

IgE-mediated mast cell responses are inhibited by thymol-mediated, activation-induced cell death in skin inflammation

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Background: Mast cells play a critical role in inflammatory skin diseases through releasing proinflammatory mediators; however, few therapies directly target these cells. In 1878, the use of topical thymol, a now recognized potent agonist for transient receptor potential channels, was first described to treat eczema and psoriasis.

Objective: We sought to determine the mechanisms through which thymol can alter skin inflammation.

Methods: We examined the effect of topical thymol on IgE-dependent responses using a mast cell-dependent passive cutaneous anaphylaxis (PCA) model, as well as *in vitro*-cultured mast cells.

Results: Thymol dose-dependently inhibited PCA when administered topically 24 hours before antigen challenge but provoked an ear-swelling response directly on application. This direct effect was associated with local mast cell degranulation and was absent in histamine-deficient mice. However, unlike with PCA responses, there was no late-phase swelling. *In vitro* thymol directly triggered calcium flux in mast cells through transient receptor potential channel activation, along with degranulation and cytokine transcription. However, no cytokine protein was produced. Instead, thymol induced a significant increase in apoptotic cell death that was seen both *in vitro* and *in vivo*.

Conclusions: We propose that the efficacy of thymol in reducing IgE-dependent responses is through promotion of activation-induced apoptotic cell death of mast cells and that this likely explains the clinical benefits observed in early clinical reports. (J Allergy Clin Immunol 2014;■■■:■■■-■■■.)

Key words: Mast cell, thymol, calcium, passive cutaneous anaphylaxis

Abbreviations used

AD: Atopic dermatitis
AICD: Activation-induced cell death
BMMC: Bone marrow-derived mast cell
FITC: Fluorescein isothiocyanate
IP₃R: Inositol trisphosphate receptor
PCA: Passive cutaneous anaphylaxis
TRP: Transient receptor potential

Mast cells have an important role in the pathogenesis of allergic disease.^{1,2} As tissue-resident cells, they are strategically situated at host-environment interfaces, such as the skin, airway, and gastrointestinal tract, ready to respond to immunogenic stimuli.³ In a variety of acute and chronic conditions, mast cell accumulation, activation, and release of proinflammatory mediators are critical to initiate and propagate inflammation.⁴ Several approaches to treating allergic disease have targeted pathways that involve mast cells and their mediators, including desensitization through immunotherapy, leukotriene and histamine receptor inhibitors, tyrosine kinase inhibitors, and anti-IgE antibodies.⁵⁻⁷ Mast cells are long-lived cells and survive repeated activation⁸; altering their proliferation and survival with a view to reducing their numbers has recently been proposed as a potential approach for therapeutic intervention for allergic diseases.^{8,9}

Clinically, these cells are well recognized for their participation in the pathogenesis of inflammatory skin diseases, such as atopic dermatitis (AD). Indeed, AD is characterized by a dramatic increase in the number of IgE⁺ mast cells present in the skin.^{10,11} In the setting of atopic disease, their activation occurs in part by cross-linking the high-affinity IgE receptor FcεRI, leading to histamine-containing granule release and *de novo* production of arachidonic acid metabolites, cytokines, and chemokines that alter vascular permeability and promote skin inflammation.¹²⁻¹⁷ In addition to IgE receptors and other activating receptors, mast cells have recently been shown to express several transient receptor potential (TRP) channels that function to sense environmental changes, including temperature, pressure, and other sensations. Because the skin is the one of the primary barriers interacting with environmental stressors, regulating TRP channel signals might allow modulation of mast cell-mediated skin inflammation.

In the *British Journal of Medicine* in 1878, Henry Radcliffe Crocker reported that topical thymol, now a known TRP channel agonist, could be used as a remedy for patients with eczema, with improvement noted in advanced lesions unresponsive to conventional therapy.¹⁸ Crocker applied topical thymol either as an ointment dissolved in Vaseline or a lotion dissolved in a mixture of ethanol and glycerin and referred to it as “stimulant therapy” because tingling occurred on initial application; this

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was followed by rapid improvement of the skin lesions. Thymol is a monocyclic phenolic compound found in thyme (*Thymus vulgaris*), part of the Lamiaceae family of plants.¹⁹ It is widely used at low concentrations in antiseptic mouthwashes²⁰ in part for its antibiotic,^{21,22} antifungal,²³ and antioxidant^{24,25} properties. In the last few years, studies revealed that thymol is a ligand for the TRPV3 and TRPA1 channels,^{26,27} members of this diverse family of temperature-sensitive cation channels.²⁸ Despite this very early clinical report, the use of thymol in treating skin inflammation has not been further pursued, perhaps because the underlying mechanisms are unknown. Studies investigating the effect of thymol *in vitro* have shown that low thymol concentrations can promote calcium mobilization²⁹⁻³¹ and protect cells from DNA damage,^{32,33} radiation-induced cytotoxicity,²⁵ and oxidative stress.³⁴ Conversely, at higher concentrations, it inhibits cell proliferation and can induce apoptosis in human and murine cancer cell lines.^{29,35-38} Thinking about the effects of thymol in the context of TRP-expressing mast cell functions, we sought to examine the effects of thymol on allergen-triggered skin inflammation.

Our findings demonstrate that thymol treatment leads to sustained calcium flux in mast cells and a significant reduction in their survival. Uncontrolled calcium signaling is a hallmark mechanism that diminishes cell survival by promotion of activation-induced cell death (AICD)-associated apoptosis.³⁹ Although calcium flux is also a hallmark of IgE stimulation through FcεRI, mast cells are resistant to AICD because of the concomitant production of nitric oxide production.⁴⁰ Conversely, thapsigargin, a calcium pump inhibitor that robustly mobilizes calcium, has been shown to drive AICD in mast cells.⁴¹ Here we show that thymol promotes calcium signaling in mast cells through TRP activation and that thymol-activated mast cells undergo apoptosis, likely through AICD. Functionally, this induced death is sufficient to prevent anaphylactic responses on antigen exposure in IgE-primed animals. Taken together, our findings suggest that promoting mast cell death could be a novel approach to limiting atopic disease. Furthermore, our study provides the first mechanistic insights into the previously observed clinical benefits of topical thymol.

METHODS

Reagents

Thymol, Ruthenium red, HC-030031, 2-APB, ionomycin, anti-DNP-IgE, DNP-HSA, and probenecid were purchased from Sigma-Aldrich (St Louis, Mo). Annexin V, SYTOX, and Fluo-4 AM were purchased from Invitrogen (Carlsbad, Calif). Anti-CD117 and anti-CD16/32 were purchased from BD PharMingen (San Diego, Calif), and anti-FcεRI was purchased from eBioscience (San Diego, Calif).

Animals

C57BL/6 and BALB/c mice (4-8 weeks old) were obtained from Taconic Farms (Hudson, NY). HDC^{-/-} mice, which are deficient in histamine, were previously described.⁴² All animal studies were performed under guidelines for care and welfare by the Institutional Animal Care and Use Committee under protocols approved by the Northwestern University Animal Care and Use Committee.

Ear swelling

For thymol-induced ear swelling, 10 μL of thymol or dimethyl sulfoxide was administered to both sides of the ear, and for passive cutaneous

anaphylaxis (PCA), anti-DNP-IgE (100 ng) was intradermally injected into a mouse ear, followed 24 hours later by topical thymol (20 μL per ear), and finally followed 24 hours later by intravenous injection of DNP-HSA (100 μg). Ear swelling was measured with thickness gauge calipers.

Histology

Mice were killed 12 and 24 hours after thymol-induced ear swelling. Ear tissue was fixed in formalin and embedded in paraffin. Tissue sections were stained with pinacyanol erythrosinate, as previously described.⁴³ Mast cell degranulation was determined by counting cells with dense granules and compact shape versus those with dispersed granules outside the cell. Twenty high-power fields were assessed per sample in a blinded fashion.

Mast cell cultures

MC/9 cells were obtained from the American Type Culture Collection, and bone marrow-derived mast cells (BMMCs) were obtained from C57BL/6, as previously described.⁴⁴

β-Hexosaminidase assay

MC/9 cells were incubated with thymol or Compound 48/80 (50 μg/mL) for 40 minutes at 37°C. The supernatants and cell lysates were collected. Degranulation was assessed by measuring the release of β-Hexosaminidase, as previously described.⁴⁵

Real-time RT-PCR

Total RNA and cDNA were prepared, as previously described.⁴⁶ Gene expression was determined by using specific TaqMan probes (Applied Biosystems, Foster City, Calif). β-Actin was used as a housekeeping gene for analysis of changes in cycle threshold. Fold induction for treated samples was determined based on vehicle-treated samples.

Cytokine measurement

MC/9 cells were incubated with thymol or ionomycin (1 μg/mL) for 18 hours. The cells were centrifuged, and supernatant was collected and analyzed by using a standard ELISA.

Annexin V/SYTOX

BMMCs were incubated with thymol for 6 hours and stained with Annexin V, according to the manufacturer's protocol (Invitrogen). The cells were costained with SYTOX and analyzed by means of flow cytometry.

Intraperitoneal thymol

C57BL/6 mice were injected intraperitoneally with thymol (100 μg in 200 μL PBS plus 0.5% EtOH). At the indicated time points, mice were killed, and the peritoneal cavity was lavaged with cold PBS (6 mL) and collected. Cells (10⁷) from this lavage fluid were blocked with anti-CD16/32, stained with anti-c-kit (CD117)/anti-FcεRI and then with Annexin V/SYTOX, and analyzed by using flow cytometry.

Caspase-3 activation

BMMCs were incubated with thymol for 6 or 24 hours and stained for activated caspase-3, according to the manufacturer's instructions (BD PharMingen), and then analyzed by using flow cytometry.

DNA fragmentation/clumping

BMMCs were incubated with thymol for 24 hours. For DNA clumping, cells were labeled with Hoechst 33342 and assessed by using fluorescence microscopy. For DNA fragmentation, cells were processed and analyzed, as previously described.⁴⁷

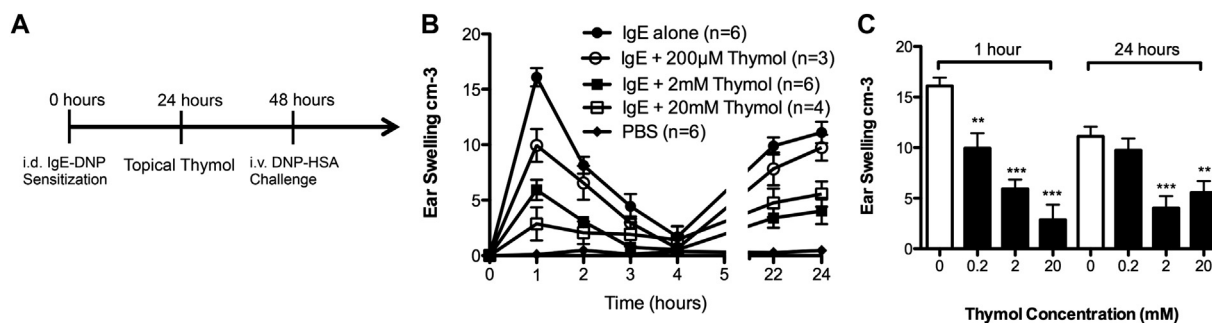


FIG 1. Thymol inhibits PCA. **A**, Schematic of PCA with thymol treatment. *i.d.*, Intradermal; *i.v.*, intravenous. **B**, Ear thickness measurements after DNP-HSA challenge. **C**, Comparison of PCA-induced ear thickness at 1 and 24 hours. Results are from 3 to 6 mice per group from 2 independent experiments. ** $P < .01$ and *** $P < .005$, 2-way ANOVA.

Calcium flux

BMMCs were loaded with 1.5 $\mu\text{mol/L}$ Fluo-4 for 30 minutes at 37°C in loading buffer ($\text{Ca}^{2+}/\text{Mg}^{2+}$ -free HBSS plus 2 mmol/L probenecid plus 0.1% BSA). The cells were washed and incubated at 37°C for 25 minutes in loading buffer plus 1.8 mmol/L anhydrous CaCl_2 . The cells were allowed to equilibrate to room temperature and analyzed by using flow cytometry for 25 seconds to establish a baseline before thymol was added and analyzed for 60 seconds. Data were analyzed with the FlowJo kinetics platform (TreeStar, Ashland, Ore) to visualize a change in fluorescein isothiocyanate (FITC) over time. Pharmacological inhibitors were incubated with mast cells for 1 hour at 37°C. For dose-curve and inhibitor studies, flux data were divided into prestimulation and poststimulation subsets, and median values were subtracted from each other to determine Δ median FITC. Relative calcium flux was determined by comparison with maximal flux for wild-type BMMCs.

Statistics

Data provided as means $\pm$ SEMs. Statistical significance was determined by using the 2-tailed Student *t* test, ANOVA (Dunnett test), or nonlinear regression, as appropriate. All analysis was done with GraphPad Prism software (La Jolla, Calif).

RESULTS

Thymol suppresses mast cell-mediated PCA

To first test whether thymol could modulate mast cell-mediated skin inflammation *in vivo*, we investigated the effect of thymol on responses during PCA, an animal model of skin inflammation mediated by antigen-specific IgE, which we and others have demonstrated to be highly mast cell dependent.^{44,48} PCA was induced by means of intradermal sensitization of anti-DNP-IgE in the ear, followed by systemic intravenous DNP-HSA challenge to cross-link the anti-DNP-IgE bound on the surface of mast cells. This is where a biphasic inflammatory response occurs, with initial ear swelling 1 hour after challenge from histamine release, followed by a cytokine-mediated secondary response approximately 24 hours later.⁴⁹ The previously published protocol was modified to allow for topical thymol treatment 24 hours before antigen-specific DNP-HSA challenge at 48 hours (Fig 1, A). Topical thymol dose-dependently suppressed PCA (Fig 1, B), with significant differences at both the early (1 hour) and late (24 hours) phases of the PCA response (Fig 1, C), suggesting that topical thymol treatment could modulate mast cell function in such a way as to diminish mast cell responses on triggering by antigen-specific IgE.

Thymol induced histamine-dependent ear swelling associated with mast cell degranulation

To next test the direct effect of thymol on cutaneous mast cells in the absence of other stimuli, such as antigen-specific IgE in the previous experiment, we measured the ear-swelling response after topical thymol administration. When administered alone to the mouse ear, topical thymol can directly activate mast cells because dose-dependent immediate ear swelling occurred in both C57BL/6 and BALB/c mice, resolved by 24 hours, and remained unchanged for 48 hours (Fig 2, A); these results suggest that thymol can directly activate mast cells. It is not clear why there were strain-to-strain differences in the threshold concentration of thymol needed to elicit ear swelling, with BALB/c mice requiring a 10-fold greater concentration.

We next assessed the ratio of intact mast cells to degranulated mast cells from pinacyanol-erythrosinate-stained paraffin-embedded ear tissue 24 hours after topical thymol and observed a significant increase in the percentage of degranulated mast cells at higher concentrations of thymol (Fig 2, B and C), suggesting that thymol activates mast cells *in vivo* to degranulate. Because immediate ear swelling is dependent on histamine release from degranulated mast cells, we further examined the requirement for histamine in thymol-induced ear swelling by examining histidine decarboxylase knockout ($\text{HDC}^{-/-}$) mice, which lack the ability to convert histidine to histamine.⁴² Thymol had a minimal effect on ear swelling in $\text{HDC}^{-/-}$ mice compared with wild-type mice, which was similar to the effect of vehicle alone (Fig 2, D), suggesting that histamine was necessary for thymol-induced ear swelling. Similar results were found for mast cell-deficient mice (data not shown). No discernable abnormality was observed either grossly or histologically 24 or 48 hours after topical thymol, suggesting a targeted effect on cutaneous mast cells. Interestingly, despite inducing acute cutaneous mast cell activation within an hour of administration, no sustained late-phase activation was observed after thymol stimulation alone, unlike seen with PCA, suggesting that mast cell activation might have been halted prematurely.

Thymol induced calcium flux in BMMCs

Mast cell degranulation and histamine production is Ca^{2+} dependent, and thymol has previously been shown to mobilize calcium stores in glioblastoma,²⁹ osteoblastoma,³¹ and pituitary GH3 cells.³⁰ BMMCs were loaded with Fluo-4 AM and analyzed by means of flow cytometry before and after thymol stimulation to

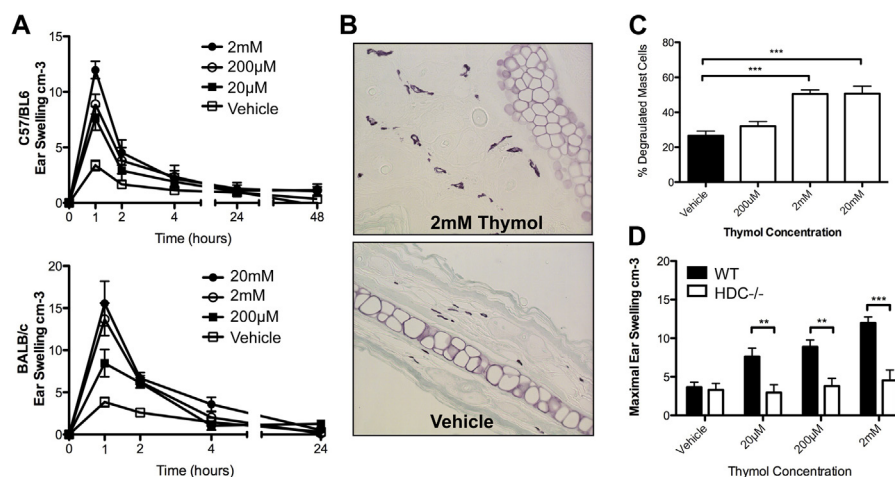


FIG 2. Topical thymol directly promotes local mast cell activation. **A**, Topical thymol-induced ear swelling in C57/BL6 and BALB/c mice. **B**, Representative ear histology. **C**, Quantification of degranulated mast cells by using pinacyanol erythrosinate stain in BALB/c mice. **D**, Ear thickness 1 hour after topical thymol in C57/BL6 and HDC^{-/-} mice. WT, Wild-type. Results are from 6 to 21 mice per group from 3 (Fig 2, A and D) and 2 (Fig 2, C) independent experiments. ***P* < .01 and ****P* < .005, 1-way ANOVA.

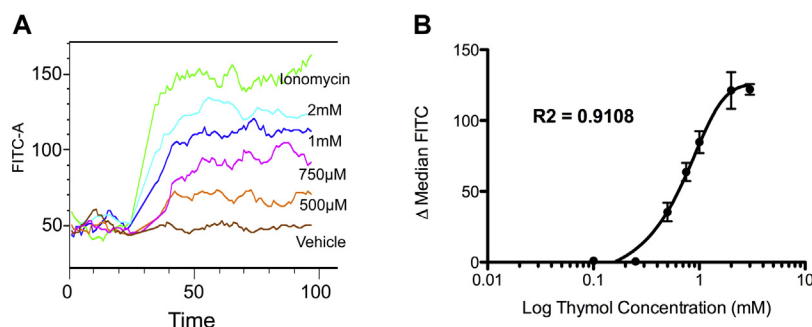


FIG 3. Thymol induced calcium flux in mast cells. **A**, Representative kinetics plot. **B**, Graphic representation of the difference between median prestimulation and poststimulation FITC signal. Results are from 4 to 6 samples per group from 4 independent experiments.

test whether thymol directly affects mast cell Ca²⁺ flux. Calcium flux was determined on FlowJo software by using the kinetics platform to assess changes in FITC over time. Indeed, thymol induced a dose-dependent calcium flux in mast cells (Fig 3), which is consistent with previous experiments suggesting that thymol activates mast cells.

Thymol-induced calcium flux is partially mediated by triggering TRPA1

Our data show that thymol regulates calcium flux in mast cells. Because thymol is known to transduce signaling through TRP channels and several TRP channels are expressed on mast cells, we focused on testing whether thymol-induced calcium flux was activated by the thymol-activated TRPA1 channel,^{26,27} which has previously been shown to be expressed on mast cells.⁵⁰ First, we assessed thymol-induced calcium flux after preincubation with Ruthenium red, a nonspecific TRP channel inhibitor. Ruthenium red dose-dependently decreased thymol-induced calcium flux (Fig 4, A), suggesting that a TRP channel might be partially responsible for the interaction of thymol with mast cells. We then tested a previously described selective antagonist for TRPA1,

HC-030031,⁵¹ along with 2-APB, a pharmacologic antagonist of the inositol trisphosphate receptor (IP₃R)-mediated Ca²⁺ release.⁵² After preincubation with mast cells, HC-030031 and 2-APB dose-dependently decreased thymol-induced calcium flux (Fig 4, B and C). Importantly, ionomycin-induced calcium flux was maintained after inhibitor treatment, suggesting a targeted effect (data not shown). Therefore thymol acts through TRPA1 and IP₃R to intracellularly mobilize calcium within the mast cell.

Thymol induced mast cell degranulation and gene transcription *in vitro* but prevented protein production

To better understand the downstream effect of direct thymol activation on mast cell degranulation and cytokine production, we examined *in vitro* degranulation, as well as cytokine mRNA expression and protein production, after treating MC/9 cells, a murine fetal liver-derived IL-3-dependent mast cell line. As shown in Fig 5, A, 2 mmol/L thymol or greater induced comparable degranulation to the control secretagogue 48/80, as assessed based on β-hexosaminidase release after a 40-minute incubation with thymol. We also observed a dose-dependent

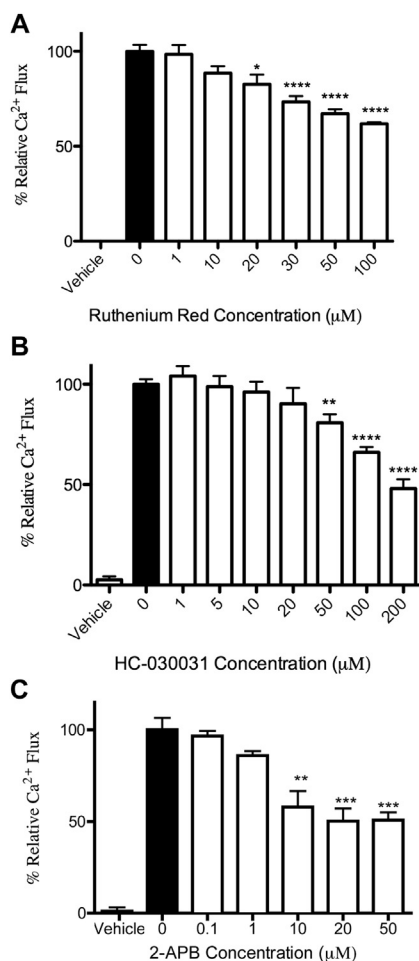


FIG 4. Thymol induced calcium flux through TRPA1 and not TRPV3. **A**, Relative calcium flux from 800 $\mu\text{mol/L}$ thymol in BMMCs after a 1-hour incubation with Ruthenium red. **B**, Relative calcium flux from 800 $\mu\text{mol/L}$ thymol in BMMCs after a 1-hour incubation with HC-030031. **C**, Relative calcium flux from 800 $\mu\text{mol/L}$ thymol in BMMCs after a 1-hour incubation with 2-APB. Results are from 3 samples per group from 3 independent experiments. ** $P < .01$ and *** $P < .005$, 1-way ANOVA.

increase in IL-6 and IL-13 gene transcription (Fig 5, B) after a 3-hour incubation. Although we expected that we would also observe increased IL-6 and IL-13 protein production, as is typical with upregulated mRNA transcripts, we surprisingly observed no increases in IL-6 and IL-13 protein levels in the supernatants of thymol-treated cells, unlike on treatment with the Ca²⁺ ionophore ionomycin (Fig 5, C). Although thymol was able to activate mast cells *in vitro*, as characterized by degranulation and gene transcription, the lack of cytokine production in the supernatant suggested that thymol might also function to inhibit the cytokine protein production/release pathway from mast cells or perhaps affect mast cell numbers at this later time point by altering their survival.

Thymol decreased BMMC viability through apoptosis

To distinguish between the possibilities that thymol inhibited cytokine production/release or affected mast cell survival after initial activation, we next examined the effect of thymol on mast

cell viability. Here we used BMMCs as opposed to MC/9 cells because of their low turnover and high relative baseline viability. Thymol was incubated with BMMCs and analyzed by means of flow cytometry for viability by using SYTOX labeling. At 24 hours (Fig 6, A), thymol induced a dose-dependent decrease in mast cell viability.

To assess whether thymol induced mast cell apoptosis, we examined the effect of thymol on cell membrane scrambling, which occurs early in apoptosis, by measuring phosphatidylserine exposure on the cell surface. As shown in representative flow plots (Fig 6, B) and graphically (Fig 6, C), 6-hour incubation with thymol led to a dose-dependent increase in numbers of early apoptotic cells (Annexin V⁺/SYTOX⁻). In addition, thymol induced a significant and time-dependent increase in intracellular caspase-3 activation, another readout of apoptosis induction (Fig 6, D). Two hallmarks of late apoptosis, DNA fragmentation (Fig 6, E) and clumping (Fig 6, F), were also both seen after 24 hours of thymol. Therefore thymol decreased mast cell viability *in vitro* by inducing apoptosis.

Thymol decreased intraperitoneal mast cell viability through apoptosis

Considering that we observed a therapeutic suppression of specific antigen-dependent mast cell responses by thymol in our PCA model, even though thymol itself activated mast cells, we postulated whether thymol-induced mast cell apoptosis by means of AICD might underlie the suppression of the PCA reaction we observed. We initially examined the density of cutaneous mast cells 12 hours after topical thymol (20 mmol/L) in BALB/c mice from pinacyanol erythrosinate-stained paraffin-embedded ear tissue. We observed a significant decrease in mast cell density, which suggested that mast cells were depleted by thymol (Fig 7, A). To further examine whether thymol induced mast cell apoptosis *in vivo*, we chose to focus on intraperitoneal mast cells, given that this is a rich source of mast cells and because of limitations in isolating and analyzing functional cutaneous mast cells. For this experiment, 100 μg of thymol was administered intraperitoneally to C57BL/6 mice. The mice were killed at various time points, and the peritoneum was lavaged with cold PBS. The recovered fluid was analyzed for mast cell content based on the percentage of viable double-positive c-kit/FcεRI cells. Thymol induced a time-dependent statistical decrease in mast cell numbers over a 12-hour period (Fig 7, B). By 1 hour, a significant increase in Annexin V⁺/SYTOX⁻ mast cell numbers was detectable, suggesting apoptosis had occurred (Fig 7, C). Therefore thymol reduced mast cell viability *in vivo* through AICD-induced apoptosis, providing an explanation for how thymol can suppress mast cell-dependent inflammation *in vivo*.

DISCUSSION

In this study we sought to elucidate a possible mechanism for the beneficial effects of thymol on mast cell-associated skin inflammation, such as in eczema.¹⁸ Severe AD is often refractory to standard topical therapy,⁵³ and for these patients, cyclosporine, tacrolimus, systemic steroids, azathioprine, and omalizumab are treatment considerations.^{53,54} One limitation of these medications is their broad immunomodulatory effect with additional side effects and risks, particularly with systemic

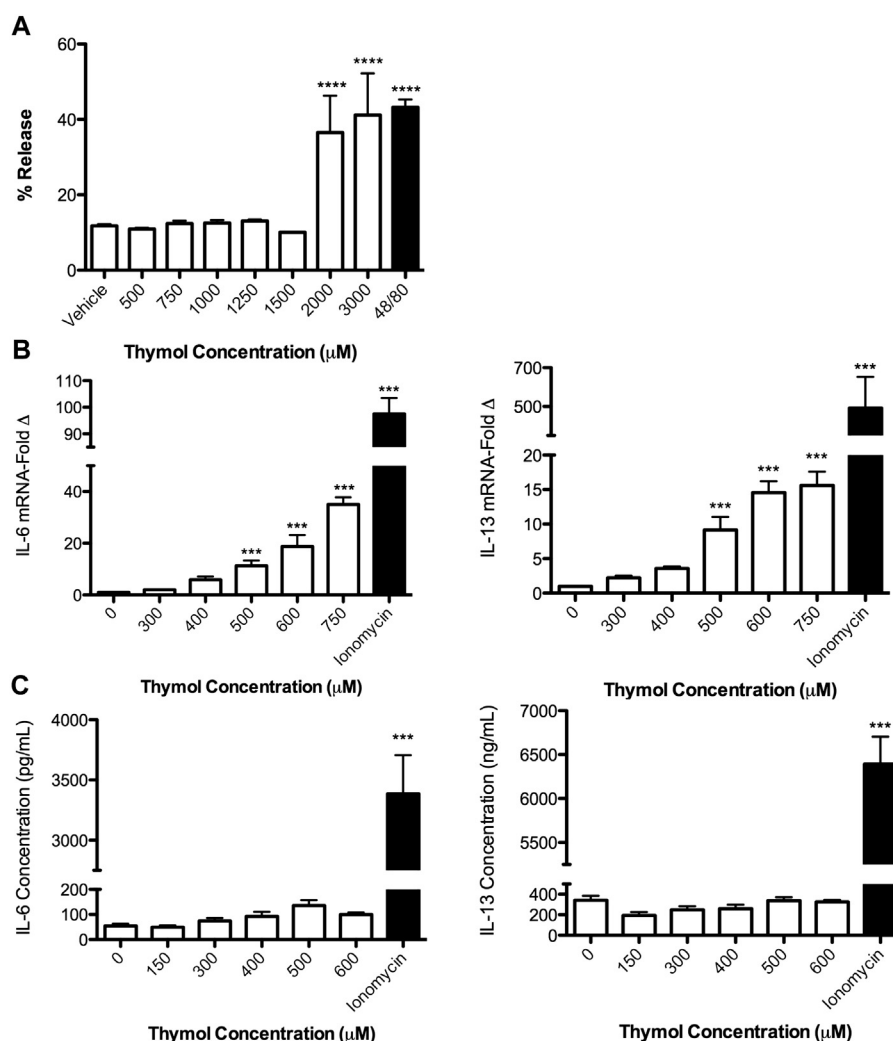


FIG 5. Thymol activated mast cells *in vitro* but prevented secretion of protein. **A**, Percentage release of β -hexosaminidase in MC/9 cells after a 40-minute stimulation with thymol. **B**, Gene expression after a 3-hour stimulation with thymol or ionomycin (1 μ g/mL) expressed as fold induction over vehicle. **C**, Cytokine quantification of supernatants after an 18-hour stimulation with thymol or ionomycin (1 μ g/mL). Results are from 3 samples per group from 3 independent experiments. *** P < .005 and **** P < .001, 1-way ANOVA.

administration, driving a need for new therapies. The effector phase of many allergic diseases is characterized by antigen-driven mast cell activation, causing degranulation with histamine release and *de novo* production of proinflammatory cytokines. Therefore we used PCA to better understand the efficacy of thymol because it is a useful model to evaluate localized antigen-driven mast cell-mediated allergic reactions, a recognized component in eczema pathogenesis.¹⁴⁻¹⁷ Our data demonstrate a novel antiallergic property of thymol to induce mast cell apoptosis, limiting their subsequent ability to elicit IgE-mediated responses.

Interestingly, thymol clearly promotes initial mast cell activation characterized by a robust increase in intracellular calcium levels, which was inhibited by the nonselective TRP channel antagonist Ruthenium red, the selective TRPA1 antagonist HC-030031, and the IP₃R antagonist 2-APB, defining this as a likely pathway through which thymol elicits effects on mast cells. Although mast cells are known to express TRPA1,

the functional effects of TRPA1 ligands on mast cells have not been shown previously. It remains possible that additional mechanisms are important because we were unable to fully ablate the entire calcium flux response with any of these well-characterized inhibitors. Indeed, we were unable to reverse the decreased cell viability using Ruthenium red, HC-030031, or 2-APB (data not shown), although this experimental approach is confounded by the intrinsic inhibitory effects of these agents on cell viability generally because Ca²⁺ signaling is necessary for cell homeostasis. Additionally, it is possible that ablation of PCA by thymol could occur through depletion of granule contents before antigen challenge; however, our data demonstrate that thymol induces cell death in mast cells that is preceded by sustained calcium flux through TRPA1.

Several studies have assessed different mechanisms for targeted induction of apoptosis in mast cells.⁸ Much of the mechanistic research has focused on the intrinsic pathway and the role of the Bcl-2 family,⁴ as well as the death receptors

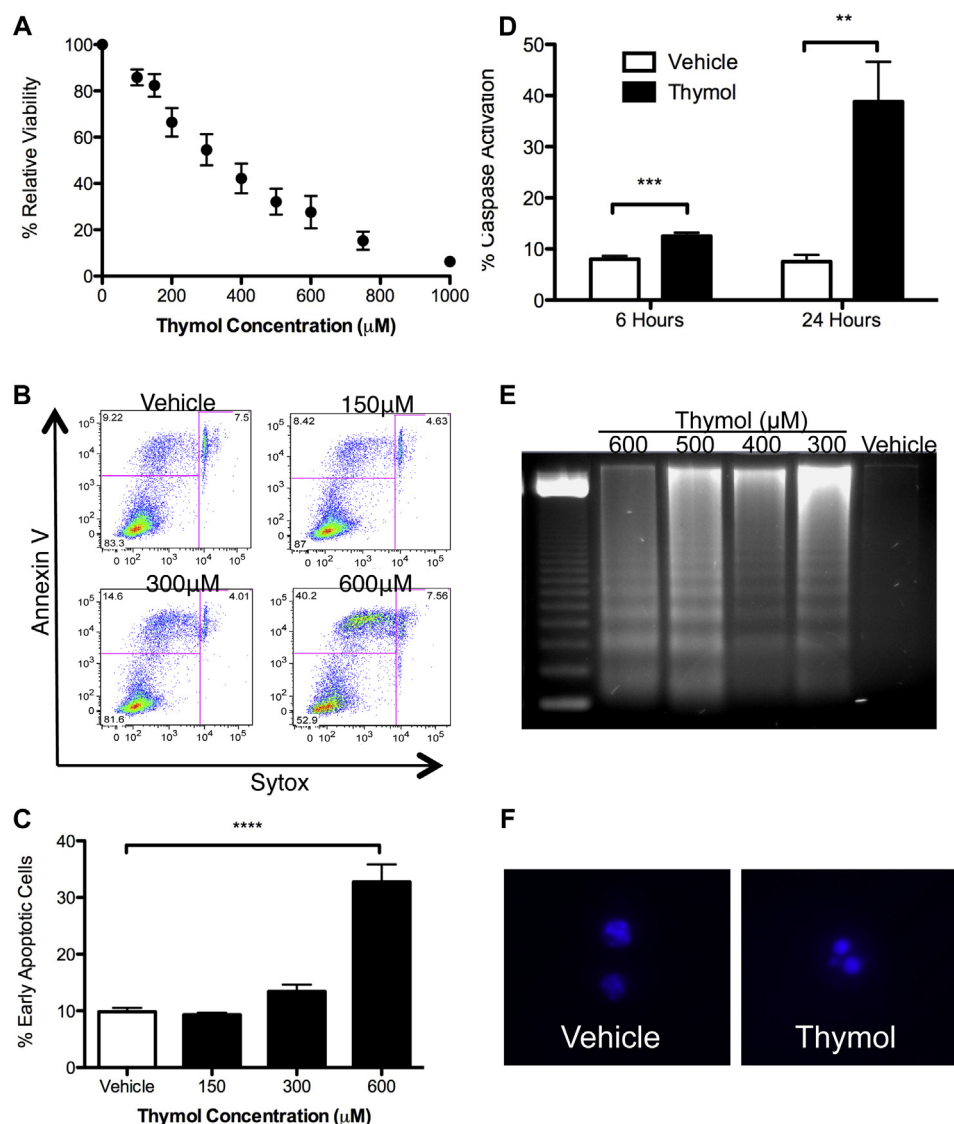


FIG 6. Thymol decreased mast cell viability *in vivo* and *in vitro* through apoptosis. **A**, Viability of BMMCs after 24 hours of thymol treatment. **B**, Flow cytometry for early (Annexin V⁺/SYTOX⁻) and late (Annexin V⁺/SYTOX⁺) apoptotic BMMCs after thymol incubation for 6 hours. **C**, Quantification of early apoptotic cells after 6 hours of thymol treatment. **D**, Intracellular staining for active caspase-3 after 6- and 24-hour thymol incubation. **E**, DNA fragmentation after 24 hours of thymol treatment. **F**, Hoechst 33342 staining after 24 hours of thymol incubation. Results are from 3 samples per group in Fig 6, A, C, and D, from 3 to 4 independent experiments. ****P* < .005 and *****P* < .001, 1-way ANOVA.

CD95R⁵⁵ and TRAIL-R⁵⁶ and the inhibitory receptor CD300a⁵⁷ in the extrinsic pathway. Recent human and animal model studies support the concept that apoptosis carries important therapeutic considerations.⁵⁸⁻⁶⁰ AICD acts a homeostatic mechanism for immune cleanup that is classically associated with the removal of activated T cells after clonal expansion in an immune response.⁶¹ Mast cells possess a long lifespan in part because of their resistance to AICD and apoptotic death through several counterregulatory mechanisms, including nitric oxide-mediated Ca_v1.2 L-type Ca²⁺ channel activation⁶² and Src homology domain 2-containing protein tyrosine phosphatase 1-directed upregulation of extracellular signal-regulated kinase 1/2 and Bcl-xL.⁶³ However, mast cells have been shown to exhibit AICD in response to thapsigargin, a stimulator of

store-operated Ca²⁺ channel entry and selective inhibitor of the sarcoplasmic/endoplasmic Ca²⁺ ATPase pump that is necessary for replenishing endoplasmic reticulum stores when sustained cytosolic calcium is required.⁴¹ It remains to be determined whether thymol targets similar calcium stores to induce AICD, but in pituitary GH₃ cells thymol was shown to induce an increase in intracellular Ca²⁺ levels by triggering both external Ca²⁺ influx through store-operated Ca²⁺ channel entry and internal Ca²⁺ release from the endoplasmic reticulum.³⁰ Our data suggest that mast cell stimulation with thymol leads to mobilization of Ca²⁺ that activates degranulation and begins gene transcription; however, continued exposure to thymol leads to a sustained increase in cytosolic calcium levels and thus AICD-associated apoptosis.

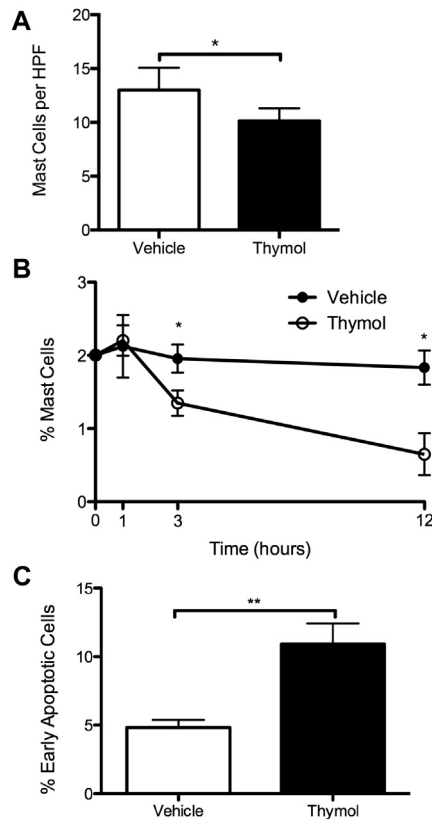


FIG 7. Thymol decreased mast cell viability *in vivo* through apoptosis. **A**, Quantification of mast cell density by using pinacyanol erythrosinate staining in BALB/c mice 12 hours after topical thymol. *HPF*, High-power field. **B**, Percentage of mast cells (c-kit⁺/FcεRI⁺) detectable after intraperitoneal injection of thymol (100 μg per animal) in peritoneal lavage fluid. **C**, Frequency of early apoptotic (Annexin V⁺/SYTOX⁺) mast cells from peritoneal lavage fluid at 1 hour. Results are from 3 to 7 mice per group. **P* < .05 and ***P* < .01, 1-way ANOVA.

Although the role of mast cells in eczema is not entirely clear, our mechanistic findings would seem to explain the 2 key observations made by Crocker¹⁸: the “stimulatory” nature of thymol is likely explained by the initial activation of mast cells and the release of preformed granules into surrounding tissue, whereas the benefits of prolonged exposure might be explained by loss of tissue-resident mast cells that confer IgE-mediated allergen sensitivity because of apoptosis. Crocker also made reference to the benefit of thymol therapy in patients who were refractory to other therapies of that time. Importantly, many patients with AD today remain difficult to treat, even with use of topical glucocorticoids or immunosuppression.⁶⁴ Anti-IgE therapy has recently been shown to be therapeutically beneficial in such patients,⁶⁵ suggesting that targeting the IgE/mast cell pathway might be clinically advantageous.

In conclusion, our study demonstrates that thymol promotes an activation-induced apoptotic death of mast cells heralded by a robust mobilization of calcium, which is likely mediated by TRPA1. The consequence of this response is a depletion of mast cells, leading to a therapeutic suppression of subsequent IgE-dependent responses to antigen. On the basis of these mechanistic findings, topical thymol might be a potential therapeutic for the ablation of mast cell–associated skin inflammation, including eczema.

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Key messages

- Topical application of thymol reduces IgE-mediated responses to antigen.
- Mechanistically, mast cells are sent into activation-induced death as a result of sustained calcium activation.

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