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## Distinct roles of Ca<sup>2+</sup> mobilization and G protein usage on regulation of Toll-Like Receptor Function in Human and Murine Mast Cells

### Abstract

Toll-like receptors (TLRs) expressed in mast cells play important roles in orchestrating host defence against bacterial pathogens. Previous studies demonstrated that TLR2 agonist tripalmitoyl-S-glycero-Cys-(Lys)<sub>4</sub> (Pam<sub>3</sub>Cys) stimulates both degranulation and cytokine production in human mast cells but only induces cytokine production in murine mast cells. To determine the molecular basis for this difference, we utilized a human mast cell line LAD 2, murine lung and bone marrow-derived mast cells (MLMC and BMMC). We found that Pam<sub>3</sub>Cys caused a sustained Ca<sup>2+</sup> mobilization and degranulation in LAD 2 mast cells but not in MLMC or BMMC. Despite these differences, Pam<sub>3</sub>Cys stimulated equivalent chemokine CCL2 generation in all mast cell types tested. Cyclosporin A (CsA), an inhibitor of Ca<sup>2+</sup>/calcineurin-mediated nuclear factor of activated T cells (NFAT) activation, blocked chemokine production in LAD 2 but not in MLMC or BMMC. In contrast, inhibitors of nuclear factor kappa B (NF-κB) completely blocked CCL2 production in MLMC and BMMC but not in LAD 2 mast cells. Pertussis toxin and U0126, which, respectively, inhibit Gα<sub>i</sub>, extracellular signal-regulated kinase (ERK) phosphorylation substantially inhibited Pam<sub>3</sub>Cys-induced CCL2 generation in LAD 2 mast cells but had little or no effect on chemokine generation in MLMC and BMMC. These findings suggest that TLR2 activation in human LAD 2 mast cells and MLMC/BMMC promotes the release of different classes of mediators via distinct signalling pathways that depend on Ca<sup>2+</sup> mobilization and G protein usage. © 2006 Blackwell Publishing Ltd.

### Keywords

Chemokine; Cytokine; Mast cells; Signal transduction; TLR

### Disciplines

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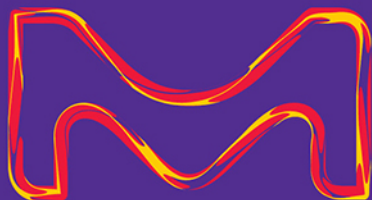
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# Distinct roles of Ca<sup>2+</sup> mobilization and G protein usage on regulation of Toll-like receptor function in human and murine mast cells

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## Introduction

Mast cells are located throughout the body in close proximity of external environment, blood vessel and nerve endings.<sup>1</sup> The role of mast cells in immediate hypersensitivity reactions and allergic diseases is well established.<sup>2–5</sup> Mast cells also express Toll-like receptors (TLRs) that allow them to respond to diverse microbial, viral and fungal-derived products to mediate innate immunity.<sup>1,6</sup> To date, 13 TLR family members have been identified in the human and murine genome and mast cells express TLR1, -2, -3, -4, -6, -7 and -9 but not -5.<sup>7–12</sup> TLR2 is critical for

## Summary

Toll-like receptors (TLRs) expressed in mast cells play important roles in orchestrating host defence against bacterial pathogens. Previous studies demonstrated that TLR2 agonist tripalmitoyl-S-glycero-Cys-(Lys)<sub>4</sub> (Pam<sub>3</sub>Cys) stimulates both degranulation and cytokine production in human mast cells but only induces cytokine production in murine mast cells. To determine the molecular basis for this difference, we utilized a human mast cell line LAD 2, murine lung and bone marrow-derived mast cells (MLMC and BMMC). We found that Pam<sub>3</sub>Cys caused a sustained Ca<sup>2+</sup> mobilization and degranulation in LAD 2 mast cells but not in MLMC or BMMC. Despite these differences, Pam<sub>3</sub>Cys stimulated equivalent chemokine CCL2 generation in all mast cell types tested. Cyclosporin A (CsA), an inhibitor of Ca<sup>2+</sup>/calcineurin-mediated nuclear factor of activated T cells (NFAT) activation, blocked chemokine production in LAD 2 but not in MLMC or BMMC. In contrast, inhibitors of nuclear factor kappa B (NF-κB) completely blocked CCL2 production in MLMC and BMMC but not in LAD 2 mast cells. Pertussis toxin and U0126, which, respectively, inhibit G<sub>αi</sub>, extracellular signal-regulated kinase (ERK) phosphorylation substantially inhibited Pam<sub>3</sub>Cys-induced CCL2 generation in LAD 2 mast cells but had little or no effect on chemokine generation in MLMC and BMMC. These findings suggest that TLR2 activation in human LAD 2 mast cells and MLMC/BMMC promotes the release of different classes of mediators via distinct signalling pathways that depend on Ca<sup>2+</sup> mobilization and G protein usage.

**Keywords:** mast cells; TLR; signal transduction; chemokine; cytokine

the propagation of inflammatory response to components of Gram-positive, Gram-negative bacteria, mycobacteria, yeast and protozoa.<sup>13–19</sup> TLR2 are predominantly expressed in cells involved in first line of host defence including mast cells, neutrophils, dendritic cells and macrophages. Mice deficient in TLR2 have been used to demonstrate an important role of this receptor in mast cell-dependent-innate immunity.<sup>20</sup>

One feature that distinguishes mast cells from other immune cells is the marked phenotypic and functional heterogeneity that exists between cell types obtained from different tissues and species.<sup>21–23</sup> Thus, TLR2 agonists

Abbreviations: BMMC, murine bone marrow derived mast cells; CCL2, chemokine CC ligand 2; ERK, extracellular-regulated kinase; GPCR, G protein coupled receptor; LAD 2, Laboratory of allergic diseases 2; MLMC, murine lung mast cells; NFAT, nuclear factor of activated T cells; PTX, pertussis toxin; TLR, Toll-like receptor.

induce degranulation in human cord blood-derived mast cells (CMBC)<sup>7,8,10</sup> but not in murine skin or bone-marrow derived mast cells (BMMCs).<sup>9,20,24</sup> In contrast, TLR2 ligands induce chemokine/cytokine production in all mast cell types tested.<sup>7,8,10,24–26</sup> The molecular basis for the differences in TLR2 function in humans and murine mast cells, however, remains unknown.

It is generally accepted that all TLRs activate a common signalling pathway which culminates in the activation of nuclear factor kappa B (NF- $\kappa$ B).<sup>27,28</sup> Fan *et al.*<sup>29,30</sup> recently showed that while TLR2-mediated tumour necrosis factor- $\alpha$  production in splenocytes does not require G $\alpha_{i2}$ , this G protein is essential for cytokine generation in macrophages. TLR activation also promote other signalling pathways such as mitogen activated protein kinases (extracellular signal-regulated kinase (ERK), p38 and c-Jun NH<sub>2</sub>-terminal kinase) and phosphatidylinositol-3 kinase (PI3K) to induce the expression of proinflammatory cytokines.<sup>31</sup> Nuclear factor of activated T cells (NFAT) is a family of transcription factors expressed in a wide variety of cell types.<sup>32–35</sup> Ca<sup>2+</sup> mobilization in response to Fc $\epsilon$ RI cross-linking<sup>36,37</sup> G-protein coupled receptor activation<sup>38</sup> nerve growth factor activation<sup>39</sup> in mast cells leads to NFAT activation and chemokine gene expression. Waters *et al.*<sup>40</sup> recently showed that TLR2 agonist tripalmitoyl-S-glycero-Cys-(Lys)<sub>4</sub> (Pam<sub>3</sub>Cys), which induces Ca<sup>2+</sup> mobilization in human airway epithelial cells, requires NFAT but not NF- $\kappa$ B for cytokine production. The possibility that TLR could utilize different signalling pathways to induce chemokine generation in different mast cell types has not been tested.

This study was undertaken to better understand the signalling pathway via which TLR2 agonist tripalmitoyl-S-glycero-Cys-(Lys)<sub>4</sub> (Pam<sub>3</sub>Cys) activates human and murine mast cells. For this study, we utilized a human mast cell line LAD 2, murine lung mast cell (MLMC) and BMMC. Here, we demonstrate the novel finding that Pam<sub>3</sub>Cys activates human LAD 2 and murine mast cells to promote the release of different classes of mediators via distinct signalling pathways that depend on Ca<sup>2+</sup> mobilization and G protein usage.

## Materials and methods

### Materials

Pam<sub>3</sub>Cys was obtained from Invivogen (San Diego, CA). Recombinant human stem cell factor (rhSCF), recombinant murine stem cell factor (rmSCF) and interleukin-3 (rmIL-3) were purchased from Peprotech (Rocky Hill, NJ). DNP-BSA (2, 4-nitrophenyl hapten conjugated to bovine serum albumin) was purchased from Biosearch Technologies (Novato, CA). Anti-DNP-immunoglobulin E (IgE) was a generous gift from Dr Juan Rivera (NIH). Reagents for enzyme-linked immunosorbent assay

(ELISA) kits were purchased from R & D Systems (Minneapolis, MN). Phosphoplus p44/42 mitogen-activated protein kinase (ERK; Thr202/Tyr204) and Phosphoplus Akt (Ser 473) kits were from Cell Signalling (Beverly, MA). All tissue culture reagents and pertussis toxin (PTX) were purchased from Invitrogen (Gaithersburg, MD). Indo-1 and pluronic F-127 were from Molecular Probes (Eugene, OR). Supersignal Western blotting analysis kits were purchased from Pierce (Rockford, IL). LY294002, U0126, pyrrolidine dithiocarbamate (PDTC) (E)3-[(4-methylphenyl)sulphonyl]-2-propenenitrile (Bay 11-7082) and cyclosporin A (CsA) were from Calbiochem (La Jolla, CA).

### Cell culture

Human LAD 2 mast cells was isolated from the bone marrow of a patient with mast cell leukemia.<sup>41</sup> These cells were cultured in serum-free media StemPro-34 (Invitrogen) supplemented with L-glutamine (2 mM), penicillin (100 IU/ml), streptomycin (100  $\mu$ g/ml) and rhSCF (100 ng/ml). Hemidepletions were performed weekly with media containing rhSCF (100 ng/ml).<sup>41,42</sup> MLMC were cultured as described by Zhong *et al.*<sup>43</sup> Briefly, lung mast cells were cultured from the upper airways of C57BL6 mice by cutting the tissue into  $\sim$ 3-mm<sup>3</sup> pieces, which were then incubated in Dulbecco's modified Eagle's minimal essential medium containing 10% fetal bovine serum, glutamine (2 mM), HEPES (10 mM), penicillin (100 units/ml), streptomycin (100  $\mu$ g/ml), rmSCF (10 ng/ml) and rmIL-3 (10 ng/ml). Culture medium was changed every other day for the first week. Non-adherent cells were collected and passaged in the same manner for additional 2–3 weeks. Cells that had been in culture for 4–8 weeks were used. Murine BMMC were obtained by flushing bone marrow cells from the femurs of C57BL6 mice, then culturing for 4–6 weeks in Iscove's medium supplemented with 10% fetal calf serum, glutamine (2 mM),  $\alpha$ -monothioglycerol (150  $\mu$ M), penicillin (100 units/ml), streptomycin (100  $\mu$ g/ml), and rmIL-3 (10 ng/ml) and rmSCF (10 ng/ml). The homogeneity of the mast cells was confirmed by acid toluidine blue staining.<sup>43</sup> More than 95% pure MLMC and BMMC population was used for these studies.

### Calcium measurement

Ca<sup>2+</sup> mobilization was determined as described previously.<sup>38,44</sup> Briefly, cells ( $0.25 \times 10^6$  for LAD 2 or  $1 \times 10^6$  for MLMC and BMMC) were loaded with 1  $\mu$ M indo-1 AM in the presence of 1  $\mu$ M pluronic F-127 for 30 min at room temperature. Cells were washed and resuspended in 1.5 ml of HEPES-buffered saline. Ca<sup>2+</sup> mobilization was measured in a Hitachi F-2500 spectrophotometer with an excitation wavelength of 355 nm and an emission wavelength of 410 nm.<sup>38</sup>

### Assay of degranulation

Mast cells ( $5 \times 10^3$  for LAD 2 or  $2 \times 10^4$  for MLMC and BMMC) were seeded into 96-well plates in a total volume of 50  $\mu$ l of buffer containing 0.1% BSA and exposed to different concentrations of Pam<sub>3</sub>Cys or DNP-BSA for 30 min. For total  $\beta$ -hexosaminidase release, control cells were lysed in 50  $\mu$ l of 0.1% Triton-X-100. Aliquots (15  $\mu$ l) of supernatants or cell lysates were incubated with 15  $\mu$ l of 1 mM *p*-nitrophenyl-*N*-acetyl- $\beta$ -D-glucosamine for 1.5 hr at 37°. Reaction was stopped by adding 250  $\mu$ l of a 0.1 M Na<sub>2</sub>CO<sub>3</sub>/0.1 M NaHCO<sub>3</sub> buffer and absorbance was measured at 405 nm.<sup>44</sup>

### Gel electrophoresis and immunoblotting

Mast cells ( $0.5 \times 10^6$ ) were stimulated with Pam<sub>3</sub>Cys (10  $\mu$ g/ml) for 0, 1, 3, 5, 10 or 30 min and were lysed in sample buffer. Proteins were separated by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) and Western blotting was performed with anti-phospho-ERK and anti-phospho-Akt antibody. Blot was stripped and reprobed with anti-ERK and anti-Akt antibody. Immunoreactive bands were visualized by SuperSignal West Femto maximum sensitivity substrate (Pierce).

### Assay of CCL2 and IL-6 production

LAD 2 mast cells, MLMC or BMMC ( $0.2 \times 10^6$  per well in 125  $\mu$ l basal medium) were stimulated with Pam<sub>3</sub>Cys (30  $\mu$ g/ml for LAD 2 cells, 1  $\mu$ g/ml for MLMC and BMMC) for 6 hr. For inhibitor studies, cells were preincubated with PTX (100 ng/ml, 18 hr), U0126 (1  $\mu$ M, 30 min), LY294002 (20  $\mu$ M, 30 min), PDTC (100  $\mu$ M; 1 hr), Bay 117082 (5  $\mu$ M, 1 hr) or CsA (100 nM; 1 hr) and then stimulated with Pam<sub>3</sub>Cys for 6 hr. Supernatants were collected and stored frozen at  $-80^\circ$ , until analysis. Human CCL2 or JE (murine chemokine equivalent to human CCL2) and interleukin-6 (IL-6) were quantified using a sandwich ELISA kit as described in the manufacturer's protocols. The reaction was read at 450 nm in a micro plate reader (Molecular Devices).

## Results

### TLR2 agonist Pam<sub>3</sub>Cys-induced degranulation in LAD 2 mast cells requires Ca<sup>2+</sup> mobilization and heterotrimeric G protein activation

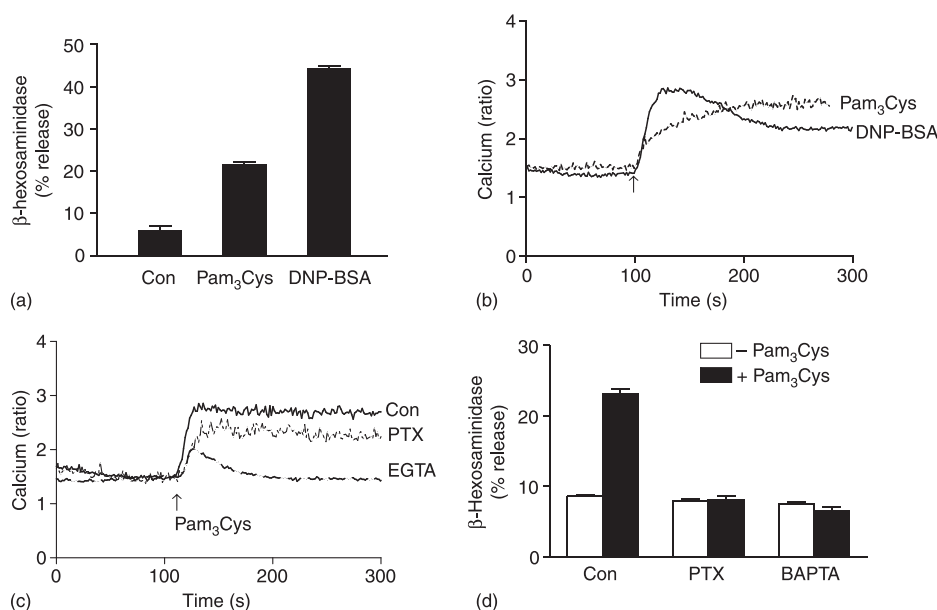
TLR2 is expressed in human CD34<sup>+</sup>-derived mast cells and cord blood derived mast cells (CBMC). A synthetic TLR2 agonist Pam<sub>3</sub>Cys induces degranulation in human CBMC.<sup>8</sup> LAD 2 is a relatively new and highly differentiated human mast cell line, which we have recently used as a model to study G protein coupled receptor

signalling.<sup>42,45</sup> Because LAD 2 cells endogenously express TLR2<sup>10</sup> we first sought to determine whether Pam<sub>3</sub>Cys causes degranulation in this cell line. We found that, as in CBMCs<sup>8</sup> Pam<sub>3</sub>Cys caused  $\sim 15\%$  degranulation in LAD 2 mast cells (Fig. 1a). This response was two- to threefold less than that induced by antigen (DNP-BSA) (Fig. 1a). Previously, it has been shown that another TLR2 agonist peptidoglycan (PGN) also induces approximately twofold degranulation in CBMCs.<sup>8</sup> Similarly, we found that PGN (50  $\mu$ g) induces  $\sim 2.4$ -fold degranulation in LAD 2 mast cells ( $6.4 \pm 1.0\%$   $\beta$ -hexosaminidase release in the presence of PGN compared to  $2.6 \pm 0.8\%$  in its absence). Given that Ca<sup>2+</sup> mobilization is required for mast cell degranulation<sup>46</sup> we tested effects of Pam<sub>3</sub>Cys on the kinetics of Ca<sup>2+</sup> response in mast cells. As shown in Fig. 1(b), Pam<sub>3</sub>Cys induced a slow but sustained Ca<sup>2+</sup> mobilization. In contrast, antigen induced a less sustained Ca<sup>2+</sup> response.

Fan *et al.*<sup>29,30</sup> showed that TLR2 activates G-protein dependent signalling pathway in murine macrophages but not in splenocytes. To test the role of G protein signalling on Pam<sub>3</sub>Cys-induced responses, LAD 2 mast cells were pretreated with pertussis toxin (PTX, 100 ng/ml) and Ca<sup>2+</sup> mobilization and degranulation were determined. PTX partially inhibited Pam<sub>3</sub>Cys-induced Ca<sup>2+</sup> mobilization (Fig. 1c) but completely blocked degranulation (Fig. 1d). We found that in the absence of extracellular Ca<sup>2+</sup>, Pam<sub>3</sub>Cys-induced Ca<sup>2+</sup> mobilization was substantially reduced. (Fig. 1c). Furthermore, chelation of intracellular Ca<sup>2+</sup> with (1,2-bis 2-aminophenoxy) ethane-*N,N,N',N'*-tetraacetic acid-acetoxymethyl ester (BAPTA) completely blocked Pam<sub>3</sub>Cys-induced degranulation (Fig. 1d). These findings suggest that Pam<sub>3</sub>Cys-induced degranulation in LAD 2 mast cells requires both G $\alpha_i$ -independent and G $\alpha_i$ -dependent signalling pathways.

### Pam<sub>3</sub>Cys-induced chemokine production in human mast cells requires Ca<sup>2+</sup>-mediated NFAT and G $\alpha_i$ -mediated ERK and PI3 kinase activation

It is generally accepted that TLRs induce cytokine/chemokine gene expression via the activation of NF- $\kappa$ B. In contrast, Waters *et al.*<sup>40</sup> recently demonstrated that Pam<sub>3</sub>Cys causes Ca<sup>2+</sup> mobilization, NFAT activation and cytokine production in human airway epithelial cells. Furthermore, CsA, an inhibitor of NFAT activation blocked Pam<sub>3</sub>Cys-induced cytokine production but NF- $\kappa$ B inhibitor had no effect. Because Pam<sub>3</sub>Cys induces Ca<sup>2+</sup> mobilization in LAD 2 cells, we hypothesized that chemokine production in this cell line may be mediated via NFAT activation. To test this possibility, we determined the effect of CsA on Pam<sub>3</sub>Cys-induced CCL2 generation in LAD 2. As shown in Fig. 2(a), CsA almost completely inhibited Pam<sub>3</sub>Cys-induced CCL2 generation. In contrast, PDTC or Bay 11-7082 (NF- $\kappa$ B inhibitors) had no effect on Pam<sub>3</sub>Cys response. These findings suggest that Pam<sub>3</sub>Cys causes



**Figure 1.** Role of G protein signalling on Pam<sub>3</sub>Cys-induced degranulation and Ca<sup>2+</sup> mobilization in human LAD 2 mast cells. (a) Cells were exposed to buffer (Con), Pam<sub>3</sub>Cys (30  $\mu$ g/ml) or DNP-BSA (20 ng/ml) for 30 min  $\beta$ -hexosaminidase release was determined. (b) Cells were loaded with Indo-1AM and stimulated with Pam<sub>3</sub>Cys (1  $\mu$ g/ml) or DNP-BSA (20 ng/ml) and intracellular Ca<sup>2+</sup> was measured. (c) Cells cultured in the absence or presence of PTX (100 ng/ml, 16 hr), loaded with Indo-1AM and Pam<sub>3</sub>Cys (10  $\mu$ g/ml)-induced intracellular calcium mobilization. For experiments in the absence of Ca<sup>2+</sup>, indo-1 loaded cells were exposed to EGTA (4 mM, 3 min) before stimulation with Pam<sub>3</sub>Cys. (d) Cells were pretreated with or without PTX (100 ng/ml, 16 hr) or BAPTA (100  $\mu$ M, 30 min), exposed to with buffer (Con) or Pam<sub>3</sub>Cys (30  $\mu$ g/ml) for 30 min and  $\beta$ -hexosaminidase release was determined. Data shown are representative of three similar experiments.

chemokine production in LAD 2 mast cells via Ca<sup>2+</sup>-mediated NFAT but not NF- $\kappa$ B activation.

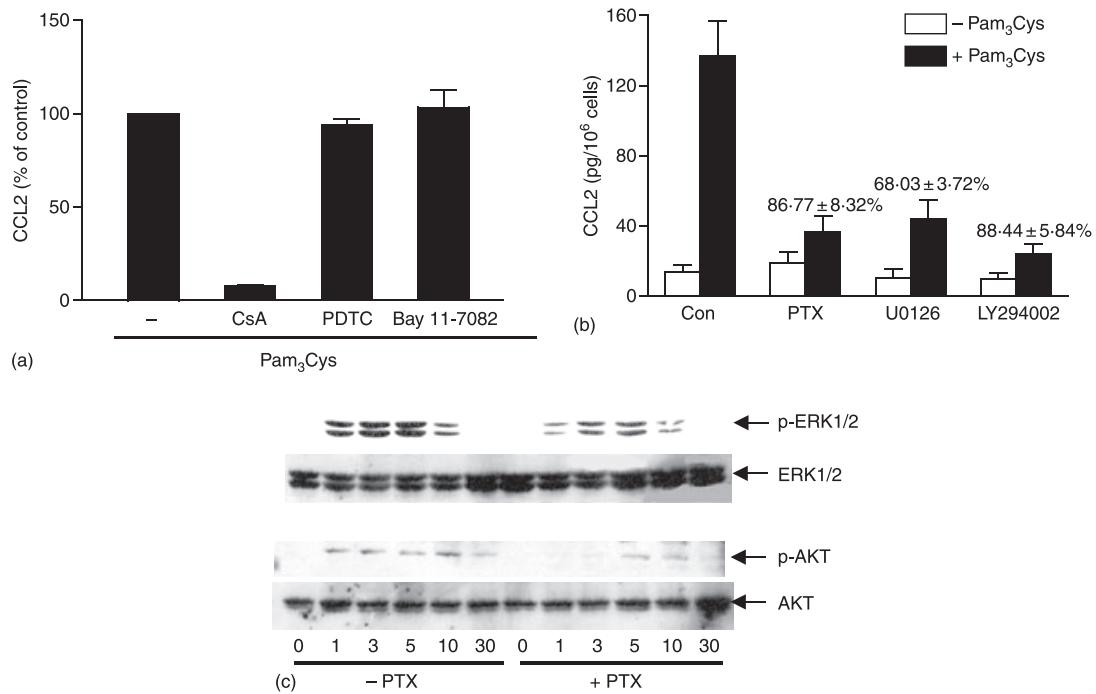
To determine whether G $\alpha_i$ -mediated signalling pathway interacts with G $\alpha_i$  independent Ca<sup>2+</sup> mobilization, we tested the effect of PTX on Pam<sub>3</sub>Cys-induced CCL2 generation. As shown in Fig. 2(b), PTX caused substantial inhibition of Pam<sub>3</sub>Cys-induced CCL2 generation. Pam<sub>3</sub>Cys caused robust ERK and Akt phosphorylation in LAD 2 mast cells (Fig. 2c). Furthermore, PTX substantially inhibited both ERK and Akt phosphorylation (Fig. 2c). In addition, inhibitors of ERK (U0126) and PI3 kinase (LY294002) blocked Pam<sub>3</sub>Cys-induced CCL2 production by (68.03  $\pm$  3%) and LY294002 (88.4  $\pm$  5%), respectively (Fig. 2b). These findings demonstrate that Pam<sub>3</sub>Cys-induced CCL2 production in LAD 2 mast cells requires both Ca<sup>2+</sup> mobilization and G $\alpha_i$ -mediated ERK and PI3 kinase activation.

#### **Pam<sub>3</sub>Cys does not induce Ca<sup>2+</sup> mobilization or degranulation but induces chemokine and cytokine production in murine mast cells via NF- $\kappa$ B activation**

While TLR2 activation leads to degranulation in human CBMC<sup>8</sup> and LAD 2 mast cells (Fig. 1a), it has no effect on degranulation in murine BMMC and fetal-skin derived mast cells.<sup>9,24</sup> All murine mast cells are, however, highly responsive to TLR2 agonists for cytokine generation.

MLMC display some properties that are distinct from BMMC. Thus, activation of MLMC via adenosine A<sub>3</sub> receptors leads to degranulation whereas the same receptor expressed in BMMC does not induce degranulation.<sup>43,47</sup> We therefore sought to determine the effects of TLR2 activation on mediator release and signalling pathways in MLMC and BMMC. As shown in Fig. 3, Pam<sub>3</sub>Cys did not cause Ca<sup>2+</sup> mobilization or degranulation in MLMC and BMMC. However, these responses were induced by Ag in both MLMC and BMMC. In contrast, Pam<sub>3</sub>Cys induced chemokine, JE as well as IL-6 in murine mast cells (Figs 4 and 5). There were, however, important differences in the mechanism by which Pam<sub>3</sub>Cys induced chemokine/cytokine production in MLMC/BMMC when compared to human LAD 2 mast cells that reflects differences in Ca<sup>2+</sup> mobilization and G protein coupling. Thus, in contrast to LAD 2 cells (Fig. 2a), CsA did not inhibit either JE (Fig. 4a) or IL-6 (Fig. 4b) in murine mast cells. Furthermore, inhibitors of NF- $\kappa$ B (PDTC and Bay 11-7082), which had no effect on chemokine generation in LAD 2 cells, almost completely inhibited both JE and IL-6 in murine mast cells (Fig. 4). Unlike the situation in LAD 2 cells, PTX and U0126 had no effect on JE or IL-6 induction in murine mast cells (Fig. 5). Although LY294002 blocked Pam<sub>3</sub>Cys-induced responses, there were differences in the magnitude of inhibition between MLMC and BMMC (Fig. 5).





**Figure 2.** Pam<sub>3</sub>Cys-induced CCL2 production in human LAD 2 mast cells requires Ca<sup>2+</sup>-mediated NFAT and Gα<sub>i</sub>-mediated ERK and PI3 kinase activation (a) Cells were pretreated with vehicle or cyclosporine A (CsA, 100 nM; 1 hr), PDTC (100 μM, 1 hr) or Bay 11-7082 (5 μM; 1 hr) and exposed to buffer or Pam<sub>3</sub>Cys (30 μg/ml) for 6 hr and chemokine/cytokine production was determined by ELISA. Data are expressed as percent stimulated with Pam<sub>3</sub>Cys in the absence of inhibitor (% of control). Data shown is mean ± SEM of three experiments performed in triplicate. (b) Cells were pretreated with vehicle or PTX (100 ng/ml, 16 hr), U0126 (1 μM, 30 min) or LY294002 (20 μM, 30 min) and exposed to buffer (Con) or Pam<sub>3</sub>Cys (30 μg/ml) for 6 hr and CCL2 production was determined by ELISA. Data shown are mean ± SEM of three experiments performed in triplicate. *P* < 0.05 in the absence or presence of inhibitors. (c) Cells pretreated with vehicle (-PTX) or PTX (+PTX; 100 ng/ml, 16 hr) were stimulated with Pam<sub>3</sub>Cys (10 μg/ml) for 0, 1, 3, 5, 10 and 30 min. Cell lysates were separated on SDS-PAGE and Western blotting was performed with anti-phospho-ERK1/2 and anti-phospho-Akt antibody. Blot was stripped and reprobbed with anti-ERK and anti-Akt antibody followed by anti-rabbit-IgG-horseradish peroxidase. Immunoreactive bands were visualized by SuperSignal West Femto maximum sensitivity substrate. Data shown are representative of three similar experiments.

## Discussion

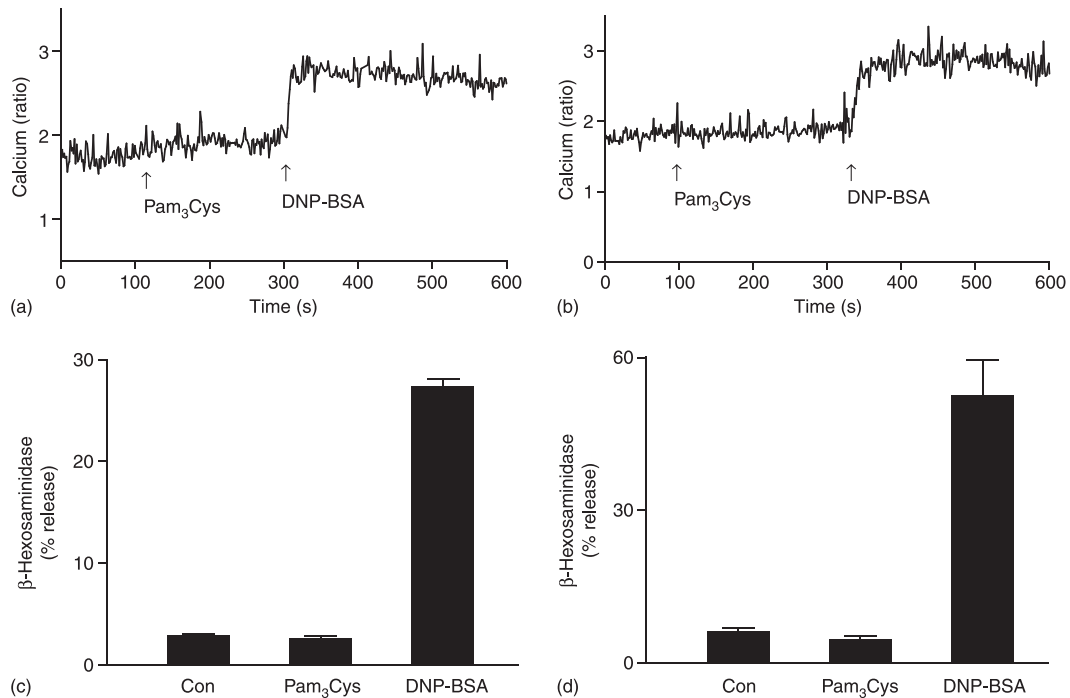
Mast cells are multifunctional immune cells that play important roles in host defence<sup>1,6</sup> but also mediate allergic and inflammatory diseases.<sup>2-5</sup> One feature that distinguishes mast cells from other immune cells is the marked phenotypic and functional heterogeneity that exists between cell types obtained from different tissues and species.<sup>21-23</sup> Recent studies demonstrated important differences in the ability of TLR2 ligands to induce the release of early and late mediators in human and murine mast cells.<sup>8-10,24</sup> Thus, TLR2 ligands induce degranulation in human CBMCs<sup>8</sup> but not in murine BMMCs, fetal skin mast cells or a murine mast cell line, MC/9.<sup>9,24,48</sup> In contrast, TLR2 activation promotes cytokine generation in all mast cell types tested. The molecular basis for the differences in TLR2 function in human and murine mast cells, however, remains unknown. In the present study, we utilized a human mast cell line LAD 2, murine lung as well as BMMC and demonstrated the novel findings that Pam<sub>3</sub>Cys induces the release of different classes of

mediators with a specificity that depends on Ca<sup>2+</sup> mobilization and G protein usage.

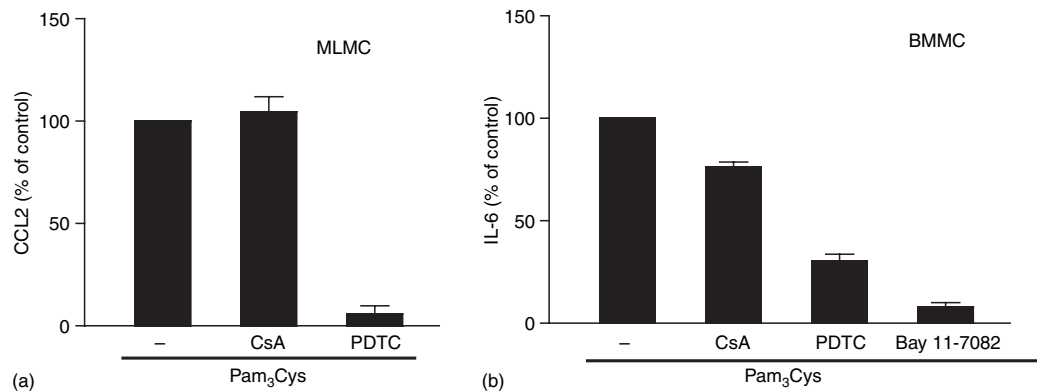
An important finding of the present study was that Pam<sub>3</sub>Cys induced degranulation in LAD 2 mast cells equal in magnitude to that previously reported for human CBMCs.<sup>8</sup> We also showed that Pam<sub>3</sub>Cys caused a sustained Ca<sup>2+</sup> mobilization in LAD 2 mast cells and that this response was dependent on the presence of extracellular Ca<sup>2+</sup>. Furthermore, chelation of Ca<sup>2+</sup> with BAPTA completely inhibited Pam<sub>3</sub>Cys-induced degranulation. Consistent with previous reports<sup>9,24</sup> we failed to detect any degranulation by Pam<sub>3</sub>Cys in murine lung or BMMC. Furthermore, unlike the situation in LAD 2 mast cells, Pam<sub>3</sub>Cys did not induce Ca<sup>2+</sup> mobilization in murine lung or BMMCs. These findings suggest that susceptibility of human CBMC<sup>8</sup> and LAD 2 mast cells to undergo degranulation in response to Pam<sub>3</sub>Cys and the resistance of murine mast cells reflect differences in Ca<sup>2+</sup> mobilization.

Interestingly, despite the differences in Pam<sub>3</sub>Cys-induced Ca<sup>2+</sup> mobilization and degranulation in human LAD 2 and murine mast cells, it caused chemokine CCL2





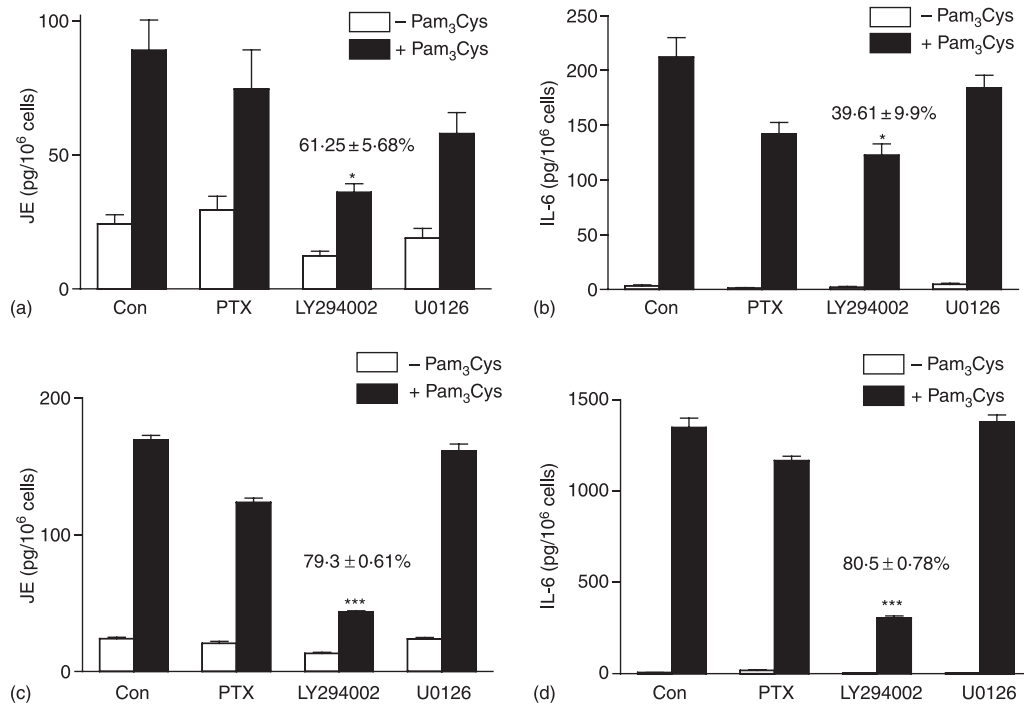
**Figure 3.** Pam<sub>3</sub>Cys does not induce Ca<sup>2+</sup> mobilization or degranulation in MLMC and BMMC. (a) MLMC or (b) BMMC were loaded with Indo-1AM, stimulated with Pam<sub>3</sub>Cys (10 μg/ml) or DNP-BSA (20 ng/ml) and intracellular calcium was measured. (c) MLMC and (d) BMMC were stimulated with Pam<sub>3</sub>Cys (10 μg/ml) or DNP-BSA (20 ng/ml) for 30 min and β-hexosaminidase release was determined. Data shown are representative of three similar experiments.



**Figure 4.** Pam<sub>3</sub>Cys induces JE and IL-6 production in murine mast cells via NF-κB activation. (a) MLMC or (b) BMMC were pretreated with vehicle or CsA (100 nM; 1 hr), PDTC (100 μM; 1 hr) or Bay 11-7082 (5 μM; 1 hr) and exposed to buffer or Pam<sub>3</sub>Cys (1 μg/ml) for 6 h and chemokine/cytokine production was determined by ELISA. Data are expressed as percent stimulated with Pam<sub>3</sub>Cys in the absence of inhibitor (% of control). Data shown is mean ± SEM of three experiments performed in triplicate.

production in all mast cell types tested. While NF-κB is the major transcription factor that mediates TLR2-induced cytokine production in a variety of cell types, a recent report indicated that Ca<sup>2+</sup>-induced phosphatase calcineurin activates NFAT, but not NF-κB, to induce cytokine production in human lung epithelial cells.<sup>40</sup> Qiao *et al.*<sup>24</sup> have also shown that FcεRI-mediated cytokine production in BMMC and MC/9 cells requires Ca<sup>2+</sup>

mobilization and NFAT activation. These findings raise the interesting possibility that Pam<sub>3</sub>Cys induces chemokine in different mast cell populations via the utilization of NFAT or NF-κB depending on its ability to induce Ca<sup>2+</sup> mobilization. Indeed, we found that CsA, an inhibitor of calcineurin-mediated NFAT activation, blocked Pam<sub>3</sub>Cys-induced CCL2 production in human LAD 2 mast cells but had no effect on murine mast cells. In



**Figure 5.** Pam<sub>3</sub>Cys-induced JE and IL-6 production in MLMC and BMMC require PI3 kinase but not G protein, ERK activation. (a and b) MLMC and (c and d) BMMC were pretreated with vehicle (Con) or PTX (100 ng/ml, 16 hr), LY294002 (20  $\mu$ M, 30 min) or U0126 (1  $\mu$ M, 30 min) and exposed to buffer (Con) or Pam<sub>3</sub>Cys (1  $\mu$ g/ml) for 6 h and (a and c) JE as well as (b and d) IL-6 production was determined by ELISA. Data shown are mean  $\pm$  SEM of two experiments performed in triplicate. \* $P$  < 0.05 or \*\*\* $P$  < 0.0001 in the absence or presence of inhibitors.

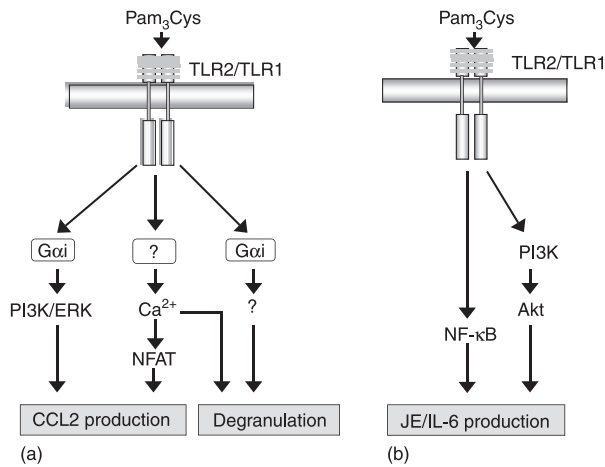
contrast, inhibitors of NF- $\kappa$ B completely inhibited CCL2 as well as IL-6 production in murine but not in LAD 2 mast cells. Another important difference was that while Pam<sub>3</sub>Cys-induced chemokine generation in LAD 2 mast cells required G $\alpha_i$ -mediated ERK phosphorylation, these signalling pathways were not required for chemokine/cytokine generation in MLMC or BMMC. These findings suggest that Pam<sub>3</sub>Cys causes chemokine production in human LAD 2 and MLMC/BMMC via distinct signalling pathways that depend on Ca<sup>2+</sup> mobilization and G protein usage.

The reason for the differences in Pam<sub>3</sub>Cys-induced chemokine production in human LAD 2 and murine mast cells is not clear. LAD 2 cells are highly differentiated connective tissue type mast cells whereas murine mast cells tested in this study are immature mucosal mast cells. It is therefore possible that this phenotypic variation could explain the observed differences in mechanism of TLR2 signalling. Matsushima *et al.*<sup>9</sup> recently compared the properties of murine skin (connective tissue) and BMMC (mucosal) with respect to TLR function. They found no difference in TLR2 signalling in skin mast cells and BMBCs. However, skin mast cells express TLR3, TLR7 and TLR9 that are not present in BMBCs. These findings suggest that phenotypic variation in mast cells regulate expression profile of different TLRs but may not

explain the differences in TLR2 signalling observed in the present study. In general, it is extremely difficult to extrapolate data from one mast cell type to another. Therefore, the further experiments are required to delineate the relevance of the studies described herein with mast cell line.

TLR2 is not the only TLR that displays differences between human and murine mast cells. Thus, human CBMCs do not respond to TLR4 ligand unless the cells are primed with interleukin-4.<sup>7</sup> In contrast, murine BMMC are fully responsive to the endotoxins in the absence of priming.<sup>9,49,50</sup> We confirmed that while lipopolysaccharide stimulates the cytokine production in murine mast cells but not in LAD 2 mast cells (data not shown). This raises the interesting possibility that the observed differences in human LAD 2 and murine mast cells reflect species-specific differences in TLR2 signalling. Future studies with human mast cells from different locations are needed to confirm the validity of this contention.

In summary, we have used a human mast cell line LAD 2, MLMC and BMMC to delineate the molecular basis for the previously described differences in TLR2 signalling in human and murine mast cells.<sup>8,9,24</sup> We propose that the ability of Pam<sub>3</sub>Cys to induce degranulation in LAD 2 mast cells, MLMC and BMMC reflects difference in Ca<sup>2+</sup> mobilization and G $\alpha_i$  activation. We have shown that



**Figure 6.** Schematic representation of the signalling pathways involved in Pam<sub>3</sub>Cys-induced degranulation and cytokine/chemokine production in human LAD 2, MLMC and BMMC. We propose that the ability of Pam<sub>3</sub>Cys to induce degranulation in (a) LAD 2 mast cells, (b) MLMC and BMMC reflects differences in Ca<sup>2+</sup> mobilization and G<sub>αi</sub> activation. Pam<sub>3</sub>Cys-induced CCL2 production in (a) human LAD 2 mast cells requires Ca<sup>2+</sup>-mediated NFAT and G<sub>αi</sub>-mediated ERK and PI3 kinase activation. However, in (b) MLMC and BMMC Pam<sub>3</sub>Cys does not induce Ca<sup>2+</sup> mobilization therefore JE production is mediated via NF-κB activation. PI3 kinase pathway is also required for JE production in MLMC and BMMC. (→ stimulation).

Pam<sub>3</sub>Cys-induced Ca<sup>2+</sup> mobilization synergizes with G<sub>αi</sub>-mediated ERK and Akt phosphorylation to induce chemokine generation in LAD 2 mast cells. In contrast, Pam<sub>3</sub>Cys, which does not induce Ca<sup>2+</sup> mobilization in MLMC or BMMC, causes chemokine production via a different mechanism involving NF-κB and Akt phosphorylation (see Model Fig. 6).

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