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Report

The Inflammasome Mediates UVB-Induced Activation and Secretion of Interleukin-1β by Keratinocytes

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Summary

It has long been known that human keratinocytes are a potent source of the proinflammatory cytokines proIL-1α and -1β [1], which are activated and released in response to UV irradiation [2]. However, the intracellular pathways, which regulate maturation and secretion of IL-1 in keratinocytes, are unknown. Here we show that the UVB-mediated enhancement of cytoplasmic Ca^{2+} is required for activation of the IL-1β-converting enzyme caspase-1 by the inflammasome, a multiprotein innate immune complex [3, 4]. Caspase-1 in turn activates proIL-1β, and keratinocytes secrete the cytokine as well as inflammasome components. These results demonstrate the presence of a proIL-1β-processing inflammasome in nonprofessional immune cells and the necessity of inflammasome components for the UVB-induced secretion of IL-1β. This supports the concept that keratinocytes are important immunocompetent cells under physiological and pathological conditions [5].

Results and Discussion

Keratinocytes Express and Secrete Inflammasome Components and Proinflammatory Cytokines

Recently, we identified the estrogen-responsive B box protein (EBBP, TRIM16) as a novel proIL-1β-binding protein in macrophages. Most importantly, EBBP enhanced IL-1β secretion [6]. EBBP also bound to different inflammasome proteins, indicating that it associates with this complex [6]. Interestingly, a similar function of EBBP/TRIM16 was found in keratinocytes. EBBP is highly expressed in these cells [7], and siRNA-mediated knockdown of EBBP reduced IL-1β secretion after UVB irradiation [6]. This finding suggested that keratinocytes express other inflammasome proteins that are responsible for proIL-1β maturation and its subsequent secretion. The latter occurs via a poorly characterized pathway that is independent of the endoplasmic reticulum/Golgi apparatus [8, 9].

We used exponentially growing, confluent, or differentiated human primary keratinocytes to study the expression of inflammasome components. In inflammasomes, oligomeric NOD-like receptors such as NALP1, NALP3, or IPAF act as stress sensors [10]. Assembly of the complexes, activation of caspase-1, processing of proIL-1β and proIL-18, and finally secretion of the active cytokines occurs after stimulation with certain bacterial compounds and various molecules released by injured cells [4]. Indeed, several components of three types of inflammasomes were expressed in keratinocytes and also in differentiated and activated monocytes/macrophages (Figure S1A in the Supplemental Data available online): IPAF, the NALP1 inflammasome components NALP1, Asc, caspases-1, and -5, as well as the NALP3 inflammasome components NALP3, Asc, and caspase-1. Although mRNA levels of NALP3 were low in exponentially growing keratinocytes, TPA strongly enhanced its expression (data not shown). These results are consistent with recent data, which also show expression of inflammasome components in cultured keratinocytes [11].

In accordance with the mRNA data, caspase-1, Asc, EBBP, and NALP1 proteins were present in the lysates of keratinocytes and U937 cells (Figure S1B). Although they lack a signal peptide, caspase-1 and EBBP were secreted from keratinocytes in a differentiation-specific manner. NALP3 was almost undetectable in keratinocytes and monocytes (data not shown). Expression and secretion of the lysosomal protease cathepsin D, which is secreted together with caspase-1 from human primary macrophages, was analyzed as a control [9]. It is unlikely that the proteins detected in the supernatant are unspecifically released because of cell lysis, because the lactate dehydrogenase (LDH) activity in the supernatant, which served as a control for cell lysis and unspecific release, was less than 4% of the total extracellular and intracellular activity (data not shown).

We also found constitutive expression of proIL-1α, -1β, and -18 mRNAs and proteins in human keratinocytes, although secretion was not observed under normal culture conditions, and activation of proIL-1β was also hardly detectable (Figure S1). Expression of EBBP, caspase-1, proIL-1α, and -1β as well as Asc was confirmed in normal human epidermis via immunofluorescence (Figure S2), indicating that inflammasomes are present in the skin in vivo.

UVB Irradiation of Keratinocytes Induces Secretion of IL-1 and Inflammasome Proteins

To determine the activity of inflammasomes in human primary keratinocytes, we used UVB irradiation, which is known to induce secretion of IL-1β [2, 12, 13]. Irradiation was performed with 50 mJ/cm^2 UVB, which is within the physiological range. Already 4 hr after irradiation, considerable amounts of IL-1β were released (Figure 1A). After 24 hr, all IL-1β and more than half of the

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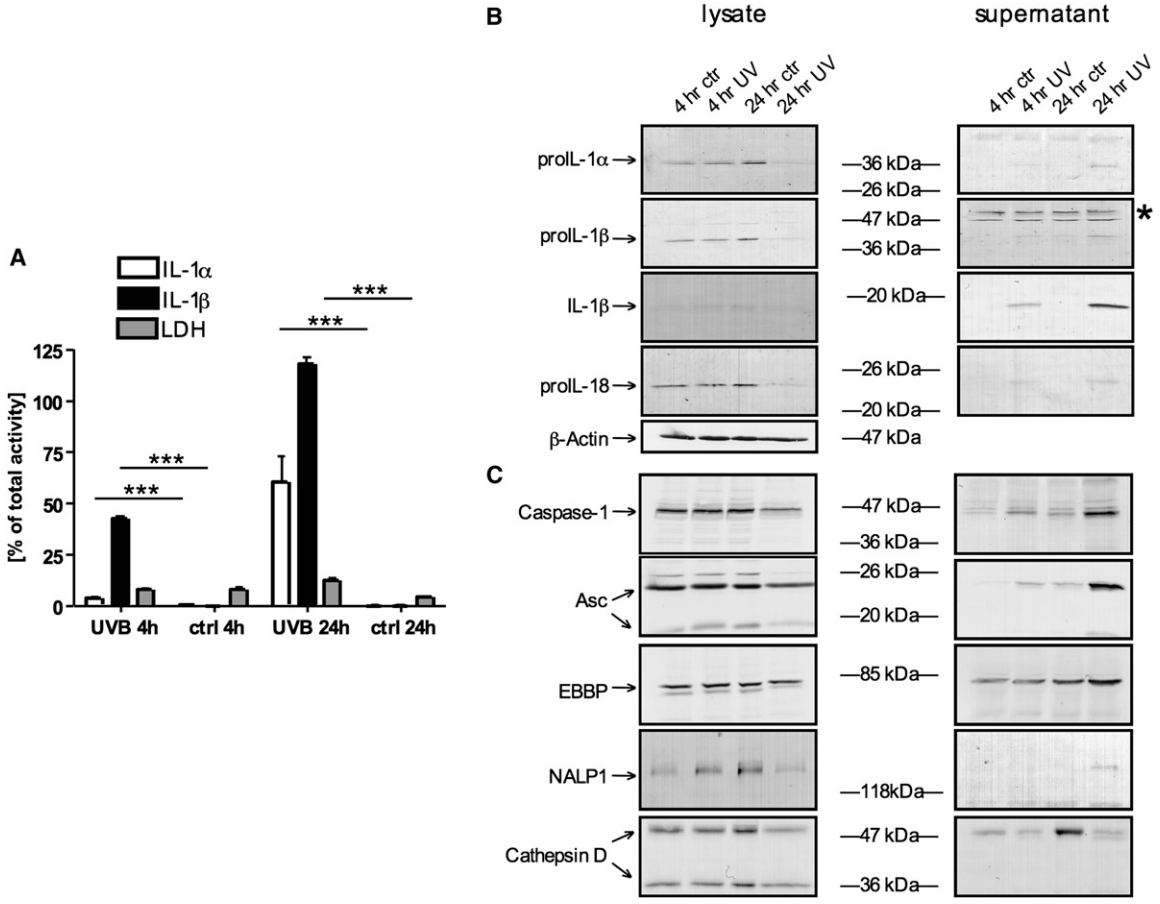

Figure 1. UVB Irradiation Induces Secretion of IL-1 and Inflammasome Proteins

HFKs were irradiated with 50 mJ/cm^2 UVB without (UVB) or with (ctrl) covering as described [6, 24]. Cells and supernatants were harvested 4 and 24 hr after treatment.

(A) ELISAs for IL-1α and -1β were performed as described (R&D Systems, Minneapolis, MN), and the amount of LDH activity was determined in the supernatants (kit from Promega, Madison, WI). Values were normalized to those of the supernatant of cells lysed by the addition of Triton X-100. Bars represent mean ± SD, n = 3, ***p < 0.001 by one-tailed unpaired t test. Absolute values of IL-1α and -1β 24 hr after irradiation are 332 and 253 pg/ml supernatant of 1.5 × 10^5 cells.

(B and C) Western blots with 20 μg protein lysate or concentrate of 300 μl supernatant are shown. Unspecific bands are labeled with asterisks.

available proIL-1α was released. The amount of secreted cytokines is comparable to the one described for activated macrophages [4]. In contrast, LDH levels were only slightly increased. Western blot analysis of the supernatants confirmed that keratinocytes also activate proIL-1β in response to UVB irradiation (Figure 1B). ProIL-1α and -18 were also secreted from keratinocytes, but processing of these cytokines could not be detected. As a control, we verified that the antibody recognizes caspase-1-cleaved IL-18 (Figure S4). This finding is consistent with previous data showing that keratinocytes cannot activate proIL-18 [14]. In contrast, caspase-1 activation in immune cells always results in activation of both proIL-1β and -18 [4].

To our surprise, UVB-induced secretion was also observed for inflammasome proteins, but not for the lysosomal protein cathepsin D (Figure 1C). Levels of caspase-1, Asc, EBBP/TRIM16, and NALP1 were increased in the supernatant of UVB-irradiated cells, particularly after 24 hr. It had previously been shown that caspase-1, Asc, and EBBP can be secreted by macrophages or transfected cells [3, 6, 15], but secretion of NALP1 had not been demonstrated yet. Interestingly, binding of Asc to caspase-1 had only been shown in the supernatant of activated macrophages but not inside the cell [15], suggesting that activation and secretion are coupled or that secretion occurs rapidly after activation. This hypothesis is consistent with our results showing that not only Asc but also other inflammasome proteins are secreted together with the activated inflammasome substrate IL-1β.

Caspase-1 Regulates UVB-Induced ProIL-1β Maturation in Keratinocytes

The proIL-1β cleavage suggests that keratinocytes can activate caspase-1, although controversial findings have been reported regarding this capacity [16, 17]. To further address this question, keratinocytes were treated before irradiation with the broad-range caspase inhibitor VAD or with the more specific caspase-1 inhibitor YVAD. Both inhibitors completely abolished IL-1β secretion after UVB treatment (Figure 2A). The UVB-mediated secretion of IL-1β was not due to induction of gene expression, which had been described after UV irradiation

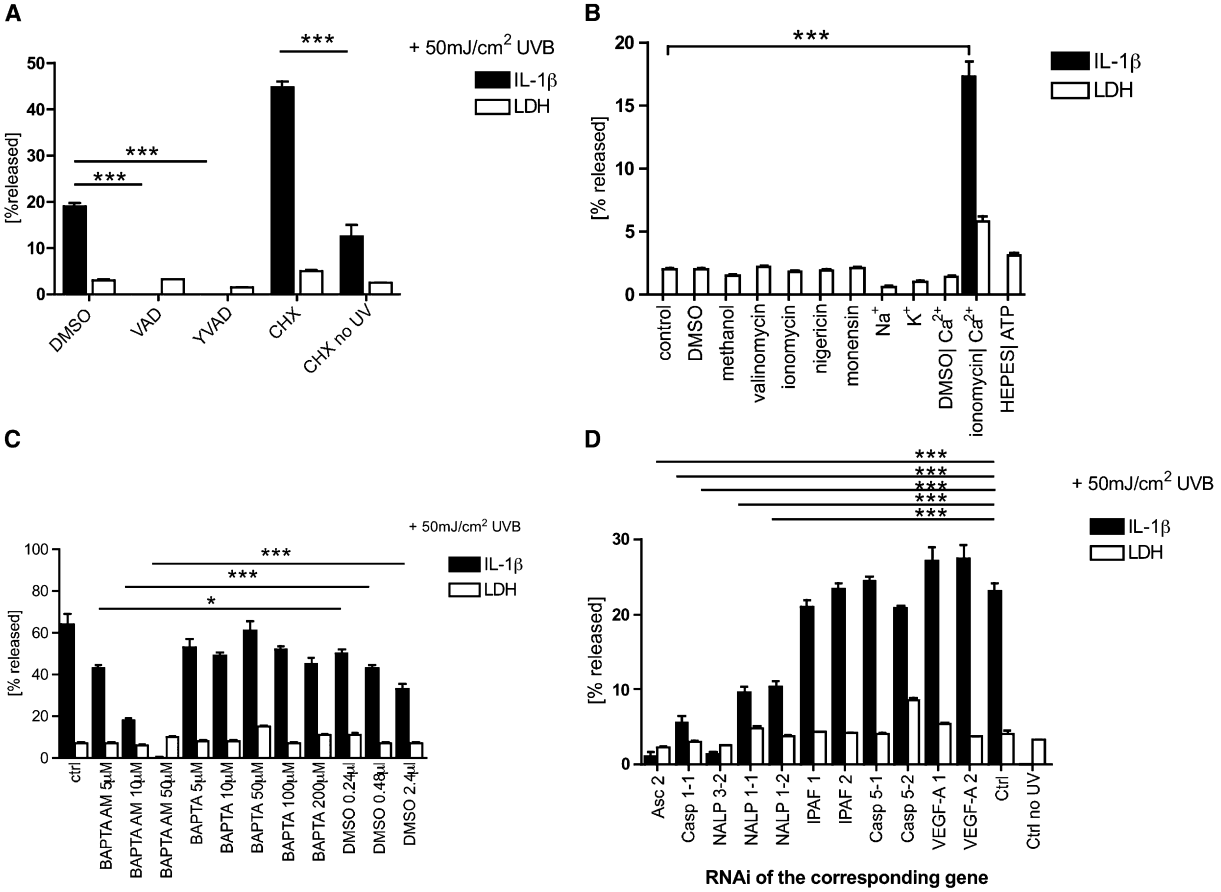

Figure 2. Active Caspase-1, Intracellular Ca^{2+}, and Inflammasome Components Are Required for UVB-Induced IL-1β Secretion

(A) Keratinocytes were treated with the broad spectrum caspase inhibitor Z-VAD-FMK (VAD) (5 μM), the caspase-1 inhibitor Ac-YVAD-chloromethylketone (YVAD) (5 μM), cycloheximide (CHX, 5 nM), or DMSO (solvent) and subsequently irradiated with UVB. IL-1β ELISA and LDH activity assay were performed with the supernatant 4 hr after irradiation. Representative results of four independent experiments are shown. For normalization, see legend to Figure 1. Bars represent mean ± SD, n = 3, ***p < 0.001 by one-tailed unpaired t test.

(B) Keratinocytes were treated for 2 hr with valinomycin (10 μM), ionomycin (5 μM), nigericin (10 μM), monensin (2.5 μM), Na^+ medium (for induction of K^+ efflux) [18], K^+ medium (control) [18], Ca^{2+} (1.5 mM), or HEPES (50 mM)/ATP (1 mM) as indicated. LDH activity and IL-1β levels in the supernatant were determined and normalized as described in the legend to Figure 1.

(C) Keratinocytes were treated with different concentrations of BAPTA-AM (cell permeable), BAPTA (not permeable), or DMSO (control for BAPTA-AM, BAPTA is water-soluble) as indicated and irradiated with UVB. LDH activity and IL-1β levels in the supernatant were determined 4 hr after irradiation. Bars represent mean ± SD, n = 3, ***p < 0.001 by one-tailed unpaired t test. Representative results of three independent experiments are shown.

(D) Keratinocytes were transfected with siRNAs for inflammasome proteins, a scrambled siRNA (control), or siRNA against the unrelated VEGF-A (Supplemental Experimental Procedures). 24 hr after transfection, keratinocytes were irradiated, and 4 hr later, IL-1β and LDH activity were measured in the supernatant and normalized as described in the legend to Figure 1. Bars represent mean ± SD, n = 3, ***p < 0.001 by one-tailed unpaired t test. Representative results of three independent experiments are shown.

[12, 13], because a 1 hr pretreatment of keratinocytes with the protein synthesis inhibitor cycloheximide did not reduce IL-1β secretion upon UVB irradiation but rather enhanced it (Figure 2A). Cycloheximide treatment alone also induced IL-1β secretion. This may indicate the existence of a caspase-1 inhibitor with a short half-life, which can no longer be produced in the presence of cycloheximide, or an enhancement of intracellular Ca^{2+} by cycloheximide (see below). We also detected cleaved caspase-1 in the supernatant of UVB-irradiated cells, but neither in the cell lysate nor in the supernatant of control cells or irradiated and YVAD-treated cells (data not shown). To examine whether a secreted factor is responsible for the activation of caspase-1 after UVB irradiation, we incubated keratinocytes with conditioned medium of UVB-irradiated cells. However, this treatment did not result in secretion of IL-1β, demonstrating that caspase-1 is not activated by a secreted factor but rather via an intracellular mechanism (Figure S3).

ProIL-1β Maturation Is Triggered by an Increase in Free Cytoplasmic Ca^{2+}

Exogenous ATP is a trigger for IL-1β secretion by macrophages. This treatment results in an efflux of K^+ followed by an influx of Ca^{2+} [9]. Toxin-induced K^+ efflux also triggers the assembly of the inflammasome in CHO or HeLa cells, but an increase in intracellular Ca^{2+} is not required in this model [18]. Therefore, we analyzed whether a potassium or calcium ionophore, ATP, or low-potassium medium, which induces K^+ efflux, can induce IL-1β secretion (Figure 2B). Surprisingly, we could detect IL-1β secretion only after addition of the calcium ionophore

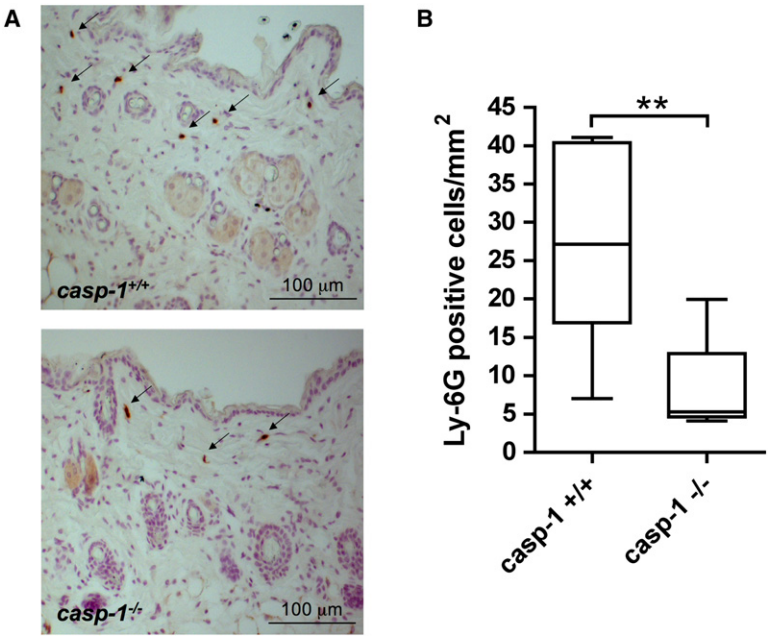

Figure 3. Caspase-1 Enhances UVB-Induced Skin Inflammation in Murine Skin

Caspase-1 knockout mice and wild-type littermates were shaved and irradiated with 75 mJ/cm^2 UVB as described [24]. 1 day after irradiation, sections from irradiated skin were stained with an antibody against the neutrophil marker Ly-6G and an alkaline phosphatase-coupled secondary antibody.

(A) Sections were counterstained with hematoxylin/eosin. Arrows indicate Ly-6G-positive cells.

(B) The number of Ly-6G-positive cells/mm^2 dermis was counted from n = 6 mice of each genotype. **p = 0.0022 by one-tailed Mann-Whitney test. In untreated skin, the number of Ly-6G-positive cells was about 2/mm^2 dermis (not shown).

ionomycin in the presence of additional Ca^{2+} (1.5 mM) in the medium. Keratinocytes grow only in low Ca^{2+} medium (90 μM); higher concentrations induce terminal differentiation. Interestingly, UVB irradiation also causes a rise in intracellular Ca^{2+} [19]. To determine whether this rise is required for inflammasome activation, we irradiated keratinocytes in the presence of the calcium chelator BAPTA. Even at high concentrations of BAPTA (200 μM), secretion of IL-1β was not significantly reduced (Figure 2C). However, in the presence of low concentrations (10–50 μM) of BAPTA-AM, which can enter the cell, IL-1β secretion was reduced or even abolished in a concentration-dependent manner (Figure 2C). These experiments demonstrate a crucial role of intracellular Ca^{2+} in the UVB-induced secretion of IL-1β, most likely via activation of caspase-1. It may well be that the sudden increase in intracellular Ca^{2+} that occurs upon UV irradiation serves as a danger signal for keratinocytes and triggers an inflammatory response. The fact that an extracellular chelator cannot inhibit IL-1β secretion suggests that UVB increases cytoplasmic Ca^{2+} through release from intracellular stores and that Ca^{2+} acts intracellularly, resulting in proIL-1β maturation. Therefore, caspase-1 seems to be generally activated through changes in intracellular ion concentrations. However, whether K^+ (CHO, HeLa) or Ca^{2+} (keratinocytes) or both (macrophages) are involved depends on the cell type ([9, 18], this study).

UV-Induced IL-1β Secretion Is Inflammasome Dependent

Finally, we determined whether the inflammasome is responsible for IL-1β secretion from keratinocytes. Although it was recently published that cultured murine keratinocytes express IL-1β protein [11], other reports argue against this possibility [20, 21]. Furthermore, we could detect IL-1α but not IL-1β in the supernatant and in the cell lysate of UVB-irradiated primary mouse keratinocytes by using ELISAs specific for the murine proteins (data not shown). Therefore, we could not use keratinocytes from knockout mice to study the role of different inflammasome components in proIL-1β maturation and secretion. To overcome this problem, we used siRNA to test the involvement of inflammasome proteins in UVB-induced caspase-1 activation and subsequent IL-1β secretion from human keratinocytes. We knocked down the components of three different inflammasome complexes. Knockdown of vascular endothelial growth factor A, which does not act on keratinocytes, as well as scrambled siRNA served as controls. The knockdown of all genes was verified at the mRNA level (Figure S5A) and also at the protein level for caspase-1, Asc, and NALP1 (Figure S5B). Knockdown of Asc, NALP3, or caspase-1 drastically reduced the amount of secreted IL-1β after UVB irradiation (Figure 2D). siRNA-mediated knockdown of IPAF, which is a sensor for bacterial flagellin [4], had no significant influence. This was also true for caspase-5, a component of the NALP1 inflammasome [3]. In contrast, a reduction in NALP1 expression itself clearly reduced IL-1β secretion. These results suggest that the NALP3 inflammasome (NALP3, Asc, caspase-1), which is activated by different bacterial substances and by ATP in macrophages [4, 22, 23], is predominantly involved in UVB-induced IL-1β secretion by human keratinocytes. However, NALP1 also contributes to this effect.

Caspase-1 Enhances UVB-Mediated Skin Inflammation In Vivo

To determine the in vivo relevance of our findings, caspase-1 knockout mice and wild-type littermates were irradiated with a physiological dose of UVB [24]. 24 hr later, the number of neutrophils in the irradiated skin was determined by staining of skin sections with an antibody against Ly6G. Interestingly, neutrophil infiltration was strongly reduced in the absence of caspase-1, demonstrating that this enzyme is important for UVB-induced skin inflammation (Figure 3). Because UVB is

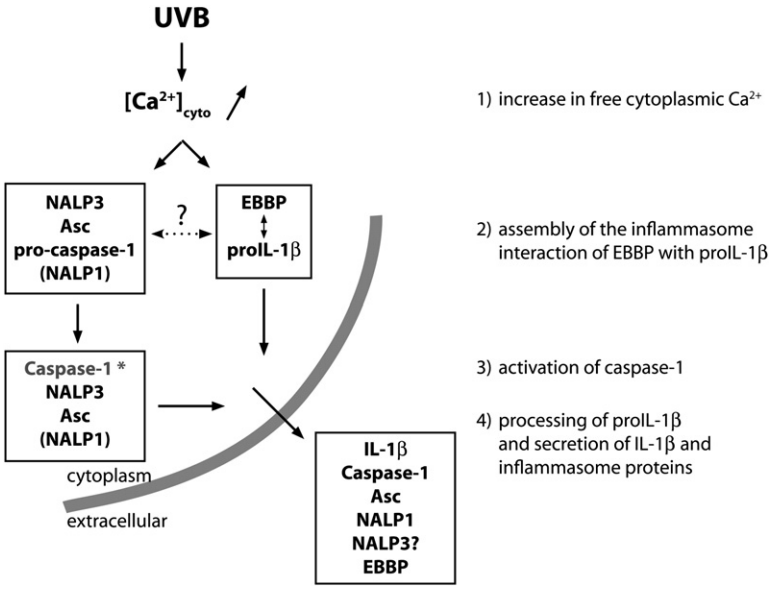

Figure 4. Mechanism of UVB-Induced ProIL-1β Maturation

UVB irradiation of human keratinocytes results in an increase in free cytoplasmic Ca^{2+}. This may trigger the assembly of the NALP3 inflammasome, but involvement of NALP1 was also demonstrated. EBBP/TRIM16 interacts with proIL-1β and possibly presents it to the inflammasome through interaction with these proteins [6]. Active caspase-1 cleaves proIL-1β. Caspase-1, Asc, EBBP, NALP1, and perhaps also NALP3 are secreted independent of the ER/Golgi together with IL-1β. In vivo, IL-1β secretion induces inflammation.

predominantly absorbed by cells in the epidermis, and because keratinocytes are the major cell type in this compartment of the skin, the different inflammatory response is most likely due to the lack of caspase-1 in keratinocytes. Secretion of IL-1α by keratinocytes is probably affected, because this cytokine is abundant in murine keratinocytes [21] and is secreted from murine macrophages in an inflammasome-dependent manner [23]. However, we cannot fully exclude the possibility that the lack of caspase-1 in Langerhans cells or even in cells of the dermis is responsible for the phenotype. To distinguish between these possibilities, it will be essential to generate conditional caspase-1 knockout mice that specifically lack this enzyme in keratinocytes.

Conclusions and Perspectives

In this study we show that human keratinocytes constitutively express inflammasome proteins together with proIL-1α, -1β, and -18. UVB irradiation enhances intracellular free Ca^{2+}, resulting in the activation of the NALP3 inflammasome (Figure 4). Active caspase-1 then processes proIL-1β, whereas proIL-18 is not or is only inefficiently cleaved. These findings demonstrate that the inflammasome-dependent activation and secretion of proIL-1β is not restricted to macrophages.

The activation and secretion of IL-1 by keratinocytes in vitro raises the possibility of a direct involvement of this cell type in different pathologic situations, which are characterized by IL-1-dependent inflammation in the skin. One example is sunburn (Figure 3), and another may be Muckle-Wells Syndrome, an autosomal-dominant fever syndrome with skin involvement, which results from activating mutations in the *NALP3* gene [4]. Therefore, it will be interesting to identify other factors that activate the inflammasome in keratinocytes and thus induce secretion of IL-1β. These studies will not only shed light on the mechanisms underlying innate immunity in the skin but also provide the basis for the development of novel treatments of inflammatory skin diseases.

Supplemental Data

Five figures and Experimental Procedures are available at http://www.current-biology.com/cgi/content/full/17/13/1140/DC1/.

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