Inflammatory Cytokines Break Down Intrinsic Immunological Tolerance of Human Primary Keratinocytes to Cytosolic DNA


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Immunological Tolerance of Human Primary Keratinocytes to Cytosolic DNA

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Keratinocytes are involved in protecting the body from infections and environmental challenges, but also in inflammatory conditions like psoriasis. DNA has emerged as a potent stimulator of innate immune responses, but there is largely no information of how keratinocytes respond to cytosolic DNA. However, if treated with inflammatory cytokines, keratinocytes gained the capacity to respond to DNA through a mechanism antagonized by the antimicrobial peptide LL37, proposed to be involved in activation and regulation of skin inflammation. The DNA sensor IFN-inducible protein 16 (IFI16) colocalized with DNA and the signaling molecule stimulator of IFN genes (STING) in the cytoplasm only in cytokine-stimulated cells, correlating with recruitment of the essential kinase TANK-binding kinase 1. Moreover, IFI16 was essential for DNA-driven innate immune responses in keratinocytes. Finally, IFI16 was upregulated in psoriasis skin lesions and localized to the cytoplasm in a subpopulation of cells. Collectively, this work suggests that inflammatory environments in the skin can lead to breakdown of tolerance for DNA in keratinocytes, which could contribute to the development of inflammatory diseases. The Journal of Immunology, 2014, 192: 2395–2404.
Materials and Methods

Cells
Normal adult human keratinocytes were obtained by trypsinization of skin samples from patients undergoing plastic surgery as previously described (20). Second-passage keratinocytes were grown in serum-free keratinocyte medium supplemented with human recombinant epidermal growth factor, bovine pituitary extract, and gentamicin (all from Life Technologies; Invitrogen Carlsbad, CA). Cells were grown at 37˚C and 5% CO₂ in a humidified incubator. We have previously reported that this protocol resulted in very pure keratinocyte cultures with >95% keratin 14–positive cells (21). Fresh medium was added every second day. By the time the cells achieved 60–80% confluence, the medium was changed to growth-factor-free keratinocyte medium, and the cells were incubated for various stimulations. To generate monocyte-derived macrophages (MDMs), buffy coats from Aarhus University Hospital blood bank were used to collect PBMCs by Ficoll-Paque (GE Healthcare) gradient centrifugation. Monocytes were purified by plastic adherence using 1 × 10⁶ PBMCs seeded in 6-cm Cell culture dishes (Nunc, Thermo-Scientific), precoated with poly-L-lysine (0.01% w/v; Cultrex) and allowed to stabilize overnight in IMDM supplemented with 10% FCS, 600 µg/ml glucose, 200 IU/ml penicillin, and 100 µg/ml streptomycin. The following day, unattached cells were washed away, and adherent cells were differentiated into MDMs by culturing for an additional 5 d in culture media: IMDM supplemented with 10% FCS, 600 µg/ml glucose, 200 IU/ml penicillin, 100 µg/ml streptomycin (Life Technologies), and 20 ng/ml M-CSF (Sigma-Aldrich).

Reagents
Cells were transfected with oligo dsDNA and FITC-dsDNA (2 µg/ml) synthesized chemically (DNA Technology, Risskov, Denmark) and stimulated with either TNF-α (50 ng/ml) or IL-1β (50 ng/ml), both from R&D Systems (Abingdon, U.K.). The transfection was done by Lipofectamine 2000 (Invitrogen) and LL37 (Innovagen, Lund, Sweden). ODN2216 and Lipofectamine 2000 (Invitrogen) were also employed.

Western blotting
Cytosolic and nuclear fractions of keratinocytes were separated and isolated using a two-step process involving cDNA synthesis by using M-MLV RT (Invitrogen) followed by QuantiFast SYBR Green Kit (Qiagen) with IFIT1 forward primer 5'-TACTTACACCTCTGGCCGGAATCAGAA-3' and reverse primer 5'-GTGAA-ACCTCACAACCCACAGGAAAGT-3'; IFI16: forward, 5'-TAGGCC-AGCCGGAGGCATCC-3' and reverse, 5'-TGGAGCTACCTGCGGA-CTGTCTT-3' (both from DNA Technology); and CXCL10: forward primer 5'-ACTGATACCTGGGTCGTCTACA-3' and reverse primer 5'-GGCTGATATCTCTGGGCTCTCA-3' and GAPDH forward primer 5'-TCTTGGCTGCGACGCGAG-3' and reverse primer 5'-ACGGAGGCC- AAATAGCAGCACA-3' (DNA Technology). For detection of IFI16 mRNA in the psoriasis biopsies, TaqMan primer-probe sets from Life Technologies were used (catalog number Hs00999902_ml for IFI16 and catalog number Hs00999902_ml for RPLP0 used for normalization).

ELISA
Human CXCL10 protein was measured on culture supernatants using the Duoset ELISA Development System (R&D systems) and following the instructions of the manufacturer.
Confocal microscopy

Cells were fixed at the indicated time point posttreatment, permeabilized with methanol for 5 min at −20°C, labeled with Abs against IFI16 (sc-8023; Santa Cruz Biotechnology), STING (Imgenex IMG-6485A), or TBK1 (sc-9910; Santa Cruz Biotechnology). Images were acquired on a Zeiss LSM 710 confocal microscope using a 63× 1.4 oil-immersion objective (Zeiss). Image processing was performed using Zen 2010 (Zeiss) and ImageJ (National Institutes of Health). All images are representative of at least two or three independent experiments.

Immunofluorescence analysis

Four-micrometer paraffin-embedded sections of lesional and nonlesional psoriatic skin were deparaffinized and rehydrated. Ag retrieval was performed by boiling in TEG buffer (pH 9) in a microwave. Blockage of the nonspecific Ab binding sites was achieved by incubating the samples for 30 min with Image-IT FX Signal Enhancer (Invitrogen). Sections were then incubated overnight at 4°C with mouse monoclonal anti-IFI16 (sc-8023; Santa Cruz Biotechnology), diluted in TBS buffer with 5% goat serum. Secondary staining was obtained incubating with Alexa Fluor 488 Dye (Invitrogen); 1:300 diluted in TBS buffer with 5% goat serum for 1 h. Finally, the samples were washed, and nuclear staining was performed by embedding samples in Prolong Gold antifade reagent with DAPI (Invitrogen). Samples were evaluated by confocal microscopy. As a negative control, sections were incubated with blocking buffer without primary Ab and as an isotype control with normal mouse IgG (Santa Cruz Biotechnology) instead of primary Ab.

Biopsies

Biopsies were obtained from nonlesional and lesional psoriatic skin. Keratome and 4-mm punch biopsies from untreated lesional plaque-type psoriatic skin were taken from the center of the chronic plaque from either the upper or lower extremities. For each patient, biopsies were taken from only one anatomical site, and the biopsies from nonlesional skin were taken at a distance of at least 5 cm from a lesional plaque. For quantitative PCR, 4-mm punch biopsies were immediately snap-frozen in liquid nitrogen and stored until further use. For immunofluorescence and immunohistochemical analysis, biopsies were embedded in paraffin. The study was conducted according to the Declaration of Helsinki Principles. The regional ethical committee, Region Midtjylland, approved all described studies, and signed informed consent was obtained from each patient (permission numbers M-20090102 and M-20110027).

Donor variations

All in vitro work with primary human keratinocytes was done with cells from two to three different donors. Data included in the article are all from series of experiments in which the conclusions drawn were independent of the donor used.

Statistical analysis

The data are shown as mean ± SD. The statistical significance was determined by two-tailed Student t test or Wilcoxon rank sum test. The p values <0.05 were considered to be statistically significant.

Results

Intracellular DNA alone is a weak inducer of innate immune responses in keratinocytes

To test whether synthetic DNA localizes to the cytoplasm in primary human epidermal keratinocytes, we transfected such cells with a dsDNA oligonucleotide labeled with FITC. As shown in Fig. 1A, synthetic dsDNA localized to the cytoplasm when transfected by either Lipofectamine or LL37, and for both DNA delivery methods, >95% of the DNA-positive spots localized to the cytoplasm. We also observed that the DNA localized to specific foci in the cytoplasm, consistent with what has previously been found in macrophages (10, 23).

To evaluate the cellular response to cytosolic DNA, we measured expression of the chemokines CCL20 and CXCL10. CCL20 is reported to play a key role in recruitment of Th17 CD4+ T cells (24), and CXCL10 is a signature of IFN-stimulated genes, also reported to be involved in skin inflammation (25). For comparison, we examined the expression of chemokines in response to TNF-α and LL37. Surprisingly, DNA delivered into the cytoplasm of keratinocytes did not evoke significant expression of CCL20 or CXCL10 (Fig. 1B, 1C), whereas TNF-α stimulation did lead to expression of the two chemokines. Expression of IFN-β was also not induced by DNA, and the highly induced IFN-stimulated gene IFIT1 was induced only marginally (Supplemental Fig. 1A, 1B). Treatment with LL37 alone did modestly stimulate expression of CCL20 and CXCL10. To examine whether the observed lack of response to DNA in keratinocytes was due to the use of a too low concentration of DNA or harvest of RNA at the wrong time point, we performed dose-response and kinetics experiments and also collected supernatants after 24 h of treatment to allow accumulation of chemokine in the supernatant to occur. DNA stimulation did not evoke a chemokine response at any of the DNA concentrations tested (Fig. 1D), and up to 24 h of treatment did not lead to production of CXCL10 mRNA or protein (Fig. 1E, 1F). By contrast, primary human macrophages responded to DNA stimulation with strong induction of CXCL10 at both the RNA and protein level (Fig. 1D, 1E). In contrast to the modest response of the keratinocytes to DNA stimulation, we found that RNA delivery into the cytoplasm of keratinocytes, by use of a virus that stimulates though the RNA-sensing RIG-I pathway (26), led to potent induction of CXCL10 (Fig. 1F, 1G).

Despite the lack of response to cytoplasmic DNA in keratinocytes, these cells did express the DNA sensors IFI16 and cGAS (Fig. 1H), with cGAS localizing exclusively to the cytoplasm and IFI16 localizing mainly to the nucleus but with a distinct and easily detectable pool of IFI16 localizing to the cytoplasm. For AIM2, we were able to detect the mRNA in the keratinocytes, but did not detect AIM2 protein (data not shown), similar to what has been reported previously (6, 27). Thus, DNA alone is a weak inducer of innate immune responses in keratinocytes.

DNA synergizes with TNF-α and IL-1β to induce gene expression in keratinocytes

Cytosolic DNA has been reported to be an important danger-associated molecular pattern (DAMP) in keratinocytes (6). In a recent study, it was reported that skin inflammation induced by synergistic action of IL-17A, IL-22, IL-1α, and TNF-α recapitulates some features of psoriasis (28). To address the potential role of cytosolic DNA and its synergy with proinflammatory cytokines in inflamed keratinocytes, we stimulated keratinocytes with transfected synthetic dsDNA together with TNF-α or IL-1β. Interestingly, this dual treatment led to very potent induction of the chemokines CCL20 and CXCL10 (Fig. 2A–D) as well as IFN-β and IFIT1 (Supplemental Fig. 1A, 1B). Interestingly, the strong synergistic induction of CXCL10 expression after stimulation with DNA and inflammatory cytokines was observed irrespective of whether the cytokine stimulation was given before or after DNA (Supplemental Fig. 1C, 1D), although we observed the synergy to be strongest when cytokine stimulation was given after DNA transfection. The observed chemokine response was independent of endosomal acidification (Supplemental Fig. 1E, 1F), as known for the TLR9 pathway (29). Furthermore, a TLR9 agonist induced only limited gene expression, and the response evoked by dsDNA in cytokine-treated cells was not affected by cotreatment with the TLR9 antagonist ODN4084F (Supplemental Fig. 1G, 1H). By the use of a series of well-described small-molecule inhibitors of the signaling pathways through IKK–NF-κB, TBK1–IFN regulatory factor 3 (IRF3), and MAPK, we found that cytokine/DNA-driven expression of CCL20 was dependent on the IKK–NF-κB pathway but not the TBK1, IRF3, and MAPK pathways.

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whereas induction of CXCL10 was dependent on all pathways (Supplemental Fig. 2).

To investigate whether human genomic DNA could induce analogous synergistic responses, we isolated genomic DNA from primary keratinocytes and transfected keratinocytes cultures with this DNA. Keratinocytes transfected with genomic human DNA and stimulated with TNF-α induced significant chemokine responses with a very strong synergy between the stimuli (Fig. 2E, 2F). Altogether, these results suggest that inflammatory cytokines enable keratinocytes to respond to cytoplasmic DNA with a potent innate immune response.

**LL37 selectively inhibits DNA-driven gene expression**

It has been previously reported that LL37 transports extracellular self-DNA fragments into TLR9-containing endosomal compart-
ments of pDCs, in turn inducing type I IFN production (5). To test the effect of LL37-mediated DNA delivery into the cytosol on chemokine responses, we used the primary epidermal keratinocytes and compared with the response evoked by DNA delivered with Lipofectamine. In contrast to what was observed after DNA delivery with Lipofectamine, cytokine-treated keratinocytes transfected with synthetic DNA using LL37 as delivery agent induced only marginal expression of CCL20 and CXCL10 (Fig. 3A, 3B).

LL37 couples with DNA and converts it into a potent DAMP, a trigger of type I IFN in pDCs (5). A recent study also reported LL37-mediated DNA delivery to be able to activate type I IFN responses in monocytes (30). On the contrary, Dombrowski et al. (6) suggested an anti-inflammatory function of LL37 by interfering with DNA-triggered inflammasome activation. Based on these data, we were interested in evaluating whether the ability of LL37 to inhibit DNA-driven gene expression in keratinocytes was dependent on the antimicrobial peptide being the DNA transfection agent. To address this, we transfected DNA into keratinocytes using Lipofectamine and stimulated in parallel with LL37, which had not been preincubated with DNA. Interestingly, chemokine responses induced by the combined treatment of Lipofectamine-delivered DNA and TNF-α were found to be significantly inhibited by LL37 (Fig. 3C, 3D). To assess the LL37 activity on self-DNA, genomic DNA was used as stimulus. Similar to what was found when stimulating with synthetic DNA, LL37 inhibited chemokine expression induced by combined stimulation with Lipofectamine-delivered genomic DNA and TNF-α (Fig. 3E, 3F). The ability of LL37 to inhibit DNA-driven gene expression was not dependent on the antimicrobial peptide being given at the same time as the DNA, because LL37 treatment 4 h post–DNA stimulation still led to strong inhibition of the cellular response (Supplemental Fig. 3). We also investigated the effect of LL37 on the modest gene induction stimulated by TNF-α alone to exclude the cytokine being the target of LL37. As expected, LL37 did not modulate TNF-α–induced gene expression, hence demonstrating DNA or DNA-stimulated activities to be the target of LL37 (Fig. 3G, 3H).

Assembly of the DNA-activated signalsome in keratinocytes is supported by cytokine treatment

The chemokine expression data presented above suggested that stimulation with inflammatory cytokines endowed keratinocytes

FIGURE 2. DNA synergizes with TNF-α and IL-1β to induce gene expression in keratinocytes. Human primary keratinocytes were treated with synthetic dsDNA (Lipofectamine [Lipo] 2 μg/ml) (A–D), human keratinocyte genomic DNA (gDNA; Lipo, 2 μg/ml) (E, F), TNF-α (50 ng/ml) (A, B, E, F), or IL-1β (10 ng/ml) (C, D) as indicated for 6 h. Total RNA was isolated, and mRNA levels of CCL20 and CXCL10 were quantified by RT-qPCR. The data were normalized to β-actin and are presented as mean fold induction of triplicate cultures ± SD relative to untreated (UT). Similar results were obtained in at least three independent experiments. *p < 0.05. NR, Normalized ratio.
with the ability to respond to intracellular DNA and that LL37 inhibited this response. To get molecular information on what may be underlying these observations, we fixed keratinocytes treated with FITC-dsDNA (Lipofectamine transfection) and TNF-α for 2 or 6 h and stained with Abs specific for IFI16, STING, and TBK1. In the untreated cells, STING was broadly distributed in the cytoplasm, and IFI16 was primarily found in the nucleus (Fig. 4A), consistent with the data presented in Fig. 1H. At 2 h posttreatment, STING was found to be mobilized to specific foci colocalizing with DNA in the cytoplasm (Fig. 4A). This was strongly stimulated by TNF-α treatment. However, only in keratinocytes treated with cytokine did we observe mobilization of IFI16 to the DNA and colocalization among DNA, IFI16, STING, and TBK1, indicative of assembly of a functional signalsome (Fig. 4A, Supplemental Fig. 4). At 6 h posttreatment, the DNA foci in the cytoplasm in the cytokine-treated cells remained positive for IFI16 and STING (Fig. 4B). Moreover, abundant colocalization between STING and TBK1 was observed, often in foci not containing DNA or IFI16. If the Lipofectamine-delivered DNA was given to cells also receiving LL37, colocalization between DNA and IFI16 in the cytoplasm was still observed in TNF-α-treated cells (Fig. 4B). However, we found significantly less colocalization between

**FIGURE 3.** LL37 selectively inhibits DNA-driven gene expression. Human primary keratinocytes were treated with synthetic dsDNA (delivered with Lipofectamine [Lipo] or LL37, 2 μg/ml) (A–D), human keratinocyte genomic DNA (gDNA; Lipo, 2 μg/ml), LL37 (5 μg/ml), or TNF-α (50 ng/ml) (E, F), as indicated for 6 h. Total RNA was isolated, and mRNA levels of CCL20 and CXCL10 were quantified by RT-qPCR. The data were normalized to β-actin and are presented as mean fold induction of triplicate cultures ± SD relative to untreated (UT). Similar results were obtained in at least three independent experiments. *p < 0.05. NR, Normalized ratio.
Given the correlation between IFI16–DNA association and DNA-driven gene induction (Figs. 2, 4A, Supplemental Fig. 4), we were also interested in evaluating how cytokine treatment may allow the association between IFI16 and DNA. To address this, we treated keratinocytes with TNF-α and IL-1β for 2 h and isolated cytoplasmic extracts. Interestingly, cytosolic extracts from cells stimulated with IL-1β, and to a lesser extent also TNF-α, contained more IFI16 than extracts from untreated keratinocytes (Fig. 4C). Collectively, these data suggest that treatment of keratinocytes with inflammatory cytokines induces translocation of a small pool of IFI16 into the cytoplasm, which enables the cells to assemble signaling complexes including IFI16, STING, and TBK1 and suggests that LL37 inhibits assembly of a functional STING signalsome.

IFI16 is essential for DNA-driven innate immune responses in cytokine-treated keratinocytes. Given the correlation between association of DNA with IFI16 and formation of STING foci, we were interested in evaluating whether IFI16 was essential for DNA-stimulated gene expression in cytokine-treated cells. For this purpose, we performed siRNA-mediated knockdown of IFI16 expression in primary human keratinocytes and were able to reduce levels of IFI16 mRNA by ~70% (Fig. 5A). Importantly, the strong DNA-mediated induction of CCL20 and CXCL10 in TNF-α-treated cells was significantly reduced in cells with knockdown of IFI16 (Fig. 5B, 5C). These data demonstrate an essential role for IFI16 in DNA-driven innate immune responses in human primary keratinocytes.

IFI16 exhibits subcellular localization in psoriasis lesions similar to what is observed after DNA transfection of cytokine-treated keratinocytes

With the aim to examine the potential relevance of the findings described above in human skin diseases, we isolated skin biopsies...
from nonlesional and lesional psoriasis skin and stained tissue sections for IFI16 and DNA. As expected, and in contrast to the nonlesional skin, the lesional psoriasis skin contained a thickened cell layer with nucleated cells in the epidermis (Fig. 6A). IFI16 was expressed at low levels in the nonlesional skin and localized primarily to specific areas resembling nucleoli in the nuclei in the keratinocytes (Fig. 6A). However, because we have previously observed that extensive sample handling and harsh protocols (like e.g., fluorescence in situ hybridization) affect the apparent localization of IFI16 as measured by immunofluorescence (31), it is not clear at this stage whether this is a real biological phenomenon due to the protocol used, including deparaffinization and rehydrated of paraffin-embedded sections. In the lesional psoriasis skin, IFI16 was upregulated at both the mRNA and protein level (Fig. 6A, 6B). More importantly, in between 5 and 8% of the cells, IFI16 could be found in the cytoplasm, where it was observed to either distribute throughout the cytoplasm or to localize to specific perinuclear foci (Fig. 6A, zoom panels). The latter pattern exhibited remarkable similarity to what was observed after DNA transfection of cytokine-treated keratinocytes in vitro (Fig 4). Unfortunately, attempts to stain the tissue sections for STING showed that the Ab was not compatible with the protocol used.

FIGURE 5. DNA-driven gene expression in cytokine-treated keratinocytes is dependent on IFI16. (A) Human primary keratinocytes were transfected with control (Ctrl) and IFI16-specific siRNA. Total RNA was harvested 48 h later, and levels of IFI16 mRNA were measured by RT-qPCR. (B and C) Keratinocytes treated with siRNA for 48 h were stimulated with synthetic dsDNA (Lipofectamine [Lipo], 2 μg/ml) or TNF-α (50 ng/ml) as indicated for 6 h. Total RNA was isolated, and mRNA levels of CCL20 and CXCL10 were quantified by RT-qPCR. The data were normalized to β-actin and are presented as mean fold induction of triplicate cultures ± SD. Similar results were obtained in at least three independent experiments. *p < 0.05. NR, Normalized ratio.

FIGURE 6. IFI16 is upregulated in psoriasis lesions and exhibits a cellular localization pattern similar to keratinocytes receiving dual stimulation with DNA and inflammatory cytokines. (A) Tissue sections of punch biopsies from nonlesional and lesional psoriatic skin were stained for IFI16 and DNA (DAPI) and analyzed by confocal microscopy. Arrows indicate cytosolic IFI16 foci. Scale bar, 100 μm. (B) RNA was isolated from nonlesional (NLS) and lesional psoriatic skin (LS), and levels of IFI16 mRNA were quantified by RT-qPCR. Data shown as means of normalized values relative to nonlesional psoriasis skin ± SD. n = 9. (C) Tissue section of punch biopsies from lesional psoriatic skin stained for DNA (DAPI) and analyzed by confocal microscopy. Arrows indicate DAPI staining (DNA) in the cytosol. Scale bar, 10 μm. *p < 0.05. DIC, Differential interference contrast; NR, Normalized ratio.
Finally, we used the DAPI-stained tissue sections to look for the potential presence of DNA in the cytosol. Interestingly, we observed DAPI-positive foci outside the nucleus in between 3 and 5% of the skin cells, but with no significant difference between cells from psoriasis lesions and nonlesional skin (Fig. 6C).

Collectively, the presented data demonstrate that keratinocytes do not evoke innate immune responses to cytoplasmic DNA unless present in an inflammatory environment. The antimicrobial peptide LL37, the expression of which is upregulated by several treatments known to ameliorate psoriasis symptoms (17–19), inhibits DNA-driven gene expression and interferes with assembly of functional signaling complexes downstream of DNA sensing. In cells treated with TNF-α, the DNA sensor IFI16 colocalizes with DNA, which correlates with DNA-driven gene expression. Finally, IFI16 is upregulated in lesional psoriasis skin and can be found to localize to specific spots in the cytoplasm, similar to what is observed after stimulation with synthetic DNA in vitro.

Discussion
Skin inflammation can be caused by autoimmune reactions, infections, or physical injury. DNA has recently emerged as an important stimulator of innate immune responses (9) and is known to evoke immune responses in keratinocytes (6). Given the abundant exposure of keratinocytes to DNA-associated danger signals, it is important that immune activation by DNA is tightly regulated in these cells. In this study, we report that immunological reactions of human primary keratinocytes after DNA exposure require parallel stimulation with inflammatory cytokines. This stimulation enables keratinocytes to assemble functional signaling complexes acting upstream of gene expression. The antimicrobial peptide LL37 interferes with this process, hence specifically inhibiting DNA-driven immune activation. Finally, we provide data on material from psoriasis patients demonstrating that IFI16 is upregulated in psoriasis lesions and that IFI16 and DNA under such conditions can be found in the cytoplasm of keratinocytes in specific perinuclear foci, similar to what is seen after DNA stimulation of keratinocytes in vitro.

A key finding of the present project is that human keratinocytes normally do not respond to cytoplasmic DNA with an innate immune response and that stimulation with inflammatory cytokines enables the cells to induce gene expression in response to this DAMP. This indicates that cytokine treatment activates an essential factor in the DNA sensing pathway acting upstream of STING, hence breaking down the intrinsic immunological tolerance of keratinocytes for cytosolic DNA. We found that the DNA sensor IFI16 was primarily expressed in the nucleus in keratinocytes not treated with cytokines, and this was not affected by delivery of DNA into the cytoplasm. By contrast, in keratinocytes treated with TNF-α, IFI16 was found to localize with DNA in the cytoplasm, which correlated with DNA-induced gene expression. Interestingly, although DNA treatment stimulated some degree of STING redistribution to the well-described punctuate foci (14), even in the absence of cytokine treatment, recruitment of the IRF3 kinase TBK1 was seen only in cytokine-treated cells in which IFI16 was also recruited to the STING foci. We also observed that cytokine treatment led to accumulation of IFI16 in the cytoplasm. This could indicate that redistribution of a small pool of cellular IFI16 from the nucleus to the cytoplasm is a critical event in stimulation of DNA sensitivity in keratinocytes. The authors do acknowledge that this conclusion could have been strengthened with higher quality data on the distribution of IFI16 and also if more data addressing this key issue had been included. This is now an area of focus in the laboratory. The concept of IFI16 redistribution in response to inflammatory stimuli is in agreement with a previous publication by Landolf and associates (32), demonstrating that exposure of human keratinocytes and human skin explants for high doses of UVB irradiation induced IFI16 localization to the cytoplasm. It has been reported that acetylation of two arginine residues in the nuclear localization signal of IFI16 inhibits the nuclear localization of the protein (33), and in this respect, it is interesting that inflammatory cytokines induce acetyl transferase activity (34), which could provide a mechanistic explanation for the observed phenomenon.

The antimicrobial peptide LL37 has intrinsic DNA-binding function and has been reported to be able to deliver DNA into endosomes and the cytoplasm (5, 6). Moreover, LL37 is upregulated in psoriasis skin (5), which could indicate a role for LL37 in promotion of disease. Paradoxically, LL37 expression is further upregulated in response to some treatments known to ameliorate psoriasis (17–19). With focus on chemokines of relevance for type I IFN function and Th17 cell recruitment, we found that DNA delivered with LL37 did not stimulate expression of CCL20 and CXCL10 in keratinocytes and that the antimicrobial peptide also inhibited gene expression induced by Lipofectamine-delivered DNA, even when given 4 h after DNA treatment. The peptide did not affect chemokine expression induced by TNF-α. Regarding the mechanism of action of LL37, our data demonstrate that treatment with LL37 has no impact on recruitment of IFI16 to cytosolic DNA STING-positive foci, but does inhibit recruitment of TBK1 to the signaling complex. This could indicate that LL37 inhibits DNA signaling by acting at a level downstream of IFI16 and STING but upstream of TBK1. The idea of LL37 acting within the cells is consistent with previous data demonstrating LL37 to inhibit inflammasome activation by DNA delivered with lipofection (6). It should be mentioned that the mode of action of LL37 may be cell-type specific, because pDCs and monocytes have been reported to respond potently to LL37-delivered DNA, through the TLR9–MyD88 and STING–TBK1 pathways, respectively (5, 30). Collectively, the data could indicate a regulatory function for LL37 in keratinocytes by controlling DNA-driven amplification of several inflammatory pathways involved in the pathogenesis of psoriasis.

Psoriasis has for many years been considered a possible autoimmune-driven inflammatory skin disease, but an endogenous trigger of the inflammatory responses has never been identified (2). The disease is characterized by hyperproliferation of keratinocytes and an excessive Th17 response (2). In addition, expression of IFN-stimulated genes is elevated in early psoriasis plaques (4). The data presented in this work point toward a role for the DNA-sensing pathway in evoking some of these responses and could also indicate that DNA may serve as an autoantigen in psoriasis. We found that IFI16 was upregulated in psoriasis lesions and was localized to specific foci in the cytoplasm in a fraction of cells, reminiscent of what is observed after immunostimulatory DNA recognition in vitro (10). Interestingly, it has been suggested that aberrant DNA replication intermediates, as may be found in hyperproliferating keratinocytes, can trigger innate immune responses (35). Physical injury, which is a classical activator of cutaneous inflammation in psoriasis and known as the Köbner phenomenon, has also been reported to induce release of DNA to the cytoplasm in keratinocytes (6). Thus, it is possible that release of DNA into the cytoplasm, induced by physical damage, DNA replication byproducts, or even infection, could amplify inflammatory reactions in keratinocytes in which tolerance to DNA has been broken due to low-grade constitutive cytokine expression. Although the focus of this study has
been on cytosolic DNA sensing, it should be noted that the elevated expression of IFI16 could also impact on exacerbation of psoriasis by other mechanisms. Most notably, nuclear IFI16 has been proposed to stimulate inflammasome activation (36) and modulate expression of a range of inflammatory genes (37).

Altogether, we report that human keratinocytes have intrinsic immunological tolerance to cytoplasmic DNA. However, treatment with inflammatory cytokines leads to breakdown of this tolerance. This correlates with cytosolic colocalization between DNA and the DNA sensor IFI16 and assembly of a STING-TBK1-containing signaling complex. The antimicrobial peptide LL37 inhibits DNA-driven innate immune responses and acts by preventing assembly of a functional signaling complex. Finally, in psoriasis skin lesions, IFI16 is upregulated and can be found in the cytoplasm in foci resembling DNA recognition/signaling complexes. Collectively, these data have revealed the immunological DNA-sensing pathway as a regulatory switch in keratinocytes and suggest a role for host or microbial DNA as danger signals in skin inflammation.

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Disclosures
The authors have no financial conflicts of interest.

References


Data supplement

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Supplemental Fig. 1. Effect of cytokine stimulation on DNA-induced gene expression.

Supplemental Fig. 2. Signaling pathways involved in chemokine induction by DNA and cytokines in keratinocytes.

Supplemental Fig. 3. LL37 inhibits DNA-driven immune responses even when given after DNA stimulation.

Supplemental Fig. 4. Assembly of the DNA-activated signalsome in keratinocytes is supported by cytokine treatment.
Supplemental Figure 1. Effect of cytokine stimulation on DNA-induced gene expression.

Human primary keratinocytes were treated with dsDNA (lipofectamine, 2 µg/ml), TNFα (50 ng/ml), IL-1β (50 ng/ml), bafilomycin A (100 nM, 30 min prior to further treatment), ODN2216 (5 µM), ODN4084F (1 µM) as indicated. (C, D) The timing of cytokine stimulation relative to DNA stimulation was varied as indicated between -4 hours and +4 hours. Total RNA was isolated 6 hours after DNA treatment and mRNA levels of (A) IFN-β, (B) IFIT1, (C, D, F, G, H) CXCL10, and (E) CCL20 mRNA were quantified by RT-qPCR. The data were normalized to β-actin or GAPDH and are presented as mean fold induction of triplicate cultures +/- st.dev. relative to untreated (UT). Similar results were obtained in at least three independent experiments. Ns, not significant. *, p<0.05.
Supplemental Figure 2. Signaling pathways involved in chemokine induction by DNA and cytokines in keratinocytes. Human primary keratinocytes were treated with dsDNA (lipofectamine, 2 µg/ml), TNFα (50 ng/ml), (A,B) BX-795 (1 µM), (C, D) Manumycin (10 µM), or (E, F) SB202190 (20 µM) as indicated. Total RNA was isolated 6 hours after DNA treatment and mRNA levels of (A, C, E) CCL20 and (B, D, E) CXCL10 mRNA were quantified by RT-qPCR. The data were normalized to β-actin and are presented as mean fold induction of triplicate cultures +/- st.dev. relative to untreated (UT). Similar results were obtained in at least three independent experiments. Ns, not significant. *, p<0.05.
**Supplemental Figure 3.** LL37 inhibits DNA-driven immune responses even when given after DNA stimulation. Human primary keratinocytes were treated with synthetic dsDNA (delivered with lipofectamine, 2 µg/ml), LL37 (5 µg/ml), or TNFα (50 ng/ml), as indicated. The treatment was either at the same time of or 4 hours after stimulation with DNA. Total RNA was isolated 6 hours after DNA stimulation and mRNA levels of (A) CCL20 and (B) CXCL10 were quantified by RT-qPCR. The data were normalized to β-actin and are presented as mean fold induction of triplicate cultures +/- st.dev. relative to untreated (UT). Similar results were obtained in at least three independent experiments. *, p<0.05.
**Supplemental Figure 4.** Assembly of the DNA-activated signalsome in keratinocytes is supported by cytokine treatment. Images of the same cells as presented in Figure 4A (lower two rows), with omission of the stainings with anti-STING antibody. The cells were treated for 2 hours with FITC-dsDNA (delivered with lipofectamine) and TNFα (50 ng/ml), as indicated. The cells were fixed and stained with specific antibodies and analyzed by confocal microscopy. White box indicates area displayed in zoom column. Scale bar 10 μm.