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Insight box

Previous studies have implicated that heterotrimeric guanine nucleotide binding regulatory (G_{i/o}) proteins are involved in signaling to TLRs. Participation of G_{i} protein in TLRs has been proved. However, the role of G_{o} protein in TLR signaling has not been identified. In the present study, genetic depletion of G_{o} proteins reduced TLR2-mediated IL-8 release in human mast cell line LAD2 cells. G_{o}-GTP protein complex was precipitated upon TLR2 stimulation from LAD2 cells. Furthermore, G_{o} proteins were required for the activation of MAPKs and NF-κB, but not PI3K-Akt or Ca^{2+}-calcineurin-NFAT in TLR2-mediated LAD2 cells. These results suggested that TLR2 activation in human mast cells partially depends on G_{o} protein action.
Toll-like receptor 2-mediated MAPKs and NF-κB activation requires GNAO1-dependent pathway in human mast cell

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**Key words:** mast cells; $G_\alpha$ protein; GNAO1; TLR2

**Abbreviations:**

- **Akt:** protein kinase B; **ERK:** extracellular signal-regulated kinase; **G protein:** guanine nucleotide-binding protein; **JNK:** c-Jun N-terminal kinase; **LAD2 cells:** laboratory of allergic disease 2 human mast cells; **MyD88:** myeloid differentiation 88 factor; **NFAT:** nuclear factor of activated T cells; **TLRs:** Toll-like receptors; **PI3K:** phosphatidylinositol-3 kinase; **PTX:** pertussis toxin
Abstract

Toll-like receptors (TLRs) expressed on mast cells are essential for effective host defense against a wide variety of pathogens. Previous studies have demonstrated that TLR2 agonists Pam3CSK4 and PGN both stimulated IL-8 release in human mast cells. To determine the molecular basis for this phenomenon, we utilized a human mast cell line LAD2 cells. We found that only release of IL-8 stimulated by Pam3CSK4 was TLR2-mediated, which was confirmed by specific TLR2 shRNA. Heterotrimeric G proteins have been previously implicated in TLRs signaling in macrophages and monocytes. In the current study, we showed that PamCSK4 induced the activation of MAPKs, NF-κB, PI3K-Akt and Ca^{2+}-calcineurin-NFAT signaling cascades in LAD2 cells. G_{o} proteins were required for the activation of MAPKs and NF-κB in TLR2 stimulated LAD2 cells. Therefore, genetic depletion of G_{o} proteins also leaded to reduction of IL-8 release in LAD2 cells. Taken together, the data presented here suggest that TLR2 activation in human mast cells promotes the release of inflammatory mediators via distinct signaling pathways that partially depends on G_{o} protein action.
Introduction

Mast cells are non-circulating cells located throughout the body and are usually in proximity to small arterioles, venules, lymph vessels, and nerve endings. The roles of mast cells in immunity especially in immediate hypersensitivity reactions or allergic reactions are well demonstrated. Mast cells are also critical for host defense against parasites and bacteria dependent on their expression of Toll-like receptors (TLRs). TLRs belong to the pattern-recognition molecules/receptors (PRMs/PRRs) which serve as innate immune sensors for invading microorganisms in vertebrates. TLRs recognize microbial components and evoke diverse immune responses. Up to present, 13 members of TLR family have been found in human being where mast cells express TLR1,-2, -3, -4, -6, -7 and -9. Among all the TLRs, TLR2 are mainly expressed in immune cells including mast cells, dendritic cells and macrophage, which are important in the induction of inflammatory response to Gram-positive bacteria. TLR2 deficient mice have been used to demonstrate an important role of this receptor in mast cell-dependent innate immunity.

It is generally accepted that all TLRs activate a common signaling pathway which includes the activation of myeloid differentiation (MyD) 88 factor. Activation of MyD88 in turn promotes other signaling pathways such as NF-κB, mitogen activated protein kinases (MAPKs including extracellular signal-regulated kinase (ERK), p38 and c-Jun N-terminal kinase (JNK)) and phosphatidylinositol-3 kinase (PI3K), leading to the gene expression of inflammatory cytokines and other inflammatory mediators. In addition, Activation of TLRs also induces other signaling pathways, including the
calcium-calcineurin-nuclear factor of activated T cells (NFAT) signaling through MyD88-independent pathways in some cell types. NFAT is a family of transcription factors expressed in a wide variety of cell types. TLR2 agonist Pam3Cys activated PTX-sensitive $G_{i/o}$-protein in mast cell leading to calcium mobilization and cytokine/chemokine expression. Thereby, the possibility that TLR2 could activate different signaling pathways to induce inflammatory mediator generation in mast cell has been partially proved. However, the upstream signaling molecules which involved in the MyD88-independent pathways for TLR2 activation remain much unknown.

Previous studies have implicated that heterotrimeric guanine nucleotide binding regulatory ($G_{i/o}$) proteins are involved in signaling to TLR2. The first direct evidence of $G_i$ proteins involved in TLRs signaling was showed in the study of TLR4 signaling cascades. The group precipitated the $G_{i}$-GTP protein complex from Raw 264.7 cells stimulated by LPS, which was a classical ligand for TLR4. However, the effects of G protein in TLRs signaling were on the contrary. Inhibition of $G_{i/o}$ protein activity with pertussis toxin (PTX) augmented LPS-induced inflammation in vitro and in vivo, but inhibited TLR2 ligand Pam3CSK4-induced human mast cells activation. Moreover, genetic deletion of $G_{i}$ protein differentially regulated murine TLR-mediated inflammatory cytokine production in different cell type upon stimulated with both TLR4 ligand LPS and TLR2 ligand *staphylococcus aureus*. These studies indicate that function of $G_{i/o}$ protein on the regulation of TLRs activation could be TLR type- and cell type-specific.
The role of Gα protein in TLR signaling has not been identified yet. Gα, encoded by GNAO1, is extremely abundant in brain tissue, constituting up to 0.5% of membrane protein. Pivotal roles of Gα protein in the participation of neurite outgrowth and in the regulation of neuron function have been addressed. Recent data also demonstrated aberrant Gα signaling in cancer development. However, the roles of Gα signaling in immune system have seldom been investigated. In our previous study, we have demonstrated that PGN (ligand for TLR2/6) and Pam3CSK4 (ligand for TLR2/1) induced the release of IL-8 by employing different signaling pathways in LAD2 cells. In the present study, we aim to identify the effect of Gα protein on cytokine production upon TLR2 stimulation in human mast cells. The downstream signaling pathways regulated by Gα protein in TLR2 activation mechanism are further investigated.
Materials and methods

Culture of human mast cells

The Laboratory of Allergic Disease 2 (LAD2) human mast cells were kindly provided by A. Kirshenbaum and D. Metcalfe (NIH, USA). 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. Culture medium was replaced every 2 weeks and the cells were kept at a density of 2 x 10⁵ cells/ml.

Chemical Reagents

PGN from *S aureus* was purchased from Sigma (St Louis, MO). Pam3CSK4 was from Invivogen. Antibodies against NFAT, NF-κB, total and phosphorylated Erk, p38, JNK and Akt were purchased from Cell Signaling (Danvers, MA). Antibodies against alpha-tubulin and Lamin B1 were purchased from ProteinTech (Chicago, IL).

Lentivirus-mediated Knockdown of TLR2 or GNAO1 in Human Mast Cell

TLR2- or GNAO1-targeted shRNA lentivirus and a scrambled control non-target lentivirus were purchased from Genechem (Montreal, Quebec). The shRNA that gave the highest knockdown efficiency (23382-1 for TLR2 and 24124-1 for GNAO1) was used. Cell transduction was conducted by mixing 1 ml of viral supernatant with 1 ml of
LAD2 (1x 10^6 cells). Medium was changed to virus-free complete medium eight hours post-infection. Puromycin (2µg/ml) was added to select cells with stable virus integration into the genome after a recovery period of 24h. Cells were analyzed for TLR2 or GNAO1 knockdown after two weeks of antibiotic selection.

**IL-8 measurement**

LAD2 cells were incubated with PGN or Pam3CSK4 for 24h to allow synthesis and release of IL-8. The release of IL-8 in the supernatants was measured by ELISA assay (BD Biosciences) according to the manufacturer’s instructions. All results were corrected for spontaneous IL-8 release that was less than 22 pg/10^6 cells.

**Western blotting**

Following stimulation, cells were washed twice with cold phosphate buffered saline and lysed in lysis buffer (0.05M Tris-HCl, pH 7.4, 0.15M NaCl, 0.25% deoxycholic acid, 1% NP-40, 10mM EDTA, protease inhibitor cocktail and phosphatase inhibitor cocktail). Individual proteins were separated on 4–12% SDS-PAGE gels and transferred onto nitrocellulose membranes. The membranes were blocked with 5% non-fatted milk or 5% BSA in Tris buffered saline (TBS)-0.1% Tween for 1h, then probed with primary antibodies against phosphorylated form of Erk, JNK, p38, and Akt overnight at 4°C. The membranes were then incubated with the appropriate horseradish peroxidase-linked secondary antibody for 1h, followed by detection using an ECL system according to the manufacturer’s instructions. The membranes were
then stripped with stripping buffer containing 2-Mercaptoethanol for 45min and re-probed with primary antibody against total Erk, JNK, p38, and Akt overnight at 4°C and went on the subsequent process as described above.

**Preparation of nuclear and cytoplasmic extract**

Nuclear and cytoplasmic fractions were separated by Nuclear and Cytoplasmic Protein Extraction Kit (Sangon Biotech) as instructed by the manufacturer. 30µg proteins were then loaded to the 8% SDS-PAGE. Translocation of NFAT and NF-κB was determined by western blotting as described above.

**Immunoprecipitation**

LAD2 cells were stimulated with Pam3CSK4 for 1min. Activated Gαo protein was immunoprecipitated with a Gαo protein activation assay kit (New East Biosciences, Malvern PA). Briefly, cells were lysed with lysis buffer provided in the kit and Gαo-GTP complex was immunoprecipitated with an antibody that specifically bound to the Gαo-GTP complex. The immunoprecipitated proteins were subjected to Western blot with anti-Gαo protein antibody.

**Statistical analysis**

Statistical significance was determined by student t-test. Differences were considered significant at a P value of less than 0.05. All data are expressed as means ± standard error of means (SEMs).
Results

Pam3CSK4 induced release of IL-8 through TLR2 activation from human mast cells

We initially confirmed that mast cells do produce and secrete IL-8 through TLR2 activation. To explore this, human mast cell line LAD2 and TLR2 agonists PGN and Pam3CSK4 were used. LAD2 cells were infected with shRNA construct targeting TLR2. A scrambled shRNA construct was used as the control. After infection and selection with puromycin, western blotting was performed to determine the extent of TLR2 knockdown (Fig. 1A). Knockdown of TLR2 significantly inhibited Pam3CSK4-induced IL-8 release from LAD2 cells (Fig. 1B), while demonstrated no effect on IL-8 release stimulated by PGN (Fig. 1C).

Gα₀ involved in TLR2-mediated IL-8 release in LAD2 cells

To confirm the involvement of Gₒ protein in TLR2 mediated LAD2 cells activation, we used lentiviral shRNA to knock down the expression of Gₒ protein in LAD2 cells. Silencing Gₒ protein expression in LAD2 cells significantly inhibited the release of IL-8 induced by Pam3CSK4 and PGN (Fig. 2A, B). To further confirm the participation of Gₒ protein in TLR2 mediated signaling pathways, LAD2 cells was stimulated with Pam3CSK4 and activated Gₒ proteins were immunoprecipitated. Before immunoprecipitation, the input proteins were immunoblotted with Gₒ protein to show that equal amounts of protein were loaded for immunoprecipitation. As shown in figure 2C, Gₒ proteins were rapidly activated by Pam3CSK4 within 2
minutes of stimulation.

**Gαo-dependent activation of MAPKs upon TLR2 stimulation in human mast cells**

In the following study, we investigated the signaling events activated by the administration of Pam3CSK4 to mast cells. Activation of MAPKs and PI3K-Akt is essential for the expression of a variety of cytokines in TLR2 and G proteins signaling mechanism\(^9,22\). We hypothesized that Gαo may participate in the regulation of MAPKs and PI3K-Akt activity in TLR2 signaling transduction in human mast cells. To test this hypothesis, LAD2 cells were stimulated with 20µg/mL Pam3CSK4 for 10min. We showed that Pam3CSK4 induced ERK1/2, JNK, P38 and Akt phosphorylation (Fig. 3A, B). Silencing Gαo protein expression significantly attenuated the phosphorylation of ERK1/2, JNK and P38 (Fig. 3A), while demonstrated no effect on the phosphorylation of Akt in LAD2 cells treated with Pam3CSK4 (Fig. 3B).

**Gαo-dependent activation of NF-κB upon TLR2 stimulation in human mast cells**

NFAT and NF-κB regulatory pathways were examined. NF-κB is essential for the expression of proinflammatory factors, such as iNOS, COX-2, TNF-α, IL-1β, and IL-8\(^23\). Through western blotting, we found that within 30min of stimulation, LAD2 cells showed enhanced levels of NF-κB expression in the nucleus, as compared to their respective basal levels, while Pam3CSK4 did not induce significantly translocation of NFAT (Fig. 4). Furthermore, translocation of NF-κB was inhibited by
knockdown of $\alpha_o$ protein in TLR2-stimulated LAD2 cells (Fig 4).
Discussion

Our studies provide the first evidence that $G_o$ protein coupled signaling pathways are activated by TLR2 stimulation in human mast cells. These data indicate that TLR2-mediated signaling could occur through divergent heterotrimeric $G_o$ protein-coupled signaling leading to MAP kinases and NF-κB activation. Silencing of $G_\alpha_o$ protein expression significantly suppresses downstream inflammatory gene expression.

Mast cells are multifunctional immune effector cells that play important roles in first defense system as well as allergic and inflammatory diseases\textsuperscript{24}. Mast cells can be selectively activated by pathogens via direct and indirect pathways\textsuperscript{25}. Among key sensors for pathogen-associated molecular patterns (PAMPs), TLRs are critical for mast cell to recognize a wide variety of pathogens\textsuperscript{11}. Recent studies demonstrated essential roles of TLR2 ligands in inducing release of inflammatory mediators in human and murine mast cells\textsuperscript{19, 26}. TLR2 signaling is important in activation of mast cells in response to Gram-positive bacteria\textsuperscript{27}. Further, TLR2 deficient-mast cells showed significantly impairment in their capacity to release inflammatory cytokines such as IL-6 and TNF-α\textsuperscript{28}. TLR2 activation promoted cytokine generation in all mast cell types tested\textsuperscript{19}. Our observations have shown that TLR2 could be selectively activated by Pam3CSK4 and PGN. Silencing of TLR2 expression only decreased Pam3CSK4-induced IL-8 release in LAD2 cells, while reduced PGN-induced response without significant difference. The susceptibility of mast cells to undergo
activation in response to Pam3CSK4 and PGN reflect differences in TLR2-dependent and independent mechanism. Indeed, PGN was also reported to activate by nucleotide oligomerization domain receptor 2 (NOD2) and the complement 5a receptor (C5aR) expressed on mast cells\textsuperscript{29,30}.

Both TLRs and G protein coupled receptors (GPCRs) signaling play pivotal roles in the regulation of immune effector cells, such as macrophage, dendritic cells and mast cells\textsuperscript{9,31}. Crosstalk between TLRs and G protein signaling has also been revealed. Activation of TLR induced change in the expression of several $\alpha_i$ subunits, such as increasing the expression of $\alpha_{i2}$ in macrophages\textsuperscript{32}. Critical evidences indicate that different subunits of the $\alpha_i$ are functional difference in inflammatory responses, such as $\alpha_{i2}$ vs. $\alpha_{i1/3}$. While $\alpha_{i2}^{-/-}$ mice displayed suppression of TNF-\(\alpha\) production in macrophage, the $\alpha_{i1/3}^{-/-}$ mice exhibited increase of TNF-\(\alpha\) production in responses to LPS challenge, which is the classical ligand for TLR4\textsuperscript{32}. The role of heterotrimeric $\alpha_i$ proteins in TLR4 signaling has been well demonstrated\textsuperscript{33}. However, whether G proteins participate in TLR2 signaling remains much unknown. In the present study, we suggested for the first time that $\alpha_o$ protein involved in the TLR2 mediated signaling pathways in LAD2 cells activated by Pam3CSK4 (Fig.2). Immunoprecipitation assay provided the direct evidence for the activation of $\alpha_o$ proteins upon TLR2 stimulation in LAD2 cells (Fig.2). Furthermore, our observations demonstrated that release of IL-8 induced by Pam3CSK4 and PGN was significantly inhibited by knockdown of $\alpha_o$ proteins. These results strongly confirmed that $\alpha_o$ signaling pathway is implicated in TLR2-mediated IL-8 production
in LAD2 mast cells.

Previous studies have demonstrated that G protein coupled signaling pathways were involved in the TLRs stimulation mechanism to induce the release of inflammatory mediators in innate immune responses\(^\text{34}\). Collaboration between TLR4 and heterotrimeric G\(\alpha_{i/o}\) protein signaling has been described in cells of the immune system\(^\text{13, 35}\). Some reports have shown that activation of G proteins was links to the signaling cascade leading to NF-\(\kappa\)B activation to regulate the transcription of various inflammatory genes\(^\text{36}\). Previous study revealed that TLR4-mediated MAPKs and NF-\(\kappa\)B activation was G\(\alpha_{i/o}\) proteins-dependent\(^\text{37}\). Furthermore, NF-\(\kappa\)B has been suggested essential for TLR2-induced cytokine release in mast cells\(^\text{38}\). These findings suggested that G\(\alpha_{i/o}\) proteins were important upstream mediators of multiple signaling events downstream of TLRs\(^\text{19, 37}\). In line with these reports, our results further provided evidence that activation of NF-\(\kappa\)B upon TLR2 stimulation was G\(\alpha_{o}\)-dependent, which was confirmed by specific G\(\alpha_{o}\) shRNA. Silencing of G\(\alpha_{o}\) protein expression resulted in reduction of NF-\(\kappa\)B translocation to the nucleus in LAD2 cells. It has been reported that Pam3CSK4 triggers MAPKs activation through TLR2\(^\text{36}\). In the present study, knockdown of G\(\alpha_{o}\) protein expression significantly inhibited phosphorylation of Erk1/2, JNK and p38 in TLR2-stimulated LAD2 cells. On the other hand, activation of Ca\(^{2+}\)-calcineurin-NFAT and PI3K-Akt has been demonstrated to involve in TLR and G protein signaling in mast cells. Pam3Cys induced CCL2 production required Ca\(^{2+}\)-calcineurin-NFAT activation and Akt phosphorylation in a G\(\alpha_{i/o}\) protein-dependent manner from LAD2 mast cells\(^\text{11}\).
However, genetic depletion of G\textsubscript{\alpha\text{lo}} protein did not inhibit the activation of NFAT or Akt in LAD2 cells. These results indicate that G\textsubscript{o} protein partially regulates TLRs signaling cascades in human mast cells.

In summary, we have shown for the first time that heterotrimeric G\textsubscript{o} proteins are involved in the upstream of TLR2 signaling cascades through regulating the MAPKs and NF-\kappaB activation in human mast cells. These findings are important in the understanding of mast cell responses to bacterial products. We further expand the roles of G\textsubscript{o} protein in immune responses except for its classical roles in never system. Mast cells are well known to play critical roles in system anaphylaxis and anaphylactic shock by secretion of a wide variety of inflammatory mediators including preformed mediators (histamine, tryptase, carboxypeptidase A, and proteoglycans, heparin and chondroitin sulfates) and newly synthesized mediators (prostaglandin D2, leukotriene D4, platelet-activating factor, cytokines and chemokines)\textsuperscript{39}. Targeting G\textsubscript{o} proteins with pharmacological agents may be an effective method for controlling pathological inflammation such as sepsis upon gram-positive bacteria infection.
Acknowledgements

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Conflict of Interest:

We disclose no conflicts of interests.
References


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Figure legends

**Fig. 1** Knockdown of TLR2 expression inhibited Pam3CSK4-induced release of IL-8. LAD2 cells were stably transduced with shRNA lentivirus targeted against TLR2 or scrambled shRNA control lentivirus. (A), representative immunoblot of LAD2 cells with TLR2 knockdown is shown. (B), Relative level of TLR2 protein expression between TLR2-targeted or scrambled shRNA lentivirus transfected cells were compared. (C, D), LAD2 cells were incubated with 20µg/ml Pam3CSK4 or 50µg/ml PGN for 24h and release of IL-8 was measured. Statistical significance was determined by *t*-test (B, C, D). * indicates p < 0.05, *** indicates p < 0.001. All data were mean ± S.E.M of three experiments.

**Fig. 2** Gαo involved in TLR2-mediated IL-8 release in LAD2 cells. LAD2 cells were stably transduced with shRNA lentivirus targeted against GNAO1 or scrambled shRNA control lentivirus. (A), LAD2 cells were incubated with 20µg/ml Pam3CSK4 for 24h and release of IL-8 was measured. (B), LAD2 cells were incubated with 50µg/ml PGN for 24h and release of IL-8 was measured. (C), LAD2 cells were stimulated with 20µg/ml Pam3CSK4 for 2 minutes. Gαo-GTP complex was immunoprecipitated and subjected to Western blot analysis (left gel). Total Gαo proteins were shown in right gel. (D), Relative level of Gαo-GTP complex between Pam3CSK4 -treated or -untreated cells were compared. Statistical significance was determined by *t*-test (B, C, D). * indicates p < 0.05, ** indicates p < 0.01. All
Fig. 3 Knockdown of Gαo protein inhibited Pam3CSK4-induced Erk, JNK and P38 phosphorylation. LAD2 mast cells were stably transduced with shRNA lentivirus targeted against GNAO1 or scrambled shRNA control lentivirus. Cells were incubated with 20µg/ml Pam3CSK4 for 10min. Total cell lysate (30µg/mL) was analyzed by immunoblotting against phosphorylated Erk1/2, JNK, p38 (A) and Akt (B). The blots were also stripped and re-probed for total Erk1/2, JNK, p38 (A) and Akt (B). A representative immunoblot from three similar experiments is shown. (C), Relative level of phosphorylated protein expression between GNAO1-targeted or scrambled shRNA lentivirus transfected cells were compared. Statistical significance was determined by t-test (C). ** indicates p < 0.01, *** indicates p < 0.001. All data were mean ± S.E.M of three experiments.

Fig. 4 Knockdown of Gαo protein inhibited Pam3CSK4-induced NF-κB translocation. LAD2 mast cells were stably transduced with shRNA lentivirus targeted against GNAO1 or scrambled shRNA control lentivirus. Cells were stimulated with 20µg/ml Pam3CSK4 for 30 min. (A, B) Cytoplasmic and nuclear extract were prepared separately and probed with indicated antibodies. A representative immunoblot from three individual experiments is shown. Lamin B and α-tubulin are served as internal reference for nucleus and cytoplasm respectively. (C)
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