Effects of Several Flavonoids and Tryptanthrin from the Brazilian Herbal Medicine *Cissus sicyoides* on Antigen-Induced Release of Cytokines, and Their Target Molecules in Rat Basophilic Leukemia Cells (RBL-2H3)

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Abstract

In our previous study, we have reported inhibitory effects of flavonoids and benzo-furan type stilbenes from Brazilian herbal medicine, the aerial parts of *Cissus sicyoides*, on antigen-induced degranulations in rat basophilic leukemia cells (RBL-2H3). In the present study, we examined effects of the active constituents on antigen-induced release of tumor necrosis factor-α (TNF-α) and interleukin-4 (IL-4) in the cells. The result revealed that flavonols (kaempferol, quercetin), flavones (lanceolatin B, 7,3',4'-trihydroxyflavone), chalcones (isoliquiritigenin, *E*-7-*O*-methylpongamol) inhibited the release of TNF-α and IL-4. With regard to the effects of active constituents on signaling transduction, these constituents (except for *E*-7-*O*-methylpongamol) suppressed the phosphorylation of ERK1/2, JNK/SAPK in the cells, and inhibition of spleen tyrosine kinase (Syk) and myocin light-chain kinase (MLCK) may, at least in part, be involved in the mechanism of action.

Keywords   Flavonoids, *Cissus sicyoides*, Cytokine, Target molecule, Spleen tyrosine kinase, Myocin light chain kinase

1. Introduction

Although excellent anti-allergic agents have been developed, recent increases in the number of patients in Japan with allergies, such as pollinosis and asthma, necessitate more effective and safe anti-allergic agents. Mast cells and basophils play important roles in both immediate and late-phase reactions of type-1 allergy. Aggregation of FcεRI by antigens results in tyrosine phosphorylations, Ca^{2+} release from intracellular Ca^{2+} stores and influx *via* Ca^{2+} release-activated Ca^{2+} channels. Elevation of intracellular free Ca^{2+} levels plays an essential role in the degranulation process.[1–3] Furthermore, mast cells and basophils concomitantly synthesize and release a variety of cytokines including interleukin (IL)-3, IL-4, granulocyte-macrophage colony-stimulating factor (GM-CSF), tumor necrosis factor α (TNF-α) to induce the late-phase reactions, and production of IgE, *etc*.[1]

Vitaceae plant, *Cissus sicyoides* (Brazilian name: Insulina) is widely distributed in Brazil, and is used in Brazilian folk medicine to treat diabetes mellitus, while the leaves are used externally for the treatment of rheumatism and wounds.

[4] Bioactive studies have indicated that the water extract of *C. sicyoides* improves hyperglycemia after an oral administration of maltose in KK-A^y mice.[5] Previous phytochemical investigations of this plant yielded coumarins, flavonoids, and anthocyanins.[4,6] In a previous study, we have reported three new flavonoid glycosides, cissosides I, II, and III, and a new benzofuran-type stilbene cissusin, together with 39 known constituents.[7] Furthermore, the inhibitory effects of isolated constituents on the release of β-hexosaminidase as a marker of degranulation in RBL-2H3 cells were examined. As a result, cissusin, flavonols [kaempferol (**2**), quercetin (**4**)], flavones [lanceolatin B (**5**), 7,3',4'-trihydroxyflavone (**6**)], a pterocarpanes (homopterocarpin), chalcones [isoliquiritigenin (**3**), *E*-7-*O*-methylpongamol (**7**)], and tryptanthrin (**1**) substantially inhibited the release of β-hexosaminidase.

As a continuing study, we investigated the effects of seven degranulation inhibitors (**1**–**7**) on antigen-induced release of TNF-α and IL-4, and effects on FcεRI signal transduction after stimulation of antigen in RBL-2H3 cells
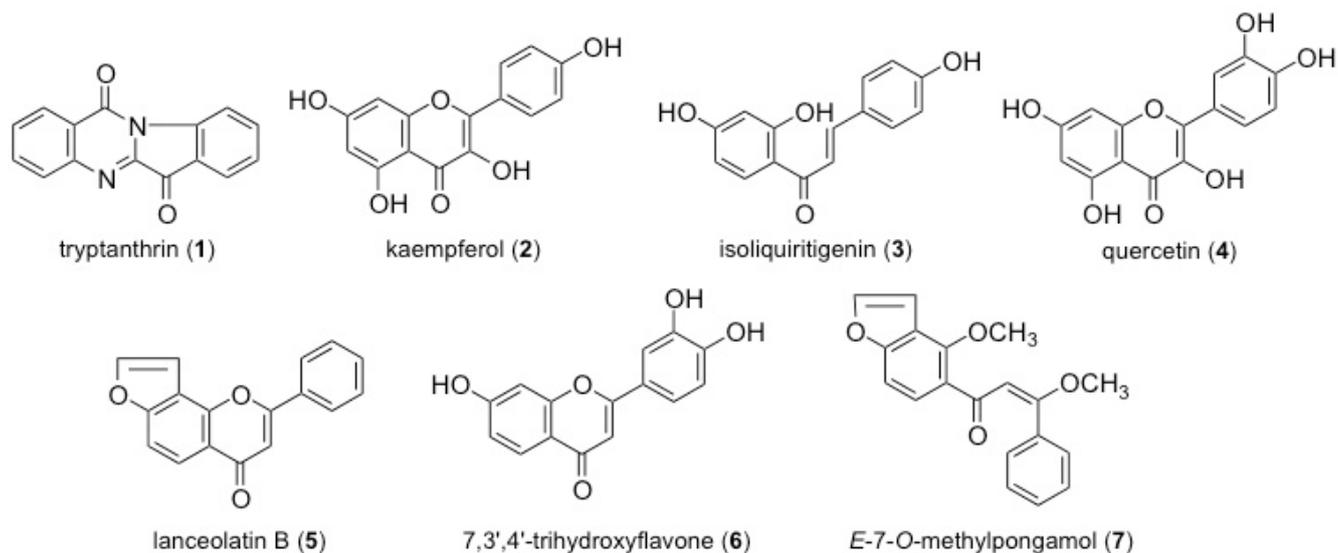
Fig. 1. Chemical structures of the isolated compounds (1–7) from the aerial parts of C. sicyoides

Table 1. Effects of 1–7 on release of TNF-α and IL-4 from RBL-2H3 cells

Test samples	TNF-α IC$_{50}$ (μM)	IL-4 IC$_{50}$ (μM)
Tryptanthrin (1)	9.0	9.2
Kaempferol (2)	7.6	33
Isoliquiritigenin (3)	5.4	29
Quercetin (4)	9.0	>10
Lanceolatin B (5)	9.3	>100
7,3',4'-Trihydroxyflavone (6)	14	58
E-7-O-Methlpongamol (7)	5.3	10

were partly examined.

2. Results and Discussion

2. 1 Effects of the constituents (1–7) on the release of TNF-α and IL-4 in RBL-2H3 cells

In type-1 allergy, IgE antibodies bind to FcεRI on the surface of tissue mast cells and blood basophils. After sensitization with IgE antibodies, exposure to the same allergen crosslinks the bound IgE on sensitized cells, resulting in degranulation and the secretion of pharmacologically active mediators such as histamine, leukotrienes (LTs), and prostaglandins (PGs) to cause inflammation on the surrounding tissues. Type-1 allergy is further classified as immediate and late-phase reactions. The immediate-phase reaction occurs minutes after exposure and involves the release of vasoactive amines and lipid mediators, whereas the late-phase reaction occurs 2–4 hr after exposure and includes the release of cytokines such as ILs-3, 4, 5, 6, TNF-α, GM-CSF. The late-phase reaction is caused by release of mediators such as major basic protein (MBP), eosinophil cationic protein (ECP), LTC$_4$ from eosinophils and are dependent on activity of Th$_2$ cells.[1,8]

Several flavonoids, such as apigenin, luteolin, kaempferol (2) and quercetin (4), inhibit the antigen-induced release of TNF-α in sensitized RBL-2H3 cells.[9,10] However, the effects of anti-degranulation constituents of roots of *C. sicyoides* (except for 2 and 4) have not been examined to date. In the present study, effects of 1–7 on antigen-induced release of TNF-α and IL-4 from the RBL-2H3 cells were examined. As shown in Table 1, kempferol (2) and quercetin (4) inhibited the release of TNF-α consistent with the previous reports.[9,10] In addition, tryptanthrin (1), isoliquiritigenin (3), lanceolatin B (5), 7,3',4-trihydroxyflavobe (6), and *E*-7-*O*-methlpongamol (7) also showed similar effects except for 5. These findings suggest that these active constituents can be effective for late-phase reactions as well as immediate-phase reactions.

2.2 Effects of the constituents on the FcεRI signal transduction in RBL-2H3 cells

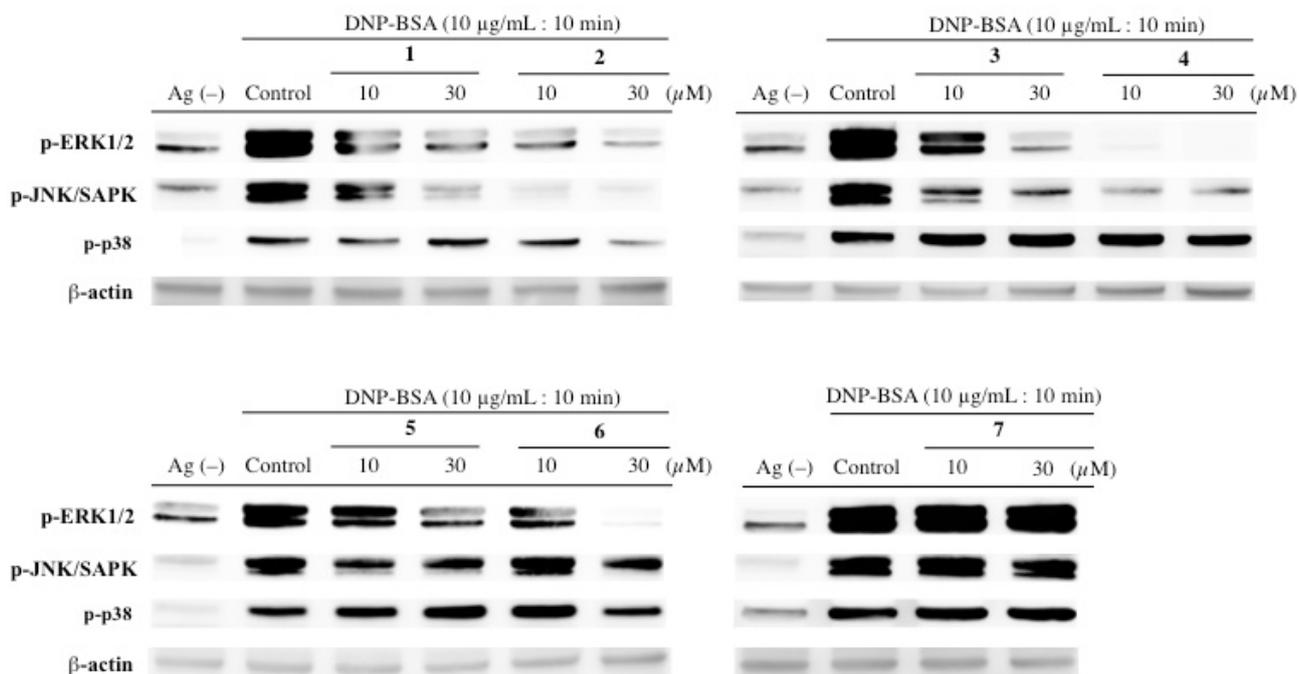#### 2.2.1. Effects of 1–7 on phosphorylation of ERK1/2, JNK/SAPK, and p38

Fig. 2. Effects of 1–7 on phosphorylations of ERK1/2, JNK/SAPK, and p38 MAPK

In the FcεRI signal transduction, the nuclear factor of activated T-cells (NFAT) and activator protein-1 (AP-1), which is composed of c-fos and c-jun, induce the transcription of target genes of cytokines by binding at gene promoters and enhancers.[1–3,11] Kimata *et al*. have reported that several flavonoids, such as luteolin and quercetin (**4**), inhibit IgE-mediated Ca^{2+} influx, and the activation of protein kinase C, extracellular signal-regulated kinases (ERKs) and c-jun NH_2-terminal kinase (JNK) without affecting the activation of p38 mitogen-activated protein kinase (p38 MAPK). These compounds further inhibit the release of leukotrienes and prostaglandin D_2 and expression of GM-CSF mRNA in cultured human mast cells.[12] Hirano *et al*. have also demonstrated that luteolin inhibit phosphorylation of c-jun and DNA binding activity of AP-1 in a human basophilic cell line, KU812, stimulated by A23187 and 12-myristate 13-acetate (PMA).[13]

We therefore examined the effects of **1**–**7**, which showed significant inhibition of release of TNF-α and/or IL-4 in RBL-2H3 cells (Table 1), on the phosphorylation of ERK1/2, JNK/SAPK, and p38 MAPK to obtain information about their mechanism of action.

As a result, tryptanthrin (**1**), kaempferol (**2**), quercetin (**4**), and 7,3',4'-trihydroxyflavone (**6**) markedly inhibited phosphorylation of ERK1/2 and JNK/SAPK, without obvious inhibitions against phosphorylation of p38 MAPK (Fig. 2). Note that *E*-7-*O*-methlpongamol (**7**) did not inhibit phosphorylation at 30 μM. These results

are not consistent with the IC_{50} values against the release of TNF-α and/or IL-4. Further study about the relevant mechanism of action is necessary.

2.2.2 Effects on the protein kinases, Syk and MLCK2

Shichijo *et al*. have reported that flavonoids inhibit activation of spleen tyrosine kinase (Syk), which plays a pivotal role in FcεRI-mediated degranulation of mast cells.[14] In addition, luteolin and quercetin (**4**) were reported to inhibit calcium ionophore A2318-induced histamine release in human cultured mast cells (HCMCs),[15] and calmodulin pathway. At least myosin-light chain kinase (MLCK), calmodulin-dependent protein kinases (CaMKs), and calcineurin positively regulate the Ca^{2+}-induced degranulation in RBL-2H3 cells.[15] Ludowyke *et al*. have observed that Ca^{2+}-induced degranulation correlates with the phosphorylation of myosin light chain *via* protein kinase C,[16] albeit kaempferol (**2**) is reported to inhibit bovine aorta MLCK.[17]

In the present study, we examined the enzyme inhibitions of **1**–**7** on Syk and MLCK2, which are commercially available kinases, using the **Z'-LYTE™ assay kit (Invitrogen)**. As shown in Tables 2 and 3, compounds **1**–**7** inhibited the enzyme activity of Syk and/or MLCK2 partly correlated with their effects on the degranulation in RBL-2H3 cells. Furthermore, kaempferol (**2**) and quercetin (**4**) inhibited the phosphorylations of LAT which is a target protein of Syk,

and myosin regulatory light chain (MRLC, myosin light chain 2) which is a target protein of MLCK (Fig. 3, 4).[15] These findings suggest that their possible target molecules with anti-allergic activity in RBL-2H3 are Syk and MLCK, although further studies are need to clarify the mechanism of action.

In conclusion, we examined effects of the active constituents on antigen-induced release of TNF-α and IL-4 in the cells. Based on the results, flavonols [kaempferol (**2**), quercetin (**4**)], flavones [lanceolatin B (**5**), 7,3',4'-trihydroxyflavone (**6**)], chalcones [isoliquiritigenin (**3**), *E*-7-*O*-methylpongamol (**7**)], and tryptanthrin (**1**) inhibited the release of TNF-α and IL-4. These constituents except

Table 2. Effects of **1–7** on Syk using Z'-LYTE^{TM} kinase assay

Compounds	Conc. (μM)	1	3	10	30	100	IC$_{50}$ (μM)
			Inhibition (%)				
Tryptanthrin (**1**)		66 ( )	60 (19)	68 (86)	(89)		<3.0 (19)
Kaempferol (**2**)		33 ( )	75 (31)	100 (80)	(93)		5.0 (15)
Isoliquiritigenin (**3**)			64 (28)	81 (55)	(91)		<10 (24)
Quercetin (**4**)	70 (6.7)	80 (46)	88 (79)	(87)	(90)		<1.0 (3.3)
Lanceolatin B (**5**)		51 (21)	61 (53)	83 (56)	(69)		3.4 (11)
7,3',4'-Tetrahydroxyflavone (**6**)		36 (1.6)	72 (30)	94 (72)	(91)		4.8 (17)
E-7-*O*-Methlpongamol (**7**)			44 (8.6)	69 (52)	86 (81)		13 (29)

Values in ( ) are effects of samples on antigen-induced release of β-hexosaminidase from RBL-2H3 cells.[7]

Table 3. Effects of **1–7** on MLCK2 using Z'-LYTE^{TM} kinase assay

Sample	Conc. (μM)	0.3	1	3	10	30	100	IC$_{50}$ (μM)
				Inhibition (%)				
Tryptanthrin (**1**)				20	18 (4.7)	30 (36)	36 (95)	>100 (42)
Kaempferol (**2**)	40 ( )		43	64 (19)	96 (64)	(87)		1.1 (7.1)
Isoliquiritigenin (**3**)			29	48 (3.0)	70 (40)	74 (84)		3.0 (12)
Quercetin (**4**)	31 ( )		57 (-4.8)	63 (37)	62 (78)			0.6 (4.5)
Lanceolatin B (**5**)			31	72 (-7.1)	(44)	(62)		1.6 (17)
7,3',4'-Tetrahydroxy flavone (**6**)			8.9	18 (2.5)	-3 (41)	(77)		(13)
E-7-*O*-Methlpongamol (**7**)			28	28 (35)	34 (86)	60 (89)		21 (13)

Values in ( ) are effects of samples on calcium ionophore A23187-induced release of β-hexosaminidase from RBL-2H3 cells.[7]
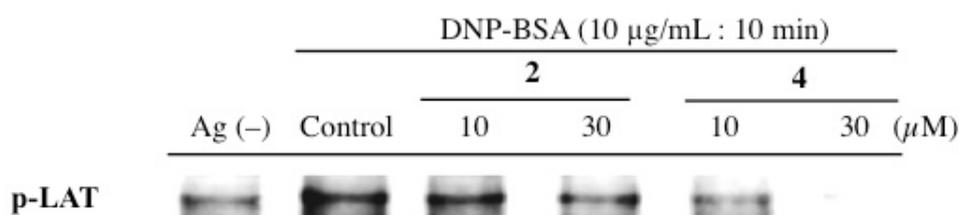

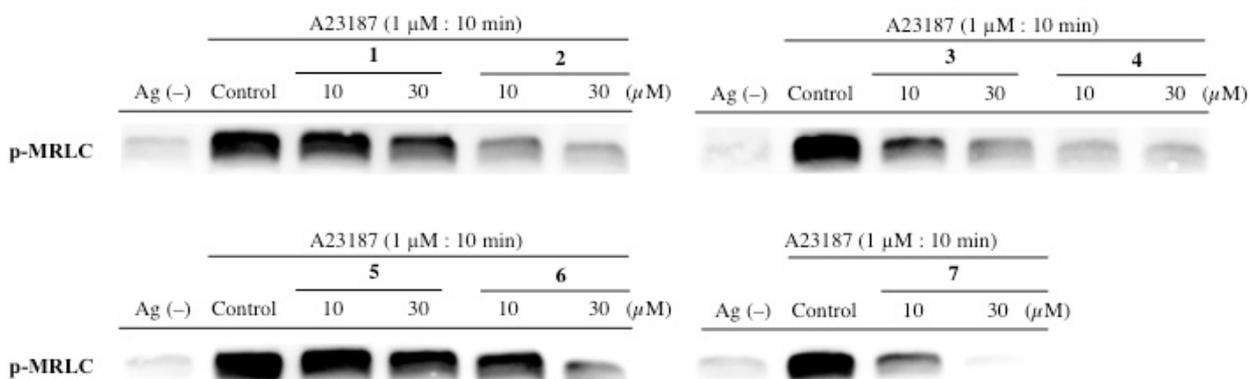Fig. 3. Effects of 12 and 13 on antigen-induced phosphorylations of LAT in RBL-2H3

Fig. 4. Effects of 1–7 on A23187-induced phosphorylation of MRLC in RBL-2H3 cells

for **7** also suppressed the antigen-induced phosphorylation of ERK1/2, JNK/SAPK in the cells, and the inhibition of Syk and MLCK may be involved.

3. Materials and methods

3.1 Test compounds

Test compounds were isolated and purified as described in our previous report.[7]

3.2 Inhibitory effects on antigen-induced releases of TNF-α and IL-4 from RBL-2H3 cells

RBL-2H3 cells [Cell no JCRB0023, obtained from Health Science Research Resources Bank (Osaka, Japan)] in Minimum Essential Medium Eagle (MEM, Sigma) containing 10% fetal bovine serum (FBS) as well as penicillin (100 units/mL) and streptomycin (100 μg/mL) were seeded into 24-well multiplates at the density of 2×10^5 cells/well before incubating with anti-DNP IgE antibody (0.45 μg/mL, Monoclonal Anti-DNP, Sigma) for cell sensitization. The cells were washed twice with 500 μL of MEM containing 10% FBS, pencillin (100 units/mL), and streptomycin (100 μg/mL), and exchanged with 320 μL fresh medium. Then, 40 μL of test sample (0.1% DMSO) and 40 μL of antigen (DNP-BSA, final conc. 10 μg/mL) were added to each well and incubated at 37°C for 4 hr. The supernatant (50 μL) was transferred into a 96-well ELISA plate and TNF-α and IL-4 concentrations were determined using commercial kits [Rat TNF-α ELISA Ready-SET-Go! (eBioscience), Quantikine ELISA Kit Rat IL-4 Immunoassay (R&D Systems)]. The test-samples were dissolved in DMSO, and the solution was added to MEM (final DMSO concentration was 0.1%). The inhibition (%) of the releases of TNF-*α* and IL-4 by the test-samples was calculated by the following equation, and IC$_{50}$ values were determined graphically:

$$\text{Inhibition (\%)} = [1 - (T - N) / (C - N)] \times 100$$
Control (C): DNP-BSA (+), test sample (–);
Test (T): DNP-BSA (+), test sample (+);
Normal (N): DNP-BSA (–), test sample (–).

3.3 Western blot analysis of signal transduction factors

RBL-2H3 cells (1 x 10^6 cells/2 mL/well) in 6-well muiltiplates were sensitized with anti-DNP IgE antibody (0.45 µg/mL) for 24 hr. The cells were washed twice with Siraganian buffer supplemented with 5.6 mM glucose, 1 mM CaCl$_2$, and 0.1 % bovine serum albumin (BSA), and

incubated in 1.6 mL of the buffer for 10 min at 37°C. Then, 0.2 mL of test compound solution or vehicle (DMSO final conc. 0.1%) was added and the cells were incubated for 10 min before being stimulated with 0.2 mL of DNP-BSA (final conc. 10 µg/mL) or calcium ionophore A23187 (final conc. 1 µM) for 10 min. The cells were washed twice with ice-cold phosphate-buffered saline [Ca^{2+}(-), Mg^{2+}(-)](PBS (-)), and then 0.14 mL of lysis buffer [150 mM NaCl, 10 mM Tris, 0.5% Triton-X, 2 mM EGTA, 20 mM β-glycerophosphate, protease inhibitor Cocktail (Thermo), phosSTOP (Roche), pH 7.4] was added to collect the cells. After sonication of cells, half-volume of Laemili buffer [composed of 0.9 mM EGTA, 8% glycerol, 6% SDS, 3 mM Tris, 6% mercaptoethanol and 0.03% bromophenol blue (pH 6.8)] was added before heating in boiling water for 5 min. After quantification of the total protein concentration in the lysate using the BCA Protein Assay Kit (Thermo), proteins (50 µg/lane) were separated by 10% SDS polyacrylamide gels (10% READY GELS J, Bio-Rad) and blotted onto PVDF membranes (Bio-Rad). The membrane was blocked overnight at 4°C using Blocking One or Blocking One-P (Nacalai tesque,). After washing with Tris-buffered saline containing 0.1% Tween 20 (T-TBS), the blots were probed with primary antibody. The membranes were washed three times with T-TBS and incubated with horseradish peroxidase-conjugated secondary antibody for 1 hr at room temperature. The detection of immunoreactive protein was performed using an enhance chemiluminescence kit (ECL Plus Western Blotting Detection System, GE Healthcare) according to the manufacturer's instructions.

For detection of p-LAT, an immunoprecipitation using Protein A/G PLUS-Agarose (Santa Cruz) and anti-LAT antibody (Cell Signaling Technology) was performed. Briefly, the cells were collected in a lysis buffer [150 mM NaCl, 10 mM , HEPES 20 mM, NP-40 1%, glycerol 10%, octyl β-glucoside 60 mM, protease inhibitor Cocktail (Thermo) and phosSTOP (Roche), pH 7.4] was cooled by ice for 15 min before the lysate was subjected to centrifugation. Protein A/G PLUS-Agarose was then added to the 250 µg/mL protein of supernatant and incubated at 4°C for 1 hr. After centrifugation of the mixture, anti-LAT antibody was added to the supernatant and incubated at 4°C for 1 hr, and then Protein A/G PLUS-Agarose was added again and incubated overnight at 4°C. After centrifugation, the precipitation was washed twice using PBS (-) supplemented with protease inhibitor cocktail (Thermo) and phosSTOP (Roche). Ten µL of

Electrophoresis Sample Buffer (Santa Cruz) was added, and heated in boiling water for 5 min. Protein solution (10μL/lane) were separated by the 10% SDS polyacrylamide gels.

The following antibodies and dilutions were used for the western blots: rabbit polyclonal antibody (Cell Signaling Technology) against phospho-ERK1/2, phospho-JNK/SAPK (1:1000), phospho-p38 (1:1000), β-actin (1:1000), p-MRLC (Thr18/Ser19) (1:1000); anti-rabbit IgG horseradish peroxidase (HRP)-conjugate secondary antibody (Cell Signaling Technology) (1:5000); mouse polyclonal antibody against anti-phospho-tyrosine antibody (1:1000) for detection of p-LAT; anti-mouse IgG HRP-conjugated secondary antibody (1:5000) (Cell Signaling Technology).

3.4 Effects on the protein kinases, Syk and MLCK2

Effects on the protein kinases, Syk and MLCK2, were examined using Z'-LYTE™ assay kit (Invitrogen) according to the manufacturer's instruction. The final concentrations of ATP were 25 μM for Syk and 300 μM for MLCK2, and Tyr 2 Peptide (final conc. 2 μM) and Ser/Thr 13 Peptide (final conc. 2 μM)(Invitrogen) were used for substrates of Syk and MLCK2, respectively. Final conc. of Syk and MLCK2 was 1 ng/μL.

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