

Honey bee (*Apis mellifera*) venom induces AIM2 inflammasome activation in human keratinocytes

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Keywords

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Abstract

Background: Following allergen exposure, cytokines and other pro-inflammatory signals play an important role in the immunological cascade leading to allergic sensitization. Inflammasomes sense exogenous and endogenous danger signals and trigger IL-1 β and IL-18 activation which in turn shape Th2 responses. Honey bee venom (BV) allergies are very common; however, the local inflammatory cascade leading to the initiation of allergic sensitization is poorly understood. In this study, the local inflammatory cascades in skin after exposure to BV were investigated.

Methods: The mechanisms of inflammasome activation in human skin and in cultured keratinocytes upon BV exposure were analyzed by ELISA, Western blot, flow cytometry, siRNA techniques, and immunofluorescence.

Results: In an *ex vivo* bee sting model, BV induced IL-1 β release suggesting the activation of inflammasomes. Indeed, in cultured keratinocytes, the BV component melittin triggered IL-1 β and IL-18 release via the AIM2 inflammasome. AIM2 is a cytosolic DNA receptor, and mitochondrial as well as genomic DNA was detected in the cytosol of melittin-treated keratinocytes as triggers of inflammasome activation. As a mechanism, melittin mediated destruction of mitochondrial membranes leading to the leakage of mitochondrial DNA into the cytosolic compartment.

Conclusion: These data suggest that upon BV exposure, keratinocytes are involved in an innate immune response by the activation of the AIM2 inflammasome and subsequent IL-1 β and IL-18 release triggered by endogenous DNA. As IL-1 β and IL-18 are involved in Th2- and IgE-mediated immune reactions, these results could add to the understanding of the role of the tissue microenvironment to subsequent allergic responses.

The complex immunological cascade leading to allergic sensitization is incompletely understood. In an individual at risk, naïve CD4⁺ T cells differentiate into allergy-mediating Th2 cells if allergens are presented in MHC class II molecules by antigen-presenting cells (APCs) such as dendritic cells (DCs). Still, DCs will only initiate a T-helper response if there is some sort of adjuvant activity present at the time of allergen exposure (1). This activity can be provided by the presence of

pathogen or danger-associated molecular patterns (PAMPs, DAMPs) but also by the cytokines released from resident cells upon tissue damage (2).

Inflammasomes are frontline danger recognition systems expressed by epithelial cells in the skin (3). They recognize an array of danger signals that range from macromolecules to changes in the microenvironment (e.g. during tissue injury) (4, 5). Upon activation, a caspase-1 activator protein, for example NLRP3, the adaptor protein ASC, and caspase-1 form the multiprotein inflammasome complex. Subsequently, activated caspase-1 processes IL-1 β and IL-18 that are secreted as active cytokines (6). IL-1 β and IL-18 are key players in the pathogenesis of autoinflammatory skin diseases, but also in allergic reactions in predisposed individuals. As an example, the dust mite allergen triggers IL-1 β secretion through the

Abbreviations

NHEK, normal human epidermal keratinocytes; BV, bee venom; PLA2, phospholipase A2; DAMPs, danger-associated molecular patterns; PAMPs, pathogen-associated molecular patterns; mtDNA, mitochondrial DNA; gDNA, genomic DNA; i.c., intracutaneous; AIM2, absent in melanoma 2.

NLRP3 inflammasome in keratinocytes and thus potentially augments atopic skin inflammation (7, 8). Pro-inflammatory IL-1 β directs Langerhans cell migration and antigen presentation and is involved in T-cell priming (9). Also, IL-1 β is required for Th2 cell activation, Th2 cytokine production and epicutaneous sensitization in mice, while IL-18 increases IgE serum levels (10, 11). Furthermore, the ability of the prototypical Th2 adjuvant alum to boost DCs to induce Th2 responses is associated with the production of bioactive IL-1 β (12, 13). Therefore, inflammasome activation, Th2 polarization, and allergic sensitization seem to be linked. Consequently, mice deficient in inflammasome activity have a defect in alum-induced IL-1 β secretion and fail to mount Th2-mediated inflammation (14).

Honey bee (*Apis mellifera*) venom allergies affect approximately 3% of the general population (15). Bee venom (BV) consists of various proteins and enzymes with potential pro-inflammatory activity. While phospholipase A2 (PLA2; 10–12% of protein content in BV) is presumed to be the major allergen in BV, the cytotoxic effects are mainly attributed to the lytic activity of melittin (50–60%). Further biologically active components are apamin (1–3%), a neurotoxin, mast-cell-degranulating peptide (1–3%), and hyaluronidase (1–3%), a secondary allergen (16).

During a bee sting, BV is injected into the epidermis and it is most likely that epidermal keratinocytes as the major resident cells in skin are involved in the initial BV-mediated inflammatory response. Keratinocytes play a pivotal role in the defense against microbial infections and in sterile inflammation triggered by endogenous ligands by sensing danger signals, activation of inflammasomes, and secretion of IL-1 β (17). Importantly, keratinocyte-derived IL-1 β is then involved in further T-cell-mediated responses (18).

In this study, we investigated the innate immune response triggered by *Apis mellifera* venom in the skin and in primary keratinocytes. As keratinocyte-derived IL-1 β and IL-18 might be critical for allergic sensitization, inflammasome activation and cytokine secretion triggered by BV were characterized.

Materials and methods

Ex vivo bee sting model

4-mm full-thickness punch biopsies obtained from healthy skin were injected i.c. with Reless® BV (100 μ g; ALK Abello, Wedel, Germany; diluted in 100 μ l dest. H₂O) or an equal volume of dest. H₂O. IL-1 β secretion into the culture medium was determined by ELISA. All sample acquisitions were approved by the committee on investigations involving human subjects at the LMU Munich.

Cell culture and stimulation

Normal human epidermal keratinocytes (NHEK) were cultured as described previously (17). Cells were stimulated with BV (1–100 μ g/ml), melittin (0.5–5 μ g/ml), apamin (1 μ g/ml), PLA2 (1 μ g/ml, all: Sigma Aldrich, Seelze, Germany, diluted in dest.

H₂O), Lipofectamine™ RNAiMAX (Invitrogen, Darmstadt, Germany) or DNase I (1 U/ μ g; Fermentas, St. Leon-Rot, Germany).

Isolation of mitochondrial and genomic DNA

Mitochondrial DNA (mtDNA) was isolated with the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) and the Mitochondria Isolation Kit (Pierce, ThermoFischer Scientific, Rockford, IL, USA); genomic DNA (gDNA) was isolated with the Quick-gDNA™ MiniPrep Kit (Zymo Research, Irvine, CA, USA). Isolated mtDNA and gDNA were verified by PCR using COX3 primers and primers specific for a 300-bp fragment of gDNA (MWG Biotech, Ebersberg, Germany).

Isolation of cytosolic fractions

Cytosolic fractions were isolated using the Mitochondria Isolation and Nuclear and Cytoplasmic Extraction Kit (Pierce). DNA in the isolated cytosol was purified with Qiaquick PCR Purification Kit (Qiagen).

Immunoblotting

Gel electrophoresis and immunoblotting were performed as described (17). Membranes were stained with antibodies detecting caspase-1 p20 or β -actin (Cell Signaling, Danvers, MA, USA) overnight and with respective HRP-conjugated secondary antibodies (Dako, Hamburg, Germany).

Immunofluorescence

Normal human epidermal keratinocytes were labeled with Mitotracker® (Invitrogen), mounted in ProLong Anti-Fade (Invitrogen), and analyzed with a TissueFAXS microscope (TissueGnostics, Vienna, Austria).

DNA and RNAi transfection

siRNAs and DNA transfections were performed as described earlier (17). gDNA, mtDNA, 'cytosolic' DNA, and poly(dA:dT) (Sigma) were transfected at 2.5 ng/ml–4 μ g/ml as indicated.

IL-1 β ELISA

Interleukin-1 β release was determined by ELISA in cell-free supernatants of NHEK (R&D Systems, Minneapolis, MN, USA).

Flow cytometry

Normal human epidermal keratinocytes treated with melittin and stained with Mitotracker® were analyzed in a FACSCanto with FACSDiva Software (BD Bioscience, Heidelberg, Germany).

Statistical analysis

All statistical analyses were performed using GRAPHPAD PRISM 4.0 (GraphPad Software Inc., San Diego, CA, USA).

Student's *t*-test was used to calculate statistical differences. Values of $P < 0.05$ were considered significant, and all data are displayed as means $\pm$ SD.

Results

BV activates an inflammasome in human keratinocytes

To investigate *Apis mellifera* venom-induced inflammasome activation, a bee sting was simulated in an *ex vivo* model. During a bee sting, 50–140 μ g of venom is injected into the skin (19, 20). Therefore, 100 μ g BV was injected i.c. into skin biopsies. This resulted in increased secretion of IL-1 β into the culture medium, which suggested inflammasome activity (Fig. 1A). IL-1 β release was concentration dependent, while the control showed only minor effects possibly attributed to sterile inflammation following mechanical injury (Fig. 1A and Fig. S1A) (21).

Subsequently, primary human keratinocytes (NHEK) were exposed to BV, which resulted in a dose-dependent IL-1 β secretion again indicating inflammasome activation (Fig. 1B). Pro-IL-1 β mRNA transcription was not affected (Fig. S1B). Additionally, caspase-1 p20 subunit was detected in the supernatants of keratinocytes exposed to BV (Fig. 1C).

When testing the different BV components, only melittin mediated IL-1 β secretion from NHEK, while PLA2 and apamin had no effect (Fig. 1D). Additionally, BV and melittin induced the secretion of active IL-18, which – similar to IL-1 β – is dependent on inflammasome activity (Fig. S1C).

Bee venom has cytotoxic effects in different cell types (22). As a consequence, BV- and melittin-treated keratinocytes had diminished metabolic activity demonstrated by reduced WST-1 substrate turnover as compared to controls (Fig. S1D). In addition, cellular viability was reduced and apoptosis was induced, while IL-1 β release was increased at the same time (Fig. S1D,E).

Interestingly, IL-1 β release from keratinocytes was not limited to BV as wasp venom (WV) also induced IL-1 β release (Fig. S2A). However, while BV mediated a strong IL-1 β release after 6 h, WV mediated a much weaker IL-1 β release that was not detectable before 24 h incubation.

The AIM2 inflammasome mediates IL-1 β secretion induced by BV and melittin

To investigate whether BV-mediated IL-1 β release in keratinocytes was dependent on inflammasome activation or simply unspecific release associated with cell damage,

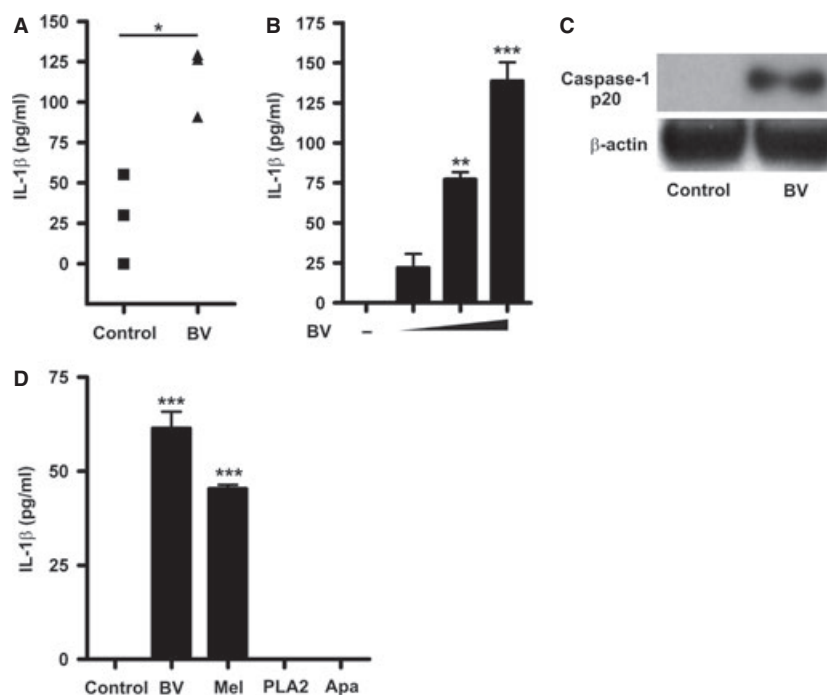


Figure 1 The bee venom component melittin mediates inflammasome activation in human keratinocytes. (A) 100 μ g bee venom (BV) or an equal volume of sterile water was injected i. c. into skin punch biopsies from healthy donors. After 6 h, IL-1 β secretion into the culture medium was determined by ELISA ($*P < 0.05$; Student's *t*-test, $n = 3$). (B) IL-1 β secretion of primary normal human epidermal keratinocytes (NHEK) exposed to BV (1, 2.5, 5 μ g/ml) for 24 h. (C) Western blot of active caspase-1

p20 from supernatants of NHEK treated with 2.5 μ g/ml BV for 24 h. β -Actin from lysates of corresponding cells confirmed equal cell density. (D) IL-1 β release of NHEK treated for 6 h with whole BV (2.5 μ g/ml), melittin (Mel, 5 μ g/ml), phospholipase A2 (PLA2, 1 μ g/ml), and apamin (Apa, 1 μ g/ml). Values in (B) and (D) represent one of three independent experiments with similar results performed in triplicates (means $\pm$ SD $**P < 0.01$; $***P < 0.001$, Student's *t*-test).

the role of distinct inflammasome components was investigated. NHEK were transfected with caspase-1-siRNA to block general inflammasome activity or with NLRP3- or AIM2-siRNA to block distinct skin-associated inflammasomes (23) and stimulated with BV. IL-1 β secretion of keratinocytes exposed to BV or melittin was dependent on caspase-1 and AIM2, but not on NLRP3 (Fig. 2A–C). In contrast, unspecific membrane lysis showed negligible effects (Fig. S2B). Nevertheless, AIM2 blockade did not affect the cytotoxic effects of BV and melittin (data not shown). Thus, BV and melittin induced IL-1 β release in keratinocytes by the activation of the AIM2 inflammasome independent of their cytotoxic effects.

Mitochondrial and genomic DNA activate the AIM2 inflammasome

Recently, double-stranded DNA located in the cytosol was identified as the trigger for AIM2-dependent inflammasome formation (24). As BV and melittin mediated AIM2 inflammasome activation in NHEK, we hypothesized that intracellular DNA leaking from the nucleus or from mitochondria through BV-induced pores gains access to the cytosol and activates AIM2. To initially test whether mitochondrial DNA (mtDNA) is capable of inflammasome activation, isolated mtDNA from untreated keratinocytes was transfected into NHEK. mtDNA induced IL-1 β release in a concentration-dependent manner, while DNase pretreatment blocked mtDNA-mediated inflammasome activation (Fig. 3A). siRNA blockade confirmed that mtDNA-mediated IL-1 β release was dependent on caspase-1 and AIM2, but not on NLRP3 expression (Fig. 3B).

Genomic DNA (gDNA) leaking from the nucleus into the cytosol might possibly be another danger signal that could activate AIM2. Therefore, isolated gDNA was transfected into NHEK in which NLRP3 and AIM2 expression was blocked by siRNA. Similarly, IL-1 β release mediated by gDNA was dependent on AIM2 inflammasome activity in keratinocytes (Fig. 3C).

Melittin mediates the leakage of endogenous DNA into the cytosol which induces IL-1 β release in keratinocytes

These data suggested that endogenous DNA could activate the AIM2 inflammasome if it gains access to the cytosol. As melittin is known to interact with cellular membranes (25) and might harm their integrity, we next investigated whether leaking self-DNA could be responsible for melittin-induced AIM2 inflammasome activity in keratinocytes.

Cytosolic fractions from melittin-treated keratinocytes were isolated and used as PCR templates for the detection of mitochondrial (mtDNA) and genomic DNA (gDNA) fragments. A specific cytosolic signal was observed in PCR for mtDNA and gDNA, while no signal was observed when cytosol from untreated keratinocytes was analyzed (Fig. 4A, B). This suggested that melittin triggered the appearance of mtDNA and gDNA in the cytosol of NHEK.

Next, we tested whether the DNA present in the cytosol of melittin-exposed NHEK (designated 'cytosolic' DNA) is sufficient to induce IL-1 β release in keratinocytes. Therefore, 'cytosolic' DNA was isolated from melittin-treated NHEK and transfected into fresh keratinocytes which resulted in a strong IL-1 β release (Fig. 4C). Again, IL-1 β secretion was dependent on AIM2 inflammasome activation as shown by siRNA blockade (Fig. 4D). These results suggested that in melittin-exposed keratinocytes, endogenous mtDNA and gDNA are present in the cytosol which is sensed by the AIM2 inflammasome as a danger signal.

Melittin disintegrates mitochondrial membranes

To eventually test this, NHEK were stained with Mitotracker[®], which accumulates in active mitochondria with stable membrane potential. In contrast to untreated keratinocytes that showed bright mitochondrial staining, melittin-exposed NHEK displayed reduced staining intensity (Fig. 5A). This was confirmed in flow cytometry indicating a decreased number of active mitochondria (Fig. 5B). At the same time, inflammasome activation resulted in the release of IL-1 β (Fig. 5B).

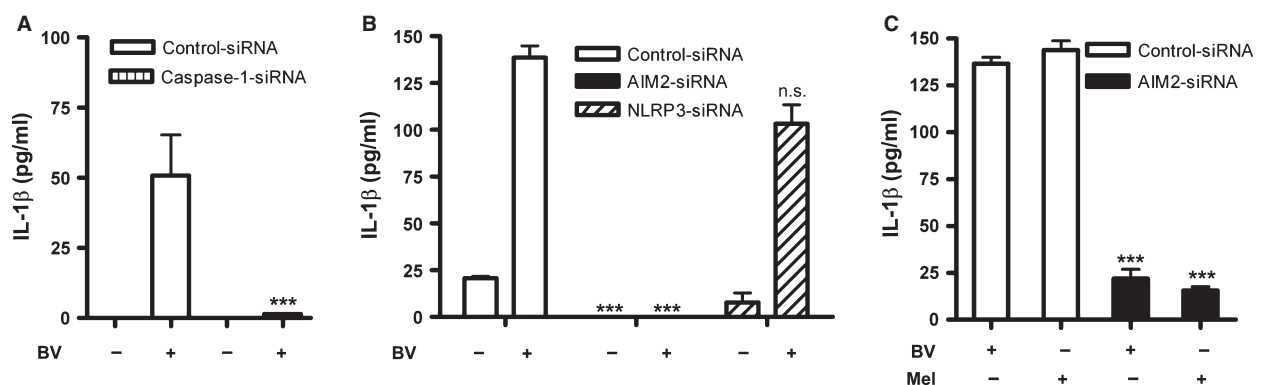


Figure 2 Bee venom and melittin mediate IL-1 β release in keratinocytes through the AIM2 inflammasome. Caspase-1 (A), NLRP3 (B), or AIM2 (B, C) was downregulated by siRNA in NHEK before stimulation with either whole BV (2.5 μ g/ml) (A, B, C) or

melittin (5 μ g/ml) (C) for 6 h. IL-1 β release was determined by ELISA. Values in A–C represent one of at least three independent experiments with similar results performed in triplicates (mean $\pm$ SD, *** P < 0.001, Student's t -test).

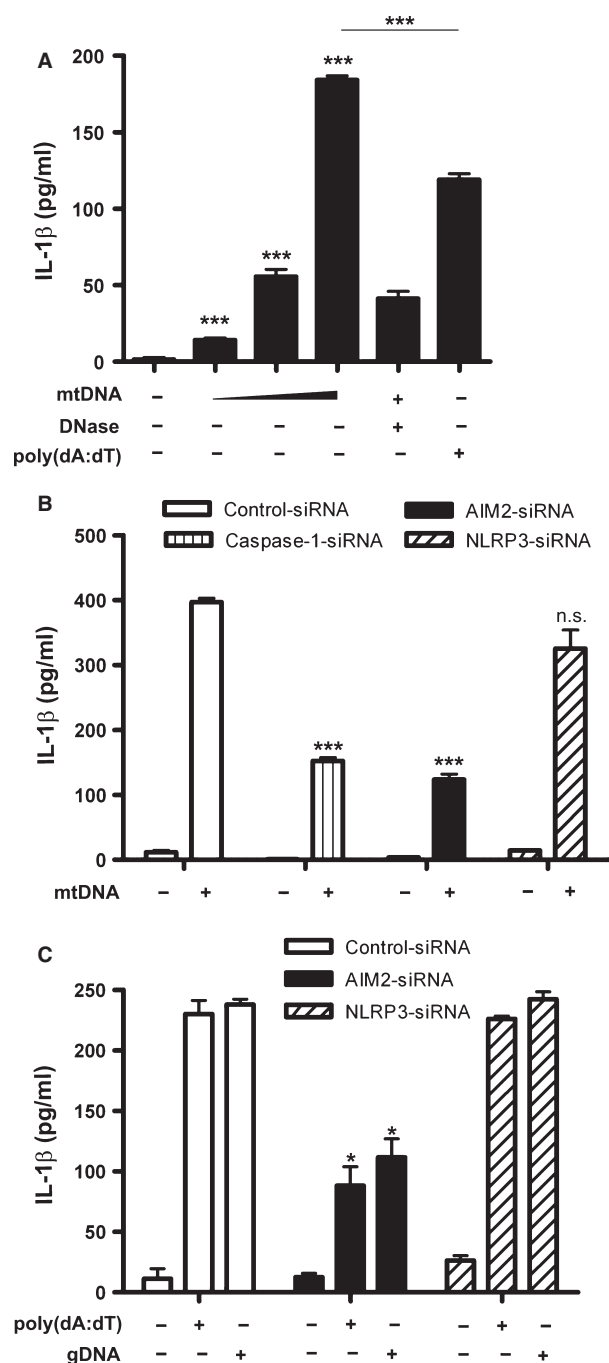


Figure 3 Mitochondrial and genomic DNA mediate AIM2-dependent inflammasome activation and IL-1 β release in primary keratinocytes. (A) IL-1 β secretion from NHEK transfected with poly(dA:dT) (250 ng/ml) or mitochondrial DNA (mtDNA; 2.5, 25, 250 ng/ml) after 24 h; DNase (1 U/ μ g DNA) was added to 250 ng/ml mtDNA in a control setting before transfection. mtDNA (B), poly(dA:dT), or genomic (g)DNA (C) (all: 250 ng/ml) were transfected into keratinocytes after downregulation of caspase-1, NLRP3, or AIM2 by siRNA. IL-1 β secretion was determined after 24 h. Values in A–C represent one experiment of at least three independent experiments with similar results performed in triplicate (mean $\pm$ SD; *** P < 0.001, Student's t -test).

Discussion

Activation of inflammasomes leads to the maturation of the IL-1 family members IL-1 β and IL-18 through caspase-1. Resident cells in the skin such as keratinocytes secrete IL-1 β and IL-18, which in turn activate innate and adaptive immune cells (26). In particular, both IL-1 β and IL-18 are involved in Th2 responses. IL-18 mediates the activation of IL-4, IL-13, and IgE production (11, 27). Consequently, caspase-1 transgenic mice show increased serum levels of IL-18 and IgE (28). IL-1 β propagates allergic priming by the activation of APCs and – together with TNF α and IL-18 – mediates the migration of Langerhans cells from the epidermis to draining lymph nodes in response to allergens (29, 30). Together with observations that inflammasome activation and IL-1 β /IL-18 secretion are involved in Th2 responses *in vivo*, these data suggest that both cytokines could play a role in the pathogenesis of allergic reactions (13, 14). As inflammasome activity is mainly necessary at the initiation of Th2 responses, the identification of those mechanisms might lead to novel therapies (31).

In the skin, barrier-forming keratinocytes are the first cells to sense and respond to danger signals. In order to do so, they are equipped with various danger recognition systems such as the NLRP3 and AIM2 inflammasomes (17, 24, 32, 33): NLRP3 senses various danger signals that are involved in allergic sensitization and/or Th2 responses such as dust mite allergen or the adjuvant alum (7, 12). AIM2 activation can be triggered not only by microbial or viral cytosolic dsDNA (34) but also by endogenous DNA if it gains access to the cytosol (17).

In this study, exposure of human keratinocytes to BV resulted in the unexpected activation of the AIM2 inflammasome and secretion of IL-1 β and IL-18. As a mechanism, the BV component melittin mediated the release of DNA from mitochondria and/or the nucleus into the cytosol of keratinocytes. There, endogenous DNA was recognized as a danger signal by AIM2 which triggered the formation of an inflammasome complex resulting in the secretion of pro-inflammatory IL-1 β and IL-18. Melittin is known to interact with cellular membranes, and melittin-induced pores have an approximate diameter of 20 Å (35), which could allow DNA fragments to passively diffuse into the cytosol. Indeed, melittin treatment resulted in disintegration of mitochondrial membranes, prompted the detection of mtDNA in the cytosol, and triggered AIM2 inflammasome activation.

Nevertheless, treatment with BV or melittin also exhibited cytotoxic effects on keratinocytes. Untreated primary human keratinocytes store large quantities of the proform pro-IL-1 β , which could have been released as a consequence of the cytotoxic activity of melittin. Still, inhibition of caspase-1 and AIM2 by siRNA completely blocked IL-1 β release induced by melittin, suggesting that indeed active inflammasome formation was required. Also, detergent-mediated lysis of keratinocyte membranes in control experiments resulted in only slight IL-1 β release. Still, inhibition of AIM2 did not block the cytotoxic effect of melittin, which suggests that AIM2 inflammasome formation with subsequent IL-1 β /IL-18 activation and cytotoxic effects occur independently but coincidentally.

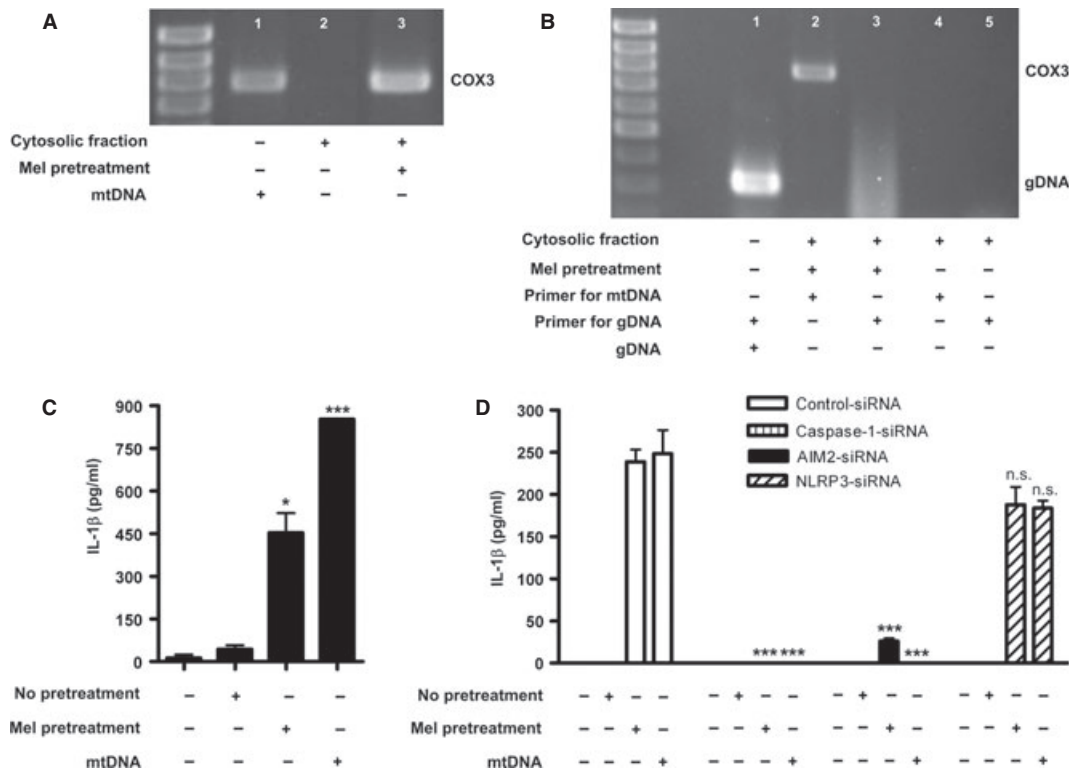


Figure 4 Melittin mediates the activation of the AIM2 inflammasome by release of mtDNA and gDNA into the cytosol of keratinocytes. Cytosolic fractions from untreated NHEK or from NHEK treated with melittin (5 μ g/ml) for 6 h were isolated. (A) 5 μ l of the cytosolic fractions was used as template in a COX3-specific PCR (lane 1 = mtDNA-template control, lane 2 = cytosol/untreated cells, lane 3 = cytosol/melittin-treated cells). (B) Cytosolic fractions were used as templates in a COX3- (lanes 2, 4) or gDNA-specific PCR (lanes 3, 5) (lane 1 = gDNA template control) ($n = 3$). (C) DNA was isolated from cytosolic fractions from melittin-treated NHEK. DNA

isolated from cytosolic fractions from NHEK without melittin pretreatment served as controls. Isolated cytosolic DNA was transfected into keratinocytes (control: transfected mtDNA). After 24 h, IL-1 β secretion was determined by ELISA. (D) Cytosolic DNA isolated from melittin-treated NHEK was transfected into fresh NHEK after caspase-1-, NLRP3-, or AIM2-siRNA knockdown (control: transfected mtDNA). After 24 h, IL-1 β secretion was determined. Values in C and D are from one representative of at least three independent experiments with similar results performed in triplicate (mean $\pm$ SD; * $P < 0.05$, *** $P < 0.001$, Student's t -test).

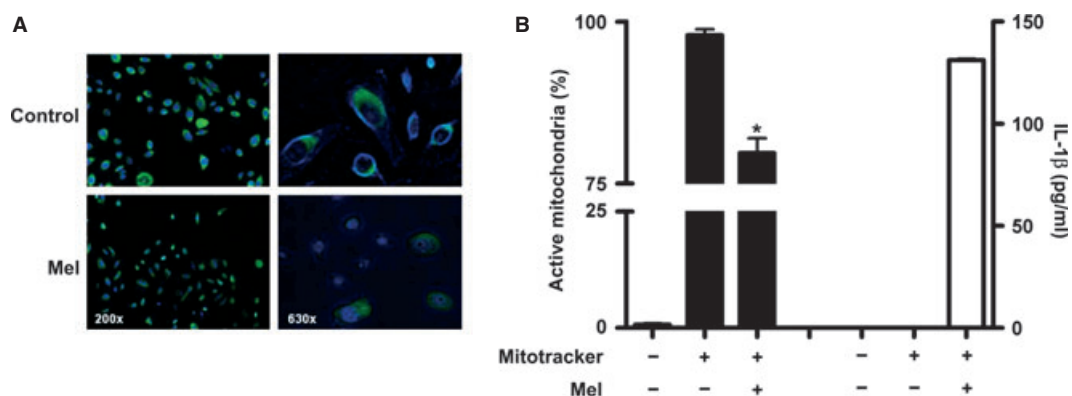


Figure 5 Melittin-induced mitochondrial disintegration is paralleled by inflammasome activation. (A) NHEK were treated with 5 μ g/ml melittin for 6 h before mitochondria were stained with 200 nM Mitotracker[®] (Invitrogen). Cells were analyzed by TissueFAX[®] ($n = 3$ –

5). (B) NHEK were treated as described in (A) and analyzed by flow cytometry for Mitotracker[®]-positive cells. IL-1 β secretion from these cells was determined in supernatants (* $P < 0.05$, *** $P < 0.001$; Student's t -test).

Noteworthy, inflammasome activation in keratinocytes was not restricted to BV as wasp venom (WV) also induced IL-1 β release. However, WV-mediated IL-1 β release was weaker and appeared later, suggesting a different mechanism.

This study provides the first evidence that venom from *Apis mellifera* triggers AIM2 inflammasome activation in human keratinocytes resulting in IL-1 β and IL-18 release. *In vivo*, both cytokines might enhance the local inflammatory response in skin following a bee sting. Moreover, this local inflammation mediated by IL-1 β and IL-18 might possibly be relevant for the initial steps of allergic sensitization toward BV in predisposed individuals. Eventually, understanding the mechanisms of activation and the role of the tissue cytokine microenvironment in allergic reactions could lead to novel approaches in the prevention of or treatment for allergic disease.

Authors' Contributions

YD designed the study, performed the experiments, analyzed the data, and wrote the manuscript; MP designed the study and performed the experiments; SK, NK, VS, and MR performed the experiments; TR revised the manuscript; JS designed the study, analyzed the data, and wrote the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Data S1. Methods.

Figure S1. (A) One, ten or hundred microgram BV dissolved in sterile water were injected i.c. into punch biopsies from healthy skin obtained from volunteers. Biopsies were cultured for 6 h and IL-1 β concentration in the medium was determined by ELISA (** $P < 0.01$; Mann–Whitney test, $n = 3$).

Figure S2. (A) Normal human epidermal keratinocytes were exposed to wasp venom (WV; 0, 2.5, 5, 10 μ g/ml), BV (2.5 μ g/ml) or were treated with 5% RIPA lysis buffer – an unspecific lysis control (B) for 6 and 24 h before IL-1 β secretion was determined by ELISA.

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Supporting Information

Honey bee (*Apis mellifera*) venom induces

AIM2 inflammasome activation in human keratinocytes

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Supplementary Methods

mRNA extraction and quantification

mRNA was extracted and transcript abundance was measured by quantitative
real-time RT-PCR using specific primers as described previously (18).

IL-18 ELISA

Supernatants of NHEK were analyzed for release of IL-18 by ELISA according
to the manufacturer's protocol (IL-18 ELISA, MBL).

Cytotoxicity and cell metabolism assay

NHEK treated with BV or melittin were washed with PBS and stained with
crystal violet for 20 min. Living cells attached to the surface stain violet while dead or
dying cells detach. After washing with H₂O and removal of excess crystal violet cells
were dried for 24 h. Crystal violet was resolved with 0.1 M sodiumcitrate/50 % EtOH
and OD_{570nm} was determined. In another setting, WST-1 was added to BV or melittin
treated NHEK in a 1/10 dilution and WST-1 conversion was analyzed 1 h later in a
photometer at OD_{450nm}. In a further experimental setting, BV exposed keratinocytes

were stained with Annexin-V-FITC and propidium iodide (BD Bioscience). Cells were detached and washed three times with PBS. Samples were measured in a FACSCanto and analyzed with the corresponding FACSDiva Software (BD Bioscience).

Wasp venom stimulation and lysis control

NHEK were stimulated with wasp venom (WV) (1-10 µg/ml, ALK Abello, resolved in dest. water) for 6 - 24 h and IL-1β secretion was determined by ELISA. As a lysis control 5% RIPA buffer (10 mM Tris-Cl, 1 mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS, 140 mM NaCl, 1 mM PMSF) was added to NHEK for 6 - 24 h before further analyses.

Supplementary Figure Legend

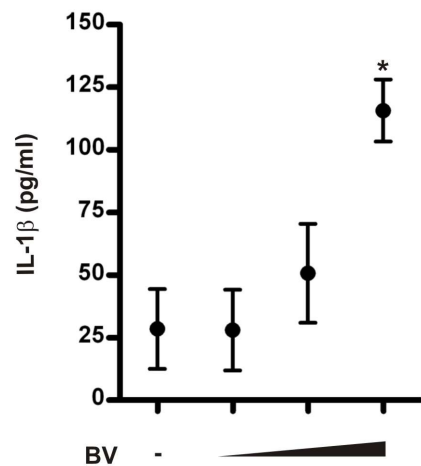
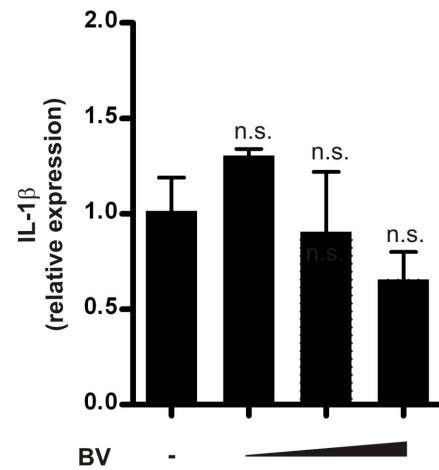
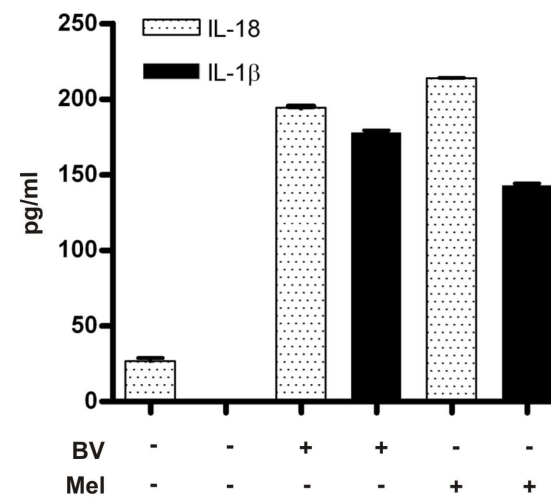
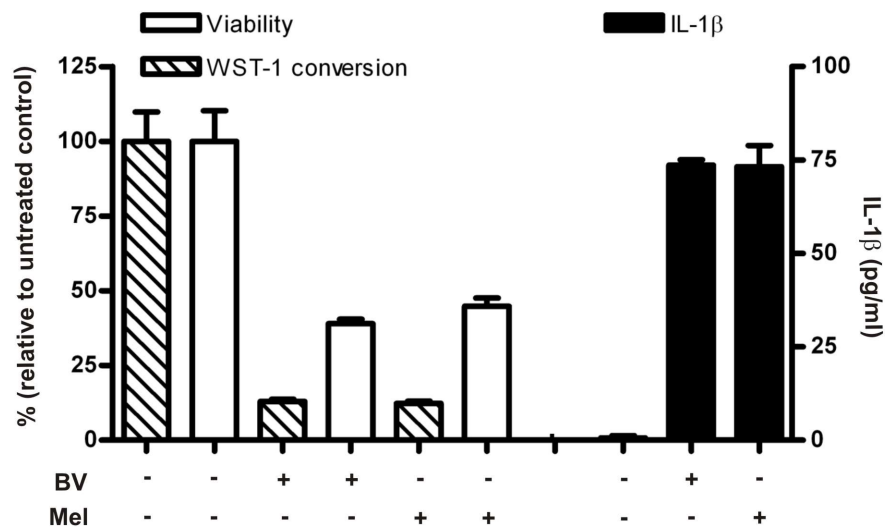
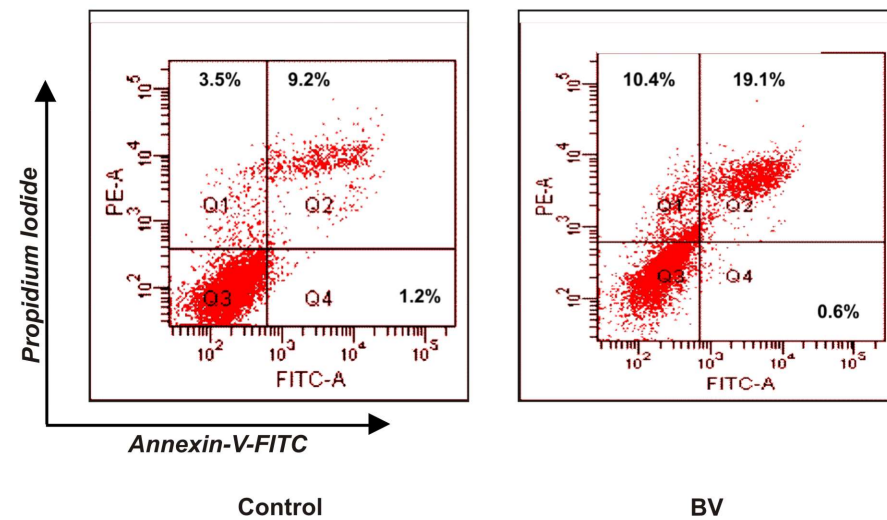
Supplementary Figure 1

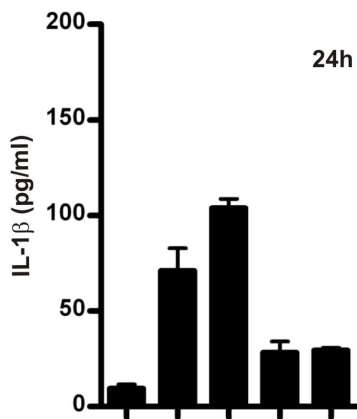
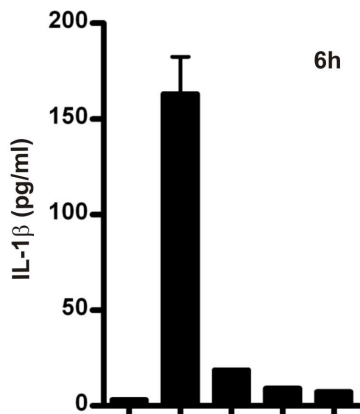
(A) 1, 10 or 100 µg BV dissolved in sterile water were injected i.c. into punch biopsies from healthy skin obtained from volunteers. Biopsies were cultured for 6 h and IL-1β concentration in the medium was determined by ELISA (**p < 0.01; Mann-Whitney test, n=3). (B) NHEK were treated with BV (0, 0.5, 1, 2.5 µg/ml) for 24 h before mRNA was isolated. Pro-IL-1β expression was determined by real-time qPCR. Values are representative of at least three independent experiments performed in triplicates (mean ± SD; n.s. not significant). (C) NHEK were treated with BV (2.5 µg/ml) or melittin (5 µg/ml) for 6 h before IL-1β and IL-18 secretion was determined by ELISA. Data shown are from one representative of at least three independent experiments performed in triplicate (mean ± SD) (D) NHEK were treated with whole BV (2.5 µg/ml) or melittin (5 µg/ml) for 6 h. IL-1β secretion was analyzed by ELISA and cells were stained with crystal violet. Cytotoxicity was determined according to

the staining intensity and calculated relative to untreated keratinocytes. Additionally, NHEK treated with BV (2.5 µg/ml) or melittin (5 µg/ml) for 6 h were incubated for 1 h with WST-1 reagent. WST-1 conversion was determined relative to untreated keratinocytes. One representative example of each experiment is displayed (n=3). (E) NHEK were exposed to BV (2.5 µg/ml) for 6 h, stained with Annexin-V-FITC and propidium iodide and analyzed by flow cytometry (one representative staining of n=3 is displayed).

Supplementary Figure 2

(A) NHEK were exposed to wasp venom (WV; 0, 2.5, 5, 10 µg/ml), BV (2.5 µg/ml) or were treated with 5% RIPA lysis buffer – an unspecific lysis control (B) for 6 and 24 h before IL-1β secretion was determined by ELISA. Values represent one of at least three independent experiments with similar results performed in triplicates.

A**B****C****D****E**

A

BV - + - - -
WV - - 

B