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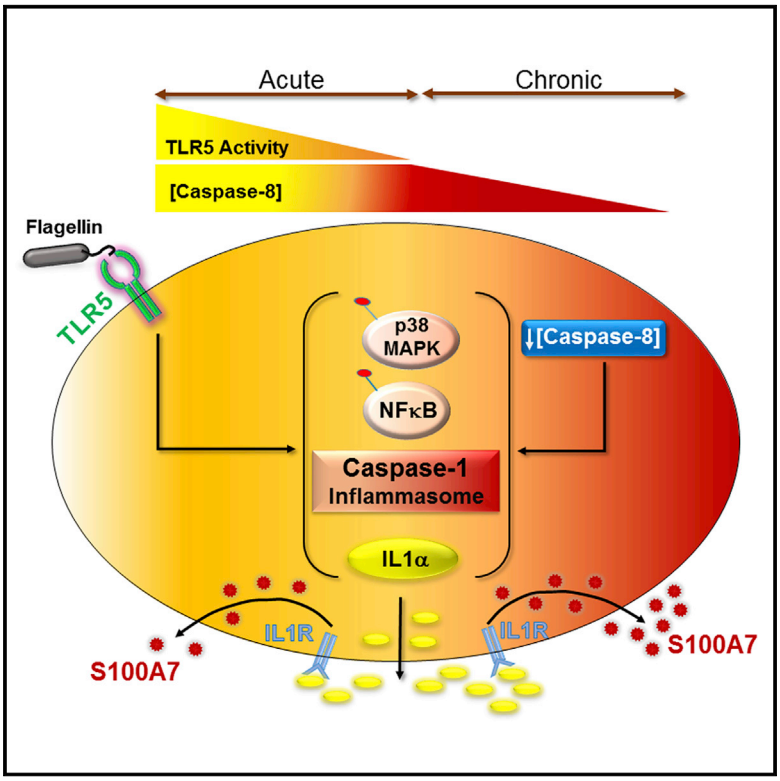
Sustained Secretion of the Antimicrobial Peptide S100A7 Is Dependent on the Downregulation of Caspase-8

Graphical Abstract



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In Brief

The global explosion of antibiotic-resistant microorganisms has spurred interest in alternative strategies to combat these "superbugs." Antimicrobial peptides (AMPs) have emerged as a promising solution. Bhatt et al. show downregulation of epidermal caspase-8 can mediate sustained release of AMPs from the skin and provide an effective defense against infection.

Highlights

- S100A7 secretion is released in a biphasic manner
- The acute phase is mediated by TLR5 signaling
- The chronic phase is mediated by the downregulation of epidermal Caspase-8
- The core machinery of both phases is composed of p38MAPK/NFκB/Caspase-1/IL-1α

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Sustained Secretion of the Antimicrobial Peptide S100A7 Is Dependent on the Downregulation of Caspase-8

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SUMMARY

Antimicrobial peptides (AMPs) are the body's natural innate immune defense against a spectrum of pathogens and can also modulate cell proliferation, chemotaxis, angiogenesis, wound healing, and immune cell activity. Harnessing these diverse functions for prophylactic use is contingent upon understanding the regulatory mechanisms governing their unconventional secretion from cells. Analysis of the secretion of S100A7 (Psoriasin), an abundant AMP stored in differentiated keratinocytes of the skin, has revealed an unexpected biphasic secretory response to bacterial exposure. The core components regulating S100A7 secretion are NFκB/p38MAPK, caspase-1, and interleukin (IL)-1α. The initial activation of this core machinery is mediated by Toll-like receptor signaling, whereas the chronic response is mediated by Caspase-8 downregulation. Interestingly, there is a concomitant downregulation of Caspase-8 in inflammatory skin diseases wherein S100A7 is constitutively released. These results highlight the potential of targeting these components to control the release of AMPs from the skin in both homeostatic and disease conditions.

INTRODUCTION

The skin is the body's first line of defense against the external environment. This is mainly accomplished via three modes: (1) the layer of elaborate cross-linked proteins in a lipid matrix that form a physical barrier (Madison, 2003); (2) beneficial commensal bacteria on the surface of the skin that counteract pathogenic bacteria (Sanford and Gallo, 2013); and (3) a rich reservoir of antimicrobial peptides (AMPs) with bactericidal activity that form a "biochemical barrier" (Harder and Schröder, 2005). With the rapid emergence of antibiotic-resistant infections globally, there is an increasing emphasis to develop strategies to boost production and secretion of AMPs, which are known to have a wide spectrum of targets including viruses, bacteria, fungi, and parasites (Bahar and Ren, 2013). AMPs are a diverse class of naturally occurring, cationic, amphipathic molecules that utilize varied modes of bactericidal activity, which may be harnessed as a strategy to avoid the development of bacterial resistance to these compounds (Bechinger and Gorr, 2017). They not only act as endogenous broad-spectrum inhibitors of microbes, but also possess various immunomodulatory functions, which include promoting chemotaxis of immune cells (Agerberth et al., 2000; Chertov et al., 1996; Niyonsaba et al., 2002), stimulating the production of cytokines/chemokines (Niyonsaba et al., 2007), regulating cell proliferation and differentiation (Choi et al., 2013; Niyonsaba et al., 2007), and accelerating wound healing (Mangoni et al., 2016). Given these varied activities, AMPs have emerged as an attractive therapy in the post-antibiotic era.

Among various classes of AMPs, certain members of the S100 protein family have gained heightened interest, in large part due to their emerging roles in modulating infections and inflammatory conditions (Zackular et al., 2015). Recent studies have focused on one such S100 family member, namely the S100A7 peptide, due to its robust antimicrobial properties, and constitutively high basal expression in the skin (Watson et al., 1998). Moreover, it exhibits selectivity toward harmful bacteria such as *Escherichia coli*, and at higher concentrations, Gram-negative *Pseudomonas aeruginosa* and Gram-positive *Staphylococcus aureus*, while having no effect on beneficial commensal bacteria (Gläser et al., 2005).

In addition to its role in homeostasis, S100A7 has been well-characterized for its overexpression in psoriatic skin, hence earning the moniker of Psoriasin. In addition to psoriasis, S100A7 is known to be overexpressed in various hyperproliferative skin conditions including atopic dermatitis, Darier's disease, mycosis fungoides, wound healing, and skin cancer (Broome et al., 2003; Hagens et al., 1999; Madsen et al., 1991; Semprini et al., 2002). S100A7 is encoded in the epidermal differentiation complex locus, which contains genes that mediate epidermal

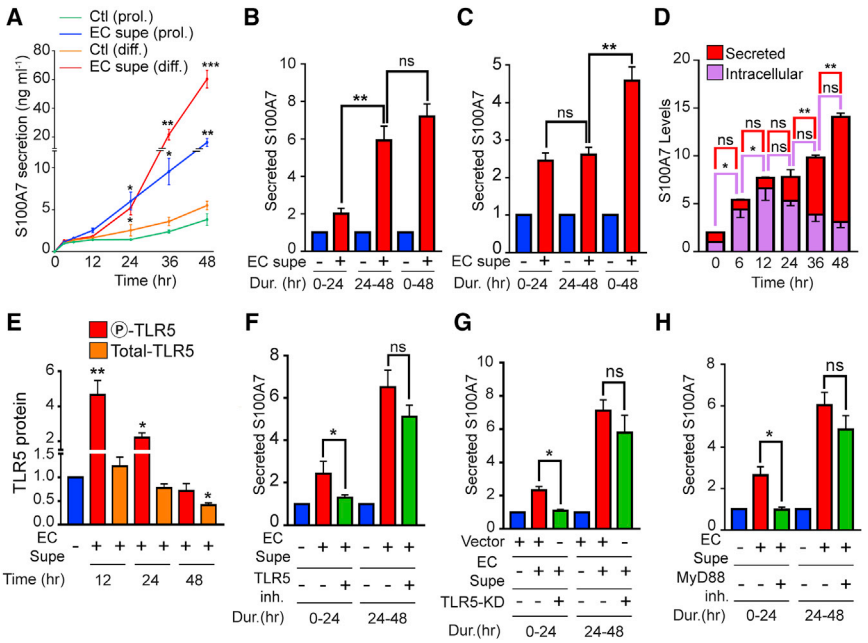

Figure 1. Differentiated Keratinocytes Secrete Significantly Higher Levels of S100A7 in the Chronic Phase of EC Supe Treatment Independent of TLR5 Activity

(A) Secretion of human S100A7 was quantified by ELISA for proliferating (prol) and differentiated (diff) human keratinocytes treated with buffer control (Ctl) or EC supe. Conditioned media (CM) was collected for analysis at different time points following treatment (n = 4).

(B and C) Fold change of S100A7 secretion was calculated at different time durations for (B) differentiated and (C) proliferating keratinocytes post-EC supe treatment. CM was collected after 24 h (0–24) duration (Dur.), then replaced with fresh media and conditioned for another 24 h (24–28), which was subsequently collected for analysis. To determine the cumulative secretion of S100A7, media was conditioned over the entire time course of the EC-supe treatment (0–48 h) (n = 4).

(D) Relative change in the intracellular (Intra) and secreted (Sec) levels of S100A7 following EC supe treatment of differentiated human keratinocytes. CM was collected, and cell lysates were extracted for analysis at different time points following treatment (n = 3).

(E) Cell lysates were extracted for quantification of phosphorylated and total TLR5 levels at the indicated time points post EC supe treatments. Results are from three different western blots. Band intensity values were normalized with β-tubulin and represented as fold change compared to the buffer-treated cells normalized to 1 (control, blue bar).

(F–H) Following the workflow in Figure S2B, the CM was analyzed for S100A7 secretion after (F) TLR5 inhibition (TLR5 inh), (G) TLR5 knockdown (TLR5-KD), and (H) MyD88 inhibition (n = 3).

Ⓟ, phosphorylated. Data are shown as mean ± SEM. The p values were calculated using two-tailed t test: *p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001; ns, p > 0.05.

differentiation, and is produced and secreted from the differentiated layer of the epidermis (Broome et al., 2003; Marenholz et al., 2001; Martinsson et al., 2005; Ruse et al., 2003).

The importance of S100A7 in both physiological and pathological scenarios has stimulated intense interest in the regulation of its expression. Recent studies have indicated that S100A7 expression is upregulated by proinflammatory cytokines (e.g., interleukin 1 [IL-1]) (Bando et al., 2007; West and Watson, 2010), bacterial components referred to as pathogen-associated molecular patterns (PAMPs) (Abtin et al., 2008; Reithmayer et al., 2009), as well as UV-B radiation (Gläser et al., 2009a). One PAMP in particular, the protein flagellin (a principal component of the flagellum which the bacteria utilizes for motility) has been shown to upregulate S100A7 expression via Toll-like receptor 5 (TLR5) on the target cell (Abtin et al., 2008). In contrast to the number of studies focusing on S100A7 expression, relatively little is known about the secretion of this AMP from differentiated keratinocytes in the granular layer of the epidermis. This is a particularly intriguing area of research given the large number of proteins that are secreted in the unconventional secretory pathway. It is worth noting that S100 proteins lack a secretory signal sequence and thus are not secreted via the conventional secretory pathway comprising the ER-Golgi-plasma membrane route (Donato et al., 2013; Leclerc and Heizmann, 2011). While the majority of the literature emphasizes the regulation of AMPs at the transcriptional level, our investigations focused on the relatively understudied issue of the regulation of S100A7 peptide secretion.

RESULTS

Analysis of S100A7 Secretion Kinetics from Differentiated Human Epidermal Keratinocytes

Although S100A7 is produced by and secreted from the differentiated layers of the epidermis, the majority of *in vitro* studies have utilized proliferating keratinocytes of the basal layer to understand the regulation and biology of this AMP (Gläser et al., 2005; Martinsson et al., 2005). Given the fundamental differences between the differentiated cells of the granular layer versus the proliferating cells of the basal layer, we investigated whether the kinetics and the quanta of S100A7 secretion would be impacted by the differentiation state of the cell (Figure S1A). Previous studies have clearly established that the protein flagellin is an active component in the supernatant of *E. coli* cultures (EC supe) to induce S100A7 expression and secretion (Abtin et al., 2008; Gläser et al., 2005). We therefore measured the effect of either EC supe (Figure 1A) or purified flagellin (Figure S1B) on the amount of S100A7 secreted from primary human epidermal keratinocytes. There was a significant increase in S100A7 released from both proliferating and differentiated keratinocytes treated with EC supe over a 48-h time course. Surprisingly, out of all the conditions tested, the secretion of S100A7 was far more pronounced in differentiated keratinocytes during the 24- to 48-h EC supe treatment. This phenomenon of even higher AMP secretion upon sustained bacterial stimulation was unexpected since most studies have been conducted over relatively short timescales (2–24 h post EC supe treatment), thereby

exploring only an acute response of S100A7 secretion (Abtin et al., 2008). This window of analysis would lead one to assume that later responses would simply maintain a similar quantum of release.

To understand whether the increased S100A7 secretion in the 24- to 48-h time window is due to the accumulation of the peptide in the media, we quantified the amount of S100A7 in freshly conditioned media over the course of 24-h intervals. Surprisingly, we found that even though there is a ~2-fold increase of S100A7 secretion within 24 h of EC supe exposure, there is an even higher amount of this AMP released in the 24- to 48-h period (Figure 1B). In fact, the amount of S100A7 released in the 24- to 48-h interval accounts for nearly all of the secreted S100A7 that can be measured over the 0- to 48-h time interval. In contrast, proliferating keratinocytes exposed to EC supe exhibited roughly the same amount of secreted S100A7 in the 0- to 24- and the 24- to 48-h time intervals (Figure 1C). As such, in case of proliferating keratinocytes, the 0- to 48-h accumulation of secreted S100A7 appears to be the additive result of the two 24-h time intervals. Thus, the use of differentiated keratinocytes as an experimental platform has uncovered a novel regulatory node in the secretion of AMPs.

We then interrogated whether the increase in EC supe-induced S100A7 secretion is partially a reflection of an increased production of this peptide. We found that S100A7 mRNA is increased after 6 h of EC supe treatment, plateaus after 24 h, and maintains that level up to 48 h (Figure S1C). Similarly, the intracellular pool of S100A7 peptide is markedly increased within 6 h and is maximal at 12 h (Figure 1D). Since the ratio of the secreted versus intracellular pools of S100A7 increases over time, we hypothesized that an unconventional transport machinery has been stimulated above basal activity to accommodate the excess amount of S100A7 produced in response to EC supe. This led us to inquire how S100A7 secretion is enhanced and sustained beyond 24 h post bacterial stimulation.

The Acute Phase of S100A7 Is Dependent on TLR5 Signaling, whereas the Chronic Phase Is TLR5 Independent

We then focused our efforts on understanding the molecular mechanisms regulating S100A7 release from differentiated keratinocytes. Since flagellin in the EC supe is a ligand for TLR5 (Abtin et al., 2008), we assessed the kinetics of TLR5 signaling activity by monitoring its phosphorylation status (Ivison et al., 2007) at various time intervals (12, 24, and 48 h). Interestingly, we observed that TLR5 phosphorylation (activity) is maximal by 12 h and starts to decrease at 24 h (Figures 1E and S2A). We therefore considered 0–24 h as an acute response to bacterial exposure in differentiated keratinocytes. Surprisingly, we observed that phosphorylated and total levels of TLR5 are decreased in the 24- to 48-h interval (Figure 1E) despite the fact that S100A7 secretion is highest in this chronic phase (Figure 1B).

To probe the functional role of TLR5 in S100A7 secretion in the acute and chronic phases, we inhibited TLR5 activation using a neutralizing antibody (Figures S2B and S2C) or downregulated TLR5 using small hairpin RNA (shRNA)-mediated knockdown (Figure S2D). Both of these approaches resulted in a significant decrease of S100A7 secretion during the acute phase (0–24 h) (Figures 1F and 1G). However, these strategies of impairing TLR5 signaling had a negligible effect on S100A7 secretion during the chronic phase (24- to 48-h time period). Another approach to interrogate the transient requirement of TLR signaling in S100A7 secretion is to inhibit MyD88, which is downstream of many TLR family members (including TLR5). Consistent with our previous results, generic inhibition of TLR signaling was sufficient to reduce secretion of S100A7 in the acute phase but not during chronic phase (Figure 1H). Utilizing multiple modalities of impairing TLR5 signaling have all pointed to the fact that TLR5 signaling is functional and required during the acute phase, but an additional signaling pathway(s) is responsible for the increase of S100A7 secretion during the chronic phase.

p38MAPK, NFκB, and Caspase-1 Comprise the Core Cytosolic Machinery Mediating S100A7 Secretion

The canonical downstream pathway of the TLR5 signaling cascade is composed of both p38MAPK and NFκB (Yu et al., 2003). Surprisingly, although TLR5 signaling is required only during the acute phase, we observed that both p38MAPK and NFκB remain active even during the chronic phase of S100A7 secretion (Figures 2A, 2B, and S3A). Short-circuiting multiple TLR signaling pathways through the inhibition of MyD88 further supported the notion that TLR signaling is only required in the acute phase, whereas p38MAPK/NFκB activities are TLR independent in the chronic phase (Figure S3B). This finding was validated by analyzing the target genes of NFκB (CCL5/RANTES, CXCL2, CXCL5, IL-1α, and IL-18) in the presence of MyD88 inhibitor or TLR5-neutralizing antibody (Figure S3C). Consistent with our previous observations, the expression of these target genes was dependent on TLR signaling during the acute phase but remained active during the chronic phase in a TLR-independent fashion. In line with the sustained activity of both proteins, pharmacological inhibition of either p38MAPK or NFκB results in the suppression of EC supe-mediated S100A7 secretion in both the acute and chronic phases (Figures 2C and S3D).

Of particular note, we previously discovered that both p38MAPK and NFκB are critical regulators of a macromolecular complex that is responsible for the secretion of many non-canonical secretory proteins (Lee et al., 2009, 2015). This complex is known as the inflammasome, and its active component is the protein Caspase-1 (Feldmeyer et al., 2010). To test whether the activation of Caspase-1 impacts the secretion of S100A7, we analyzed the activity of Caspase-1 in differentiated keratinocytes treated with EC supe. We found that Caspase-1 proteolytic activity is elevated in both the acute and chronic phases, which can be specifically abrogated by its pharmacological inhibitor (YVAD-CHO) (Figure 2D). It is important to note that Caspase-1 activity remains significantly higher compared to buffer treated samples in the chronic phase, despite the fact that the TLR5 signaling pathway is not operational during this phase (Figure 1E). Similar to the kinetic profile of p38MAPK and NFκB activity, Caspase-1 is dependent on TLR5 signaling in the acute phase but is TLR5 independent in the chronic phase of

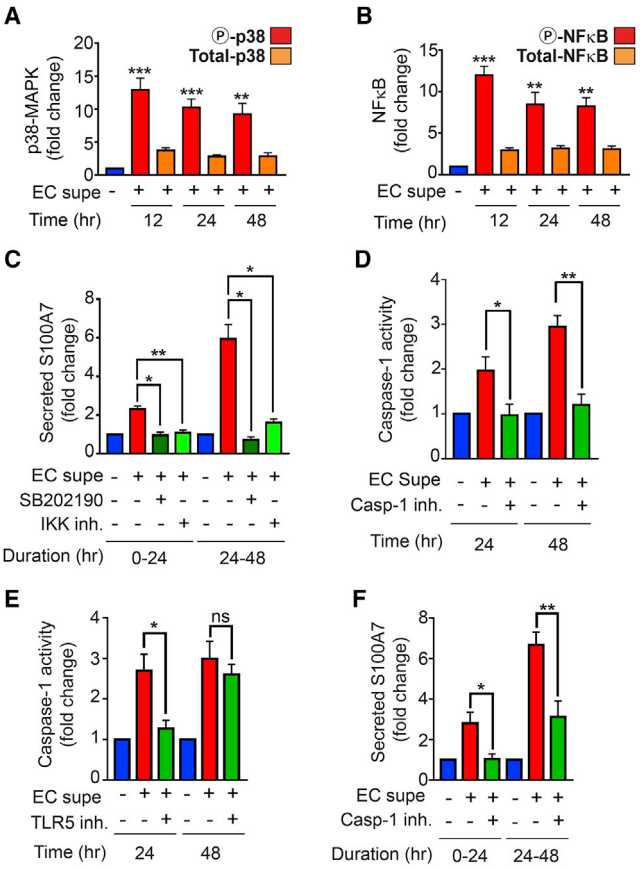

Figure 2. p-38MAPK, NFκB, and Caspase-1 Activity Are Required in Both the Acute and Chronic Phases of S100A7 Secretion

(A and B) Cell lysates were extracted for quantification of phosphorylated and total protein levels of (A) p38-MAPK and (B) NFκB at the indicated time points post EC supe treatment. Results are from three different western blots. Band intensity values were normalized with β-actin and represented as fold change compared to the buffer-treated control (blue bar).

(C) Effect of p38-MAPK (SB202190) and NFκB (IKK inh.) inhibitors on S100A7 secretion. The experiment was conducted as outlined in Figure S2B (n = 3).

(D and E) Fold change in Caspase-1 activity at different time points post EC supe treatment in presence or absence of (D) Caspase-1 inhibitor (Casp-1 inh) and (E) TLR5 inhibitor (n = 4).

(F) Fold change in S100A7 secretion at different time intervals post EC supe treatment in presence or absence of Caspase-1 inhibitor. The experiment was conducted as outlined in Figure S2B (n = 3).

Ⓟ, phosphorylated. Data are shown as mean ± SEM. The p values were calculated using one-way ANOVA with Dunnett's test (A and B) or two-tailed paired t test (C–F): *$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$; ns, $p > 0.05$.

S100A7 secretion (Figure 2E). This indicates that TLR5 is dispensable in activating the inflammasome pathway in the chronic phase and is reminiscent of previous findings of TLR5-independent inflammasome activation in macrophages (Matusiak et al., 2015). Regardless of TLR5 dependency, Caspase-1 activity is required for the EC supe-stimulated release of S100A7 (Figure 2F). Altogether, these findings suggest that p38MAPK, NFκB, and Caspase-1 form the core cytosolic signaling node that is utilized in both the acute and chronic phases of S100A7 secretion.

IL-1α Is Necessary and Sufficient to Induce S100A7 Secretion

These core components are well known to regulate the non-canonical secretion of proinflammatory cytokines such as IL-1α. IL-1α has been shown to upregulate the expression of the S100 family of AMPs (Bando et al., 2007; Guilloteau et al., 2010; West and Watson, 2010), but whether this cytokine also has an effect on AMP secretion is unknown. We therefore proceeded to determine whether IL-1α is necessary and sufficient to induce S100A7 secretion. Interestingly, the kinetics of IL-1α secretion (Figure 3A) closely tracked that of S100A7 release from differentiated epidermal keratinocytes (Figures 1A and 1B). IL-1α secretion was elevated at 12 h and was substantially increased by 48 h post EC supe treatment. In line with Bando et al., 2007, we also observed that an increase in IL-1α mRNA (Figure S4A) correlates with an increase in S100A7 mRNA (Figure S1C). Importantly, we observed that inhibition of p38MAPK and NFκB activity resulted in a significant decrease of IL-1α secretion in the acute and chronic phases, suggesting that IL-1α is downstream of these proteins during both phases. (Figures S4B and S4C).

We further found that recombinant IL-1α is sufficient to induce S100A7 expression (Figure S4D) and secretion (Figure 3B). Likewise, IL-1α signaling is necessary for EC supe-mediated secretion of S100A7, since cells pretreated with an IL-1R antagonist (IL-1RA) significantly reduced the secretion of S100A7 in both the acute and chronic phases (Figure 3C). To determine whether IL-1α secretion in the acute phase is a product of TLR5 signaling, we examined the effect of the TLR5 inhibition on the release of IL-1α in keratinocytes treated with EC supe. We found that antibody-mediated neutralization (Figure 3D) or knockdown of TLR5 (Figure 3E) reduces IL-1α secretion during the acute phase but not in the chronic phase. We have also tested the possibility reported in the literature that S100A7 can itself induce IL-1α secretion (Lei et al., 2017). In contrast to the reported effect in proliferating keratinocytes, we found that S100A7 treatment of differentiated keratinocytes does not significantly elevate IL-1α mRNA or its secretion from the cell (Figures S4E and S4F). Furthermore, treatment of recombinant S100A7 does not result in the upregulation of its own mRNA in differentiated keratinocytes (Figure S4G).

Altogether, these observations suggest that IL-1α secretion is a common contributor to the release of S100A7 in both the acute and chronic phases. These findings are consistent with the inflammatory skin disease psoriasis, which is marked by elevated levels of both IL-1α and S100A7 (also known as Psoriasin) (Mee et al., 2006). Overall, these data suggest a signaling axis composed of NFκB/p38MAPK/Caspase-1/IL-1α, which regulates S100A7 secretion in both the acute and chronic phases, but what differs between the phases are the upstream stimuli.

The Downregulation of Caspase-8 Mediates the Chronic Release of S100A7

We therefore investigated the TLR-independent mechanism that activates this signaling node in the chronic phase of S100A7 secretion. We previously found that the wound-induced downregulation of Caspase-8 leads to the activation of the inflammasome complex (Lee et al., 2009). Moreover, this mode of

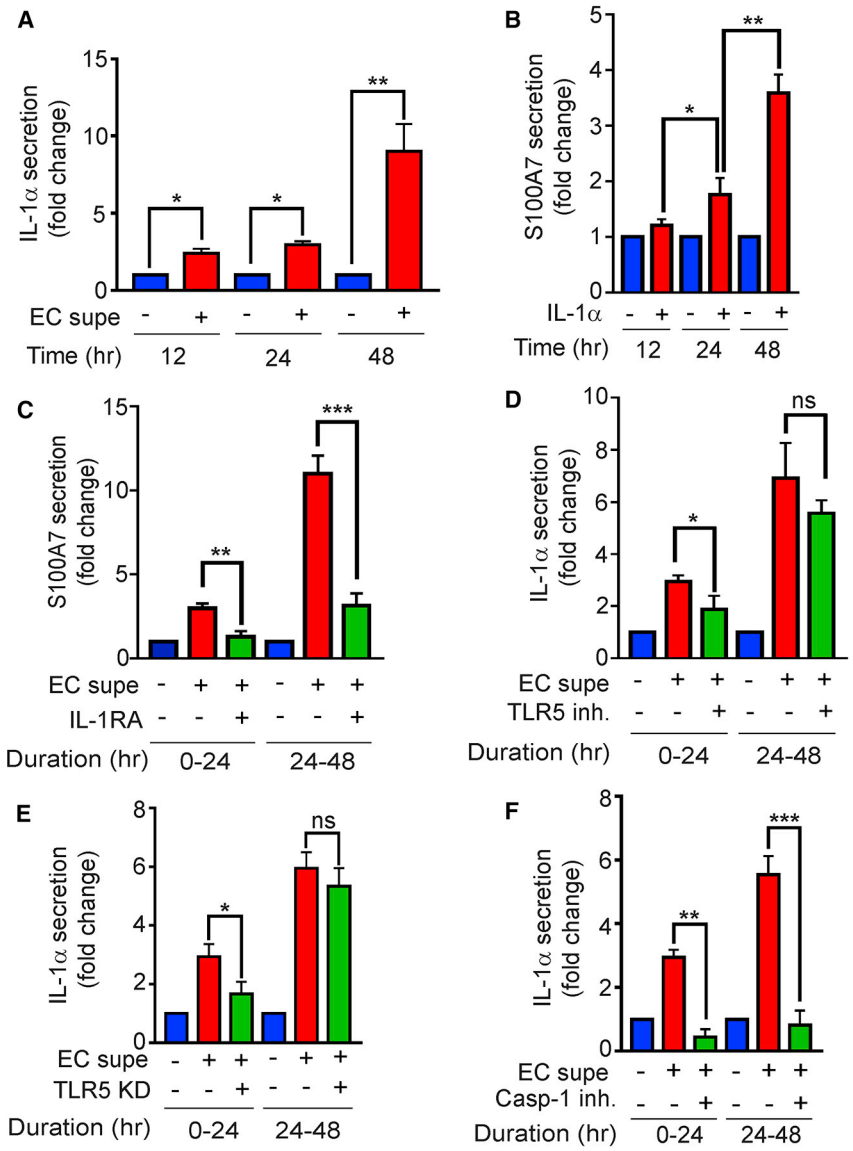

Figure 3. Role of IL-1α Signaling in the Acute and Chronic Phases of S100A7 Secretion

(A) Secretion of IL-1α was quantified by ELISA from EC supe-treated differentiated keratinocytes and converted to fold change relative to buffer-treated cells at the respective time points (blue bar) (n = 3).

(B) Effect of IL-1α on S100A7 secretion was assessed at different time points (n = 4).

(C) Effect of IL-1 receptor antagonist (IL-1RA) on S100A7 secretion was assessed at different durations (Dur.) post EC supe treatments (n = 3).

(D) Effect of TLR5 inhibitor (TLR5 inh) on IL-1α secretion was assessed at different time points post EC supe treatment (n = 3).

(E) Effect of TLR5 knockdown on IL-1α secretion was assessed at different time points post EC supe treatment (n = 3).

(F) Effect of Caspase-1 inhibitor (Casp-1 inh) on IL-1α secretion was assessed at different time points post EC supe treatment (n = 3).

For (C–F), the experiment was conducted as outlined in Figure S2B. Data are shown as mean ± SEM. The p values were calculated using two-tailed t test: *p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001; ns, p > 0.05.

inflammasome activation and function utilizes the same complex of proteins: p38MAPk/NFκB/Caspase-1/IL-1α (Lee et al., 2015). Thus, we hypothesized that the downregulation of Caspase-8 might play a role in the chronic phase of S100A7 secretion. Consistent with our hypothesis, caspase-8 mRNA levels significantly decrease as early as 12 h post EC supe treatment and remained low through the 48-h time point (Figure 4A). However, the protein level of Caspase-8 is significantly reduced only within the 24- to 48-h time interval, which happens to correspond to the chronic phase of S100A7 secretion (Figures 4B and S5A). It is important to note that EC supe treatment does not result in the catalytic cleavage of Caspase-8 but only in the reduction of its full-length form (Figure S5A), which is consistent with the emerging concept of a non-apoptotic role for Caspase-8 (Lee et al., 2009; Maelfait and Beyaert, 2008).

Furthermore, we investigated the mode by which EC supe treatment reduces caspase-8 expression: does it accelerate the turnover of caspase-8 RNA and protein or inhibit its *de novo* production? We observed that EC supe treatment mirrors the effect of inhibiting *de novo* synthesis of caspase-8 RNA and protein (Figures S5B and S5C). This suggests that bacterial stimulation does not induce the acceleration of caspase-8 mRNA or protein turnover but rather relies on its natural half-life (approximately 24 h) to reduce Caspase-8 levels. This may explain why Caspase-8-mediated signaling operates only during the chronic phase (24–48 h) of S100A7 secretion.

To test the functional role of Caspase-8 protein downregulation in inducing S100A7 secretion, we performed shRNA-mediated knockdown of the human caspase-8 gene (Figure S5D). Merely knocking down caspase-8 is sufficient to significantly increase the secretion of S100A7 (Figure 4C), as well as IL-1α (Figure 4D). Moreover, inhibition of the IL-1 signaling in the caspase-8 knockdown cells perturbed the elevated secretion of S100A7 (Figure 4E). Consistent with our previous report of a non-canonical role for Caspase-8 in the wound-healing response (Lee et al., 2009), pharmacological inhibition of Caspase-8's enzymatic activity did not alter S100A7 secretion (Figure S5E).

To test the necessity of Caspase-8 downregulation for S100A7 secretion in the chronic phase, we ectopically expressed a catalytically dead form of this protein. This approach restored the intracellular pool of Caspase-8 to control levels even in the presence of EC supe (Figure S5F). Surprisingly, the failure to reduce Caspase-8 expression ameliorates the surge in the S100A7 and IL-1α secretion only in the chronic phase. However, their

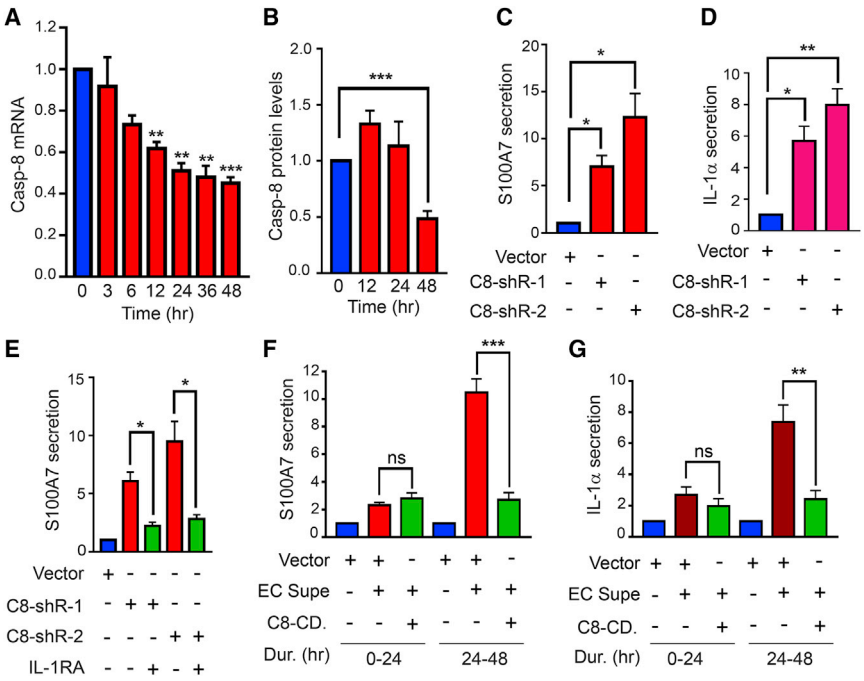

Figure 4. Downregulation of Caspase-8 Protein Regulates the Chronic Release of S100A7

(A) qPCR analysis of caspase-8 mRNA expression at different time points post EC supe treatment. Data are represented as fold change relative to 0 h EC supe treatment (blue bar) (n = 3).

(B) Quantification of Caspase-8 protein levels at the indicated time points post EC supe treatments from three different western blots. Band intensity values were normalized with GAPDH and represented as fold changes compared to the control (0 h EC supe treatment, blue bar) (n = 4).

(C and D) Effect of shRNA-mediated caspase-8 knockdown using two different constructs (C8-shR-1 or C8-shR-2) on the secretion of (C) S100A7 and (D) IL-1α (n = 3).

(E) Effect of IL-1 receptor antagonist (IL1-RA) on the secretion of S100A7 in the caspase-8 knockdown keratinocytes. Data are represented as fold change relative to cells transfected with empty vector (n = 3).

(F and G) Effect of Caspase-8 ectopic expression (C8-CD) on the secretion of (F) S100A7 and (G) IL-1α at 24- and 48-h durations (Dur.) post EC supe treatment.

Data are shown as mean ± SEM. The p values were calculated using one-way ANOVA with Dunnett's tests (A) or two-tailed t test (B–G): *$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$; ns, $p > 0.05$.

secretion in the acute phase, which is TLR5 dependent, remains unaltered (Figures 4F and 4G). These data implicate the downregulation of Caspase-8 as a sufficient as well as necessary mechanism to sustain the long-term secretion of S100A7.

The Correlation between S100A7 Expression and Caspase-8 Downregulation Is Relevant in Normal and Diseased Human Skin

Based on the *in vitro* results, we tested whether there is a connection between Caspase-8 downregulation and human skin conditions marked by elevated levels of AMP secretion. We first examined whether EC supe is capable of affecting S100A7 levels in human skin explants. We observed that compared to their buffer treated controls, both acute and chronic phases showed increased levels of S100A7 in the epidermis (Figures 5A and S6A). Interestingly, the 48-h treatment showed qualitatively higher levels of S100A7 compared to the 24-h EC supe treatment, which is consistent with the kinetics of S100A7 secretion observed *in vitro* (Figure 1B). In parallel, we also observed that epidermal Caspase-8 protein levels were reduced in a time-dependent fashion upon EC supe treatment (Figure 5B).

Since heightened S100A7 (also known as Psoriasin) secretion is a signature for psoriatic skin (Figure S6B) (Lai and Gallo, 2009), we examined whether this is concomitant with a change in epidermal Caspase-8 levels. Along with epidermal hyperplasia, we found that the lesional skin from a psoriatic patient exhibited a dramatic downregulation of Caspase-8 in the differentiated layers of epidermis, compared to non-lesional areas (Figure 5C). This correlation can also be extended to other pathological conditions such as cancers, which exhibit both a downregulation of Caspase-8 and elevated levels of AMPs (Fulda, 2009; Kaur et al., 2014; Moubayed et al., 2007). These results suggest that the chronic phase of S100A7 secretion is under the regulatory control of epidermal Caspase-8 levels. Furthermore, they support the increasingly popular paradigm that pathological and pathogen-induced conditions are marked by a wound signature.

DISCUSSION

Altogether, our data suggest that PAMPs induce AMP secretion in a biphasic manner (Figure S7A). The early acute phase of S100A7 secretion is largely dependent on TLR5 signaling, whereas chronic and sustained release utilizes Caspase-8 downregulation. Although the upstream regulators differ, the downstream core components namely p38MAPK, NFκB, Caspase-1, and IL-1α are the common node for the secretion of S100A7. The correlative evidence of these core components in regulating AMP secretion in various pathological and physiological scenarios suggests that modulating these signaling cascades may be an effective therapeutic strategy. Atopic dermatitis and psoriasis (Figure S6B) are such examples, in which S100A7 secretion correlates with the activation of p38MAPK, NFκB, Caspase-1, and IL-1α (Mavropoulos et al., 2013; Niebuhr et al., 2014). In addition, UV irradiation, which also results in the activation of Caspase-1, is likewise accompanied by increased S100A7 secretion from epidermal keratinocytes (Dittlein et al., 2016; Gläser et al., 2009a). Our results are consistent with a secretome analysis, which revealed that among the plethora of cytokines whose release from proliferating keratinocytes was dependent on caspase-1 were several AMPs (Keller et al., 2008).

Interestingly, the core components remain active in the chronic phase but are mediated by the downregulation of Caspase-8. We have previously shown that caspase-8 downregulation in the epidermis is a normal response to tissue damage

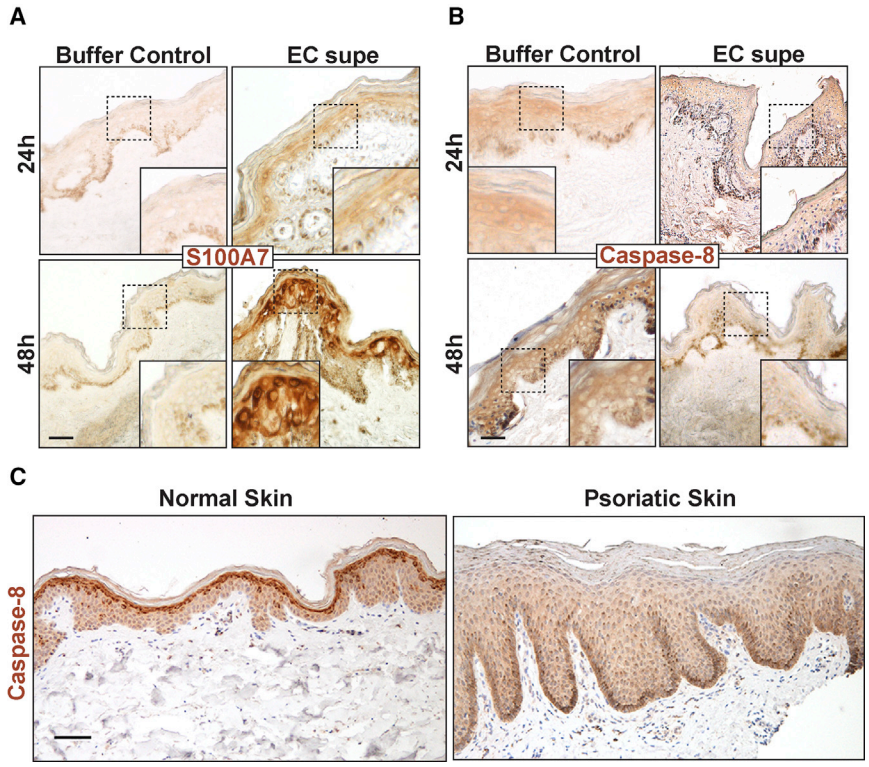

Figure 5. Correlation between S100A7 Upregulation and Caspase-8 Downregulation in EC Supe-Treated Human Skin Explants

(A and B) Immunohistochemical staining of (A) S100A7 and (B) Caspase-8 on human skin explants with EC supe or buffer treatments for 24 and 48 h (representatives of n = 3).

(C) Immunohistochemistry of Caspase-8 expression in normal and psoriatic human skin sections (representatives of n = 3). Insets are magnified views of the area outlined by the dotted box. Scale bar: 20 μm.

and its genetic ablation is sufficient to induce the wound healing program in the absence of injury (Lee et al., 2009). Similarly, we have found that merely reducing the levels of Caspase-8 in differentiated human keratinocytes is sufficient to induce the secretion of S100A7 (Figure 4C). Furthermore, the downregulation of Caspase-8 can function as an initiator of the NLRP3 inflammasome, which regulates IL-1α secretion in a wound microenvironment (Yazdi and Drexler, 2013). This may be the mechanistic basis by which the wound-healing response achieves the heightened release of AMPs to combat microbial infection through the compromised skin barrier (Lee and Eckert, 2007). In addition, there are several reports of pathological conditions exhibiting heightened AMP secretion that utilizes this same intracellular wound-healing machinery. For instance, we have previously shown that atopic dermatitis exhibits Caspase-8 downregulation (Li et al., 2010), and it has been reported that AD skin secretes elevated levels of S100A7 (Gläser et al., 2009b). In this manuscript, we demonstrate that psoriatic skin, which also releases excessive levels of S100A7 (Figure S6B), likewise exhibits epidermal Caspase-8 downregulation (Figure 5C). This correlation also extends to several cancers, where Caspase-8 downregulation is observed along with secretion of S100A7 (Fulda, 2009; Kaur et al., 2014; Moubayed et al., 2007).

In part, due to these activities, cancer has been portrayed as an over-healing wound (Schäfer and Werner, 2008). In all, these scenarios suggest that the wound-healing machinery is reiteratively utilized in both physiological and pathological conditions. While skin diseases that perturb epidermal integrity like atopic dermatitis (Li et al., 2010) and skin wounds (Lee et al., 2009) activate Caspase-8 mediated signaling, routine skin cleansing with commercial bars and body washes had no impact and Caspase-8 levels remained generally unchanged (Figure S7B). How caspase-8 downregulation is achieved upon PAMP stimulation, wound healing, or in inflammatory skin diseases remains an intriguing but unresolved question.

The use of a differentiated keratinocyte system has allowed us to elucidate a sustained and robust secretion of AMP release, which has not been reported for proliferating keratinocytes. Our observation that the chronic phase is TLR5 independent is consistent with reports in other cell types. Matusiak et al. (2015) have shown in bone marrow-derived macrophages that flagellin-mediated NLRC4 and NAIP5 inflammasome activation is TLR5 independent. This was found to be due to cytosolic flagellin, which could be the result of the phagocytic activity of macrophages. Keratinocytes, on the other hand, do not show such phagocytic activity. Moreover, TLR5 is a surface-bound receptor (Lee and Barton, 2014), which has not been shown to mediate flagellin uptake in differentiated keratinocytes. Given the relative instability and susceptibility for degradation by proteases that are prevalent on the skin surface (Mahlapuu et al., 2016; de Veer et al., 2014), the sustained release of AMPs may address the deficits posed by the transient nature of this peptide. Sustained release of AMPs may also reflect their diverse functions that range from mediating chemotaxis, angiogenesis, antigen presentation, and immunomodulation that extends over multiple phases of the wound-healing response (Mandal et al., 2007; Mangoni et al., 2016; Wolf et al., 2008). The chronic release of AMP also raises the question of how this response is ultimately terminated. Since keratinocytes in the granular layer are near the end of the terminal differentiation program, it is likely that cell death (cornification) would limit the production and secretion of this peptide.

Here, we have used the differentiated keratinocytes as an experimental system and have observed that differentiation status indeed impacts the S100A7 expression and secretion upon PAMP stimulation. A previous report on lipopolysaccharide (LPS)-mediated stimulation suggests an increase in several differentiation markers, mainly tight junction proteins in epidermal

keratinocytes (Yuki et al., 2011). In parallel, our observations suggest that EC supe treatment has a modest effect on the differentiation status of pre-differentiated keratinocytes (Figure S7C). It has also been reported that S100A7 protein can modulate the differentiation status of epidermal keratinocytes (Hattori et al., 2014; Son et al., 2016). Similar to Hattori et al., S100A7 treatment increased the differentiation markers in proliferating keratinocytes (Figure S7D) but had a negligible effect on differentiated keratinocytes (Figure S7E). It is thus emerging that the differentiation state of human epidermal keratinocytes affects its response to PAMP exposure.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.celrep.2019.10.090.

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AUTHOR CONTRIBUTIONS

Conceptualization, T.B., A.M., and C.J.; Methodology, T.B., A.M., and C.J.; Investigation, T.B., A.B., B.B., M.S.M., and A.R.; Writing – Original Draft, T.B., A.B., B.B., A.M., and C.J.; Writing – Review & Editing, T.B., A.B., B.B., A.M., and C.J.; Funding Acquisition, C.J. and A.M.; Resources, M.S.M., A.R., G.S., and A.M.; Supervision, T.B., C.J., and A.M.

DECLARATION OF INTERESTS

C.J. receives consultancy fees from Unilever R&D Bangalore. M.S.M. and A.M. are employees of Unilever R&D Bangalore.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
anti-hTLR5-IgG	InvivoGen	Cat # pab-hstlr5; RRID:AB_11124931
Caspase-8	CST	Cat #9746; RRID:AB_2275120
phospho-p38MAPK	CST	Cat #4511; RRID:AB_2139682
p38MAPK	CST	Cat #8690; RRID:AB_10999090
phospho-NFκB	CST	Cat #3033; RRID:AB_331284
NFκB	CST	Cat #4764; RRID:AB_823578
S100A7	Novus	Cat #NB100-56559; RRID:AB_838744
phospho-TLR5 (Tyr798)	Signalway Antibody	Cat #12648
TLR5	Thermo	Cat #MA5-16237; RRID:AB_2537755
β-catenin	Sigma	Cat #C7082; RRID:AB_258995
β-tubulin	CST	Cat #2146; RRID:AB_2210545
β-Actin	CST	Cat #4970; RRID:AB_2223172
GAPDH	Abcam	Cat #ab8245; RRID:AB_2107448
Bacterial and Virus Strains		
E.coli	ATCC	10536
Biological Samples		
Ex-vivo Treated Skin Explants (Indian Origin)	Sapien Biosciences, Hyderabad	N/A
Normal skin sections, Psoriatic skin sections, and Cleanser treated skin explants (Caucasian Origin)	Genoskin, Toulouse	N/A
Chemicals, Peptides, and Recombinant Proteins		
MyD88 inhibitory peptide	InvivoGen	Cat# tlrl-pimyd
Caspase-1 inhibitor, YVAD-CHO	Merck Millipore	Cat# 400011
p38-MAPk inhibitor, SB202190	Sigma	Cat# S7067
p38-MAPk inhibitor, PD169316	Merck	Cat# 513030
IKK inhibitor VII	Merck Millipore	Cat# 401486
NFκB inhibitor, BAY 11-7085	Cayman	Cat#14795
Caspase-8 Inhibitor	Calbiochem	Cat#218773
IL-1 Receptor Antagonist (IL1RA)	Sigma	Cat#SRP3327
Flagellin protein (recombinant)	Abcam	Cat#ab201366
Human Recombinant IL-1α	R&D	Cat#200LA002
Human S100A7	MBL	Cat #CY-R2257
EpiLife Media	GIBCO	Cat #MEPI500CA
Growth Suplement (HKGS)	GIBCO	Cat #S0015
Transfection Reagent (Keratinocytes)	Mirus	Cat #MIR 2804
Transfection Reagent (Lipofectamine3000)	Invitrogen	Cat #L3000008
G418	GIBCO	Cat #10131035
SsoFast (qPCR master mix)	BioRad	Cat #172-5201
Critical Commercial Assays		
ELISA for S100A7	CircuLex	# CY-8073
ELISA for IL-1α	R&D	# DLA50
Caspase-1 Activity Assay	Abcam	# ab39412
HRP Anti-Mouse Polymer Detection Kit	Vector	# MP-7402

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Experimental Models: Cell Lines		
Human Epidermal Keratinocytes (Caucasian Origin)	Invitrogen	#C0015C
Human Epidermal Keratinocytes (Indian Origin)	Lonza	#192907
293T	ATCC	# CRL-3216
Oligonucleotides		
qPCR primers for various genes	This paper	Table S2
Recombinant DNA		
Plasmid: caspase-8 shRNA (SHCLNG-NM_033356) 1	Sigma	Clone NM_033356.3-415s21c1
Plasmid: caspase-8 shRNA (SHCLNG-NM_033356) 2	Sigma	Clone NM_033356.3-547s21c1
Plasmid: TLR5 shRNA (SHCLNG-NM_003268)	Sigma	Clone NM_003268.5-1546s21c1
Plasmid for Caspase-8 catalytic mutant (pcDNA3-Casp8 C360A)	Addgene – Dr. Guy Salvesen	Cat #11818
Plasmid: pMD2.G	Addgene – Dr. Didier Trono	Cat #12259
Plasmid: psPax2	Addgene – Dr. Didier Trono	Cat #12260
Software and Algorithms		
ImageJ (Fiji)	Schindelin et al., 2012	https://fiji.sc/
Excel 2010	Microsoft	https://products.office.com/en-us/previous-versions/microsoft-excel-2010?rtc=1
Prism version 7.0	GraphPad	https://www.graphpad.com/

LEAD CONTACT AND MATERIALS AVAILABILITY

This study did not generate new unique reagents. Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Colin Jamora (colinj@instem.res.in)

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Human Subjects

Human skin samples were obtained from Sapien Biosciences (Hyderabad, India) and Genoskin (Toulouse, France). All the skin samples and sections were from anonymized female donors from 30 to 60 years of age. With regards to Sapien Biosciences Ethics approval, their company website states that they work within ICMR (Indian Council of Medical Research) and international guidelines and follows high ethical norms with respect to transparency and patient privacy. Sapien banks samples after hospital ethics committee approval and with informed consent (the language of the consent form follows ICMR guidelines). Sapien's Institutional Ethics Committee (IEC) reviews and approves each project. With regards to Genoskin, their protocol involves the following (extracted from the company website): Before the start of the study, research protocols must be submitted to the Ethics Committee for approval. The functioning of the committee should be transparent, members should be duly qualified and be independent of the researcher, the sponsor, and any other undue influences. Individuals can participate in medical research on an entirely voluntary basis by signing an Informed Consent Form that lists aims, methods, sources of funding, any possible conflicts of interest, institutional affiliations of the researcher, the anticipated benefits and potential risks of the study and the discomfort it may entail, post-study provisions as well as any other relevant aspects of the study. Informed Consent is equally required to collect, store or use material or data contained in biobanks or similar repositories. There may be exceptional situations where consent would be impossible or impracticable to obtain for such research, in which case the research needs to be considered and approved by a research ethics committee. Genoskin has Biological Sample Transfer Contracts with hospitals and clinics with regard to the skin tissue we use for our human skin models. The Declaration of Helsinki also sets the framework for the ethical and unbiased publication of results.

Work in the Jamora lab with human skin samples was approved by the Institutional Ethics Committee of inStem [Certificate No. inStem/IEC-5/002 (valid from September 2018 – August 2021)].

Primary Cell Cultures

Primary neonatal human epidermal keratinocytes were purchased from commercial vendors noted in the Key Resources Table.

Microbe Strains

Escherichia coli strain was purchased from ATCC (Catalog number 10536)

METHOD DETAILS

EC Supe Preparation and Keratinocyte Stimulation

Bacterial culture (strain ATCC 10536) supernatants (EC supe) were prepared as described elsewhere (Abtin et al., 2008). Briefly, bacteria were grown in tryptic soy broth (TSB) with agitation at 37°C until an optical density of 1.0 (OD600) was reached. 1 mL of this culture was combined with 9 mL of TSB and incubated overnight in T75 flasks (BD Falcon) at 37°C without agitation. Optical density (OD600) of overnight cultures of *E. coli* was set with TSB to 1.7. Subsequently, bacteria were heat-killed in a water bath at 65°C for 60 min. After heat-killing, cultures were centrifuged at 5000xg for 15 min. The resulting supernatants were diluted 1:100 in culture media and used to stimulate keratinocytes. Sterilized TSB without any bacterial component was used as a control buffer.

Cell Culture and Treatments

Experiments were carried out using neonatal human epidermal keratinocytes (see Key Resources Table). Keratinocytes were cultured as proliferating cells in T75 flasks (BD, falcon) using EpiLife (GIBCO #MEPI500CA) medium with growth supplement (HKGS, GIBCO #S0015). Keratinocyte differentiation was achieved by growing them to 100% confluence followed by increasing calcium concentration to 2 mM in EpiLife (without HKGS) media for 48 hours. Keratinocytes were treated with the inhibitors/antibodies, and conditioned media were collected as shown in Figure S2B, except for MyD88 peptide inhibitor, which was incubated for 12 hr before EC supe treatment. To induce Caspase-8 cleavage (positive control), UV-B irradiation was used (Figure S5A). As described by Takasawa et al., UV-C induces apoptosis via intrinsic pathway, involving Caspase-9, whereas UV-B induces Caspase-8 cleavage (Takasawa et al., 2005). Various inhibitors, stimulants, and their concentrations used in this manuscript are listed in Table S1.

Lentiviral Production, Transduction, and Transfection

Plasmids expressing shRNAs were obtained from Sigma (caspase-8: SHCLNG-NM_033356; Clone NM_033356.3-415s21c1, and Clone NM_033356.3-547s21c1 and TLR5: SHCLNG-NM_003268; Clone NM_003268.5-1546s21c1). To produce viruses, 293T cells were transfected with psPAX2, pMD2.G, and either empty vector or shRNA- containing plasmids, using Lipofectamine3000 (Invitrogen) transfection reagents according to the manufacturer's protocol. Following a 48 hr transfection, the virus particle-containing media was harvested and added to the differentiated cells for 24 hr. Expression of Caspase-8 and TLR5 genes were measured two to three days after viral infection. Silencing efficiency was confirmed by immunoblotting. Plasmid for Caspase-8 catalytic mutant (pcDNA3-Casp8 C360A) was a gift from Guy Salvesen (Addgene plasmid #11818; http://addgene.org/11818). The plasmid was transfected using Keratinocyte transfection reagent (Mirus) as per manufacturer protocol. Transfected cells were selected using G418 antibiotic (GIBCO).

ELISA

Secretion of S100A7 from keratinocytes was measured using the CircuLex S100A7 ELISA kit (#CY-8073) and IL-1α was measured using R&D System Human IL-1 alpha Quantikine ELISA Kit (#DLA50), as per manufacturer's protocol. The absorbance was measured at 450 nm using the microplate reader (Tecan)

Quantitative Real-Time PCR

RNA was isolated from human keratinocytes (proliferating or differentiated) using the RNAiso Plus (Takara). 1 μg of RNA was used to prepare cDNA using the PrimeScript kit (Takara). cDNA equivalent to 100 ng of RNA was used for setting up the qPCR reaction using the SsoFast 2x master mix (BioRad). All reactions were performed in technical triplicates using the CFX384 Touch Real-time PCR detection system (BioRad). The primers used for the measurements have been listed in Table S2.

Western Blot Analysis

Protein expression analysis was done as described previously (Lee et al., 2015). In brief, keratinocyte lysates were prepared in RIPA buffer with protease inhibitors (Sigma, #P2714) and sonicated at 4°C. Lysate protein was mixed with 4X Laemmli sample buffer, heated at 95°C for 3 min and loaded on 8%, 10% or 15% polyacrylamide gel, followed by transfer onto a nitrocellulose membrane. After transfer, the membrane was blocked using 6% BSA (Bovine Serum Albumin) in Tris buffer Saline containing 0.1% Tween (TBST) for 60 min. The blots were probed overnight with the respective primary antibody (see Key Resources Table). After washing with TBST, the blots were probed with HRP-tagged secondary antibodies and washed again. Signals were detected using Enhanced Chemiluminescence substrate (ECL, Merck) and iBright FL (Thermo) detector. The bands were quantified using FIJI (ImageJ) software and normalized to loading controls.

Enzyme-Substrate Assay

The enzymatic activity of Caspase-1 was determined with some modifications to the manufacturer's protocol (enzyme assay kit from Abcam, #ab39412). Briefly, keratinocytes were lysed in hypotonic lysis buffer, and supernatant was collected via centrifugation at 14,000xg for 5 min at 4°C. 30 μL supernatant from each condition was mixed with 30 μL of 2x reaction buffer (with DTT) and 3 μL of 1mM YVAD-AFC substrate. The reaction mixture was incubated at 37°C for 1-2 hr. in a 96-well dish. The Caspase-1 activity was measured (Ex/Em = 400/505 nm) using Spectramax Microplate reader (Molecular Devices).

Tissue Preparation and Immunohistochemistry Staining

Human skin explants treated with EC supe for different time periods were obtained from Sapien Biosciences, Hyderabad. Sections of lesional and non-lesional skin from psoriatic patients, and Cleanser treated skin samples were obtained from Genoskin, Toulouse. Skin samples were fixed in 10% buffered formalin for 48 hours at room temperature and processed for paraffin wax embedding. Skin sections were stained with primary antibodies post antigen unmasking with 10 mM sodium citrate (pH 6) at 95°C for 20 minutes. Antibodies used were Caspase-8 (CST #9746) and S100A7 (Novus #NB100-56559). Polymer HRP Anti-Mouse detection kit and DAB chromogen (Vector Labs) were used according to manufacturer's instructions to develop the signal. Images were obtained on Olympus IX73 or Leica DMi1 microscope and analyzed using ImageJ software.

QUANTIFICATION AND STATISTICAL ANALYSIS

Information on statistical tests are mentioned in the figure legends. In general, comparison of two groups were done using paired or unpaired, two-tailed Student's t test. One-way ANOVA followed by Dunnett's post hoc analysis was used for multiple group comparisons. GraphPad Prism or Microsoft Excel software were used for all the statistical analysis. Graphs represent mean ± SEM. Throughout the study, $p < 0.05$ were considered significant.

DATA AND CODE AVAILABILITY

This paper does not include any next-generation sequencing datasets and has not used or made any particular codes for large-scale data analysis.