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Cathepsin S is the major activator of the psoriasis-associated pro-inflammatory cytokine IL-36γ

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Abstract

The pro-inflammatory cytokine IL-36γ is highly expressed in epithelial cells and is a pivotal mediator of epithelial inflammation. In particular, IL-36γ is strongly associated with the inflammatory skin disease psoriasis. As with other IL-1 cytokines, IL-36γ is expressed as an inactive precursor and must be processed by specific proteases to become bioactive. Our aim therefore was to identify protease/s capable of IL-36γ activation and explore the importance of this activation in psoriasis. Using a keratinocyte-based activity assay in conjunction with small-molecule inhibitors and siRNA gene silencing, cathepsin S was identified as the major IL-36γ-activating protease expressed by epithelial cells. Interestingly, cathepsin S activity was strongly upregulated in samples extracted from psoriasis patients, relative to healthy controls. In addition, IL-36γ-Ser18, identified as the main product of cathepsin S-dependent IL-36γ cleavage, induced psoriasiform changes in human skin-equivalent models. Together, these data provide important mechanistic insights into the activation of IL-36γ, and highlight that cathepsin S-mediated activation of IL-36γ may be important in the development of numerous IL-36γ driven pathologies, in addition to psoriasis.

Significance statement

IL-36γ is a potent cytokine which drives and orchestrates inflammation. It is strongly expressed at barrier tissues such as the skin thus is particularly relevant to inflammatory diseases which affect these tissues, including psoriasis. IL-36γ is expressed as an inactive precursor which requires precise N-terminal truncation for activation. In these investigations, we demonstrate that cathepsin S is the major IL-36γ-activating protease expressed by barrier tissues. Moreover, we show that both cathepsin S and IL-36γ are strongly up-regulated in psoriatic inflammation. These findings are important as they both identify the mechanism of IL-36γ activation, and highlight that this mechanism may play a central role in the development of psoriatic inflammation.
Introduction

The interleukin (IL)-1 family cytokines are fundamental regulators of the innate immune system, and orchestrate multiple inflammatory responses (1, 2). IL-1 cytokines are produced rapidly following infection or injury, and are capable of potently inducing a range of beneficial pro-inflammatory processes, including additional cytokine expression, antigen-presenting cell migration and leukocyte activation and infiltration (3-5). The aberrant expression and regulation of IL-1 cytokines is associated with a broad range of immuopathologies, ranging from autoinflammatory to autoimmune disorders (6-8). Therefore, a greater insight into the regulation and function of IL-1 cytokines is not only of academic interest, but also of significant therapeutic importance.

IL-36α, IL-36β and IL-36γ are agonistic cytokines and the most recently discovered of the IL-1 family (9). Interestingly, there is growing evidence to suggest that these cytokines are important for the development of several inflammatory disorders, including psoriasis (10). In psoriatic lesions, the IL-36 cytokines have been shown to be amongst the most specific and highly up-regulated mRNAs relative to other inflammatory skin diseases and healthy controls (11-13). Moreover, hypomorphic mutations in the IL-36 receptor antagonist (IL-36Ra) cause the severe and potentially lethal subtype of psoriasis called pustular psoriasis in a number of cohorts (14, 15). Mouse models further support these observations, showing that IL-36 overexpression in keratinocytes results in a transient inflammatory skin condition resembling psoriasis (16). In addition, IL-36 receptor-deficient mice have been found to be resistant to Imiquimod-induced psoriasiform dermatitis (17). Interestingly, recent studies have also demonstrated a role for the IL-36 receptor in mucosal wound healing, suggesting that IL-36 cytokines have an important physiological role in epithelial repair and homeostasis, as well as the more established, pathological role in the initiation and maintenance of psoriatic inflammation (18).
Unlike many other cytokines, IL-1 family members are expressed without a signal peptide and therefore are not secreted via the classical secretory pathway (19). In addition, most IL-1 cytokines are expressed as inactive precursors and must be processed to become biologically active (20). The activation of IL-1β and IL-18 for instance, is dependent upon caspase-1, a proteolytic enzyme that is regulated by large multiprotein inflammasome complexes (21, 22). This cascade can be induced by a range of endogenous and exogenous triggers and serves as an important sensor of danger (23). In contrast, IL-1α does not contain a caspase-1 cleavage motif and is instead processed by the calcium-dependent protease calpain (24). In short, these IL-1-processing proteases are central to the activation of IL-1 family cytokines and thus are essential mediators of inflammation. In a previous report it was demonstrated that a precise N-terminal truncation (9 amino acids upstream of a conserved A-X-D motif) was also required for IL-36α, IL-36β and IL-36γ to gain biological activity (25). Specifically, activation of IL-36α was demonstrated when cleaved at amino acid K6, IL-36β at R5 and IL-36γ at S18. In these in vitro studies, precise cleavage was found to increase receptor affinity over 10,000 fold, suggesting that processing is likely to be significant in an in vivo setting.

Given the potential importance of IL-36 cleavage in epithelial biology, there is a large and growing interest in the proteases that drive IL-36 processing. There is a particular focus on IL-36-activating proteases in the context of psoriatic inflammation, where the therapeutic potential is evident (26). IL-36γ is of particular interest here, as this cytokine is the only IL-36 cytokine constitutively expressed in the skin (13), and is the most strongly up-regulated in psoriasis plaques (12). In a recent report by Henry et al, it was found that the neutrophil-derived proteases neutrophil elastase and proteinase 3 were capable of activating IL-36γ (27). Although the findings of this report are of interest, the expression of these IL-36γ activating proteases by neutrophils would likely be limited to sites of acute inflammation. Furthermore, despite neutrophil proteases generating IL-36γ activity, the study showed that the majority of cleavage by neutrophil proteases generated inactive forms of IL-36γ and only minor undetectable amounts of bioactive cytokines were responsible for activity. Due to their lack of specificity and expression profile, it is therefore unlikely that these proteases represent the major
processors of IL-36γ within barrier tissues. In this study, we show that IL-36γ processing is independent of caspase-1 and that the cysteine protease cathepsin S precisely cleaves and activates IL-36γ. Both intracellular and secreted extracellular cathepsin S activity was detectable in a range of skin resident cells, including fibroblasts and keratinocytes, suggesting that this protease represents the major regulator of IL-36γ at barrier tissues. Furthermore, it was also shown that cathepsin S levels and activity are elevated in psoriatic skin lesions, and that IL-36γ Ser(S)18, the product of cathepsin S-dependent IL-36γ processing, drives hyperkeratosis in skin equivalents. Together, these data demonstrate that the precise cleavage of IL-36γ by cathepsin S is critical in the activation of its bioactivity, and that this processing may be pivotal in the generation of a psoriatic phenotype.

Results

Epithelial cell-derived proteases activate IL-36γ

Initial experiments confirmed that the addition of truncated IL-36γ (IL-36γ S18) induces the secretion of the pro-inflammatory cytokine IL-8 in a human keratinocyte cell line (HaCaT cells; Fig 1A). In contrast, the addition of full-length IL-36γ (IL-36γ FL) had no effect on IL-8 levels, supporting previous evidence suggesting that IL-36 cytokines require processing to elicit their pro-inflammatory activity (25). These effects were shown to be IL-36 dependent as they were ablated by addition of the IL-36 receptor antagonist (Ra). Importantly, these results also validate the use of HaCaT cells as a bioassay for measuring IL-36γ processing.

It is well established that other IL-1 cytokines, most notably IL-1β, can be cleaved and activated by caspase-1 (21). By incubating IL-36γ FL or SUMO-tagged IL-36γ FL (both polyhistidine tagged) with recombinant caspase-1, and then running the samples on an anti-His Western blot, it was confirmed that caspase-1 does not cleave IL-36γ FL or SUMO-tagged IL-36γ FL, suggesting that this caspase is not capable of IL-36γ activation (Fig 1B). Whilst some IL-36-activating proteases have been identified previously, we propose that these do not represent the major tissue resident activators...
of IL-36γ. Recent evidence suggests that IL-36 cytokines play important roles in inflammatory responses at epithelial surfaces. Therefore, we hypothesised that IL-36γ-activating proteases may also be expressed within the cells of such barrier tissues. To test this hypothesis, the epithelial cell line A549 was lysed using a hypotonic buffer and incubated with HaCaTs for 24 hr. Neither the addition of the lysate nor IL-36γ FL alone had an effect on IL-8 expression (Fig 1C). However, when both the lysate and IL-36γ FL were added together, there was a modest but significant increase in IL-8 expression, suggesting that there are IL-36γ-activating proteins within the lysate. Importantly, the addition of IL-36Ra ablated this effect, demonstrating that the increase in IL-8 expression was due to IL-36γ activity.

To determine the subcellular localization of the IL-36γ-processing activity, the A549 cell lysates were separated into cytosolic and lysosomal fractions and the activity assay was repeated. Firstly, successful separation of cellular fractions was demonstrated by Western blot analysis. Here it was shown that the cytosol fraction contained the cytosol marker GAPDH but not the lysosome marker LAMP-1, whereas the lysosomal fraction contained LAMP-1 but not GAPDH (Fig.1D). In the activity assay experiments, the addition of both IL-36γ FL and A549 cytosol had no effect on IL-8 levels, suggesting that the IL-36γ-processing activity did not reside within this fraction (Fig. 1E). In contrast, the addition of both the lysosomal fraction and IL-36γ FL caused a strong and significant up-regulation in IL-8 secretion, relative to both IL-36γ FL alone and lysosome alone treated cells. Again, this effect was almost completely ablated when IL-36Ra was added, excluding non-specific effects on IL-8 secretion. To investigate whether there is IL-36γ-processing in other cell types, the above experiment was repeated using lysosomal fractions from HaCaT cells (Fig. 1F), primary human fibroblasts (Fig. 1G) and keratinocytes (Fig. 1H). As observed with A549 cells, the incubation of cells with both IL-36γ FL and lysosome caused a significant and IL-36-specific upregulation in IL-8 secretion, relative to samples treated with IL-36γ FL or lysosome alone. Together these data highlight that IL-36γ-activating cytokines are expressed ubiquitously, at least within the lysosomes of cells in barrier tissues.
Activation of IL-36γ is dependent on cathepsin S

The lysosome contains a broad range of proteases, most of which are either serine or cysteine proteases (28). To identify the IL-36γ-activating protease, the class of this protease was first determined using broad-range molecular inhibitors. As previously shown, the incubation of HaCaT cells with both IL-36γ FL and A549 cell lysosome caused a significant increase in IL-8 secretion (Fig. 2A). Whilst this IL-8 secretion was unaffected by addition of the serine protease inhibitor AEBSF, IL-8 secretion was almost completely ablated by the addition of the cysteine protease inhibitor E64. Importantly, E64 had no effect on IL-36γ S18-induced IL-8 secretion, demonstrating that the inhibitor does not simply inhibit IL-8 secretion. Thus, it was concluded that the IL-36γ-activating lysosomal protease is a cysteine protease/s.

In the activity assay used, HaCaT cells were cultured in a neutral buffered media and therefore the IL-36γ-activating lysosomal protease must be both stable and active at pH 7. Whereas most lysosomal cysteine proteases only function at an acidic pH, cathepsin S can function at a neutral pH (29) and so this protease was identified as a candidate IL-36γ-activating lysosomal protease. To test this, HaCaT cells were incubated with A549 cell lysosome and IL-36γ FL, both in the absence and presence of the specific cathepsin S inhibitor CATSi. IL-8 secretion induced in response to lysosome and IL-36γ FL was completely abrogated by the addition of CATSi (Fig. 2B). Again, the inhibitor had no effect on S18-induced IL-8 secretion, highlighting that the CATSi functions to inhibit IL-36γ FL processing specifically. To support these findings and rule out any non-specific effects of the CATSi, the activity assay was repeated using lysosome extracted from A549 cells that had been transfected with cathepsin S siRNA or scrambled siRNA. Downregulation of cathepsin S lysosomal protein expression in the cathepsin S siRNA transfected cells was significant, relative to the scrambled siRNA control (Fig. 2C). Importantly, the downregulation in cathepsin S expression resulted in a strong and significant decrease in the lysosome and IL-36γ FL-induced IL-8 secretion, again relative to the scrambled
siRNA control (Fig. 2D). Therefore, there is robust evidence to suggest that the activation of IL-36γ by lysosomal proteases is dependent on cathepsin S.

*Secreted cathepsin S activates IL-36γ*

It is well established that cathepsin S can be secreted by a range of different cell types. Thus, we postulated that cathepsin S may also function extracellularly to induce IL-36γ FL activation. In this investigation, cathepsin S was shown to accumulate in the supernatants of A549 cells, primary keratinocytes and primary fibroblasts (Fig. 3A). To test whether this secreted cathepsin S could activate IL-36γ, supernatants were collected and added to HaCaT cells. In these experiments, 0.1mM DTT was added to the supernatants to facilitate cathepsin S activity. Importantly, the addition of IL-36γ FL to DTT supplemented OptiMEM did not have any effect on IL-8 production, relative to DTT supplemented OptiMEM alone, highlighting that the *de novo* cathepsin S production by HaCaT cells is not significant enough to affect IL-8 secretion (Fig. 3B). Here, the addition of both IL-36γ FL with either A549 cell conditioned media (Fig. 3B), keratinocyte conditioned media (Fig. 3C) or fibroblast conditioned media (Fig. 3D) caused an increase in IL-8 secretion, relative to IL-8 secretion induced by the addition of conditioned media alone. These data indicate that there is IL-36γ FL processing activity within the secretome of A549 cells, primary keratinocytes and primary fibroblasts. Of note, activity was ablated by the addition of IL-36Ra, proving that the increase in IL-8 secretion was specifically due to the processing of IL-36γ FL.

To determine whether this extracellular IL-36γ FL processing activity was dependent on cathepsin S, A549 cells were transfected with cathepsin S siRNA or scrambled siRNA. Downregulation of cathepsin S protein levels were observed in the supernatant of the cathepsin S siRNA transfected cells, and were found to be significantly lower than cathepsin S levels in supernatants extracted from the scrambled siRNA treated cells (Fig. 3E). Importantly, the downregulation of cathepsin S resulted in a strong and significant decrease of IL-8 secretion induced by IL-36γ FL in combination with
conditioned media, relative to the scrambled siRNA control (Fig. 3F). Therefore, these results demonstrate that secreted cathepsin S also activates IL-36γ FL.

Recombinant cathepsin S cleaves and activates IL-36γ

To explore the nature of cathepsin S-dependent IL-36γ cleavage, recombinant cathepsin S and recombinant IL-36γ FL were utilised. In these experiments, the recombinant IL-36γ FL was expressed with an N-terminal SUMO tag so that IL-36γ FL processing could be visualised by electrophoresis. When both IL-36γ FL and cathepsin S were incubated together at 37 °C, IL-36γ FL was rapidly cleaved (within 5 min) into a protein of approximately 17 kDa (Fig. 4A). The identity of the cleaved protein was interrogated by mass spectrometry and was found to have a mass of 17031, corresponding exactly to the predicted mass of the active form IL-36γ cleaved between residue glutamine 17 and serine 18 (figure 4A). This was also confirmed via N-terminal sequencing (data not shown).

To demonstrate that recombinant cathepsin S can activate IL-36γ FL, both proteins were incubated together or separately for 10 min at 37 °C, and the products of this incubation were added to cultured HaCaT cells. Whilst the addition of IL-36γ FL or cathepsin S did not have any effect on IL-8 secretion when added individually, the addition of both together caused a strong and significant upregulation in IL-8 secretion, supporting previous evidence suggesting that cathepsin S potently activates IL-36γ FL (Fig. 4B). Finally, it was important to demonstrate that cathepsin S could activate native as well as recombinant IL-36γ FL. We have shown that primary keratinocytes constitutively express high levels of IL-36γ protein (data not shown). To determine whether cathepsin S can activate the IL-36γ contained within primary keratinocytes, HaCaT cells were incubated with either cathepsin S alone, the cytosolic fraction of primary keratinoctye alone or both cytosolic fraction and cathepsin S. Whereas the addition of cathepsin S alone had no effect on IL-8 secretion, the addition of the cytosolic fraction caused a strong up-regulation in IL-8 secretion (Fig. 4C). Importantly, IL-8 secretion was significantly higher when cells were incubated with both cathepsin S and cytosolic
fraction together, relative to cells incubated with the cytosolic fraction alone, suggesting that cathepsin S can activate the native IL-36γ protein contained within the cytosol of primary keratinocytes. Importantly, the addition of IL-36Ra completely abrogated this effect, demonstrating that the observed effect was IL-36 dependent.

*Neutrophil proteases do not cleave IL-36γ into the potent IL-36γ S18 isoform*

As discussed previously, a recent study by Henry et al. has shown that the neutrophil proteases cathepsin G, neutrophil elastase and proteinase 3 all cleave IL-36γ, with neutrophil elastase and proteinase 3 increasing the activity of the protein (Henry et al., 2016). Thus it was important to assess the relevance of cathepsin S-dependent IL-36γ in the context of these neutrophil proteases. In this study, SUMO tagged IL-36γ was incubated with recombinant cathepsin G, neutrophil elastase or proteinase 3 for 30 minutes at 37°C. The processing of IL-36γ was then visualised by electrophoresis (Fig. 5A) and the identity of the cleaved products analysed by N terminal sequencing. From these analyses, it was shown that proteinase 3 and cathepsin G truncated IL-36γ to Y16 and Q17 respectively, whilst elastase generated both truncations. To compare the activity of these IL-36γ truncations to IL-36γ S18 (the truncation produced by cathepsin S), recombinant versions of these proteins were added to HaCaT cells and incubated for 24h (Fig. 5B). Here, it was shown that S18 induced a potent expression of IL-8 at all concentrations tested. In contrast the Y16 truncation and the Q17 truncation had no effect on IL-8 expression. To support these data, HaCaT cells were incubated with IL-36γ FL and various concentrations of either recombinant cathepsin S, cathepsin G, neutrophil elastase or proteinase 3 (Fig. 5C). Here IL-8 secretion was markedly higher when the cells were incubated with cathepsin S, further demonstrating that the product of cathepsin S-dependent IL-36γ processing is a much more potent inducer of inflammation, relative to the products of neutrophil protease-dependent IL-36γ processing.
To investigate whether the processing of IL-36γ by neutrophil proteases has a down-regulatory effect on the activation of IL-36γ by cathepsin S, HaCaT cells were incubated for 24 hr with IL-36γ and various concentrations of cathepsin S, both with and without the neutrophil proteases cathepsin G, neutrophil elastase and proteinase 3 (Fig. 5D). In these experiments, the addition of neutrophil proteases had a down-regulatory impact upon IL-8 expression, suggesting that neutrophil proteases serve to downregulate the activation of IL-36γ by cathepsin S. To determine whether neutrophil proteases also downregulate the IL-8 expression induced by IL-36γ S18, HaCaT cells were incubated for 24 hr with various concentrations of IL-36γ S18, both in the presence and absence of neutrophil proteases (Fig. 5E). Again, the addition of neutrophil proteases reduced the IL-8 expression induced by IL-36γ S18. In contrast with previous studies, these data suggest that neutrophil proteases serve to dampen IL-36γ-mediated pro-inflammatory responses by non-specifically processing both the full length and active cytokines into inactive or only partially active truncations.

**IL-36γ and cathepsin S are strongly up-regulated in psoriasis**

Previous studies have shown that IL-36γ RNA is strongly up-regulated in psoriatic biopsies. Importantly, using the tape stripping methodology we were able demonstrate the corresponding increase in IL-36γ protein in psoriatic lesions, relative to healthy controls (Fig. 6D). However, as we were unable to distinguish the active and inactive forms of IL-36γ in psoriatic lesions, the presence and activity of the IL-36γ-activating protease cathepsin S by skin resident cells was investigated. Cathepsin S secretion was up-regulated by the psoriasis associated cytokines TNFα or IFNγ in primary fibroblasts and was strongly upregulated by IFNγ in primary keratinocytes (Fig. 6A). Cathepsin S secretion was up-regulated further by the addition of both TNFα and IFNγ together, suggesting that these cytokines have a synergistic effect on cathepsin S expression. Neither IL-22 nor IL-17 had an effect on cathepsin S secretion in these experiments.
Interestingly, a previous study using cathepsin S staining of biopsies has suggested that cathepsin S may be upregulated in psoriatic lesions (30). To consolidate and quantify these findings, cathepsin S protein levels were analysed in tape-strip samples acquired from the skin of psoriasis patients and healthy controls. Here, cathepsin S levels and activity were significantly higher in the samples acquired from psoriasis patients, suggesting that cathepsin S is up-regulated in psoriasis (Fig. 6B/C). Together, these data serve to show that both cathepsin S and IL-36\(\gamma\) are strongly up-regulated in psoriatic lesions, implicating the processing of IL-36\(\gamma\) by cathepsin S as a component of psoriatic inflammation.

**IL-36\(\gamma\) S18 causes hyperkeratosis in a skin equivalent model**

Having shown that cathepsin S activates IL-36\(\gamma\) by cleaving it into IL-36\(\gamma\) S18, and having shown that both cathepsin S and IL-36\(\gamma\) are strongly up-regulated in psoriasis, it was important to investigate whether IL-36\(\gamma\) S18 drives any changes that culminate in a psoriatic phenotype. To address this, reconstituted human epidermis skin equivalents were treated with either growth media alone, IL-36\(\gamma\) S18 and IL-36Ra. Intriguingly, the addition of IL-36\(\gamma\) S18 caused psoriasiform changes including stratum corneum thickening (hyperkeratosis) and epidermal cornification, the process whereby living keratinocytes differentiate into non-living corneocytes (Fig. 7A). The thickness of the stratum corneum was measured and shown to be significantly thicker in the S18 treated samples (Fig. 7B). IL-8 secretion was also measured and shown to be significantly higher in the S18 treated samples, suggesting that S18 drives pro-inflammatory and differentiation related changes in the epidermal compartment (Fig. 7C). Importantly, these changes were blocked when the samples were treated with the receptor antagonist, confirming that the effects observed were dependent on IL-36\(\gamma\) S18. This would explain the S18-induced thickening of the stratum corneum and concomitant loss of normal differentiation pattern.

**Discussion**
Activated IL-1 family cytokines play a fundamental role in innate immune responses (31, 32). Thus, the proteases involved in IL-1 cytokine activation are considered to be critical mediators of inflammation. The importance of cytokine processing is highlighted by regulatory defects in caspase-1 activation, which cause the recurrent, systemic and severe inflammatory episodes observed in cryopyrin-associated periodic syndromes and other auto-inflammatory diseases such as Familial Mediterranean Fever (2, 33). The correct balance of inflammation versus tissue maintenance is also of particular relevance at cutaneous and epithelial borders, which are constantly exposed to complex pathogenic and other environmental stresses. (34). This is evident in the rare auto-inflammatory diseases DIRA and DITRA, where patients present with severe pustular skin eruptions due to deficiencies in the receptor antagonists of IL-1 and IL-36 cytokines, respectively (35). As well as its apparent pathological role in inflammation, IL-36γ is also involved wound repair within epithelial compartments (9, 36, 37). Thus, a comprehensive understanding of IL-36γ activation in the epithelium is imperative. In this investigation, we show that cathepsin S is the major IL-36γ activating protease produced by skin resident cell types, implicating this protease as a central mediator of IL-36γ-driven cutaneous inflammation.

The mechanisms involved in the processing and secretion of IL-1 cytokines are of longstanding academic interest, both because of their clinical significance and their evident complexity (38, 39). Despite this interest, many of the processes involved are still poorly understood. In general, most IL-1 cytokines require 2 independent signals for secretion; an initial signal to induce expression of the precursor and a second signal to drive cleavage and secretion (40). Most of the proteases that process IL-1 precursors have been identified as cytosolic proteases and thus many of the current models of IL-1 secretion indicate that processing occurs prior to secretion (21, 24, 41). In contrast, cathepsin S is readily secreted by a range of skin resident cells, suggesting that this IL-36γ-activating protease is abundantly expressed in the extracellular space of epithelial tissue. Given that IL-36γ is constitutively expressed in keratinocytes and is further induced by microbial derived stimuli (42), we hypothesise that IL-36γ released following necrotic cell death is rapidly activated by extracellular cathepsin S.
Therefore, IL-36γ may act as an important damage associated molecular pattern (DAMP), akin to IL-1α and IL-33 mediating inflammation and wound repair within epithelial compartments (43).

Although necrotic cell death is likely to be an important route for IL-36γ release and activation, it may not be the only route. Many IL-1 cytokines have various mechanisms of release and thus it is probable that IL-36γ also has multiple pathways of activation, especially given that cathepsin S is not secreted by the cells of many other tissues. Intriguingly, previous studies have shown that a fraction of cytosolic pro-IL-1β is sequestered into early lysosomes and secreted in monocytes (44). Given that cathepsin S is contained within lysosomes, it is tempting to speculate that IL-36γ may also be sequestered into these secretory lysosomes. As lysosomal cathepsin S has been shown to activate IL-36γ, this process would not only facilitate IL-36γ release, but would also allow for concomitant activation.

In a previous study by Henry et al., it was proposed that the neutrophil proteases elastase and proteinase-3 are responsible for driving IL-36γ-dependent inflammatory responses (27). In more detail, it was suggested that the trauma induced necrosis at the epidermis caused both IL-36γ release and neutrophil recruitment, culminating in a neutrophil protease-dependent activation of IL-36γ. Whilst it is clear that the exposure of IL-36γ to neutrophil proteases results in the generation of inflammatory responses, this research questions the relative importance of neutrophil protease-dependent IL-36γ cleavage in an in vivo setting, especially in light of the evidence herein. We show that the neutrophil protease-dependent cleavage of IL-36γ results in the production of truncations that are significantly less potent than the S18 truncation. Moreover, whereas neutrophil-dependent IL-36γ activation requires cellular recruitment followed by subsequent activation and degranulation (45), cathepsin S is strongly expressed at the site of IL-36γ release and so can instantaneously cleave the cytokine following its release. Thus, our data suggest that cathepsin S is the major protease responsible for driving IL-36γ-dependent responses in the skin. Interestingly, neutrophil proteases
down-regulate cathepsin-S mediated IL-36γ activation, suggesting that neutrophil proteases may actually serve to dampen IL-36γ-driven inflammation at epithelial borders. This is supported by previous work in our laboratory, which demonstrated that neutrophil proteases activate IL-36Ra (46).

In recent studies, a role for IL-36γ in the development of psoriasis has been postulated, not least because IL-36γ has been identified as one of the most strongly and specifically up-regulated genes in psoriatic lesions (12-14). Therefore, having demonstrated a role for cathepsin S in IL-36γ activation, and having demonstrated that the expression levels of both proteins are elevated in psoriatic lesions, it is highly likely that cathepsin S plays an important role in the pathogenesis of psoriasis. Furthermore, given that the product of cathepsin S dependent IL-36γ cleavage induces stratum corneum thickening (hyperkeratosis) in a skin equivalent model, the results herein suggest that the activation of IL-36γ by cathepsin S may even be pivotal in driving a psoriatic phenotype. This hypothesis is supported by previous work, which shows that IL-36 signalling is required in the Imiquimod-induced model of psoriasis (17). Future studies, involving inducible cathepsin S overexpression or ablation in mice, should provide further credence to these findings and thus provide a basis for future in vivo investigations and therapeutic targeting.

At present anti-TNFα, anti-IL-17 and anti-IL-23 treatment of severe psoriasis patients has been shown to be of high clinical benefit, with higher efficacy regarding IL-23/IL-17 pathway blockage (47). IL-36γ has been shown to be induced by both IL-17 and TNF and they act synergistically to upregulate its expression and secretion. Therefore targeting downstream IL-36γ could potentially be more beneficial than targeting IL-17 or TNF alone (48). Psoriasis susceptibility genes that impact on the NF-kB pathway (e.g. CARD14, A20) have also been suggested to cause higher IL-36 expression and activity (49). Once expressed and activated, IL-36 acts in a positive feedback manner activating skin tissue cells including keratinocytes and fibroblasts. Thus, under circumstances of limited IL-36Ra availability and/or excessive activating cathepsin S, IL-36 could be a central molecule, serving to
maintain a T cell-independent psoriatic skin phenotype within the epidermal compartment. Although further work is required to clarify the extent to which IL-36γ contributes to the development of psoriasis, these data indicate that IL-36γ could represent major therapeutic targets in its treatment. Furthermore, although previous clinical trials targeting cathepsin S have been discontinued (50), these studies were conducted based on the role of cathepsin S in MHCII antigen presentation. Therefore, it may be of benefit to revisit this therapeutic target, given the evidence that cathepsin S is required for the activation of IL-36γ.

To conclude, this study demonstrates that cathepsin S is the major protease of IL-36γ at barrier tissues. These findings are not only significant from a mechanistic perspective, but may also provide new therapeutic strategies for the treatment of psoriasis, especially as both IL-36γ and cathepsin S are strongly up-regulated in psoriatic lesions. These results now pave the way for in vivo studies to investigate the extent to which cathepsin S activation of IL-36γ contributes to the maintenance of epithelial homeostasis, and to the initiation and development of psoriasis.

Methods

Reagents and antibodies

The protease inhibitors E64 and cathepsin S inhibitor (CATSi) were purchased from Merck Millipore (Billerica, MA). 4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF) was obtained from Sigma (Poole, UK). Human recombinant Cathepsin S was purchased from Bio Vision (Milpitas, CA). Cathepsin S siRNA and scrambled siRNA was purchased from Dharmacon (Lafayette, CA). For Western-blot analysis, the primary antibodies were a rabbit anti-SUMO antibody (AB14405; AbCam, Cambridge, UK), a mouse anti-GAPDH antibody (GT239; Genetex, Irvine, CA), a mouse anti-LAMP-1 antibody (SC18822; Santa Cruz Biotechnology; Santa Cruz, CA) and a goat anti-IL-36γ antibody BAF2320; R&D Systems; Minneapolis, MN). The HRP-conjugated secondary antibodies used were an anti-rabbit IgG antibody (64405; Southern Biotech; Birmingham, AL), an anti-mouse
IgG antibody (A9917; Sigma) and an anti-goat antibody (A3919; Sigma). Human Recombinant pro-IL-1β was purchased from Sino Biological (Beijing, China) and human recombinant caspase-1 was purchased from Biovision (Milpitas, CA). The human recombinant proteins TNFα, IFNγ, IL-17 and IL-22 were all purchased from R&D Systems.

**Generation of recombinant proteins**

To generate IL-36 fusion proteins possessing N-terminal SUMO domains, cDNA of full length IL-36γ, IL-36Ra V2 and IL-36γ S18 proteins were cloned into a Champion™ pET SUMO expression vector (Invitrogen, UK). Proteins were subsequently expressed in BL21-CodonPlus (DE3)-RIL E. coli overnight at 25°C and soluble proteins purified via Ni²⁺-affinity and size exclusion chromatography. Proteins used for stimulations were purified by Ni²⁺-affinity chromatography prior to overnight cleavage of N-terminal SUMO by the Ulp1 protease, followed by subsequent ion exchange and size exclusion chromatography into 20mM Tris pH7.4, 300mM NaCl.

**Caspase-1 activity assay**

1µg of SUMO-tagged recombinant IL-36γS18 (biologically active form), IL-36γ full length (FL) or pro-IL-1β (all polyhistidine tagged) were diluted in assay buffer (50mM HEPES, pH 7.4, 100mM NaCl, 0.1% CHAPS, 1mM EDTA, 10% glycerol, 10mM DTT) and incubated with recombinant caspase-1 (4 units) at 37°C for 6 hr.

**Gel electrophoresis and Western blotting**

In preparation for Western blot analysis, samples were diluted in sample buffer (50mM Tris HCl pH 6.8, 2% SDS, 10% glycerol, 0.02 % bromophenol blue) and heated at 90°C for 5 min. Samples were resolved on a 15% acrylamide gel and proteins transferred to a nitrocellulose membrane. Specific
proteins were detected using anti-SUMO, anti-GAPDH or anti-LAMP-1 antibodies (all 1 µg/ml. Subsequently, blots were incubated with either HRP-labelled anti-mouse IgG antibody (for GAPDH and LAMP-1) or HRP-labelled anti-rabbit IgG antibody (for SUMO; all 1µg/ml). Proteins were visualized using enhanced chemiluminescence reagents (GE Healthcare; Little Chalfont, UK).

**Cells, cell lines and skin equivalents**

Primary keratinocytes and fibroblasts were purchased from Promocell (Heidelberg, Germany) and Lonza (Castleford, UK) respectively. A549 cells, HaCaT cells and primary fibroblasts were cultured in FCS-supplemented culture medium (DMEM), containing 100 U penicillin/ 0.1 mg/ml streptomycin and 10% FCS (all Life technologies). Primary keratinocytes were cultured in keratinocyte growth medium-2 (Promocell). Epidermal skin equivalents (EpiSkin; Lyon, France) were purchased from SkinEthics and maintained according to the manufacturer’s protocol. Skin equivalents were treated on day 11 with either control medium or a, IL-36γ S1 or IL-36γ S18, or IL-36Ra for 96 hours. Following incubation, supernatants were removed and frozen at -80°C. Skin reconstructs were fixed in 4% formaldehyde for 1 hr at RT, removed from their inserts and embedded in paraffin. Sections were cut at 4 µm thickness on a Leica RM2235 microtome (Leica; Wetzlar, Germany) transferred onto PlusFrost microscope slides (Solmedia UK) and subjected to HE-staining at RT. Prior to analysis, the operator was blinded to the identity of the samples. Slides were imaged using a Leica Aperio AT2 scanner and analysed using Image J (Bethesda, MA).

**Cell lysis and fractionation**

Whole cell lysates, lysosomal extracts and cytosolic extracts were generated from 10^7 growing cells. Cells were harvested by centrifugation at 800g. To generate whole cell lysates, cells were resuspended in 500µl cell extract buffer (20mM Heps, pH 7.5, 10mM KCl, 1mM EDTA, 1mM EGTA, 1mM DTT) and incubated for 20 min on ice. Cells were then transferred to a Dounce-type homogeniser and homogenised with 15 strokes of a B-type pestle. Lysates were centrifuged at 15,000 g for 30 min to
remove cellular debris and frozen at -80°C. To generate cytosolic and lysosomal extracts, cells were resuspended in 500µl cell fractionation buffer (Tris HCl, pH 7.4, 0.25M sucrose, 1mM EDTA, 1mM EGTA, 1mM DTT), transferred to a Dounce-type homogeniser and homogenised with 15 strokes of a B-type pestle. The homogenate was centrifuged at 1000 g for 10 min to remove the nuclei and other cellular debris. The supernatant was then collected and centrifuged at 15,000 g to separate the lysosome from the cytosol. The cytosol was then frozen down at -80°C and the lysosome resuspended in cell extract buffer 500µl. Following a 20 min incubation on ice and repeated freeze thaw cycles, the lysosomal fraction was centrifuged at 15,000 g for 30 min and supernatants frozen at -80°C.

**IL-36 cleavage assay**

For activity assay experiments, HaCaTs were plated at $10^5$ cells/well (24-well plate) in complete culture media, and incubated for 24 hours. The media was then removed and replaced with OptiMEM (Life technologies). For most activity assay experiments, indicated treatments were added in conjunction with OptiMEM and cells incubated for 24 hr at 37°C. For activity assay experiments involving protease inhibitors, lysosome fractions were preincubated with E64, AEBSF or CATSi for 3 hr at 4°C before being added to HaCaT cells. For the activity assay experiments involving cell supernatants, A549 cells, primary keratinocytes or primary fibroblasts were plated at $0.5x10^5$ cells/well (6-well plate) in complete culture media. Once confluent, the media was replaced with OptiMEM and cells incubated for a further 24 hr. Following incubation, the conditioned media was removed and frozen at -80°C. In preparation for the activity assay, the conditioned media was supplemented with 0.1mM DTT. The activity assay for these experiments was performed as previously described, the only difference being that the media on the HaCaT cells was replaced with the conditioned media and not OptiMEM. Following incubation, cell supernatants were removed and frozen at -80°C.

**siRNA knockdown**
A549 cells (0.5 x 10⁶/well) were plated in 6-well plates and incubated for 24 hr at 37°C in culture media without penicillin/streptomycin. For each well, 50pmol Cathepsin S siRNA or scrambled siRNA was diluted in 200µl of OptiMEM and incubated at room temperature for 5 minutes. 8µl Lipofectamine (Thermo Scientific, Waltham, MA) was also diluted in 200µl of OptiMEM and incubated at room temperature for 5 min. The lipofectamine and siRNA preparations were then combined and incubated for 20 min at room temperature. 1.6ml of culture media (without penicillin/ streptomycin) was added to the lipofectamine/siRNA mixture. The culture media was removed from each well and the siRNA preparations added. Cells were incubated for 24 hr at 37°C. The culture media was then replaced with OptiMEM and cells incubated for a further 48 hr. Following incubation, cell supernatants were removed and frozen at -80°C.

**ELISA**

Supernatants were analyzed for IL-8 protein using a specific ELISA kit from Biolegend (San Diego, CA). Supernatants and lysates were analyzed for cathepsin S protein using a specific ELISA kit from R&D Systems. ELISA were performed following the manufacturer’s instructions. The lower limits of accurate detection for IL-8 and cathepsin S were 15.6 pg/ml. For the conditioned media experiments, levels of IL-8 in the media prior to the activity assay were deducted from levels recorded after the activity assay to give an accurate measure of de novo IL-8 synthesis.

**Molecular mass confirmation by LC-MS**

1 µg protein was loaded onto a MassPREP micro desalting column (Waters; Elstree, UK) and washed for 5 min with 10% acetonitrile/ 0.1% formic acid. Following a 1 min gradient to 85% acetonitrile/ 0.1% formic acid the protein was eluted into a Xevo G2-XS QToF (Waters) using electrospray ionisation for molecular mass measurement.
**Tape stripping**

All samples were collected with written informed consent in place of the patients, taken in accordance with the Declaration of Helsinki (REC 14/NE/1199). Healthy volunteers were also recruited from the University of Leeds and written consent was also sought (BIOSCI09-001). The severity of lesions was clinically assessed before sample collection using D-squame adhesive discs of 3.8 cm² (Cuderm; Dallas TX). Samples from non-lesional skin were taken preferably from the ventral lower arm area. Location of lesional samples was guided by lesion appearance. Only non-erosive, non-oozing lesions were tape stripped. The tapes were placed on the skin for 5 sec with gentle pressure. The first tape was discarded and 10 subsequent tapes collected were put in an empty container and immediately stored on dry ice for transportation or storage at -80°C until processing for protein extraction. To extract the protein, tapes were placed in lysis buffer (20mM Tris pH 7.4, 150mM NaCl, 1mM DTT, 5mM EDTA, 1mM PMSF, 1% Triton X-100) for 30 min at 4 °C. Samples were sonicated for 3 times 20 sec with a 20 sec interval on ice between each sonication. Samples were centrifuged for 10 min at 15,000g. Supernatants were removed and frozen at -80°C.

**BCA**

Total protein concentrations were quantified using a reducing agent compatible BCA assay (Biovision). The BCA assay was performed following the manufacturer’s instructions.

**Cathepsin S activity assay**

Samples (1µg total protein/ sample) were analysed for cathepsin S activity using a fluorometric kit obtained from Biovision. The activity assay was performed following the manufacturer’s instructions.

**Statistics**
Statistical analysis was performed using the software Graphpad Prism 6. Data were analyzed by one-way ANOVA to determine overall differences and a Tukey post-hoc test was performed to determine statistically significant differences between treatment groups. Data were also analysed using an unpaired t-test.

**Abbreviations**

AEBSF- 4-(2-Aminoethyl) benzenesulfonfyl fluoride hydrochloride  
DTT- Dithiothreitol  
IL-36γ FL- IL-36γ full length  
IL-36Ra- IL-36 receptor antagonist  
IL-36γ S18- truncated IL-36γ  

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**Figures**
**Fig 1. Epithelial cell proteases activate IL-36γ.** HaCaT cells (10^5/well) were incubated with media alone (U), IL-36γ FL (36γ; 10nM), IL-36γ S18 (S18; 10nM), or both IL-36γ S18 and IL-36Ra (RA; 50nM) for 24 hr (A). In addition, 1µg of SUMO-tagged recombinant IL-36γ (suIL-36γ), IL-36γ FL or pro-IL-1β (all polyhistidine tagged) were incubated with recombinant caspase-1 at 37°C for 6 hr. These samples were then analysed by Western blot using an anti-His-HRP antibody (B). A protein marker lane on each gel was used to determine molecular weight. HaCaT cells (10^5/well) were also incubated for 24 hr with media alone, IL-36γ FL, whole cell lysates from A549 cells (W), or a combination of IL-36γ FL and whole cell lysates with and without IL-36Ra (50nM) (C).

HaCaT cells (10^5/well) were incubated for 24 hr with IL-36γ FL (10nM), the cytosolic fraction of A549 cells (c), a combination of IL-36γ FL and cytosol with and without IL-36Ra (10nM), the lysosome fraction from A549 cells (L), or a combination of IL-36γ FL and lysosome with and without IL-36Ra (E). HaCaT cells (10^5/well) were also incubated with IL-36γ FL (10nM), the lysosome fraction of either HaCaT cells (F), primary fibroblasts (G) or primary keratinocytes (H), or a combination of IL-36γ FL and lysosome with and without IL-36Ra (50nM). The supernatants from these experiments were analysed for the presence of IL-8 using cytokine-specific ELISA (A, C, E, F, G, H). The cytosolic and lysosomal fractions from A549 cells were analysed by Western blot using either an anti-LAMP-1 or anti-α tubulin antibody (D). A one-way ANOVA was used to determine statistical significance of differences between treatment groups. *, p < 0.05; **, p < 0.01, ***, p < 0.001, ****, p < 0.0001. Data shown are mean ± SEM (n=3).

**Fig 2. Activation of IL-36γ is dependent on cathepsin S.** HaCaT cells (10^5/well) were incubated for 24 hr with media alone, IL-36γ S18 (S18; 10nM), or IL-36γ FL (FL; 10nM) with the lysosome fraction of A549 cells, all with and without the cysteine protease inhibitor E64 (0.2μM) or the serine inhibitor AEBSF (2μM; A). In these experiments, the inhibitors were preincubated with the IL-36γ S18 or lysosome at 4°C for 3 hr to give an initial concentration of 0.1mM for AEBSF and 10μM for E64. HaCaT cells (10^5/well) were also incubated for 24 hr with media alone, IL-36γ S18 (10nM), or IL-36γ FL (10nM) with the lysosome fraction of A549 cells, all with and without the cathepsin S
inhibitor (CATSi; 100ng/ml; B). Again, the CATSi was preincubated with the IL-36γ S18 or lysosome at 4 °C for 3 hr to give an initial concentration of 5μg/ml. To inhibit expression of cathepsin S within A549 lysosomes, A549 cells were transfected with cathepsin S siRNA (CATS) or scrambled siRNA (SCRAM; both 25nM) for 24 hr and lysosome fractions extracted. The lysosome fractions were analysed for the presence of cathepsin S using a specific ELISA (C). Finally, HaCaT cells (10⁵/well) were incubated for 24 hr with the lysosome of SCRAM-transfected or CATS-transfected A549 cells, both with or without IL-36γ FL (10nM; D). The supernatants from these experiments were analysed for the presence of IL-8 using cytokine-specific ELISA (A, B, D). A one-way ANOVA (A, B, D) or unpaired t-test (C) was used to determine statistical significance of differences between treatment groups. **, p < 0.01, ****, p < 0.0001. Data shown are mean ± SEM (n=3).

Fig 3. Secreted cathepsin S activates IL-36γ. A549 cells, primary keratinocytes, or primary fibroblasts were incubated in 6-well plates until confluent, at which point the media was replaced with OptiMEM and cells incubated for a further 24 hr. Supernatants were analysed for the presence of cathepsin S using cytokine-specific ELISA (A). HaCaT cells (10⁵/well) were incubated for 24 hr with 0.1mM DTT-supplemented conditioned media from A549 cells (B), primary keratinocytes (C), primary fibroblasts (D). Cells were incubated with either media alone, with IL-36γ FL (10nM), or with both IL-36γ FL and IL-36Ra (50nM). Supernatants were analysed for the presence of IL-8 using cytokine-specific ELISA (B, C, D). To inhibit expression of cathepsin S, A549 cells were transfected with cathepsin S siRNA (CATS) or scrambled siRNA (SCRAM; both 25nM) for 24 hr and the supernatants extracted. Supernatants were analysed for the presence of cathepsin S using a specific ELISA (E). Finally, HaCaT cells (10⁵/well) were incubated for 24 hr with the supernatants from SCRAM-transfected or CATS-transfected A549 cells, either with or without IL-36γ FL (10nM; F). The supernatants from these experiments were analysed for the presence of IL-8 using cytokine-specific ELISA (F). A one-way ANOVA (B, C, D, F) or unpaired t-test (E) was used to determine statistical significance of differences between treatment groups. *, p < 0.05, **, p < 0.01. Data shown are mean ± SEM (n=3).
Fig 4. **Recombinant cathepsin S can cleave and activate IL-36γ.** 2μg of SUMO-tagged IL-36γ was incubated with 20ng of recombinant cathepsin S at 37°C for 30 minutes. Several time points were taken and analysed by coomassie stained SDS-PAGE gel. Diagram depicts the truncation generated by cathepsin S cleavage identified by mass spectrometry analysis (A). HaCaT cells (10⁵/well) were incubated for 24 hr with media alone, IL-36γ (10nM), cathepsin S (Cat S; 10ng/ml) or a combination of IL-36γ and cathepsin S (B). In addition, HaCaT cells were incubated with media alone, the cytosol of keratinocytes, cathepsin S (10ng/ml), a combination of cytosol and cathepsin S or a combination of cytosol, cathepsin S and IL-36Ra (C). Supernatants were analysed for the presence of IL-8 using a specific ELISA (B, C). A one-way ANOVA was used to determine statistical significance of differences between treatment groups. *, p < 0.05. Data shown are mean ± SEM (n=3).

Fig 5. **Neutrophil proteases do not cleave IL-36γ into the potent IL-36γ S18 bioactive form.** 2μg of SUMO-tagged IL-36γ was incubated with 20pg of recombinant cathepsin G, neutrophil elastase or proteinase 3 at 37°C for 30 minutes. Samples were analysed by coomassie stained SDS-PAGE gel. Diagram depicts the truncation generated by cleavage identified by mass spectrometry analysis (A). HaCaT cells (10⁵/well) were incubated for 24 hr with media alone (U), IL-36γ FL, IL-36γ Y16, IL-36γ Q17or IL-36γ S18 (all either 10nM, 50nM or 100nM; B). HaCaT cells (10⁵/well) were also incubated with IL-36γ FL (10nM) and various concentrations of either recombinant cathepsin S, cathepsin G, neutrophil elastase or proteinase 3 for 24hr (C). In addition, HaCaT cells (10⁵/well) were incubated for 24 hr with IL-36γ (10nM) and cathepsin S (1nM-0.001nM), both with (black line) and without (red line) the neutrophil proteases cathepsin G, neutrophil elastase and proteinase 3 (all 1nM; D). HaCaT cells (10⁵/well) were also incubated for 24 hr with IL-36γ S18 (0.01-10nM) both with (black line) and without (red line) the neutrophil proteases cathepsin G, neutrophil elastase and proteinase 3 (all 1nM; E). Supernatants were analysed for the presence of IL-8 using a specific ELISA (B, C, D, E). A two-way ANOVA was used to determine statistical significance of differences between NS and other treatment groups. **** p < 0.0001. Data shown are mean ± SEM (n=3).
Fig 6. **IL-36γ protein expression and cathepsin S activity are elevated in psoriasis.** Primary fibroblasts (white bar) or primary keratinocytes (black bar; both 10⁶/well) were incubated for 48hr with either TNFα, IFNγ, IL-17 or IL-22 (all 100ng/ml) and supernatants analysed for the presence of cathepsin S using a specific ELISA (A). In addition, elutes were acquired from the skin of healthy and psoriasis patients using tape stripping methodology. Samples were analysed for the presence of cathepsin S using a specific ELISA (B). Cathepsin S activity was measured using a fluorometric cathepsin S activity assay (C). Samples were also analysed by Western blot using an anti-IL-36γ antibody (n=5; D). For Western blot analysis, samples were normalised so that equal amounts of total protein were loaded in each well. A one-way ANOVA was used to determine statistical significance of differences between treatment groups. *, p < 0.05. Data shown are mean ± SEM (n = 3 unless previously stated).

Fig 7. **IL-36γ S18, the main product of cathepsin S cleavage, induces hyperkeratosis in a skin equivalent model.** Reconstructed human epidermis skin equivalents were grown for 96 hours in growth medium either untreated (U) or containing IL-36γ S18 (100nM) with and without IL-36Ra (100nM). Sections were stained with H&E and annotated in ImageJ software to display epidermal thickness (A). Stratum corneum thickness was measured from 4 points along each skin slice (n=4) (B). Supernatants were also collected at the end of the experiment and analysed for the presence of IL-8 using a specific ELISA (C). A one-way ANOVA was performed to determine statistical significance of differences between treatment groups. *, p < 0.05. **** p < 0.0001. Data shown are mean ± SEM.
A

(U) 78.6 μm
(U) 70.5 μm
(S18) 141.3 μm
(S18 + RA) 84.8 μm

B

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C

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