

## Research Article

# Cross-Linking Mast Cell Specific Gangliosides Stimulates the Release of Newly Formed Lipid Mediators and Newly Synthesized Cytokines

Edismauro Garcia Freitas Filho,<sup>1</sup> Elaine Zayas Marcelino da Silva,<sup>1</sup>  
Camila Ziliotto Zanotto,<sup>2</sup> Constance Oliver,<sup>1</sup> and Maria Célia Jamur<sup>1</sup>

<sup>1</sup>Department of Cell and Molecular Biology and Pathogenic Bioagents, Ribeirão Preto Medical School, University of São Paulo, Avenida Bandeirantes 3900, 14049-900 Ribeirão Preto, SP, Brazil

<sup>2</sup>Department of Pharmacology, Ribeirão Preto Medical School, University of São Paulo, Avenida Bandeirantes 3900, 14049-900 Ribeirão Preto, SP, Brazil

Correspondence should be addressed to Maria Célia Jamur; [mjamur@fmrp.usp.br](mailto:mjamur@fmrp.usp.br)

Received 25 April 2016; Accepted 13 July 2016

Academic Editor: Dianne Cooper

Copyright © 2016 Edismauro Garcia Freitas Filho et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Mast cells are immunoregulatory cells that participate in inflammatory processes. Cross-linking mast cell specific GD1b derived gangliosides by mAbAA4 results in partial activation of mast cells without the release of preformed mediators. The present study examines the release of newly formed and newly synthesized mediators following ganglioside cross-linking. Cross-linking the gangliosides with mAbAA4 released the newly formed lipid mediators, prostaglandins D<sub>2</sub> and E<sub>2</sub>, without release of leukotrienes B<sub>4</sub> and C<sub>4</sub>. The effect of cross-linking these gangliosides on the activation of enzymes in the arachidonate cascade was then investigated. Ganglioside cross-linking resulted in phosphorylation of cytosolic phospholipase A<sub>2</sub> and increased expression of cyclooxygenase-2. Translocation of 5-lipoxygenase from the cytosol to the nucleus was not induced by ganglioside cross-linking. Cross-linking of GD1b derived gangliosides also resulted in the release of the newly synthesized mediators, interleukin-4, interleukin-6, and TNF- $\alpha$ . The effect of cross-linking the gangliosides on the MAP kinase pathway was then investigated. Cross-linking the gangliosides induced the phosphorylation of ERK1/2, JNK1/2, and p38 as well as activating both NF $\kappa$ B and NFAT in a Syk-dependent manner. Therefore, cross-linking the mast cell specific GD1b derived gangliosides results in the activation of signaling pathways that culminate with the release of newly formed and newly synthesized mediators.

## 1. Introduction

Gangliosides are sialic acid containing glycosphingolipids that are present in the outer leaflet of the plasma membrane as well as in the membranes of some organelles [1, 2]. Gangliosides play a role in diverse physiological processes including growth, differentiation, cell-cell interactions, and cell signaling. They are also involved in many pathological processes, acting as receptors for viruses and toxins, and are implicated in tumor progression, atherosclerosis, and neurodegenerative disorders [3].

Gangliosides are present on the surface of mast cells and are critical for mast cell function [1]. Mast cells are

multifunctional immune cells that participate in various biological events, such as inflammation and allergy. Mast cell functions are directly related to their activation and subsequent release of biologically active mediators [4, 5]. Mast cell activation via the high affinity IgE receptor (Fc $\epsilon$ RI) is the best characterized form of activation. It occurs when multivalent antigens cross-link antigen-specific immunoglobulin E (IgE) bound to Fc $\epsilon$ RI on the mast cell surface. Cross-linking Fc $\epsilon$ RI initiates a signal transduction cascade that is dependent on the tyrosine kinase Syk [6]. This activation results in the release of three classes of mediators: preformed mediators such as histamine, proteases, cytokines, and enzymes; newly formed lipid mediators which are

comprised of prostaglandins (PG), leukotrienes (LT), and platelet activating factor; and newly synthesized mediators which include cytokines and chemokines [4, 7, 8].

Mast cell specific gangliosides derived from GD1b are present on the surface of rodent mast cells [9, 10]. Cross-linking the GD1b derived gangliosides with a ganglioside specific monoclonal antibody (mAbAA4) or its F(ab)<sub>2</sub> fragment results in partial activation of RBL-2H3 mast cells without degranulation or release of preformed mediators [11, 12]. Although cross-linking GD1b derived gangliosides activates mast cells, whether or not cross-linking these gangliosides stimulates release of newly formed and newly synthesized mediators has not been investigated. Therefore, it was of interest to determine if newly formed lipid mediators and newly synthesized mediators were released following ganglioside cross-linking and whether or not this release was Syk-dependent.

## 2. Materials and Methods

**2.1. Cells.** RBL-2H3 cells, a rat mast cell line [13], a Syk-negative variant of RBL-2H3 cells (C4A2) [14], the stable RBL-2H3 cell lines expressing NFκB-GFP reporter (NFκB2; [15]); NFAT-GFP reporter (VB9; [16]); and Syk-negative cell lines expressing NFκB-GFP reporter (IC2; [6]); and NFAT-GFP reporter (IH10; [16]) were generously provided by Dr. Reuben P. Siraganian (National Institute of Dental and Craniofacial Research, National Institutes of Health, Bethesda, MD). Cells were grown as monolayers at 37°C in Dulbecco's modified Eagle's medium (DMEM) supplemented with 15% fetal calf serum, 0.434 mg/mL glutamine, and an antibiotic-antimycotic mixture containing 100 U/mL penicillin, 100 μg/mL streptomycin, and 0.25 μg/mL amphotericin B (all from Life Technologies, Gibco, Carlsbad, CA) in a humidified incubator with 5% CO<sub>2</sub> in air. Transfected cells were selected with Geneticin (0.4 mg/mL) (Sigma-Aldrich; St. Louis, MO).

**2.2. Antibodies.** Mouse monoclonal antibody anti-rat GD1b derived gangliosides (mAbAA4) were purchased from BD Biosciences (San Jose, CA). Rabbit polyclonal antibody anti-human phospho-cPLA<sub>2</sub>; rabbit polyclonal antibody anti-human cPLA<sub>2</sub>; rabbit mAb anti-human phospho-ERK1/2; rabbit mAb anti-rat ERK1/2; rabbit mAb anti-human phospho-JNK1/2; rabbit polyclonal antibody anti-human JNK1/2; rabbit mAb anti-human phospho-p38; rabbit polyclonal antibody anti-human anti-p38, and rabbit polyclonal antibody anti-human α/β-tubulin were purchased from Cell Signaling Technology Inc. (Danvers, MA). Rabbit polyclonal antibody anti-rat cyclooxygenase-2 (COX-2), rabbit mAb anti 5-lipoxygenase (5-LO), and rabbit polyclonal antibody anti-mouse Lamin B1 were purchased from Abcam (Cambridge, MA). Donkey anti-rabbit IgG conjugated to horseradish peroxidase (HRP) (Jackson ImmunoResearch Laboratories Inc., West Grove, PA) was used as the secondary antibody.

**2.3. Mast Cell Activation and Mediator Release.** In order to cross-link the GD1b derived gangliosides, RBL-2H3 cells

and C4A2 cells were incubated with mAbAA4 at various concentrations (1, 2.5, 5, or 10 μg/mL) depending on the experiment. For stimulation via FcεRI, cells were sensitized overnight (ON) with mouse IgE anti-TNP ascites fluid (1:5,000 dilution) and then stimulated with 50 ng/mL of DNP<sub>48</sub>-HSA (Sigma-Aldrich) for 30 min or for 1 h and then rinsed and cultured for an additional 3 h, for the release of newly formed lipid mediators (PGD<sub>2</sub>, PGE<sub>2</sub>, LTB<sub>4</sub>, and LTC<sub>4</sub>). In order to examine the release of newly synthesized mediators (IL-4, IL-6, and TNF-α), cells were stimulated for 1 h, rinsed, and cultured for an additional 11 h. For FcεRI independent stimulation, cells were incubated with 0.1 μg/mL of calcium ionophore A23187 (Sigma-Aldrich). PGD<sub>2</sub>, PGE<sub>2</sub>, LTB<sub>4</sub>, and LTC<sub>4</sub> in culture supernatants were analyzed using EIA kits (Cayman Chemical, Ann Arbor, MI). IL-4, IL-6, and TNF-α in the culture supernatants were measured using ELISA kits (BD Biosciences) according to the manufacturer's instructions. Nonstimulated cells were used as controls.

**2.4. NFκB and NFAT Activation.** NFκB2 cells, VB9 cells, IC2 cells, and IH10 cells were incubated with mAbAA4, stimulated via FcεRI, or with calcium ionophore for 1 h (as described in Section 2.3), rinsed, and cultured for an additional 5 h (NFκB activation) or 15 h (NFAT activation). Cells were analyzed by flow cytometry and the percent of GFP positive cells was determined using a Guava Easy Cyte Mini System and Cytosoft Blue software (Guava Technologies Inc., Hayward, CA).

**2.5. Immunoblotting.** Total cells lysates were obtained as previously described [17]. For some experiments, nuclear and cytosolic extracts were obtained as previously described [18]. The proteins were separated electrophoretically on 8% or 12% polyacrylamide gels and transferred to Hybond membranes (GE Healthcare Life Sciences, Marlborough, MA). After transfer, the membranes were blocked for 1 h at RT in TTBS (0.05 M Tris-HCl, 0.15 M NaCl, pH 7.5, and 0.05% Tween 20) containing 4% BSA (Sigma-Aldrich). After blocking, the membranes were incubated ON at 4°C with the primary antibodies diluted in TTBS. The membranes were then washed, incubated for 30 min with secondary antibody, and developed using enhanced chemiluminescence (ECL Kit; GE Healthcare). The images were obtained with ImageQuant LAS 4000 (GE Healthcare). Mean optical density of the target protein was determined using ImageJ software (NIH).

**2.6. Statistical Analyses.** Results were analyzed using GraphPad Prism (GraphPad Software, Inc., San Diego, CA). The results were expressed as mean ± SD and differences between experimental samples were assessed by one-way analysis of variance (ANOVA) with Bonferroni's post hoc test; *P* < 0.05 was considered statistical significant.

## 3. Results

**3.1. Cross-Linking GD1b Derived Gangliosides with mAbAA4 Induced the Release of Newly Formed Lipid Mediators PGD<sub>2</sub> and PGE<sub>2</sub>.** RBL-2H3 cells and C4A2 Syk-negative cells

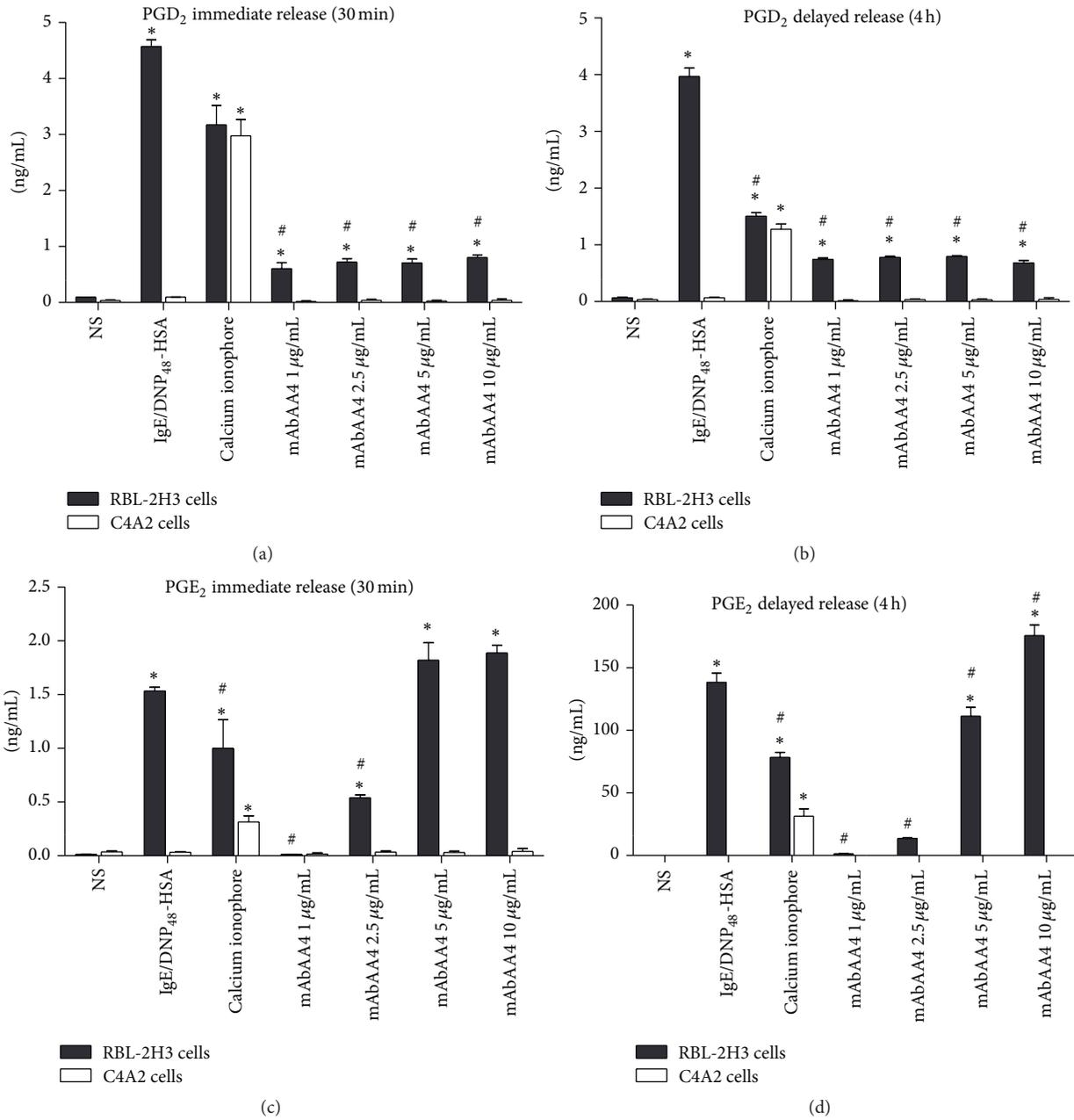


FIGURE 1: Cross-linking GD1b derived gangliosides by mAbAA4 induced Syk-dependent release of PGD<sub>2</sub> and PGE<sub>2</sub>. For stimulation via FcεRI, RBL-2H3 cells and C4A2 Syk-negative cells were sensitized with IgE anti-TNP and stimulated with DNP<sub>48</sub>-HSA (50 ng/mL). For FcεRI independent stimulation, the cells were incubated with calcium ionophore (0.1 μg/mL). To cross-link GD1b derived gangliosides, cells were incubated with mAbAA4 (1, 2.5, 5, and 10 μg/mL). Nonstimulated (NS) cells were used as negative controls. Culture supernatants were collected after 30 min of incubation to evaluate immediate release (a and c) or cells were incubated for 1 h and rinsed and cultured for an additional 3 h and culture supernatants were collected to evaluate delayed release (b and d). PGD<sub>2</sub> (a and b) and PGE<sub>2</sub> (c and d) were measured in the culture supernatant by EIA. Data is expressed as the mean ± SD of three independent experiments. \* *P* < 0.05 between experimental samples and the nonstimulated (NS) cells. # *P* < 0.05 between experimental samples and FcεRI stimulated RBL-2H3 cells.

were incubated with mAbAA4 for either 30 min or 1 h and then rinsed and cultured for an additional 3 h to evaluate both immediate and delayed release of lipid mediators. The cross-linking of GD1b derived gangliosides by mAbAA4 induced both immediate and delayed release of PGD<sub>2</sub> (Figures 1(a) and 1(b)) and PGE<sub>2</sub> (Figures 1(c) and 1(d)) by RBL-2H3 cells, but not by Syk-negative C4A2

cells (Figures 1(a)–1(d)). Furthermore, the amount of PGE<sub>2</sub> released following ganglioside cross-linking was higher when compared to that found after FcεRI stimulation. Interestingly, cross-linking GD1b derived gangliosides did not induce the release of the LT, LTB<sub>4</sub>, and LTC<sub>4</sub> (see Supplementary Figures 1(A) and 1(B) in Supplementary Material available online at <http://dx.doi.org/10.1155/2016/9160540>).

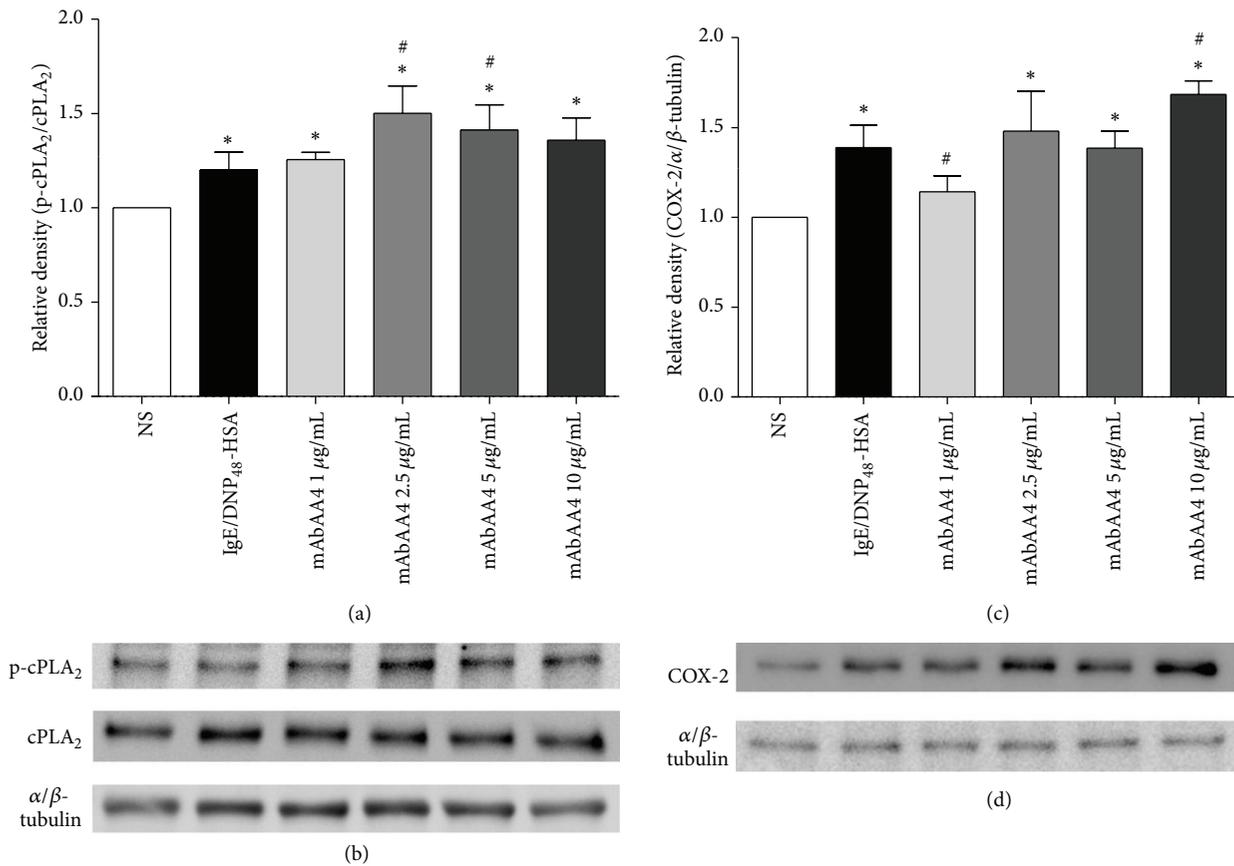


FIGURE 2: Cross-linking GD1b derived gangliosides by mAbAA4 increased cPLA<sub>2</sub> phosphorylation and induced COX-2 expression. Either RBL-2H3 mast cells were stimulated via FcεRI, by sensitizing the cells with IgE anti-TNP and stimulating with DNP<sub>48</sub>-HSA (50 ng/mL), or cells were incubated with mAbAA4 (1, 2.5, 5, and 10 μg/mL) for 5 min (cPLA<sub>2</sub> phosphorylation) or for 1 h and then rinsed and cultured for an additional 3 h (COX-2 expression). Total cell lysates were immunoblotted with antibodies against p-cPLA<sub>2</sub>, cPLA<sub>2</sub>, COX-2, and α/β-tubulin (housekeeping protein) and the mean optical density of the bands was determined. Densitometry of the changes in expression and phosphorylation of proteins were corrected for α/β-tubulin. Data were expressed as the fold of nonstimulated (NS) cells. (a) Ratio of phosphorylated cPLA<sub>2</sub>/total cPLA<sub>2</sub>; (b) a representative blot from (a); (c) ratio of COX-2/α/β-tubulin; (d) a representative blot from (c). Data is expressed as the mean ± SD of three independent experiments. \*  $P < 0.05$  between experimental samples and the nonstimulated (NS) cells. #  $P < 0.05$  between experimental samples and FcεRI stimulated cells.

**3.2. Cross-Linking GD1b Derived Gangliosides with mAbAA4 Resulted in Phosphorylation of Cytosolic Phospholipase A<sub>2</sub> (cPLA<sub>2</sub>) and Cyclooxygenase-2 (COX-2) Expression.** Cross-linking GD1b derived gangliosides resulted in the release of PGs but not LTs. Therefore, cPLA<sub>2</sub> phosphorylation and induction of COX-2 expression, which are required for PG generation, were investigated. An increase in cPLA<sub>2</sub> phosphorylation was observed after incubation of RBL-2H3 cells with mAbAA4 for 5 min and the levels of cPLA<sub>2</sub> phosphorylation were higher than those observed in cells stimulated via FcεRI (Figures 2(a) and 2(b)). COX-2 expression was also induced in cells incubated with mAbAA4 for 1 h and rinsed and cultured for an additional 3 h (Figures 2(c) and 2(d)). In contrast, translocation of 5-LO from the cytosol to the nucleus, a requirement for LT generation, was not induced by ganglioside cross-linking (Supplementary Figures 1(C)–1(F)). Therefore, the cross-linking of GD1b derived gangliosides specifically induces the activation of the arachidonic pathway responsible for PG generation in mast cells.

**3.3. Cross-Linking GD1b Derived Gangliosides with mAbAA4 Induced the Release of Newly Synthesized Cytokines.** Mast cell activation via FcεRI leads to transcription factor activation resulting in the production and release of cytokines [19]. Therefore, it was of interest to investigate whether cytokines are released after cross-linking GD1b derived gangliosides by mAbAA4. RBL-2H3 cells and Syk-negative C4A2 cells were incubated with mAbAA4 for 1 h and rinsed and cultured for an additional 11 h. Ganglioside cross-linking resulted in a Syk-dependent release of the newly synthesized mediators, interleukin-4 (IL-4) (Figure 3(a)), interleukin-6 (IL-6) (Figure 3(b)), and tumor necrosis factor-α (TNF-α) (Figure 3(c)). Interestingly, the amount of cytokines released after ganglioside cross-linking, with the exception of IL-6, was lower than that observed after stimulation via FcεRI.

**3.3.1. Cross-Linking GD1b Derived Gangliosides with mAbAA4 Induced MAP Kinase Phosphorylation.** MAP kinases are involved in signaling pathways that lead to production of newly synthesized mediators [20]. Since incubation of mast

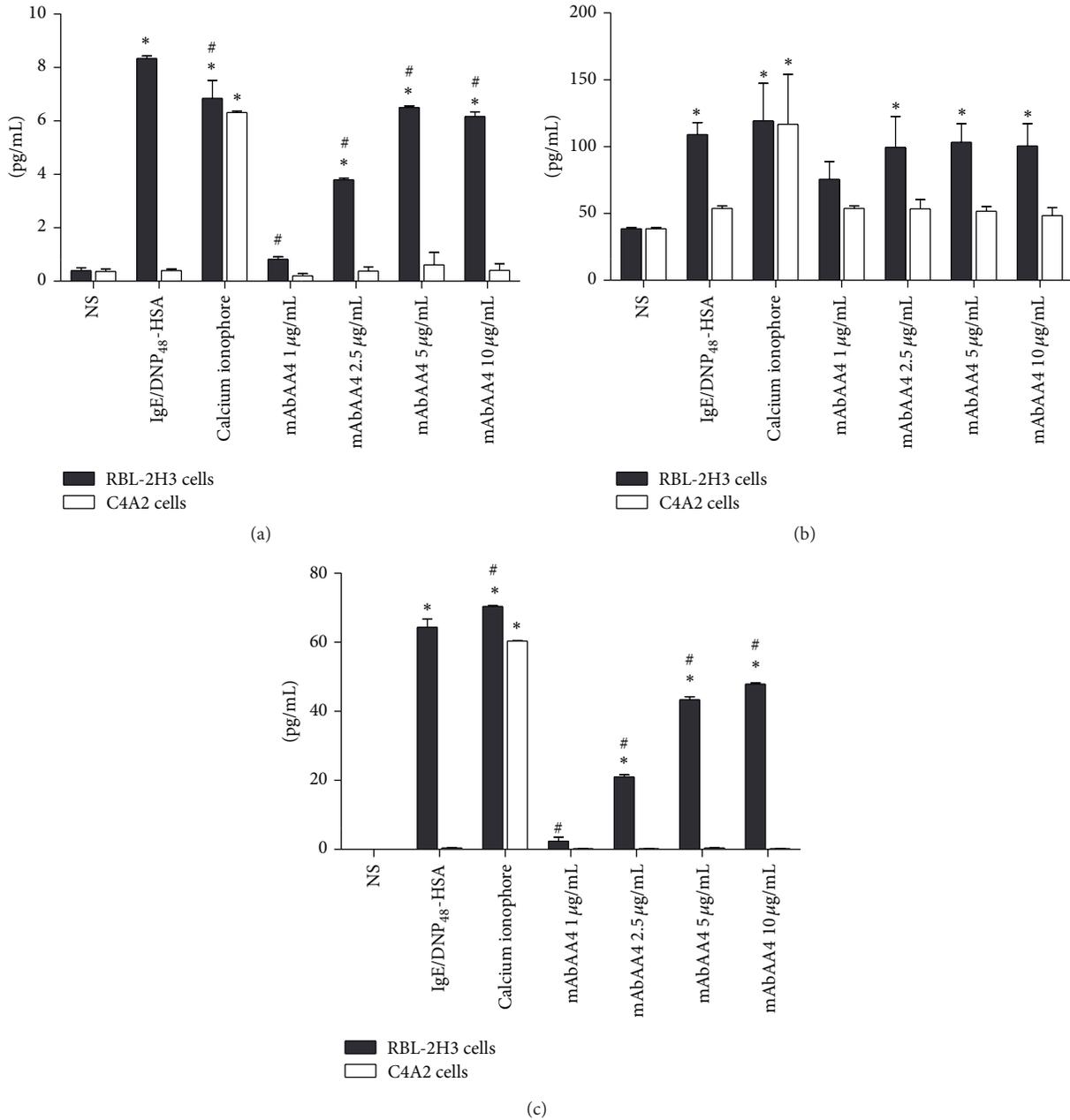


FIGURE 3: Cross-linking GD1b derived gangliosides with mAbAA4 induced the release of IL-4, IL-6, and TNF- $\alpha$  in a Syk-dependent manner. For stimulation via Fc $\epsilon$ RI, RBL-2H3 cells and C4A2 Syk-negative cells were sensitized with IgE anti-TNP and stimulated with DNP<sub>48</sub>-HSA (50 ng/mL). For Fc $\epsilon$ RI independent stimulation, the cells were incubated with calcium ionophore (0.1  $\mu$ g/mL). To cross-link GD1b derived gangliosides, cells were incubated with mAbAA4 (1, 2.5, 5, and 10  $\mu$ g/mL). Nonstimulated (NS) cells were used as negative controls. Culture supernatants were collected 1 h after stimulation and the cells were rinsed and cultured for an additional 11 h to evaluate cytokine release. IL-4 (a), IL-6 (b), and TNF- $\alpha$  (c) were measured in the culture supernatants by ELISA. Data is expressed as the mean  $\pm$  SD of three independent experiments. \*  $P < 0.05$  between experimental samples and the nonstimulated (NS) cells. #  $P < 0.05$  between experimental samples and Fc $\epsilon$ RI stimulated RBL-2H3 cells.

cells with mAbAA4 resulted in the release of IL-4, IL-6, and TNF- $\alpha$ , it was of interest to investigate the degree of MAP kinase phosphorylation induced by cross-linking the GD1b derived gangliosides. When RBL-2H3 cells were incubated with mAbAA4 for 10 min, MAP kinases ERK1/2, JNK1/2, and p38 were phosphorylated (Figure 4). The degree of MAP kinase phosphorylation in mast cells incubated with

mAbAA4 was less than that observed in cells stimulated via Fc $\epsilon$ RI, which agrees with the amount of cytokine released.

**3.3.2. Cross-Linking GD1b Derived Gangliosides with mAbAA4 Induced the Activation of Transcription Factors.** Transcription factor activation is the ultimate requirement for the production of newly synthesized mediators [21]. Therefore,

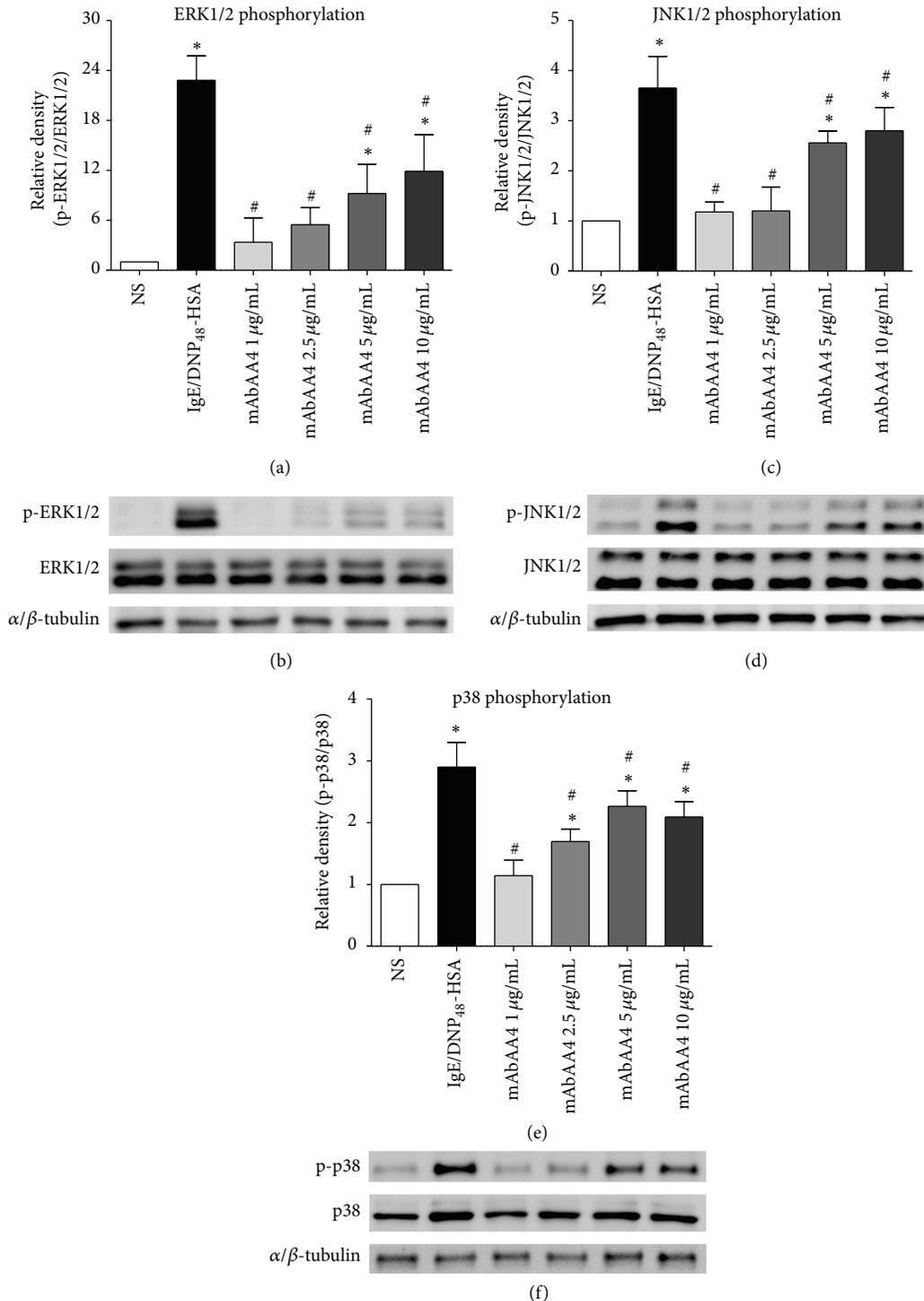


FIGURE 4: Cross-linking GD1b derived gangliosides with mAbAA4 induced MAP kinase phosphorylation in mast cells. RBL-2H3 cells were either stimulated via FcεRI, where cells were sensitized with IgE anti-TNP and stimulated with DNP<sub>48</sub>-HSA (50 ng/mL) or incubated with mAbAA4 (1, 2.5, 5, and 10 μg/mL) for 10 min. Total cell lysates were immunoblotted with antibodies against phospho-ERK1/2 (p-ERK1/2), ERK1/2, phospho-JNK1/2 (p-JNK1/2), JNK1/2, phospho-p38 (p-p38), p38, and α/β-tubulin (housekeeping protein) and the mean optical density of the bands was determined. Densitometry of the changes in expression and phosphorylation of proteins were corrected for α/β-tubulin. Data is expressed as the fold of nonstimulated (NS) cells. (a) Ratio of phosphorylated ERK1/2/total ERK1/2; (b) a representative blot from (a); (c) ratio of phosphorylated JNK1/2/total JNK1/2; (d) a representative blot from (c); (e) ratio of phosphorylated p38/total p38; (f) a representative blot from (e). Data is expressed as the mean ± SD of three independent experiments. \*  $P < 0.05$  between experimental samples and the nonstimulated (NS) cells. #  $P < 0.05$  between experimental samples and FcεRI stimulated cells.

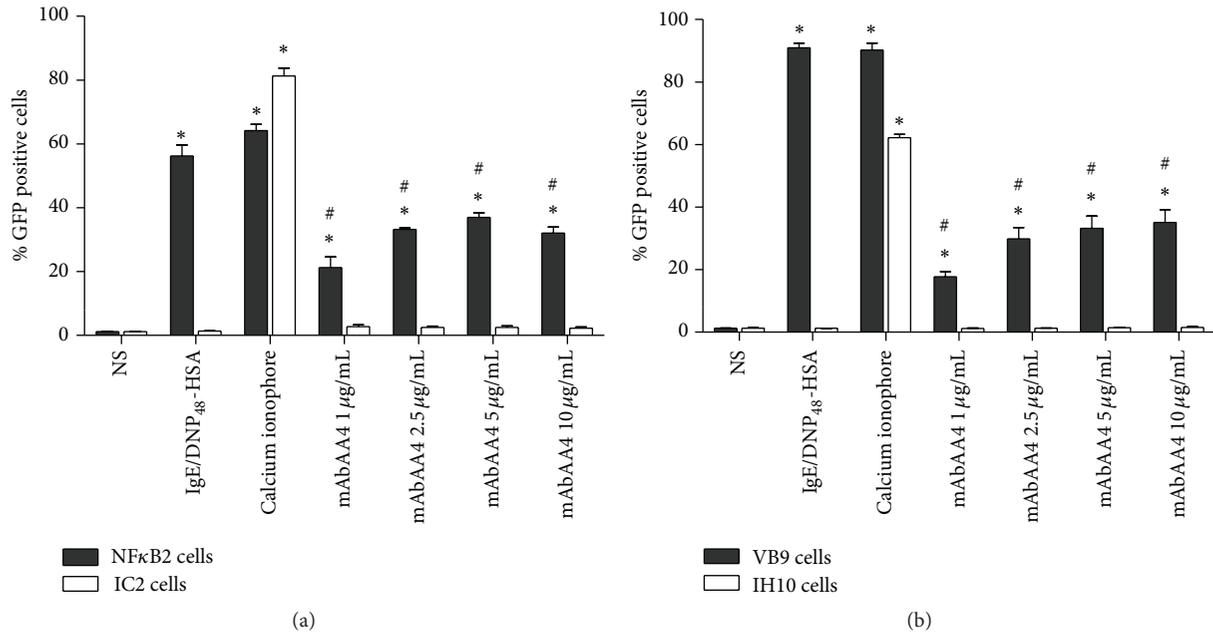


FIGURE 5: Cross-linking GD1b derived gangliosides by mAbAA4 induced the activation of the transcription factors NFκB and NFAT. NFκB2 cells, VB9 cells, IC2 cells, and IH10 cells were sensitized with IgE anti-TNP and stimulated with DNP<sub>48</sub>-HSA (50 ng/mL) for 1 h and rinsed and cultured for additional 5 h ((a); NFκB) or 15 h ((b); NFAT). For FcεRI independent stimulation, the cells were incubated with calcium ionophore (0.1 μg/mL). To cross-link GD1b derived gangliosides, cells were incubated with mAbAA4 (1, 2.5, 5, and 10 μg/mL). GFP expression was analyzed by flow cytometry. Data is expressed as the mean ± SD of three independent experiments. \**P* < 0.05 between experimental samples and the nonstimulated (NS) cells. #*P* < 0.05 between experimental samples and FcεRI stimulated cells.

RBL-2H3 derived GFP reporter cell lines were used to assess NFκB and NFAT activation. Cross-linking GD1b derived gangliosides by mAbAA4 induced activation of both NFκB (Figure 5(a)) and NFAT (Figure 5(b)) in a Syk-dependent manner. However, transcription factor activation by ganglioside cross-linking was less prominent than that observed by stimulation via FcεRI.

#### 4. Discussion

The present study demonstrates that cross-linking the mast cell specific GD1b derived gangliosides induces the release of newly formed and newly synthesized mediators. Furthermore, this release is Syk-dependent. However, previous investigations have demonstrated that mast cell activation by cross-linking these gangliosides does not induce the release of preformed mediators [11, 12]. Moreover, other studies have shown that cross-linking gangliosides can also activate a variety of cell types [22–26]. Antibodies to gangliosides have been shown to activate PKC and increase proliferation in lymphocytes [27, 28], stimulate calcium influx in oligodendrocytes [29], and induce leukocyte degranulation [30]. The molecular mechanisms by which cross-linking gangliosides can activate cells are poorly understood.

Eicosanoids (prostaglandins, thromboxane, leukotrienes, and lipoxins) are the most important lipid mediators generated by mast cells [31]. The results of the present study show that cross-linking GD1b derived gangliosides induces release of prostaglandins, but not leukotrienes. Incubation with mAbAA4 stimulated cPLA<sub>2</sub> phosphorylation and incubation

with mAbAA4 for extended periods of time increased COX-2 expression. The first step in eicosanoid generation is Ca<sup>2+</sup>-dependent phosphorylation of cPLA<sub>2</sub> through the MAP kinase pathway. Phosphorylated cPLA<sub>2</sub> translocates to cellular membranes, principally to the nuclear envelope, where arachidonic acid (AA) is released from membrane phospholipids by the action of cPLA<sub>2</sub>. AA is then metabolized either by COX-2 or CYP2E1 to produce PGs such as PGE<sub>2</sub> or by 5-LO to produce LTs in concert with 5-lipoxygenase-activating protein (FLAP) on the nuclear envelope [32, 33]. Previous studies have shown that the immediate phase of PG generation (5–30 min) requires the action of constitutively expressed COX-1 and phosphorylation of cPLA<sub>2</sub>, while the delayed phase of PG generation (4–6 h) depends on the induced expression of COX-2 [34]. In addition, cross-linking GD1b derived gangliosides did not induce 5-LO translocation from the cytosol to the nuclear membrane. These results agree with the findings that ganglioside cross-linking induces release of prostaglandins, but not leukotrienes, and indicate that ganglioside cross-linking selectively stimulates the eicosanoid biosynthetic pathway to induce PG generation.

Cross-linking GD1b derived gangliosides induces the release of the newly synthesized mediators IL-4, IL-6, and TNF-α. In mast cells, newly synthesized mediator expression depends on the activation of signaling pathways that ultimately leads to transcription factor activation [21]. These events culminate with cytokine production and release and can occur even in the absence of mast cell degranulation [16]. A variety of studies investigating FcεRI independent mast cell activation also revealed that release of proinflammatory

mediators can occur in the absence of degranulation [35–37]. The production of newly synthesized mast cell mediators following FcεRI activation relies on MAP kinase signaling pathways as well as on the activation of the transcription factors NFκB and NFAT.

The MAP kinase signaling pathway participates in activation, differentiation, proliferation, and migration of mast cells. Cross-linking GD1b derived gangliosides results in ERK1/2, JNK1/2, and p38 MAP kinase phosphorylation. ERK1/2 is an essential signal in the production of the newly synthesized mediators IL-5, IL-3, IL-13, and TNF-α in mast cells [38]. JNK1/2 is responsible, at least partially, for the expression and production of several cytokines, including IL-6 and TNF-α in mast cells [39]. Additionally, activation of p38 MAP kinase was shown to stimulate IL-4 production in bone marrow derived mast cells [40]. When mast cells are stimulated via FcεRI, the transcription factors NFκB and NFAT are translocated to the nucleus and initiate the transcription of genes for proinflammatory and regulatory cytokines. This results in the expression and release of cytokines [19, 41, 42]. Cross-linking GD1b derived gangliosides by mAbAA4 activates the transcription factors NFκB and NFAT. However, the degree of activation by ganglioside cross-linking was less than that observed by stimulation via FcεRI. This reduction in activation is expected since the degree of MAP kinase phosphorylation after ganglioside cross-linking was less than that observed in mast cells stimulated via FcεRI. Similar results have been reported for phosphorylation of Lyn, Syk, PLCγ1, and the β- and γ-subunits of FcεRI [12].

The lower phosphorylation of MAP kinase resulted in a reduction in NFκB and NFAT activation leading to a decrease in IL-4 and TNF-α release. In FcεRI stimulated mast cells, activation of NFκB depends on PKC activation [43]. On the other hand, NFAT is activated by calcineurin induced dephosphorylation, a Ca<sup>2+</sup>-calmodulin dependent serine/threonine phosphatase that is activated by an increase in intracellular calcium [44, 45]. mAbAA4 binding to RBL-2H3 mast cells results in a modest increase in intracellular calcium as well as in a partial redistribution of PKC [11], which could explain the reduced activation of NFκB and NFAT seen in the present study. Additionally, cross-linking GD1b derived gangliosides in Syk-negative cells did not stimulate the release of either newly formed or newly synthesized mediators. This is in agreement with previous studies that have shown that the inhibition or the lack of Syk results in the failure of mast cells to produce and release any mediators [46, 47]. Syk-negative mast cells are also unable to activate NFκB and NFAT in response to FcεRI activation [6, 16].

The exact mechanism by which cross-linking the GD1b derived gangliosides causes the various effects observed both previously and in this study is still unknown. Several intracellular signals induced by mAbAA4 binding are very similar to those induced by FcεRI activation. Binding of mAbAA4 to mast cells is known to stimulate protein tyrosine phosphorylation, including phosphorylation of Lyn, Syk, PLCγ1, and the β- and γ-subunits of FcεRI. However, the rate of phosphorylation of Lyn, Syk, and PLCγ1 was slower with ganglioside cross-linking than with FcεRI stimulation [12]. In addition to these effects of mAbAA4, preincubation

of RBL-2H3 cells with mAbAA4 selectively inhibits the degranulation induced by FcεRI stimulation at a very early step of upstream receptor tyrosine phosphorylation. This inhibition is unrelated to mAbAA4 blocking IgE-binding to the cells [48, 49]. Moreover, the GD1b derived gangliosides coimmunoprecipitate with FcεRI [48] as well as with the tyrosine kinase Lyn [49]. Oliver et al. [50] demonstrated that in RBL-2H3 cells stimulated via FcεRI, the gangliosides and FcεRI are internalized together and follow the same intracellular endocytic pathway suggesting that the GD1b derived gangliosides are involved in the organization of the signaling complex.

## 5. Conclusions

The present study has demonstrated that cross-linking the GD1b derived gangliosides stimulates the release of newly formed and newly synthesized mediators. Although these gangliosides are intimately associated with FcεRI, the ability of the gangliosides to activate mast cells is not dependent on FcεRI cross-linking. The present study helps to explain the extremely broad spectrum of potential mechanisms by which mast cells might act in suppressing, amplifying, and modulating the non-FcεRI mediated immune responses. Furthermore, an understanding of the role of gangliosides in mast cell activation may lead to new therapeutic targets for allergic and inflammatory processes.

## Competing Interests

The authors declare no competing financial interests.

## Acknowledgments

The authors would like to thank Dr. Rita de Cassia Aleixo Tostes Passaglia from the Department of Pharmacology, FMRP-USP, Ribeirão Preto, SP, Brazil, for kindly providing the IL-6 ELISA kit and antibodies against phosphorylated proteins. This work was supported by Fundação de Amparo à Pesquisa do Estado de São Paulo, FAPESP (2014/17671-8, MCJ; 2013/12861-0, EGFF; 2012/06373-0, EZMS).

## References

- [1] A. M. M. Silveira e Souza, M. C. Jamur, and C. Oliver, “The importance of gangliosides in mast cell biology,” in *Glycolipids: New Research*, D. Sasaki, Ed., pp. 53–78, Nova Science, New York, NY, USA, 2008.
- [2] R. K. Yu, Y.-T. Tsai, T. Ariga, and M. Yanagisawa, “Structures, biosynthesis, and functions of gangliosides—an overview,” *Journal of Oleo Science*, vol. 60, no. 10, pp. 537–544, 2011.
- [3] J. L. Daniotti, R. D. Lardone, and A. A. Vilcaes, “Dysregulated expression of glycolipids in tumor cells: from negative modulator of anti-tumor immunity to promising targets for developing therapeutic agents,” *Frontiers in Oncology*, vol. 5, article 300, 2016.
- [4] E. Z. M. da Silva, M. C. Jamur, and C. Oliver, “Mast cell function: a new vision of an old cell,” *Journal of Histochemistry and Cytochemistry*, vol. 62, no. 10, pp. 698–738, 2014.

- [5] S. J. Galli, "The mast cell-IgE paradox: from homeostasis to anaphylaxis," *The American Journal of Pathology*, vol. 186, no. 2, pp. 212–224, 2016.
- [6] R. O. de Castro, J. Zhang, M. C. Jamur, C. Oliver, and R. P. Siraganian, "Tyrosines in the carboxyl terminus regulate Syk kinase activity and function," *The Journal of Biological Chemistry*, vol. 285, no. 34, pp. 26674–26684, 2010.
- [7] M. Krystel-Whittemore, K. N. Dileepan, and J. G. Wood, "Mast cell: a multi-functional master cell," *Frontiers in Immunology*, vol. 6, article 620, 2016.
- [8] J. Rivera, N. A. Fierro, A. Olivera, and R. Suzuki, "New insights on mast cell activation via the high affinity receptor for IgE," *Advances in Immunology*, vol. 98, pp. 85–120, 2008.
- [9] N. H. Guo, G. R. Her, V. N. Reinhold, M. J. Brennan, R. P. Siraganian, and V. Ginsburg, "Monoclonal antibody AA4, which inhibits binding of IgE to high affinity receptors on rat basophilic leukemia cells, binds to novel  $\alpha$ -galactosyl derivatives of gangliosides G(D1b)," *The Journal of Biological Chemistry*, vol. 264, no. 22, pp. 13267–13272, 1989.
- [10] L. A. K. Basciano, E. H. Berenstein, L. Kmak, and R. P. Siraganian, "Monoclonal antibodies that inhibit IgE binding," *Journal of Biological Chemistry*, vol. 261, no. 25, pp. 11823–11831, 1986.
- [11] C. Oliver, N. Sahara, S. Kitani, A. R. Robbins, L. M. Mertz, and R. P. Siraganian, "Binding of monoclonal antibody AA4 to gangliosides on rat basophilic leukemia cells produces changes similar to those seen with fee receptor activation," *Journal of Cell Biology*, vol. 116, no. 3, pp. 635–646, 1992.
- [12] W. D. Swaim, K. Minoguchi, C. Oliver et al., "The anti-ganglioside monoclonal antibody AA4 induces protein tyrosine phosphorylations, but not degranulation, in rat basophilic leukemia cells," *Journal of Biological Chemistry*, vol. 269, no. 30, pp. 19466–19473, 1994.
- [13] E. L. Barsumian, C. Isersky, M. G. Petrino, and R. P. Siraganian, "IgE-induced histamine release from rat basophilic leukemia cell lines: isolation of releasing and nonreleasing clones," *European Journal of Immunology*, vol. 11, no. 4, pp. 317–323, 1981.
- [14] J. Zhang, K. Suzuki, T. Hitomi, and R. P. Siraganian, "TOMIL1 is a Lyn substrate involved in Fc $\epsilon$ RI signaling in mast cells," *Journal of Biological Chemistry*, vol. 282, no. 52, pp. 37669–37677, 2007.
- [15] C. X. R. Valim, E. Z. M. da Silva, M. A. Assis et al., "RPbPgal from paracoccidioides brasiliensis activates mast cells and macrophages via NF $\kappa$ B," *PLoS Neglected Tropical Diseases*, vol. 9, no. 8, 2015.
- [16] A. C. G. Grodzki, K. D. Moon, E. H. Berenstein, and R. P. Siraganian, "Fc $\epsilon$ RI-induced activation by low antigen concentrations results in nuclear signals in the absence of degranulation," *Molecular Immunology*, vol. 46, no. 13, pp. 2539–2547, 2009.
- [17] A. M. M. Silveira e Souza, V. M. Mazucato, R. O. de Castro et al., "The  $\alpha$ -galactosyl derivatives of ganglioside GD1b are essential for the organization of lipid rafts in RBL-2H3 mast cells," *Experimental Cell Research*, vol. 314, no. 13, pp. 2515–2528, 2008.
- [18] Y. Lu, Y. Li, C.-S. Seo, M. Murakami, J.-K. Son, and H. W. Chang, "Sauceroneol D inhibits eicosanoid generation and degranulation through suppression of Syk kinase in mast cells," *Food and Chemical Toxicology*, vol. 50, no. 12, pp. 4382–4388, 2012.
- [19] A. M. Gilfillan and C. Tkaczyk, "Integrated signalling pathways for mast-cell activation," *Nature Reviews Immunology*, vol. 6, no. 3, pp. 218–230, 2006.
- [20] R. P. Siraganian, "Mast cell signal transduction from the high-affinity IgE receptor," *Current Opinion in Immunology*, vol. 15, no. 6, pp. 639–646, 2003.
- [21] U. Blank, I. K. Madera-Salcedo, L. Danelli et al., "Vesicular trafficking and signaling for cytokine and chemokine secretion in mast cells," *Frontiers in Immunology*, vol. 5, article 453, 2014.
- [22] P. L. Triozzi, J. J. Shah, W.-Q. Wang et al., "Anti-GD3 monoclonal antibody effects on lymphocytes and antibody-dependent cellular cytotoxicity," *Cancer Biotherapy and Radiopharmaceuticals*, vol. 21, no. 6, pp. 553–560, 2006.
- [23] J. Wang, Z.-H. Lu, H.-J. Gabius, C. Rohowsky-Kochan, R. W. Ledeen, and G. Wu, "Cross-linking of GM1 ganglioside by galectin-1 mediates regulatory T cell activity involving TRPC5 channel activation: possible role in suppressing experimental autoimmune encephalomyelitis," *Journal of Immunology*, vol. 182, no. 7, pp. 4036–4045, 2009.
- [24] G. Wu, Z.-H. Lu, A. G. Obukhov, M. C. Nowycky, and R. W. Ledeen, "Induction of calcium influx through TRPC5 channels by cross-linking of GM1 ganglioside associated with  $\alpha$ 5 $\beta$ 1 integrin initiates neurite outgrowth," *The Journal of Neuroscience*, vol. 27, no. 28, pp. 7447–7458, 2007.
- [25] K. Nohara, M. Kunimoto, and H. Fujimaki, "Antibody against ganglioside GD1c containing NeuGc $\alpha$ 2-8NeuGc cooperates with CD3 and CD4 in rat T cell activation," *The Journal of Biochemistry*, vol. 124, no. 1, pp. 194–199, 1998.
- [26] T. O. Nashar, N. A. Williams, and T. R. Hirst, "Cross-linking of cell surface ganglioside GM1 induces the selective apoptosis of mature CD8<sup>+</sup> T lymphocytes," *International Immunology*, vol. 8, no. 5, pp. 731–736, 1996.
- [27] P. Hersey, S. Schibeci, and D. Cheresch, "Augmentation of lymphocyte responses by monoclonal antibodies to the gangliosides GD3 and GD2: the role of protein kinase C, cyclic nucleotides, and intracellular calcium," *Cellular Immunology*, vol. 119, no. 2, pp. 263–278, 1989.
- [28] Y. Norihisa, D. W. McVicar, P. Ghosh et al., "Increased proliferation, cytotoxicity, and gene expression after stimulation of human peripheral blood T lymphocytes through a surface ganglioside (GD3)," *Journal of Immunology*, vol. 152, no. 2, pp. 485–495, 1994.
- [29] C. A. Dyer and J. A. Benjamins, "Glycolipids and transmembrane signaling: antibodies to galactocerebroside cause an influx of calcium in oligodendrocytes," *Journal of Cell Biology*, vol. 111, no. 2, pp. 625–633, 1990.
- [30] N. M. van Sorge, L. H. van den Berg, K. Geleijns et al., "Anti-GM1 IgG antibodies induce leukocyte effector functions via Fc $\gamma$  receptors," *Annals of Neurology*, vol. 53, no. 5, pp. 570–579, 2003.
- [31] J. A. Boyce, "Mast cells and eicosanoid mediators: a system of reciprocal paracrine and autocrine regulation," *Immunological Reviews*, vol. 217, no. 1, pp. 168–185, 2007.
- [32] B. L. Diaz, H. Fujishima, A. Sapirstein, J. V. Bonventre, and J. P. Arm, "Participation of cytosolic phospholipase A2 in eicosanoid generation by mouse bone marrow-derived mast cells," *Advances in Experimental Medicine and Biology*, vol. 507, pp. 41–46, 2002.
- [33] T. Hirabayashi and T. Shimizu, "Localization and regulation of cytosolic phospholipase A<sub>2</sub>," *Biochimica et Biophysica Acta (BBA)—Molecular and Cell Biology of Lipids*, vol. 1488, no. 1–2, pp. 124–138, 2000.
- [34] S. T. Reddy and H. R. Herschman, "Prostaglandin synthase-1 and prostaglandin synthase-2 are coupled to distinct phospholipases for the generation of prostaglandin D2 in activated mast

- cells," *The Journal of Biological Chemistry*, vol. 272, no. 6, pp. 3231–3237, 1997.
- [35] S. J. Galli and M. Tsai, "Mast cells: versatile regulators of inflammation, tissue remodeling, host defense and homeostasis," *Journal of Dermatological Science*, vol. 49, no. 1, pp. 7–19, 2008.
- [36] T. C. Theoharides, D. Kempuraj, M. Tagen, P. Conti, and D. Kalogeromitros, "Differential release of mast cell mediators and the pathogenesis of inflammation," *Immunological Reviews*, vol. 217, no. 1, pp. 65–78, 2007.
- [37] T. C. Moon, A. D. Befus, and M. Kulka, "Mast cell mediators: their differential release and the secretory pathways involved," *Frontiers in Immunology*, vol. 5, article 569, 2014.
- [38] A. Lorentz, I. Klopp, T. Gebhardt, M. P. Manns, and S. C. Bischoff, "Role of activator protein 1, nuclear factor- $\kappa$ B, and nuclear factor of activated T cells in IgE receptor-mediated cytokine expression in mature human mast cells," *Journal of Allergy and Clinical Immunology*, vol. 111, no. 5, pp. 1062–1068, 2003.
- [39] Y. Kawakami, S. E. Hartman, P. M. Holland, J. A. Cooper, and T. Kawakami, "Multiple signaling pathways for the activation of JNK in mast cells: involvement of Bruton's tyrosine kinase, protein kinase C, and JNK kinases, SEK1 and MKK7," *Journal of Immunology*, vol. 161, no. 4, pp. 1795–1802, 1998.
- [40] B. Frossi, J. Rivera, E. Hirsch, and C. Pucillo, "Selective activation of Fyn/PI3K and p38 MAPK regulates IL-4 production in BMMC under nontoxic stress condition," *Journal of Immunology*, vol. 178, no. 4, pp. 2549–2555, 2007.
- [41] S. Monticelli, D. C. Solymar, and A. Rao, "Role of NFAT proteins in *IL13* gene transcription in mast cells," *The Journal of Biological Chemistry*, vol. 279, no. 35, pp. 36210–36218, 2004.
- [42] H. Okamura, J. Aramburu, C. García-Rodríguez et al., "Concerted dephosphorylation of the transcription factor NFAT1 induces a conformational switch that regulates transcriptional activity," *Molecular Cell*, vol. 6, no. 3, pp. 539–550, 2000.
- [43] S. Klemm and J. Ruland, "Inflammatory signal transduction from the Fc $\epsilon$ RI to NF- $\kappa$ B," *Immunobiology*, vol. 211, no. 10, pp. 815–820, 2006.
- [44] Y. Gwack, S. Feske, S. Srikanth, P. G. Hogan, and A. Rao, "Signalling to transcription: store-operated Ca<sup>2+</sup> entry and NFAT activation in lymphocytes," *Cell Calcium*, vol. 42, no. 2, pp. 145–156, 2007.
- [45] M. Klein, S. Klein-Hessling, A. Palmethofer et al., "Specific and redundant roles for NFAT transcription factors in the expression of mast cell-derived cytokines," *Journal of Immunology*, vol. 177, no. 10, pp. 6667–6674, 2006.
- [46] J. Zhang, T. Kimura, and R. P. Siraganian, "Mutations in the activation loop tyrosines of protein tyrosine kinase Syk abrogate intracellular signaling but not kinase activity," *Journal of Immunology*, vol. 161, no. 8, pp. 4366–4374, 1998.
- [47] A. B. Rossi, E. Herlaar, S. Braselmann et al., "Identification of the Syk kinase inhibitor R112 by a human mast cell screen," *Journal of Allergy and Clinical Immunology*, vol. 118, no. 3, pp. 749–755, 2006.
- [48] V. Stephan, A. Seibt, D. Dukanovic et al., "Anti-ganglioside monoclonal antibody AA4 selectively inhibits IgE-induced signal transduction pathways in rat basophilic leukemia cells," *Molecular Immunology*, vol. 34, no. 3, pp. 227–235, 1997.
- [49] K. Minoguchi, W. D. Swaim, E. H. Berenstein, and R. P. Siraganian, "Src family tyrosine kinase p53/56lyn, a serine kinase and Fc $\epsilon$ RI associate with  $\alpha$ -galactosyl derivatives of ganglioside GD1b in rat basophilic leukemia RBL-2H3 cells," *Journal of Biological Chemistry*, vol. 269, no. 7, pp. 5249–5254, 1994.
- [50] C. Oliver, A. Fujimura, A. M. M. Silveira E Souza, R. O. De Castro, R. P. Siraganian, and M. C. Jamur, "Mast cell-specific gangliosides and Fc $\epsilon$ RI follow the same endocytic pathway from lipid rafts in RBL-2H3 cells," *Journal of Histochemistry and Cytochemistry*, vol. 55, no. 4, pp. 315–325, 2007.



**Hindawi**  
Submit your manuscripts at  
<http://www.hindawi.com>

