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Title Page

Methoxyluteolin inhibits neuropeptide-stimulated TNF, CXCL8 and VEGF release via mTOR activation from human mast cells

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Running Title Page

Neuropeptide-stimulation of human mast cells via mTOR

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corticotropin-releasing hormone (CRH); C-X-C motif chemokine ligand 8 (CXCL8); dimethyl sulfoxide (DMSO); Disodium cromoglycate (cromolyn); enzyme linked immunosorbent assay (ELISA); high affinity IgE receptor (FcεRI); ; luteolin (Lut); LY294002 (LY); mammalian target of rapamycin complex 1 (mTORC1); mammalian target of rapamycin complex 2 (mTORC2); mast cell (MC); ; 3',4',5,7-tetramethoxyluteolin (Methlut); neurotensin (NT); nuclear factor-kappa B (NF-κB); phosphatase and tensin homolog (PTEN); phosphorylation (p); phosphoinositide 3-kinase (PI3K); Rapamycin (Rap); Recombinant human stem cell factor (rhSCF); ribosomal p70S6 kinase (p70S6K); substance P (SP); tris-buffered saline (TBS); tumor necrosis factor (TNF); vascular endothelial growth factor (VEGF); soluble N-ethylmaleimide-sensitive factor attachment proteins (SNARE complexes); signal transducer and activator of transcription (STAT); 4E-binding protein 1 (4EBP1)

Abstract

Mast cells (MC) are critical for allergic reactions, but are also important in inflammatory processes. Stimulation by neuropeptides, such as substance P (SP) and neurotensin (NT) leads to release of pre-formed molecules stored in numerous MC secretory granules and newly-synthesized pro-inflammatory mediators, including tumor necrosis factor (TNF), interleukin 8 (CXCL8) and vascular endothelial growth factor (VEGF). Here, we investigate the role of mammalian target of rapamycin (mTOR) signaling in the stimulation of cultured human LAD2 MC by NT or SP, and the inhibitory effect of the natural flavonoids 3',4',5,7-tetrahydroxyflavone (luteolin) and its novel structural analog 3',4',5,7-tetramethoxyluteolin (methoxyluteolin). Stimulation by NT (10 μM) or SP (1 μM) increases ($p<0.0001$) the gene expression (after 6 h) and release (after 24 h) of TNF, CXCL8 and VEGF. This occurs via activation of both mTOR complexes, as denoted by the increased phosphorylated (p) protein levels ($p<0.0001$) of the downstream mTORC1 substrate pp70S6KThr389 and mTORC2 component pmTORSer2448. Pre-treatment of human MC using the mTORC1 inhibitor rapamycin or the mTORC1/mTORC2 inhibitor Torin1 or the two flavonoids decreases both gene expression and release ($p<0.0001$) of all three mediators. Methoxyluteolin is more potent human MC inhibitor than luteolin or Torin1, implicating other MC protein targets in addition to the mTOR complex. The present findings indicate that mTOR is partially involved in the neuropeptide-stimulation of MC, but the novel flavonoid methoxyluteolin inhibits the response entirely, suggesting that it may be developed for treatment of allergic and inflammatory diseases.

Introduction

Mast cells (MC) are unique immune cells that derive from hematopoietic precursors and mature in vascularized tissues (Rodewald, et al., 1996;Kitamura and Ito, 2005;Schmetzer, et al., 2016). In addition to stimulation by the high affinity IgE receptor (FcεRI) (Rivera, et al., 2008), MC are also activated by bacterial endotoxins, such as lipopolysaccharides via the toll-like receptors (Palaska, et al., 2016) and neuropeptides such as neurotensin (NT) (Lazarus, et al., 1977;Kulka, et al., 2007) and substance P (SP) (Church, et al., 1991;Theoharides, et al., 2010), Stimulated MC release pre-stored molecules, including histamine, heparin and tryptase, as well as *de novo* synthesized pro-inflammatory mediators, such as tumor necrosis factor (TNF), interleukin-8 (CXCL8) and vascular endothelial growth factor (VEGF) (Theoharides, et al., 2012). As a result, MC are not only involved in allergies (Galli and Tsai, 2012), but also in immunity (Galli, et al., 2008), mast cell disorders, including mastocytosis (Theoharides, et al., 2015), and inflammatory diseases (Theoharides and Cochrane, 2004;Theoharides, et al., 2012).

The neuropeptides SP (Chang and Leeman, 1970) and NT (Carraway and Leeman, 1973), initially characterized by Leeman and colleagues, are implicated in inflammatory processes (Mashaghi, et al., 2016). Increased levels of SP have been associated with inflammatory diseases (O'Connor, et al., 2004) and the cross-talk between endocrinology system and skin (Caraffa, et al., 2016), leading to disorders, such as psoriasis (Remröd, et al., 2007).Circulating levels of NT have been reported in patients with psoriasis and atopic dermatitis (Vasiadi M, et al., 2012). MC-derived CXCL8 enhances recruitment of immune cells to the site of inflammation (Salamon, et al., 2005), while VEGF can increase vascular permeability (Donelan, et al., 2006). Even though the allergic stimulation of MC is fairly well characterized (Rivera, et al., 2008), less is known about the stimulation of MC by neuropeptides. A receptor (Mrgprx2) was recently identified as being critical in pseudo-allergies, some of which

are due to MC activation by cationic drugs and molecules (McNeil, et al., 2015), but NT and SP were not studied.

The phosphatidylinositol-3-kinase (PI3K)-dependent mammalian target of rapamycin (mTOR) kinase pathway (Dibble and Cantley, 2015) has been implicated in the regulation of normal MC homeostasis and function (Kim, et al., 2008a), including FcεRI-mediated allergic responses (Smrz, et al., 2011), but has not been studied in neuropeptide-stimulated MC. The mTOR catalytic subunits exists in two complexes: (a) mTORC1, involved in protein translational control by phosphorylation of ribosomal S6 kinases (S6K) and the eukaryotic initiation factor 4E-binding proteins (4EBP1), and (b) mTORC2 implicated in cytoskeleton reorganization (Laplante and Sabatini, 2012). It was previously shown that mTORC1 is involved in the allergic stimulation of MC (Kim, et al., 2008b) and is constitutively activated in cultured human LAD2 MC (Kim, et al., 2008a). Other studies have revealed that mTORC2 mediates MC chemotaxis (Kuehn, et al., 2011) and proliferation of neoplastic human MC (Smrz, et al., 2011). Downregulation of the upstream mTOR inhibitory protein phosphatase and tensin homolog (PTEN) leads to increased MC activation (Furumoto, et al., 2006) and a mastocytosis-like state (Furumoto, et al., 2011).

There are still no clinically effective MC inhibitors (Theoharides, et al., 2012;Finn and Walsh, 2013). Disodium cromoglycate (cromolyn) is known as a “MC stabilizer” because it inhibits rodent peritoneal MC histamine release (Theoharides, et al., 1980), but it does not effectively inhibit either murine MC (Oka, et al., 2012) or human MC (Weng, et al., 2012). Even though the local application of an experimental cromolyn ointment reduced histamine-induced pruritus in human skin, it apparently did so via modulation of sensory nerves and not by inhibiting MC (Vieira Dos, et al., 2010). Moreover, inhibitors of the tyrosine kinase c-kit receptor that reduce MC proliferation (Heinrich, et al., 2000) do not inhibit MC activation (Gotlib, et al., 2016). Tacrolimus was developed as an mTOR inhibitor for skin inflammatory diseases (Michel, et al., 1996), but it has been associated with allergic responses (Beck, 2005). Hence, there is an

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urgent need for developing effective inhibitors of human MC. The natural flavonoid 3,5,7,3',4'-pentahydroxyflavone (quercetin) has been shown to be more potent than cromolyn in inhibiting photosensitivity in humans (Weng, et al., 2012). Quercetin (Mlcek, et al., 2016) and 3',4',5,7-tetrahydroxyflavone (luteolin) are natural anti-oxidant and anti-inflammatory flavonoids (Middleton, et al., 2000), which also inhibit MC histamine, TNF and VEGF (Kempuraj, et al., 2005), as well as release of leukotrienes and prostaglandin D2 (Kimata, et al., 2000). We recently showed that its structural analog, 3',4',5,7-tetramethoxyflavone (methoxyluteolin) is a more potent MC inhibitor (Weng, et al., 2015) and is also metabolically more stable (Walle, 2007).

In the present study, we report that mTOR activation is involved in the stimulation of human MC by the neuropeptides NT and SP, and that this mechanism is inhibited by the flavonoids luteolin and methoxyluteolin.

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Methods

Materials

SP (S6883) and NT (N6383) were obtained from Sigma-Aldrich (St Louis, MO). PI3K inhibitor LY294002 (Cell Signaling Technology/CST, Danvers, MA) and mTOR inhibitors (rapamycin and Torin 1 (TOCRIS biosciences, Bristol, UK) were purchased. The flavonoids luteolin and methoxyluteolin were obtained from Pharmascience Nutrients (Clear Water, FL). RNeasy Mini (Qiagen Inc., Valencia, CA) and iScript cDNA synthesis kits (BioRad, Hercules, CA) were purchased. Taqman gene expression primers/assays for TNF (Hs99999043_m1), CXCL8 (Hs00174103_m1), VEGFA (Hs00900055_m1) and GAPDH endogenous control (4310884E) were purchased from Applied Biosystems (ThermoFisher Scientific, Foster City, CA). ELISA kits for TNF (DY210), CXCL8 (DY208) and VEGF (DY293B) were purchased from R&D Biosystems (Minneapolis, MN). Rabbit mAb for mTOR (7C10), pmTORSer2448 (D9C2), pmTORSer2481, mTORC1 substrates p70S6K (49D7), pp70SK Thr389 (108D2), 4EBP1 (53H11) and p4EBP1Thr37/46 (236B4), as well as the loading control β-actin (D6A8) were purchased from Cell Signaling Technology.

Human MC Culture

Human LAD2 MC (kindly supplied by Dr. A.S. Kirshenbaum, National Institutes of Health, NIH), derived from human MC leukemia (Kirshenbaum, et al., 2003), were cultured in StemPro®-34 SFM medium (Invitrogen, Carlsbad, CA) supplemented with 100 $U.mL^{-1}$ penicillin/streptomycin and 100 $ng.mL^{-1}$ recombinant human stem cell factor (rhSCF, kindly supplied by Biovitrum AB, Stockholm, Sweden). These cells have been used numerous times in our laboratory and shown to behave like primary human umbilical-cord blood derived MC. Cell viability was determined by trypan blue (0.4 %) exclusion.

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LAD2 MC Treatments

LAD2 MC were stimulated with NT (1-10 µM, Sigma-Aldrich, St. Louis, MO) or SP (1 µM, Sigma-Aldrich) and/or pre-incubated with DMSO or the following inhibitors: (a) PI3K inhibitors (LY294002 (LY), 1-10 µM, 2h, Cell Signaling Technology), (b) mTOR inhibitors (rapamycin (Rap), 0.005-0.2 µM or Torin1, 0.005-0.2 µM TOCRIS biosciences, Bristol, UK) and (c) the flavonoids (luteolin or methoxyluteolin, 1-50 µM, 2, 12 or 24 h, PharmaScience Nutrients, Clearwater, FL). All inhibitors were dissolved in water or DMSO with final concentration of < 0.1 %.

Pro-inflammatory Mediator Release by ELISA

Mediator release in cell-conditioned culture medium/ supernatant fluid was determined by using commercial available ELISA kits (R&D Systems, Minneapolis, MN) as per manufacturer's instructions. LAD2 MC (0.5×10^6 cells/well) were seeded in 96-well flat-bottom Falcon Culture plates (Becton Dickinson) prior to pretreatment with inhibitors/flavonoids (for 30 min) and NT- or SP-stimulation for 24 h. MC supernatant fluids were collected and TNF, CXCL8 and VEGF mediator release was measured. For all experiments, the control cells were treated with equal volume of culture medium or DMSO, and the minimum detectable level for all by ELISA was 5 $pg.mL^{-1}$.

MC Degranulation

LAD2 MC were stimulated with the neuropeptides, NT (10 µM, Sigma-Aldrich) or substance P (SP 1 µM, Sigma-Aldrich) for 30 min. Beta-hexosaminidase release was assayed using a fluorometric method as previously reported (Weng, et al., 2015). Briefly, beta-hexosaminidase activity in the supernatant fluid and cell lysates (0.5×10^5 cells per tube, were lysed with 1% Triton X-100 to measure residual cell-associated beta-hexosaminidase) were incubated with substrate solution (p-nitrophenyl-N-acetyl-beta-D-glucosaminide from Sigma-

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Aldrich) in 0.1 M NaOH/0.2 M glycine. Absorbance was read at 405 nm in a plate reader (Lab Systems Multiskan RC, Thermo Fisher), and the results were expressed as percentage of beta-hexosaminidase released over the total.

Pro-inflammatory Mediator Gene Expression by qRT-PCR

Total RNA from LAD2 MC was isolated using RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Reverse transcription (RT) was performed with 300 ng of total RNA using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA). Gene expression analysis of TNF, CXCL8 and VEGF mediators in LAD2 MC (1 $\times 10^6$ cells per 6-well plate), those pre-incubated with inhibitors/flavonoids and/or those stimulated with NT or SP, was measured after 6 h. Quantitative real-time PCR (qRT-PCR) was performed using Taqman gene expression assays to assess the gene expression of mediators using validated oligonucleotide primers (Applied Biosystems, Carlsbad, CA). Samples were run at 45 cycles using Applied Biosystems 7300 Real-Time PCR System. Relative mRNA abundance was determined from standard curves run for each experiment. Gene expression was normalized to GAPDH endogenous control.

Assessing mTOR Activation by Western blot & ELISA

The activation of mTOR was assessed by phosphorylation (p) of downstream mTORC1 and mTORC2 substrates by Western blot analysis and using Pathscan phospho-ELISA kits (R&D Systems). LAD2 MC (1 $\times 10^6$ cells per flask) were pretreated with inhibitors or flavonoids for the indicated doses and times, then stimulated with NT or SP for 0-60 mins. Thereafter, cells were pelleted and lysates harvested in radioimmunoprecipitation assay buffer (Sigma Aldrich), containing Halt Protease and Phosphatase Inhibitor Cocktails (Thermo Fisher Sci., Rockford, IL). The total protein concentrations were determined by the bicinchoninic acid assay (Thermo Fisher Sci.) using bovine serum albumin protein as standards. The total proteins (20 or 40 µg)

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were separated using 4-20 % Mini-PROTEAN TGX™ precast gels (BioRad) under SDS denaturing conditions and electro-transferred onto PVDF membranes (EMD Millipore). Blocking was carried out with 5% (wt/vol) BSA in Tris-buffered saline (TBS) containing 0.1 % Tween-20. The membranes were probed with the following primary antibodies: mTOR (7C10), pmTOR (Ser2448), mTORC1 substrates p70S6K, pp70SK (Thr389), 4EBP1 and p4EBP1 (Thr-37/46), while β-actin served as the loading control (Cell Signaling Technology/ CST, Danvers, MA). All proteins were visualized with horseradish peroxidase-conjugated secondary antibodies and then by SuperSignal West Pico enhanced chemiluminescence (Thermo Fisher Sci). To quantitate changes in protein phosphorylation, the films were scanned and densitometric analysis will be carried out using Image J software. In parallel experiments, using the CST's PathScan mTOR, pmTOR (Ser2448), p70S6K, pp70S6K (Thr389), 4EBP1 and p4EBP1 sandwich ELISA kits, the levels of total and phosphorylated mTORC1 substrates were measured in MC after treatments described.

Statistical Analysis

All conditions were performed in triplicate and all experiments were repeated at least three times (n=3). Results from cultured cells are presented as mean ± SD. Comparisons were made between (1) control and stimulated cells using the unpaired 2-tailed, Student's *t*-test, with significance denoted by the horizontal lines and by $p<0.05$ (*), $p<0.001$ (**) and $p<0.0001$ (***). In addition, multiple comparisons were made between (2) stimulated cells without inhibitors/flavonoids (DMSO or buffer control) and those with inhibitors/flavonoids using one-way ANOVA, followed by post-hoc analysis by Dunnett's Multiple Comparison Test; significance is denoted by horizontal lines and indicated values $p<0.001$ or $p<0.0001$ and (3) all the condition with inhibitor/flavonoid among themselves using one-way ANOVA, followed by post-hoc analysis by Tukey's Multiple Comparison Test; those conditions for which there was significance is denoted by the horizontal brackets and by the corresponding $p<0.05$ (*), $p<0.001$ (**) and

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p<0.0001 (***). All statistical analyses were performed by using the GraphPad Prism version 5.0 software (GraphPad Software, San Diego, CA, USA). Representative images for western blots were scanned and analyzed using Image J (NIH; https://imagej.nih.gov/ij/).

Results

Neuropeptide-stimulated human MC pro-inflammatory mediator release is inhibited by PI3K or mTOR inhibitors.

Initially, we investigated the optimal concentrations of NT or SP (1-10 μM) that stimulated the *de novo* pro-inflammatory mediator release from cultured human LAD2 MC. Stimulation by NT (10 μM) and SP (1 μM) significantly increased the release of TNF, CXCL8 and VEGF from human LAD2 MC after 24 h. Using these optimal concentrations, we next evaluated the involvement of mTOR signaling by employing pharmacological inhibitors of mTOR, which included the bacterial macrolide and more selective mTORC1 inhibitor rapamycin and the more potent ATP-competitive dual inhibitor of mTOR, Torin1, as well as the upstream PI3K inhibitor LY294002.

Pre-treatment of LAD2 MC (24 h) with the inhibitors of mTOR signaling, rapamycin and Torin1, dose-dependently (0.05-0.2 μM) decreases SP-stimulated TNF **(Fig. 1A)** and CXCL8 **(Fig. 1B)** release from human MC. At the optimal inhibitory concentration of 0.2 μM, Torin1 significantly decreases ($p<0.001$) pro-inflammatory mediator release from SP-stimulated LAD2 MC, as compared to rapamycin that is more selective for mTORC1 inhibition. Pre-treatment with the PI3K inhibitor, LY294002 (1-10 μM, 2 h) decreases ($p<0.0001$) TNF and CXCL8 release, with optimal inhibitory concentration of 10 μM **(Fig. 1C)**.

Neuropeptide-stimulated human MC degranulation is decreased by the PI3K inhibitor and the flavonoids luteolin and methoxyluteolin, but not mTOR inhibitors

Unlike their effect on *de novo* synthesized mediator release, pre-treatment of LAD2 MC with the mTOR inhibitors (rapamycin or Torin1, 0.2 μM, 24 h), followed by stimulation with either NT **(Suppl. Fig. 1A)** or SP **(Suppl. Fig. 1B)** for 30 min had no effect on β-hexosaminidase release from LAD2 MC. The upstream PI3K inhibitor (LY294002, 10 μM, 2 h) or the flavonoids

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luteolin and methoxyluteolin also had no effect on β-hexosaminidase release from LAD2 MC at 10 μM; however, all three compounds significantly decrease ($p<0.001$), β-hexosaminidase release at 50 μM, when used for 24 h **(Suppl. Fig. 1)**.

Neuropeptide-stimulated human MC pro-inflammatory mediator release is inhibited by the flavonoids luteolin and methoxyluteolin.

LAD2 MC were first pre-treated with luteolin and methoxyluteolin at various doses (1-50 μM, 2 h), then stimulated by NT (10 μM) for 24 h to identify the most effective concentration inhibiting release of pro-inflammatory mediators. Methoxyluteolin more potently than luteolin ($p<0.001$) decreases the release of all mediators at equimolar flavonoid concentrations **(Suppl. Fig. 2)**. We further compared the effects of the PI3K/mTOR inhibitors to the flavonoids luteolin and methoxyluteolin on LAD2 MC mediator release after neuropeptide stimulation for 24 h. LAD2 MC were pre-treated with inhibitors of mTOR (rapamycin or Torin 1, 0.2 μM, 24 h) or PI3K (10 μM, 2h) and the flavonoids luteolin or methoxyluteolin (50 μM, 2 h), and then stimulated by NT (10 μM) or SP (1 μM) for 24 h. The release of TNF, CXCL8 and VEGF significantly decreases in the presence of the flavonoids ($p<0.0001$), after stimulation by either NT **(Fig. 2A-C)** or SP **(Fig. 2D-F)**.

Neuropeptide-stimulated pro-inflammatory mediator gene expression in human MC is decreased by the PI3K or mTOR inhibitors, as well as luteolin and methoxyluteolin.

We further investigated whether mTOR signaling is involved in the gene transcription of pro-inflammatory mediators in human MC stimulated by either NT or SP. LAD2 MC were pretreated with the mTOR inhibitors rapamycin and Torin1 (0.2 μM, 24 h) and the upstream PI3K inhibitor LY294002 (10 μM, 2 h), prior to stimulation with NT (10 μM) or SP (1 μM) for 6 h. Stimulation of LAD2 MC by either NT **(Fig. 3A-C)** or SP **(Fig. 3D-F)** significantly increases

($p<0.001$) the gene expression of TNF, CXCL8 and VEGF, which decreases ($p<0.0001$) after treatment with the PI3K/mTOR inhibitors **(Fig. 3)**.

Pre-treatment with either luteolin or methoxyluteolin also significantly decreases ($p<0.0001$) gene expression of TNF, CXCL8 and VEGF in response to NT or SP **(Fig. 3)**. Methoxyluteolin (50 µM) is more potent ($p<0.05$) than luteolin or the PI3K inhibitor LY294002 ($p<0.001$) for inhibition of TNF and CXCL8, but not for VEGF gene expression **(Fig. 3C and 3F)**.

Neuropeptide stimulation of human MC activates signaling via mTOR that is inhibited by methoxyluteolin.

To investigate the specific mTOR complex activated in response to NT or SP stimulation, Western blot analysis was performed to detect the total and phosphorylated (p) levels of mTOR (pmTORSer2448, an indicator of signaling via mTORC2) and the mTORC1 substrates p70S6K (pp70S6KThr389) and 4EBP1 (p4EBP1Thr37/46) proteins **(Fig. 4A)**. Densitometric analysis revealed that stimulation of LAD2 MC with either NT (10 µM) or SP (1 µM) increases ($p<0.0001$) the levels of pmTORSer2448 **(Fig. 4A and 4D)** and the downstream mTORC1 substrate, pp70S6KThr389 **(Fig. 4B and 4E)** after 20 min, when compared to control cells. Noteworthy, stimulation with NT or SP had no effect on the levels of p4EBP1Thr37/46, as shown by Western blot **(Fig. 4C and 4F)**.

Since, PI3K upstream of mTOR signaling has been implicated in activation of MC (Kim, et al., 2008b), we further evaluated the inhibitory effects of luteolin and methoxyluteolin on mTOR in NT or SP stimulated LAD2 MC. LAD2 MC were pre-incubated with rapamycin or Torin 1 (0.2 µM, 24 h) or LY294002 (10 µM, 2 h) or luteolin and methoxyluteolin (50 µM, 2 h) prior to NT (10 µM) **(Fig. 5A-C)** or SP (1 µM) **(Fig. 5D-F)** stimulation for 20 min. The PI3K/mTOR and the flavonoids inhibitors significantly decrease levels of pmTORSer2448 and pp70S6KThr389, compared to those of neuropeptide-stimulated LAD2 MC.

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To further quantify the levels of phosphorylated mTOR and its substrates, phospho-ELISAs were also performed on LAD2 MC pre-treated with the PI3K/mTOR inhibitors or luteolin and methoxyluteolin, prior to stimulation with NT or SP. Levels of pmTOR Ser2448 (**Fig. 5A and 5D)** and pp70S6K Thr389 proteins **(Fig. 5B and 5E)** increase in response to stimulation with NT or SP, but significantly decrease ($p<0.001$) after pre-treatment with all the inhibitors. Noteworthy, methoxyluteolin shows greater reduction of pmTORSer2448 and pp70S6KThr389 levels, when compared to the PI3K inhibitor LY294002 ($p<0.0001$) or luteolin at equimolar flavonoid concentrations ($p<0.001$) **(Fig. 5).**

Discussion

A novel finding reported here is the involvement of mTOR signaling in the synthesis and release of TNF, CXCL8 and VEGF from cultured human MC in response to stimulation by neuropeptides. Stimulation of LAD2 MC by either NT or SP markedly increases levels of pmTORSer2448, the mTOR site phosphorylated by upstream PI3K signaling and the mTORC1-dependent p70S6K Thr389 site, indicating activation of mTOR. In addition, the dual mTORC1 and mTORC2 inhibitor, Torin1 is more potent than the predominant mTORC1 inhibitor rapamycin suggesting that both complexes may be involved in TNF, VEGF and CXCL8 gene expression in LAD2 MC. Our results are also in agreement with the previous report that rapamycin blocks FcεRI-mediated PI3K-dependent activation of mTORC1 signaling and *de novo* synthesized IL-6 and CXCL8 release from murine MC (Kim, et al., 2008b).

Signaling via mTORC1 had previously been implicated in FcεRI-mediated allergic cytokine release (Smrz, et al., 2011) and in the regulation of normal MC homeostasis (Kim, et al., 2008a).In fact, constitutively activated mTORC1 (Kim, et al., 2008a) is critical for the survival of LAD2 MC (Smrz, et al., 2011). Even though LAD2 MC derive from a patient with MC leukemia (Kirshenbaum, et al., 2003), these cells have repeatedly been shown to behave like primary human MC (Guhl, et al., 2010;Zhang, et al., 2012a;Weng, et al., 2015). One could argue that the findings presented here should be validated in primary human MC and in some mouse model. However, normal primary MC derived from skin were recently shown to have enormous variability in the responsiveness (Theoharides TC, 2016) and murine models are now considered to poorly mimic human inflammatory diseases (Seok, et al., 2013).

In order to allow for the de novo synthesis of TNF, CXCL8 and VEGF in human mast cells stimulated by either SP or NT, pro-inflammatory mediator gene expression was measured after 6 h, while mediator protein release was measured after 24 h to enable protein translation

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and secretion from MC. An important new finding reported here is that the flavonoid methoxyluteolin significantly inhibits *gene* expression and release of all the pro-inflammatory mediators, as well as activation of mTOR. Moreover, methoxyluteolin is more potent than luteolin, rapamycin or Torin1. These findings suggest that mTOR signaling is involved in the transcriptional regulation of mediator induction in human LAD2 MC in response to stimulation by NT and SP. Luteolin had been shown to inhibit nuclear factor-kappa B (NF-κB)-mediated pro-inflammatory TNF synthesis in murine macrophages (Xagorari, et al., 2001), while our laboratory had previously shown that methoxyluteolin inhibits the SP-stimulated TNF release from LAD2 MC and induction of nuclear factor-kappa B (NF-κB) (Weng, et al., 2015). Hence, we speculate that methoxyluteolin could target inhibition of PI3K/mTOR signaling that is upstream of NF-κB (Dan, et al., 2008) and/or the signal transducer and activator of transcription (STAT) (Laplante and Sabatini, 2013;Saleiro and Platanias, 2015), and in effect inhibit these critical transcriptional regulators of pro-inflammatory cytokines and chemokines (Weichhart, et al., 2008). In fact, PI3K/mTOR, NF-κB and STAT have all been implicated in FcεRI-mediated allergic activation of human MC (Kim, et al., 2008b;Blatt, et al., 2012;Siegel, et al., 2013), while rapamycin was previously shown to inhibit TNF gene expression in rat MC (Park, et al., 2012).

Unlike the mTOR inhibitors, luteolin and methoxyluteolin also inhibit MC degranulation. This inhibition is even better than that of the PI3K inhibitor LY294002, which is known to be involved in the regulation of MC degranulation (Takayama, et al., 2013). Instead, the preferential mTORC1 inhibitor, rapamycin and the dual mTORC1/mTORC2 inhibitor, Torin 1 did not inhibit NT or SP-stimulated human MC degranulation, which is in agreement with the recent report showing that FcεRI-mediated allergic MC degranulation is regulated by the rictor protein alone, and not via the intact mTORC2 signaling (Smrz, et al., 2014). Our findings suggest that the flavonoids luteolin and methoxyluteolin could target inhibition of MC degranulation in an mTOR-independent manner. One such mechanism could involve inhibition of intracellular calcium

levels in human MC, which is required for activation of PI3K and other signaling proteins, such as phospholipase C (Gilfillan and Rivera, 2009) and contributes to the regulation of granule exocytosis (Holowka, et al., 2012). We had previously shown that methoxyluteolin inhibits intracellular calcium levels in human MC (Weng, et al., 2015). Additionally, the flavonoids could target specific proteins involved in vesicle fusion, such as soluble N-ethylmaleimide-sensitive factor attachment proteins (SNARE complexes) (Yang, et al., 2015), implicated in MC degranulation.

Patients with systemic mastocytosis (Metcalfe and Akin, 2001;Theoharides, et al., 2015) have increased mTOR gene expression in bone marrow mononuclear cells (Smrz, et al., 2011) and it has been suggested that selectively targeting mTOR complexes could effectively reduce proliferation of MC associated with inflammation and MC disorders. Rapamycin had already been reported to inhibit the survival of KIT D816V mutated MC in culture (Gabillot-Carre, et al., 2006), a mutation characteristic in almost 80% of patients with systemic mastocytosis, rendering the encoded tyrosine kinase receptor and mTOR constitutively active. However, treatment using everolimus, an oral mTOR inhibitor, resulted in toxicity and side-effects in patients with systemic mastocytosis (Parikh, et al., 2010). More recently, dual inhibitors of PI3K/mTOR that inhibit activation of neoplastic human MC (Blatt, et al., 2012) have been proposed as a treatment of systemic mastocytosis, but could have and poor tolerability. In addition to treating mastocytosis (Metcalfe and Akin, 2001;Theoharides, et al., 2015), methoxyluteolin could also be used for asthma (Galli and Tsai, 2012), atopic dermatitis, (Vasiadi M, et al., 2012) and psoriasis (Theoharides, et al., 2012).

The present findings of neuroimmunoendocrine interactions involving the neuropeptides NT and SP, and activation of MC could be important in the pathology of skin disorders (Caraffa, et al., 2016). For instance, activation of human MC via FcεRI up-regulates the surface expression of the SP neurokinin receptors (Kulka, et al., 2007). We had shown that SP (Asadi,

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et al., 2012) and NT (Alysandratos, et al., 2012) induce the expression of corticotropin-releasing hormone (CRH) receptor-1 in human MC, through which NT synergistically with CRH stimulates VEGF release (Donelan, et al., 2006) and MC degranulation (Pang, et al., 1998). LAD2 MC express the NT receptor 1 (Alysandratos, et al., 2012), while other human MC lines also synthesize precursor NT peptides (Cochrane, et al., 2011). MC can also degrade NT (Piliponsky, et al., 2008), indicating tight regulation. Moreover, SP induces adhesion molecules on endothelial cells resulting in infiltration of other immune cells such as neutrophils and eosinophils (Quinlan, et al., 1999). Hence, the elevated circulating levels of SP and/or NT in patients with psoriasis and atopic dermatitis (Vasiadi M, et al., 2012) could have relevance in pathophysiological settings. Furthermore, we recently reported increased levels of TNF, SP and the SP-structural analog hemokinin A in patients with fibromyalgia syndrome (Tsilioni, et al., 2016). Interestingly, hemokinin A was reported to be secreted from rodent MC and have an autocrine action on MC (Sumpter, et al., 2015).

The ability of NT to stimulate mTOR activation in MC reported here is additionally intriguing given the increased levels of NT in patients with ASD (Angelidou, et al., 2010;Tsilioni, et al., 2014), who also have skin allergies (Theoharides, et al., 2016). Communication between MC and microglia has been invoked in inflammation of the brain (Skaper, et al., 2014) and increasing evidence indicates that MC and inflammation of the brain (Esposito, et al., 2002) are involved in the pathogenesis of ASD (Theoharides, et al., 2016). Microglia, the resident immune cells of the brain are stimulated by MC-derived histamine (Dong, et al., 2014) and tryptase (Zhang, et al., 2012b). Moreover, about 1-5% of ASD cases have gene mutations in regulatory proteins upstream of mTOR (Willsey and State MW, 2015), removing any innate inhibition of this signaling complex. We recently showed that NT can stimulate cultured human microglia, an action inhibited by methoxyluteolin (Patel, et al., 2016).

Our present findings of mTOR activation in neuropeptide-stimulated MC and its inhibition by methoxyluteolin have clear therapeutic potential. In fact, two open-label clinical studies have reported that a luteolin-containing dietary formulation significantly improved attention and sociability in children with ASD (Theoharides T.C., et al., 2012;Taliou, et al., 2013), while a combination of luteolin with palmitoylethanolamide was further reported to have significant benefit in cerebral ischemia in humans (Caltagirone, et al., 2016). Therapeutically, methoxyluteolin could be superior to luteolin for treating patients with mast cell disorders not only because it is a more potent inhibitor of MC and mTOR activation, but its four additional methyl groups, as compared to luteolin, increases its solubility, absorption and metabolic stability (Walle, 2007). Moreover, since flavonoids typically have low oral bioavailability of less than 10 % in humans (Williamson and Manach, 2005), utilizing novel drug delivery systems, such as encapsulation of methoxyluteolin into liposomes coated with a targeting ligand could improve bioavailability and enhance delivery to sites of inflammation, where activated MC are present.

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Authorship contributions

Participated in research design: Patel, Theoharides

Conducted experiments & performed data analysis: Patel

Wrote or contributed to the writing of the manuscript: Patel, Theoharides

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Footnotes

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Legends for Figures

Figure 1. Inhibitors of mTOR signaling dose-dependently decrease SP-stimulated pro-inflammatory mediator release from human MC. LAD2 MC ($0.5x10^6$ cells) were pre-treated with the mTOR inhibitor, rapamycin (0.05-0.2 μM, 24 h), the ATP-competitive mTOR inhibitor, Torin1 (0.05-0.2 μM, 24 h) prior to stimulation with SP (1 μM) for 24 h to measure release of **(A)** TNF and **(B)** CXCL8 by ELISA. LAD2 MC ($0.5x10^6$ cells) were also pre-treated with the upstream **(C)** PI3K inhibitor LY294002 (1-10 μM, 2 h), prior to stimulation with SP (1 μM) for 24 h to measure release of TNF and CXCL8 mediators. All inhibitors were dissolved in water or DMSO with final concentration < 0.1 %. All conditions were performed in triplicates for each data set and were repeated three times (n=3), with results presented as mean ± SD. Significance of comparisons were made for stimulated cells without any inhibitor (control) and those with inhibitor/flavonoid, as denoted by the horizontal lines, which indicate significance at $p<0.001$ for each comparison made. All inhibitor/flavonoid treatments were also compared among themselves and the vertical brackets indicate the corresponding levels of significance, when present [$p<0.05$ (*), $p<0.001$ (**) and $p<0.0001$ (***)].

Figure 2. Human MC pro-inflammatory mediator release in response to NT or SP is attenuated by PI3K/mTOR inhibitors and the flavonoids, luteolin and methoxyluteolin. LAD2 MC ($0.5x10^6$ cells) were pre-treated with the mTOR inhibitors [rapamycin (Rap) and Torin1, 0.2 μM, 24 h] or the upstream PI3K inhibitor [LY294002 (LY), 10 μM, 2 h] or the natural flavonoids [luteolin (Lut) and methoxyluteolin (Methlut), 50 μM, 30 mins], then stimulated with NT (10 μM) **(A-C)** or the positive control trigger SP (1 μM) **(D-F)** for 24 h to measure release of TNF, CXCL8 and VEGF mediators by specific enzyme-linked immunosorbent assays (ELISA). All conditions were performed in triplicates for each data set and were repeated three times

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(n=3), with results presented as mean ± SD. Significance of comparisons were made for stimulated cells without any inhibitor (control) and those with inhibitor/flavonoid, as denoted by the horizontal lines, which indicate significance at $p<0.0001$ for each comparison made. All inhibitor/flavonoid treatments were also compared among themselves and the horizontal brackets indicate the corresponding levels of significance, when present [$p<0.05$ (*), $p<0.001$ (**) and $p<0.0001$ (***)].

Figure 3. Human LAD2 MC pro-inflammatory mediator gene expression in response to NT or SP is inhibited by the PI3K/mTOR inhibitors, luteolin and methoxyluteolin. LAD2 MC ($1x10^6$ cells) were pre-treated with the mTOR inhibitors [rapamycin (Rap) and Torin1, 0.2 µM, 2 or 24 h] or the upstream PI3K inhibitor [LY294002 (LY), 10 µM, 2 h] or the natural flavonoids [luteolin (Lut) and methoxyluteolin (Methlut), 50 µM, 30 mins], prior to stimulation with NT (10 µM) **(A-C)** or the positive control trigger SP (1 µM) **(D-F)** for 6 h to measure gene expression of TNF, CXCL8 and VEGF by RT-qPCR. All inhibitors were dissolved in water or DMSO with final concentration < 0.1 %. All conditions were performed in triplicates for each data set and were repeated three times (n=3), with results presented as mean ± SD. Significance of comparisons were made for stimulated cells without any inhibitor (control) and those with inhibitor/flavonoid, as denoted by the horizontal lines, which indicate significance at $p<0.0001$ for each comparison made. All inhibitor/flavonoid treatments were also compared among themselves and the horizontal brackets indicate the corresponding levels of significance, when present [$p<0.05$ (*), $p<0.001$ (**) and $p<0.0001$ (***)].

Figure 4. NT and SP stimulate mTOR activation in human LAD2 MC. LAD2 MC ($1x10^6$ cells) were stimulated with NT (10 µM) **(A-C)** or the positive control trigger SP (1 µM) **(D-F)** for 0-60

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mins to probe for the total and phosphorylated levels of mTOR, and substrates p70S6K and 4EBP1 by Western blot analysis; peak mTOR activation was denoted at 20 min for pmTORSer2448 and pp70S6KThr389 proteins, while the levels of p4EBP1Thr37/46 protein remained unchanged. Results were quantified using densitometric analyses for pmTORSer2448 to mTOR levels **(B, D)** and pp70S6KThr389 to p70S6K levels **(B, E)** in response to NT (10 μM) or SP (1 μM). All conditions were performed in triplicates for each data set, repeated three times (n=3), with results presented as mean ± SD. Significance of comparisons were made for unstimulated cells without NT or SP treatment and those with stimulation by the neuropeptides, as denoted by $p<0.05$ (*), $p<0.001$ (**) and $p<0.0001$ (***).

Figure 5. NT- or SP-stimulated mTOR activation in LAD2 MC is inhibitable by luteolin and methoxyluteolin. LAD2 MC ($1x10^6$ cells) were pre-treated with the mTOR inhibitors [rapamycin, (Rap) and Torin1, 0.2 μM, 24 h] or the upstream PI3K inhibitor [LY294002 (LY), 10 μM, 2 h] or the natural flavonoids [luteolin (Lut) and methoxyluteolin (Methlut), 10 or 50 μM, 30 mins], prior to stimulation with **(A-C)** NT (10 μM) or **(D-F)** SP (1 μM) for 20 min to probe for the total and phosphorylated levels of mTOR, and substrates p70S6K and 4EBP1 by both phospho-ELISA kits and Western blot analysis (lower panel). The protein levels of mTOR and pmTOR Ser2448 **(A, D)** and the downstream mTORC1 substrates, p70S6K and pp70S6K Thr389 **(B, E)** and, 4EBP1 and p4EBP1 Thr37/46 **(C, F)** were measured using specific total or phospho-ELISA kits for equal amounts of protein lysates, with ratios of phosphorylated to total proteins normalized. All conditions were performed in triplicates for each data set and were repeated three times (n=3), with results presented as mean ± SD. Significance of comparisons were made for stimulated cells without any inhibitor (control) and those with inhibitor/flavonoid, as denoted by the horizontal lines, which indicate significance at $p<0.0001$ for each comparison made. All inhibitor/flavonoid treatments were also compared among themselves and the horizontal

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brackets indicate the corresponding levels of significance, when present [$p<0.05$ (*), $p<0.001$ (**) and $p<0.0001$ (***)].

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Authors: Arti B. Patel and Theoharis C. Theoharides

Figures

Figure 1

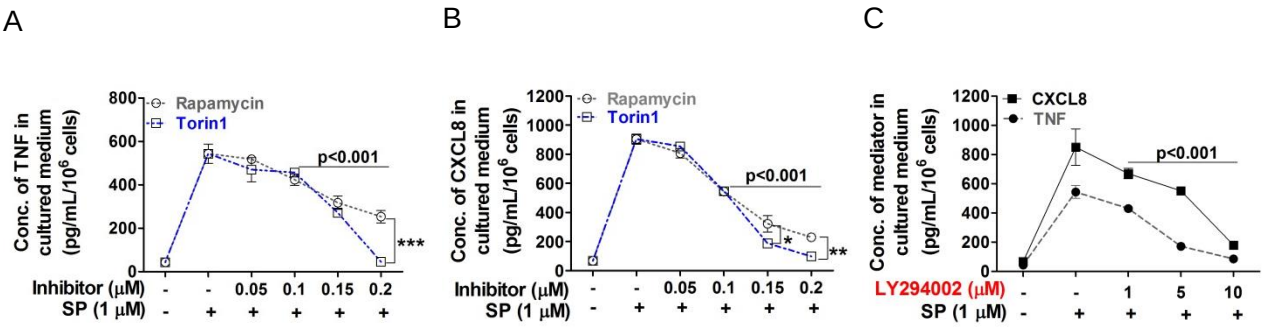

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Figure 2

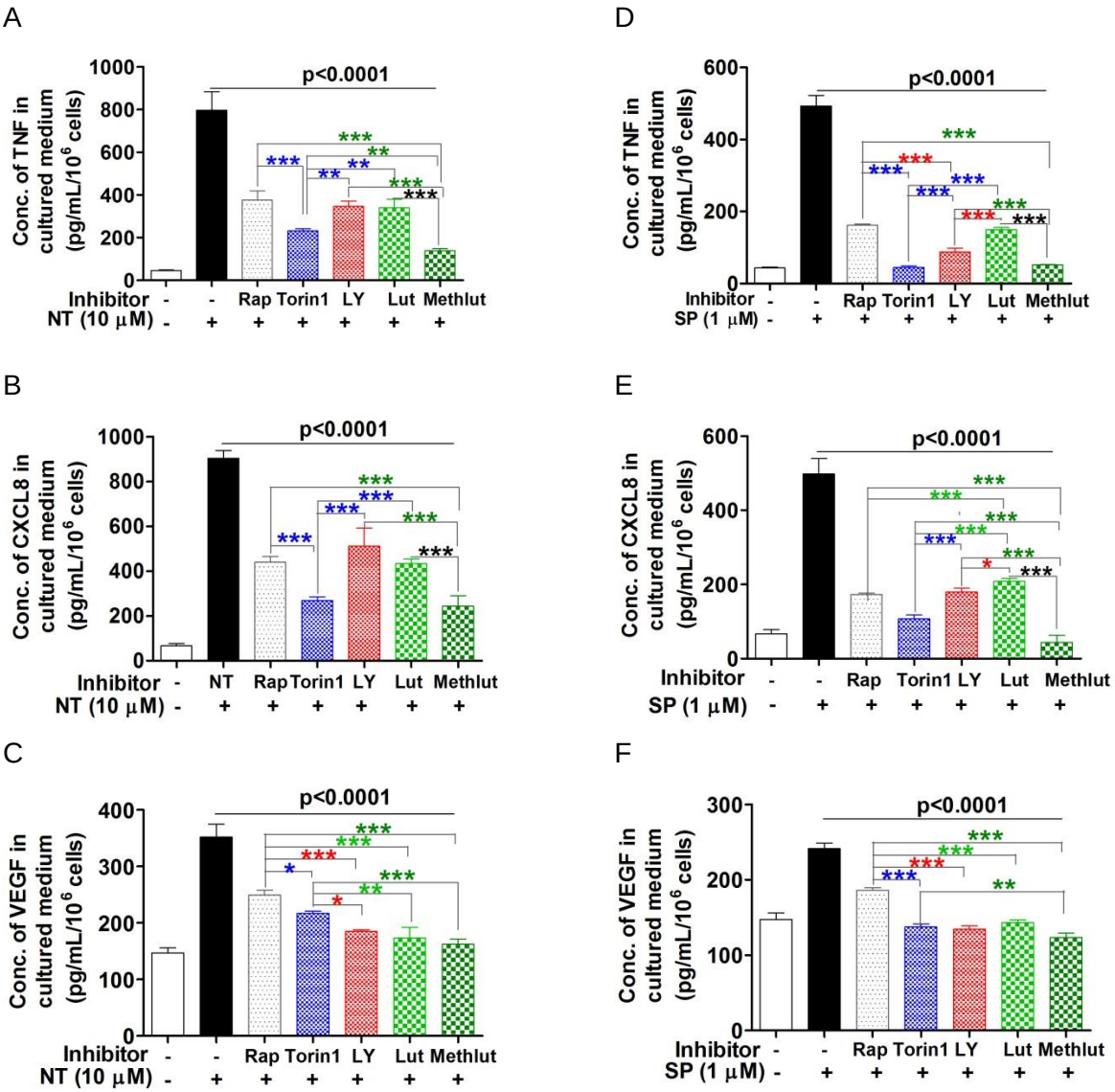

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Figure 3

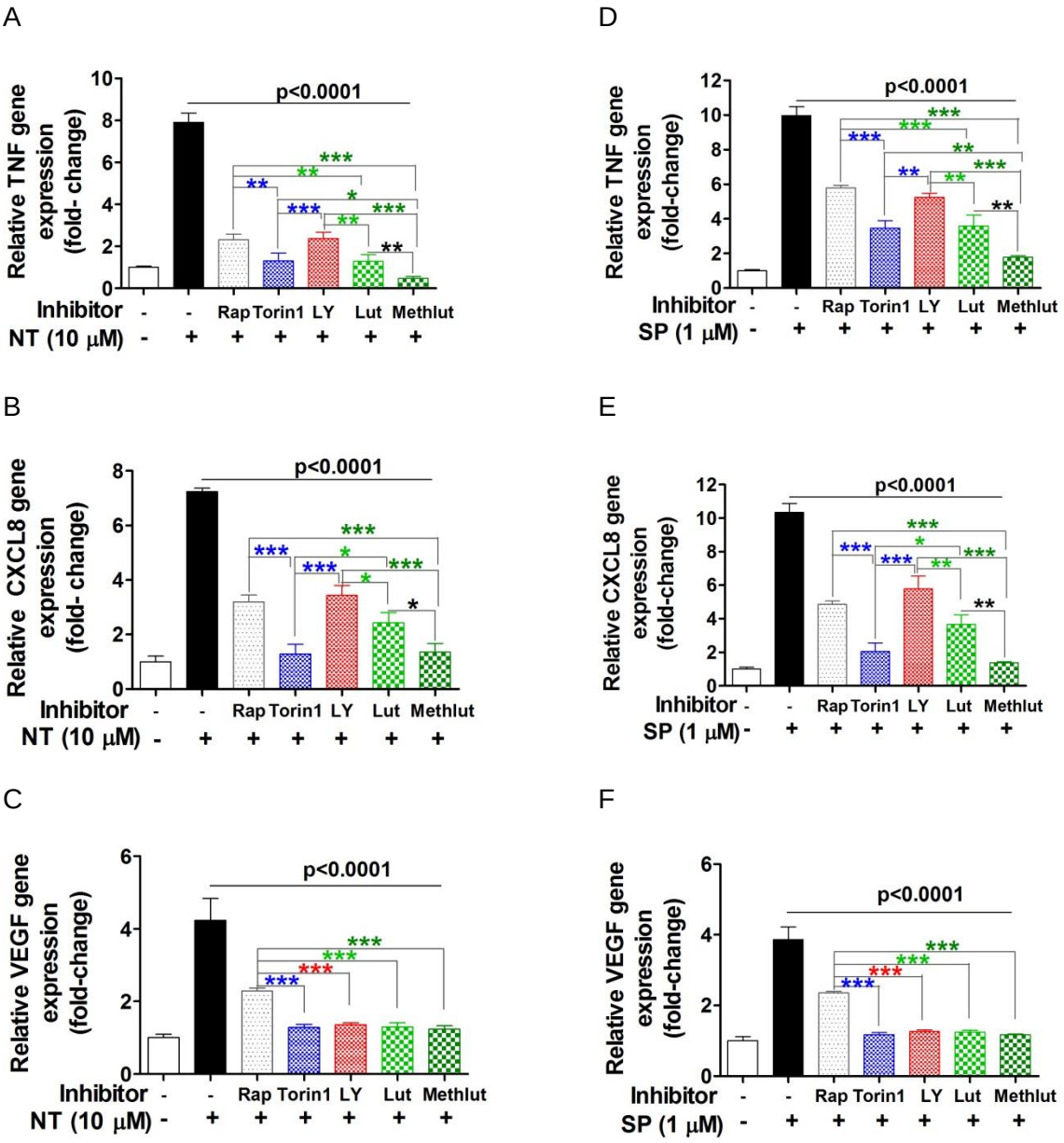

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Figure 4

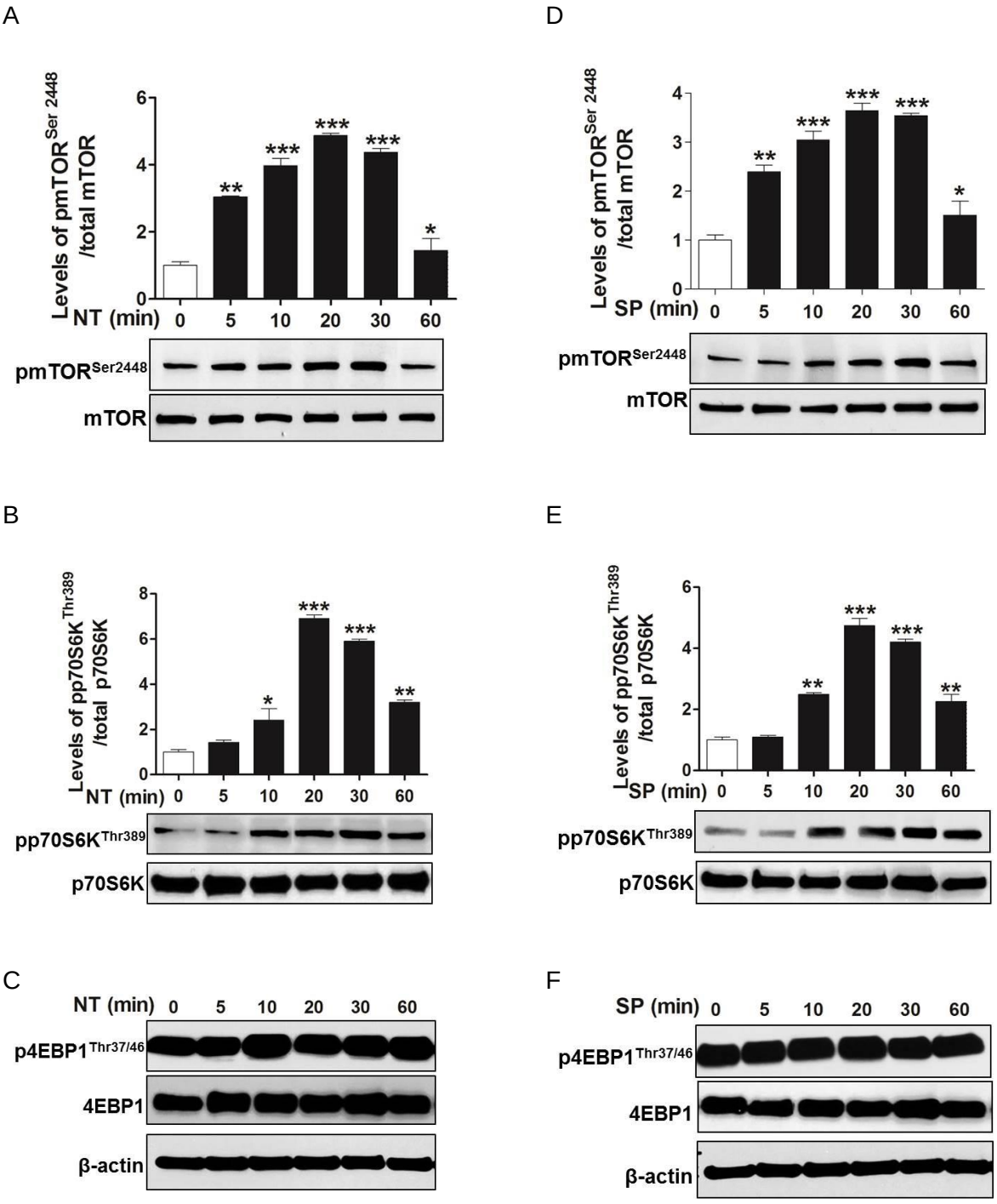

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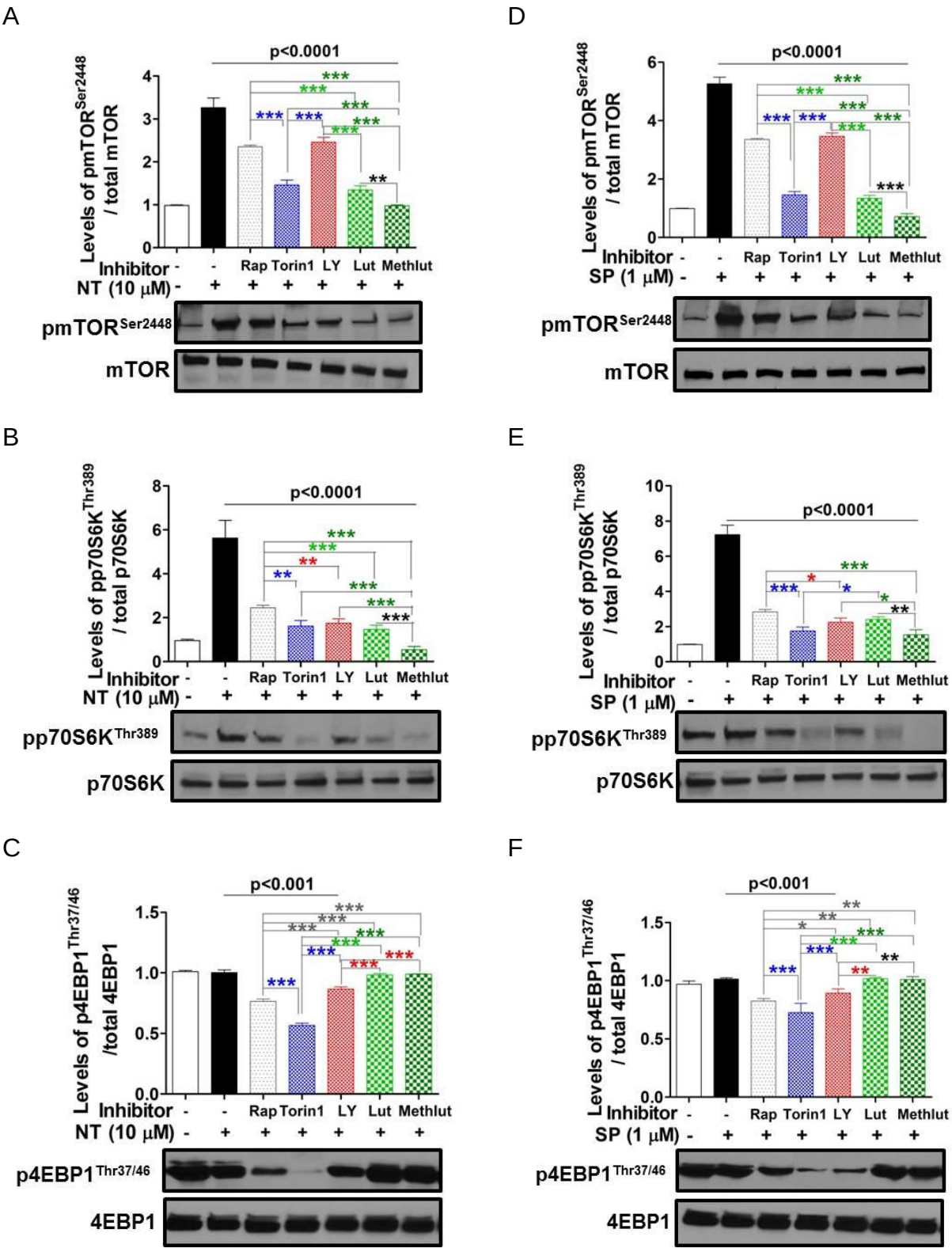
Figure 5



Legends for Supplementary Figures

Supplementary Figure 1. Human MC degranulation in response to NT or SP is decreased by the PI3K inhibitor LY294002, luteolin and methoxyluteolin. LAD2 MC ($0.5x10^6$ cells) were pre-treated with the mTOR inhibitors (rapamycin, Rap and Torin1, 0.2 μM, 24 h), the upstream PI3K inhibitor (LY294002, 50 μM, 2 h) or the natural flavonoids (luteolin, Lut methoxyluteolin, Methlut, 50 μM, 2 h), then stimulated with A) NT (10 μM) or B) the positive control trigger SP (1 μM) for 30 min. β-hexosaminidase release was measured to assess pre-stored mediator release via degranulation. All conditions were performed in triplicates for each data set and were repeated three times (n=3), with results presented as mean ± SD. Significance of comparisons were made for stimulated cells and those with inhibitors/flavonoids, as denoted by the horizontal lines ($p<0.0001$) and also among each of the inhibitors/flavonoids treatments shown by the horizontal brackets and by corresponding $p<0.05$ (*), $p<0.001$ (**) and $p<0.0001$ (***).

Supplementary Figure 2. Methoxyluteolin more potently than luteolin inhibits NT-stimulated pro-inflammatory mediator release from human MC. LAD2 MC ($0.5 x10^6$ cells) were pre-treated the flavonoids [luteolin (Lut) and methoxyluteolin (Methlut), 1-50 μM] for 30 min, then stimulated with NT (10 μM) for 24 h to measure release of (A) TNF, (B) CXCL8 and (C) VEGF by ELISA. All inhibitors were dissolved in water or DMSO with final concentration < 0.1 %. All conditions were performed in triplicates for each data set and were repeated three times (n=3), with results presented as mean ± SD. Significance of comparisons were made for stimulated cells and those with flavonoids, as denoted by the horizontal lines ($p<0.0001$) and also among each of the inhibitors treatments shown by the vertical brackets and by corresponding $p<0.05$ (*) and $p<0.001$ (**).

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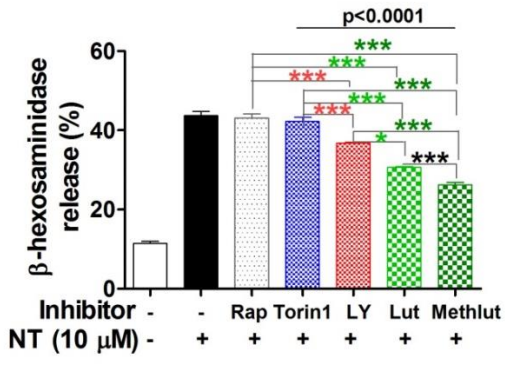
Supplementary Figures

Supplementary Figure 1

A



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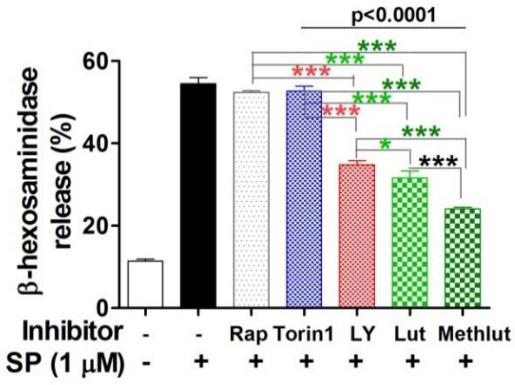

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Supplementary Figure 2