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The modulatory effect of TLR2 on LL-37-induced human mast cells activation



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ABSTRACT

The sole and endogenous anti-microbial peptide LL-37 is a significant effector molecule in the innate host defense system. Apart from its broadly direct anti-microbial activity, the peptide also activates mast cell in respect of allergic diseases and inflammation. On the other hand, mast cell can be activated by Toll-like receptors (TLRs) which are at the center of innate immunity. It was the aim of the study to illustrate the modulatory effect of TLR2 ligands peptidoglycan (PGN) and tripalmitoyl-S-glycero-Cys-(Lys)4 (Pam3CSK4) on LL-37 induced LAD2 cells (a human mast cell line) activation. LL-37 induced LAD2 cells degranulation and the release of IL-8. TLR2 ligands didn't induce LAD2 cells degranulation, but triggered the release of IL-8. Incubation with PGN or Pam3CSK4 significantly suppressed LL-37-induced degranulation through inhibition of calcium mobilization from LAD2 cells. Similarly, the release of IL-8 was inhibited when LAD2 cells were co-stimulated with TLR2 ligands and LL-37. Studies with inhibitors of key enzymes involved in mast cell signaling indicated that the release of IL-8 induced by TLR2 ligands and LL-37 involved the activation of the PI3K, ERK, JNK and calcineurin signaling pathways. In contrast, p38 activation down-regulated the release of IL-8 induced by TLR2 ligands and LL-37. Taken together, these observations suggest that activation of human mast cells by LL-37 could be modified by TLR2 ligands and the function of human mast cells could be switched from allergic reactions to innate immune response.

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1. Introduction

Mast cells derived from CD34⁺ hematopoietic progenitor cells play critical roles in allergic inflammatory response [1]. Mature mast cells express high level of FcεRI (high-affinity IgE receptor) and act as the key immune effector cells in allergic associative diseases such as asthma and atopic dermatitis. Despite the well-defined role of FcεRI in mast cells activation [5], increasing

evidences have suggested that mast cells are involved in the pathogenesis of a number of chronic inflammatory diseases via IgE-independent mechanisms [2–4,6–8].

Toll-like receptors (TLRs), which constitute a family of mammalian cell-surface sensors, are the principal pattern recognition receptors in innate immune system. TLRs could be widely expressed in various types of immune cells including mast cells [9–11]. Previous studies have demonstrated that mast cells could be activated by TLR2 ligands through IgE-independent manner. Akira S and his colleagues have illustrated that the activation of TLR2 induces the recruitment of the adaptor molecules myeloid differentiation primitive-response protein 88 (MyD88) and successive IL-1R-associated protein kinases (IRAKs), which lead to the activation of nuclear factor kappa B (NF-κB) and trigger the transcription of pro-inflammatory cytokine genes [12].

CAMP (also renowned as hCAP-18) is the only member of anti-microbial peptide cathelicidins identified in human beings [13]. The proteolysis of the C-terminal end of CAMP results in the generation

Abbreviations: ERK, extracellular regulated protein kinases; JNK, c-Jun N-terminal kinase; MAPKs, mitogen-activated protein kinases; PKC, protein kinase C; PLC, phospholipase C.

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of a small peptide with 37 amino acid residues, therefore named LL-37. LL-37 can be found in its unprocessed form (hCAP-18) in the granules of neutrophils at high concentrations and released in response to pathogenic microbial challenge. Moreover, previous studies have demonstrated that LL-37 could be up-regulated by TLR2 activation. Release of LL-37 directly participates and modulates an extensive array of innate immune reactions including mast cells associated immune responses [14,15]. However, to date, the influence of TLR2 on LL-37-induced mast cell activation has not yet been analyzed.

Based on previous studies, in real situation *in vivo*, mast cells could be activated by both TLR2 ligands and LL-37 upon invasion of pathogenic microorganisms. In the present study, we sought to determine whether TLR2 ligands might alter the responses of mast cells to LL-37. Two classical TLR2 ligands are employed in the study. Peptidoglycan (PGN) is a primary cell wall constituent of Gram-positive bacterial which activates TLR2 and TLR6 heterodimer and Tripalmitoyl-S-glycero-Cys-(Lys)4 (Pam3CSK4) is a synthetic tri-palmitoylated lipopeptide which stimulates TLR2 and TLR1 heterodimer.

2. Materials and methods

2.1. Human mast cells culture

The Laboratory of Allergic Disease 2 (LAD2) human mast cells were kindly provided by A. Kirshenbaum and D. Metcalfe (NIH, USA) [16]. Cells were maintained in StemPro-34 medium supplemented with 10 ml/l StemPro nutrient supplement, 1:100 penicillin-streptomycin, 2 mmol/l L-glutamine, 100 ng/ml human stem cell factor, and 50 ng/ml interleukin-6 in an atmosphere containing 5% CO₂ at 37 °C. The culture medium was replaced every 2 weeks and the cells were kept at a density of 2×10^6 cells/ml.

2.2. Chemical reagents

LL-37 was purchased from GL Biochem. Pam3CSK4 was bought from Invivogen. SB203580, ciclosporin A, U73122, BOC-MLF and Bay 11-7821 were from Tocris. Peptidoglycan (PGN) from *S. aureus*, Ro31-8220 and KN-62 were bought from Sigma. Wortmannin was from Cayman. PD98059 and JNK Inhibitor II were from Calbiochem. The ultimate concentration of DMSO did not change the normal response of LAD2 cells when chemicals were dissolved in DMSO.

2.3. Degranulation assay

β -hexosaminidase (β -hex) is an enzyme contained in the cytoplasmic granules of mast cells and the degree of release of such enzyme into the supernatant indicates the degranulation process as the consequence of mast cell activation. LAD2 cells were in turns incubated with different stimuli for 30 min and the release of β -hex was measured. β -hex in supernatants and cell lysates was determined by a colorimetric assay where release of p-nitrophenol from 4-nitrophenyl N-acetyl- β -D-glucosaminide was measured [17]. The absorbance was measured at 405 nm by using a multiplate reader. 605 nm reading was regarded as a reference. The percentage of β -hex release was computed as the percentage of the whole β -hex content. All results were calibrated for spontaneous β -hex release which was less than 5%.

2.4. IL-8 measurement

LAD2 cells were pre-incubated with different inhibitors for the corresponding time courses prior to incubation with distinct stimulants for 24 h to allow production and release of IL-8. The

release of IL-8 in the supernatants was measured by ELISA assay (BD Biosciences) in accordance with the manufacturer's instructions. All results were corrected for spontaneous IL-8 release which was less than 22 pg/10⁶ cells.

2.5. Intracellular Ca²⁺ mobilization assay

LAD2 cells were loaded with 2 μ M Fura-3 AM (Invitrogen) for 30 min at 37 °C. The cells were then washed three times with HEPES buffer with human albumin and resuspended in it and then stimulated with various stimuli. Fura-2-loaded mast cells were viewed with an Olympus inverted IX51 microscope. They were captured with a CCD camera at each 10-s interval. Fluorescence images were gained at wavelengths of 340 and 380 nm with an emission wavelength of 510 nm. The fluorescence ration of 340–380 nm was measured and analyzed. F1/F0 was the fluorescence ration of time-point X divided by the ratio of time-point zero.

2.6. Statistical analysis

Statistical significance was determined by student's t-test, one-way or two-way ANOVA. Differences were considered significant at a P value of less than 0.05. The whole data are expressed as means $\pm$ standard error of means (SEMs).

3. Results

3.1. TLR2 ligands suppressed LAD2 cells degranulation induced by LL-37

PGN and Pam3CSK4 did not induce the release of β -hex on their own (Fig. 1A, B) while LL-37 induced a release of around 30% of total β -hex after 30 min incubation with LAD2 cells. Dose-dependent inhibition of LL-37 induced β -hex release from LAD2 cells was observed when LAD2 cells were pre-incubated with the TLR2 ligands PGN and Pam3CSK4 for 24 h or when LAD2 cells were simultaneously incubated with either TLR2 ligand and LL-37 (Fig. 1A, B).

3.2. TLR2 ligands modified LL-37 induced IL-8 release from LAD2 cells

Both TLR2 ligands and LL-37 induced IL-8 release from LAD2 cells (Fig. 1C, D). When LAD2 cells were co-stimulated with TLR2 ligands and LL-37, the value of the release of IL-8 was smaller than that of the expected combination value of TLR2 ligands and LL-37 (Fig. 1C, D).

3.3. TLR2 ligands modulated LL-37-induced intracellular calcium increase in LAD2 cells

Increase in concentration of cytosolic calcium is necessary for both degranulation and the release of de novo synthesized mediators in mast cells [18]. We showed that LL-37 caused a swift increase of [Ca²⁺]_i with peak elevation at about 3 min after activation followed by gradual reduction during the observation period and that both PGN and Pam3CSK4 induced a much lower and transient level of [Ca²⁺]_i from LAD2 cells (Fig. 2A, B). When LAD2 cells were co-stimulated with TLR2 ligands and LL-37, the value of increase of [Ca²⁺]_i was lower than that of LL-37 alone (Fig. 2A, B). Similarly, pre-incubation of TLR2 ligands significantly inhibited LL-37 induced calcium mobilization from LAD2 cells (Fig. 2C, D).

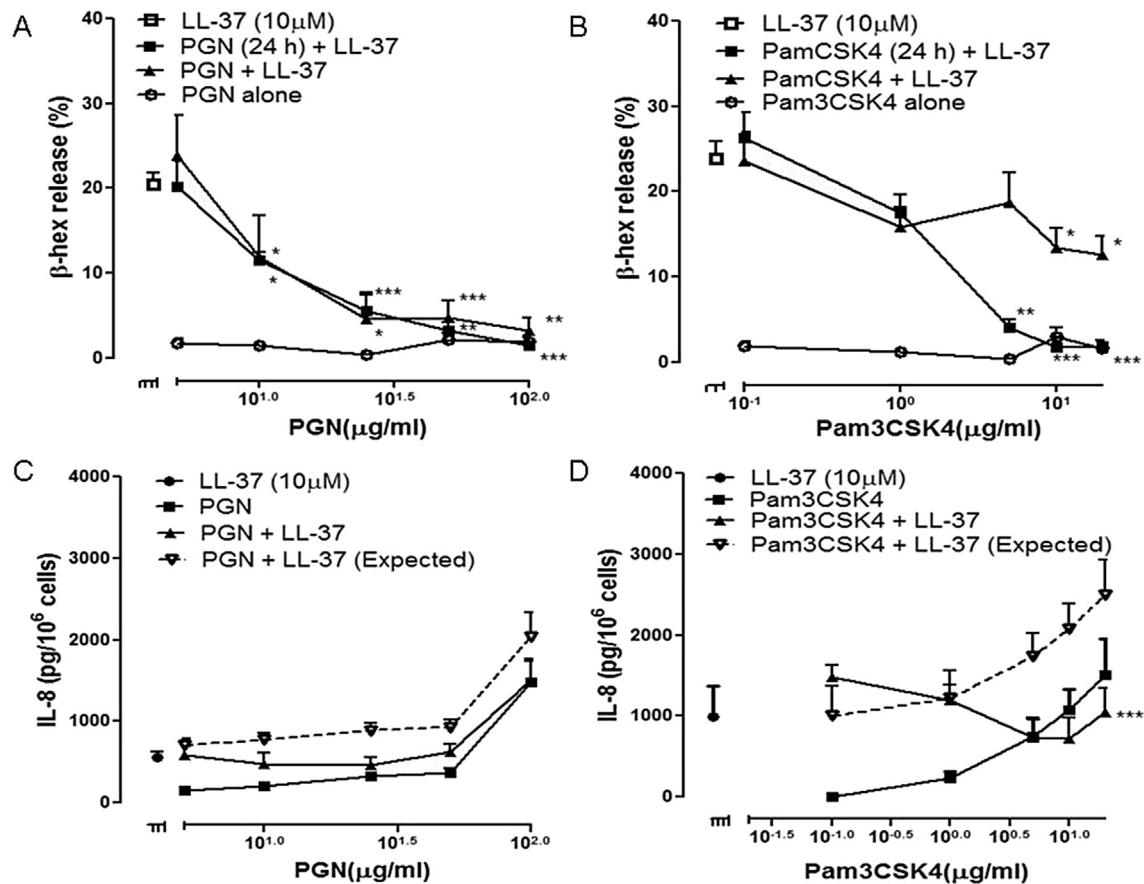


Fig. 1. Effect of TLR2 ligands on LL-37-induced degranulation and IL-8 release from LAD2 cells. A, B) LAD2 cells were incubated with only PGN/Pam3CSK4 (○) or co-stimulated with TLR2 ligands and LL-37 (10 μ M) at the same time (▲) for 30 min or with LL-37 after 24 h pre-incubation with either of the TLR2 ligands (■). The amount of β -hex release induced by LL-37 alone and in the presence of TLR2 ligands were compared with one-way ANOVA and Dunnett's multiple comparison tests ($n = 5$). C, D) LAD2 cells were incubated with LL-37 (10 μ M, ●), PGN/Pam3CSK4 (■) or combination of LL-37 with PGN/Pam3CSK4 (▲) for 24 h. The actual amount of IL-8 release and the expected value gained by adding the individual amounts released by LL-37 and PGN/Pam3CSK4 (▽) was compared with two-way ANOVA and Bonferroni post tests. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ ($n = 5-6$).

3.4. Release of IL-8 induced by concomitant activation of LAD2 cells by LL-37 and TLR2 ligands was inhibited by blockers of calcineurin and PI3K

In this series of experiments, LAD2 cells were pre-incubated with a signaling molecule inhibitor for 30 min prior to the addition of various mast cells activators. It's reported that the activation of calcineurin induced by $[Ca^{2+}]_i$ contributes to the release of cytokines from mast cells [19]. As presented in Fig. 3A, IL-8 release induced by Pam3CSK4, LL-37 and their co-stimulation were potently inhibited by the specific inhibitor of calcineurin, cyclosporin A (0.1 μ g/ml). Nonetheless, cyclosporin A did not alter PGN induced IL-8 release from LAD2 cells (Fig. 3A), though it significantly decreased the release of IL-8 induced by co-stimulation of PGN with LL-37 (Fig. 3A).

The study done by Arbibe et al. has suggested that PI3K is necessarily required in TLR2 signaling activation [20]. The secretion of IL-8 induced by PGN, LL-37 and their combination was inhibited by the PI3K inhibitor wortmannin (1 μ M). In contrast, wortmannin did not inhibit IL-8 release from LAD2 cells stimulated with Pam3CSK4 alone or combination with LL-37 (Fig. 3B).

3.5. Nonparticipation of PKC and PLC in TLR2 ligands and LL-37 induced mast cells activation

It's well known that both PKC and PLC involve in the release of

cytokines and chemokines in human mast cells activation [25,26]. While both the PKC inhibitor Ro31-8220 (1 μ M) and the PLC inhibitor U73122 (10 μ M) significantly inhibited LL-37 induced IL-8 release from LAD2 cells, no significant inhibition was observed in the case of TLR2 ligands alone or their combination with LL-37 from LAD2 cells (Fig. 3C, D).

3.6. Involvement of MAPKs in TLR2 ligands and LL-37 induced mast cells activation

It has been demonstrated that both TLR2 ligands and LL-37 could induce the tyrosine phosphorylation of MAPKs which are critical signaling molecules in regarding to the production of a wide variety of cytokines and chemokines [21–24] in various cell types including mast cells. ERK inhibitor PD98059 (10 μ M) significantly inhibited the release of IL-8 from LAD2 cells induced by PGN, Pam3CSK4, LL-37 and their combination (Fig. 4A). The JNK Inhibitor II (10 μ M) only inhibited the secretion of IL-8 induced by PGN, LL-37 and their combination from LAD2 cells but had no effect on Pam3CSK4 or Pam3CSK4 co-stimulated with LL-37 induced IL-8 release from LAD2 cells (Fig. 4B). In contrast, the P38 inhibitor SB203580 (10 μ M) significantly enhanced IL-8 release induced by PGN, Pam3CSK4, LL-37 or their combination from LAD2 cells (Fig. 4C).

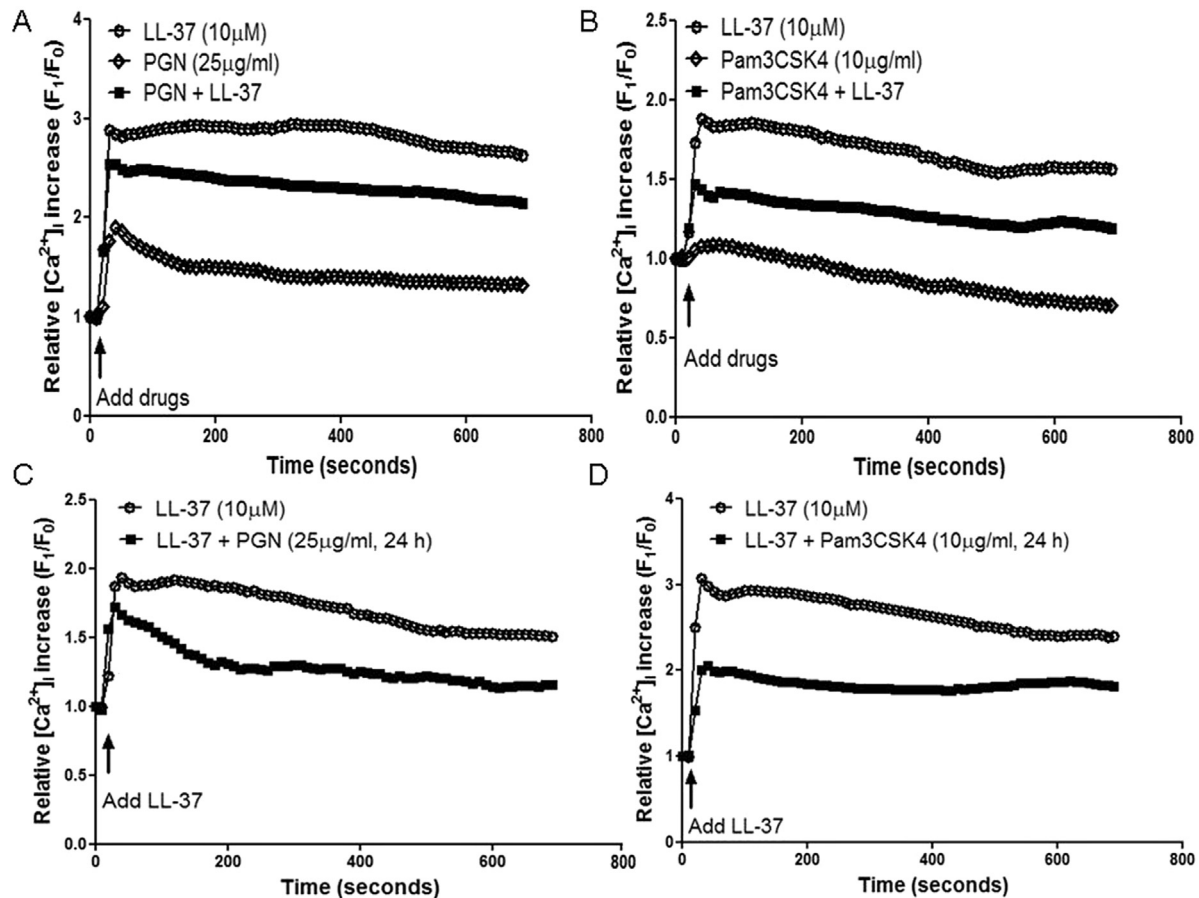


Fig. 2. Effect of TLR2 ligands on LL-37-induced calcium mobilization in LAD2 cells. A, B) LAD2 cells were incubated with LL-37 ($\circ$), PGN/Pam3CSK4 ($\diamond$), LL-37 with PGN/Pam3CSK4 ($\blacksquare$), and calcium mobilization was measured at the same time. C, D) LAD2 cells were incubated with PGN or Pam3CSK4 for 24 h prior to being stimulated with LL-37 ($\blacksquare$). Changes in $[Ca^{2+}]_i$ were compared in the presence or absence of PGN or Pam3CSK4. Error bars were eliminated for the sake of being clarity of the graph ($n = 4-7$).

4. Discussion

Antimicrobial peptide (LL-37) secreted by mast cells and other immune cells is involved in a wide variety of inflammatory reactions through activating mast cells via IgE-independent peptidergic pathways [27–29]. Meanwhile, previous studies have reported that mast cells participate in innate immunomodulatory activities through specific membrane receptors such as TLR2 [30]. Activation of TLR2 heterodimers could either amplify or limit neuropeptide substance P-induced mast cells cytokine release [31]. In the present study, we aim to investigate the manners/mechanisms in which TLR2 make an effect on LL-37-induced mast cells activation. The work presented here demonstrates that LL-37-induced human mast cells activation could be modified by TLR2 ligands, to be more precisely, which was TLR2 ligands inhibited human mast cells degranulation and suppressed IL-8 release triggered by LL-37.

Pre-incubation with PGN or Pam3CSK4 for 24 h both suppressed LL-37-induced degranulation. It has been widely believed that degranulation in mast cells involves the calcium reliable membrane fusion and exocytosis [32,33]. Thus the increase in $[Ca^{2+}]_i$ is necessary for the complete activation of mast cells, we found that both Pam3CSK4 and PGN were effective in inhibiting LL-37-induced $[Ca^{2+}]_i$ influx. While Pam3CSK4 and PGN could induce sustained low level of $[Ca^{2+}]_i$ influx, which were inadequate to trigger degranulation from LAD2 cells. This phenomenon implies that TLR2 ligands might suppress the activation mechanisms

triggered by LL-37 through competing for the identical sources of calcium rather than through initiating calcium-independent inhibitory mechanisms to prohibit the degranulation induced by LL-37.

Despite previous report has demonstrated that different TLR2 ligands played distinct roles in the IL-8 release from mast cells activation, with PGN amplifying the release of IL-8 but Pam3CSK4 limiting that from substance P-induced mast cells activation, respectively [31], our results showed that the value of IL-8 was suppressed by both PGN and Pam3CSK4 separately from mast cells induced by LL-37, with the amount of IL-8 less than the respectively expected values. To further investigate the regulatory actions of PGN and Pam3CSK4 in mast cells activation, we employed inhibitors of enzymes in varied signaling pathways involved in pro-inflammatory cytokine synthesis in mast cells. The production and release of IL-8 induced by PGN resulting in the activation of ERK, JNK and PI3K signaling cascades, while the release of IL-8 induced by Pam3CSK4 only required ERK and calcineurin signaling systems. At the same time, we initially demonstrated that ERK, JNK, PKC, PLC, PI3K and calcineurin were all responsible for LL-37-induced IL-8 release. Moreover, the disability of Bay11-7821 to decrease IL-8 release induced by LL-37 (data not shown) suggested that NF- κ B was not simultaneously activated when mast cells were activated by the LL-37 relative pathways, which may because the activation of PI3K/Akt signaling pathway, ERK were insufficient to trigger the NF- κ B system activation since both are well renowned upstream molecules for NF- κ B activation [18] and that may

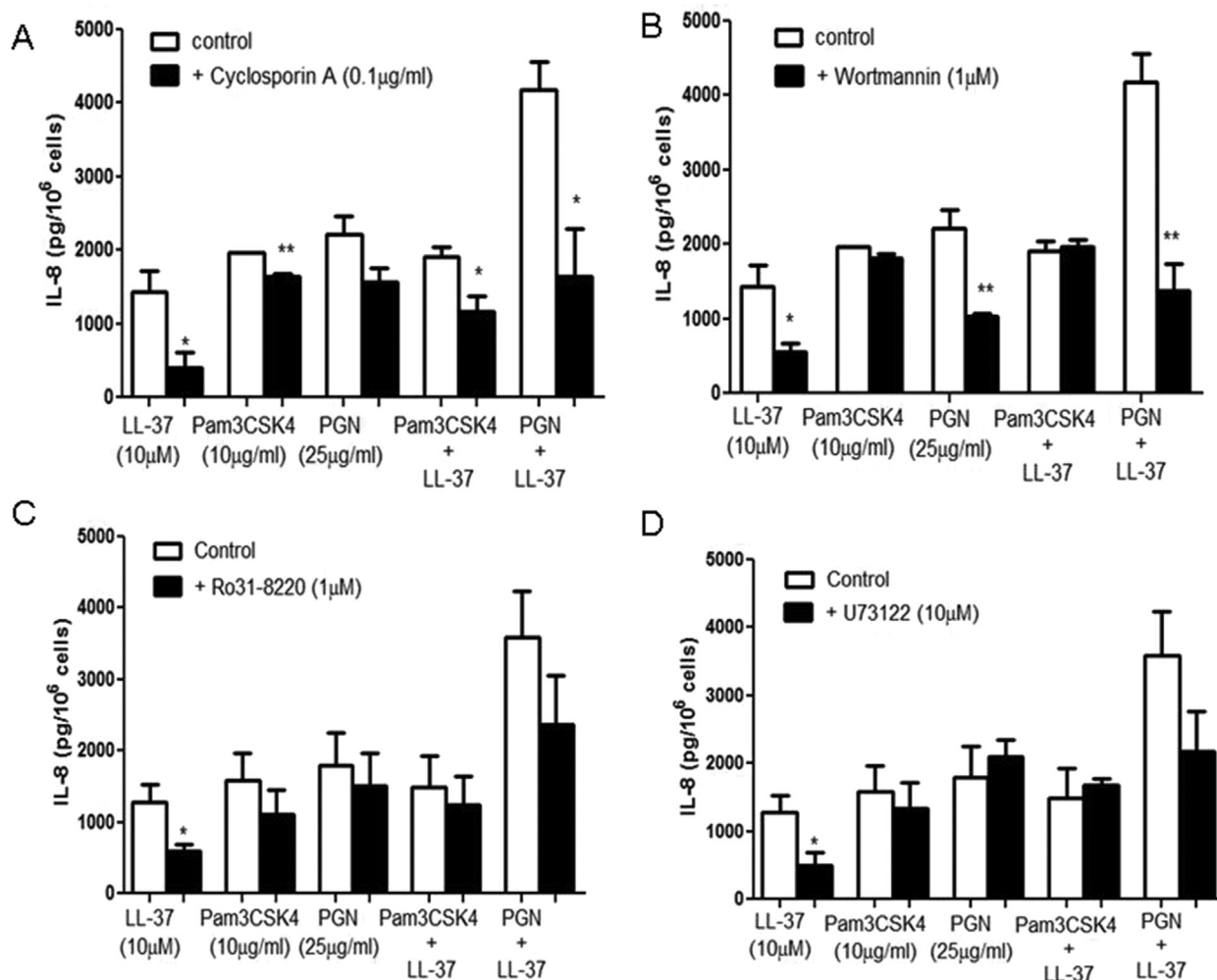


Fig. 3. Effect of inhibitor of calcineurin, PI3K, PKC and PLC on TLR2 ligands and LL-37-induced IL-8 release from LAD2 cells. A, B, C, D) LAD2 cells were incubated with cyclosporin A (0.1 μg/ml), Wortmannin (1 μM), Ro31-8220 (1 μM) and U73122 (10 μM) for 30 min prior to the addition of LL-37 (10 μM), PGN (25 μg/ml), Pam3CSK4 (10 μg/ml), or their combination for 24 h to induce the release of IL-8. The amount of IL-8 released from activated LAD2 cells pre-incubated with an inhibitor and the relevant control pre-incubated in culture medium were compared with student's t-test. * $p < 0.05$, ** $p < 0.01$ ($n = 4$).

further demonstrated LAD2 cells demanded more of MAPKs than NF- κ B for cytokines secretion. Except for interpreting the need of different signaling pathways involved in PGN and Pam3CSK4 stimulation in human mast cells, our findings are consistent with previous studies demonstrated that TLR2/TLR6 and TLR2/TLR1 heterodimers would activate distinctive signaling mechanisms [31,34,35] in immune cells including mast cells respectively [36].

The amount of IL-8 release following stimulation with LL-37 and PGN together was almost the same with that induced by PGN alone, indicating that the interplay between LL-37 and LAD2 cells was perhaps straightforwardly intruded by PGN. Obviously, the release of IL-8 induced by PGN or LL-37 or their combination was decreased significantly by the inhibitors of ERK, JNK, PI3K. The failure of the P38 inhibitor to reduce the IL-8 release indicated that ERK and JNK were the major MAPK contributing to interaction of TLR2/TLR6 heterodimer and LL-37-induced pathways for IL-8 release.

Meanwhile, the value of IL-8 release following co-stimulation with LL-37 and Pam3CSK4 was less than that of the expected value of IL-8 release triggered by LL-37 and Pam3CSK4 together, but was similar to that induced by LL-37 or Pam3CSK4, especially at high concentrations. Signaling pathways modulated by Pam3CSK4 and LL-37 were quite different as presented by modulatory actions of signaling molecular inhibitors. Only cyclosporine A and PD98059

both inhibited the IL-8 release induced by LL-37 or Pam3CSK4 or their combination. The wortmannin, JNK inhibitor II, Ro31-8220 and U73122 which significantly inhibited LL-37 induced IL-8 release did not influence that triggered by Pam3CSK4 alone or their combination, though the P38 inhibitor intriguingly failed to reduce the IL-8 release from mast cells stimulated with LL-37 or Pam3CSK4 or their combination. This phenomenon indicates that ERK was possibly the major MAPK contributing to the interplay of TLR2/TLR1 heterodimer and LL-37-induced pathways for IL-8 release and that some cross-talk perhaps exist between the signaling pathways triggered by Pam3CSK4 and LL-37 separately which needs further investigations.

In conclusion, mast cells play significant role in innate immunity, which involves initiating the local inflammatory responses to the defense of pathogenic bacteria. TLR2 can be extensively expressed in mast cells and has been widely regarded as one of the most significant pattern receptors in innate immunity. LL-37 can be recruited or secreted by mast cells upon pathogenic invasion. This study demonstrates, for the first time, that TLR2 was able to suppress the degranulation and IL-8 release from mast cells induced by LL-37. Given that degranulation and IL-8 release from mast cells are closely associated with allergic inflammatory reactions and immune responses respectively, TLR2, therefore, appears to

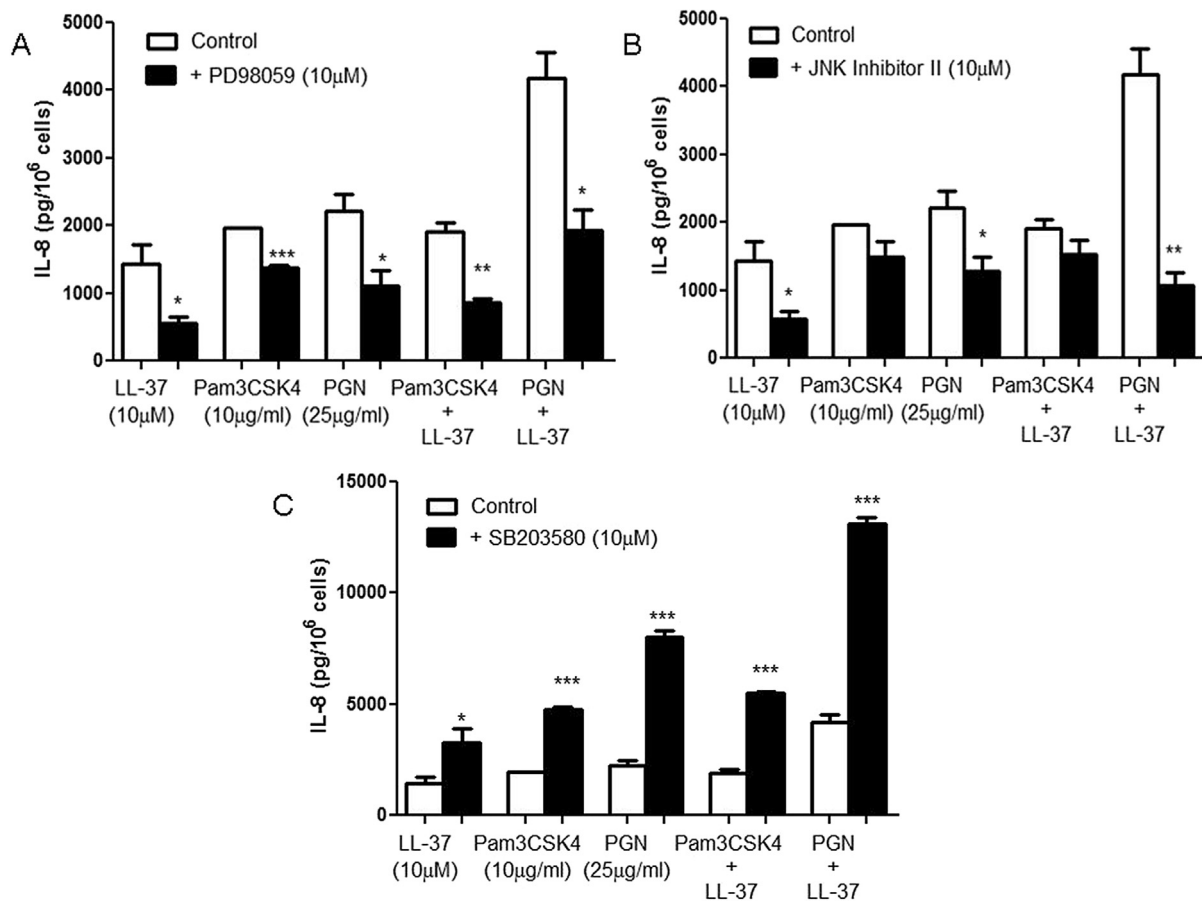


Fig. 4. Effect of inhibitors of MAPKs on TLR2 ligands and LL-37-induced IL-8 release from LAD2 cells. A) ERK inhibitor, PD98059 (10 μM), (B) JNK inhibitor, JNK inhibitor II (10 μM) or (C) p38 inhibitor, SB203580 (10 μM) was incubated with LAD2 cells for 30 min prior to the addition of LL-37 (10 μM), PGN (25 μg/ml), Pam3CSK4 (10 μg/ml), LL-37 with PGN or LL-37 with Pam3CSK4 for 24 h to induce the release of IL-8. The levels of IL-8 release from activated LAD2 cells pre-incubated with an inhibitor and the relevant control pre-incubated in culture medium were compared with student's t-test. *p < 0.05, **p < 0.01, ***p < 0.001 (n = 4).

manipulate complex mechanisms that need to be further investigated by further molecular studies. To better clarify that whether TLR2 directly or indirectly modify the activation of human mast cells and switch the function of human mast cells from allergic reactions to innate immunity, which perhaps not only opens up a new field for natural immunity, but has great significance for seeking for new approaches to prevent and control the allergy and bacterial infectious diseases.

Conflicts of interest disclosure

None declared.

Acknowledgments

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