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Cytosolic DNA Triggers Inflammasome Activation in Keratinocytes in Psoriatic Lesions

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Abstract

The proinflammatory cytokine interleukin-1β (IL-1β) plays a central role in the pathogenesis and the course of inflammatory skin diseases, including psoriasis. Posttranscriptional activation of IL-1β is mediated by inflammasomes; however, the mechanisms triggering IL-1β processing remain unknown. Recently, cytosolic DNA has been identified as a danger signal that activates inflammasomes containing the DNA sensor AIM2. In this study, we detected abundant cytosolic DNA and increased *AIM2* expression in keratinocytes in psoriatic lesions but not in healthy skin. In cultured keratinocytes, interferon-γ induced *AIM2*, and cytosolic DNA triggered the release of IL-1β via the AIM2 inflammasome. Moreover, the antimicrobial cathelicidin peptide LL-37, which can interact with DNA in psoriatic skin, neutralized cytosolic DNA in keratinocytes and blocked AIM2 inflammasome activation. Together, these data suggest that cytosolic DNA is an important disease-associated molecular pattern that can trigger AIM2 inflammasome and IL-1β activation in psoriasis. Furthermore, cathelicidin LL-37 interfered with DNA-sensing inflammasomes, which thereby suggests an anti-inflammatory function for this peptide. Thus, our data reveal a link between the AIM2 inflammasome, cathelicidin LL-37, and autoinflammation in psoriasis, providing new potential targets for the treatment of this chronic skin disease.

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INTRODUCTION

Psoriasis is a chronic inflammatory skin disease of unknown etiology that affects ~2% of the general population (1). The cytokine interleukin-1β (IL-1β) has been identified as a key player in the pathogenesis and course of cutaneous inflammation in psoriasis (1). In particular, the IL-23–triggered development of T helper 17 (T_H17) cells, which are pathogenic in psoriasis, depends on the presence of IL-1β (2). Furthermore, IL-1β enhances T_H17 cell maturation and cytokine production (3, 4). Constitutive IL-1β activation or the lack of the IL-1 receptor antagonist in mice leads to a T_H17 cytokine profile in skin and an inflammatory phenotype resembling human psoriasis (5, 6). Moreover, excess IL-1 signaling causes psoriasis-like skin disease in mice even in the absence of T cells (7).

In humans, the transcriptional changes in psoriatic skin lesions resemble IL-1β–induced changes in skin cells, which further implicates IL-1β in cutaneous inflammation (8). Among other cells, keratinocytes produce IL-1β, and amounts of IL-1β are elevated in lesional psoriatic skin (1, 9). In inflamed skin, keratinocytes are a main source of IL-1β, and IL-1β derived from these cells fosters further T cell–dependent cutaneous inflammation (10). These observations have prompted physicians to target IL-1β as a therapy for psoriasis and psoriasis arthritis. Although larger clinical studies have not been reported to date, therapies targeting IL-1β show beneficial effects in patients suffering from severe psoriasis in smaller trials (11).

The biologic activity of IL-1β is regulated on both the transcriptional and posttranscriptional level. Proinflammatory signals such as tumor necrosis factor–α (TNF-α) induce transcription of IL-1β through activation of nuclear factor κB (NF-κB) (12). On the posttranscriptional level, IL-1β is activated by proteolytic processing of its inactive proform; defects in the posttranscriptional control of IL-1β are sufficient to cause an inflammatory skin disease (5). Proteolytic cleavage of IL-1β is regulated by inflammasomes, which are immune complexes that are formed upon recognition of certain molecular patterns. Inflammasome formation results in caspase-1 activation; caspase-1 then cleaves IL-1β into its active and secreted form (13). Several pathogen-, danger-, and disease-associated molecular patterns that trigger the formation of inflammasomes have been discovered (13).

Recently, cytosolic DNA has been identified as a danger signal that leads to the activation of inflammasomes containing the DNA sensor AIM2 (14–17). Physiologically, intracellular DNA is present in the nucleus or mitochondria but not in the cytosol. Extracellular DNA is normally cleared via deoxyribonuclease (DNase)–mediated degradation but can act as a proinflammatory signal in psoriasis (18). In particular, extracellular self-DNA aggregates with the antimicrobial cathelicidin peptide LL-37, and self-DNA/LL-37 complexes trigger the activation of plasmacytoid dendritic cells (pDCs) (18). LL-37 is highly expressed in lesional skin in psoriasis, and LL-37/self-DNA–triggered inflammatory responses have been implicated in the course of cutaneous inflammation (18).

In psoriasis, IL-1β plays a central role and inflammasome activation has been demonstrated (9). However, the disease-associated molecular patterns that trigger inflammasome formation in psoriasis are not known. Here, we investigated self-DNA as a trigger for AIM2 inflammasome activation in psoriasis. In addition, we studied the role of the cathelicidin peptide LL-37 in DNA-triggered inflammation.

RESULTS

IL-1β is activated in psoriatic lesions

The cytokine micromilieu in inflamed skin in psoriasis is composed of several proinflammatory mediators (1). Among these, IL-1β serves as an important proinflammatory signal. *IL-1*β mRNA was significantly increased in biopsies taken from lesional skin (Fig. 1A). Subsequent proteolytic activation of the IL-1β peptide is mediated by caspase-1, and, consequently, caspase-1 activation was also increased in psoriatic lesions but not in nonlesional skin (Fig. 1B) (9). Because sterile injury, such as biopsy sampling, results in inflammasome activation (19), active caspase-1 could already be detected in biopsies from noninflamed skin (Fig. 1B). Nevertheless, in lesional skin in psoriasis, we observed increased activation of caspase-1 and higher levels of cleaved IL-1β in psoriatic lesions, confirming enhanced inflammasome activity (Fig. 1B).

Keratinocytes in psoriatic lesions produce elevated amounts of AIM2

We hypothesized that in epidermal psoriatic keratinocytes, cytosolic DNA activates the AIM2 inflammasome, leading to IL-1β activation. Indeed, significantly increased amounts of *AIM2* mRNA were detected in lesional skin from psoriasis patients compared to healthy donors (Fig. 1C). Also, in psoriasis patients, *AIM2* mRNA levels were significantly higher in lesional skin compared to nonlesional skin (Fig. 1D). In histological sections, TissueFAXS analyses confirmed a more than 50-fold increase in AIM2 protein in psoriatic skin lesions compared to healthy tissue, with the strongest staining in the apical keratinocyte layers (Fig. 1E). To identify the factors that up-regulate *AIM2* in psoriasis, we tested proinflammatory cytokines that are typically elevated in psoriasis. Interferon-γ (IFN-γ), which induces genes of the *HIN200* family such as *AIM2*, increased the expression of *AIM2* in primary keratinocytes, whereas TNF-α, IL-17A, IL-6, IL-9, IL-21, or IL-22 showed no effect (Fig. 1F and fig. S1) (20, 21). In addition, induction of cell differentiation sensitized keratinocytes to IFN-γ–induced *AIM2* expression (fig. S2). Consequently, *IFN*-γ and *AIM2* expression correlated in lesionalskininpsoriasis (Fig. 1G, $R^2 = 0.75$).

Cytosolic DNA induces the release of IL-1β in keratinocytes via the AIM2 inflammasome

To test whether cytosolic DNA activates IL-1β release via an AIM2-dependent inflammasome, we studied primary human epidermal keratinocytes. Stimulation of keratinocytes with TNF-α or IFN-γ or transfection of DNA [polydeoxyadenylic acid–polydeoxythymidylic acid double-stranded homopolymer, poly(dA:dT)] did not increase IL-1β secretion. In contrast, when keratinocytes were primed with IFN-γ and TNF-α, transfection of DNA increased IL-1β release by four times (Fig. 2A). DNase treatment before stimulation blocked IL-1β activation, confirming DNA as the trigger (Fig. 2B). Shorter DNA fragments induced stronger IL-1β release from keratinocytes than longer fragments, and isolated genomic DNA containing a mixture of different DNA lengths induced the strongest response (fig. S3). In addition, cytokine-primed keratinocytes secreted higher amounts of active caspase-1 subunits (p20) in response to cytosolic DNA, which confirmed inflammasome activation (Fig. 2C). To test whether AIM2 is involved in this DNA-dependent IL-1β release in keratinocytes, we targeted *AIM2* by RNA interference (RNAi). Two different small interfering RNAs (siRNAs) efficiently down-regulated *AIM2* expression (Fig. 2D). In addition, induction of *AIM2* by IFN-γ was blocked by siRNA-mediated knockdown (fig. S4). Knockdown of *AIM2* completely inhibited IL-1β release in response to poly(dA:dT), indicating a crucial role of the AIM2 inflammasome in the response of keratinocytes to cytosolic DNA (Fig. 2E). Notably, AIM2-dependent IL-1β activation required IFN-γ priming, which increased *AIM2* expression (Fig. 1F), and priming with TNF-α, which increased expression of *pro–IL-1*β in keratinocytes similar to IL-17A (fig. S5). These results suggest that the specific proinflammatory cytokine

microenvironment present in lesional skin in psoriasis provides essential elements for AIM2-dependent inflammasome activation in keratinocytes.

Cytosolic DNA is present in keratinocytes in psoriasis lesions in vivo

Activation of the AIM2 inflammasome further requires the presence of DNA in the cytosol as a trigger. To detect cytosolic DNA in psoriatic skin, we stained paraffin sections using terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick end labeling (TUNEL). DNA fragments were detectable in the cytosol of keratinocytes in lesional skin of all psoriasis patients tested (three of three patients), but not in sections from healthy skin (Fig. 3, A and B) or from skin in atopic dermatitis (AD) (fig. S6). More than 80% of cells stained positive for cytosolic DNA in lesional skin in psoriasis, whereas in healthy skin, no cytosolic DNA fragments were detected (Fig. 3C). The high number of positive staining keratinocytes suggests that cytosolic DNA is a major disease-associated molecular pattern that promotes inflammation in psoriasis.

The antimicrobial cathelicidin peptide LL-37 reduces AIM2-dependent release of IL-1β by binding to cytosolic DNA

The presence of DNA in the cytosol of keratinocytes in psoriatic lesions was unexpected, and the mechanisms underlying this observation are unknown. Experimental barrier disruption by superficial skin injury could induce the presence of cytosolic DNA in epidermal keratinocytes in healthy skin (fig. S7). Also, there is evidence that the cationic antimicrobial peptide cathelicidin LL-37, which is increased in inflamed skin in psoriasis (Fig. 3D) (18, 22), can promote cellular uptake of DNA (18, 23). Confirming these earlier results, the mature cathelicidin LL-37 peptide could be detected in lesional skin (fig. S8). To analyze the role of LL-37 in the AIM2-dependent IL-1β response, we first examined whether LL-37 promotes DNA delivery into keratinocytes. Indeed, when biotin-labeled DNA was applied to keratinocytes together with LL-37, both were detected in the cytosolic compartment (Fig. 3E). These data suggested that LL-37 could serve as a proinflammatory factor in psoriasis by promoting uptake of self-DNA into keratinocytes, leading to increased IL-1β production. Therefore, we tested whether LL-37–mediated DNA uptake contributes to cutaneous inflammation and leads to inflammasome formation. Surprisingly, when DNA was delivered together with LL-37, only low levels of IL-1β release were observed (Fig. 3F). These data suggested that LL-37 is able to deliver DNA into keratinocytes, but LL-37–delivered cytosolic DNA does not activate the AIM2 inflammasome.

LL-37 binds to DNA in the cytosol and inhibits AIM2 inflammasome formation

These data suggested that LL-37 may function as a physiologic inhibitor of DNA-dependent inflammasome activation. This was unexpected because LL-37 has been identified as a proinflammatory signal in psoriasis recently: In pDCs, LL-37 complexed with self-DNA from dying cells initiates an inflammatory cascade through Toll-like receptor 9 (TLR9) activation and subsequent IFN production (18). However, although required for TLR9 signaling, such aggregates may not be recognized by AIM2. To test this hypothesis, we analyzed the effect of DNA complexed to cationic liposomes, which is protected from LL-37–mediated aggregate formation until endosomal release. Again, the addition of LL-37 diminished secretion of IL-1β, which indicates an inhibitory activity within the cell (Fig. 4A). Inhibition of IL-1β secretion was not caused by diminished DNA delivery into keratinocytes; there was no difference when DNA was incubated with LL-37 before the formation of the liposomal complex or when liposomal complexed DNA was applied together with LL-37 (Fig. 4A). Furthermore, the reduction of IL-1β release in the presence of LL-37 was not due to different amounts of delivered (cytosolic) DNA: As shown in Fig. 4B, the number of cells taking up fluorescein isothiocyanate (FITC)–labeled DNA was

similar after treatment with LL-37 (1 to 10 μg/ml; with or without liposomal transfection) or after liposomal transfection without LL-37.

Next, we tested whether LL-37 blocks the DNA-triggered formation of AIM2 inflammasomes. The assembly of the AIM2 inflammasome requires the adaptor protein ASC, which forms large complexes after its activation (24). Consequently, cyan fluorescent protein (CFP)–ASC–overexpressing macrophages form brightly fluorescent clusters of CFP-ASC in the cytoplasm in response to DNA (Fig. 4C). In contrast, the presence of intracellular cathelicidin LL-37 or its mouse homolog CRAMP blocked DNA-triggered formation of ASC-containing inflammasome complexes in these cells (Fig. 4C and fig. S9). To track intracellular localization of the LL-37 peptide, we treated cells with DNA in the presence of rhodamine-labeled LL-37. After this treatment, LL-37 was visible in the cytosol of these cells (Fig. 4C, right column).

These observations suggest that LL-37 interferes with the assembly of DNA-triggered inflammasomes. The D enantiomer of LL-37 (dLL-37) showed a similar inhibitory effect on IL-1β release. Therefore, it is conceivable that blocking IL-1β activation with either LL-37 peptide is due to their cationic charge and subsequent binding to negatively charged DNA (Fig. 4D). Also, smaller cathelicidin peptide fragments showed similar blocking activity, whereas other antimicrobial peptides involved in psoriasis pathogenesis such as S100A7 and S100A15 did not (fig. S10). In addition, cathelicidin LL-37 significantly inhibited IL-1β release induced by cytosolic DNA with different lengths (fig. S11).

Next, we investigated whether LL-37 interacts with DNA in the cytosol. Confocal microscopy of keratinocytes treated with rhodamine-labeled LL-37 and FITC-labeled DNA demonstrated colocalization of DNA and LL-37 in the cytosol (Fig. 4E). In further experiments, biotinylated DNA was applied to keratinocytes together with LL-37, and cytosolic extracts were prepared. Biotinylated DNA in cytosolic fractions was captured by streptavidin-coated magnetic beads. LL-37 was present in the captured fraction, which demonstrates that LL-37 directly interacts with DNA in the cytosol (Fig. 4F). Notably, transfer of LL-37 to the cytosol of keratinocytes was independent of cell surface receptors such as P2X7, which had been shown to interact with LL-37 earlier (fig. S12) (25).

Together, these data suggest that LL-37 is a physiologic inhibitor of AIM2 inflammasome formation in keratinocytes. LL-37 binds to DNA within cells, which may disable AIM2-mediated DNA recognition. Subsequently, DNA-dependent inflammasome formation is abrogated and IL-1β release is blocked.

DISCUSSION

Here, we observed elevated expression of the cytosolic DNA sensor AIM2 and increased inflammasome activity in psoriasis. Cytosolic DNA was identified as a trigger for AIM2 activation in keratinocytes. Because current studies directly link inflammasome activation and increased IL-1β production with T_H17 responses, these observations might contribute to the current understanding of the pathogenesis of psoriasis. Furthermore, we observed that the antimicrobial cathelicidin peptide LL-37 interferes with AIM2 activation by interacting with cytosolic DNA in keratinocytes. Because cathelicidin expression in human skin is under the control of the vitamin D pathway, these observations could possibly be translated into novel therapies for this chronic skin disease.

There is strong evidence that the IL-1β pathway contributes to the initiation of T cell–mediated autoinflammatory skin diseases such as psoriasis (6, 26). In lesional psoriatic skin, keratinocytes are a major source of IL-1β; however, the mechanisms involved in IL-1β activation remained unclear (9, 10). Proteolytic activation of IL-1β is regulated by

inflammasomes, and keratinocytes in psoriatic lesions had increased expression of *AIM2*—a recently identified inflammasome that senses cytosolic DNA (16). Indeed, *AIM2* was increased both by IFN-γ in keratinocytes and upon activation by cytosolic DNA–mediated IL-1β secretion. These data suggested that cytosolic DNA could be an important disease-associated molecular pattern in psoriasis, leading to activation of IL-1β in keratinocytes by the AIM2 inflammasome.

Indeed, whereas cytosolic DNA fragments could not be detected in healthy skin, cytosolic DNA fragments were found in keratinocytes in skin lesions of psoriasis patients. Physical injury, which is a classical activator of cutaneous inflammation in psoriasis, induced cytosolic DNA and could thereby trigger the development of skin lesions. AD is accompanied by significant itching, which triggers subsequent scratching. Thus, one would expect to find cytosolic DNA in keratinocytes in lesional skin in AD. However, the stratum corneum of the AD sample shown in fig. S6 is intact, which indicates that the biopsy site was not physically injured before sampling. Thus, in contrast to physical injury, cutaneous inflammation per se seems not to be sufficient to induce cytosolic DNA in keratinocytes in inflammatory skin diseases.

Cathelicidin peptide LL-37, which can be induced by topical anti-inflammatory therapy or ultraviolet B (UVB) phototherapy, interfered with the activation of the AIM2 inflammasome by neutralizing cytosolic DNA in keratinocytes. Cathelicidin LL-37 was initially identified as an antimicrobial peptide with defense functions in cutaneous innate immunity (27). Our results suggest that LL-37 might also be a regulator of autoinflammation. Consequently, therapeutic targeting of the AIM2 inflammasome by endogenous inhibitors such as cathelicidin LL-37 may be sufficient to ameliorate cutaneous inflammation in this chronic skin disease.

The role of T_H17 responses in the pathogenesis of psoriasis is firmly established, and the pathogenic role of IL-1β in psoriasis development is increasingly well understood. Indeed, recent evidence links T_H17 and IL-1β pathways in psoriasis pathogenesis. Patients with mutations in the gene encoding the IL-1 receptor antagonist (*IL-1Ra*) develop an inflammatory skin phenotype that resembles psoriasis, including up-regulation of IL-17 cytokines and infiltration of neutrophils (28, 29). Mice carrying a mutation in the *NLRP3* gene, which leads to hypersensitive inflammasome formation, spontaneously develop a psoriasis-like skin phenotype with a T_H17 cytokine-dominant microenvironment and histological features characteristic for the human disease (5). Moreover, the IL-23–triggered development of pathogenic human T_H17 cells depends on the presence of IL-1β (2).

The observation that defective regulation of IL-1β cleavage is sufficient to contribute to skin inflammation underscores the importance of posttranscriptional control of IL-1β. In contrast to transcriptional control, posttranscriptional regulation of IL-1β by inflammasome formation requires specific danger-associated patterns as a trigger (13). The identification of such molecular patterns in inflammatory diseases is crucial and could lead to novel therapies targeting these patterns.

Our observations identified cytosolic DNA as a disease-associated trigger of IL-1β activation in psoriasis. Although eukaryotic cells store DNA primarily in the nucleus and in the mitochondria, we detected DNA in the cytosol of keratinocytes in psoriatic lesions. Genomic DNA is sensed by AIM2 and activates the inflammasome, similar to bacterial DNA detected during infection with cytosolic bacteria or DNA viruses (30). The source of cytosolic DNA in psoriatic keratinocytes remains unknown; however, it is possible that DNA may leak from the nucleus to the cytosol or that extracellular DNA from dying surrounding cells may be taken up.

This proinflammatory mechanism in keratinocytes is different from other pathogenic mechanisms recently observed in psoriasis involving self-DNA: Complexes of extracellular DNA with cathelicidin peptide LL-37 were shown to activate TLR9 in pDCs, which results in a type I IFN response (18). Because cathelicidin LL-37 interacts with DNA and is strongly expressed in skin in psoriasis, we hypothesized that LL-37–DNA interactions might contribute to AIM2-dependent inflammasome activation. Instead, we observed that LL-37–DNA complexes internalized into the cytosol by keratinocytes lost their ability to activate the AIM2 inflammasome. Indeed, LL-37 is able to translocate into the cytosol of keratinocytes, where it binds directly to cytosolic DNA and neutralizes its proinflammatory effect (fig. S13).

The DNA binding and inhibition of AIM2 activity by cathelicidin could potentially be due to the cationic charge of the peptide. Other cathelicidin peptide forms with comparable electrostatic charge but different structure had similar anti-inflammatory effects as LL-37. However, antimicrobial peptides with lower cationic charge, such as S100 peptides that are also overexpressed in psoriasis, showed no effect (31).

These data suggested that the cathelicidin peptide LL-37 acts as a physiologic inhibitor of AIM2 inflammasome formation. However, LL-37 is up-regulated in untreated lesional psoriaticskin, wherethe AIM2 inflammasome is still active. The cutaneous concentrations of LL-37 in psoriasis may be insufficient to inhibit AIM2-mediated inflammasome activity in untreated patients. This hypothesis is supported by clinical observations that treatment of psoriasis patients with UVB irradiation, which increases cutaneous vitamin D synthesis, or topical vitamin D analogs strongly induces cathelicidin expression in lesional skin while ameliorating cutaneous inflammation (22, 32). Notably, vitamin D is the only factor known to date that up-regulates cathelicidin in human skin (27).

Thus, our observations may provide another molecular mechanism for the mode of action of current psoriasis therapies. Because cathelicidin LL-37 exerts diverse immune functions such as chemotaxis, increased cytokine release from immune cells, and effects on angiogenesis, possible side effects of an uncontrolled cathelicidin increase have to be considered (27). However, because cathelicidin expression in keratinocytes is increased downstream of vitamin D signaling, drugs that specifically target cathelicidin expression may be beneficial for treating psoriasis.

MATERIALS AND METHODS

Patients and skin samples

Punch biopsies (4 mm) were taken from untreated lesional and nonlesional psoriatic skin. Skin biopsies from healthy (nonpsoriatic) volunteers and a patient with AD served as controls. For mRNA analyses, biopsies were directly transferred to 1 ml of Trizol (Invitrogen) and homogenized. To analyze protein expression, we transferred biopsies to 200 μl of radioimmunoprecipitation assay (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 phenylmethylsulfonyl fluoride (PMSF)] and homogenized. All sample acquisitions were approved by the committees on investigations involving human subjects at the Faculty of Medicine, Ludwig-Maximilian-University (Munich, Germany) and the University of Kiel (Kiel, Germany). For all procedures, informed written consent was obtained.

Cell culture and stimuli

Normal human epidermal keratinocytes (NHEKs) were grown in EpiLife cell culture medium (Cascade Biologics) containing 0.06 mM Ca^{2+} and 1× EpiLife defined growth supplement (EDGS) at 37°C under standard tissue culture conditions. Stock cultures were

maintained for up to five passages in this medium with the addition of gentamicin (10 μg/ml) and amphotericin B (0.25 μg/ml). Cells at 30 to 60% confluency were stimulated for different time periods with cathelicidin peptide LL-37 (1 to 10 μg/ml; Innovagen), cathelicidin peptide dLL-37 (an enantiomer containing D-amino acids) (10 μg/ml), S100A15 (10 μg/ml), S100A7 (10 μg/ml), $ZnCl_2$ (100 nM), IFN-γ (1 to 100 ng/ml; Biomol), TNF-α (10 ng/ml; Biomol), IL-17A, IL-22 (10 to 100 ng/ml; R&D Systems), IL-6, IL-9, IL-21 (10 to 100 ng/ml; ImmunoTools), Lipofectamine RNAiMAX Reagent (Invitrogen), DNase I (1 IU/μg; Ambion), and/or poly(dA:dT) (4 μg/ml; Sigma). C57/Bl6 wild-type macrophages overexpressing CFP-ASC were cultured as previously described (33). Mouse cathelicidin peptide CRAMP was used at 10 μg/ml (Innovagen).

Synthesis of biotinylated or FITC-labeled DNA

Biotinylated, FITC-labeled, or nonlabeled 300– or 1000–base pair (bp) DNA fragments were generated by polymerase chain reaction (PCR) from human genomic DNA with forward and reverse primers conjugated with FITC, biotin, or unconjugated primers (MWG Biotech), respectively. PCR products were analyzed in an agarose gel and extracted with the QIAquick Gel Extraction Kit (Qiagen). FITC-DNA or biotin-DNA was used at 2 to 4 μg/ml.

mRNA extraction and quantification

Total RNA was extracted from cells or skin biopsies with Trizol (Invitrogen). About 1 μg of RNA was reverse-transcribed with the DyNAmo cDNA Synthesis Kit (Finnzymes). mRNA expression was measured with a LightCycler 2.0 system and the Universal Probe Library System (Roche). Porphobilinogen deaminase (*PBGD*) was used as a housekeeping gene in a duplex quantitative PCR reaction. *PBGD* was chosen because *PBDG*, *AIM2*, and *cathelicidin* belong to a low-abundance class of mRNAs and expression levels in untreated keratinocytes are low. Induction relative to the vehicle-treated control was calculated with the comparative C_t method, where $\Delta\Delta C_t$ is $\Delta C_{t(\text{stimulant})} - \Delta C_{t(\text{vehicle})}$, ΔC_t is $C_{t(\text{gene})} - C_{t(\text{PBGD})}$, and C_t is the cycle at which an arbitrary detection threshold is crossed.

Immunofluorescence

For immunofluorescence, primary human keratinocytes or C57/Bl6 wild-type macrophages overexpressing CFP-ASC were grown on chamber slides. For detection, cathelicidin LL-37 peptide was labeled with rhodamine with the Lightning-Link Rhodamine conjugation kit (Innova Biosciences). After three washing steps with phosphate-buffered saline (PBS), slides were mounted in ProLong Gold Anti-Fade reagent (Invitrogen) containing 4′,6-diamidino-2-phenylindole (DAPI). For evaluation, a TissueFAXS System microscope (Zeiss) and the corresponding TissueQuest software (Tissue Gnostics) or an Olympus IX81 laser confocal microscope was used.

Immunohistochemistry

Sections of lesional psoriatic skin were incubated overnight with a polyclonal AIM2 antibody, followed by incubation with a horseradish peroxidase (HRP)–conjugated anti-rabbit antibody (Dako) for 1 hour, and visualized by adding DAB reagent (Dako). Cells were counterstained with hematoxylin, and sections were analyzed with a TissueFAXS System microscope.

TUNEL staining

Paraffin sections of psoriatic lesions, healthy skin, or healthy skin after tape stripping were deparaffinized and rehydrated, and antigen retrieval was performed in citrate buffer (10 mM, pH 6). For positive controls, some sections were treated with DNase I (50 IU/ml; Invitrogen) before staining. TUNEL was performed according to the manufacturer's instructions (In Situ

Cell Death Detection Kit, AP; Roche), and for signal conversion, FastRed (Sigma) was used. Counterstainings were performed with hematoxylin.

Preparation of cytosolic extracts

Cells were detached, washed, and incubated for 15 min in a digitonin-containing buffer (200 μg/ml in PBS) on ice to lyse cell membranes, leaving mitochondria and nuclei intact. Lysates were centrifuged (1000*g*) for 5 min at 4°C to pellet membranes, nuclei, and mitochondria. Protein concentration was determined as described above, and 10 μg of the supernatant (cytosolic fraction) was analyzed by immunoblotting.

Isolation of biotinylated DNA and determination of DNA-bound LL-37

NHEKs were treated with biotinylated DNA in the presence of LL-37. Cytosolic fractions containing biotinylated DNA were isolated and mixed with streptavidin-coated magnetic beads (Dynabeads M-280 Streptavidin; Invitrogen) for 1 hour and separated. Unbound material (flow-through) was collected. Repeated washing steps with PBS containing 0.1% bovine serum albumin (BSA) and 0.05% Tween 20 were performed. Washed beads were incubated with PBS containing 0.1% SDS at 100°C for 5 min to elute DNA and associated proteins from beads. Beads were removed by magnetic separation. Flow-through and eluate were spotted onto a nylon membrane (Roche). After UV cross-linking, the membrane was washed with PBS and blocked in tris-buffered saline (TBS) diluted 1% Western Blocking Reagent for 1 hour. Membranes were stained with an HRP-conjugated antibody detecting biotin or LL-37 and corresponding secondary HRP-conjugated antibodies. For detection, the Amersham ECL Plus Western blotting Detection System was used.

Transfection procedures

NHEKs at about 30% confluence were transfected with siRNAs (20 nM) with Lipofectamine RNAiMAX. The following siRNA sequences were used: untargeted control siRNA 1 (5′-GCGCAUUCCAGCUUACGUAUU), untargeted control siRNA 2 (5′-GCGCUAUCCAGCUUACGUAUU), AIM2 siRNA 1 (5′-GAAACGAGGACACAAUGAATT), AIM2 siRNA 2 (5′-GCACCAUAAAGGUUAUUAA), P2X7 siRNA 1 (5′-AUCCAGAGCAUGAAUUAUG), and P2X7 siRNA 2 (5′-AGAAACGGACUCUGAUAAA). For down-regulation of all known P2X7 isoforms (A-J), a mixture of P2X7 siRNA 1 and 2 was applied. poly(dA:dT) was transfected at 4 μg/ml with Lipofectamine RNAiMAX. Biotin- or FITC-labeled DNA fragments were transfected at 2 to 4 μg/ml.

IL-1β enzyme-linked immunosorbent assay

NHEKs were seeded in 12-well plates and grown to 60% confluence. After siRNA transfection, priming, and stimulation, supernatants were collected and subsequently centrifuged (2000 rpm, 3 min, room temperature) to pellet cell debris. Supernatants were analyzed for IL-1β by enzyme-linked immunosorbent assay (ELISA) (IL-1β ELISA Duo Set; R&D Systems).

Flow cytometry

Cells were detached and washed three times with PBS. Samples were measured in a FACSCanto and analyzed with the corresponding FACSDiva Software (Becton-Dickinson).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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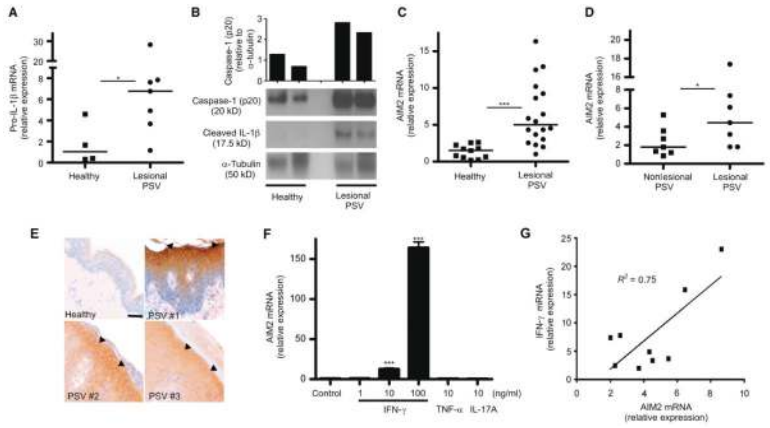

Fig. 1.

IL-1β production and *AIM2* expression are increased in keratinocytes in psoriatic lesions. (**A**) Expressionof *IL-1*β mRNA in skin lesions of psoriasis vulgaris (PSV) patients ($n = 7$) compared to skin from healthy donors ($n = 4$). *$P < 0.05$, Mann-Whitney test. (**B**) Western blot analysis of protein extracts from biopsies taken from lesional psoriatic skin ($n = 2$) or from skin of healthy donors ($n = 2$) with antibodies specific for active caspase-1 p20 subunit or cleaved IL-1β (lower panel). Caspase-1 p20 protein levels were quantified by densitometry with α-tubulin as a control (upper panel). (**C** and **D**) *AIM2* mRNA expression in skin biopsies from healthy donors ($n = 11$) compared to lesional skin of PSV patients ($n = 18$) (C) or from lesional and nonlesional skin obtained from individual psoriasis patients ($n = 7$) (D). *$P < 0.05$; ***$P < 0.001$, Mann-Whitney test (C) or Wilcoxon matched-pairs test (D). (**E**) AIM2 protein levels analyzed by immunohistochemistry in skin of healthy donors or in lesional psoriatic skin ($n = 3$). Arrowheads, apical keratinocyte layer. Scale bar, 20 μm. (**F**) Primary human keratinocytes were stimulated with the indicated cytokines for 24 hours and *AIM2* mRNA was quantified. Experiments were performed in triplicate; data are expressed as means ± SD ($n = 3$). ***$P < 0.001$, Student's *t* test. (**G**) Correlation of *IFN*-γ and *AIM2* mRNA abundance in lesional psoriatic skin ($n = 9$) ($R^2 = 0.75$). In (A) to (E) and (G), *n* refers to the number of patients investigated. In (F), *n* refers to the number of repeated experiments with similar results.

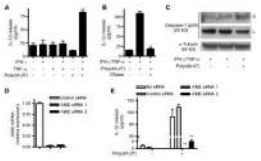

Fig. 2.

Cytosolic DNA induces the release of IL-1β in keratinocytes via the AIM2 inflammasome. (**A**) Primary human keratinocytes were primed for 24 hours with IFN-γ and TNF-α as indicated and then transfected with poly(dA:dT). IL-1β secretion was determined after 24 hours. (**B**) DNase treatment before transfection blocks IL-1β secretion from cells treated as described in (A). Data in (A) and (B) are expressed as means ± SD (n = 3 to 5). $^{**}P < 0.01$, Student's t test. (**C**) Western blot demonstrating active caspase-1 (p20 subunit) in lysates (L) and supernatants (S) of keratinocytes treated as described in (A) (n = 3). (**D**) Keratinocytes were transfected with *AIM2*-specific or nontargeting control siRNAs, and *AIM2* transcript abundance wasanalyzedafter 24 hours (n = 3). (**E**) TNF-α/IFN-γ–primed keratinocytes were transfected with two different *AIM2*-targeting siRNAs or a nontargeting control siRNA and treated with poly(dA:dT). IL-1β release was measured after 24 hours. Data are expressed as means ± SD (n = 3 to 5). $^{**}P < 0.01$, Student's t test. In (A) to (E), n refers to the number of repeated experiments with similar results.

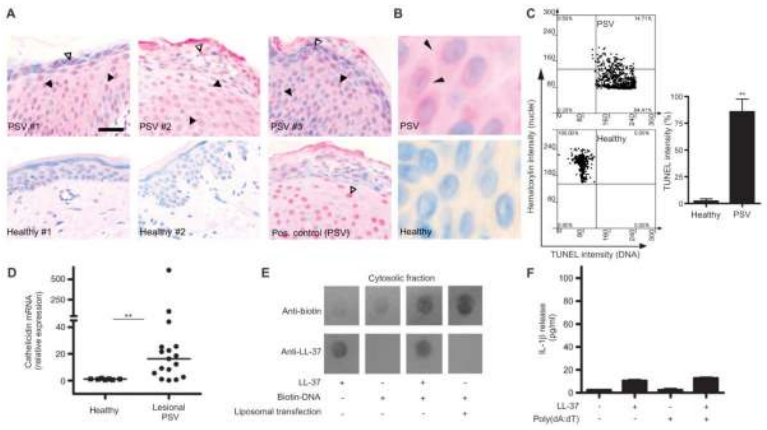

Fig. 3.

The cathelicidin antimicrobial peptide LL-37 promotes uptake of cytosolic DNA, which is present in psoriatic lesions but does not lead to release of IL-1β.(**A**) Detection of cytosolic DNA fragments by TUNEL staining in psoriatic lesions ($n = 3$) or in skin of healthy donors ($n = 2$). DNase I–treated tissue served as a positive control. Closed arrowheads, cytosolic DNA in keratinocytes; open arrowheads, nuclear DNA staining. Scale bar, 20 μm. (**B**) Cytosolic DNA fragments visualized as described in (A) at a higher magnification. Closed arrowheads, cytosolic DNA in keratinocytes. (**C**) TissueFAXS quantification of TUNEL intensity of PSV and healthy tissue sections. Hematoxylin staining representing nuclei (*y* axis) is depicted versus TUNEL staining representing cytosolic DNA fragments (*x* axis). Staining was quantified with HistoQuest software (Tissue Gnostics) by defining colors for nuclear staining (hematoxylin) and TUNEL staining (FastRed), respectively, with TUNEL-negative healthy samples for gate settings as displayed. Representative example (left panel) and graph summary (right panel) ($n = 3$). $^{**}P = 0.002$. (**D**) Cathelicidin mRNA expression in lesional skin in PSV patients ($n = 17$) andinskinof healthy donors ($n = 7$). $^{**}P < 0.01$, Mann-Whitney test. (**E**) Keratinocytes were treated with biotinylated DNA in the presence of LL-37, cytosolic fractions were isolated, and LL-37 and biotinylated DNA were detected by immunodot blot ($n = 3$). (**F**) TNF-α/IFN-γ–primed keratinocytes were incubated with poly(dA:dT) in the presence of LL-37 (10 μg/ml) for 24 hours, and IL-1β secretion was analyzed. Data are expressed as means ± SD ($n = 3$ to 6). In (A) to (D), *n* refers to the number of patients investigated. In (E) and (F), *n* refers to the number of repeated experiments with similar results.

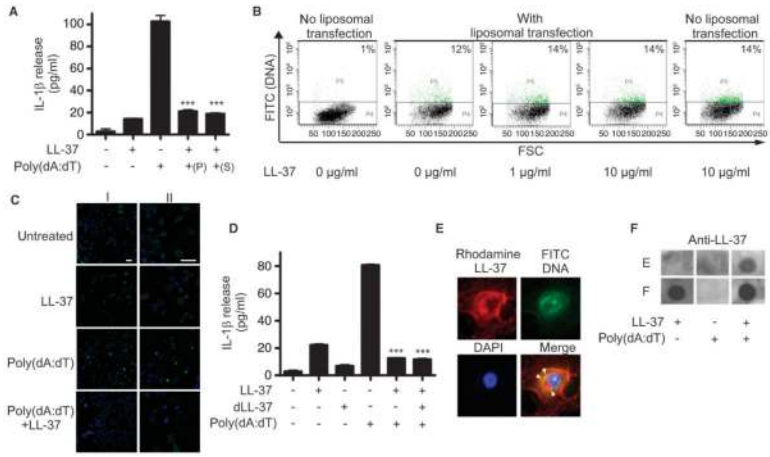

Fig. 4.

LL-37 binds to DNA in the cytosol and inhibits AIM2 inflammasome formation. (**A**) TNF-α/IFN-γ–primed keratinocytes were transfected with liposomal-complexed poly(dA:dT) for 24 hours in the presence or absence of LL-37 (10 μg/ml), and IL-1β release was measured. LL-37 either was added simultaneously with liposomal-complexed DNA (S) or was incubated with DNA for 2 hours before liposomal complexation (P). Data are expressed as means ± SD (n = 3 to 6). ***P < 0.001, Student's t test. (**B**) Keratinocytes were treated with FITC-labeled DNA and LL-37 with or without liposomal complexation as indicated. Twenty-four hours after treatment, DNA uptake was quantified by flow cytometry. Stained cells were gated from forward scatter (FSC) versus side scatter (SSC) in FSC versus FL1/FITC delineation. Gates were set with untreated keratinocytes (FITC-negative control) and keratinocytes transfected with FITC-labeled DNA (FITC-positive control) [P4 = FITC-negative (lower gate), P5 = FITC-positive (upper gate)] (n = 3). (C) Confocal microscopy of CFP-ASC–expressing macrophages, which formed large cytoplasmic ASC-containing oligomers in response to DNA, indicating inflammasome activation. The changes in the cytoplasmic fluorescence pattern were inhibited by LL-37 (left column I). In the right column II, cells that were treated with rhodamine-labeled LL-37 to track intracellular localization of the peptide are displayed. A higher magnification was chosen in the right column II to improve the visibility of fluorescence-labeled LL-37. Scale bar, 25 μm. (n = 3). (**D**) TNF-α/IFN-γ–primed keratinocytes were transfected with DNA in the presence or absence of LL-37 or its D enantiomer (dLL-37), and IL-1β secretion was measured after 24 hours. Data are expressed as means ± SD (n = 3 to 5). ***P < 0.001, Student's t test. (**E**) Keratinocytes were treated with FITC-labeled DNA and rhodamine-labeled LL-37 for 24 hours and analyzed by confocal microscopy (n = 3). (**F**) Keratinocytes were incubated with biotinylated DNA and LL-37 for 24 hours, and cytosolic fractions were prepared. Biotinylated DNA was captured in the cytosolic preparation by streptavidin-coated magnetic beads. The captured fraction E (eluate, DNA binding) and the supernatant fraction F (flow-through, no DNA binding) were analyzed for LL-37 by immunodot blot (n = 3). In (A) to (F), n refers to the number of repeated experiments with similar results.