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A common signaling pathway leading to degranulation in mast cells and its regulation by CCR1-ligand

Short title: Common signaling pathway leading to degranulation

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Abstract

Background: Signal transduction pathways mediated by various receptors expressed on mast cells are thought to be complex and inhibitory signals that turn off activating signals are not known.

Methods: Upstream signaling cascades mediated by several known receptors in bone marrow derived mast cells that lead to degranulation and mediator release were studied by immunoblotting and immunoprecipitation. Small interfering RNAs and knockout mice were used to confirm findings.

Results: All ligands tested including IgE/Ag, SCF, HSP70, CCL3 and its valiant eMIP induced phosphorylation of linker-for-activation-of-T-cells (LAT), which triggered their receptor mediated downstream signaling cascades that controlled degranulation and mediator release. Phosphorylation of lymphocyte-specific-protein-kinase (Lck) was induced by each ligand, which commonly played an indispensable role in LAT phosphorylation. In contrast, phosphorylation of spleen tyrosine kinase was additionally induced in cells stimulated only with IgE/Ag and SCF, which is also associated with LAT phosphorylation in part. Degranulation and mediator release induced by IgE/Ag, SCF or HSP70 were enhanced by nanomolar doses of CCR1 ligands CCL3 and eMIP via enhanced LAT phosphorylation. On the other hand, micromolar doses of CCR1 ligand inhibited degranulation and mediator release from mast cells stimulated with IgE/Ag, SCF or HSP70 by dephosphorylation of phosphorylated Lck with Src homology region 2 domain-containing phosphatase-1.

Conclusions: LAT plays a central role in signal transduction pathways in mast cells stimulated with any ligand tested. Dose-dependent alternate co-stimulation and inhibition of CCR1 ligands in IgE/Ag-, SCF- or HSP70- stimulated mast cells occur at the level of Lck-LAT phosphorylation.

Key words: CCR1, FcεRI, KIT, SHP-1, TLR4

Abbreviations:

Ag: antigen

BMMC: bone marrow-derived mast cells

β -Hex: β -hexosaminidase

DNP-HSA: dinitrophenyl-human serum albumin

ERK/Erk1/2: extracellular signal regulated kinase1/2

Fexo: fexofenadine

GPCR: G protein coupled receptor

HSP: heat shock proteins

IKK: I κ B kinase

IgE: immunoglobulin E

IL-6: interleukin-6

IRF: interferon regulatory factor

JNK: c-Jun N-terminal kinase

KIT: ckit receptor

KO: knockout

Lck: lymphocyte-specific protein tyrosine kinase

LAT: linker for activation of T cells

LTC₄: leukotriene C₄

MAPK: mitogen-activated protein kinase

MyD88: myeloid differentiation factor 99

M-dose: micro-molar doses

N-dose: nano-molar doses

NF- κ B: nuclear factor-kappa B

NK: natural killer

PCA: passive cutaneous anaphylaxis or anaphylactic

PGD₂: prostaglandin D₂;

Phospho-Lck; phosphorylated Lck

Phospho-Syk; phosphorylated Syk

PI3K: phosphatidylinositol 3-kinase

PKC: protein kinase C

PLC γ : phospholipase C γ

SCF; stem cell factor

SHP-1: Src homology region 2 domain-containing phosphatase-1

Syk: spleen tyrosine kinase

TRAF6: TNF receptor associated factor 6;

TIR domain: Toll/interleukin-1 receptor homology domain

TIRAP: TIR domain containing adaptor protein

TLR4: Toll-like receptor 4

TNF- α : tumor necrosis factor- α

1 Introduction

Mast cells play a key role in the inflammatory process, being recognized effector cells of innate immunity and have a central role in allergic diseases. By antigen (Ag)-dependent cross-linking of IgE-loaded FcεRI, mast cells are activated to rapidly release granules of histamine, serotonin and β-hexosaminidase (β-Hex). Mast cells also synthesize and secrete the inflammatory lipid mediators LTC₄ and PGD₂, as well as pro-inflammatory cytokines TNFα and IL-6. The signaling cascade induced by cross-linking of FcεRI with Ag in mast cells is known to lead to tyrosine phosphorylation of the Src family protein kinases Fyn and Lyn, which in turn recruits spleen tyrosine kinase (Syk) to the receptor.¹ The resulting tyrosine phosphorylation of Syk triggers phosphorylation of linker-for-activation-of-T-cells (LAT) and PLCγ1, and degranulation. In parallel, phosphorylation of phosphatidylinositol 3-kinase (PI3K)/Akt/MAPK results in eicosanoid and cytokine production.

Besides FcεRI, stem cell factor (SCF) receptor, KIT (c-kit receptor, CD117), chemokine receptor CCR1 and toll like receptor 4 (TLR4) are also expressed on mast cells, some of which co-stimulate IgE/Ag-induced degranulation.² Among them, co-stimulatory activity of CCR1 in mouse mast cells has been studied extensively. Degranulation and pro-inflammatory cytokine release from IgE/Ag-stimulated mast cells are enhanced by co-stimulation of CCR1, with its ligand CCL3 (MIP-1α), both *in vitro* and *in vivo*.^{3,4} Deficiency of CCR1 or its ligand reduces allergic symptoms in a murine model system.^{4,5} Simultaneous engagement of FcεRI and CCR1 appears to be important for optimal degranulation of mast cells.

CCR1 is a G protein coupled receptor (GPCR) and its ligand can activate a series of protein kinases, including PI3K/Akt/MAPK,⁶ although the precise mechanism of its co-stimulatory effect has not been elucidated and upstream signaling cascades remain unknown. Signal transduction pathways mediated by other co-stimulatory receptors such as KIT, one of the class III receptor tyrosine kinases, and the pathways

that contribute to co-stimulation have not been studied extensively. Known pathways besides the PI3K/Akt/MAPK and PLC γ 1 pathways include those of Ras/Erk and Src kinase.⁷ In the case of TLR4 signaling, the myeloid differentiation factor 88 (MyD88)/IRF/PKD1 pathway is reported to be activated and the adapter protein TIRAP is required to mediate interaction between TLR4 and MyD88.⁸

We studied upstream signal transduction pathways mediated by various receptors expressed in bone marrow-derived mast cells (BMMC), specially focusing on phosphorylation of LAT and lymphocyte-specific-protein-kinase (Lck). These signaling molecules were recently found by us to play a key role in HSP70-induced degranulation and mediator release from mast cells.⁹ In this paper we show that not only HSP70, but also other agonists such as IgE/Ag, SCF, CCL3 and eMIP, a human CCL3 variant carrying a single amino acid substitution¹⁰, induce degranulation and mediator released via phosphorylation of Lck and LAT in BMMC. We also show that nano-molar doses of CCR1 ligands, CCL3 and eMIP, enhanced degranulation and mediator release induced by IgE/Ag, SCF and HSP70, whereas micromolar doses of the ligands inhibited these activities by de-phosphorylation of phosphor-Lck, mediated by Src homology region 2 domain-containing phosphatase-1(SHP-1).

2 METHODS

2.1 Reagents

Recombinant human CCL3 (hMIP-1 α) was obtained using a *Brevibacillus choshinensis* expression system (ProteinExpress)¹⁰ or purchased from PeproTech. Recombinant eMIP, a 69-amino acid variant of human CCL3 carrying a single amino acid substitution of Asp27 to Ala was generated as described previously.¹⁰ Because of its better pharmaceutical properties (compared with CCL3, eMIP has a lower tendency for self-aggregation,¹⁰) eMIP was employed in this study and its activity compared with CCL3. Mouse SCF was obtained from Miltenyi Biotec GmbH (Bergisch, Gladbach) and recombinant mouse HSP70-A1 (low endotoxin) was from Enzo Life Sciences (Farmingdale, NY). Primary polyclonal rabbit antibodies used for immunoblotting were those against phosphorylated form of PI3K, Ras, Raf, PLC γ 1 (Tyr783), Akt (Ser473), ERK1/2, p38, JNK IKK or IKK, and those against total Akt, ERK1/2, p38 or JNK, all of which were from Cell Signaling Technology (Danvers, MA). Antibodies against HRP-conjugated goat anti-rabbit IgG and rabbit anti-goat IgG, and those against total Fyn, Lyn, Syk, Lck, LAT, PLC γ 1, PI3K, Ras, Raf or SHP-1 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phospho-tyrosine antibody (pY) was purchased from Millipore (Darmstadt, Germany). Anti-phospho-Lck (Tyr505 and Tyr395) antibodies were from Cell Signaling Technology and Thermo Fisher Scientific, respectively. Following inhibitors were used in this study; histamine H1 antagonist Fexo (Korea Pharma, Seoul), Src family protein kinase inhibitor PP2 (Cabochem, La Jolla), SHP-1 inhibitor NSC-87877 (Cabochem), and Lck inhibitor A770041 (Axon Medchem, VA). Mouse Lck siRNA was obtained from OriGene (Rockville, MD), and mouse SHP-1 siRNA and nonspecific siRNA were from Santa Cruz Biochemistry.

2.2 Animal studies and *in vitro* experiments.

Works presented here, including preparation, culture, activation of BMMC isolated from normal and Syk-gene-knockout (KO) mice, evaluation of degranulation, release of eicosanoids and pro-inflammatory cytokines, immunoblotting and immunoprecipitation, and Lck- and SHP-1-specific siRNA transfection were carried out as described previously.^{9,11,12} IgE/Ag mediated, mast cell-dependent passive cutaneous reaction (PCA reaction) was performed as described previously.⁹ In brief, PCA reaction was induced by intradermal injection of 60 ng Ag (DNP-HAS) into one ear of a mouse (n=6), which had been sensitized for 24 hours with intravenously administered 80 ng Ag. The reaction was visualized by dye extravasation 1 hour after simultaneous injection of Evans Blue with Ag and quantitated by extraction of dye from ear tissues. Serum levels of LTC₄ and PGD₂ were determined 1 hour after Ag challenge.

2.3 Statistical analysis

All *in vitro* experiments were repeated at least three times in triplicate, each time using BMMC derived from a different mouse. Calculation and statistical analyses were performed using GraphPad Prism 3.0 software. The data are presented as means $\pm$ SEM from three independent experiments with different BMMC unless otherwise stated. The Student's *t*-test was used for the comparison of two independent groups. For all tests, a *P* value < 0.05 (*) or (#) was considered significant.

3 Results

3.1 LAT plays a common and essential role in signaling cascades mediated by various receptors on mast cells

Although several receptors are known to be expressed on mast cells, FcεRI is the only receptor whose signal transduction pathways, leading to degranulation and mediator release, have been extensively studied. In the FcεRI-mediated signaling cascade, phosphorylation of LAT plays a key role (Figure 1A). LAT is a membrane protein known to facilitate the recruitment of signaling proteins to the plasma membrane of mast cells activated with IgE/Ag.¹³ We found that not only IgE/Ag but other stimuli such as SCF (Figure 1B), HSP70 (Figure 1C) and CCR1-ligands, eMIP and CCL3 (Figure 1D), induced degranulation from BMNC via phosphorylation of LAT. Furthermore, eMIP enhanced IgE/Ag-induced degranulation, which was accompanied by increased phosphorylation of LAT and downstream signaling molecules including PI3K and PLCγ1 (Fig. 1E). These results suggest that co-stimulation of the CCR1 ligand with IgE/Ag takes place at the level of LAT phosphorylation in the signaling cascade. Simultaneous engagement of IgE receptor FcεRI and CC chemokine receptor CCR1 is important for optimal, immediate hypersensitivity reactions and mast cell degranulation *in situ*.⁴

3.2 Lck is responsible for stimulation induced LAT phosphorylation

Based on our present findings regarding LAT phosphorylation upon exposure to any of IgE/Ag, SCF, HSP70, eMIP and CCL3, we deduced that the same tyrosine kinase is associated with LAT phosphorylation in mast cells. LAT is reported to be phosphorylated by phosphorylated-Syk (phospho-Syk) in IgE/Ag-stimulated mast cells.¹⁴ However, we found previously that Fyn, Lyn and Syk were not

phosphorylated upon exposure of BMMC to HSP70. Instead, Lck and LAT were phosphorylated, by which degranulation and mediator release were induced.⁹ This, together with reports of LAT phosphorylation by phosphorylated Lck (phospho-Lck) as well as by phospho-Syk in T cells,¹⁵ led us examine if Lck phosphorylation could be induced by different stimuli. As shown in Figure 2A, Lck is indeed phosphorylated in BMMC stimulated with IgE/Ag, SCF, eMIP, CCL3 in addition to HSP70.

To confirm role of Lck in the signaling cascade that leads to degranulation and mediator release in stimulated mast cells, we examined the effect of A770041, a selective inhibitor of Lck,¹⁶ on degranulation and mediator release. As shown in Figure 2B, phosphorylation of Lck and downstream signaling molecules in IgE/Ag-stimulated BMMC were inhibited by this compound, whereas phosphorylation of Fyn and Syk, (red box) was not. These results suggest that phospho-Lck triggers activation of signal transduction pathways initiated by LAT phosphorylation. Degranulation and eicosanoid release from IgE/Ag-stimulated BMMC were markedly inhibited by this compound (Figure 2C). Inhibition of Lck and LAT phosphorylation, as well as degranulation and LTC₄ release, by A770041 was also observed in SCF-stimulated BMMC (Figures 2D), whereas A770041 did not inhibit phosphorylation of Fyn, Lyn and Syk (red box).

In contrast to these stimuli (IgE/Ag and SCF), neither eMIP (Figure 2E top) nor CCL3 (Supplementary Figure1A) induced phosphorylation of Fyn, Lyn or Syk (red box), though they induced phosphorylation of Lck (and LAT). As shown in Figure 2E, inhibition of Lck phosphorylation by A770041 (top) accompanied by inhibition of degranulation and LTC₄ release (bottom). Likewise, we have shown previously that HSP70 did not induce phosphorylation of Fyn, Lyn or Syk but did induce degranulation and mediator release.⁹ These results support the notion that activation of Lck by phosphorylation plays an essential role in phosphorylation of LAT and downstream signaling proteins leading to degranulation and mediator release induced by multiple stimuli. When mast cells are stimulated with either IgE/Ag or SCF, but not with eMIP, CCL3 or HSP70, phospho-Syk also works together with phospho-Lck in LAT

phosphorylation as shown below.

3.3 Partial inhibition of degranulation and mediator release from IgE/Ag- or SCF-stimulated mast cells by siRNA-induced Lck knockdown or mast cell-specific Syk-gene inactivation

We next tested the effect of Lck depletion on degranulation and mediator release in stimulated BMMC. Lck-specific siRNA was transiently transfected into BMMC and the cells were activated with IgE/Ag. As shown in Figure 3A, induction of degranulation and mediator release in the specific siRNA-transfected cells was 50% diminished compared to the cells transfected with the control (mock) siRNA. Under these conditions, Lck-siRNA transfection reduced Lck expression (Supplemental Figure 1B) without affecting expression of other signaling molecules and, upon stimulation with IgE/Ag, the reduced amount of phospho-Lck was accompanied by reduced phosphorylation of LAT, PI3K, Ras and Raf. A similar observation was made when the cells were stimulated with SCF and degranulation was diminished by 50% in the Lck-siRNA transfected cells (Figure 3B). In this case also, the reduced amount of phospho-Lck was accompanied by reduction of LAT phosphorylation (Supplemental Figure 1C). In contrast to IgE/Ag- or SCF-stimulation where degranulation was diminished by half by Lck-siRNA, degranulation decreased to control level when Lck-siRNA transfected cells were stimulated with HSP70, eMIP or CCL3 (Figure 3C). These results indicate that both phospho-Lck and phospho-Syk work together for LAT phosphorylation when mast cells are stimulated with either IgE/Ag or SCF, whereas only phospho-Lck works for LAT phosphorylation in the cells stimulated with eMIP, CCL3 or HSP70.

The relationship between Syk and Lck in IgE/Ag- and SCF-stimulated mast cells was further examined using mast cell-specific Syk-gene knockout (KO, *Syk*^{-/-}) mice. As shown in Figure 3D, levels of degranulation and eicosanoid release were greatly reduced in IgE/Ag-stimulated KO mast cells as compared with those in wild type cells. However, significant levels of these activities were retained in the

KO cells. A similar observation was made when SCF was used as stimulus instead of IgE/Ag. As shown in Figure 3E, reduced but significant levels of degranulation and LTC₄ release were observed in Syk-KO BMMC stimulated with SCF. Similarly, a weaker but significant PCA reaction was induced in Syk-KO mice, and reduced but significant serum levels of eicosanoids in the peripheral blood was detected (Figure 3F).

3.4 Inhibition of degranulation and mediator release from mast cells by CCR1-ligands

In the above experiments, we used eMIP and/or CCL3 as a ligand for CCR1. These ligands induced degranulation from BMMC in a dose-dependent manner, with a peak value of 12.5 nM (0.1 µg/ml) and enhanced IgE/Ag-induced degranulation. We found that if concentrations rose above 0.125 µM (1 µg/ml), eMIP inhibited degranulation from IgE/Ag-, SCF or HSP70-stimulated BMMC dose-dependently (Figure 4A). Release of eicosanoids and inflammatory cytokines from IgE/Ag- (Supplementary Figure 2A) and HSP70-stimulated BMMC was also inhibited dose-dependently (Supplementary Figure 2B) and percent inhibition by 5 µM eMIP of degranulation and release of LTC₄, PGD₂, TNF-α and IL-6 from IgE/Ag-simulated mast cells was 97, 95, 85, 82 and 90, respectively. Inhibition by eMIP was found at the level of Lck phosphorylation (Figure 4B) and phosphorylation of Fyn, Lyn and Syk was not affected (Figure 4C). Fyn and Lyn (and downstream signaling protein Syk) phosphorylation was inhibited by Src family-selective tyrosine kinase inhibitor PP2, used as a positive control. These results suggest that CCR1 acts as a common inhibitory receptor for FcεRI and other receptors where and when more than a certain amount of its ligand is present. This negative regulation system is controlled by micromolar doses of (M-dose) CCR1 ligand via Lck-phosphorylation inhibition. In the concentration-dependent sinusoidal regulation by CCR1-ligand, therefore, the convergence between CCR1 and other receptors including FcεRI occurs at the level of phosphorylation of Lck and LAT.

In vivo, mast cell dependent PCA reaction was found to be inhibited by *i.v.* administration of eMIP. As shown in Figure 4D, dye extravasation was significantly reduced by 35.4% in mice treated with eMIP 1 hour prior to Ag injection, as for treatment with 50 mg/ml of fexofenadine-HCl (Fexo), a histamine H1 inhibitor. At the same time, serum levels of LTC₄ and PGD₂ in the peripheral blood were markedly reduced (Figure 4D). This inhibitory effect was not observed when eMIP was administered 1 hour after injection of Ag.

3.5 Lck-phosphorylation inhibition by eMIP or A770041 is explained by de-phosphorylation of phospho-Lck with SHP-1

Although eMIP (above 0.125 μ M) and A770041 both inhibit degranulation and mediator release phenomenologically by inhibiting Lck phosphorylation, it is possible that these compounds stimulate de-phosphorylation of Phospho-Lck by activating a tyrosine phosphatase rather than inhibiting phosphorylation of Lck. Since the protein-tyrosine phosphatase SHP-1 is known to be a negative regulator of signaling pathways in hematopoietic cells and is reported to de-phosphorylate phospho-Lck,¹⁷ we examined if eMIP and A770041 could induce SHP-1-mediated de-phosphorylation of phospho-Lck. Immunoprecipitation studies (Figures 5A and B) show that phospho-Lck formed in IgE/Ag stimulated BMMC was, in fact, de-phosphorylated dose dependently by the presence of eMIP or A770041 (red box). These figures also show, at the same time, that SHP-1 and phospho-Lck but not free Lck form a complex (SHP-1/Lck-P(Tyr)) and co-precipitate (blue box) when IgE/Ag-stimulated BMMC are exposed to 5 μ M eMIP or 1 μ M A770041. Figure 5C shows that eMIP induced de-phosphorylation of phospho-Lck at Tyr-394, which is known to activate Lck,¹⁷ but not at Tyr-505.

To confirm binding between SHP-1 and phospho-Lck, we examined if decreased levels of SHP-1 by specific siRNA treatment increases free phospho-Lck levels in eMIP- or A770041-treated cells. As shown

in Figure 5D, no free phospho-Lck was found in the cells treated with eMIP (Column 3, red box) due to complex formation with SHP-1 (Column 3, blue box), whereas free phospho-Lck appeared in the siRNA treated cells (Column 6, red box) in parallel with a reduced amount of co-precipitated SHP-1/phospho-Lck complex (SHP-1-Lck-P(Tyr): Column 6, blue box). Like eMIP, Lck-inhibitor A770041 induced complex formation between SHP-1 and phospho-Lck (SHP-1-P(Tyr): Figure 5E, Column 3, blue box). No free phospho-Lck was found (Column 3 red box). The complex was decreased with SHP-1 siRNA (Column 6, blue box) and free phospho-Lck appeared in the cells (Column 6, red box). Similarly, the SHP-1 inhibitor, NSC-87877 gave similar results as SHP-1 siRNA and free phospho-Lck appeared (Figure 5F, Column 4, red box) in parallel with a reduced amount of co-precipitated SHP-1-phospho-Lck (Column 4, blue box). Inhibited degranulation from IgE/Ag-stimulated BMMC by eMIP (Figure 5G) or A770041 (Figure 5H) was significantly restored by SHP-1-siRNA treatment or by NSC87877.

4 DISCUSSION

Up to now the signal transduction pathways mediated by various receptors expressed on mast cells were thought to be complex and redundant, except in the case of FcεRI mediated signaling. The crosstalk picture between FcεRI and other receptors was not complete and considered to occur relatively downstream of the signal transduction pathways.² As summarized in Figure 6, we demonstrated that phosphorylation of LAT is induced by any one of IgE/Ag, SCF, HSP70 or CCL3/eMIP (N-dose/nanomolar doses). LAT contains a short extracellular region, a single transmembrane spanning region and a long intracellular region.¹⁸ The molecule functions as an adapter protein by facilitating the formation of a multiprotein complex with various signaling proteins and is indispensable for T cell activation via its receptor.¹⁸ In mast cells, we found that LAT phosphorylation also plays a central, common, role in all receptor-mediated activation systems examined in addition to FcεRI.

Upon activation of mast cells by IgE/Ag, tyrosine residues of FcεRI subunits are phosphorylated by associated Fyn and Lyn, resulting in recruitment of Syk.^{14,19} This kinase in turn triggers LAT phosphorylation. In this paper, we show that phosphorylation of Lck occurs in IgE/Ag-stimulated BMMC, which in turn mediates LAT phosphorylation. It is important to note that the Lck-LAT phosphorylation system is also activated in mast cells treated with other stimuli including SCF, eMIP/CCL3 and the reported HSP70.⁹ This system, therefore, plays an indispensable role commonly in the FcεRI-, KIT-, CCR1- and TLR4-mediated signal transduction pathways leading to degranulation and mediator release. In contrast, the Fyn-Lyn-Syk phosphorylation system plays a role only in FcεRI- or KIT- mediated signaling. The role of phospho-Lck and phospho-Syk in LAT phosphorylation in mast cells was further examined by Lck depletion from BMMC by specific siRNA or Syk-gene inactivation. Degranulation (and eicosanoid release) induced by IgE/Ag or SCF was inhibited by 50% following Lck depletion or Syk-gene knockout, whereas that induced by eMIP/CCL3 or HSP70 decreased to the control level upon Lck depletion. The findings

again confirmed that both phospho-Syk and phospho-Lck are associated with LAT phosphorylation in IgE/Ag- or SCF-stimulated cells, whereas only phospho-Lck is associated with N-dose eMIP/CCR3- or HSP70-stimulated cells.

In this paper, we show for the first time that CCR1 can act as a negative receptor for mast cell activation when concentrations of its ligand rise above a certain level. *In vitro*, mast cell dependent PCA reaction was inhibited by *i.v.* administration of eMIP, and serum levels of LTC₄ and PGD₂ in the peripheral blood were reduced. Although the present results derive from model animals, it is conceivable that CCR1 ligands generated at inflammation sites could suppress mast cell activities induced by IgE/Ag and/or other stimuli, and attenuate allergy symptoms in human. On the other hand, co-stimulation of eMIP/CCL3 with IgE/Ag was observed in the nanomolar range, suggesting a concentration-dependent sinusoidal regulation of allergy by CCR1 ligands where CCR1 is closely associated with inflammation mediated by mast cells, from release through subsidence. Since CCR1 is known to be expressed by various leukocytes including neutrophils, monocytes, T and B lymphocytes, natural killer cells, and CD34⁺ bone marrow cells,²⁰ it would be significant and important to test if a similar regulatory system by CCR1 ligands (viz. enhanced Lck phosphorylation and dephosphorylation of phospho-Lck by SHP-1) exists in other CCR1 expressing cells.

Receptors expressed on the surface of mast cells including FcεRI, KIT, TLR4 and CCR1, are known to become internalized upon exposure to appropriate ligands, before being recycled back to the membrane.^{19,21-24} At least FcεRI was shown to be colocalized with CCR1 at membrane rafts, by which their internalization is mutually enhanced,²⁵ and other receptors are considered also to have a special structural relationship with CCR1. To maintain mast cells in an activated state, continuous endocytosis of receptors and their re-expression on the cell surface is essential. The process requires activation of associated tyrosine kinase(s) by phosphorylation and deactivation by de-phosphorylation. Activated Lck is reported to be associated with ligand-induced receptor internalization, surface redistribution and receptor destruction^{26,27}

and SHP-1 plays a role in de-phosphorylation of phospho-Lck in T cells.¹⁷ In the present findings, coupled receptors (FcεRI, KIT, TLR4 and CCR1) with their ligands (IgE/Ag, SCF, HSP70 and eMIP/CCL3, respectively) mediated phosphorylation of Lck and, in the presence of either M-dose eMIP or A770041, phospho-Lck was de-phosphorylated by coupled and activated SHP-1. The results strongly suggest that FcεRI, KIT, TLR4 and CCR1 are internalized upon exposure to their ligands with the aid of activated Lck, and M-dose eMIP or A770041 terminate receptor recycling by stimulating SHP-1 de-phosphorylation activity against phospho-Lck (Figure 6, inset). The termination of receptor recycling by de-phosphorylation of phospho-Lck may also explain the extensive inhibition by A770041 or M-dose eMIP of degranulation and mediator release in IgE/Ag- and SCF-stimulated mast cells (see Graphs in Figures 2C, 2D and 4A), in which phospho-Syk also mediates degranulation and mediator release.

In conclusion, signal transduction pathways in mast cells stimulated with IgE/Ag, SCF, HSP70 and N-dose eMIP/CCL3 can be unified by the theory summarized in Figure 6. In all cases tested, LAT phosphorylation triggers each receptor-mediated downstream signaling cascade that controls all cellular responses, while Lck plays a significant role in LAT phosphorylation. CCR1 ligands can control these processes either positively or negatively in a dose-dependent manner both *in vitro* and *in vivo*. Our findings may open new avenues in chemokine/chemokine receptor research, since CCR1 is expressed widely by leukocytes in the bone marrow as well as in the peripheral blood.

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CONFLICT-OF-INTEREST

The authors declare that they have no conflicts of interests.

AUTHOR CONTRIBUTIONS

The conception and design are attributable to SK, HWC and TT. FJ, YD, ZY, JHC, DYK and MT performed experiments. HWC supervised the experiments and acquired data. JRK and YJL contributed to data analysis. SK, HWC, NT-S and TT interpreted data and prepared the manuscript. All authors approved the final version of the manuscript.

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FIGURE Legends

FIGURE 1 Phosphorylation of LAT and downstream signaling proteins in BMMC stimulated with IgE/Ag, SCF, HSP70, eMIP or CCL3. Except for tyrosine phosphorylation of LAT and PI3K, total cell lysate was used for immunoblotting. For LAT and PI3K, an immuno-precipitate was used. Cells were stimulated for 15 min unless otherwise stated. Note that in all cases tested, LAT was phosphorylated (red box). (A) Phosphorylation of signaling molecules in BMMC stimulated with IgE/Ag (incubated with 500 ng/ml of anti-DNP IgE antibody overnight, then stimulated with 100 ng/ml DNP-HSA). (B) Time course of degranulation induced by 0.1 μ g/ml SCF and protein phosphorylation. As an indicator of degranulation, released β -hexosaminidase (β -Hex) in the supernatant was evaluated by spectrophotometry. (C) Time course of degranulation induced by 10 μ g/ml HSP70 and protein phosphorylation. (D) Time course of degranulation induced by IgE/Ag, eMIP or CCL3 and phosphorylation of LAT and PLC γ 1. (E) Enhanced degranulation and LAT phosphorylation in IgE/Ag-stimulated BMMC by eMIP. Data in graphs are expressed as means $\pm$ SEM of three independent experiments with different BMMC. Significant differences from control (open column: B-D) and between $\pm$ eMIP (E) are indicated by * $P < 0.05$ and ** $P < 0.01$.

FIGURE 2 Lck phosphorylation in BMMC stimulated with IgE/Ag, SCF, HSP70 or eMIP and its inhibition by A770041, a Lck inhibitor. A770041 (1 μ M) was added 1 hour prior to stimulation. Other experimental conditions are described in the legend for Figure 1. (A) Lck phosphorylation induced by IgE/Ag, SCF (0.1 μ g/ml), HSP70 (10 μ g/ml), eMIP or CCL3 (12.5 nM each). (B) Phosphorylation of signaling molecules in IgE/Ag-stimulated BMMC and inhibition of Lck- and downstream signaling molecule-phosphorylation by A770041. Note that phosphorylation of Fyn and Syk (red box) was not inhibited. (C) Inhibition of degranulation and eicosanoid release from IgE/Ag-stimulated BMMC by A770041. After 15 min incubation with Ag, released β -Hex and eicosanoids in supernatant were evaluated. (D) Phosphorylation of

signaling molecules in BMMC stimulated with 0.1 $\mu\text{g/ml}$ SCF and inhibition of Lck and LAT phosphorylation by A770041. Note that phosphorylation of Fyn, Lyn and Syk (red box) was not inhibited. Graphs: Inhibition of degranulation and LTC₄ release by A770041 from SCF-stimulated-BMMC. (E) Phosphorylation of signaling molecules in BMMC simulated with 12.5 nM eMIP and inhibition of Lck and LAT phosphorylation by A770041. Note that phosphorylation of Fyn, Lyn and Syk (red box) was not induced by eMIP. Graphs: Inhibition of degranulation and LTC₄ release by A770041 from eMIP-stimulated-BMMC. Significant differences from data without A770041 in graphs are indicated by *** $P < 0.001$.

FIGURE 3 Effect of Lck depletion (A-C) or Syk gene knockout (D-F) on degranulation and eicosanoid release from stimulated BMMC. Data in graphs are expressed as means $\pm$ SEM of three independent experiments with different BMMC. (A) Inhibition of degranulation and eicosanoid release from Lck-siRNA-treated BMMC (open column). Mock siRNA (M) treated BMMC were used as a control (closed column). BMMC cultured in serum-free medium were transfected with mouse Lck siRNA using a Dharma FECT transfection reagent according to the manufacturer's protocol. (B) Inhibition of degranulation from 0.1 $\mu\text{g/ml}$ SCF-stimulated BMMC by Lck depletion. (C) Inhibition of degranulation from 10 $\mu\text{g/ml}$ HSP70- or 12.5 nM each of eMIP- or CCL3-stimulated BMMC by Lck depletion. Significant differences between Lck siRNA and mock siRNA treatments in (A)–(C) are indicated by ** $P < 0.01$ and *** $P < 0.001$. (D) Inhibition of degranulation and eicosanoid release from Syk-KO BMMC stimulated with IgE/Ag. Mast cell-specific Syk-KO mice were obtained by crossing Syk-floxed mice with mast cell specific *Mast-Cmal-Cre* mice on a C57BL/6 background. (E) Inhibition of degranulation and LTC₄ release from Syk-KO BMMC stimulated with SCF. (F) PCA reaction induced in Syk-KO mice by IgE/Ag and serum levels of eicosanoids in the peripheral blood. PCA reaction was induced in one ear of a sensitized mouse (n=6) by intradermal Ag (DNP-HSA) challenge. The reaction was visualized by dye extravasation 1 hour after simultaneous injection of Evans Blue with Ag and quantitated by extraction of dye from ear tissues. Serum levels of LTC₄ and PGD₂ were

determined 1 hour after Ag challenge. In graphs of (D)–(F), significant differences of KO from WT BMMC are indicated by ** $P<0.01$ and *** $P<0.001$; and those from control by [#] $P<0.01$ and ^{###} $P<0.001$.

FIGURE 4 Effect of M-dose eMIP on mast cell activation *in vivo* and *in vitro*. (A) Dose dependent inhibition by eMIP of degranulation from IgE/Ag-, SCF- or HSP70-stimulated BMMC. eMIP was added 1 hour before stimulation. Experiments were repeated at least three times in triplicate, each time using different BMMC. (B) Inhibition of Lck phosphorylation by 5 μ M eMIP in stimulate BMMC (red box). (C) Effect of eMIP on phosphorylation of Fyn, Lyn and Syk (red box). Note that no inhibition of phosphorylation of these proteins was found. Src family-selective tyrosine kinase inhibitor PP2 was used as a positive control. PP2 was added 1 hour before stimulation. (D) Inhibition by eMIP of mast cell dependent PCA reaction and eicosanoid release to sera. PCA reaction was induced in one ear of a sensitized mouse (n=6) as described in the legend for Figure 3 an MTHODS. eMIP (50 μ g/kg; its half-life in the peripheral blood is approximately 1 hour) or Fexo was administrated intravenously or orally, respectively, 1 hour before Ag challenge. In (A) and (D), data are expressed as means $\pm$ SEM. Significant differences from control without eMIP or Fexo are indicated by * $P<0.05$, ** $P<0.01$ and *** $P<0.001$.

FIGURE 5 Complex formation between SHP-1 and phospho-Lck, and de-phosphorylation of phospho-Lck by SHP-1 activated by eMIP or A770041. (A) Dose dependent de-phosphorylation of phospho-Lck induced by eMIP or (B) by A7700421 (red box) and complex formation between phospho-Lck and SHP1 (blue box). Anti-Lck or anti-SHP-1 antibody was used for immunoprecipitation (ImPre), and anti-Lck, anti-phospho-tyrosine (P(Tyr)), and anti-SHP1 antibodies were used for immunoblotting (ImBlot). Indicated concentration of eMIP or A770041 was added 1 hour before Ag challenge. (C) De-phosphorylation of phospho-Lck at tyrosine 394 induced by eMIP in IgE/Ag simulated BMMC. Anti-phospho-tyrosine (Tyr395 and Tyr 505) antibodies were used for detection. (D) Effect of SHP-1 depletion by SHP-1-siRNA on binding

between SHP-1 and phospho-Lck induced by eMIP or (E) A770041. M indicates mock siRNA treatment. Note that free phospho-Lck levels increased in eMIP- or A770041-treated cells (column 6, red box). (F) Increased free phospho-Lck and decreased SHP-1-phospho-Lck complex levels by SHP-1 inhibitor NSC-87877. (G) Reversed activity by SHP-1-siRNA or a SHP-1 inhibitor NSC-87877 of eMIP- or A770041 (H)-induced degranulation inhibition. BMMC were incubated first with or without eMIP or A770041 for 1 hour and then with or without SHP-1 siRNA or NSC-87877 before Ag challenge for 15 min. Data in graphs are expressed as means $\pm$ SEM of three independent experiments with different BMMC. Significant differences from IgE/Ag alone (second column) are indicated by * P <0.05, ** P <0.01 and *** P <0.001 and from IgE/Ag + A770041 (third column) by #### P <0.001.

FIGURE 6 Summary of the present findings. Binding of eMIP/CCL3 (N-dose), HSP70, SCF or IgE/Ag to their receptors, CCR1, TLR4, KIT or Fc ϵ RI, respectively, induces Lck phosphorylation, which in turn phosphorylates LAT. In the cases of SCF and IgE/Ag but not eMIP/CCL3 and HSP70, phosphorylation of Fyn, Lyn and Syk, is also induced, and phospho-Syk is associated with LAT phosphorylation. Phospho-LAT triggers PLC γ and PI3K phosphorylation followed by a chain of downstream signaling-protein activation (phosphorylation). PI3K may also be activated by other parallel pathways. The processes lead to degranulation and mediator release. Inset: All receptors internalize after binding their ligands with aid of phospho-Lck.^{19,21-24} Activated SHP-1 by M-dose eMIP/CCL3 or A770041, de-phosphorylates phospho-Lck, by which receptor recycling is terminated. Dotted lines and circles indicate presence of other signaling molecule(s) in between. Lck and phospho-Lck may indirectly bind to the membrane.

SUPPLEMENTAL FIGURE Legends

SUPPLEMENTARLY FIGURE 1 Phosphorylation of signaling molecules in stimulated BMMC and its inhibition by A770041 or Lck siRNA. (A) Inhibition of Lck and LAT phosphorylation by A770041 in BMMC stimulated with 12.5 nM CCL3. Note that phosphorylation of Fyn, Lyn and Syk (red box) was not induced by CCL3. (B) Effect of Lck depletion by Lck siRNA on phosphorylation of signaling molecules in IgE/Ag- or SCF(C)-stimulated BMMC. Mock siRNA(M)-treated BMMC were used as a control. In (B) and (C), BMMC cultured in serum-free medium were transfected with mouse Lck siRNA using a Dharma FECT transfection reagent according to the manufacturer's protocol. Other experimental conditions are described in the legend for Figures 1 and 2.

SUPPLEMENTARLY FIGURE 2 Inhibition by eMIP of mediator release from IgE/Ag- or HSP70-stimulated BMMC. eMIP was added 1-hour before 15 min-stimulation of BMMC with antigen or HSP70. (A) Dose dependent inhibition of LTC₄ and PGD₂ release from IgE/Ag stimulated BMMC by eMIP (Graphs). Percent inhibition of degranulation or release of LTC₄, PGD₂, TNF- α or IL-6 from IgE/Ag-stimulated BMMC by 5 μ M eMIP (Table). (B) Dose dependent inhibition of degranulation and release of LTC₄, PGD₂, TNF- α or IL-6 from HSP70 stimulated BMMC by eMIP. In graphs, data are expressed as means $\pm$ SEM. Significant differences from data without eMIP (second column) are indicated by * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$

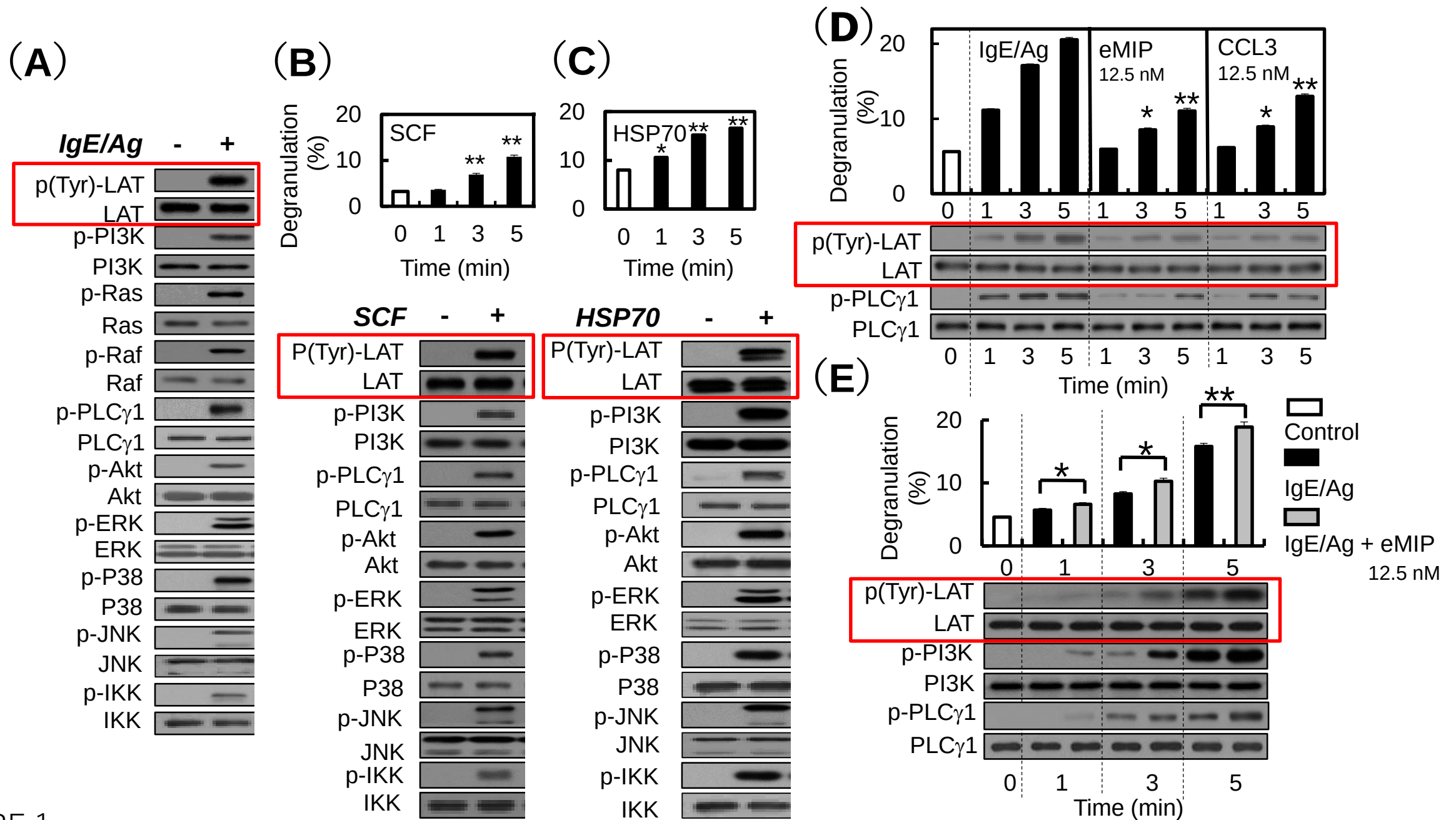


FIGURE 1

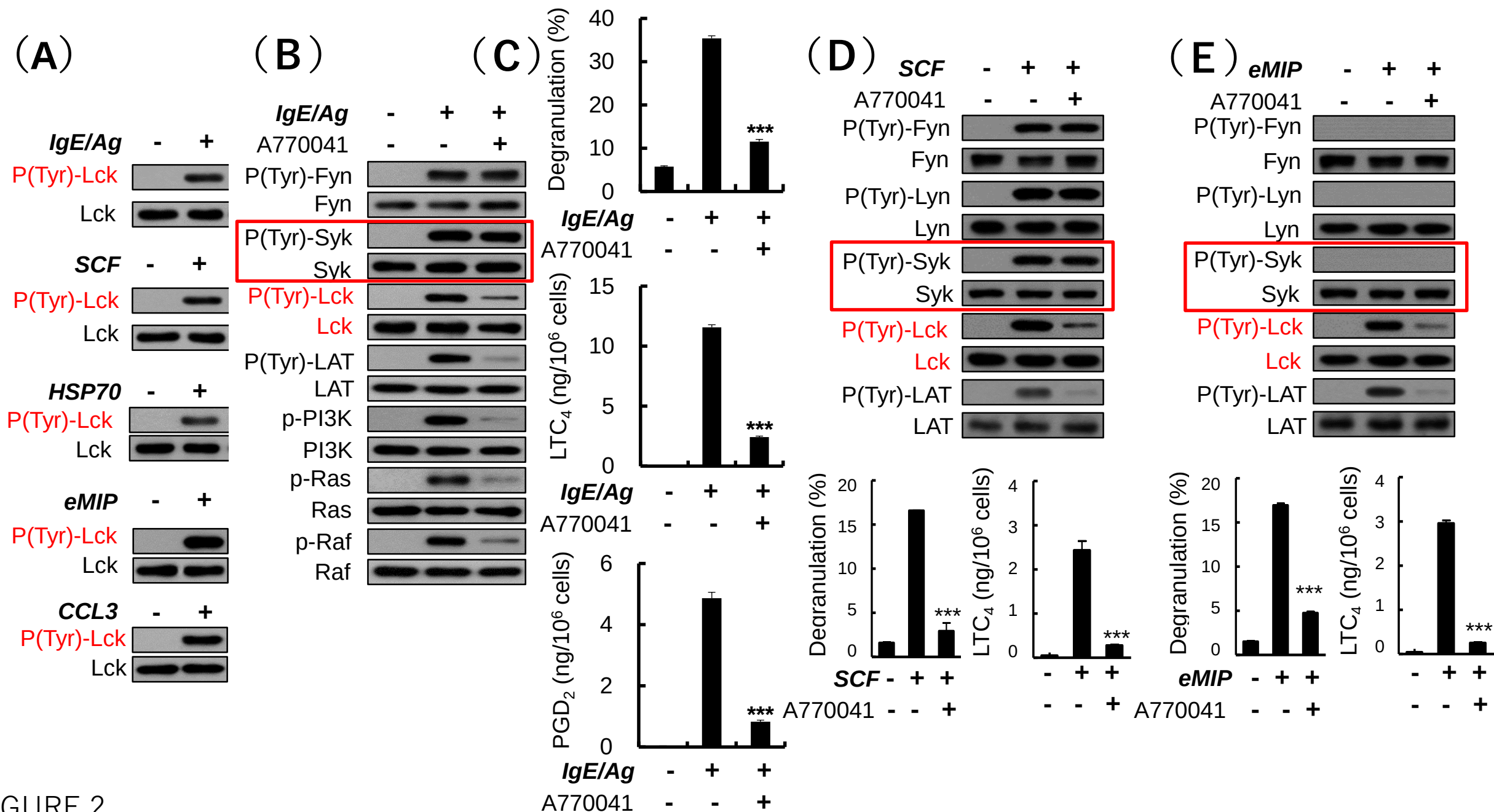


FIGURE 2

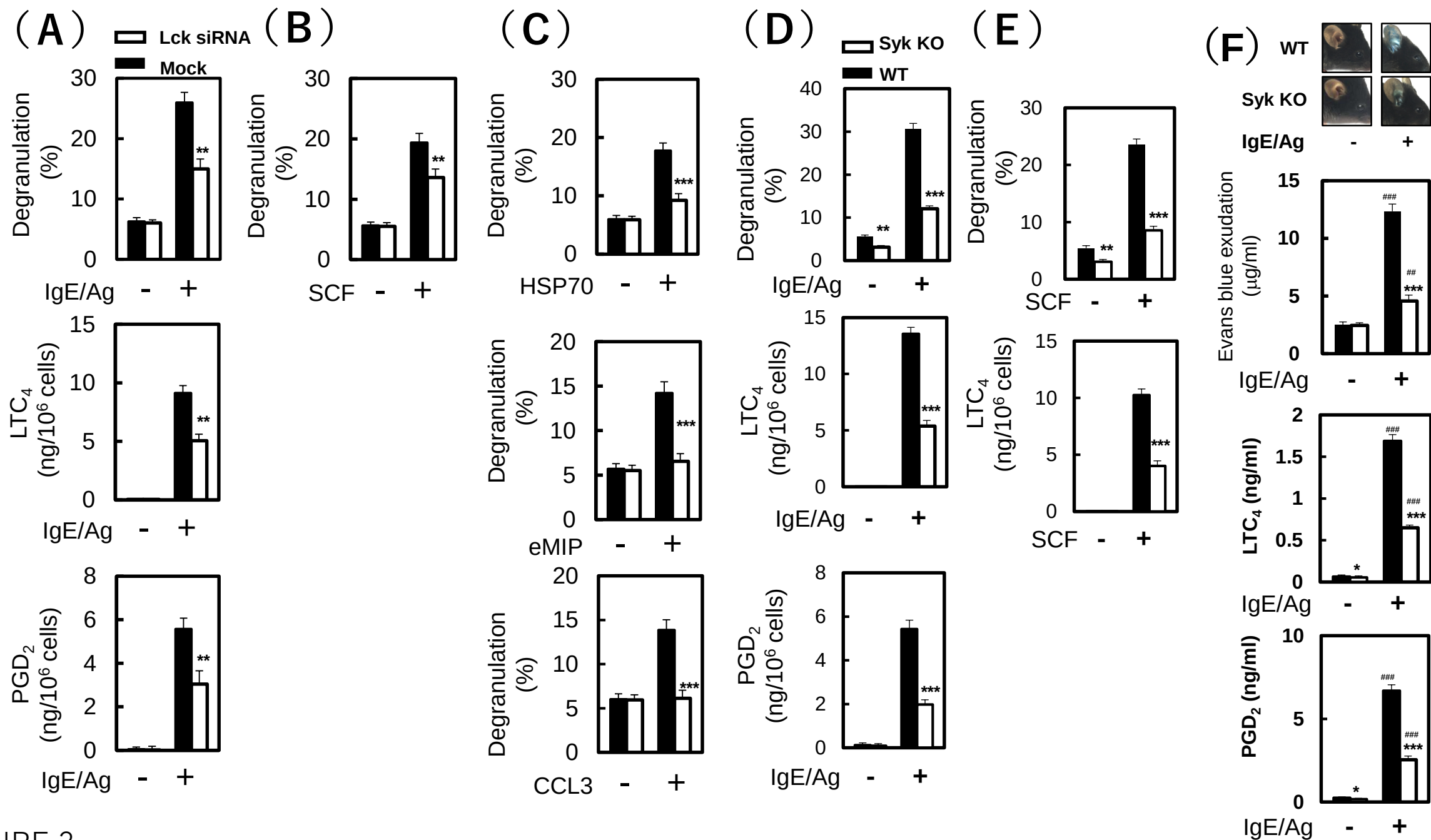


FIGURE 3

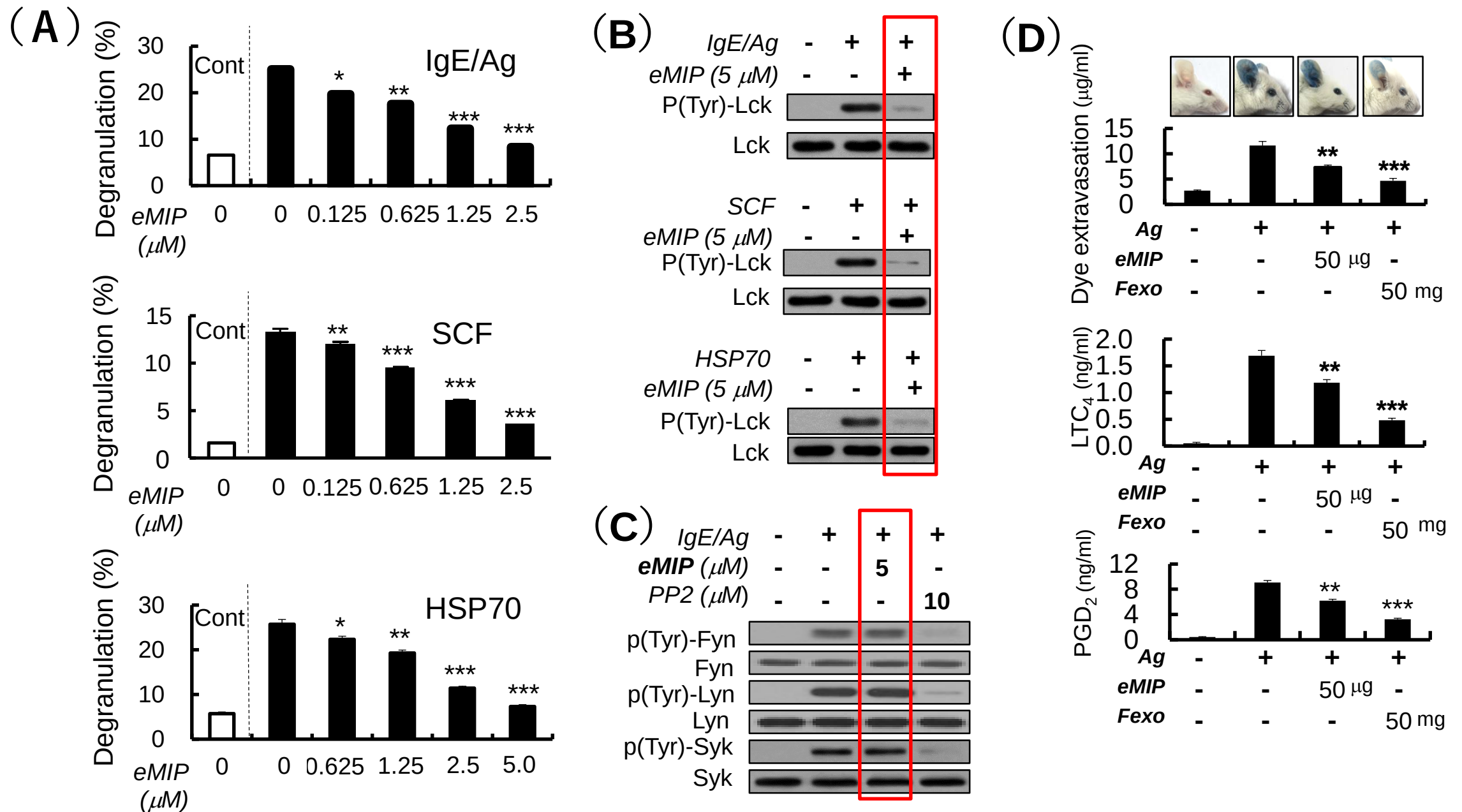


FIGURE 4

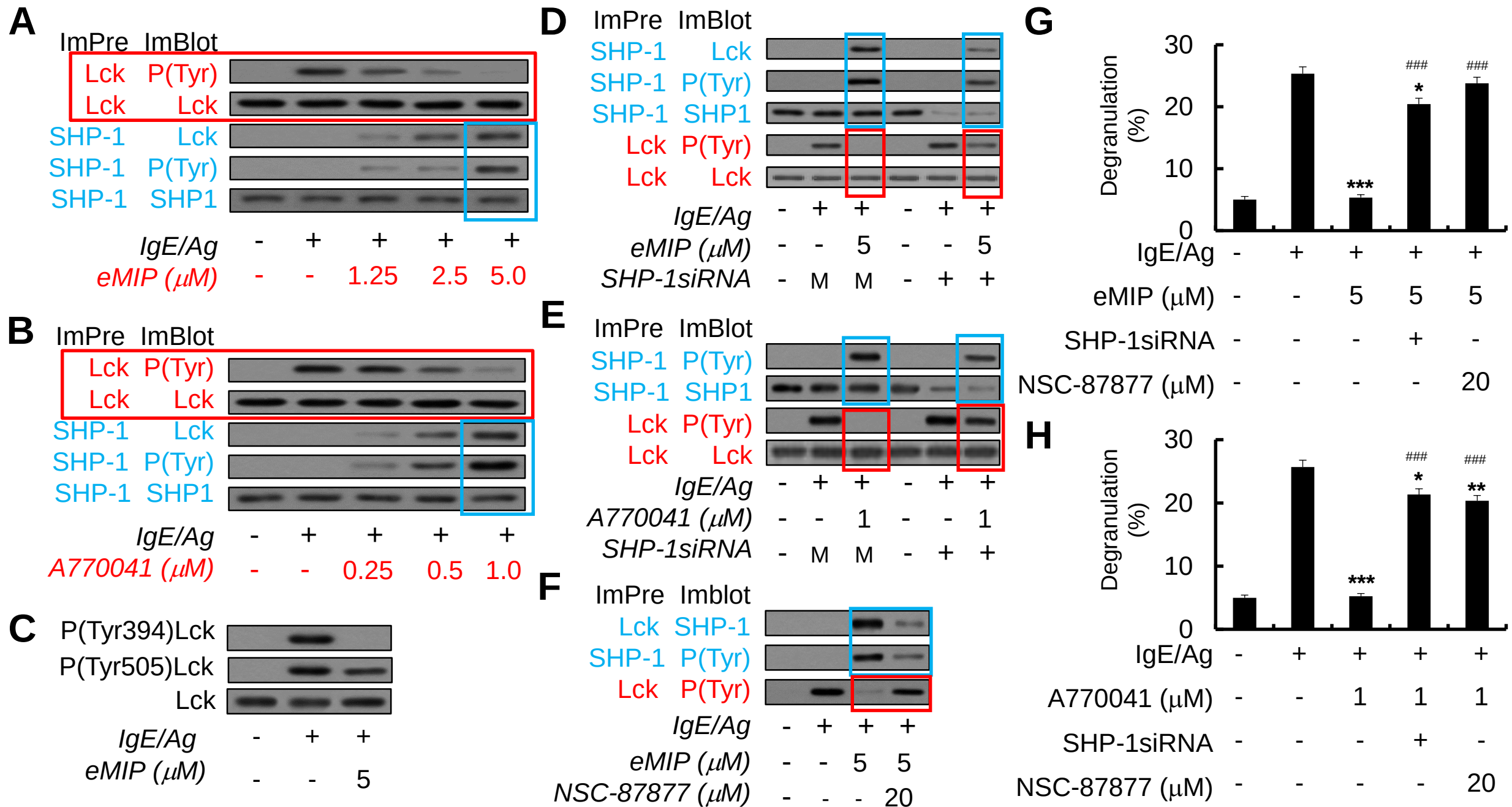


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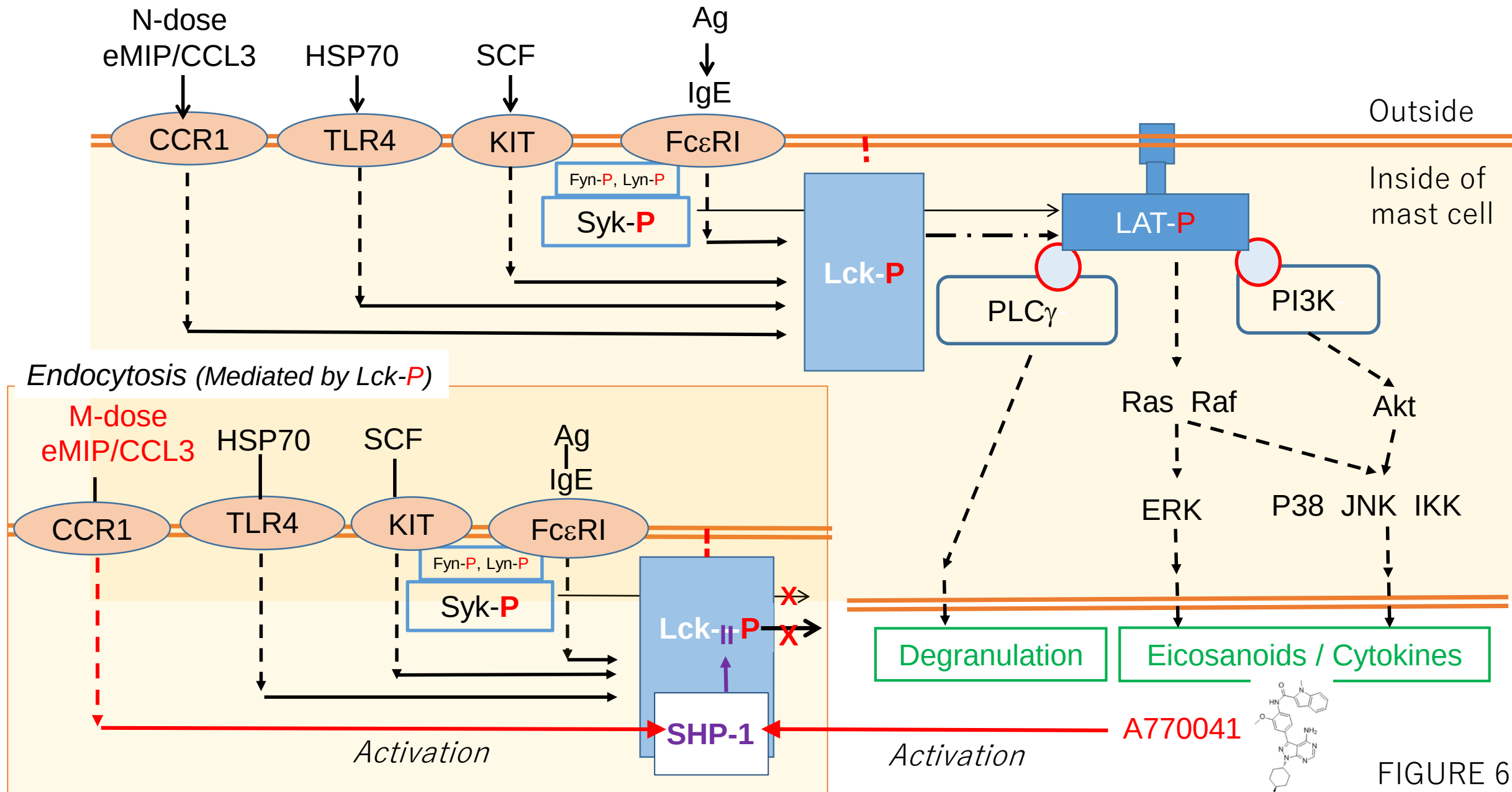
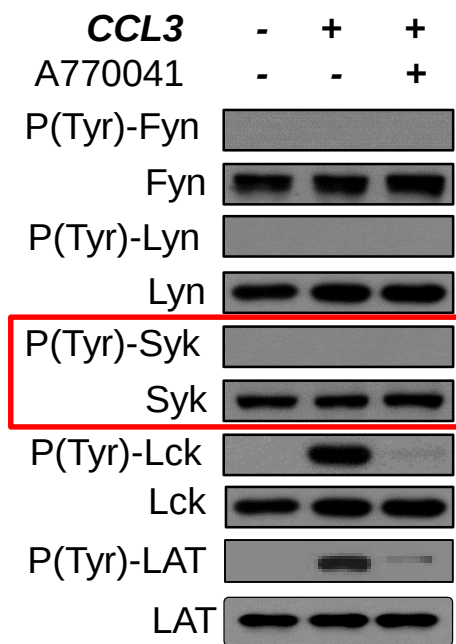
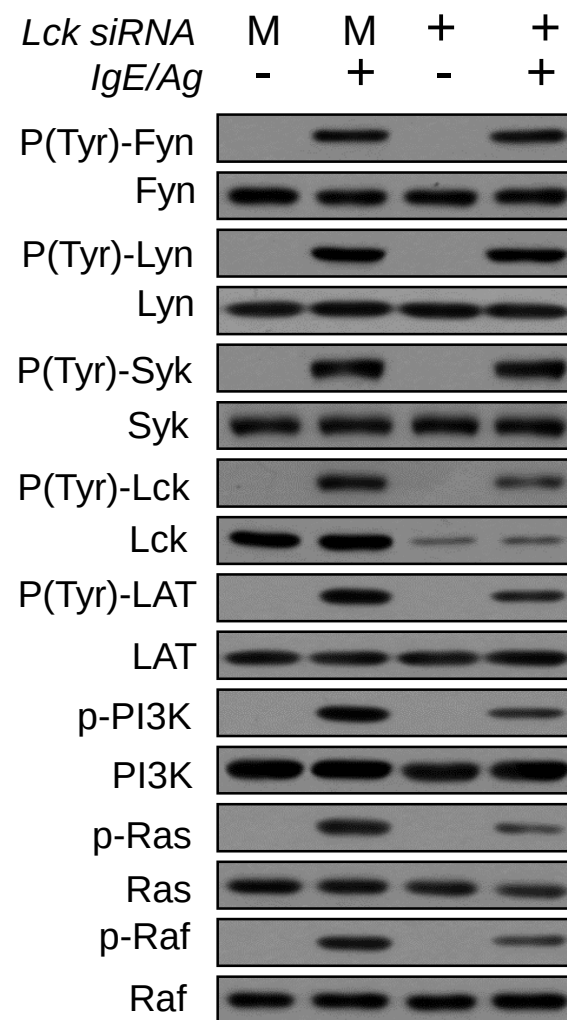
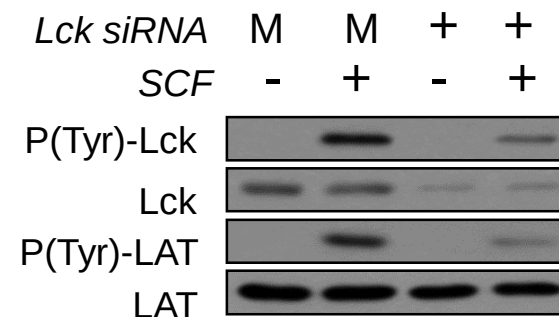
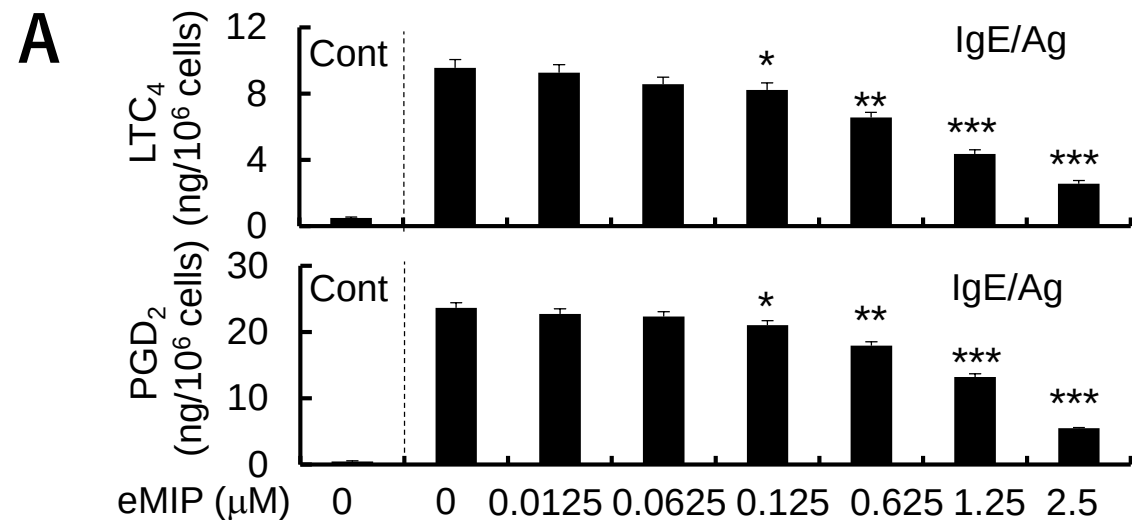
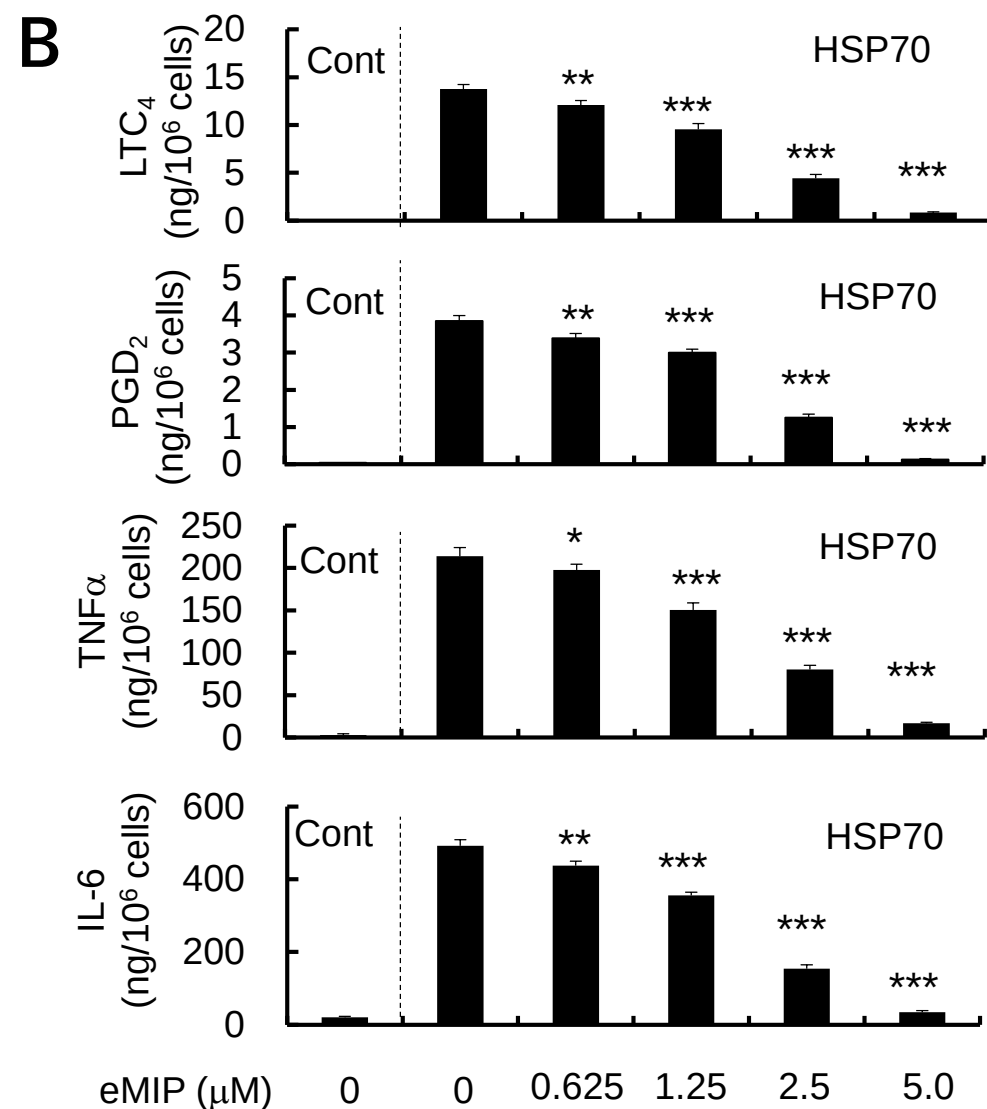


FIGURE 6

A**B****C**



	5 μM	Mean (n=3)	Inhibition
Degranulation (%)	None	12.4 ± 0.6	
	IgE/Ag	42.3 ± 2.1	
	IgE/Ag + eMIP	13.6 ± 0.7	97 %
LTC ₄ released (ng/10 ⁶ cells)	None	0.1 ± 0.0	
	IgE/Ag	21.9 ± 1.1	
	IgE/Ag + eMIP	1.2 ± 0.1	95 %
PGD ₂ released (ng/10 ⁶ cells)	None	0.1 ± 0.0	
	IgE/Ag	6.1 ± 0.3	
	IgE/Ag + eMIP	1.0 ± 0.1	85 %
TNF-α released (ng/10 ⁶ cells)	None	0.0 ± 0.0	
	IgE/Ag	357 ± 17.9	
	IgE/Ag + eMIP	65 ± 3.3	82 %
IL-6 released (ng/10 ⁶ cells)	None	0.0 ± 0.0	
	IgE/Ag	625 ± 28.1	
	IgE/Ag + eMIP	65 ± 2.9	90 %



SUPPLEMENTAL FIGURE 2