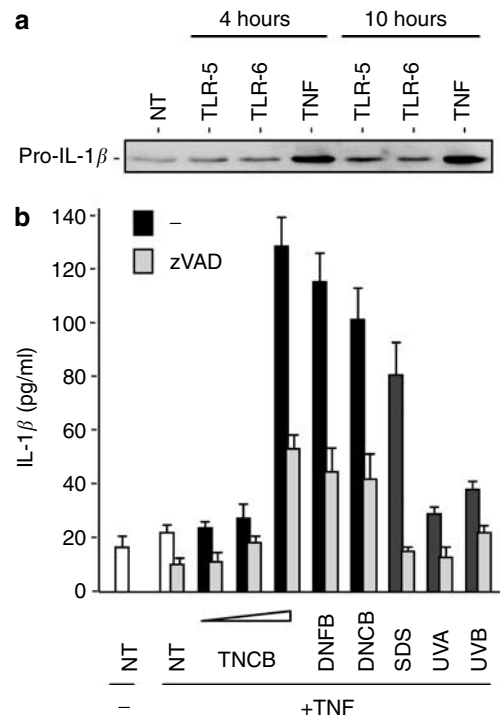
Figure 1. Danger signals induce caspase-dependent IL-1β secretion by keratinocytes. (**a**) Stimulation of primary keratinocytes by NF-κB activators such as TLR5/6 or TNFα (100 ng/ml) results in an increase in pro-IL-1β expression levels. (**b**) Exposure to TNFα itself does not result in a significant secretion of IL-1β, but TNFα-pretreated cells respond to TNCB (0.1, 1, and 5 μg/ml), SDS (15 μg/ml), or UVB (80 mJ/cm^2) for 24 hours by secreting IL-1β in the supernatant (ELISA detection, $n \geqslant 6$), as opposed to stimulation by UVA (80 mJ/cm^2) exposure. In each case, IL-1β secretion is blocked by the pan-caspase inhibitor zVAD (20 μM).

to have a similar effect on cultured keratinocytes. This increase in pro-IL-1β was not paralleled by a significant rise in IL-1β secretion (Figure 1b). However, significant secretion of IL-1β could be induced by exposure to the CS TNCB, DNFB, and dinitro-1-chlorobenzene. Likewise, SDS and UVB significantly increased IL-1β secretion, whereas UVA induced only a minor increase in IL-1β levels. IL-1β secretion was dependent on caspase activity, as it was blocked by the pan-caspase inhibitor benzyloxycarbonyl-valine-alanine-aspartate (zVAD) (Figure 1b). Apoptosis was not observed at these doses of TNCB and UV, suggesting that caspase-1 may be involved. In accordance with this, targeted overexpression of caspase-1 in mouse keratinocytes was shown to result in CHS symptoms dependent on IL-1β and IL-18 (Yamanaka et al., 2000). Keratinocytes can thus sense danger signals and subsequently activate IL-1β independently of the immune system. Although TNF prestimulation allowed an easier detection of IL-1β, it was not essential for its activation and secretion (Figure 3a).

Both keratinocytes and monocyte-derived cells contain molecular components required for inflammasome assembly

In macrophages, pro-IL-1β processing is dependent on the assembly of the inflammasome, composed of a NALP protein,

the adaptor protein ASC, and caspase-1, which cleaves it directly (Dinarello, 1998; Martinon *et al.*, 2002). We reasoned that resident skin cells may use the same machinery and assessed the presence of molecular components of the inflammasome in keratinocytes and Langerhans-like cells. Messenger RNA coding for all NALPs 1–6, 8, 10–11, and 13–14 were detectable in primary keratinocytes and human adult calcium-reduced temperature elevated keratinocyte (HaCaT) cells (Figure 2a). NALP7 was only detectable in primary keratinocytes, whereas NALP12 could only be detected in HaCaT cells. Messenger RNA for all NALPs except NALP 5, 7, and 9 were observed in Tohoku University, Department of Pediatrics-1 (THP-1) cells, a monocyte-derived cell line. Monocytes have the potential to differentiate into dendritic cells and respond to sensitizers, thus resembling LCs (Ashikaga *et al.*, 2002). Messenger RNA for ASC-1, caspase-1, caspase-5, pro-IL-1β, and IL-18 were detectable in the three cell types (Figure 2b).

Expression at the protein level was confirmed by SDS-PAGE/Western blot analysis of whole-cell proteins extracts. NALP1 and NALP3, ASC, caspase-1 and caspase-5 could be readily detected in primary keratinocytes, HaCaT cells, and THP-1 cells (Figure 2c). Levels of NALP1, NALP3, and caspase-1 were lower in keratinocytes than in THP-1 cells, whereas levels of caspase-5 and ASC were similar. This suggests that resident skin cells contain the molecular components necessary for inflammasome assembly.

TNCB induces ASC-dependent caspase-1 and IL-1β activation in keratinocytes

We next looked at the role of caspase-1 in TNCB-induced IL-1β activation by exposing primary keratinocytes to TNCB and analyzing the cellular and secreted caspase-1/IL-1β patterns. Activated (cleaved) fragments of caspase-1 (p35 and p11), IL-18 (p18), and IL-1β (p17) could be found in supernatants only after TNCB stimulation (Figure 3a, supernatants). Pretreatment with TNFα increased capase-1 cleavage and IL-1β

secretion as previously reported (Dinarello, 1998; Martinon *et al.*, 2002), but IL-18 activation was unchanged. This may be explained by the observation that both caspase-1 and pro-IL-1β expression levels are increased by TNFα but not IL-18 (Figure 4a, cell extracts).

TNCB-induced caspase-1 cleavage resulted in a decrease of the intracellular pool of full-length caspase-1 (Figure 3a, cell extracts). Shorter fragments of capase-1 were not detected in the cell extracts, which is consistent with previous reports showing that cleaved caspase-1 is secreted together with IL-1β (Martinon *et al.*, 2002). The kinetic of caspase-1 (p35 and p11) and IL-1β activation, appearing 6 hours after stimulation and increasing thereafter (Figure 3b and data not shown), was similar in monocyte-derived cells stimulated with uric acid crystals (Martinon *et al.*, 2006). Concomitant exposure to the caspase-1-specific inhibitor benzyloxycarbonyl-tyrosine-valine-alanine-aspartate (zYVAD) blocked both caspase-1 and IL-1β activation and secretion (Figure 3b). Hence, TNCB-induced caspase-1 activation in keratinocytes is necessary for IL-1β activation.

We confirmed the central role of the inflammasome by isolating mouse keratinocytes from ASC-deficient mice and exposing them to TNCB. ASC-deficient keratinocytes failed to secrete IL-1β upon *in vitro* exposure to TNCB, in contrast to keratinocytes isolated from wild-type mice (Figure 3c). Hence, TNCB-induced IL-1β activation and secretion by keratinocytes is likely to be controlled by the inflammasome.

Contact hypersensitivity to DNFB and TNCB is reduced in ASC and NALP3-deficient mice

In order to confirm the role of the inflammasome *in vivo*, we used a model of CHS to DNFB, where 6- to 7-week-old ASC-deficient mice and their wild-type littermates were sensitized either on the left ear or on their shaved belly and then challenged on day 5 at a distant site (i.e. the right ear). Ear thickness measured 24 hours after the challenge showed a significant diminution of the ear swelling in ASC-deficient
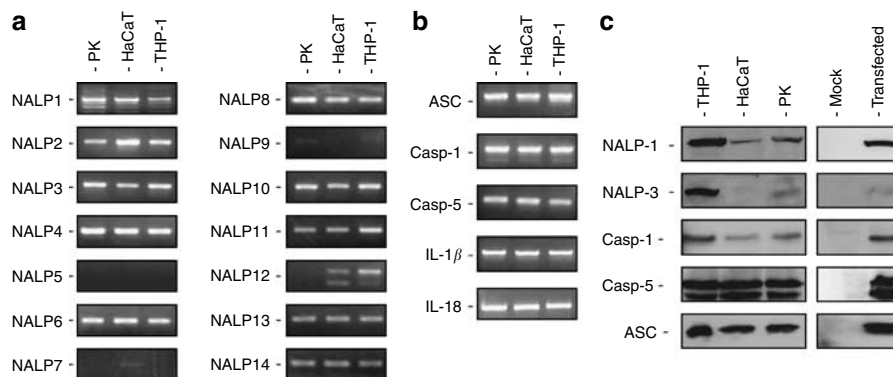

Figure 2. **Keratinocytes and monocyte-derived cells contain the molecular components necessary for inflammasome activity.** (**a**) Reverse transcription-PCR analysis shows detectable levels of mRNA coding for most members of the NALP family in primary human keratinocytes (PK), keratinocyte-derived HaCaT cells, and the monocyte-derived THP-1 cell line. (**b**) Messenger RNA of ASC, caspase-1, caspase 5, IL1-β, and IL-18 are present in primary keratinocytes (PK), HaCaT, and THP-1 cells. (**c**) Western blot analysis reveals the presence of NALP1, NALP3, caspase-1, caspase-5, and ASC at the protein level in PK, HaCaT, and THP-1 cells. The specificity of each antibody was confirmed by Western blot analysis of extracts from 293T cells transfected or not with the relevant complementary DNAs. The data presented are representative of at least three independent experiments.
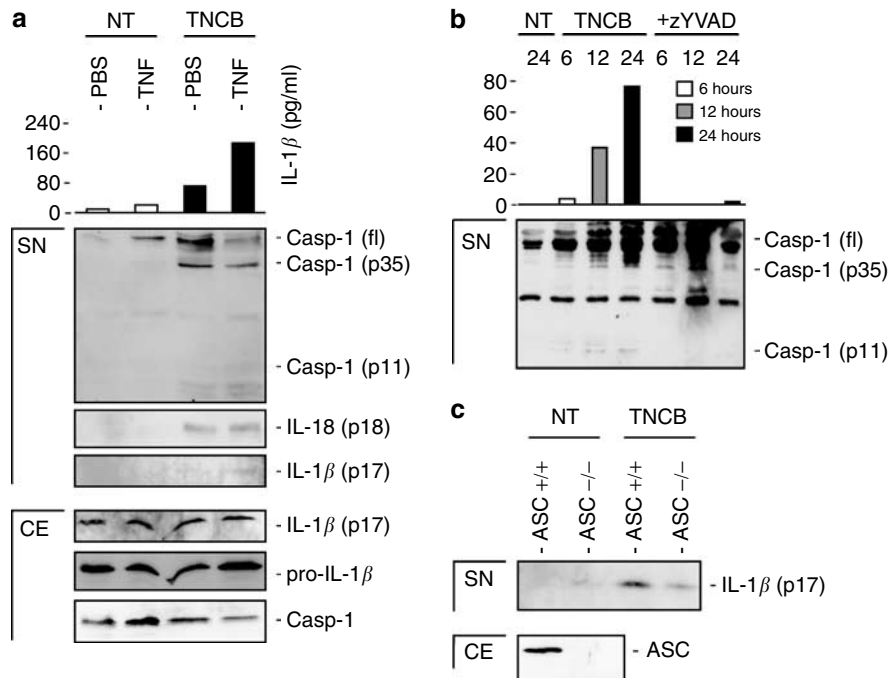
Figure 3. **TNCB induces ASC-dependent caspase-1 and IL-1β activation in keratinocytes.** (**a**) Primary keratinocytes exposed to TNCB (5 μg/ml) secrete active caspase-1 (p35 and p11), IL-18 (p18), and IL-1β (p17), as revealed by Western blot and ELISA analysis of supernatants (SN). This is paralleled by a decrease in full-length caspase-1 found in cell extracts (CE). TNFα increases the expression levels of caspase-1 and pro-IL-1β but not IL-18 (CE). TNFα pretreatment increases activation and secretion of both caspase-1 and IL-1β ΣN (**b**) Secretion of active caspase-1 (p35 and p11) in the supernatant (SN) and IL-1β (ELISA) starts 6 hours after TNCB exposure and is blocked by the caspase-1 inhibitor zYVAD (50 μM). (**c**) TNCB (1 μg/ml)-induced secretion of active IL-1β is reduced in primary keratinocytes cultures derived from neonatal ASC-deficient (−/−) mice as opposed to wild-type (WT) mice.

mice (Figure 4a). In non-sensitized mice, DNFB failed to induce a detectable ear swelling, as previously described (Riemann *et al.*, 2005). The limited ear swelling observed after sensitization on the belly may be due to the ingestion (by licking) of small amounts of DNFB, which acts as a tolerizer when applied orally (Faria and Weiner, 2005).

Contact hypersensitivity (CHS) involves priming of naïve T cells (sensitization) and activation of primed T cells upon re-exposure to the antigen (elicitation). Elicitation can be subdivided into an early and a late phase (Grabbe and Schwarz, 1998). The early phase, characterized by increased vessel permeability and local edema, peaks 8 hours after antigen re-exposure and is believed to be mediated by local release of cytokines (including IL-1β) and histamine (Kitagaki *et al.*, 1997; Grabbe and Schwarz, 1998). The late phase develops 12–36 hours after antigen re-exposure and is due to cellular infiltration.

We examined both the early and late phases of TNCB-induced CHS in IL-1R-, ASC-, and NALP3-deficient mice. Mice 6–7 week old were sensitized twice to TNCB by external application of TNCB on the ear at day 0 and 7, and ear thickness was measured 1, 2, 3.5, 5, 8, 14, 24, 32, and 48 hours after elicitation with TNCB at day 14. Consistent with previous reports, we observed a peak ear swelling 5–8 hours after TNCB application (early phase), followed by a second peak at 24 hours (late phase) (Figure 4b). IL-1R deficiency did not alter the kinetics, but resulted in a significant decrease in the intensity of the ear swelling (Figure 4b and c).

In ASC- and NALP3-deficient animals, TNCB-induced swelling was significantly lower in the early phase of elicitation (Figure 4c). The extent of this reduction was similar in ASC, NALP3, and IL-1R-deficient mice, although strain differences (BALB/c, DBA/C57BL/6, and C57BL/6, respectively) resulted in different peak levels in ear swellings, as previously reported (Bigby *et al.*, 1987). The reduction of the ear swelling was confirmed by histological analysis of ASC and NALP3 deficient mice ears 5 hours after TNCB application, which revealed a marked reduction in the swelling, subcutaneous edema, and vasodilatation that normally characterize the early phase (Figure 5). The late phase of elicitation, characterized by the recruitment of inflammatory cells in the skin, was not significantly different in ASC- and NALP3-deficient mice, suggesting that the inflammasome only plays a minor role at this stage (Figure 4c and 5).

We found that IL-1R deficiency affected the early phase as well as the late phase of elicitation, although the difference observed was not always statistically significant (Figure 4b and c). This either suggests that IL-1α in involved in the late phase of elicitation (both IL-1α and β signal thought the IL-1R) or that IL-1β can be cleaved by other proteases, such as mast cells chymases (Mizutani *et al.*, 1991). However, activation of caspase-1 was demonstrated to be essential for optimal sensitization in mice, suggesting that other proteases only play a minor role in IL-1β activation in CHS (Antonopoulos *et al.*, 2001).
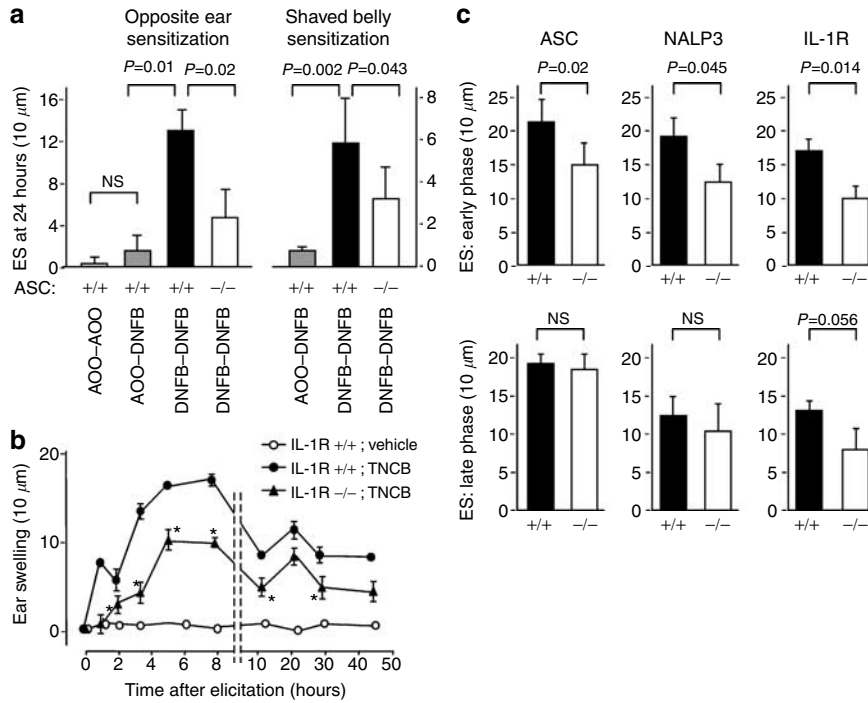
Figure 4. Contact hypersensitivity to DNFB and TNCB is reduced in ASC-, NALP3-, and IL-1R-deficient mice. (**a**) ASC deficiency blocks the ear swelling measured 24 hours after challenge (day 5) with DNFB on the right ear of mice previously sensitized at distant sites (left ear or shaved belly) at days 0 and 1 ($n=5$ per group). Non-sensitized wild-type mice show barely discernable ear swelling after DNFB challenge and the vehicle (acetone:olive oil $=$ AOO) alone has no detectable effect either. (**b–c**) Ear swelling measured after elicitation (day 14) of TNCB-sensitized mice (days 0 and 7) is reduced during both the early and late phases of CHS in IL1R-deficient ($-/-$, filled triangles) mice as compared with wild-type ($+/+$, filled circles) littermates. Asterisks denote statistical significance (*$P<0.05$; $n=5$ mice per group). (**c**) Ear swelling measured after elicitation of TNCB-sensitized mice is significantly reduced during the early phase of elicitation in ASC- and NALP3-deficient mice as compared with wild-type littermates, but not during the late phase. The extent of reduction of the ear swelling in the early phase of elicitation is similar in ASC-, NALP3-, and IL-1R-deficient mice, although strain differences result in different peak levels in ear swellings.
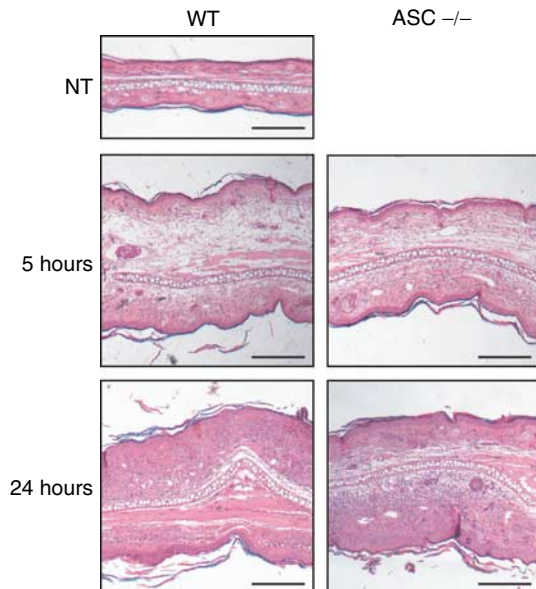

Figure 5. Contact hypersensitivity to TNCB is reduced in ASC-deficient mice. Histology of ASC-deficient and wild-type (wt) mice ears 5 hours after TNCB challenge (bar $=200\,\mu m$). Edema and vasodilatation characteristic of the early phase of elicitation of CHS are less prominent in ASC-deficient ($-/-$) mice as compared with wild-type littermates ($+/+$). No apparent histological difference is apparent 24 hours after elicitation with TNCB.

DISCUSSION

We provide evidence that primary human keratinocytes contain the necessary elements to form an inflammasome and that TNCB induces caspase-1-mediated cleavage and activation of IL-1β in an ASC-dependent manner. The kinetics of the activation of caspase-1 and IL-1β cleavage and secretion was similar to the other inflammasome activators. TNCB can thus be considered as a new ''danger signal'' sensed by the inflammasome, together with uric acid crystals and ATP (Mariathasan *et al.*, 2006; Martinon *et al.*, 2006). Interestingly, we found that other stimuli such as chemical irritants (SDS) and physical agents (UVB) could also trigger inflammasome activation, giving further support to the hypothesis that mammals evolved a common set of sensors for various danger signals, ranging form infections to physical insults (Matzinger, 2002; Shi *et al.*, 2003). It is unclear whether the capacity to sense CS is a means of defense/alarm or whether sensitizers ''highjack'' systems that have evolved to sense other target molecules such as bacterial products. In any case, inflammasome inhibitors may have the ability to prevent inflammatory reactions owing to CS.

The importance of NALP3 and ASC in the early phase, and not in the late phase, of elicitation suggests that the inflammasome has little impact on the recruitment of primed T cells, but rather initiates pro-inflammatory signals that

promote T-cell priming. This is consistent with a recent report showing that NALP3 is essential for the sensitization phase of contact hypersensitivity to trinitrophenylchloride (Sutterwala *et al.*, 2006).

Low doses of CS are sufficient to stimulate pre-primed T cells but insufficient to elicit a CHS response (Grabbe *et al.*, 1996), suggesting that in addition to hapten-specific recognition, a pro-inflammatory stimulus that is provided locally by the contact sensitizer itself is necessary (Grabbe *et al.*, 1996). We propose that this pro-inflammatory stimulus is due to NALP3 inflammasome-dependent IL-1β production in the skin. However, other cytokines/signals are likely to modulate the early phases of CHS, as IL-1R-deficiency did not totally abrogate ear swelling after elicitation with TNCB. This may be due at least in part to the presence of IL-18, which is also activated by caspase-1 (Wang *et al.*, 2002; Kawase *et al.*, 2003).

Our data suggest that keratinocytes stimulated by CS may directly provide the pro-inflammatory stimuli required for CHS. However, several other cells such as LC, dermal dendritic cells, and mast cells were also reported to play a role in CHS (Kitagaki *et al.*, 1997; Griffiths *et al.*, 2005; Kaplan *et al.*, 2005), and inflammasome activity in these cells also be implicated. Interestingly, NOD2, which plays an essential role in inflammatory bowel disease, is expressed at low levels in normal enterocytes, but is induced by TNFα in inflammatory conditions (Rosenstiel *et al.*, 2003). We found likewise that TNFα significantly increases some of the inflammasome components in keratinocyte. This increase was neither sufficient nor essential for IL-1β activation and secretion, although it potentiated the effect of CS. Interestingly, TNFα has been demonstrated to play a crucial role in the cross-talk between LC and keratinocytes that drives LC migration (Cumberbatch *et al.*, 2003; Griffiths *et al.*, 2005). Taken together, these data suggest an active role of epithelial cells, enterocytes, and keratinocytes in the surveillance of danger signals and the activation of innate immunity signals.

Blocking of NF-κB signaling using topical decoy oligonucleotide was recently reported to diminish symptoms of atopic dermatitis in a mouse model of this disease (Dajee *et al.*, 2006). Although this effect may not be related to a decrease in pro-IL-1β levels, it suggests that targeting the inflammasome with small-molecule inhibitors (which remain to be discovered) may represent a new modality for the treatment of CHS. The characterization of the role of the inflammasome in inflammatory skin disorders must be therefore be pursued as it may eventually lead to development of new treatment modalities for inflammatory skin diseases.

MATERIALS AND METHODS
Cell culture
Primary keratinocytes (HEK, Biocoba, Basel, Switzerland) were cultured in Epilife supplemented with the provided human keratinocyte growth supplement and $60\,\mu$M of Ca^{++} and HaCaT in DMEM:F-12 (1:1) (Invitrogen, NV Leeks, The Netherlands) supplemented with $5\,\mu$g/ml human insulin, $10\,$ng/ml cholera toxin, $0.4\,\mu$g/ml hydrocortisone, $10\,$ng/ml of human epidermal growth factor, and 10% fetal calf serum. THP-1 cells were cultured in RPMI-1640 medium (Invitrogen, NV Leeks, The Netherlands) containing 2×10^{-5} M of β-mercapto-ethanol and 10% fetal calf serum. Cell cultures were maintained at 37°C in humidified incubators with 5% CO$_2$. All media contained $100\,$U/ml of penicillin and $10\,\mu$g/ml of streptomycin. Purified flagellin (activating TLR5) and MALP-2 (activating TLR6) from the TLR activating kit (Alexis, Lausen, Switzerland) were used at $2\,\mu$g/ml. TNFα (human recombinant TNFα, Alexis, Lausen, Switzerland) was used at $1\,\mu$g/ml. zVAD and zYVAD (Alexis, Lausen, Switzerland) were used at a final concentration of $20\,\mu$M each. Mouse primary keratinocyte were obtained from 2-day-old mice as described (Pirrone *et al.*, 2005). Briefly, whole skins were floated overnight in 0.25% trypsin at 4°C. After mechanical separation from the dermis, the epidermis was dissociated by 50 shakes in a 15-ml tube in $5\,$ml of medium. Keratinocytes were seeded in 50% low calcium fibroblast-conditioned medium (EMEM, Cambrex, Verviers, Belgium) and preincubated for 24 hours on dermal cells and 50% Epilife (Biocoba, Basel, Switzerland) on collagen IV-coated dishes (non-keratinocyte will not adhere/grow in these conditions) for 7 days before stimulation with TNCB or DNFB or 4,6-dinitro-1-chlorobenzene at 1–$10\,\mu$g/ml.

Reverse transcription-PCR
Total RNA extracted from fresh THP-1, HaCaT, and primary keratinocytes using TRIzol reagent (Invitrogen, LV Leeks, The Netherlands) was reverse-transcribed and amplified using OneStep RT-PCR kit (Qiagen, Basel, Switzerland) according to the manufacturer's instructions. Sequences of the oligonucleotide used for RT-PCR are provided online in Table S1.

Western blot analysis and ELISA
Cell lysates and supernatants were subjected to SDS-PAGE/Western blotting as described (Agostini *et al.*, 2004). Primary antibodies: human cleaved IL-1β (D116 epitope, Cell Signaling, Danvers), human proIL-1β (R&D Systems, Abingdon, UK), human ASC/Pycard, NALP1 and 3 (Apotech, Epalinges, Swizerland), human caspase-1 (Santa Cruz Biotechnology, LabForce AG, Nunningen, Switzerland), or human caspase-5 (MBL, LabForce AG, Nunningen, Switzerland). Secondary antibodies: horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG (Jackson immunoresearch, Milan Analytica, La Roche, Switzerland). Detection: ECL (Amersham, Zurich, Switzerland) as described in the manufacturer's instructions. ELISA: undiluted cell culture supernatants were subjected to human IL-1β detection by ELISA (BD biosciences, Basel, Switzerland) according to the manufacturer's instructions.

Analysis of CHS in IL-1R, ASC, and NALP3 mice
Mice experiments were approved by the Geneva Cantonal Veterinary Office before being conducted, according to institutional and Swiss Federal Veterinary Office guidelines. IL-1R- and ASC-deficient mice were obtained from M Kopf (Molecular Biomedicine ETH, Zurich, Switzerland) and VM Dixit (Genentech, San Francisco, CA). NALP3-deficient mice were generated as described (Martinon *et al.*, 2006). Mice (7–8 week old) ($n=5$ per group) were sensitized by external applications of $20\,\mu$l of 0.5% DNFB in acetone:olive oil (3:1, v/v) on the left ear or on their shaved belly at days 0 and 1. At day 5, sensitized mice were elicited on the ventral and dorsal side of the right ear by topical application of $10\,\mu$l of 0.3% DNFB in

acetone:olive oil. Ear thickness was measured 24 hours after elicitation with a digital gauge (Mitsutoyo, Kanagawa, Japan) on both ears. Alternatively, 6- to 7-week-old mice ($\geqslant 3$ per group) were sensitized by external applications of 20 μl of 1% TNCB in acetone on the right ear at days 0 and 7. On day 14, ear thickness was measured before and after TNCB application (20 μl of 1% TNCB in acetone) to the same ear.

Statistical analysis

Groups were compared using one-tailed Student's or Aspin–Welch's *t*-tests.

SUPPLEMENTARY MATERIAL

Table S1. Oligos used for reverse transcription–PCR detection of inflammasome components.

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