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RESEARCH ARTICLE

Toll-like receptor agonists, poly(I:C) and flagellin, lead to IL-36γ induction with divergent release kinetics and differentially alter autophagy in primary human keratinocytes

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ABSTRACT. IL-36γ, a pro-inflammatory member of the IL-1 cytokine superfamily, can be induced and secreted by normal human foreskin keratinocytes (HFKs) in response to pathogenic stimuli, however, the mechanisms underlying the secretion are unknown. In this study, we demonstrate that stimulation with the TLR3 agonist, poly (I:C), led to a delayed secretion of IL-36γ compared to stimulation with the TLR5 agonist, flagellin, despite equal levels of the cytokine ($p = 0.006$). IL-36γ was shown to be released from HFKs in its inactive, uncleaved form, based on western blotting. Moreover, recombinant IL-36γ in its activated, cleaved form induced endogenous IL-36γ 10-fold ($p = 0.004$) and CXCL8 five-fold ($p = 0.003$) over baseline levels compared to unactivated full-length recombinant IL-36γ. The ratio of LC3b-II/LC3b-I was significantly higher in poly(I:C)-treated cells compared to flagellin-treated and unstimulated controls without a change in SQSTM1/p62 after 24 hours of stimulation ($p = 0.043$). Under fluorescence microscopy, poly(I:C) led to a two-fold increase at eight hours and four-fold increase at 24 hours in accumulated autophagosomes post-stimulation ($p = 0.032$). In contrast, autophagosomes were unchanged relative to baseline in response to flagellin. Bafilomycin A1 treatment enhanced poly(I:C)-mediated IL-36γ secretion ($p = 0.044$) while rapamycin led to a noticeable, but non-significant, increase in flagellin-mediated IL-36γ secretion, indicating that interrupting autophagic flux can alter IL-36γ release from HFKs. Finally, we show that, compared to clinically normal laryngeal tissue, there were significantly higher levels of LC3b-II in HPV-infected respiratory papilloma tissue, indicating a higher number of autophagosomes; a signature of disrupted autophagic flux.

Key words: interleukin-36γ, autophagy, Toll-like receptor, LC3b, keratinocyte

Keratinocytes respond to exogenous pathogenic stimuli by synthesizing and secreting specific cytokines to initiate immune responses. The IL-1 superfamily member, interleukin-36γ (IL-36γ), is a cytokine that plays a major role in pathogen detection and response and in the maintenance of epithelial barriers [1]. While the signalling axis of the IL-36 cytokines has been well described, a complete understanding of the regulation of the cytokines themselves is lacking. Dysregulated secretion, in part, contributes to various epithelial pathologies including plaque and pustular psoriasis and acneiform skin toxicity [2–4]. IL-36γ transcript and protein is significantly upregulated in human papillomavirus 11-induced lesions present/11-induced lesions present in the larynx of recurrent respiratory papillomatosis (RRP) patients, without observable inflammation, compared to clinically normal adjacent tissue [5]. The secretory mechanisms associated with IL-36γ have not yet been fully elucidated and further related progress will likely impact on therapeutic opportunities for epithelial disease.

Once in extracellular space, exogenous proteases including neutrophil elastase and cathepsin-S cleave the IL-36γ N-terminus, turning the cytokine into an active and potent inflammatory agent with activity approximately 1,000-fold greater than its

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full-length form [6, 7]. Binding of IL-36γ to its receptor, IL36R, leads to the induction of chemokines, CCL1 and CCL20, and IL-36γ itself in monocyte-derived CD1a+ CD207+Langerhan's cells; a microenvironment primed to recruit monocytes and lymphocytes [8]. Despite our understanding of this aspect of IL-36γ regulation, mechanisms of its release remain unknown. IL-36γ is described to be passively externalized across ruptured keratinocyte membranes during poly(I:C)-mediated pyroptosis, and later studies have shown that IL-36γ can be secreted in response to low doses of poly(I:C), in the absence of inflammatory cell death [9, 10]. The primary structure of IL-36γ indicates that it is a poor candidate for the classic endoplasmic reticulum/Golgi secretion mechanism and is instead likely to be externalized via a non-classic mechanism.

Autophagy is a degradative process that functions to sequester cellular components into autophagosomes for delivery to lysosomes. In addition to this classic function of autophagy, recent intriguing studies have demonstrated the secretory capabilities of this cellular process [11, 12]. Autophagy-related genes and autophagosomes contribute to the secretion of leaderless proteins with important signalling properties, in both a free form (i.e., IL-1β and HMGB1) and as a complex with extracellular vesicles (annexin A2 and α-synuclein) [13–15]. A key event during autophagy induction, autophagosome formation, and cargo sequestration is the lipidation and conversion of microtubule associated protein 1 light chain 3 beta 1 (MAP1LC3B, henceforth referred to as LC3b) into membrane-associated LC3b-II. SQSTM1/p62 targets and tags aggregated and aberrant proteins for autophagic sequestration destined for lysosomal degradation [16, 17]. The molecular analysis of LC3b (*i.e.* LC3b-II/-I ratios), SQSTM1/p62 levels and visualization of autophagosomic vesicles are often approaches utilized to monitor autophagy, and changes may indicate alterations of flux through the system.

In this study, we hypothesized that in addition to differences in poly(I:C)- or flagellin-induced IL-36γ secretion, we might also observe concurrent changes in autophagic machinery and autophagic vesicle abundance. Additionally, by manipulating autophagic flux with well described pharmaceuticals, we sought to investigate whether it is possible to alter the release of poly(I:C) or flagellin-induced IL-36γ from primary human keratinocytes. We conclude that IL-36γ secretion is not dependent on internal levels of the cytokine itself and is likely affected by broader cellular processes including autophagy in primary keratinocytes. Finally, our observation that steady state LC3b-II/LC3b-I ratios in papilloma biopsies from RRP patients are significantly lower than those of autologous, normal adjacent laryngeal biopsies provides an interesting link with IL36γ dysregulation observed in these tissues.

MATERIALS AND METHODS

Cell culture and reagents

Neonatal foreskins were obtained as anonymous surgical discards and keratinocytes were isolated and pooled from six individuals, as described previously [8]. Keratinocytes were cultured in E-media [18] on J2-3T3 fibroblast feeder layers, as previously described [10]. Cells were stimulated with 1,500 ng/mL high-molecular-weight polyinosinic-polycytidylic acid (poly [I:C]) (Invivogen, San Diego, CA) or 1 μg/mL flagellin (from *S. typhimurium,* Invivogen, San Diego, CA) for the times indicated. Conditioned media from cultured cells was cleared by centrifugation at 14,000g for 20 minutes at 4°C and stored at -80°C for subsequent analysis of secreted IL-36γ.

To investigate effects on autophagy, HFKs were stimulated with poly(I:C), flagellin or left unstimulated for 24 hours and the media was removed and replaced with media containing 1 μM rapamycin, 50 nm bafilomycin A1 (Sigma, St. Louis, MO), or 100 mM sucrose (Sigma, St. Louis, MO). Conditioned media was collected after 48 hours, at the 72-hour timepoint after stimulation. IL-36γ release was measured by ELISA in experiments with rapamycin and bafilomycin A1 after 24 and 72 hours.

Papilloma and laryngeal biopsies were snap-frozen for whole biopsy analysis. All patients provided written informed consent for use of their samples and the studies were approved by the Feinstein Institutional Review Board.

Immunoblotting

Cultured cells were lysed with TNE lysis buffer (50 mM Tris pH 8.0, 150 mM NaCl, 10 mM NaF, 1% [vol/vol] NP-40) supplemented with protease and phosphatase inhibitors (Roche Diagnostics, Indianapolis, IN) for 30 minutes on ice. Laryngeal biopsies were homogenized on ice in TNE buffer. Samples were clarified by centrifugation at 14,000g for 20 minutes at 4°C. Total protein was measured in clarified homogenates using the BCA assay (Thermo Fisher, Waltham, MA). Equal masses of protein for each sample were heated at 95°C for 5 minutes with 6× loading buffer containing SDS and loaded onto 4-20% precast, polyacrylamide gradient gels (Bio-Rad; Hercules, CA). IL-36γ recombinant full-length (2320-IL-025) and cleaved (6835-IL-010) controls were purchased from R&D Systems. Proteins were electrotransferred onto PVDF membranes (Millipore; IPFL07810; Carrigtwohill, Ireland), blocked with Odyssey PBS blocking buffer (LI-COR; Lincoln, NE) at room temperature for an hour, and incubated at 4°C overnight with primary antibodies. Primary antibodies used were reactive to IL-36γ (R&D; AF2320) at 1:1000, β-actin (Sigma; A5441) at 1:20000, LC3b (Fisher Scientific; PA1-46286) at 1:1000, LAMP1 (Abcam; ab24170) at 1:1000 and p62/SQSTM1 (Abcam; ab56416) at 1:1000. Wash buffer (1X PBS, 0.1% Tween-20) was used to wash membranes prior to incubation with near-infrared dye-conjugated secondary antibodies using LI-COR at 1:5000 (donkey anti-mouse, anti-rabbit, or anti-goat IR Dye 800CW or 680LT). Densitometry was quantified and analysed using the Odyssey Li-Cor system or quantified using the Sapphire Biomolecular Imager from Azure biosystems, followed by quantification using ImageJ. All densitometry values were normalized to β-actin.

HFK-conditioned media and qPCR

Conditioned media used in stimulation experiments was generated by stimulating approximately 2.0×10^6 nearly confluent HFKs for 48 hours with poly(I:C) (1,500 ng/mL) or flagellin (1,000 ng/mL) in E-media. Resulting conditioned media was collected and centrifuged at 14,000g for 20 minutes at 4°C to pellet debris. Clarified conditioned media was stored at -80°C until use. Separate cultures of HFKs were grown in 24-well plates on 3t3 feeder cells in E-media, as described above. HFKs were stimulated with recombinant full-length IL-36γ (R&D; 2320-IL-025; 10 ng/mL), recombinant cleaved IL-36γ (R&D; 6835-IL-010; 10 ng/mL), or poly(I:C)- or flagellin- conditioned media for 18 hours. Stimulation media was aspirated, cells were washed with cold 1x PBS, and RNA was extracted with RNeasy mini kit (Qiagen; 74106). Quantitative RT-PCR was carried out using iTaq Universal Proves 1-step kit (BioRad; 1725141). Reverse transcription was performed at 50°C for 10 minutes. cDNA was melted at 95°C for three minutes followed by amplification at 95°C for 10 seconds and 60°C for 30 seconds for 40 cycles. Primer sets used were acquired from Applied Biosystems and were specific to GAPDH (Hs01922876_u1), IL-36γ (Hs00219742_m1) or CXCL8 (Hs00174103_m1). The primer and probe sequences were not provided by the manufacturer. Quantification was performed using the 2-ΔΔCt method, normalizing to GAPDH. Statistics were performed on data prior to transformation to fold change.

Protein precipitation from HFK-conditioned media

To analyse secreted IL-36γ, 0.5 mL of poly(I:C)-treated (for 96 hours) HFK-conditioned media was concentrated using 99% trichloroacetic acid, added in a 1:4 ratio with conditioned media. The solution was incubated on ice for 10 minutes and centrifuged at 20,000g for 5 minutes. The resulting pellet was washed twice with cold acetone and re-centrifuged at 20,000g for 5 minutes. The pellet was then dried and resuspended in RIPA buffer (Fisher Scientific; Pittsburgh, PA) and analysed by immunoblotting as described above using recombinant standards.

Total membrane isolation

Cells were homogenized and centrifuged at 3000g for 10 minutes at 4°C to pellet unbroken cells and nuclei. Post-nuclear supernatant was then centrifuged at 100,000g for one hour at 4°C. Total membrane pellets were resuspended in RIPA buffer containing 1% triton X-100 and protease and phosphatase inhibitors. Membrane protein was quantified using the BCA protein assay kit (Pierce; Rockford, IL), and analysed by immunoblotting.

ELISA

IL-36γ secretion was measured in conditioned media by ELISA (Sigma; RAB0689) according to the manufacturer's protocol. IL36γ secretion was normalized to total cell protein. Samples were run undiluted in duplicate and normalized to total cell protein, as measured using the BCA assay.

Autophagy assay and fluorescence microscopy

HFKs were grown, as described above, on sterile glass cover slips on growth-arrested 3T3 feeder layers. When approximately 50% confluent, HFKs were washed once with 1x PBS and treated with poly(I:C) (1,500 ng/mL), flagellin (1,000 ng/mL), or chloroquine (CQ) (50 μM) as a positive control or left untreated for either eight or 24 hours. After stimulation, coverslips were treated with 0.68 mM EDTA in 1x PBS at 37°C for one minute with gentle agitation to remove residual fibroblast feeder layers. HFKs were washed and stained with Green Detection Reagent, optimized to accumulate in vesicles produced during autophagy, and counterstained with DAPI included in the Autophagy Assay Kit (Abcam; ab139484), according to the manufacturer's protocol for fluorescence microscopy. After staining, HFKs were fixed with 4% formaldehyde for 20 minutes at room temperature and mounted with VectaShield (Vector Laboratories; H-1000). Untreated cells were stained at time zero for each replicate.

Autophagosomal accumulated Green Detection Reagent and nuclei were visualized through FITC and DAPI filters on a ZEISS Axio Observer inverted microscope. The signal of three regions of interest per slide was quantified after background correction using imageJ. Mean relative Corrected Total Cellular Fluorescence (CTCF) was calculated by normalizing FITC/DAPI signal ratios to that measured at time zero for each replicate. Experiments were performed three times with independent passages of HFKs.

Statistical analysis

All data are presented as mean ± standard deviation. Results were analysed by two-tailed Student t-tests assuming unequal variance. P values <0.05 were considered statistically significant.

RESULTS

Poly(I:C) and flagellin treatment of HFKs leads to divergent IL-36γ release kinetics

To address the relationship between autophagy and IL-36γ secretion, we first characterized induction and secretion kinetics of IL-36γ during stimulation with either poly(I:C) or flagellin; pathogen stimuli that are known to induce expression of IL-36γ. Time course analysis by western blot revealed that IL-36γ protein levels were induced to nearly identical levels in response to poly(I:C) and flagellin; approximately four-fold above baseline by 24 hours and maintained over the course of 72 hours after stimulation (*figure 1A, B*). In contrast, poly(I:C) and flagellin treatments led to disparate IL-36γ secretion patterns. While flagellin stimulation led to a consistent and significant accumulation of IL-36γ in conditioned media at 48 hours, from 1.6 to 4.4 pg IL-36γ/μg cell protein, a 2.8-fold increase

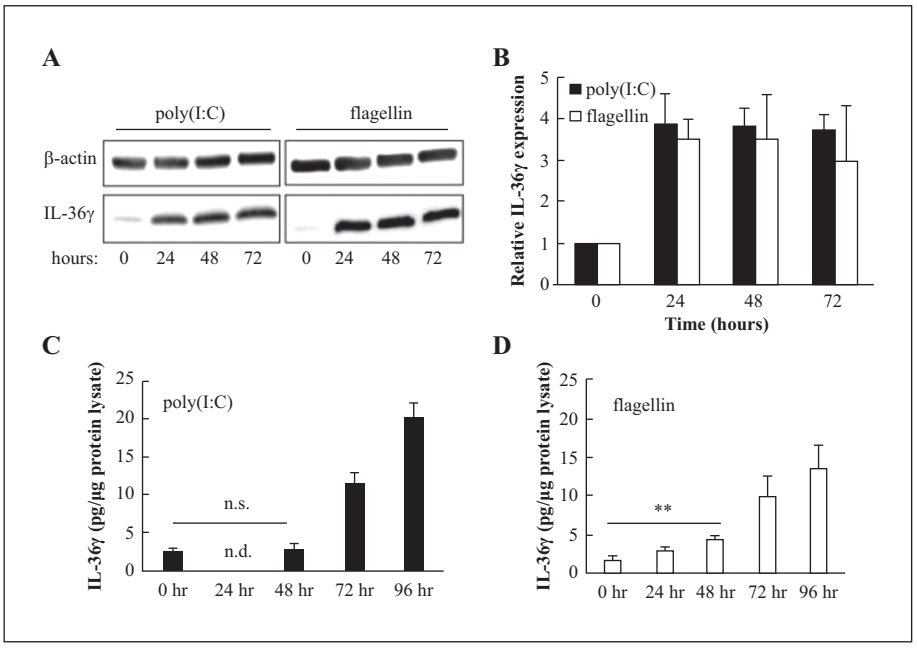

Figure 1
Induction and secretion of IL-36γ in HFKs in response to TLR agonists. HFKs were stimulated with poly(I:C) (1,500 ng/mL) or flagellin (1;000 ng/mL) over the course of three days or left unstimulated as control, after which cell lysates were subject to western blotting. **A**) Representative western blot showing full induction of IL-36γ by 24 hours and maintenance of intracellular levels up to 72 hours in response to both TLR agonists. **B**) Quantification of IL-36γ induction showing a nearly four-fold increase in IL-36γ in response to either agonist by 24 hours (bars represent mean ± SD based on three experiments). **C**) Time-dependent secretion of IL-36γ in HFKs stimulated with 1,500 ng/mL poly(I:C) measured by ELISA showing no elevation in IL-36γ by 48 hours post-stimulation. **D**) Time-dependent secretion of IL-36γ in HFKs stimulated with 1,000 ng/mL flagellin measured by ELISA showing a nearly three-fold increase in IL-36γ by 48 hours post-stimulation (bars represent mean ± SD based on three experiments). Measurement of IL-36γ in response to poly(I:C) stimulation at 24 hours was not performed as previous studies have shown baseline levels at 48 hours. ** $p<0.01$; two-tailed Student's T-test. n.s.; not significant.

over baseline ($p=0.006$), IL-36γ consistently remained at baseline levels (1.1 pg IL-36γ/μg cell protein) for the initial 48 hours post-poly(I:C) stimulation (*figure 1C,D*). HFKs continued to release IL-36γ up to four days post-stimulation during treatment with either agonist. These data suggest that treatment with poly(I:C) or flagellin induces different pathways of secretion. The observed delay in poly(I:C)-mediated secretion was independent of intracellular IL-36γ protein level due to the high level of intracellular IL-36γ at the 48-hour time point.

IL-36γ is secreted from HFKs following poly(I:C) or flagellin stimulation in its uncleaved, inactive form

The primary sequence of IL-36γ indicates that it is a candidate for non-classic protein secretion. Proteins secreted through these mechanisms are usually loaded or incorporated into intracellular vesicles in the absence of direct transport across the plasma membrane. We therefore hypothesized that IL-36γ is associated with membranes rather than residing exclusively in the cytoplasm. To assess this, we enriched intracellular membranes from lysates prepared from poly(I:C) or flagellin-stimulated HFKs via ultracentrifugation followed by western blotting. We separated intracellular membranes from the cytosol, as indicated by detection of the lysosomal marker, LAMP1, and autophagosomal marker, LC3b-II, in the membrane fractions (*figure 2A*). When probed for IL36γ, most of the cytokine was found in the cytosolic fractions, with a small pool of IL-36γ pelleting with the isolated membranes after treatment with either poly(I:C) or flagellin, indicative of an association with membrane-bound organelles (*figure 2A*).
Post-translational cleavage of IL-36γ increases its potency on epithelial immunocyte responses [19]. Since processing can precede the unconventional secretion of cytokines, as with IL-1β, we assessed the form of IL-36γ accumulating in the media in response to either poly(I:C) or flagellin treatment. HFKs were stimulated for 96 hours in order to allow maximum accumulation of IL-36γ. After total protein was precipitated from the conditioned media and subject to SDS-PAGE, the molecular weight of HFK-derived IL-36γ was compared to commercially available full-length (AA1-169) and processed (active) (AA18-169) recombinant control standards. The molecular weights of the IL-36γ secreted were 16.45 and 16.46 kDa for poly(I:C) and flagellin, respectively (*figure 2B*). The full-length IL-36γ standard was 16.45 kDa while the cleaved (biologically active) standard was 14.71 kDa (*figure 2B*). Thus, IL-36γ secreted from normal HFKs corresponds to the full-length form with either poly(I:C) or flagellin stimulation, indicating that the secretion mechanisms for either TLR agonist do not require cleavage.

The recombinant cleaved (AA18-169) but not full-length form of IL-36γ induces endogenous IL-36γ and CXCL8 in HFKs

To better understand the broader signalling context in the epithelium during the poly(I:C) or flagellin response, we treated HFKs with culture media

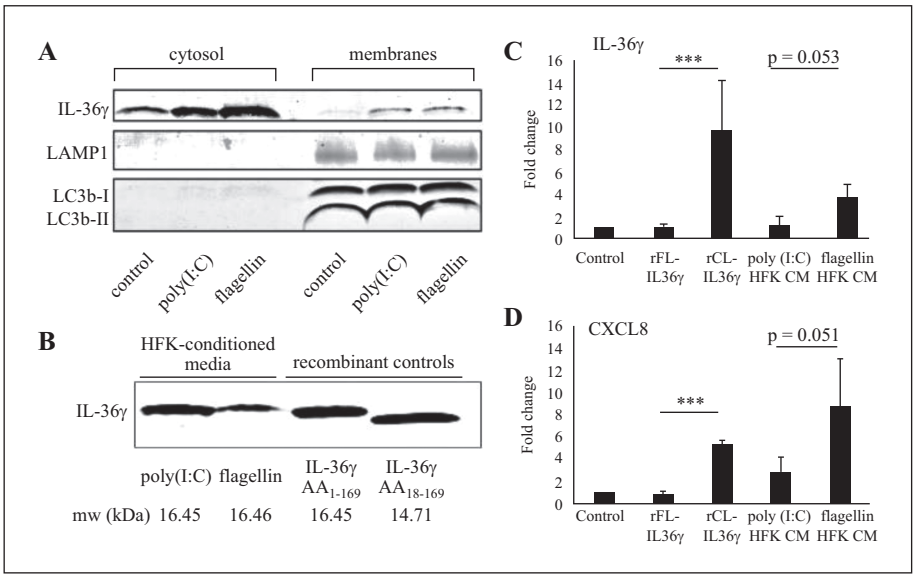

Figure 2

IL-36γ in HFKs is associated with intracellular membranes and is secreted as a full-length, unprocessed form. **A**) HFKs were stimulated for 48 hours with poly(I:C) (1,500 ng/mL) or flagellin (1,000 ng/mL) followed by homogenization and ultra-centrifugation to separate membranes from the cytosol. The representative western blot shows effective separation based on LAMP1 and LC3b-II signal in the membrane fraction. The majority of IL-36γ resides in the cytosol while a fraction pellets with internal membranes, suggesting an association with intracellular vesicles prior to secretion (data are representative of three experiments). **B**) HFKs were stimulated for 96 hours with poly(I:C) (1,500 ng/mL) or flagellin (1,000 ng/mL), conditioned media was collected, and total protein precipitated. Precipitated protein was subjected to western blotting in parallel with recombinant full-length (amino acids: 1-169) and cleaved (amino acids: 17-169) IL-36γ standards. IL-36γ released in response to poly(I:C) and flagellin was of a similar size to the full-length standard. **C-D**) HFKs were stimulated with recombinant full-length (rFL) or cleaved (rCL) IL36γ (as used in [**A**] and [**B**], at 10 ng/mL), or poly(I:C)-stimulated or flagellin-stimulated HFK-conditioned media for 18 hours, and IL-36γ (**C**) and CXCL8 (**D**) transcript levels were measured by qPCR, relative to unstimulated controls and normalized to GAPDH. *** <0.00; Student's T-test.

conditioned by HFKs exposed to poly(I:C) or flagellin, using the recombinant cleaved or full-length form of IL-36γ as controls. We measured the effect of these conditioned media and recombinant controls to induce IL-36γ and CXCL8 transcription. As expected, full-length IL-36γ did not induce endogenous IL-36γ or CXCL8 transcripts in keratinocytes compared to cleaved IL-36γ, which induced endogenous IL-36γ and CXCL8 transcripts 10-fold (p=0.004) and five-fold (p=0.003), respectively, demonstrating post-translational cleavage requirement for IL-36γ function (*figure 2C,D*). Flagellin-conditioned media induced IL-36γ by 4.4-fold over baseline, while poly(I:C)-conditioned media led to no appreciable increase (p=0.053) (*figure 2C*). Additionally, flagellin-conditioned media induced CXCL8 transcripts in HFKs 8.7-fold, compared to a 3.2-fold induction in response to poly(I:C)-conditioned media (p=0.051). Although just below significance, these data are an indication that CXCL8 and IL-36γ are selectively induced in response to the pathogenic challenge and the signalling microenvironment.

Poly(I:C) stimulation promotes the conversion of LC3b-I to autophagosome membrane-associated LC3b-II in HFKs

There is evidence that autophagy is affected differentially in response to various TLR agonists [20]. Therefore, we wanted to determine whether the TLR agonists, poly(I:C) and flagellin, would modulate autophagosome levels in HFKs differently, thus potentially contributing to an autophagy-dependent secretion mechanism for IL-36γ. To do this, we measured changes in LC3b-II and p62 levels, two well-established markers of autophagy, at eight and 24 hours after poly(I:C) or flagellin stimulation. Both conditions showed a decrease in LC3b-II/-I ratio at eight hours post stimulation, compared to time zero. This was expected, as the cells received media containing fresh serum (*figure 3A,B*). In contrast, at 24 hours post stimulation, HFKs exposed to poly(I:C) showed a rise in the LC3b-II/-I ratio, returning to levels observed at time zero (p=0.043). However, the LC3b-II/-I ratio in untreated and flagellin-treated HFKs continued to fall (*figure 3A,B*). This selective difference in LC3b conversion indicates increased levels of autophagosomal membranes in HFKs after eight hours of poly(I:C) exposure, which was not the case after flagellin stimulation. Within the same time course, no changes were observed in SQSTM1/p62 levels for any condition (*figure 3C,D*). These observations are consistent with poly(I:C)-specific formation of autophagosomes while the constant levels of SQSTM1/p62 across stimulation conditions indicated no significant acceleration nor deceleration in autophagosomal degradation. We believe this data reflects a subtle but significant alteration in autophagic flux that precedes IL-36γ secretion.

TLR agonists, poly(I:C) and flagellin, lead to differential perturbation of autophagic flux in HFKs

To better understand changes in autophagy caused by poly(I:C) or flagellin treatments, we assessed the presence of autophagic vesicles using fluorescence

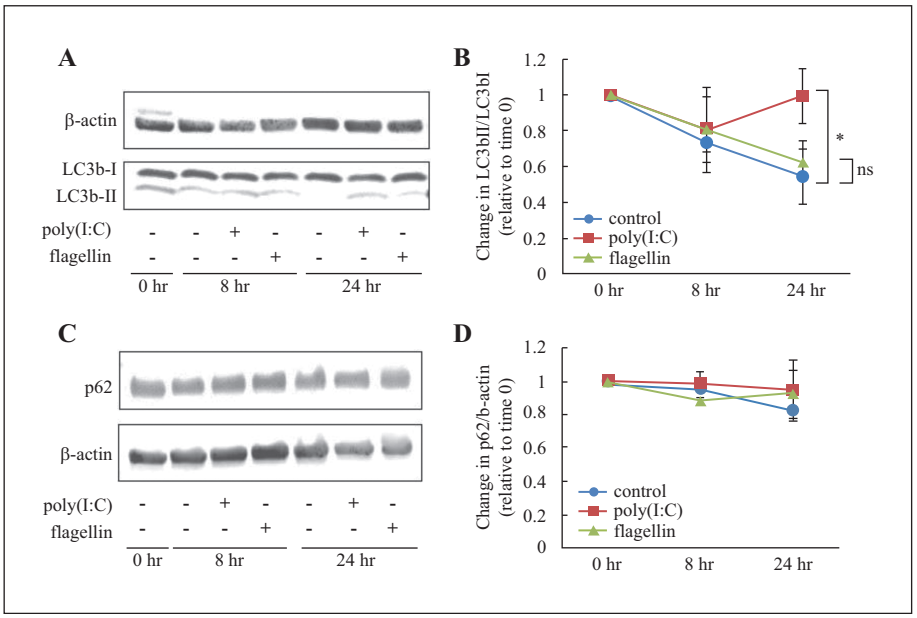

Figure 3

Differential effect of poly(I:C) and flagellin on LC3b-I to LC3b-II conversion and p62/SQSTM1. HFKs were stimulated with 1,500 ng/mL poly(I:C) or 1,000 ng/mL flagellin and lysed after 8 or 24 hours. Total protein was subjected to western blotting and probed for LC3b and p62 to assess early effects on autophagy. **A**) Representative western blot showing stimulus-specific changes in the LC3b-II/-I ratio as an early response to poly(I:C) or flagellin stimulation. **B**) Quantified changes in LC3b-II/-I ratios of unstimulated or poly(I:C) or flagellin-stimulated HFKs, relative to time zero, indicating a poly(I:C)-specific increase in autophagosome membrane formation at 24 hours. **C**) Representative western blot showing p62/SQSTM1 levels after 8- and 24-hour stimulations with poly(I:C) or flagellin along with unstimulated controls. **D**) Quantified p62/SQSTM1 levels, normalized to β-actin, in unstimulated or poly(I:C) or flagellin-stimulated HFKs relative to time zero, showing no evidence of p62/SQSTM1 decrease after 24 hours of stimulation. *$p<0.05$; two-tailed Student's T-test. n.s.; not significant.

microscopy over the course of 24 hours, in addition to assessing LC3b-II/-I ratio based on western blot. Control cells prior to treatment consistently showed low levels of green fluorescent signal, indicating a relatively low abundance of autophagic vesicles at steady state at eight and 24 hours (*figure 4A, B*). Treatment with CQ, to inhibit lysosomal function and interrupt autophagy, was used as a positive control. As expected, CQ treatment led to a drastic increase in green fluorescent punctate perinuclear signal, four-fold over baseline, indicating elevated autophagosome abundance by eight hours, which was extended to 24 hours post treatment (*figure 4A, B*). Neither eight- nor 24-hour post-flagellin treatment showed any elevation in autophagosome abundance compared to control cells (*figure 4A,B*). In contrast, poly(I:C) treatment led to a two-fold increase in autophagosome abundance by eight hours (p=0.032) (*figure 4A,B*). By 24 hours, poly(I:C) treatment resulted in an approximate four-fold increase from time zero, which was almost significant (p=0.059) (*figure 4A,B*). Moreover, poly(I:C) caused autophagosome accumulation in HFKs indicating either reduced autophagosome turnover, induced autophagosome biogenesis or a combination of both, while flagellin-treated cells showed no alterations in autophagy.

Perturbation of autophagic flux affects IL-36γ secretion in HFKs

To determine whether IL-36γ can be released through an autophagy-dependent secretory pathway, we measured IL-36γ accumulation after blocking autophagic flux with bafilomycin A1. Bafilomycin A1 inhibits lysosomal proton pumps, effectively raising lysosomal pH which prevents effective autophagosomal turnover [21, 22]. Bafilomycin treatment led to increased LC3b-I and -II, reflecting the accumulation of LC3b-II+ autophagosomes and adequate TLR-3 and TLR-5-mediated IL-36γ induction (*figure 5A*). Bafilomycin treatment, 24 hours after IL-36γ induction, enhanced poly(I:C)-mediated (p=0.044) but not flagellin-mediated (p=0.121) IL-36γ secretion (*figure 5B*). These data provide evidence that the active secretion of IL-36γ can be affected by autophagy-modulating compounds and likely involves the autophagosome.

To strengthen our notion that the autophagosome can potentially have a secretory role in IL-36γ release from HFKs, we next asked if inducing autophagy pharmacologically would alter IL-36γ secretion. Rapamycin, a well-established inducer of autophagy, acts through an mTOR-dependent mechanism [23]. Rapamycin treatment clearly induced autophagy by 48 hours, as indicated by the reduction in LC3b-I but not LC3b-II compared to vehicle controls (*figure 5C*). Rapamycin treatment following flagellin stimulation led to a noticeable increase in the amount of IL-36γ secreted compared to flagellin with vehicle control, but the increase did not reach statistical significance (*figure 5D*). This trend reinforces the observation that autophagy plays a role in IL-36γ externalization. Rapamycin treatment had no effect on poly(I:C)-mediated secretion, consistent with the fact that poly(I:C) alone can induce autophagy.

Since we noticed a positive trend with rapamycin in enhancing IL-36γ release, we investigated whether inducing autophagy via an mTOR-independent mechanism led to similar effects. We induced autophagy

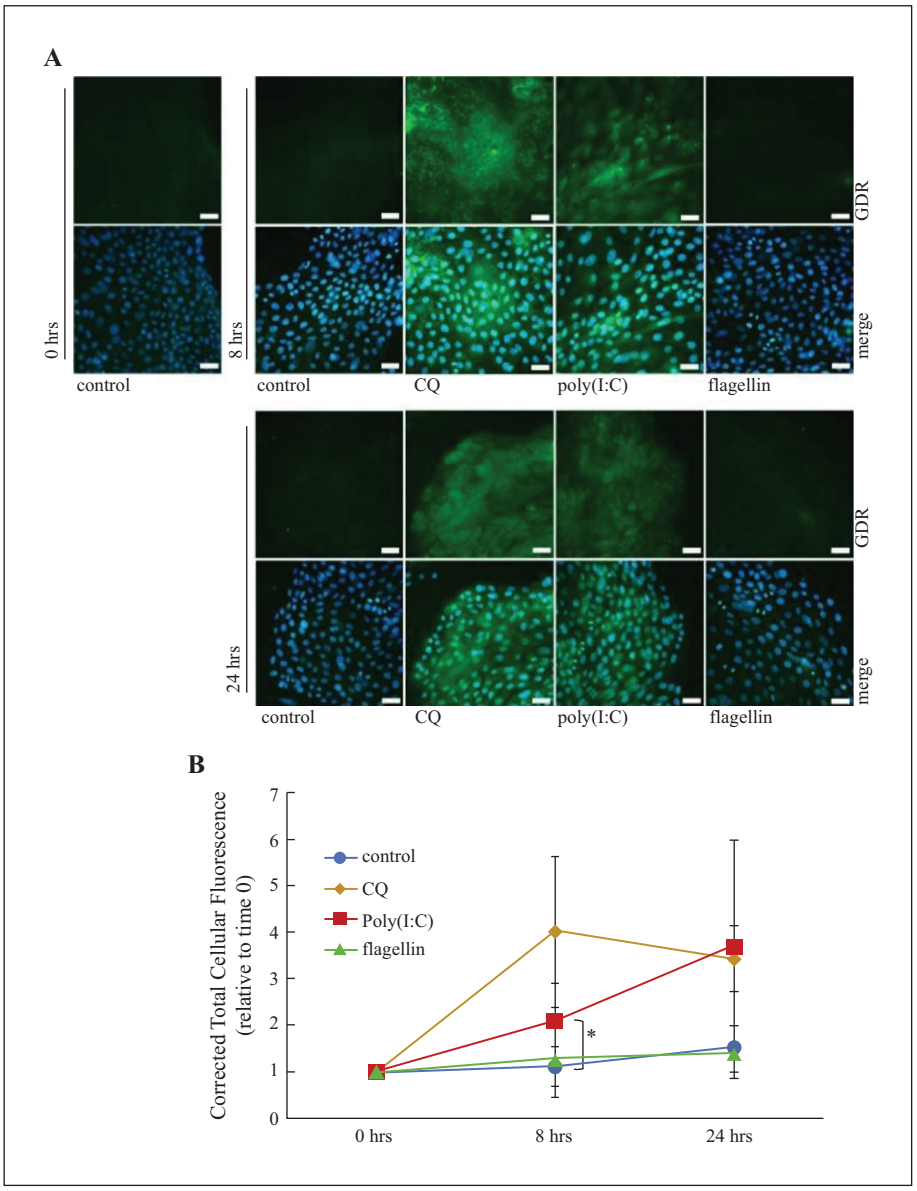

Figure 4

Poly(I:C) but not flagellin treatment leads to autophagosome accumulation. HFKs were stimulated with poly(I:C) (1,500 ng/mL) or flagellin (1,000 ng/mL), or CQ (50 μM) as a positive control, or were left unstimulated for the indicated time points. Cells were stained with an autophagy monitoring kit and fluorescent signals were visualized. **A**) Representative micrographs showing steady state, poly(I:C)-, flagellin- or CQ-induced autophagosome presence at 0, 8 and 24 hours. **B**) Quantification of fluorescent autophagosomes post-treatment, expressed as CTCF relative to time zero. Scale bars represent 40 μm. Data represent three independent experiments after passaging HFKs three times. GDR; green detection reagent; CQ; chloroquine. *$p< 0.05$; two-tailed Student's T-test.

with a high dose of sucrose 24 hours after poly(I:C) or flagellin stimulation. Sucrose has been recently shown to induce autophagy in keratinocytes, independent of the mTOR complex [24]. Sucrose induced autophagy by 48 hours, as indicated by the accumulation of LC3b-II and LC3b-I (*figure 5E*). Surprisingly, however, sucrose treatment did not lead to any significant difference in the secretion of IL-36γ after stimulation by either TLR agonist (*figure 5F*). This suggests that the modest, but noticeable, effect autophagy induction has on IL-36γ secretion is mTOR dependent.

LC3b-II is reduced in respiratory papillomas

HPV-infected laryngeal papillomas from individuals with RRP show no signs of inflammation despite over-expression of IL-36γ; one possible explanation is dysfunctional regulated release from infected keratinocytes [8]. Since dysregulated autophagy has been reported in many types of cancer, it is possible that autophagy is dysregulated in papilloma tissue. Relative to clinically normal laryngeal tissue, we confirmed IL-36γ over-expression in RRP tissues, a hallmark of the diseased tissue (*figure 6A*). As a rudimentary measure of autophagy, we compared LC3b-I and -II levels in papilloma and clinically normal tissue biopsies. Levels of LC3b-II were reduced in papillomas compared to clinically normal tissue from the same patients (*figure 6B*). While it is impossible to draw conclusions about differences in rates of autophagy flux between normal and papilloma tissues based on this measure, the data suggest that the number of autophagosomes is reduced in papillomas.

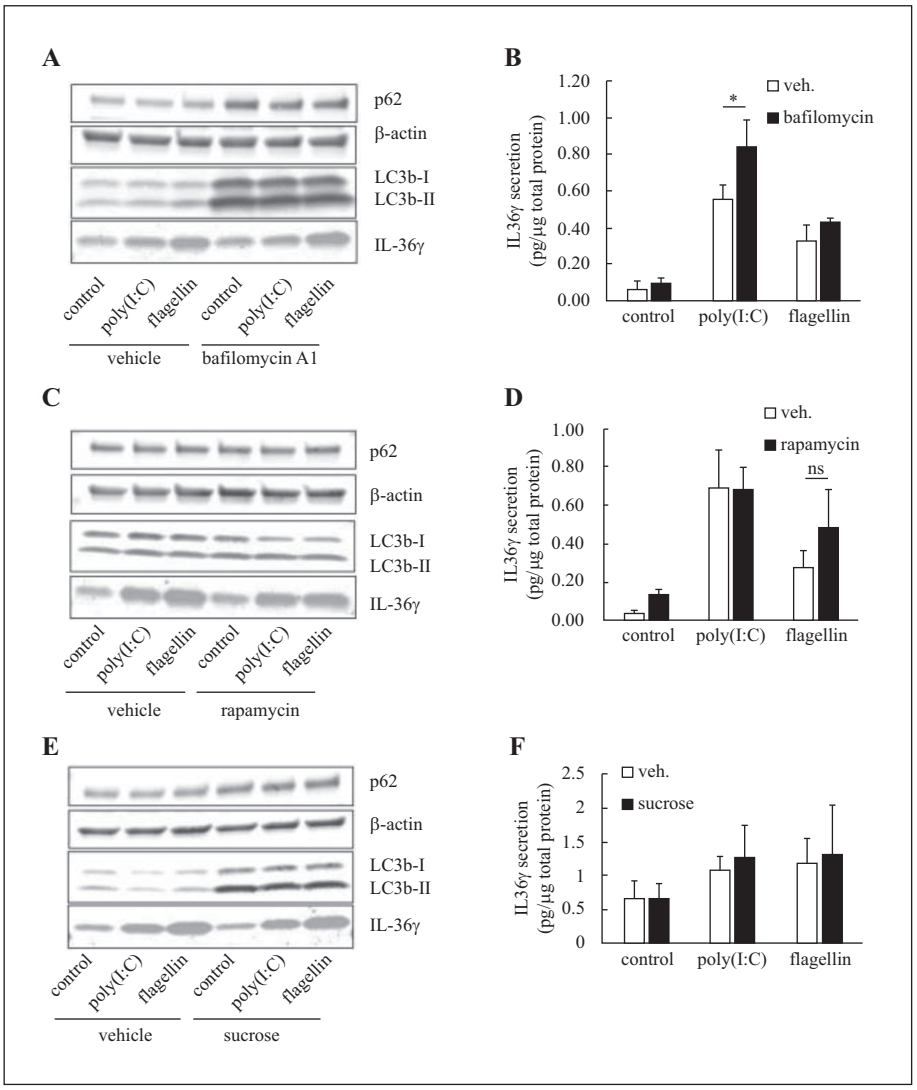

Figure 5

IL-36γ secretion in HFKs is modulated by compounds that affect autophagy. HFKs were stimulated with poly(I:C) or flagellin as described previously for 24 hours followed by a media change containing bafilomycin A1 (50 nM), rapamycin (1 μM) or sucrose (100 mM). IL-36γ accumulated for 48 hours, after which cells were lysed, and conditioned media was collected and subjected to IL-36γ-specific ELISA. Total protein was subjected to western blotting to confirm effects on autophagic protein expression and adequate IL-36γ induction. **A**) Representative western blot showing IL-36γ induction after TLR stimulation, an elevated LC3b-II/-I ratio, and accumulation of p62/SQSTM1 (indicative of autophagy blockage by bafilomycin A1). **B**) IL-36γ accumulation, measured by ELISA, showing that blocking autophagy increases poly(I:C)-mediated, but not flagellin-mediated IL-36γ secretion. **C**) Representative western blot showing IL-36γ induction after TLR stimulation as well as LC3b-I, -II, and p62/SQSTM1 levels 48 hours after rapamycin introduction. **D**) IL-36γ accumulation, measured by ELISA, showing a trend in the increase of IL-36γ secretion in the presence of flagellin , but not poly(I:C), in response to rapamycin, compared to vehicle. **E**) Representative western blot showing IL-36γ induction after TLR stimulation, and elevated LC3b-II, -I and p62/SQSTM1 levels, 48 hours after sucrose-mediated mTOR independent autophagy induction. **F**) IL-36γ accumulation, measured by ELISA, showing no evidence of altered IL-36γ secretion in response to sucrose for either agonist. Bars represent mean ± SD from three experiments. *$p < 0.05$; two-tailed Student's T-test.

DISCUSSION

IL-36γ is a potentially potent cytokine that has important roles in the epithelial immune response during inflammation [8] and wound healing [25]. While its dysregulation is implicated in diseases of the epithelium including psoriasis [26] and RRP [5, 8], the molecular mechanisms responsible for normal IL-36γ secretion are not well understood. Early work on IL-36γ release from keratinocytes described passive, unregulated release during lytic cell death during exposure to toxic doses of poly(I:C) [9]. Later work from our group demonstrated that IL-36γ could be secreted robustly and consistently in response to non-toxic doses of poly(I:C), but specific cellular processes that contribute to its externalization in the absence of pyroptotic cell death remain to be described [10].

Non-classic protein secretion mechanisms including secretory lysosomes, secretory autophagy and exosomes contribute to a cell's ability to externalize leaderless proteins. Release of leaderless IL-1β has been demonstrated through several of these mechanisms including chaperone-mediated autophagy [27]. Based on the study by Iula *et al.,* treatment with bafilomycin A1 reduces LPS- and LPS+ATP-mediated IL-1β secretion from neutrophils, suggesting that functional turnover of autophagosomes via the lysosome clearly contributes to IL-1β release [28].

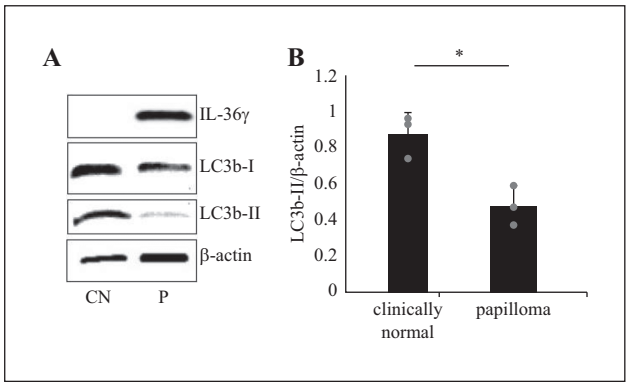

Figure 6
The level of autophagosome membrane-associated LC3b-II is altered in laryngeal papillomas compared to clinically normal laryngeal tissue. **A)** Representative western blot of extracts from clinically normal (CN) and papilloma (P) laryngeal tissue, showing IL-36γ over-expression and low levels of the autophagosomal marker, LC3b-II, in papilloma laryngeal tissue (β-actin was used as a loading control). Data are representative of three different tissue pairs analysed. **B)** LC3b-II/β-actin ratios are significantly reduced in RRP biopsies compared to clinically normal tissues as a surrogate, relative measure of autophagy. Bars represent mean ± SD based on three papilloma and three clinically normal tissues. * $p<0.05$; two-tailed Student's T-test.

In contrast to the work performed in neutrophils, HFKs treated with bafilomycin after poly(I:C) treatment resulted in an increased IL-36γ accumulation in HFK-conditioned media. It is possible that keratinocytes may be compensating for the stress of accumulating autophagosomes by fusing the organelles with the plasma membrane, releasing the contents, potentially including IL36γ. Although just below the level of significance, pharmacologically inducing autophagy with rapamycin led to a trending increase in IL-36γ release compared to vehicle; an effect specific to flagellin stimulation. Treatment of primary keratinocytes with compounds that augment autophagy can lead to differential levels of secreted IL-36γ, which seems dependent on the exogenous signal detected. Further investigation is required to tease out the details of this mechanism.

Strengthening the notion that IL-36γ is a selective cargo of a secretory autophagosome is the recently published work from our group showing that IL-36γ co-migrates with LC3b-II and markers of multivesicular bodies during subcellular fractionation of homogenates derived from poly(I:C)-stimulated HFKs [29]. This provides evidence that IL-36γ protein can become a selective cargo of autophagosomes and/or amphisomes prior to its secretion in small extracellular vesicles [29]. It may be that the differences in the intracellular handling and release of IL-36γ reflects the nature of the pathogen-associated exogenous signal detected at the epithelium, to better deal with the threat. In this study, we saw a difference between poly (I:C) and flagellin in LC3b-I to -II conversion kinetics. This result is consistent with previously published studies reporting that TLR3 activation induces autophagy (*i.e.* autophagosome formation via LC3b-II-GFP puncta) while TLR5 activation does not [20]. We propose that more than one non-classic secretion pathway mediates soluble IL-36γ release from HFKs in response to stimulation by different agonists. While LC3b-I conversion patterns and elevated fluorescent signal during autophagy perturbance indicate a greater presence of autophagosomes during poly(I:C) exposure, we clearly observe IL-36γ accumulation during the first 24 hours of flagellin exposure in the absence of LC3b-II conversion. Although further experimentation is needed, the delay in secretion observed with poly(I:C) treatment (*figure 1 C,D*) may reflect the transcription/translation of autophagic machinery and assembly of autophagosomes. Diverging IL-36γ secretion kinetics, dependent on poly(I:C) and flagellin, indicate separable secretion mechanisms. In contrast, rapamycin did not further enhance autophagosome formation during poly(I:C) stimulation or increase secretion, suggesting that the pathways used by flagellin-stimulated release are not contributing to poly(I:C)-mediated release. The absence of SQSTM1/p62 reduction during the first 24 hours of poly(I:C) stimulation suggests that the autophagosomes are primarily secretory, not degradative, but further experimentation is required to confirm this. Fluorescence microscopy showed obvious autophagosome accumulation in response to poly(I:C), a likely cellular stress, as lysosomal regeneration is a limiting step in macroautophagy. It is possible, in attempts to mitigate this stress, these cellular compartments are redirected to fuse with the plasma membrane rather than the lysosome, resulting in secretion of their cargos. This could explain why lysosomal inhibition with bafilomycin A1 enhanced secretion after stimulation with poly(I:C) and not flagellin, as saturation of the lysosomal degradation system during poly(I:C) exposure may redirect trafficking of intracellular compartments containing IL-36γ. This is consistent with IL-36γ release being independent of intracellular levels to trigger secretion during poly(I:C) detection, but dependent on broader cellular processes including autophagosome formation that are selectively affected by divergent pathogen-associated stimuli.

We also addressed whether IL-36γ may be shaping the local epithelial immune response once secreted. We and others have reported drastic differences in the activities of full-length and cleaved IL-36γ in driving proinflammatory cytokine expression in monocytes and keratinocytes [8,30]. We showed that recombinant cleaved IL-36γ can drive expression of CXCL8 and IL-36γ itself in HFKs, which primes the local microenvironment for neutrophil influx to combat a bacterial pathogen. HFK responses to flagellin-conditioned media show similarities to those driven by cleaved IL-36γ despite no evidence of cleaved IL-36γ being present. Poly(I:C)-stimulated HFK-conditioned media led to lower levels of IL-36γ and CXCL8 induction. These results suggest that during bacterial challenge at epithelial surfaces, flagellin detection may lead to neutrophil influx, independent of activated IL-36γ. Once recruited, this provides the elastase to process IL-36γ, amplifying the inflammatory response during infection; a response targeted to deal with the bacterial threat.

To our knowledge, we are the first to report that respiratory papillomas have significantly reduced levels of the autophagy marker, LC3b-II, compared to clinically normal laryngeal tissue; an indication of perturbed autophagic flux. The early HPV protein 5 (E5) of HPV-16 can manipulate autophagy to its advantage through suppression of autophagosome formation by down-regulating key autophagy-related genes, to help protect viral components from degradation [31]. When expressed in non-infected keratinocytes, HPV-16 E5 led to lower LC3b-II levels as autophagosome generation was blocked [31]. A similar mechanism could explain the lower level of LC3b-II in papillomas induced by HPV-6/11. The scarcity of, and technical challenges of obtaining and growing RRP biopsies continue to be a barrier to answering these questions but future experiments will better address alterations in HPV-infected, pre-malignant tissues.
Although utilizing bafilomycin A1 and rapamycin to manipulate autophagy provided valuable insight into the role autophagy plays in cytokine secretion, it does represent a limitation of our study. Future experiments will include knockdown of specific transcripts of crucial autophagy-related genes (*ATG5*) for more highly controlled autophagy interruption. Despite the limitations of our study, we have shown that secretion of the cytokine, IL-36γ, from normal keratinocytes is dependent on the stimulus used to induce it and likely involves multiple non-classic protein secretion pathways. IL-36γ secretion can be modulated with well-established compounds that affect autophagy, opening new avenues for therapeutic options for skin disorders due to dysregulated IL-36γ signalling.

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