The novel flavone tetramethoxyluteolin is a potent inhibitor of human mast cells

Zuyi Weng, MS, PhD,[a,b] Arti B. Patel, MS,[a,c] Smaro Panagiotidou, MA,[a] and Theoharis C. Theoharides, MS, PhD, MD[a,b,c,d] *Boston, Mass*

Background: Mast cells (MCs) are hematopoietic cells that mature in tissues and are involved in allergy, immunity, and inflammation by secreting multiple mediators. The natural flavone luteolin has anti-inflammatory actions and inhibits human mast cells (MCs).

Objective: We sought to investigate the ability of luteolin and its novel structural analog 3′,4′,5,7-tetramethoxyluteolin (methlut) to inhibit human MC mediator expression and release *in vitro* and *in vivo*.

Methods: Human LAD2 cells and umbilical primary human cord blood–derived cultured mast cells were stimulated with substance P (SP) or IgE/anti-IgE with or without preincubation with luteolin, methlut, or cromolyn (1-100 μmol/L) for 2 or 24 hours, after which mediator secretion was measured. The effect of the compounds on MC intracellular calcium levels and nuclear factor κB activation was also investigated. Pretreatment with methlut was also studied in mice passively sensitized with dinitrophenol–human serum albumin and challenged intradermally.

Results: Methlut is a more potent inhibitor than luteolin or cromolyn for β-hexosaminidase and histamine secretion from LAD2 cells stimulated by either SP or IgE/anti-IgE, but only methlut and luteolin significantly inhibit preformed TNF secretion. Methlut is also a more potent inhibitor than luteolin of *de novo*–synthesized TNF from LAD2 cells and of CCL2 from human cord blood–derived cultured MCs. This mechanism of action for methlut might be due to its ability to inhibit intracellular calcium level increases, as well as nuclear factor κB induction, at both the transcriptional and translational levels in LAD2 cells stimulated by SP without affecting cell viability. Intraperitoneal treatment with methlut significantly decreases skin vascular permeability of Evans blue dye in mice passively sensitized to dinitrophenol–human serum albumin and challenged intradermally.

Conclusion: Methlut is a promising MC inhibitor for the treatment of allergic and inflammatory conditions. (J Allergy Clin Immunol 2014;■■■:■■■-■■■.)

Key words: *Allergy, inflammation, mast cells, luteolin, tetramethoxyluteolin, calcium, nuclear factor κB*

Mast cells (MCs) are immune cells derived from hematopoietic precursors that mature in tissue microenvironments.[1-3] In addition to allergic triggers, MCs can be stimulated by neuropeptides, such as substance P (SP).[3,4] On stimulation, MCs release preformed mediators stored in their numerous secretory granules; these include β-hexosaminidase, histamine, TNF, and tryptase through rapid degranulation, as well as newly synthesized prostaglandin D_2 (PGD_2), TNF, and CCL2 (monocyte chemoattractant protein 1).[5-7]

MC-derived histamine induces bronchoconstriction and mucus secretion, contributing to asthma.[8,9] MCs are probably the only cell type that stores preformed TNF,[10] which is rapidly released and influences T-cell recruitment and activation.[11,12] MC-derived CCL2[13] and CXCL-8 (IL-8) enhance recruitment of immune cells to the site of inflammation.[6,7,14] The ability to release multiple mediators allows MCs to actively interact with other cell types in their surrounding environment and participate in the induction, propagation, or both of various immune and inflammatory responses, including mastocytosis,[15] asthma,[16] atopic dermatitis,[17] and psoriasis.[3,16,18,19] Therefore inhibition of MC activation has clear therapeutic potential.

Disodium cromoglycate (cromolyn; Fig 1, *A*) is the only clinically available MC stabilizer because it was reported to reduce gastrointestinal effects in patients with mastocytosis.[20] Even though cromolyn inhibits rat peritoneal MC histamine secretion,[21] it does not inhibit rat mucosal MCs[22,23] or mouse MCs.[24] A recent study concluded that the beneficial effect of cromolyn in reducing pruritus in human subjects might be mediated through inhibition of sensory nerve endings instead of MCs.[25-28] In addition, poor intestinal absorption of cromolyn severely limits its clinical efficacy. Consequently, there is an urgent need to develop effective inhibitors of human MCs.

While searching for potential MC inhibitors, we noticed that part of the structure of cromolyn is similar to the backbone of flavones (see Fig E1, highlighted areas, in this article's Online Repository at www.jacionline.org), which are naturally occurring compounds with potent antioxidant, anti-inflammatory, and MC-blocking activities.[29] The flavone luteolin (see Fig E1) inhibits the release of histamine and PGD_2 from human cultured MCs.[30] Luteolin also inhibits mercury-induced vascular endothelial growth factor release from human MCs[31] and activated T cells.[32] The structural analog of luteolin, 3′,4′,5,7-tetramethoxyluteolin (methlut; see Fig E1) is more lipid soluble than luteolin, more likely to penetrate cells, and less metabolized.[33] However, the action of methlut on MC activation has not been investigated.

From [a]the Molecular Immunopharmacology and Drug Discovery Laboratory, Department of Integrative Physiology and Pathobiology, Tufts University School of Medicine; the Graduate Programs in [b]Pharmacology and [c]Cell, Molecular and Developmental Biology, Sackler School of Graduate Biomedical Sciences, Tufts University; and [d]the Department of Internal Medicine, Tufts University School of Medicine and Tufts Medical Center.

Supported in part by National Institute of Health grant AR47652, a Translational Grant from the National Psoriasis Foundation, and Theta Biomedical Consulting and Development (Brookline, Mass) to T.C.T.

Disclosure of potential conflict of interest: T. C. Theoharides has received research support from the National Institutes of Health (AR47652). The rest of the authors declare that they have no relevant conflicts of interest.

Received for publication January 14, 2014; revised October 2, 2014; accepted for publication October 22, 2014.

Corresponding author: Theoharis C. Theoharides, MS, PhD, MD, Department of Integrative Physiology and Pathobiology, Tufts University School of Medicine, 150 Harrison Ave, J 304, Boston, MA 02111. E-mail: theoharis.theoharides@tufts.edu.

0091-6749/$36.00

© 2014 American Academy of Allergy, Asthma & Immunology

http://dx.doi.org/10.1016/j.jaci.2014.10.032

Abbreviations used

CCL2:	Chemokine (C-C motif) ligand 2
DMSO:	Dimethyl sulfoxide
DNP-HSA:	Dinitrophenol–human serum albumin
hCBMC:	Human cord blood–derived mast cell
IκBα:	Nuclear factor κB inhibitor α
MC:	Mast cell
Methlut:	3′,4′,5,7-Tetramethoxyluteolin
NF-κB:	Nuclear factor κB
PGD2:	Prostaglandin D2
rhSCF:	Recombinant human stem cell factor
SP:	Substance P

In this study we examined whether methlut could inhibit mediator release from human cultured MCs stimulated by an allergic or a neuropeptide trigger and compared it with luteolin and cromolyn. We also studied the effect of methlut in mice passively sensitized to dinitrophenol-human serum albumin (DNP-HSA) and challenged intradermally.

METHODS

Reagents

Recombinant human stem cell factor (rhSCF) was kindly donated by Orphan Biovitrum AB (Stockholm, Sweden). Cromolyn, luteolin, and SP were purchased from Sigma-Aldrich (St Louis, Mo). Methlut was obtained from Pharmascience Nutrients (Clear Water, Fla) and was 100% pure, as determined by means of HPLC–mass spectroscopy (see Fig E2 in this article's Online Repository at www.jacionline.org). Cromolyn, luteolin, and methlut were dissolved in dimethyl sulfoxide (DMSO). SP was prepared in distilled water. Working dilutions for all compounds were prepared in culture medium immediately before use. The final concentration of DMSO was less than 0.1% and had no effect on cell viability.

Human MC culture

The limited number of MCs obtained from normal human tissues has led to the increased use of human LAD2 cells derived from a human patient with MC leukemia[34] or primary human cord blood–derived MCs (hCBMCs).[35] Human LAD2 cells (kindly supplied by Dr A. Kirshenbaum, National Institutes of Health, Bethesda, Md) were cultured in StemPro-34 medium (Life Technologies, Carlsbad, Calif) supplemented with 2 mmol/L L-glutamine, 1% penicillin/streptomycin, and 100 ng/mL rhSCF (Orphan Biovitrum AB).

Human umbilical cord blood was obtained after normal deliveries in accordance with established institutional guidelines to culture primary hCBMCs.[36] Mononuclear cells were isolated by layering heparin-treated cord blood onto Lymphocyte Separation Medium (INC Biomedical, Aurora, Ohio). $CD34^+$ progenitor cells were isolated by means of positive selection of AC133 ($CD133^+/CD34^+$) cells by using magnetic cell sorting (CD133 Microbead Kit; Miltenyi Biotech, Auburn, Calif). For the first 6 weeks, $CD34^+$ progenitor cells were cultured in Iscove modified Dulbecco medium (Life Technologies) supplemented with 0.1% BSA, 1% insulin-transferrin-selenium, 50 ng/mL IL-6, 0.1% β-mercaptoethanol, 1% penicillin/streptomycin, and 100 ng/mL rhSCF. After 6 weeks, the cells were cultured in Iscove modified Dulbecco medium supplemented with 10% FBS, 50 ng/mL IL-6, 0.1% β-mercaptoethanol, 1% penicillin/streptomycin, and 100 ng/mL rhSCF. hCBMCs cultured for at least 15 weeks were used for experiments, and cell purity was greater than 98%. Cell viability was determined by means of trypan blue (0.4%) exclusion.

Degranulation assays

β-Hexosaminidase release was assayed as an index of MC degranulation. LAD2 cells (0.5×10^5) were preincubated with cromolyn, luteolin, or methlut (10-100 μmol/L for 30 minutes) before stimulation with SP (2 μmol/L for 30 minutes). LAD2 cells were treated with cromolyn (100 μmol/L) and SP (2 μmol/L) at the same time to test the effect of cromolyn. Control cells were treated with 0.1% DMSO. Supernatant fluids were collected, and cell pellets were lysed with 1% Triton X-100. Supernatants and cell lysates were incubated in the reaction buffer (p-nitrophenyl-N-acetyl-β-D-glucosaminide from Sigma) for 1.5 hours, and then 0.2 mol/L glycine was added to stop the reaction. Absorbance was measured at 405 nm. Results are expressed as the percentage of β-hexosaminidase released over the total amount present in LADs cells.

MC degranulation was also assessed by measuring histamine release. After the same treatment, LAD2 cells were pretreated with cromolyn, luteolin, or methlut and subsequently stimulated with SP. Supernatant fluids were collected, and histamine release was measured with a Histamine EIA Kit (Cayman Chemical, Ann Arbor, Mich).

hCBMCs (0.5×10^5) were first primed with human IgE (1 μg/mL; Millipore, Billerica, Mass) overnight and preincubated with cromolyn, luteolin, or methlut (10-100 μmol/L) for 30 minutes before stimulation with anti-IgE (10 μg/mL for 30 minutes, Life Technologies) to assay degranulation in primary MCs.

TNF and CCL2 ELISA

For TNF release, LAD2 cells (1×10^5) were preincubated with cromolyn, luteolin, or methlut (10-100 μmol/L) and subsequently stimulated with SP (2 μmol/L) for 30 minutes to measure preformed TNF release. LAD2 cells were also stimulated for 24 hours to measure *de novo*–synthesized TNF release, which occurs in addition to rapid preformed TNF release. TNF levels were measured in supernatant fluids by using a TNF ELISA assay kit (R&D Systems, Minneapolis, Minn).

For CCL2 release, primary hCBMCs (1×10^5) were primed with human IgE (1 μg/mL, Millipore) overnight and preincubated with luteolin or methlut (50 μmol/L) for 30 minutes before stimulation with anti-IgE (10 μg/mL for 2 hours, Life technologies). CCL2 was measured with a CCL2 ELISA assay kit (R&D Systems).

RNA isolation and quantitative real-time PCR

LAD2 cells and hCBMCs (5×10^5) were treated with luteolin or methlut (10-100 μmol/L for 30 minutes) before stimulation with either SP (2 μmol/L) or anti-IgE (10 μg/mL) for 6 hours. Total RNA was extracted with an RNeasy Mini kit (Qiagen, Valencia, Calif). An iScript cDNA synthesis kit (Bio-Rad Laboratories, Hercules, Calif) was used for reverse transcription of each sample. Quantitative real-time PCR was performed with TaqMan gene expression assays (Applied Biosystems, Foster City, Calif) for TNF (Hs99999043_m1) and CCL2 (Hs00234140_m1) and the 2 genes encoding different subunits of the nuclear factor κB (NF-κB) protein complex, NFKB1 (NF-κB p50 subunit, Hs00765730_m1) and RELA (NF-κB p65 subunit, Hs00153294_m1). Samples were run at 45 cycles by using a real-time PCR system (7300, Applied Biosystems). The mRNA gene expressions were normalized to human GAPDH endogenous control (4310884E, Applied Biosystems).

Intracellular calcium measurements

LAD2 cells were incubated in Tyrode buffer with the calcium indicator Fura-2 AM (30 nmol/L for 20 minutes, Life Technologies). Cells were washed and resuspended in plain Tyrode buffer and incubated for another 20 minutes. Cells were then transferred to 96-well plates (1×10^5 cells per well) and pretreated with luteolin or methlut (10 and 50 μmol/L for 30 minutes) before stimulation with SP (2 μmol/L). Changes in Fura-2 AM fluorescence was immediately read with the MDC FlexStation II (Molecular Devices, Sunnyvale, Calif) at an excitation wavelength of 340 nm/380 nm and an emission wavelength of 510 nm. Results were processed according to the Life Technologies Fura-2 AM protocol and reported as the relative ratio.

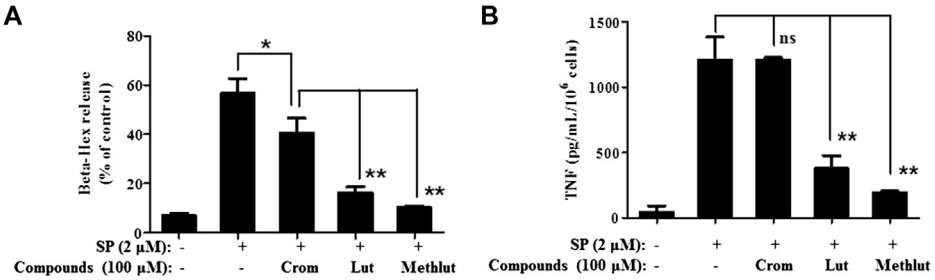

FIG 1. Methlut inhibits degranulation of LAD2 cells stimulated by SP. MCs were preincubated with either cromolyn *(Crom)*, luteolin *(Lut)*, or methlut (10-100 μmol/L for 30 minutes) before stimulation with SP (2 μmol/L for 30 minutes). **A,** β-Hexosaminidase. **B,** Preformed TNF release. $*P < .05$ and $**P < .01$. *ns,* Not significant.

NF-κB inhibitor α phosphorylation assay

The nuclear transcription factor NF-κB plays a pivotal role in the regulation of inflammatory mediator expression.[37] On stimulation, the NF-κB inhibitor α (IκBα) is rapidly phosphorylated and degraded, allowing NF-κB to translocate into the nucleus, where it binds to the promoter region of a number of target genes.[37] We investigated the effects of luteolin and methlut on IκBα phosphorylation and NF-κB DNA-binding activity, as described later. After preincubation with either luteolin or methlut (10 or 50 μmol/L for 30 minutes), LAD2 cells (4×10^6) were stimulated with SP (2 μmol/L for 15 minutes). Phosphorylation of IκBα (serine 32) was detected by using the PathScan Inflammation Sandwich ELISA kit (#7276; Cell Signaling, Danvers, Mass). Whole-cell lysates were assayed at a protein concentration of 5 mg/mL. Absorbance was read at 450 nm. Relative phospho-IκBα levels were normalized to those of control cells treated with 0.1% DMSO.

NF-κB DNA-binding activity

After luteolin and methlut preincubation (10 and 50 μmol/L for 30 minutes), LAD2 cells (4×10^6) were stimulated with SP (2 μmol/L for 15 minutes). Cells were harvested, and cytosolic and nuclear extracts were isolated by using an NE-PER nuclear extraction kit (Thermo Scientific, Rockford, Ill). DNA-binding activity of NF-κB p65 in the extracts was detected by using the NF-κB (p65) Transcription Factor Assay Kit (#10007889, Cayman Chemical). Cytosolic and nuclear extracts (each containing 10 μg of protein) were added to 96-well plates coated with a specific double-stranded DNA sequence containing the NF-κB response element. NF-κB was detected by addition of specific primary antibody directed against NF-κB followed by horseradish peroxidase (HRP)–conjugated secondary antibody to provide a colorimetric readout at 450 nm. Relative NF-κB p65 DNA-binding activities in the cytosolic and nuclear extracts were normalized to values in control cells treated with 0.1% DMSO.

Intracellular ATP measurement

Intracellular ATP content was measured to determine whether luteolin and methlut have any effect on cellular energy production. After incubation with luteolin or methlut (10-100 μmol/L for 24 hours), 1×10^6 LAD2 cells were lysed, and intracellular ATP contents were determined by using an ATP assay kit (Abcam, Cambridge, Mass).

Cell viability

Cell viability was assayed by means of trypan blue exclusion.[38] The effect of luteolin and methlut (100 μmol/L) was tested on LAD2 cells, hCBMCs, and microglial cells and was greater than 98% viable after 24 hours of incubation. Luteolin was recently reported not to affect the viability of HaCaT keratinocytes and primary cultured keratinocytes.[39]

Evans blue extravasation

Evans blue extravasation was performed, as previously described.[40] Passive cutaneous anaphylaxis was produced as follows: normal saline or anti-DNP IgE (20 μg/50 μL, Sigma) was administered intradermally at 2 skin sites on each mouse (n = 5). Two days later, dinitrophenol–human serum albumin (DNP-HSA) (200 μg) along with 1% Evans blue in sterile normal saline (100 μL) was injected into the tail vein. Male BALB/c mice (4-6 weeks old; Charles River, Wilmington, Mass) were then anesthetized by means of an intraperitoneal injection (0.1 mL) of ketamine (100 mg/kg)/xylazine (10 mg/kg), after which the dorsal subscapular skin was shaved and either 10 μg of SP or DNP-HSA (200 μg) was injected intradermally in a total volume of 50 μL in the subscapular region with a tuberculin syringe. Mice were pretreated with an intraperitoneal injection of normal saline or methlut (100 mg/kg) 30 minutes before the intradermal injections. Mice were killed, and the skin was removed, turned over, and photographed. Circular pieces of skin at each injection site (8 mm diameter) were removed with a puncher, weighted, and placed in Eppendorf tubes. Evans blue was extracted in 1 mL of N,N-dimethylformamide overnight at 55°C, and the OD was measured at 620 nm with a PerkinElmer Luminescence Spectrophotometer (PerkinElmer, Norwalk, Conn). The Evans blue concentration was calculated by using a standard curve, and values were normalized to tissue weight. Intravascular Evans blue and nonspecific effects of injection were accounted for by subtracting the Evans blue extracted after injection of normal saline.

Statistical analysis

All experiments were performed in triplicate and repeated at least 3 times (n = 3). Results are presented as means ± SDs. Data between different treatment groups were analyzed by using the unpaired 2-tailed Student *t* test.

RESULTS

Methlut inhibits SP-stimulated LAD2 cell degranulation

SP (2 μmol/L for 30 minutes) triggers significant release of β-hexosaminidase (Fig 1, *A*) and preformed TNF (Fig 1, *B*) from LAD2 cells. Luteolin and methlut inhibit β-hexosaminidase release by 74% and 85%, respectively (Fig 1, *A*). Cromolyn reduces β-hexosaminidase release (Fig 1, *A*) but does not reduce TNF release (Fig 1, *B*), whereas luteolin and methlut significantly inhibit both β-hexosaminidase and TNF release by more than 70% (Fig 1). The inhibitory effect of methlut is greater than that of luteolin.

Cromolyn reduces SP-stimulated β-hexosaminidase release by 30% (Fig 1, *A*), but it must be added together with the trigger because preincubation for 30 minutes eliminates its inhibitory activity (see Fig E3 in this article's Online Repository at www.jacionline.org). Instead, luteolin and methlut inhibit β-hexosaminidase release whether added together or 30 minutes before the trigger (Fig 1, *A*).

The inhibitory effect of the flavonoids is dose dependent (10-100 μmol/L for 30 minutes), with almost complete inhibition

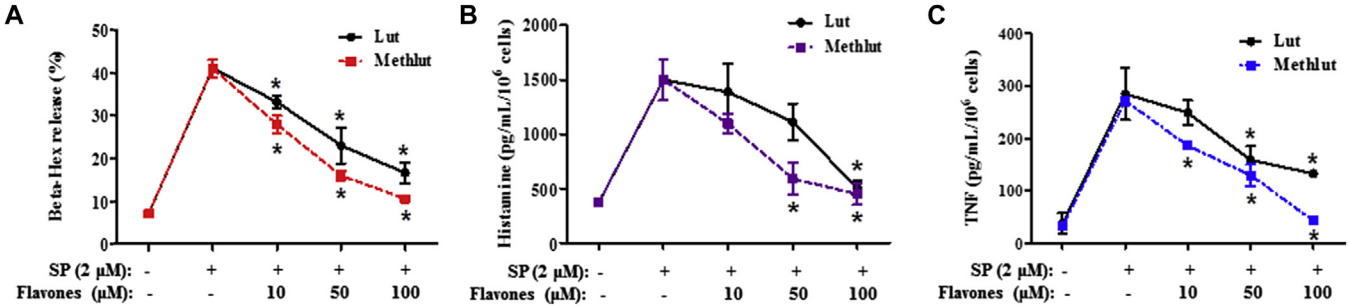

FIG 2. Dose response of the inhibitory effect of methlut on degranulation of LAD2 cells stimulated by SP. MCs were preincubated with either luteolin *(Lut)* or methlut (10-100 μmol/L for 30 minutes) before stimulation with SP (2 μmol/L for 30 minutes). **A,** β-Hexosaminidase. **B,** Histamine. **C,** Preformed TNF release. *$P < .05$.

of SP-stimulated β-hexosaminidase, histamine, and preformed TNF release at 100 μmol/L (Fig 2, *B*).

Methlut inhibits IgE/anti-IgE–stimulated β-hexosaminidase and TNF release from LAD2 cells

We also stimulated LAD2 cells with IgE/anti-IgE. Preincubation with luteolin or methlut (100 μmol/L for 30 minutes) significantly inhibits IgE/anti-IgE–stimulated β-hexosaminidase release by 70% and 99%, respectively (Fig 3, *A*). Similar inhibition is seen for the release of preformed TNF (Fig 3, *B*).

Methlut inhibits SP-stimulated *de novo* TNF synthesis and release from LAD2 cells

In addition to preformed TNF, MCs also release newly synthesized TNF 12 to 24 hours later. SP (2 μmol/L for 24 hours) triggers significant TNF release (Fig 4, *A* and *B*), which is dose-dependently inhibited by preincubation with luteolin or methlut (1-100 μmol/L for 30 minutes). Prolonged preincubation times do not increase the extent of inhibition, except for luteolin, where inhibition at 10 μmol/L becomes more prominent after 12 and 24 hours compared with 30 minutes (Fig 4, *A*). Methlut is more effective than luteolin at 10 μmol/L. Moreover, luteolin at 50 μmol/L reduces TNF release by about 50%, whereas methlut achieves 95% inhibition at the same concentration (Fig 4, *B*). Cromolyn has no inhibitory effect (results not shown).

Preincubation with luteolin or methlut for 30 minutes also significantly decreases TNF mRNA expression (Fig 4, *C* and *D*). Methlut, even at 10 μmol/L, is more effective than luteolin and reduces SP-stimulated TNF expression by 73% (Fig 4, *D*). Cromolyn has no effect (results not shown).

Methlut inhibits IgE/anti-IgE–stimulated CCL2 synthesis and release from IgE/anti-IgE–stimulated hCBMCs

We investigated the effect of the flavonoids on release of CCL2 because CCL2 is known to be synthesized by MCs[13] and stimulates recruitment of immune cells to the site of inflammation.

Stimulation of hCBMCs with IgE/anti-IgE (10 μg/mL for 24 hours) triggers CCL2 release from primary hCBMCs. This effect is blocked by preincubation with luteolin or methlut (10 or 50 μmol/L for 30 minutes; Fig 5, *A*). Anti-IgE stimulation (10 μg/mL for 6 hours) also triggers a 2-fold increase in CCL2 mRNA expression compared with that seen in control cells primed with IgE only (Fig 5, *B*); this effect is also completely inhibited by preincubation with luteolin or methlut (50 μmol/L for 30 minutes) but not by cromolyn (results not shown).

Methlut inhibits NF-κB activation

In LAD2 cells SP (2 μmol/L for 15 minutes) rapidly causes IκBα phosphorylation on serine 32 (Fig 6, *A*), which allows subsequent activation of NF-κB. Preincubation with luteolin or methlut (10 or 50 μmol/L for 6 hours) dose-dependently decreases IκBα phosphorylation compared with that seen in control cells (Fig 6, *A*). In addition, preincubation with luteolin or methlut (10 or 50 μmol/L for 6 hours) significantly decreases the DNA-binding activity of NF-κB p65 in the nuclear extract (Fig 6, *B*). The DNA-binding activity of NF-κB p65 in the cytosolic extract is not affected (data not shown). At 10 μmol/L, methlut is more effective than luteolin in reducing both IκBα phosphorylation and DNA-binding activity of NF-κB p65 in the nucleus.

Methlut inhibits mRNA expression of NF-κB–related genes

NFKB1 encodes for the NF-κB p50 subunit, and RELA encodes for the NF-κB p65 subunit. In LAD2 cells SP (2 μmol/L for 6 hours) slightly induces mRNA expression levels of NFKB1 (Fig 7, *A*) and RELA (Fig 7, *B*), which are decreased by preincubation (10-100 μmol/L for 30 minutes) with luteolin or methlut. Methlut is more effective than luteolin. Similar results are obtained in primary hCBMCs, where preincubation with methlut (50 μmol/L for 30 minutes) significantly decreases mRNA expression levels of both NFKB1 (Fig 7, *C*) and RELA (Fig 7, *D*). Preincubation with luteolin (50 μmol/L for 30 minutes) only slightly reduces the mRNA expression of NFKB1 and RELA, but this reduction is not statistically significant.

Methlut inhibits intracellular calcium increase

SP (2 μmol/L) triggers a rapid intracellular calcium increase in LAD2 cells (see Fig E4 in this article's Online Repository at www.jacionline.org). Preincubation with luteolin (50 μmol/L for 30 minutes) inhibits calcium increase by 50%. Preincubation

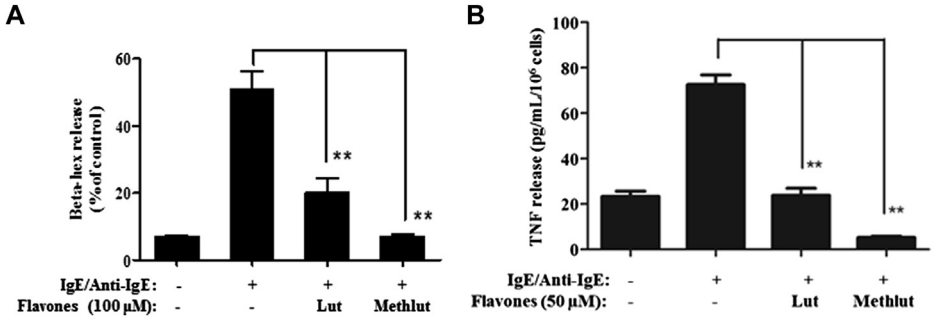

FIG 3. Methlut inhibits degranulation of LAD2 cells stimulated by IgE/anti-IgE. After overnight priming with IgE (1 μg/mL), LAD2 cells were incubated with luteolin *(Lut)* or methlut (50 or 100 μmol/L for 30 minutes) before anti-IgE stimulation (10 μg/mL) for 2 hours. **A,** β-Hexosaminidase. **B,** Preformed TNF. $^{**}P < .001$.

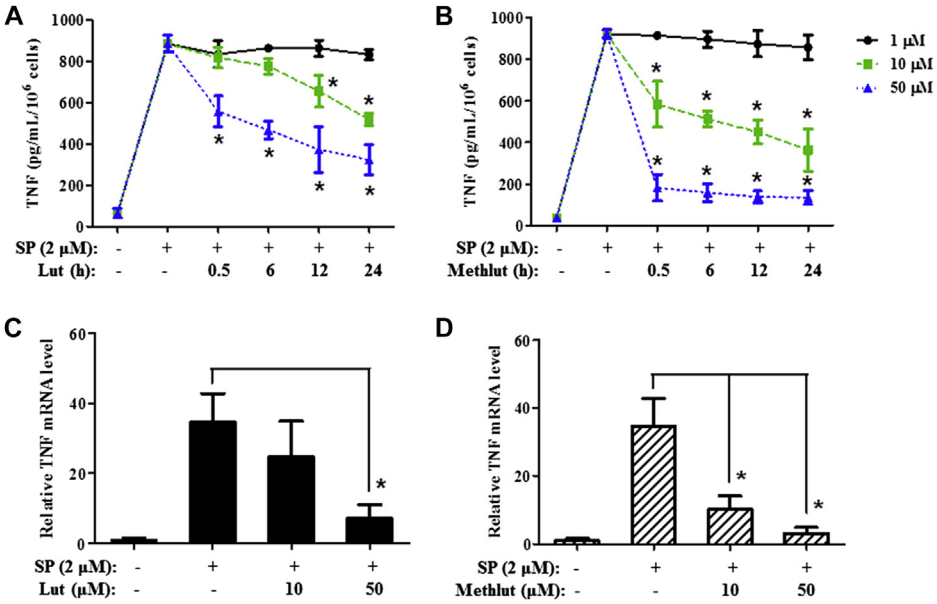

FIG 4. Methlut inhibits TNF mRNA expression in LAD2 cells stimulated by SP. LAD2 cells were incubated with luteolin (*Lut*; **A**) or methlut (**B**; 1-50 μmol/L up to 24 hours) before SP stimulation (2 μmol/L for 24 hours), and TNF release was assayed. In another set of experiments, LAD2 cells were incubated with luteolin **(C)** or methlut (**D**; 10 or 50 μmol/L for 30 minutes) before SP stimulation (2 μmol/L for 6 hours), and mRNA expression was examined. $^{*}P < .05$.

with methlut (50 μmol/L for 30 minutes) completely blocks calcium increase, which is even lower than in control cells treated with DMSO (see Fig E4).

Methlut does not alter intracellular ATP production

Neither luteolin nor methlut (100 μmol/L) reduces cell viability (results not shown). To further document the lack of any detrimental effect on cellular function, we examined the effects of luteolin and methlut on intracellular ATP production. Preincubation with luteolin or methlut (100 μmol/L for 24 hours) decreases intracellular ATP content by 10% and 15%, respectively (see Fig E5 in this article's Online Repository at www.jacionline.org), which is not statistically significant.

Methlut prevents increased vascular permeability caused by either SP or DNP-HSA

Intradermal injection of SP (10 μmol/L) or DNP-HSA in mice passively sensitized to HSA significantly increases Evans blue skin extravasation (Fig 8, *A*). Mice injected intraperitoneally with methlut (100 mg/kg for 30 minutes) before intradermal injection have significantly reduced vascular permeability to either SP or DNP-HSA (Fig 8, *B*).

DISCUSSION

Our results indicate that the novel flavone methlut is more potent than luteolin and significantly inhibits human LAD2 cell release of histamine, β-hexosaminidase, and preformed TNF. In contrast, an equimolar concentration of cromolyn, the only compound marketed as an MC stabilizer, reduces β-hexosaminidase release by about 30%, but not preformed TNF release, when LAD2 cells are stimulated by SP. In addition, both luteolin and methlut are effective when administered before the trigger, whereas cromolyn has to be added together with the trigger for any inhibition to be evident because of rapid tachyphylaxis.[38,41] We also previously showed that the flavonol quercetin, which is structurally related

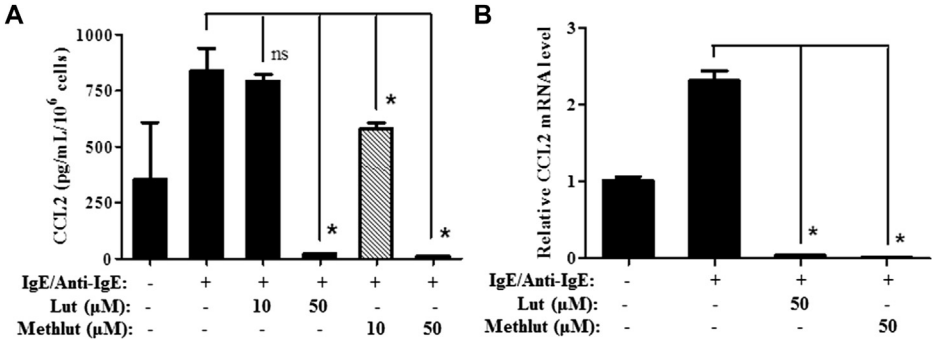

FIG 5. Methlut inhibits CCL2 synthesis and release from hCBMCs stimulated by IgE/anti-IgE. After overnight priming with IgE (1 μg/mL), hCBMCs were incubated with luteolin *(Lut)* or methlut (10 or 50 μmol/L for 30 minutes) before anti-IgE stimulation (10 μg/mL). **A,** CCL2 release was assayed at 24 hours after stimulation. **B,** CCL2 mRNA expression. hCBMCs were incubated with luteolin or methlut (50 μmol/L for 30 minutes) before stimulation with anti-IgE (10 μg/mL for 6 hours), and mRNA expression of CCL2 was examined. $*P < .05$.

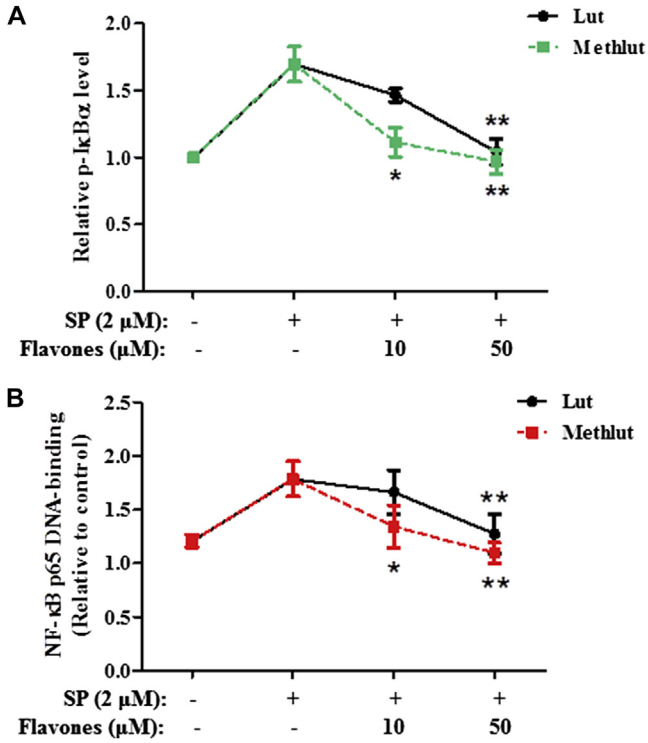

FIG 6. Methlut inhibits NF-κB activation. LAD2 cells were incubated with luteolin *(Lut)* or methlut (10 or 50 μmol/L for 6 hours) before SP stimulation (2 μmol/L for 15 minutes). **A,** IκBα phosphorylation was detected and presented as fold relative to control. **B,** NF-κB p65 DNA-binding activity in the nuclear fraction was examined and expressed as fold relative to control. $*P < .05$ and $**P < .01$.

to luteolin, is more effective than cromolyn in blocking hCBMC cytokine release.[38] Nevertheless, cromolyn (100 μmol/L) inhibited histamine release from hCBMCs by about 60%, PGD_2 release by 80%, and LTC_4 by 100%.[38] A recent publication also reported that cromolyn inhibited PGD_2 release from hCBMCs stimulated by IgE/anti-IgE by 100%.[42] Therefore it is apparent that cromolyn can inhibit the release of histamine and arachidonic acid products, but not cytokines, either due to IgE/anti-IgE or SP stimulation. In contrast, luteolin and methlut are more effective MC inhibitors than cromolyn, regardless of the trigger and the mediator measured. Moreover, luteolin and methlut were effective when MCs were preincubated, whereas cromolyn had to be added together with the trigger for its inhibition to manifest. Further incubation with luteolin up to 48 hours does not reduce TNF gene expression any more than what we observe at 24 hours (results not shown). There is no evidence the flavonoids are metabolized inside the cells. The inhibitory effect of methlut also remains the same up to 48 hours (results not shown).

We also report that both luteolin and methlut inhibit the release of CCL2 from hCBMCs. Although luteolin had been reported to inhibit histamine release from hCBMCs,[30] this is the first time that luteolin and methlut have been reported to inhibit CCL2 release. CCL2 is known to be synthesized by MCs[13] and stimulates recruitment of immune cells to the site of inflammation.

We also examined several key signaling processes involved in MC activation, including intracellular calcium[43] and ATP production[44] and activation of the nuclear transcriptional factor NF-κB.[45,46] Here we show that methlut inhibits the inducible transcription factor NF-κB, which is a protein complex that translocates from the cytoplasm into the nucleus on activation and regulates gene expression of various inflammatory mediators.[47] Methlut prevents phosphorylation of IκBα, which is an upstream inhibitor of NF-κB and is degraded on phosphorylation.[37]

We also show that methlut decreases NF-κB p65 DNA-binding activity in the nuclear extract. Apart from inhibiting NF-κB activation at the protein level, we also report, for the first time to our knowledge, that methlut decreases mRNA expression of 2 genes encoding different subunits in the NF-κB protein complex: NFKB1 (encoding NF-κB p50 subunit) and RELA (encoding NF-κB p65 subunit). By blocking NF-κB activation at both the gene and protein levels, methlut and luteolin can effectively regulate proinflammatory mediator production. Luteolin had been reported to inhibit cytokine production by blocking NF-κB in leukemic HMC-1 cells.[46] However, HMC-1 cells do not express the high-affinity FcεRI receptor for IgE,[48] and they proliferate independently of stem cell factor, which is absolutely required for proliferation of primary human MCs. In our studies we used the LAD2 cell line, which is a more mature human MC line than HMC-1, with functional FcεRI and dependence on stem cell factor.[34]

Lut and methlut effectively block stimulated intracellular calcium increase, which is required for MC degranulation.[35,49]

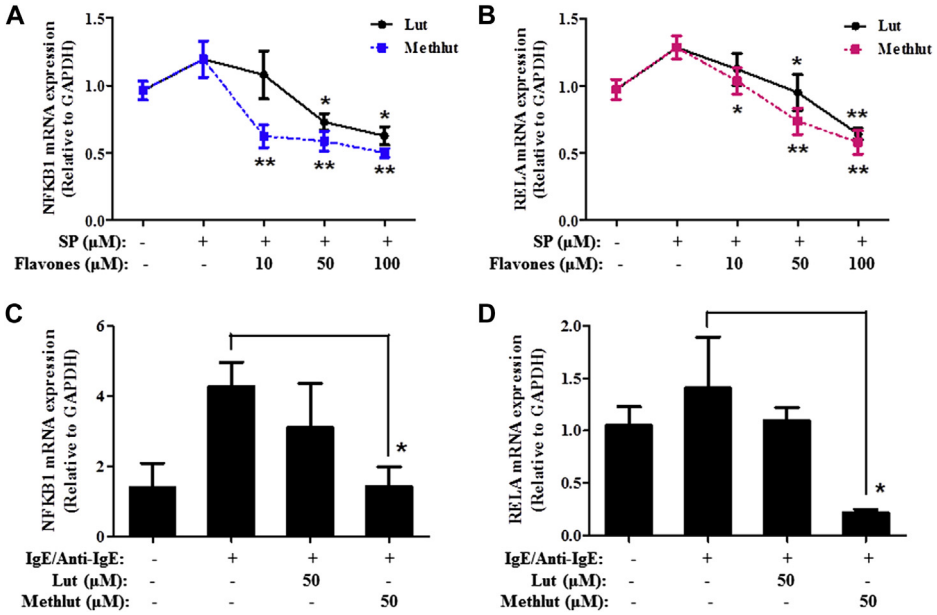

FIG 7. Methlut inhibits NF-κB mRNA expression. **A** and **B,** LAD2 cells were incubated with luteolin *(Lut)* or methlut (10-100 μmol/L for 30 minutes) before SP stimulation (2 μmol/L for 6 hours). **C** and **D,** hCBMCs were primed overnight with IgE (1 μg/mL) and then incubated with lut or methlut (50 μmol/L for 30 minutes) before anti-IgE stimulation (10 μg/mL for 6 hours). mRNA expression levels of NFKB1 and RELA were examined. $*P < .05$.

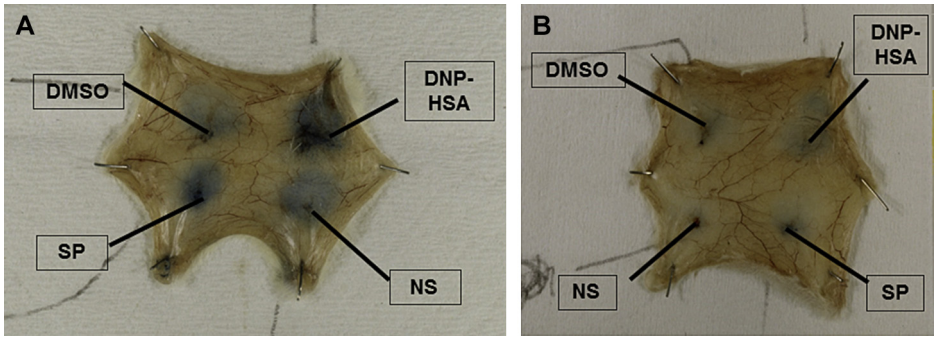

FIG 8. Methlut inhibits mouse skin Evans blue extravasation. Mice were passively sensitized to DBP-HSA and injected with Evans blue through the tail vain. MCs were pretreated with methlut (100 μmol/L) 10 minutes before intradermal challenge. Photographs of mouse skin showing extravasated Evans blue before **(A)** and after **(B)** pretreatment with methlut. *NS,* Normal saline.

Because methlut has 4 methoxyl groups substituted for the 4 hydroxyl groups on luteolin (Fig 1, *C* and *D*), the calcium-blocking action of luteolin is unlikely to be due to any polyphenolic structure, where calcium chelation can occur. These flavones might also inhibit or activate the regulatory components of calcium signaling pathways in MCs. For instance, luteolin-7-O-glucoside inhibited phospholipase C phosphorylation in mouse MCs[50] and the structurally related flavonol quercetin blocked calcium-dependant protein kinase C θ.[51] This effect on stimulated calcium increase should not be of concern for cardiac cells and neurons because the calcium spikes in these cell types are voltage gate regulated, whereas the calcium increase in MCs is not.[52] In fact, luteolin did not have any effect on calcium ion levels in neonatal cultured cardiomyocytes (results not shown).[53]

We were obviously interested in knowing whether the flavones tested affect cell viability and whether any such effect might be mediated through reduction in cellular ATP levels. Neither flavone significantly reduced the viability of human MCs. Moreover, luteolin does not affect the viability of T cells,[32] keratinocytes,[39] or microglia[54] for up to 24 hours. Both luteolin and methlut had no significant effect on intracellular ATP content in MCs.

Here we also show that methlut inhibits skin vascular permeability stimulated by intradermal SP and DNP-HSA in passively sensitized mice. This method was previously used to document the effect of other inhibitors on MC activation,[40] as well as of resveratrol on passive cutaneous anaphylaxis in mice.[55] Moreover, the flavonoid concentrations used here in this mouse model (100 mg/kg) have already been shown to have a statistically significant benefit, at least for luteolin, in children with autism,[56] many of whom have been reported to have "allergic-like" symptoms[57] implicating MC activation.[58]

Surprisingly, we show that methlut is more effective than luteolin in decreasing MC activation. The substitution of the 4

hydroxy groups in luteolin by methoxyl groups (Fig 1, *D*) apparently enhances the inhibitory activity (Fig 1, *C*). This finding seems to contradict previous studies that showed flavones containing more hydroxyl groups have greater antiasthma effects.[59] However, additional publications support the present findings. For instance, 6-methoxyluteolin significantly inhibits histamine and intracellular calcium increase in human cultured basophilic KU812F cells.[60] Two polymethoxy flavones (3′,4′,5,6,7,8-hexamethoxy flavone [nobiletin] and [4′,5,6,7,8-pentamethoxy flavone] tangeretin) prevent LPS-induced bone loss in a mouse model of periodontitis and inhibit PGE_2 production in cocultures of bone marrow cells and osteoblasts.[61]

Another possible explanation might be that the presence of the methyl groups in methlut permits greater penetration in MCs and higher intracellular concentration than luteolin. In addition, methoxy flavones are less metabolized, thereby increasing their biological activities,[33] an important attribute because flavonoids are extensively metabolized.[62]

Luteolin apparently does not have any cell selectivity for MCs because it had previously been shown to also inhibit stimulation of human T cells[32] and keratinocytes,[39] as well as microglia.[63] However, our preliminary evidence (unpublished) indicates that both methlut and luteolin are preferentially taken up by immune cells, especially those with phagocytic activity, such as MCs, macrophages, and microglia. A potential mechanism of selectivity of flavonoids, where luteolin was the best inhibitor of histamine and β-hexosaminidase release, might be disruption of distinct MC vesicle secretion by interacting with granule-dependent SNARE complexes.[64] Interestingly, the beneficial action of cromolyn might also not be selective for MCs because it could be mediated through inhibition of sensory nerve endings, as recently shown for a cromolyn-containing skin cream.[27] Other studies have shown that cromolyn might stimulate afferent nerves in human subjects.[26]

Our present results indicate that the natural flavone methlut is a potent inhibitor of human MC inflammatory mediator release. Its likely mechanism or mechanisms of action involve decreasing intracellular calcium levels and decreasing NF-κB activation at both the transcriptional and translational levels. Methlut has the potential to be developed into a promising MC blocker for the treatment of allergic and inflammatory conditions in which MC activation is involved, particularly when made into formulations that increase its solubility and absorption.

We thank Dr A. S. Kirshenbaum (National Institutes of Health) for providing the LAD2 human MCs and Swedish Orphan Biovitrum AB for their generous gift of rhSCF. We also thank Dr Errol (Department of Obstetrics and Gynecology, Tufts Medical Center) for providing the umbilical cord blood.

Key messages

- **Methlut inhibits release of inflammatory mediators from human MCs stimulated by allergic and neuropeptide triggers.**
- **Methlut is more effective than luteolin and cromolyn for inhibition of MCs.**
- **Methlut inhibits skin vascular permeability in a passively sensitized mouse model.**

REFERENCES

1. Rodewald HR, Dessing M, Dvorak AM, Galli SJ. Identification of a committed precursor for the mast cell lineage. Science 1996;271:818-22.
2. Chen CC, Grimbaldeston MA, Tsai M, Weissman IL, Galli SJ. Identification of mast cell progenitors in adult mice. Proc Natl Acad Sci U S A 2005;102:11408-13.
3. Theoharides TC, Alysandratos KD, Angelidou A, Delivanis DA, Sismanopoulos N, Zhang B, et al. Mast cells and inflammation. Biochim Biophys Acta 2010;1822:21-33.
4. Theoharides TC, Kempuraj D, Tagen M, Conti P, Kalogeromitros D. Differential release of mast cell mediators and the pathogenesis of inflammation. Immunol Rev 2007;217:65-78.
5. Theoharides TC, Bondy PK, Tsakalos ND, Askenase PW. Differential release of serotonin and histamine from mast cells. Nature 1982;297:229-31.
6. Theoharides TC, Cochrane DE. Critical role of mast cells in inflammatory diseases and the effect of acute stress. J Neuroimmunol 2004;146:1-12.
7. Wedemeyer J, Tsai M, Galli SJ. Roles of mast cells and basophils in innate and acquired immunity. Curr Opin Immunol 2000;12:624-31.
8. Deckers J, Branco MF, Hammad H. Innate immune cells in asthma. Trends Immunol 2013;34:540-7.
9. Holgate ST. The role of mast cells and basophils in inflammation. Clin Exp Allergy 2000;30(suppl 1):28-32.
10. Zhang B, Weng Z, Sismanopoulos N, Asadi S, Therianou A, Alysandratos KD, et al. Mitochondria distinguish granule-stored from de novo synthesized tumor necrosis factor secretion in human mast cells. Int Arch Allergy Immunol 2012;159:23-32.
11. Askenase PW. Mast cells and the mediation of T-cell recruitment in arthritis. N Engl J Med 2005;349:1294.
12. Tartaglia LA, Goeddel DV, Reynolds C, Figari IS, Weber RF, Fendly BM, et al. Stimulation of human T-cell proliferation by specific activation of the 75-kDa tumor necrosis factor receptor. J Immunol 1993;151:4637-41.
13. Eglite S, Morin JM, Metzger H. Synthesis and secretion of monocyte chemotactic protein-1 stimulated by the high affinity receptor for IgE. J Immunol 2003;170:2680-7.
14. Salamon P, Shoham NG, Gavrieli R, Wolach B, Mekori YA. Human mast cells release interleukin-8 and induce neutrophil chemotaxis on contact with activated T cells. Allergy 2005;60:1316-9.
15. Petra AI, Panagiotidou S, Stewart JM, Conti P, Theoharides TC. Spectrum of mast cell activation disorders. Expert Rev Clin Immunol 2014;10:729-39.
16. Sismanopoulos N, Delivanis DA, Mavrommati D, Hatziagelaki E, Conti P, Theoharides TC. Do mast cells link obesity and asthma? Allergy 2013;68:8-15.
17. Vasiadi M, Therianou A, Sideri K, Smyrnioti M, Delivani D, Sismanopoulos N, et al. Increased serum CRH levels with decreased skin CRH-R1 gene expression in psoriasis and atopic dermatitis. J Allergy Clin Immunol 2012;129:1410-3.
18. Maurer M, Theoharides TC, Granstein RD, Bischoff SC, Bienenstock J, Henz B, et al. What is the physiological function of mast cells? Exp Dermatol 2003;12:886-910.
19. Castells M. Mast cell mediators in allergic inflammation and mastocytosis. Immunol Allergy Clin North Am 2006;26:465-85.
20. Horan RF, Sheffer AL, Austen KF. Cromolyn sodium in the management of systemic mastocytosis. J Allergy Clin Immunol 1990;85:852-5.
21. Theoharides TC, Sieghart W, Greengard P, Douglas WW. Antiallergic drug cromolyn may inhibit histamine secretion by regulating phosphorylation of a mast cell protein. Science 1980;207:80-2.
22. Barrett KE, Metcalfe DD. The histologic and functional characterization of enzymatically dispersed intestinal mast cells of nonhuman primates: effects of secretagogues and anti-allergic drugs on histamine secretion. J Immunol 1985;135:2020-6.
23. Pearce FL, Befus AD, Gauldie J, Bienenstock J. Mucosal mast cells. II: effects of anti-allergic compounds on histamine secretion by isolated intestinal mast cells. J Immunol 1982;128:2481-6.
24. Oka T, Kalesnikoff J, Starkl P, Tsai M, Galli SJ. Evidence questioning cromolyn's effectiveness and selectivity as a 'mast cell stabilizer' in mice. Lab Invest 2012;92:1472-82.
25. Holgate ST. Reflections on the mechanism(s) of action of sodium cromoglycate (Intal) and the role of mast cells in asthma. Respir Med 1989;83(suppl A):25-31.
26. Collier JG, Fuller RW. Evidence for an effect of sodium cromoglycate on sensory nerves in man. Br J Clin Pharmacol 1983;16:639-43.
27. Vieira Dos SR, Magerl M, Martus P, Zuberbier T, Church MK, Escribano L, et al. Topical sodium cromoglicate relieves allergen- and histamine-induced dermal pruritus. Br J Dermatol 2010;162:674-6.

28. Kudo H, Tanaka T, Miyachi Y, Imamura S. Contact dermatitis from sodium cromoglycate eyedrops. Contact Dermatitis 1988;19:312.
29. Middleton EJ, Kandaswami C, Theoharides TC. The effects of plant flavonoids on mammalian cells: implications for inflammation, heart disease and cancer. Pharmacol Rev 2000;52:673-751.
30. Kimata M, Shichijo M, Miura T, Serizawa I, Inagaki N, Nagai H. Effects of luteolin, quercetin and baicalein on immunoglobulin E-mediated mediator release from human cultured mast cells. Clin Exp Allergy 2000;30:501-8.
31. Asadi S, Zhang B, Weng Z, Angelidou A, Kempuraj D, Alysandratos KD, et al. Luteolin and thiosalicylate inhibit HgCl(2) and thimerosal-induced VEGF release from human mast cells. Int J Immunopathol Pharmacol 2010;23:1015-20.
32. Kempuraj D, Tagen M, Iliopoulou BP, Clemons A, Vasiadi M, Boucher W, et al. Luteolin inhibits myelin basic protein-induced human mast cell activation and mast cell dependent stimulation of Jurkat T cells. Br J Pharmacol 2008;155: 1076-84.
33. Walle T. Methylation of dietary flavones greatly improves their hepatic metabolic stability and intestinal absorption. Mol Pharm 2007;4:826-32.
34. Kirshenbaum AS, Akin C, Wu Y, Rottem M, Goff JP, Beaven MA, et al. Characterization of novel stem cell factor responsive human mast cell lines LAD 1 and 2 established from a patient with mast cell sarcoma/leukemia; activation following aggregation of FcepsilonRI or FcgammaRI. Leuk Res 2003;27:677-82.
35. Kempuraj D, Huang M, Kandere-Grzybowska K, Basu S, Boucher W, Letourneau R, et al. Azelastine inhibits secretion of IL-6, TNF-a and IL-8 as well as NF-κB activation and intracellular calcium ion levels in normal human mast cells. Int Arch Allergy Immunol 2003;132:231-9.
36. Kempuraj D, Papadopoulou NG, Lytinas M, Huang M, Kandere-Grzybowska K, Madhappan B, et al. Corticotropin-releasing hormone and its structurally related urocortin are synthesized and secreted by human mast cells. Endocrinology 2004; 145:43-8.
37. Baeuerle PA, Henkel T. Function and activation of NF-kappa B in the immune system. Annu Rev Immunol 1994;12:141-79.
38. Weng Z, Zhang B, Asadi S, Sismanopoulos N, Butcher A, Fu X, et al. Quercetin is more effective than cromolyn in blocking human mast cell cytokine release and inhibits contact dermatitis and photosensitivity in humans. PLoS One 2012;7: e33805-k.
39. Weng Z, Patel AB, Vasiadi M, Theoharides TC. Luteolin inhibits human keratinocyte activation and decreases NF-κB induction that is increased in psoriatic skin. PLoS One 2014;9:e90739.
40. Donelan J, Boucher W, Papadopoulou N, Lytinas M, Papaliodis D, Theoharides TC. Corticotropin-releasing hormone induces skin vascular permeability through a neurotensin-dependent process. Proc Natl Acad Sci USA 2006;103:7759-64.
41. Wells E, Mann J. Phosphorylation of a mast cell protein in response to treatment with anti-allergic compounds; implications for the mode of action of sodium cromoglycate. Biochem Pharmacol 1983;32:837-42.
42. Yazid S, Sinniah A, Solito E, Calder V, Flower RJ. Anti-allergic cromones inhibit histamine and eicosanoid release from activated human and murine mast cells by releasing Annexin A1. PLoS One 2013;8:e58963.
43. Douglas WW. Exocytosis and the exocytosis-vesiculation sequence: with special reference to neurohypophysis, chromaffin and mast cells, calcium and calcium ionophores. In: Thorn NA, Petersen AH, editors. Secretory mechanisms of exocrine glands. Copenhagen: Munksgaard; 1974. pp. 116-36.
44. Douglas WW. Stimulus-secretion coupling: variations on the theme of calcium-activated exocytosis involving cellular and extracellular sources of calcium. CIBA Found Symp 1978;54:61-90.
45. Azzolina A, Bongiovanni A, Lampiasi N. Substance P induces TNF-alpha and IL-6 production through NFκB in peritoneal mast cells. Biochem Biophys Acta 2003;1643:75-83.
46. Kang OH, Choi JG, Lee JH, Kwon DY. Luteolin isolated from the flowers of *Lonicera japonica* suppresses inflammatory mediator release by blocking NF-kappaB and MAPKs activation pathways in HMC-1 cells. Molecules 2010; 15:385-98.
47. Muller-Ladner U, Gay RE, Gay S. Role of nuclear factor kappaB in synovial inflammation. Curr Rheumatol Rep 2002;4:201-7.
48. Butterfield JH, Weiler DA, Hunt LW, Wynn SR, Roche PC. Purification of tryptase from a human mast cell line. J Leukocyte Biol 1990;47:409-19.
49. Saito H, Ebisawa M, Tachimoto H, Shichijo M, Fukagawa K, Matsumoto K, et al. Selective growth of human mast cells induced by *Steel* factor, IL-6, and prostaglandin E_2 from cord blood mononuclear cells. J Immunol 1996;157: 343-50.
50. Jin M, Son KH, Chang HW. Luteolin-7-O-glucoside suppresses leukotriene C(4) production and degranulation by inhibiting the phosphorylation of mitogen activated protein kinases and phospholipase Cgamma1 in activated mouse bone marrow-derived mast cells. Biol Pharm Bull 2011;34:1032-6.
51. Kempuraj D, Madhappan B, Christodoulou S, Boucher W, Cao J, Papadopoulou N, et al. Flavonols inhibit proinflammatory mediator release, intracellular calcium ion levels and protein kinase C theta phosphorylation in human mast cells. Br J Pharmacol 2005;145:934-44.
52. Vig M, Kinet JP. Calcium signaling in immune cells. Nat Immunol 2009;10: 21-7.
53. Huang M, Kempuraj D, Papadopoulou N, Kourelis T, Donelan J, Manola A, et al. Urocortin induces interleukin-6 release from rat cardiomyocytes through p38 MAP kinase, ERK and NF-kappaB activation. J Mol Endocrinol 2009;42: 397-405.
54. Jang S, Dilger RN, Johnson RW. Luteolin inhibits microglia and alters hippocampal-dependent spatial working memory in aged mice. J Nutr 2010; 140:1892-8.
55. Han SY, Bae JY, Park SH, Kim YH, Park JH, Kang YH. Resveratrol inhibits IgE-mediated basophilic mast cell degranulation and passive cutaneous anaphylaxis in mice. J Nutr 2013;143:632-9.
56. Theoharides TC. Extracellular mitochondrial ATP, suramin, and autism? Clin Ther 2013;35:1454-6.
57. Theoharides TC. Is a subtype of autism “allergy of the brain”? Clin Ther 2013; 35:584-91.
58. Theoharides TC, Angelidou A, Alysandratos KD, Zhang B, Asadi S, Francis K, et al. Mast cell activation and autism. Biochim Biophys Acta 2012;1822:34-41.
59. Lee JY, Kim JM, Kim CJ. Flavones derived from nature attenuate the immediate and late-phase asthmatic responses to aerosolized-ovalbumin exposure in conscious guinea pigs. Inflamm Res 2014;63:53-60.
60. Shim SY, Park JR, Byun DS. 6-Methoxyluteolin from Chrysanthemum zawadskii var. latilobum suppresses histamine release and calcium influx via down-regulation of FcepsilonRI alpha chain expression. J Microbiol Biotechnol 2012; 22:622-7.
61. Tominari T, Hirata M, Matsumoto C, Inada M, Miyaura C. Polymethoxy flavonoids, nobiletin and tangeretin, prevent lipopolysaccharide-induced inflammatory bone loss in an experimental model for periodontitis. J Pharmacol Sci 2012;119:390-4.
62. Chen Z, Zheng S, Li L, Jiang H. Metabolism of flavonoids in human: a comprehensive review. Curr Drug Metab 2014;15:48-61.
63. Jang S, Kelley KW, Johnson RW. Luteolin reduces IL-6 production in microglia by inhibiting JNK phosphorylation and activation of AP-1. Proc Natl Acad Sci U S A 2008;105:7534-9.
64. Yang Y, Oh JM, Heo P, Shin JY, Kong B, Shin J, et al. Polyphenols differentially inhibit degranulation of distinct subsets of vesicles in mast cells by specific interaction with granule-type-dependent SNARE complexes. Biochem J 2013; 450:537-46.

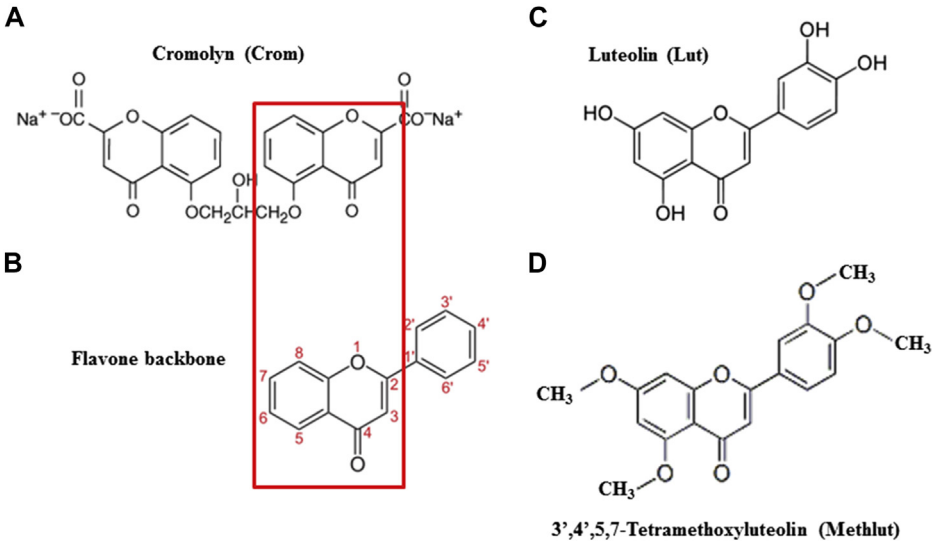

FIG E1. Structures are shown for cromolyn **(A)**, flavone backbone **(B)**, luteolin **(C)**, and methlut **(D)**.

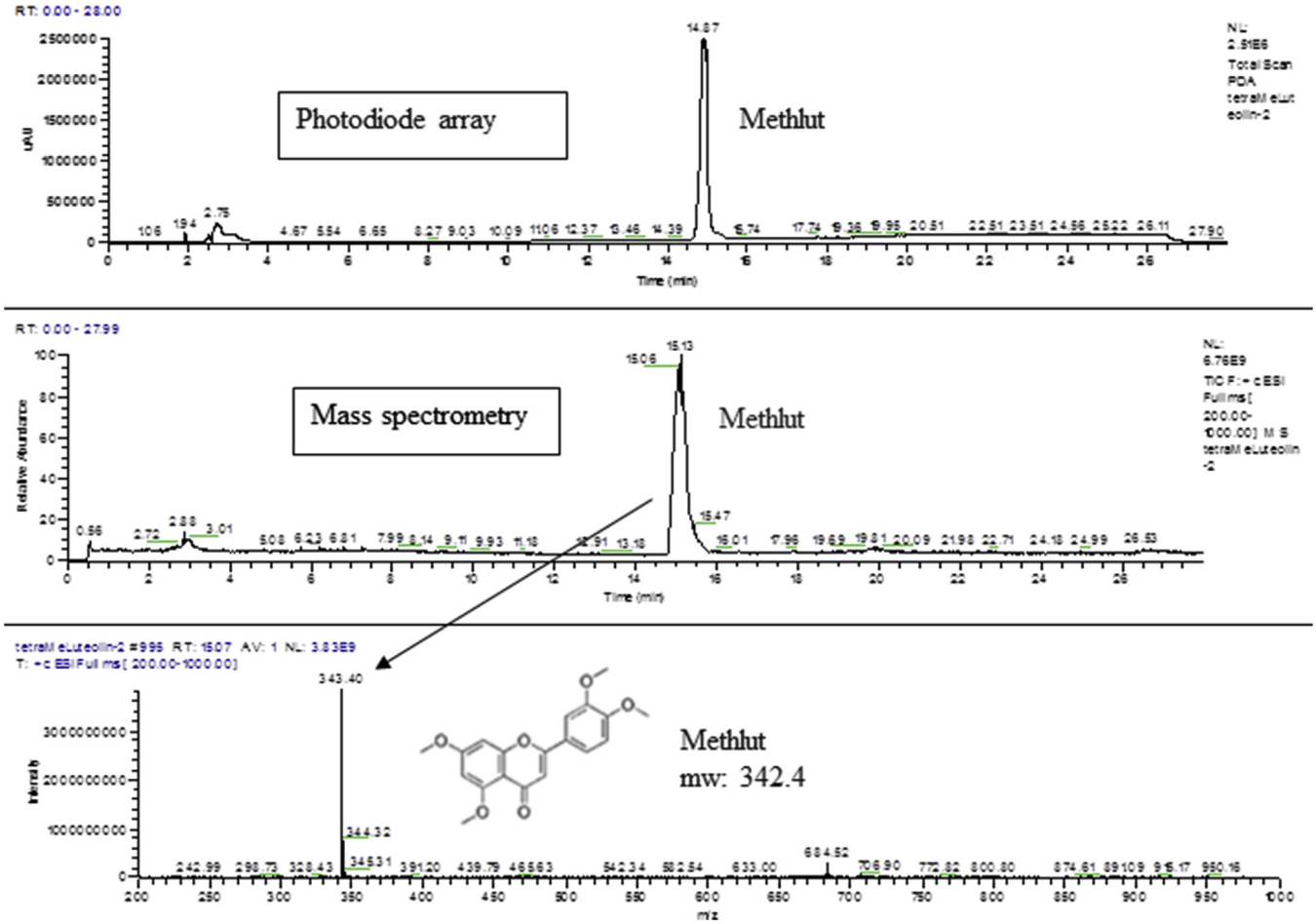

FIG E2. Purity of methlut. Analysis was done by means of mass spectrometry, as detailed in the Methods section.

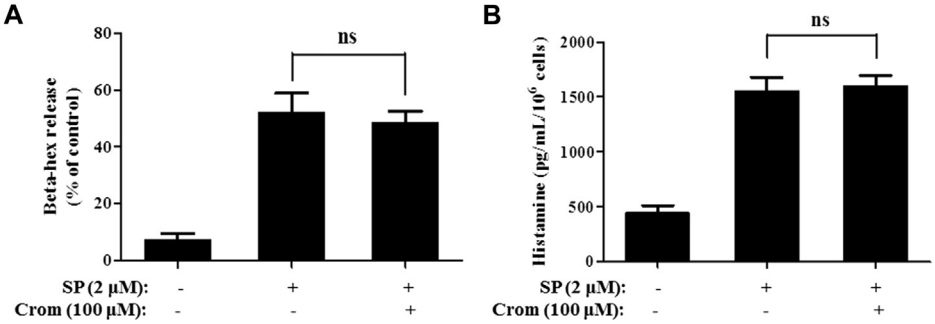

FIG E3. Cromolyn *(Crom)* loses its inhibitory effect on LAD2 cell degranulation if used as pretreatment. MCs were preincubated with cromolyn (100 μmol/L) for 30 minutes before stimulation with either SP (2 μmol/L) or anti-IgE (10 μg/mL) for an additional 30 minutes. **A,** β-Hexosaminidase release. **B,** Histamine release. *ns*, No statistical significance.

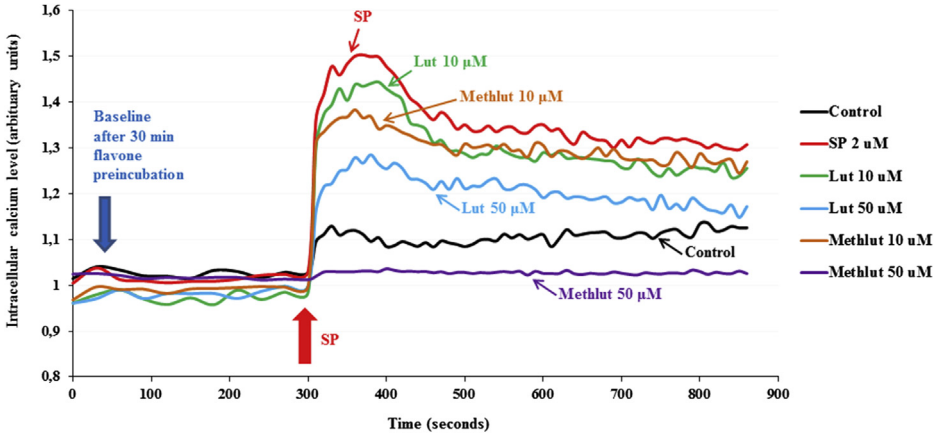

FIG E4. Methlut and luteolin *(Lut)* inhibit intracellular calcium level increase. LAD2 cells were loaded with Fura-2 AM (30 nmol/L for 20 minutes) and then preincubated with luteolin or methlut (10 and 50 μmol/L for 30 minutes). Immediately after stimulation with SP (2 μmol/L), Fura-2 AM fluorescence was monitored at an excitation wavelength of 340 nm and 380 nm and an emission wavelength of 510 nm. Control cells were treated with 0.1% DMSO. The curve shown is representative of 5 experiments with similar results.

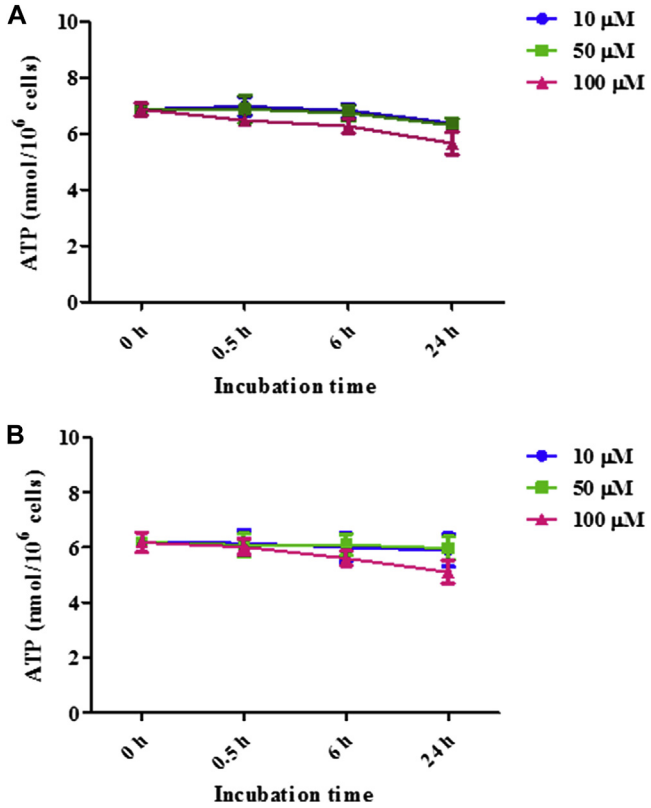

FIG E5. Luteolin and methlut do not affect intracellular ATP levels. After incubation with luteolin **(A)** or methlut (**B**; 10-100 μmol/L up to 24 h), 1×10^6 LAD2 cells were lysed, and intracellular ATP contents were determined.