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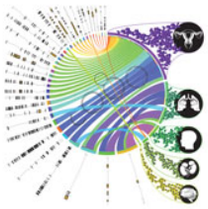

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Phenylbutyrate induces LL-37-dependent autophagy and intracellular killing of *Mycobacterium tuberculosis* in human macrophages

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Keywords: antimicrobial peptides, cathelicidin, innate immunity, P2RX7, tuberculosis, vitamin D

Abbreviations: $1,25(OH)_2D_3$, 1,25-dihydroxy vitamin D_3; AMPK, adenosine monophosphate-activated protein kinase; AMPs, antimicrobial peptides; ATG, autophagy related; BECN1, Beclin 1, autophagy related; CAMP, cathelicidin antimicrobial peptide; CFUs, colony-forming units; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; MAP1LC3/LC3, microtubule-associated protein 1 light chain 3; MDMs, monocyte-derived macrophages; *Mtb*, *Mycobacterium tuberculosis*; P2RX7, purinergic receptor P2X, ligand gated ion channel, 7; PBA, 4-phenylbutyrate; PBS, phosphate-buffered saline; PtdIns3K, phosphatidylinositol 3-kinase; *RNA18S/18S* rRNA, RNA 18S ribosomal; SQSTM1, sequestosome 1; TB, tuberculosis

LL-37 is a human antimicrobial peptide (AMP) of the cathelicidin family with multiple activities including a mediator of vitamin D-induced autophagy in human macrophages, resulting in intracellular killing of *Mycobacterium tuberculosis* (*Mtb*). In a previous trial in healthy volunteers, we have shown that LL-37 expression and subsequent *Mtb*-killing can be further enhanced by 4-phenylbutyrate (PBA), also an inducer of LL-37 expression. Here, we explore a potential mechanism(s) behind PBA and LL-37-induced autophagy and intracellular killing of *Mtb*. *Mtb* infection of macrophages downregulated the expression of both the *CAMP* transcript and LL-37 peptide as well as certain autophagy-related genes (*BECN1* and *ATG5*) at both the mRNA and protein levels. In addition, activation of LC3-II in primary macrophages and THP-1 cells was not detected. PBA and the active form of vitamin D_3 ($1,25[OH]_2D_3$), separately or particularly in combination, were able to overcome *Mtb*-induced suppression of LL-37 expression. Notably, reactivation of autophagy occurred by stimulation of macrophages with PBA and promoted colocalization of LL-37 and LC3-II in autophagosomes. Importantly, PBA treatment failed to induce autophagy in *Mtb*-infected THP-1 cells, when the expression of LL-37 was silenced. However, PBA-induced autophagy was restored when the LL-37 knockdown cells were supplemented with synthetic LL-37. Interestingly, we have found that LL-37-induced autophagy was mediated via P2RX7 receptor followed by enhanced cytosolic free Ca^{2+}, and activation of AMPK and PtdIns3K pathways. Altogether, these results suggest a novel activity for PBA as an inducer of autophagy, which is LL-37-dependent and promotes intracellular killing of *Mtb* in human macrophages.

Introduction

Tuberculosis (TB) infection poses a serious threat to human health worldwide, especially in many developing countries. It is difficult to cure TB as standard chemotherapy involves daily treatment with multiple drugs for 6 to 8 mo. The emergence of drug-resistant TB has further complicated the treatment situation around the world.[1] Complete clearance of TB infection is difficult since *Mycobacterium tuberculosis* (*Mtb*) is an intracellular human pathogen that has developed strategies to survive inside host macrophages even in the presence of inflammation. *Mtb* persists within immature phagosomes of macrophages and actively inhibits the maturation of phagolysosomes.[2] Activation of autophagy in macrophages causes mycobacterial phagosomes to mature into autolysosomes, which reduces intracellular survival of mycobacteria.[3,4]

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Autophagy is a programmed homeostatic cellular process, which plays a vital role to maintain the balance between protein synthesis and degradation in cells.[5] The most prominent role of autophagy in relation to immunity is to kill intracellular pathogens such as *Mtb*.[6] Autophagy is a highly conserved process, involving the formation of double-membrane vesicles designated autophagosomes that deliver cytoplasmic materials including macromolecules and whole organelles or intracellular pathogens to lysosomes for degradation.

Additional well-known innate immune defense mechanisms that macrophages utilize to kill intracellular *Mtb* bacilli include effects of antimicrobial peptides (AMPs) and production of nitric oxide.[7,8] AMPs kill bacteria by disrupting membrane function and also interfere with cell-wall synthesis.[9] Cathelicidin is a family of AMPs, where the only human cathelicidin identified is the human antimicrobial protein, hCAP-18, from which the C-terminal part is cleaved to form the active peptide LL-37.[10] The importance of LL-37 in human TB has been revealed partly through studies on the immunomodulatory effects of vitamin D_3, which is known to promote LL-37 expression with subsequent intracellular killing of *Mtb*.[11,12] We have shown that LL-37 expression is downregulated at the mucosal epithelial surfaces during acute diarrhea,[13] and in infection with *Neisseria gonorrhea*.[14] Furthermore, we have discovered that downregulation of a cathelicidin ortholog in a rabbit model of shigellosis can be counteracted by butyrate, a bacterial fermentation product from dietary fibers present in the colon.[15] Our findings have further established that 4-phenylbutyrate (PBA) can induce LL-37 expression at both mRNA and protein levels in a synergistic manner with the active form of vitamin D_3, $1,25(OH)_2D_3$, in lung epithelial cells.[16] Accordingly, LL-37 expression is induced in human monocyte derived macrophages (MDMs) upon oral administration of PBA and vitamin D_3 to healthy individuals.[17] Interestingly, Yuk et al. showed that $1,25(OH)_2D_3$ activates autophagy in human macrophages via an LL-37-dependent mechanism, which results in reduced intracellular survival of *Mtb*.[18] Given the potent induction of LL-37 by PBA together with vitamin D we hypothesized that these compounds, alone or in combination, could improve killing of *Mtb* in human macrophages, also by activation of autophagy. Therefore, in the present study we used an in vitro model including primary macrophages and THP-1 cells to study the role of LL-37 and the inducer PBA together with vitamin D in activation of autophagy with subsequent *Mtb* killing. We show that PBA and vitamin D-induced autophagy is dependent on LL-37 and is receptor mediated.

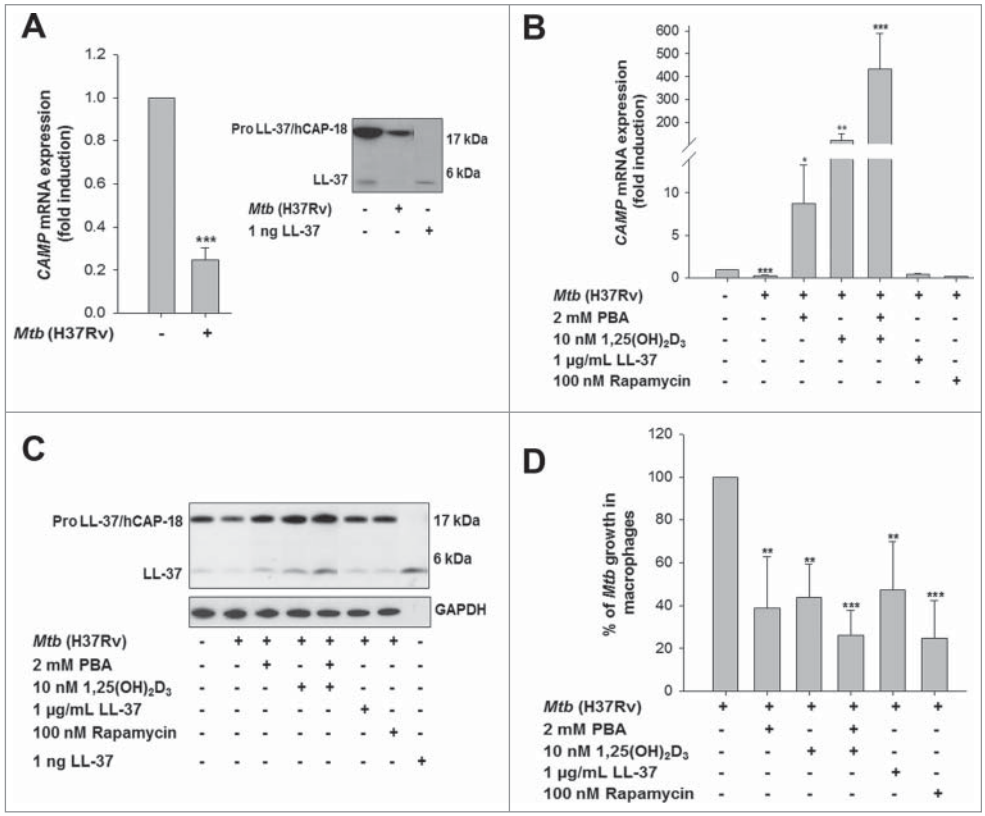

Figure 1. Phenylbutyrate (PBA)-induced LL-37 expression was associated with the control of *Mtb* growth in human monocyte-derived macrophages (MDMs). (**A**) Human MDMs were infected with the virulent strain of *Mycobacterium tuberculosis* H37Rv at a multiplicity of infection of 1:5 for 4 h, and quantitative real-time qPCR and western blot for the expression of the *CAMP* mRNA transcript (normalized to *RNA18S/18S* rRNA expression) and peptide of LL-37 was investigated, respectively. (**B**) After *Mtb* infection cells were treated with PBA (2 mM) and/or$1,25(OH)_2D_3$ (10 nM) or LL-37 (1 μg/mL) or rapamycin (100 nM) for 24 h; real-time qPCR was performed for the transcript of *CAMP* (normalized to *RNA18S/18S* rRNA expression) in 6 independent experiments (mean ± SD). (**C**) A representative western blot of LL-37 peptide and GAPDH from 6 independent experiments are shown. (**D**) Intracellular bacterial viability was determined based on the number of CFUs. Results are shown from 5 independent experiments (mean ± SD). The *P* values were ≤0.001, ≤0.01, ≤0.05 as indicated by ***, **and *, respectively.

Results

Expression of LL-37 induced by phenylbutyrate (PBA) is associated with control of *Mtb* growth in human macrophages

Innate immune effector molecules such as the human antimicrobial peptide LL-37 which is encoded by the *CAMP* (cathelicidin antimicrobial peptide) gene have been involved in the control of human TB.[12] Here, we found that *Mtb* H37Rv suppressed the expression of LL-37 in human monocyte-derived macrophages (MDMs), at both mRNA ($P < 0.001$) (**Fig. 1A**) and protein (*P*

= 0.041) levels (**Fig. 1A**). Importantly, treatment of MDMs with PBA counteracted this suppressive effect of *Mtb* and instead induced both *CAMP* mRNA ($P = 0.023$) (**Fig. 1B**) and LL-37 peptide ($P = 0.031$) (**Fig. 1C**) expression. Similar effects on *CAMP* mRNA expression were observed in THP-1 cells (**Fig. S1A and S1B**). Active vitamin D_3 ($1,25[OH]_2D_3$) is a known potent inducer of LL-37 expression and accordingly, $1,25(OH)_2D_3$ also counteracted the downregulation of *CAMP* mRNA and LL-37 peptide in MDMs (**Fig. 1B and C**). Moreover, the combination of PBA and $1,25(OH)_2D_3$ exhibited a synergistic effect on *CAMP* mRNA ($P < 0.001$) (**Fig. 1B**) and LL-37 peptide expression ($P = 0.033$) (**Fig. 1C**) in *Mtb*-infected MDMs. Treatment with synthetic LL-37 peptide or rapamycin, which is a known inducer of autophagy, had no significant effect on *CAMP* mRNA and LL-37 peptide expression (**Fig. 1B and C**).

To study whether the induction of LL-37 was associated with control of intracellular *Mtb* growth, primary MDMs were infected with virulent *Mtb* strain and treated with PBA alone or in combination with $1,25(OH)_2D_3$. PBA treatment significantly reduced the bacterial load (colony-forming units [CFU], see **Fig. S2**) of *Mtb* (H37Rv, virulent strain of *Mtb*) in MDMs (40% to 60% growth inhibition, $P = 0.002$) as did treatment with $1,25(OH)_2D_3$, synthetic LL-37 and rapamycin (positive control) (**Fig. 1D**). The most prominent effect on *Mtb*-inhibition was observed when MDMs were treated with a combination of PBA together with $1,25(OH)_2D_3$ and also the rapamycin treatment (50% to 80% growth inhibition, $P < 0.001$ for both treatments) (**Fig. 1D**). Altogether, these results demonstrate that PBA and $1,25(OH)_2D_3$ are potent inducers of LL-37, and effectively reduce the viability of intracellular *Mtb* in human macrophages.

mRNA and protein expression of the autophagy-related genes BECN1 and ATG5 are enhanced by PBA and concur with colocalization of LC3-II and LL-37 in the autophagosome

Autophagy is involved in the control of intracellular *Mtb* growth. Thus, we continued to explore if PBA also is able to induce autophagy, using real-time quantitative (q) PCR, western blot analysis and confocal microscopy of *Mtb*-infected MDMs. Two essential proteins involved in autophagosome initiation and elongation are BECN1/Beclin 1 and ATG5. Similar to the expression of *CAMP*, mRNA expression of *BECN1* and *ATG5* was significantly downregulated in *Mtb*-infected MDMs ($P = 0.046$ and $P = 0.049$, respectively; **Fig. 2A and B**). However, PBA, alone or in combination with $1,25(OH)_2D_3$, was able to overcome this effect of *Mtb* and upregulated mRNA expression of both *BECN1* and *ATG5* in MDMs ($P = 0.031$ and $P = 0.019$, respectively) (**Fig. 2A and B**). Thus, the inducers of LL-37, PBA, and $1,25(OH)_2D_3$ were able to prevent an *Mtb*-mediated downregulation of the autophagy-related genes *BECN1* and *ATG5* in human macrophages. Similar effects were observed on the protein level of BECN1 and ATG5 (**Fig. 2C and D**).

Together with BECN1 and ATG5, LC3 is also a defined marker for autophagy, which plays an essential role in autophagosome formation. Cellular localization of LC3 was cytoplasmic with faint staining in uninfected and *Mtb*-infected MDMs, however LC3 was localized in puncta structures after treatment with PBA and/or $1,25(OH)_2D_3$. The LL-37 peptide was also upregulated in PBA-treated cells and further found to be colocalized with the LC3 puncta structures (**Fig. 2E**), indicating the presence of the peptide in the autophagosome. The combined action of PBA and $1,25(OH)_2D_3$ resulted in the highest percentage of cells with LC3-positive puncta (**Fig. 2F**). Interestingly, synthetic LL-37 peptide turned out to be similarly efficient as PBA, $1,25(OH)_2D_3$ and rapamycin to induce autophagosome formation (**Fig. 2F**).

While LC3 is predominantly present in the cytosol of cells in the nonlipidated form LC3-I, a series of conjugation reactions mediated by a specific set of autophagy-related proteins leads to the covalent linkage of LC3 to phosphatidylethanolamine upon autophagy induction. This lipidated form called LC3-II is associated with the membrane of the phagophore and autophagosome. Here, western blot analysis was utilized to demonstrate that PBA treatment of *Mtb*-infected MDMs promoted the conversion of LC3-I to LC3-II. Hence, the relative ratio of LC3-II to LC3-I was substantially higher in treated cells (**Fig. 2G**). A similar effect on LC3 was observed when THP-1 cells were treated with PBA (**Fig. S3**).

Next, the SQSTM1/p62 (sequestosome 1) protein was used as a marker to monitor autophagic flux. SQSTM1 is a ubiquitin-binding scaffold protein that is degraded by autophagy. Thus, SQSTM1 accumulates when autophagy is inhibited,[19] whereas decreased levels of SQSTM1 are observed when autophagy is activated.[20] Here, the SQSTM1 protein was detected in uninfected and *Mtb*-infected cells but significantly decreased in cells treated with PBA and/or $1,25(OH)_2D_3$ as well as with LL-37 (**Fig. S4**). Altogether, these results demonstrated that PBA activates autophagy in *Mtb*-infected macrophages via induction of the autophagy-related markers BECN1 and ATG5 at both the mRNA and protein levels (**Fig. 2A to D**). Enhanced autophagic activity was evident by involving conversion of LC3-I to LC3-II (**Fig. 2E to G**) and degradation of the SQSTM1 protein (**Fig. S4**). These findings confirmed that both PBA and $1,25(OH)_2D_3$ were efficient inducers of autophagy.

PBA-mediated activation of autophagy in human macrophages is dependent on the expression of LL-37

To evaluate the role of LL-37 in autophagy, we silenced LL-37 expression by knocking down *CAMP* (cathelicidin antimicrobial peptide) gene in the THP-1 cell line. In *CAMP* short hairpin RNA (shRNA)-transfected THP-1 cells, treatment with PBA and $1,25(OH)_2D_3$, failed to induce autophagy, while LC3-II was clearly detected in nonspecific (*NS*) shRNA-transfected cells (**Fig. 3A**). Notably, autophagy was restored when *CAMP* shRNA-transfected THP-1 cells were stimulated with synthetic LL-37 peptide, but also upon treatment with rapamycin that induce autophagy in an MTOR-dependent manner (**Fig. 3A**). Western blot analysis revealed that *CAMP* shRNA-transfected THP-1 cells only contained the non-lipidated form of LC3, LC3-I, after treatment with PBA and/or $1,25(OH)_2D_3$, while the lipidated, autophagosome-associated form of LC3, LC3-II,

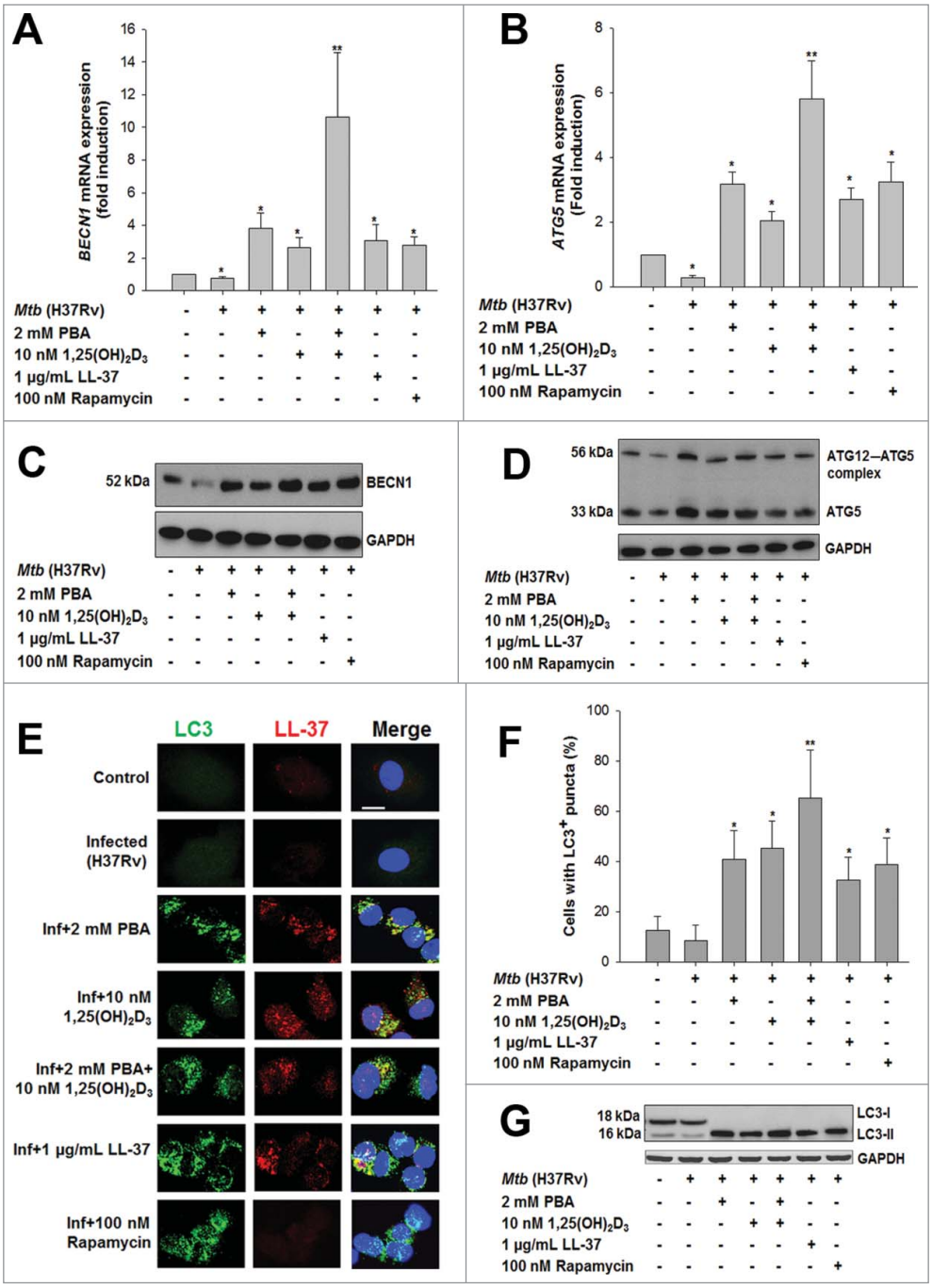

Figure 2. Phenylbutyrate (PBA)-induced autophagy in human macrophages, upregulated the expression of the autophagy-related genes *BECN1* and *ATG5*, and activated the processing of LC3. Human MDMs were infected with the virulent strain of *Mycobacterium tuberculosis* H37Rv for 4 h and treated with PBA (2 mM) and/or1,25$(OH)_2D_3$ (10 nM) or LL-37 (1 μg/mL) or rapamycin (100 nM) for 24 h. (**A and B**) The mRNA levels of autophagy-related genes *BECN1* (**A**) and *ATG5* (**B**) (normalized to *RNA18S/18S* rRNA expression) in human MDMs were measured by real-time qPCR. Results are shown from 6 independent experiments (mean ± SD). (**C and D**) A representative western blot of BECN1, ATG5 and the housekeeping protein GAPDH from 6 independent experiments are shown. (**E**) Human MDMs were infected with the virulent strain of *Mycobacterium tuberculosis* H37Rv for 4 h and treated with PBA (2 mM) and/or1,25$(OH)_2D_3$ (10 nM) or LL-37 (1 μg/mL) or rapamycin (100 nM) for 24 h. The cells were fixed and stained with DAPI to visualize the nuclei (blue), and with anti-LC3 and anti-LL-37, followed by the addition of Alexa-fluor 488- and 594-conjugated goat anti-rabbit and mouse IgG (green and red color respectively). One representative immunofluorescence image out of 6 independent replicates are shown; scale bars: 10 μm. (**F**) Quantitative analysis of the percentages of cells with $LC3^+$ puncta was measured from 6 independent experiments (mean ± SD. (**G**) A representative western blot shows the conversion of LC3-I to LC3-II and the house keeping protein GAPDH from 6 independent experiments. The *P* values were ≤0.01 and ≤0.05 as indicated by **and *, respectively.

was clearly visible in NS shRNA-transfected cells (**Fig. 3B**). Consistent with the microscopy analysis, conversion of LC3-I to LC3-II was observed when *CAMP* shRNA-transfected THP-1 cells were treated with synthetic LL-37 or rapamycin (**Fig. 3B**). The expression of the autophagy-related genes *BECN1* and *ATG5* were also quantified in the *CAMP* shRNA and *NS* shRNA-transfected THP-1 cells by real-time qPCR after PBA and/or 1,25$(OH)_2D_3$ treatment. Silencing of *CAMP* gene expression caused a significant reduction of both *BECN1* and *ATG5* mRNA levels (3–5-fold– reduction in 24 h) compared to *NS* shRNA-transfected THP-1 cells upon PBA and/or 1,25 $(OH)_2D_3$ stimulation. However, no significant differences in *BECN1* and *ATG5* expression were noted between *CAMP* shRNA and *NS* shRNA-transfected THP-1 cells when treated with LL-37 or rapamycin (**Fig. 3C and 3D**). These results support our hypothesis that LL-37 is essential for PBA-mediated induction of autophagy.

PBA and vitamin D-treated macrophages secrete factors that activate autophagy in an LL-37-dependent manner

First we showed that LL-37 was secreted from macrophages upon treatment with PBA and 1,25$(OH)_2D_3$ (**Fig. 4A and B**).

Next we showed that supernatants from macrophages stimulated with PBA and $1,25(OH)_2D_3$ significantly induced autophagy in freshly prepared macrophages measured by increased mRNA levels of *BECN1* and *ATG5* in addition to the conversion of LC3-I to LC3-II (**Fig. 4C to E**). This indicated that macrophages can release one or several soluble factors that was/were essential for activation of autophagy. In order to investigate if LL-37 was the main mediator of autophagy, we preincubated the supernatant fraction with a neutralizing monoclonal antibody against LL-37 prior to stimulation of the macrophages. Importantly, neutralization of LL-37 in the culture supernatants prevented the activation of autophagy in macrophages (**Fig. 4F to H**), while autophagy was maintained in cells treated with supernatant preincubated with an isotype control measured by the same methods as in panel 4**C to E** above (**Fig. 4F to H**). Thus, this result demonstrated that LL-37 in an autocrine or paracrine fashion can activate autophagy.

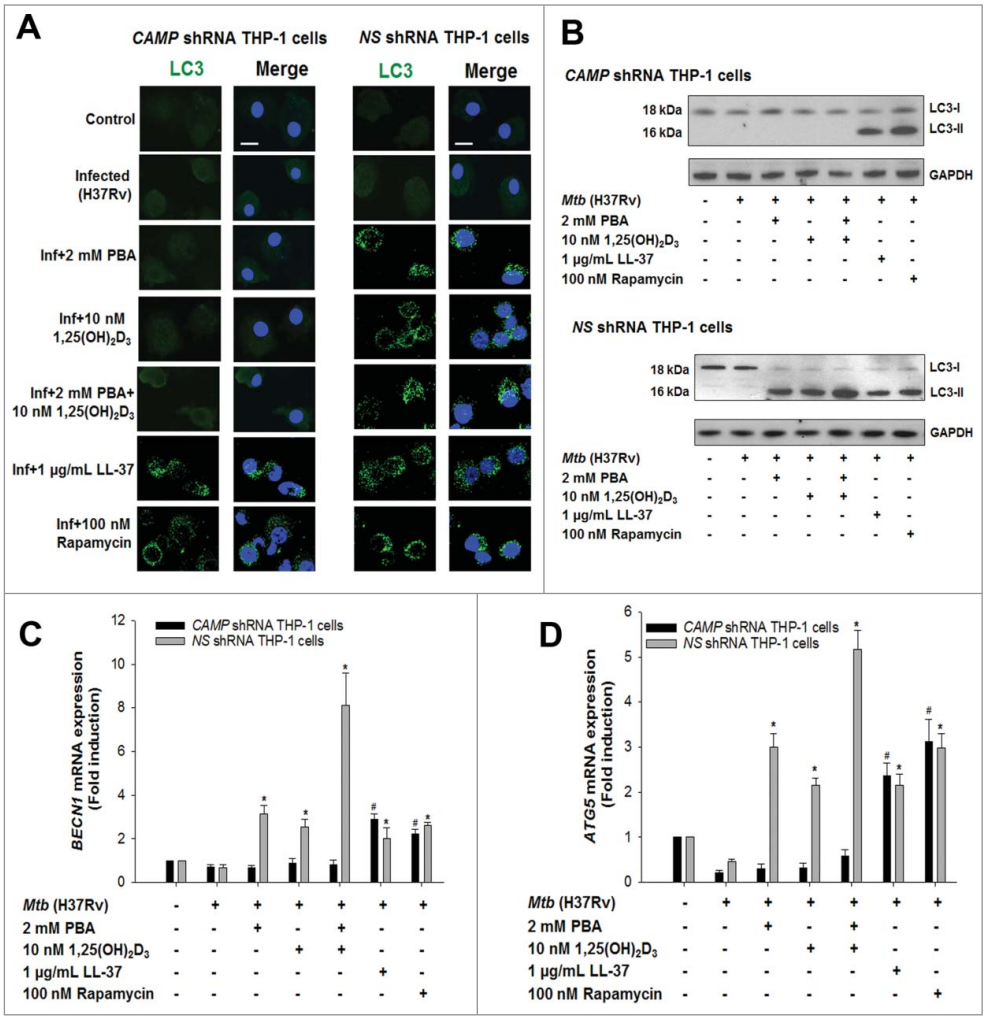

Figure 3. LL-37 was essential for PBA-induced autophagy. (**A**) The human monocytic cell-line THP-1 was transfected with a specific shRNA for *CAMP* (*CAMP* shRNA) and a nonspecific shRNA (*NS* shRNA). The cells were then infected with the virulent strain of *Mycobacterium tuberculosis* H37Rv for 4 h and stimulated with PBA (2 mM) and/or $1,25(OH)_2D_3$ (10 nM) or LL-37 (1 µg/mL) or rapamycin (100 nM) for 24 h . After that the cells were stained with anti-LC3, followed by the addition of Alexa Fluor 488-conjugated goat anti-mouse IgG (green) and DAPI to visualize the nuclei (blue). Representative immunofluorescence images of 3 independent replicates are shown; scale bars: 10 µm. (**B**) Western blot analysis of cell lysate using antibodies against LC3 and GAPDH was conducted. The experimental conditions were as outlined in **Fig. 3A** and a representative western blot of 3 independent replicates is shown. (**C and D**) Expression of *BECN1* and *ATG5* mRNA (normalized to *RNA18S/18S* rRNA expression) was measured by real-time qPCR. Results are shown from 3 independent experiments (means ± SD). The significance for *NS* shRNA THP-1 cells is indicated by * and with # for *CAMP* shRNA THP-1 cells, when comparing untreated with treated cells. The *P* values was≤0.05 as indicated by *or #.

LL-37-mediated activation of autophagy in human macrophages involves P2RX7 receptor

Next, we aimed to explore which receptor was responsible for LL-37-mediated autophagy in human macrophages. It has recently been observed that uptake of Texas-Red labeled LL-37 by macrophages involves P2RX7 (purinergic receptor P2X, ligand gated ion channel, 7) (our unpublished result). This result prompted us to investigate if P2RX7 may also be involved in LL-37-mediated autophagy. Pretreatment of MDMs with KN62, a potent inhibitor of the P2RX7 receptor, resulted in total suppression of LL-37-mediated autophagy in human MDMs (**Fig. 5**). While LC3 was detected in puncta structures with confocal microscopy of LL-37 and rapamycin-treated MDMs, LC3 was faint and spread in the cytoplasm after pretreatment with KN62 inhibitor (**Fig. 5A**). Accordingly, only LC3-I was detected upon western blot analysis of cellular extracts from KN62-treated MDMs (**Fig. 5B**). Similarly, *BECN1* mRNA expression was significantly downregulated in LL-37-treated cells, which were also exposed to the KN62 inhibitor compared to cells that were treated with only LL-37 ($P = 0.045$) (**Fig. 5C**). For *ATG5* mRNA expression was close to significant with a *P* value of 0.056 (**Fig. 5D**).

LL-37-mediated activation of autophagy in human macrophages requires the intracellular free Ca^{2+}, AMP-activated protein kinase and phosphatidylinositol 3-kinase pathways

It has been reported that enhanced intracellular free Ca^{2+}, AMP-activated protein kinase (AMPK) and the phosphatidylinositol 3-kinase (PtdIns3K) pathways play important roles in

activation of autophagy.[21,22] In order to investigate if these downstream mediators or signaling pathways were responsible for LL-37-mediated autophagy in human macrophages, MDMs were treated with LL-37 together with selected inhibitors of these different intracellular signaling pathways. We found that expression of both *BECN1* and *ATG5* mRNA was inhibited by pretreatment of MDMs with the respective inhibitors of intracellular Ca^{2+} (BAPTA-AM), AMPK (compound C) and the PtdIns3K (3-methyladenine) pathways (**Fig. 6B, C and 6F, G**). In addition, the conversion of LC3-I to LC3-II was inhibited (**Fig. 6D and 6H**) and consequently the LC3 puncta formation (**Fig. 6A and 6E**). These results suggest that all 3 signaling pathways are involved in LL-37-mediated activation of autophagy in human macrophages downstream of P2RX7 after LL-37 treatment.

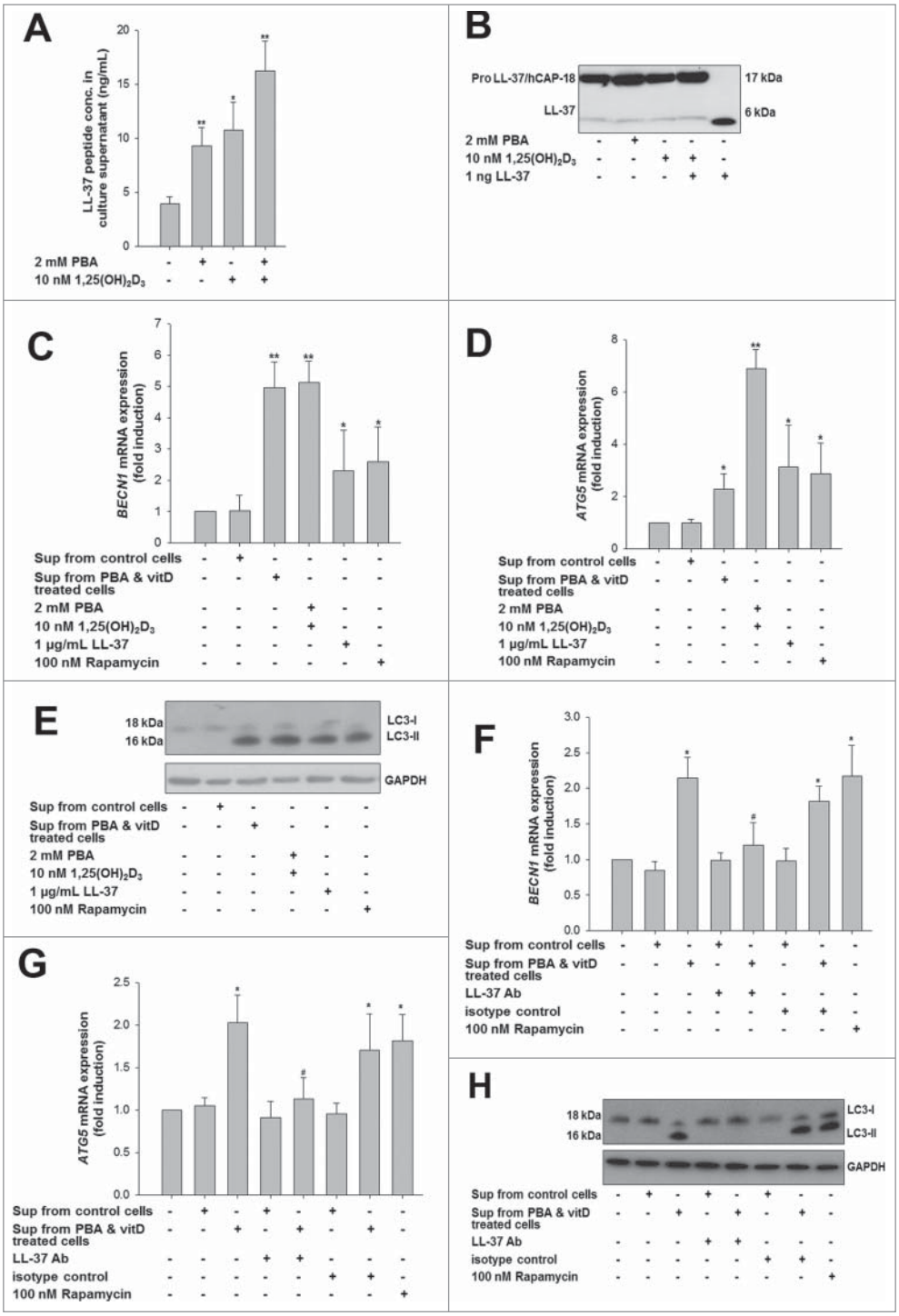

Figure 4. For figure legend, see page 1694.

Discussion

In the present study, we have revealed an important activity of PBA as a mediator of LL-37-dependent autophagy in human macrophages that contributes to intracellular killing of *Mtb*. Treatment of macrophages with PBA specifically enhances the expression of the autophagy-related genes *BECN1* and *ATG5* both at the mRNA and protein levels, in addition PBA stimulation leads to conversion of the autophagy marker LC3-I to LC3-II and promotes degradation of the protein SQSTM1. All these processes are significant and relevant for efficiently induced autophagy associated with immunity. Our results show a fundamental role of LL-37 in the PBA-induced autophagy since silencing of LL-37 in THP-1 cells prevented the induction of autophagy, which could be restored by addition of synthetic LL-37. Furthermore, a supernatant fraction from PBA and vitamin D-treated monocyte-derived macrophages (MDMs) was able to activate autophagy in an LL-37-dependent manner, which supports the conclusion that LL-37 is a key molecule of the PBA-mediated autophagic flux in human macrophages. In the present study, we also demonstrated that LL-37-mediated activation of autophagy involves signaling through P2RX7 receptor. In addition, intracellular free Ca^{2+}, the AMPK and the PtdIns3K pathways are required for LL-37-mediated activation of autophagy in human macrophages.

Altogether, these results describe a novel activity of PBA in the induction of autophagy and reveal the mechanism behind PBA-induced autophagy and killing of *Mtb* in human macrophages.

Previously, the human cathelicidin LL-37 has been shown to be essential to mediate effects of both innate and adaptive immunity, including broad-spectrum antimicrobial activities, chemotaxis, regulation of inflammatory responses, angiogenesis, and wound healing.[23] Recently, Yuk et al. have reported that vitamin D induces autophagy in human primary macrophages in an LL-37-dependent manner.[18] Our results indicate a similar activity for PBA to induce autophagy as described for vitamin D by Yuk et al.[18] However, we have extended the discovery of LL-37- autophagy, showing that the surface receptor P2RX7 mediates LL-37-induced autophagy. P2RX7 has earlier been reported to function as a low affinity receptor to LL-37,[24] and our result is supported by using an inhibitor of P2RX7, i.e., KN62. We also demonstrated that synthetic LL-37 per se activates autophagy and leads to killing of intracellular *Mtb* bacilli in human macrophages. This result may explain the in vivo situation, where LL-37 from neutrophils,[25] can activate autophagy and also enhanced phagocytosis in macrophages.[26]

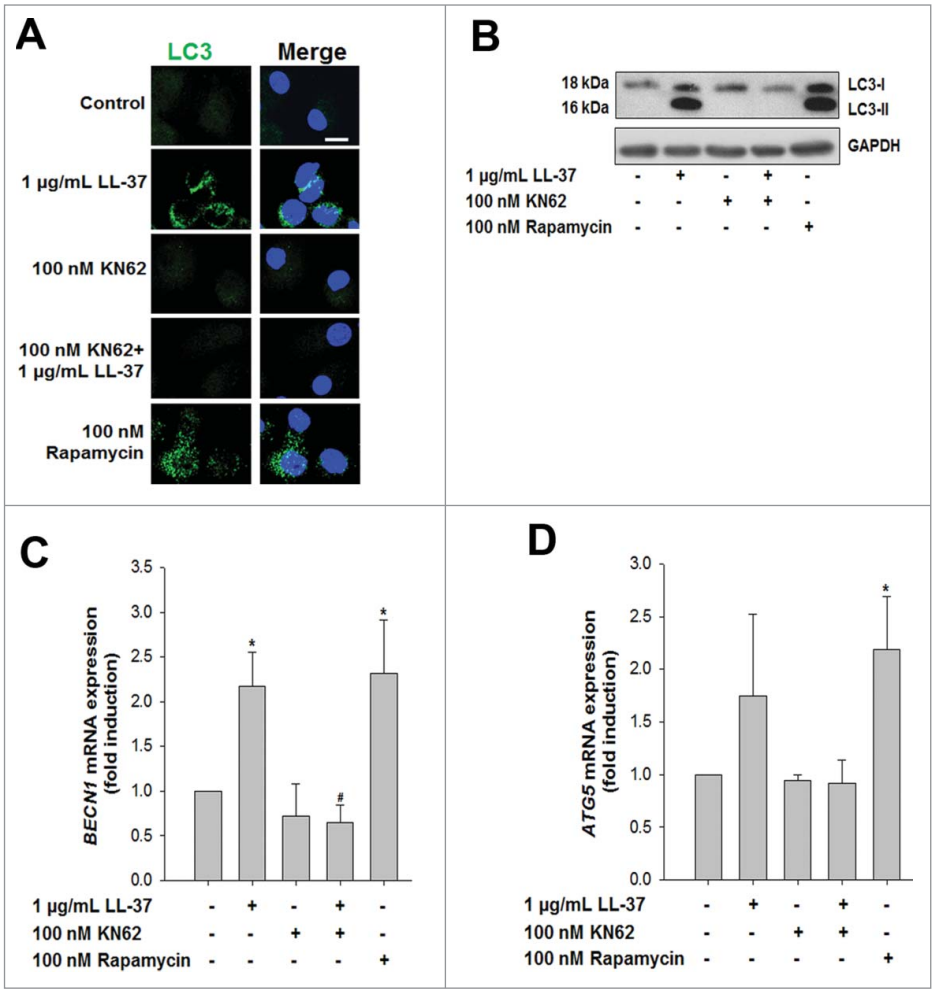

Figure 5. Activation of autophagy by LL-37 in human macrophages was mediated via the P2RX7 receptor. Human MDMs were pretreated with 100 nM KN62 for 15 min and then treated with LL-37 (1 μg/mL) for 24 h. (**A**) Cells were stained with anti-LC3, followed by the addition of Alexa-fluor 488 conjugated goat anti-mouse IgG (green) and DAPI to visualize the nuclei (blue), scale bars: 10 μm. One representative immunofluorescence images of 3 independent replicates are shown for LC3 expression. (**B**) Western blot analysis of cell lysate using antibodies against LC3 and GAPDH was performed. A representative western blot of 3 independent replicates is shown. (**C and D**) Expression of *BECN1* (**C**) and *ATG5* mRNA (**D**) (normalized to *RNA18S/18S* rRNA expression) was measured by real-time qPCR. Results are shown from 3 independent experiments (mean ± SD). * was used to indicate the comparison between untreated cells with treated cells and # was used to indicate the comparison between the cells that were stimulated with LL-37 in absence and presence of the inhibitor KN62. Significance was found for the *BECN1* mRNA expression but was not reached for *ATG5* mRNA ($P = 0.056$). The *P* value was ≤0 .05 as indicated by * or #.

Figure 4 (See previous page). Activation of autophagy by LL-37 in secreted material of PBA- and $1,25(OH)_2D_3$- stimulated macrophages. Human MDMs were stimulated with PBA (2 mM) and/or 1,25(OH)2D3 (10 nM) for 24 h (**A**) LL-37 concentration in culture supernatants was measured by a specific ELISA for LL-37. Results are shown from 3 independent experiments (mean ± SD). The *P* values were ≤0.01 and ≤0.05 as indicated by **and *, respectively. (**B**) Western blot analysis of cell culture supernatant using antibody against LL-37 was performed. Representative western blot of 3 independent replicates is shown. (**C and D**) Human MDMs were stimulated with supernatant from PBA (2 mM) and $1,25(OH)_2D_3$ (10 nM)-treated macrophages or treated with PBA (2 mM) and/or $1,25(OH)_2D_3$ (10 nM) or LL-37 (1 μg/mL) or rapamycin (100 nM) for 24 h. Expression of *BECN1* and *ATG5* mRNA (normalized to *RNA18S/18S* rRNA expression) was measured by real-time qPCR. Results are shown from 3 independent experiments (mean ± SD). The *P* values were ≤0.01 and ≤0.05 as indicated by **and *, respectively. (**E**) Western blot analysis of cell lysate using antibodies against LC3 and GAPDH was performed. The experimental conditions were as outlined in **Fig. 4C** and a representative western blot of 3 independent replicates is shown. Human MDMs were stimulated with supernatant from PBA (2 mM) and $1,25(OH)_2D_3$ (10 nM)-treated macrophages in the absence or presence of the monoclonal anti-LL-37 for 24 h. An isotypic antibody control was used as a negative control and rapamycin (100 nM) was used as a positive control. (**F and G**) Expression of *BECN1* and *ATG5* mRNA (normalized to *RNA18S/18S* rRNA expression) was measured by real-time qPCR. Results are shown from 3 independent experiments (mean ± SD). (**H**) A representative western blot showing the conversion of LC3-I to LC3-II and the level of GAPDH from 3 independent experiments. * Indicates the comparison between untreated cells with treated cells and # was used to indicate the comparison between the cells that were stimulated with supernatant from PBA and vitD-treated cells in the absence or presence of LL-37 antibody. The *P* value was ≤0.05 as indicated by * or #.

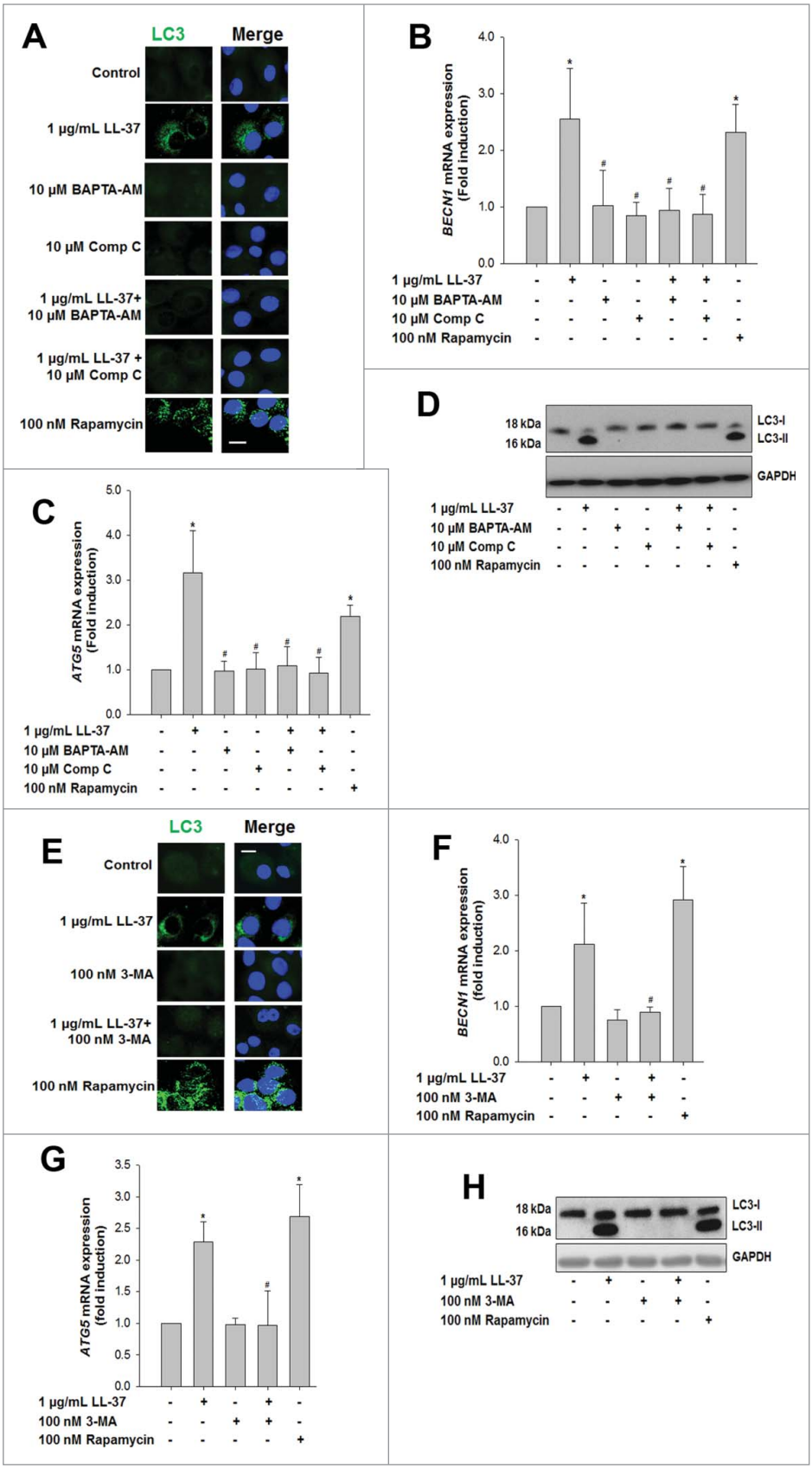

Figure 6. Intracellular free Ca^{2+}, AMP-activated protein kinase and PtdIns3K pathways were required for PBA-induced autophagy. Human MDMs were treated with LL-37 (1 μg/mL), in the absence or presence of BAPTA (10 μM), or compound C (Comp C; 10 μM) or 3-methyladenine (100 nM) for 24 h. (**A, E**) Cells were then fixed and stained with DAPI to visualize the nuclei (blue) and with anti-LC3 followed by the addition of Alexa-fluor 488 conjugated goat anti-mouse IgG (green). One representative immunofluorescence image out of 4 independent replicates is shown; scale bars: 10 μm. (**B and C, F and G**) *BECN1* and *ATG5* mRNA expression (normalized to *RNA18S/18S* rRNA expression) in human MDMs were measured by real-time qPCR. Results are shown from 3 independent experiments (mean ± SD). * was used to indicate the comparison between untreated cells with treated cells and # was used to indicate the comparison between the cells that were stimulated with LL-37 in absence and presence of the inhibitor BAPTA, compound C or 3-methyladenine. The *P* value was ≤0.05 as indicated by * or #. (**D, H**) Western blot analysis of cell lysate using antibodies against LC3 and GAPDH was performed. A representative western blot of LC3 and GAPDH from 3 independent experiments is shown.

Interestingly, we have recently shown that induction of the *CAMP* gene encoding LL-37 by PBA is dependent on VDR (vitamin D [1,25-dihydroxyvitamin D3] receptor) in human lung epithelial cells.[27] This may indicate activation of VDR as an important transcription factor for LL-37-dependent autophagy in macrophages.

PBA is known as a histone deacetylase inhibitor that induces LL-37 expression in a lung epithelial cell line.[16] Histone deacetylase inhibitors, such as butyrate and suberoylanilide hydroxamic acid/SAHA, induce autophagy in several human cancer cell lines.[28] We have recently demonstrated that oral administration of PBA alone or in combination with vitamin D, enhanced LL-37 expression and significantly improved the capacity to inhibit the growth of *Mtb* in MDMs obtained from healthy human volunteers.[17]

We have found that treatment of *Mtb*-infected macrophages with PBA and/or vitamin D can overcome

Mtb-mediated downregulation of LL-37 and the autophagy markers BECN1 and ATG5. Furthermore, a faint staining of LC3 in the cytoplasm of *Mtb* infected macrophages was detected, but treatment with PBA and/or vitamin D induced the conversion of LC3-I to LC3-II. Our results also provide evidence that a soluble factor secreted by PBA and vitamin D-treated macrophages is able to activate autophagy in the same cell type. The responsible component for activation of autophagy in the secreted material was LL-37, since neutralization of LL-37 in the culture supernatants abrogated the activation of autophagy. Thus, LL-37 could mediate its effect in an autocrine or paracrine way. Furthermore, LL-37 induced autophagy in human macrophages via the purinergic receptor P2RX7, which can activate different downstream signaling pathways such as mitogen-activated protein kinases and enhance the expression of CEBPB (CCAAT/enhancer binding protein [C/EBP], β), pathways, which contribute to transcriptional activation of both *BECN1* and *ATG5*.[18,29] Notably, these pathways are involved in enhanced expression of LL-37.[30,31]

Here, we have demonstrated that LL-37-mediated autophagy requires intracellular calcium influx together with the signaling pathways AMPK and PtdIns3K downstream of the P2RX7 receptor. Together with calcium mobilization, these pathways are essential for the induction of the autophagy process in macrophages. AMP-activated protein kinase plays an important role as a general regulator of autophagy activation in response to Ca^{2+} signaling,[21] while PtdIns3K is required in controlling the activation of the autophagy process via MTOR (mechanistic target of rapamycin [serine/threonine kinase]).[22,32] As the name indicates MTOR is a defined target of rapamycin and we have therefore included rapamycin as a control in our experiments. We have found that rapamycin activates autophagy in a different manner than PBA and/or vitamin D. Rapamycin does not induce LL-37 and its activation of autophagy is independent of P2RX7. However, both rapamycin and PBA or vitamin D induce intracellular killing of *Mtb*, indicating different activation pathways to fight this bacterium.

In the current study, we describe a mechanistic link between PBA-induced expression of the antimicrobial peptide LL-37 and autophagy, both of which are key processes in the elimination of intracellular pathogens such as *Mtb*. We hypothesize that the clinical impact of these findings can be widely explored. Here, our research collaborators and we have recently conducted 2 clinical trials in Bangladesh (www.clinicaltrials.gov ID-number NCT01580007) and Ethiopia (NCT01698476), respectively, to test the antimicrobial effects of adjunctive immunotherapy with PBA and/or vitamin D given to patients with pulmonary TB. These trials aim to explore if novel treatment with PBA and/or vitamin D can restore immune control in TB infected patients by induction of LL-37 and autophagy in human cells. The trial in Bangladesh has recently been completed and the positive results with significant reduced clinical score and faster sputum culture conversion highlighting the clinical relevance of this study.

Materials and Methods

Cell culture

Human monocytic THP-1 cells were purchased from the European Collection of Cell Cultures, (88081201) and maintained in RPMI 1640 (Invitrogen, Life Technologies, 21875–091) with 10% fetal calf serum (FCS; Invitrogen, Life Technologies, 10270–106), 2 mM L-glutamine (Invitrogen, Life Technologies, 25030–024), penicillin G (100 IU/ml)-streptomycin (100 μg/ml; Invitrogen, Life Technologies, 15140–122) and 50 μM 2-mercaptoethanol (Invitrogen, Life Technologies, 31350–010). The cells were treated with 10 ng/mL phorbol myristate acetate (Sigma-Aldrich, P8139) for 48 h to induce differentiation into macrophage-like cells. For monocyte isolation, peripheral blood mononuclear cells from healthy donors (Karolinska Hospital Blood Bank, Stockholm, Sweden) were isolated from buffy coats by density sedimentation over Ficoll-Paque Premium (GE Healthcare, 17–5442–02). After that, cells were allowed to adhere in culture plates (Nunc, 140675) for 2 h at 37°C. The nonadherent cells were removed by washing with phosphate-buffered saline (PBS; Gibco, Life Technologies, 20012–027). The recovered cells were > 95% monocytes, determined by flow cytometric analysis. Human monocyte-derived macrophages (MDMs) were prepared by culturing peripheral blood monocytes for 7 d in the presence of 50 ng/mL human macrophage colony-stimulating factor (M-CSF) (Gibco, Life Technologies, PHC9501). The viability of MDMs was determined with Trypan blue (Invitrogen, 15250–061) staining.

Cell treatments

Human MDMs and THP-1 cells were treated with 2 mM 4-phenylbutyrate (Santa Cruz Biotechnology, sc-200652), and/or 10 nM $1,25(OH)_2D_3$ (Sigma, D-1530), synthetic 1 μg/mL LL-37 (Innovagen, SP-LL37–1) or 100 nM rapamycin (Sigma, R0395).

The supernatant fraction of human primary MDMs, which were treated with PBA and $1,25(OH)_2D_3$ for 6 to 8 h, washed and cultured for additional 16 to 18 h in media was collected. This supernatant fraction was used to treat a new batch of freshly prepared MDMs for 24 h. The concentration of LL-37 in culture supernatant of MDMs was measured by ELISA. The monoclonal LL-37 antibody,[33] was utilized to neutralize LL-37 in the cell culture of MDMs. A nonspecific monoclonal isotype antibody (Santa Cruz Biotechnology, sc-3877) was used as control.

To block the expression of the P2RX7 receptor, primary MDMs were pretreated with 100 nM KN62 (1-[N,O-bis(5-Isoquinolinesulfonyl)-N-methyl-L-tyrosyl]-4-phenylpiperazine) that is an inhibitor of P2RX7 receptor (Sigma, I2142), for 15 min; and then treated with LL-37 for 24 h. To inhibit different signaling pathways, primary MDMs were pretreated with 10 μM of the intracellular calcium-specific chelator 1,2-bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid-acetoxymethyl ester (BAPTA-AM; Sigma, A1076), or 10 μM of the ATP-competitive inhibitor of AMP-activated protein kinase (compound C; Sigma, P5499) for 1 h or 100 nM of 3-methyladenine

(Invivogen, 5142–23–4) an inhibitor of the PtdIns3K pathway for 30 min and then the cells were treated with LL-37 for 24 h.

Mycobacteria culture, infection and CFU assay

The virulent *Mtb* strain H37Rv (American Type Culture Collection, 27294) was cultured in Middlebrook 7H9 broth supplemented with 10% OADC (oleic acid, albumin, dextrose and catalase) (Becton, Dickinson and Company, 271310, 245116), 0.05% Tween-80 (Sigma, P4780) and 0.5% glycerol (Sigma, G2025) for 2 or 3 wk at 37°C. The bacterial suspension was washed twice in PBS-0.05% Tween-80 before the pellet was resuspended in RPMI media. The bacteria were repeatedly vortexed and sonicated using an ultrasound sonication bath to obtain a single cell suspension. Bacterial concentration was determined from the OD of the culture at 600 nm, as a function of CFUs per milliliter. MDMs were cultured in 6-well plates (1×10^6 cells/well) and infected with the bacteria (multiplicity of infection 1:5) for 4 h before the cells were washed to remove extracellular bacteria. Human MDMs were treated with PBA and/or $1,25(OH)_2D_3$ or rapamycin or LL-37 in RPMI-10% FCS at 37°C for 24 h before the cells were harvested for mRNA analysis. For the CFUs experiment, infected cells were treated with PBA and/or $1,25(OH)_2D_3$ or rapamycin or LL-37 for 3 d After that, the intracellular bacteria were harvested and cells were lysed with 0.036 % SDS (Sigma, L3771) in water. Lysis was confirmed by fast-contrast microscopy. The lysates were then cultured on Middle Brook 7H11 agar media (Becton, Dickinson and company, 283810) and after 21 to 28 d of culture at 37°C bacterial viability was calculated by the CFU count.

Real-time qPCR

RNA was extracted from MDMs and THP-1 cells, utilizing Ribopure RNA extraction kit as described by the manufacturer (Ambion, Life Technologies, AM1924). RNA was eluted in RNase-free elution solution and cDNA was synthesized using iScript™ cDNA Synthesis Kit (Bio-Rad, 170–8891). Transcripts of the autophagy markers *ATG5* and *BECN1* as well as *CAMP* gene encoding LL-37 relative to the housekeeping *RNA18S/18S* rRNA (18S rRNA–housekeeping gene kit, Applied Biosystems, 4308329) were measured in triplicate from the cDNA samples by real-time quantitative -PCR using CFX96 Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). The results were analyzed by using the relative standard method.[14]

Western blot of LL-37, BECN1, ATG5, LC3, and SQSTM1

Expression of LL-37 and markers of autophagy were evaluated by western blot analysis. Cellular extracts and culture supernatants were used for western blot analysis. When macrophages and THP-1 cells were infected with *Mtb*, the cellular extracts were prepared according to the manufacturer protocol for protein extraction by using Trizol reagent (Ambion, Life Technologies, 10296010). In some experiments, cellular extracts were prepared by using radioimmunoprecipitation assay buffer (Sigma, R0278). Cell culture supernatants were enriched for polypeptides on OASIS 1cc reversed-phase HLB columns (Waters Corporation, 186003908). The proform of LL-37 (hCAP-18) and the active form LL-37, were detected by monoclonal anti-LL-37.[33] Autophagy-related protein BECN1 and ATG5 were detected by rabbit anti-BECN1 (Sigma, B6186) and rabbit anti-ATG5 (Sigma, A0731). The cytosolic inactive form (LC3-I) and lipidated form (LC3-II) of the autophagy marker LC3, were detected by rabbit anti-LC3 (Sigma, L7543). Furthermore, an additional marker of autophagy, SQSTM1 expression, was also detected by rabbit anti-SQSTM1 (Sigma, P0067) through western blot analysis. The western blot images were acquired by Odyssey LI-COR Imaging Systems (LI-COR, Lincoln, Nebraska, USA).

Fluorescence microscopy

For immunostaining, human MDMs and THP-1 cells were seeded on 8-well chamber slides (2×10^5 cells/well) and were differentiated by M-CSF and phorbol myristate acetate, respectively. The cells were infected with the virulent *Mtb* H37Rv for 4 h and stimulated with PBA, and/or $1,25(OH)_2D_3$, or rapamycin or LL-37 for 24 h. After that cells were fixed with 4% paraformaldehyde in PBS at room temperature (RT) for 10 to 15 min and permeabilized with 0.1% Triton X-100 (Sigma, T9284) and 0.1% sodium citrate in PBS for 5 min. Then the cells were treated with 10% goat serum (Jackson ImmunoResearch, 005–000–121) for one h at room temperature and stained with primary antibodies, including mouse anti-human LC3 (MBL International, M152–3) or rabbit anti-LC3 (Sigma, L7543), rabbit anti-SQSTM1 (Sigma, P0067) and mouse anti-human LL-37,[33] overnight at 4°C. After washing to remove excess primary antibodies, the cultures were incubated for one h at room temperature with the following fluorescently labeled secondary antibodies: anti-mouse IgG-Alexa Fluor 488 or 594 and anti-rabbit IgG-Alexa 594 or 488 (Jackson Immunoresearch, 115–545–062, 115–585–062, 111–585–045, 111–095–144), and then washed 3 times with PBS. Cells were loaded with DAPI mounting media (Vector Laboratories, H-1200) for visualization of nuclei, and imaged with an Olympus confocal microscope (Olympus FluoView™ FV1000 confocal microscope, Melville, NY, USA). Quantification of autophagy was performed based on the percentage of the cells with LC3-II punctate dots by ImageJ software. For positive control of autophagy activation, cells were incubated in complete medium that contained 100 nM rapamycin (Sigma, R0395) for 24 h.

Transient transfection of THP-1 cells with CAMP shRNA

Human monocytic cells (THP-1 cells) were seeded in 12-well plates at a density of 1×10^6 cells/well in 2 mL of RPMI 1640 with 10% FCS, 2 mM L-glutamine, penicillin G (100 IU/ml)-streptomycin (100 μg/ml) and incubated for 24 h before transfection. The next day, cells were transfected with 2 μg of either pLKO.1-based *CAMP* short hairpin RNA (Sigma-Aldrich, SHCLND-NM_004345) constructs or a control vector without any insert using 2 μl of Turbofect (Fermentas, R0531). After 24 h, medium was removed and replaced with fresh RPMI medium containing 10 μg/ml puromycin (Sigma, P8833), which was replaced every third d up to 2 wk in order to generate

knockdown cells. Cells were stained with Trypan blue to control for viable cells.

LL-37 ELISA

LL-37 levels in culture supernatants were measured by ELISA. Duplicate samples were analyzed, and the concentrations were calculated using a standard curve generated from synthetic LL-37 (Innovagen, SP-LL37–1). A brief procedure of ELISA has been described previously.[17]

Statistical analysis

Data are presented as mean ± SD and comparisons between groups were analyzed by paired Student t test and one-way ANOVA for comparing more than 2 groups. $P \leq 0.05$ was considered as statistically significant.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed

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Supplemental Material

Supplemental data for this article can be accessed on the publisher's website.

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