

Transient Receptor Potential Ankyrin 1 Enhances Ovalbumin-Induced Acute Allergic Inflammation in Murine Models

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Keywords

TRPA1 · Allergic inflammation · Paw edema · Allergic conjunctivitis · Ovalbumin

Abstract

Background: Transient receptor potential ankyrin 1 (TRPA1) is an ion channel known to mediate nociception and neurogenic inflammation, and to be activated by reactive oxygen and nitrogen species (ROS and RNS) produced at the sites of inflammation. Because neurogenic inflammation as well as the release of ROS and RNS are typical features of early stages of allergic responses, we hypothesized that TRPA1 may be involved in triggering and/or amplifying allergic inflammation. **Objective:** This study aims at exploring the role of TRPA1 ion channel in acute ovalbumin-induced allergic inflammation in applicable murine models. **Methods:** The effects of pharmacological blockade and genetic deletion of TRPA1 in ovalbumin-induced allergic conjunctivitis and acute paw inflammation were studied in mice sensitized to

ovalbumin. **Results:** Ovalbumin-induced allergic conjunctivitis was milder in TRPA1-deficient mice and alleviated in wild-type mice treated with the TRPA1 antagonist TCS 5861528. Subcutaneous challenge with ovalbumin caused a significant paw edema and interleukin (IL)-4 production in sensitized mice; these responses were attenuated in animals treated with the TRPA1 antagonist and in TRPA1-deficient mice. Interestingly, blockade of the major secondary effector of TRPA1, substance P, also resulted in attenuated ovalbumin-induced paw edema and IL-4 production. However, the splenocytes' responses to ovalbumin were similar in cells from wild-type and TRPA1-deficient mice sensitized to ovalbumin. **Conclusion:** These results introduce a novel concept that TRPA1 mediates early events in allergic inflammation, but does not seem to affect allergic sensitization, and could therefore be a novel drug target to treat conditions associated with allergic inflammation.

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Introduction

In allergic inflammation, features of neurogenic inflammation have recently been recognized as complementary mechanisms to the classical T_H2 -driven allergic responses. Release of the proinflammatory neuropeptide substance P has been shown to promote allergic inflammation in cases of experimental allergic asthma [1] and conjunctivitis [2]. Substance P induces proinflammatory responses including vascular leakage, edema formation, and proinflammatory cytokine production. It is released from peripheral nerve-endings and also from inflammatory cells such as macrophages, eosinophils, lymphocytes, and dendritic cells [3].

Transient receptor potential ankyrin 1 (TRPA1) is a cation channel involved in nociception and neurogenic inflammation, and it has been recognized as a potential drug target to treat painful and inflammatory conditions [4–7]. TRPA1 is expressed throughout the body mainly in neurons, but also in lining cells such as keratinocytes, and endothelial and epithelial cells [5, 8, 9]. As a physiological chemosensor, the function of TRPA1 is to detect potentially harmful substances presented to the body and hence it is activated by a variety of exogenous noxious compounds such as allyl isothiocyanate (AITC). Importantly, reactive oxygen and nitrogen species (ROS and RNS) produced endogenously at the sites of inflammation also activate TRPA1 [4, 5]. Activation of neuronal TRPA1 is known to induce the release of proinflammatory neuropeptides such as substance P and calcitonin gene-related peptide, and these neuropeptides are likely to contribute to the inflammatory responses induced by activation of TRPA1 [5, 7, 10].

The role of TRPA1 as a factor in inflammation is unfolding. Activation of TRPA1 by exogenous or endogenous proinflammatory substances can induce acute inflammation [4] in conditions such as experimental paw inflammation [6], dermatitis [11], colitis [12], gout [13, 14], or osteoarthritis [10]. Interestingly, activation of TRPA1 was found to mediate also the development of airway inflammation in experimental asthma in mice following a repeated allergen challenge [15, 16]. Considering that neurogenic inflammation as well as the release of ROS and RNS are characteristics of the early stages of allergic inflammation [17], and that TRPA1 is linked to both these features, we hypothesized that TRPA1 may be a general mechanism involved in triggering and/or amplifying allergic inflammation. Here, we investigated the role of TRPA1 in ovalbumin-induced allergic conjunctivitis and acute allergic paw inflammation by using TRPA1-deficient and TRPA1 blocker-treated mice.

Materials and Methods

Studied Mice

Wild-type and TRPA1 knock-out B6;129P-Trpa1(tm1Kyk)/J hybrid mice (Charles River Laboratories, Sulzfeld, Germany) were used in the experiments studying the role of TRPA1 in ovalbumin-induced allergic conjunctivitis and acute allergic paw edema. The genotype for *Trpa1* was confirmed with PCR after the experiments. In addition, C57BL/6N mice (Scanbur Research A/S, Karlslunde, Denmark) were used to study the drug effects in ovalbumin-induced acute allergic paw edema. Mice were housed under standard conditions (12/12 h light-dark cycle and $22 \pm 1^\circ\text{C}$) with food and water provided ad libitum. Animal experiments were carried out in accordance with the legislation for the protection of animals used for scientific purposes (Directive 2010/63/EU) and approved by The National Animal Experiment Board. Intraperitoneal injection of medetomidine (0.5 mg/kg, Domitor®, Orion, Espoo, Finland) and ketamine (75 mg/kg, Ketalar®, Pfizer Animal Health, Helsinki, Finland) were used for anesthesia. After the experiments, animals were sacrificed by suffocation with carbon monoxide followed by cranial dislocation.

Reagents

Reagents were purchased from Sigma Chemicals, St. Louis, MO, USA, unless otherwise indicated. The PBS used was sterile and endotoxin-free.

Sensitization to Ovalbumin

The mice were sensitized to ovalbumin with intraperitoneal (i.p.) injection of ovalbumin (100 µg), Al (OH)₃ (1 mg, Acros Organics, Geel, Belgium) and Pertussis toxin (300 ng) suspended in 550 µL of PBS on day 1. Sensitization was boosted with an i.p. injection of ovalbumin (100 µg) and Al (OH)₃ (1 mg) suspended in 500 µL of PBS on day 6. Nonsensitized control mice received i.p. injection of PBS only. The experiments were performed 14 days after the sensitization.

Ovalbumin-Induced Allergic Conjunctivitis

Ovalbumin-induced allergic conjunctivitis was triggered by topically applying DL-dithiothreitol (0.77 mg in 5 µL of PBS) and then ovalbumin (0.5 mg in 5 µL of PBS) 15 min later into both eyes of the studied mice. Both eyes were then treated topically by applying the TRPA1 blocker TCS 5861528 (25 µg in 5 µL of PBS), the control compound dexamethasone (5 µg in 5 µL, Oftan Dexa, Santen, Tampere, Finland) or vehicle (5 µL of PBS) 1 h before and 2, 4, and 6 h after the application of ovalbumin. Twenty-four hours after the ovalbumin application, the mice were sacrificed, and conjunctival tissue from both eyes was surgically removed and analyzed for eosinophil infiltration by measuring eosinophil peroxidase activity.

Eosinophil Peroxidase Activity Measurements

Conjunctivas were washed with ice-cold PBS and homogenized with disposable pellet pestle cordless motor (Kimble Chase Life Science and Research Products, Rockwood, TN, USA) in Tris-HCl buffer (50 mM, pH 8.0) containing 0.1% Triton-X100. Samples were then incubated at 4°C for 2 h and centrifuged (10 min, 10,000 g, 4°C), and the supernatants were collected for further analysis. Samples (50 µL) and eosinophil peroxidase substrate solution (100 µL) containing o-phenylenediamine (2 mM) and 0.1% Triton-

X100 in Tris-HCl buffer (50 mM, pH 8.0) were added to a 96-well microplate. The reaction mixture was incubated in the dark for 30 min at room temperature and the reaction was stopped by adding H₂SO₄ (50 µL, 2 M). Finally, eosinophil peroxidase activity, correlating with the number of eosinophils infiltrated to the tissue, was measured as absorbance at 490 nm by a Victor3 1,420 multilabel counter (Perkin Elmer, Waltham, MA, USA).

Eosinophil Isolation and Apoptosis Measurements

Blood-derived eosinophils were obtained from healthy human donors. Subjects gave a written informed consent to a study protocol approved by the Ethics Committee of Tampere University Hospital (Tampere, Finland). The eosinophils were isolated and cultured as previously described [18]. Next, the cells were incubated with a TRPA1 blocker, TCS 5861528 (10 µM) or HC-030031 (10 µM), the TRPA1 agonist AITC (10 µM), or the control compound dexamethasone (1 µM) in the presence of interleukin (IL)-5 (0.5 pM) for 40 h. Eosinophil apoptosis was determined by the relative DNA fragmentation assay in propidium iodide-stained cells by flow cytometry (FACScan, Becton Dickinson, San José, CA, USA) as previously described [18]. Eosinophils were isolated from 6 different donors and the experiments were performed with 4 replicates.

Ovalbumin-Induced Acute Allergic Edema Test

Ovalbumin-induced acute allergic paw edema was induced by subcutaneous (s.c.) injection of ovalbumin (45 µg in 45 µL of PBS) into the hind paw of anesthetized mice previously sensitized to ovalbumin. The contralateral paw was injected with vehicle and developed no measurable edema. The paw volume was measured up to 6 h with plethysmometer (Ugo Basile, Comerio, Italy) and compared to the baseline value. Mice were dosed with the selective TRPA1 blocker, TCS 5861528 (10 mg/kg orally), the anti-inflammatory control compound dexamethasone i.p. (2 mg/kg), the selective neurokinin-1 receptor blocker L703,606 i.p. (10 mg/kg, Enzo Life Sciences AG, Lausen, Switzerland) which has been shown to block the action of neuropeptide substance P [19], or with vehicle (i.p. PBS) 1 h prior to the experiments. In addition, TRPA1-deficient mice were compared to the corresponding wild-type mice.

Paw Tissue Extraction and IL-4 Measurements

After the mice were sacrificed, the inflamed paw tissue (injected s.c. with ovalbumin) and the corresponding contralateral paw tissue were collected for analysis in buffer containing Tris (50 mM, pH 7.4), NaCl (150 mM), Triton-X (0.5%), and the protease and phosphatase inhibitors phenylmethylsulfonyl fluoride (0.5 mM), sodium orthovanadate (2 mM), leupeptin (0.10 µg/mL), aprotinin (0.25 µg/mL), and NaF (1.25 mM). The tissue was minced and incubated in the lysis buffer for 20 min with shaking. The samples were centrifuged (10 min, 10,000 g) and the supernatant was collected and measured for IL-4 by ELISA (R&D Systems Europe, Abingdon, UK).

Splenocyte Isolation and IL-13 Measurements to Study Allergic Sensitization

Sensitized animals were sacrificed on day 14 and their spleens were surgically removed. The spleens were pressed through a 70-µm cell strainer and the cells were suspended in a buffer containing NH₄Cl (150 mM), KHCO₃ (10 mM), and EDTA (0.1 mM) to lyse

the erythrocytes in the suspension. The isolated splenocytes were plated at a density of 1×10^6 cells/mL, and cultured in RPMI 1640 medium (Lonza, Verviers SPRL, Verviers, Belgium) supplemented with FBS (10%), penicillin (100 U/mL), streptomycin (100 µg/mL), amphotericin B (250 ng/mL, all from Invitrogen/Life Technologies, Carlsbad, CA, USA), and β-mercaptoethanol (14 µM). The cells were incubated with T cell-activating CD3 and CD28 antibodies (CD3 antibody [5 µg/mL] coated on the wells and CD28 antibody [5 µg/mL] diluted in culture medium, both from eBioscience, San Diego, CA, USA) or with ovalbumin (100 µg/mL diluted in culture medium) both with or without dexamethasone (1 µM). After 42 h, the culture medium was harvested and analyzed for IL-13 concentration by ELISA (R&D Systems).

Ca²⁺ Influx Measurements

TRPA1-mediated Ca²⁺ influx was measured in HEK 293 cells transiently transfected with human (h)TRPA1 as described previously [6]. The intracellular free Ca²⁺ levels were assessed by Fluo-3-AM assay as described previously [20, 21]. In the experiments, the cells were first pre-incubated with the TRPA1 blocker HC-030031 (100 µM) or the vehicle for 30 min at 37 °C. Next, the cells were exposed sequentially to ovalbumin (1 mg/mL), the TRPA1 agonist AITC (50 µM) and the control ionophore ionomycin (1 µM). The results are normalized against background.

Statistical Analysis

Results are expressed as mean ± standard error of the mean (SEM). Data were analyzed with SPSS v21 software (SPSS Inc, Chicago, IL, USA) by Student's *t* test or one-way ANOVA with Bonferroni's or Dunnett's multiple-comparison test (**p* ≤ 0.05, ***p* ≤ 0.01, ****p* ≤ 0.001; Fig. 1–5).

Results

In the allergic conjunctivitis model, the mice had first been sensitized to ovalbumin, and the topical challenge with ovalbumin produced eosinophil accumulation in the conjunctival tissue seen by the increase in eosinophil peroxidase activity. Curiously, the TRPA1 blocker TCS 5861528 clearly reduced eosinophil accumulation similarly to the control compound dexamethasone (Fig. 1a). Also, TRPA1-deficient mice presented a clearly blunted response when compared to the corresponding wild-type mice (Fig. 1b).

Eosinophil accumulation in allergic inflammation is supported by delayed eosinophil apoptosis induced by cytokines such as IL-5, and the antiallergic effect of glucocorticoids lies partly with their ability to induce eosinophil apoptosis [22]. However, neither the TRPA1 activator AITC nor the TRPA1 blockers TCS 5861528 and HC-030031 had an effect on the apoptosis rate in human eosinophils; dexamethasone enhanced apoptosis as expected (Fig. 2). This suggests that the attenuating properties of TRPA1 on allergic inflammation lie elsewhere.

Fig. 1. The alleviating effects of topical TRPA1 blocker TCS 5861528, control compound dexamethasone (a) and genetic TRPA1 deficiency (b) on ovalbumin-induced allergic conjunctivitis measured as eosinophil peroxidase activity in the conjunctiva. Time point 24 h, mean $\pm$ SEM, $n = 5$ –6 mice/group.

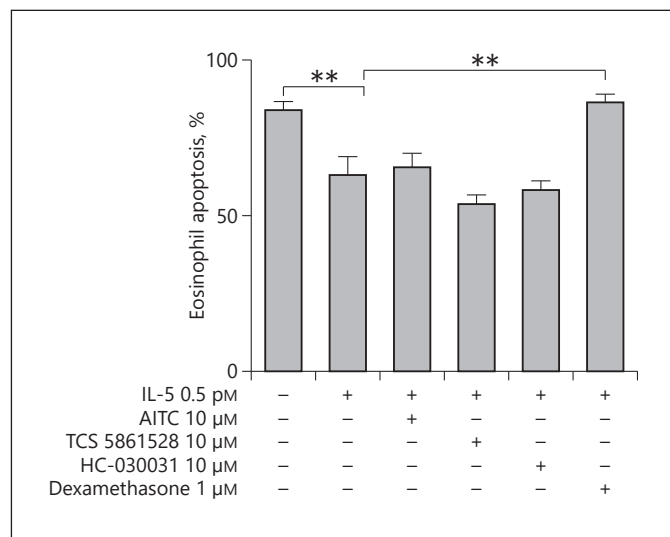
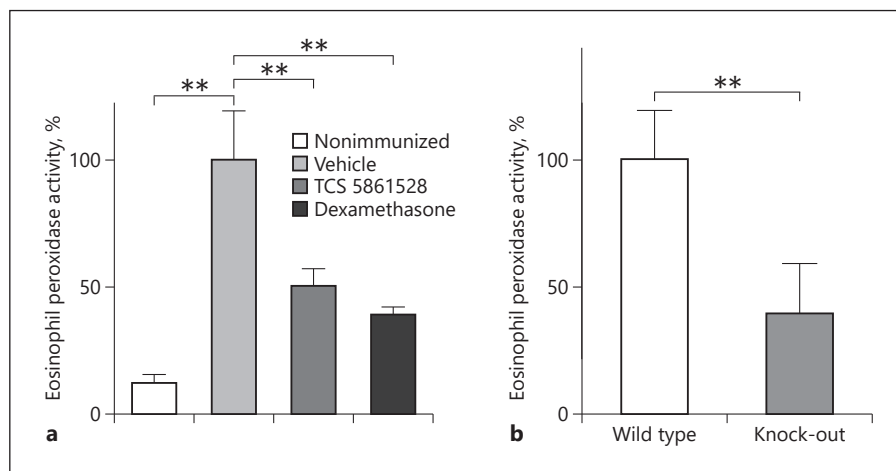


Fig. 2. The TRPA1 activator AITC and blockers TCS 5861528 or HC-030031 did not alter primary cultured human eosinophil apoptosis induced by IL-5. Time point 40 h, mean $\pm$ SEM, $n = 4$.

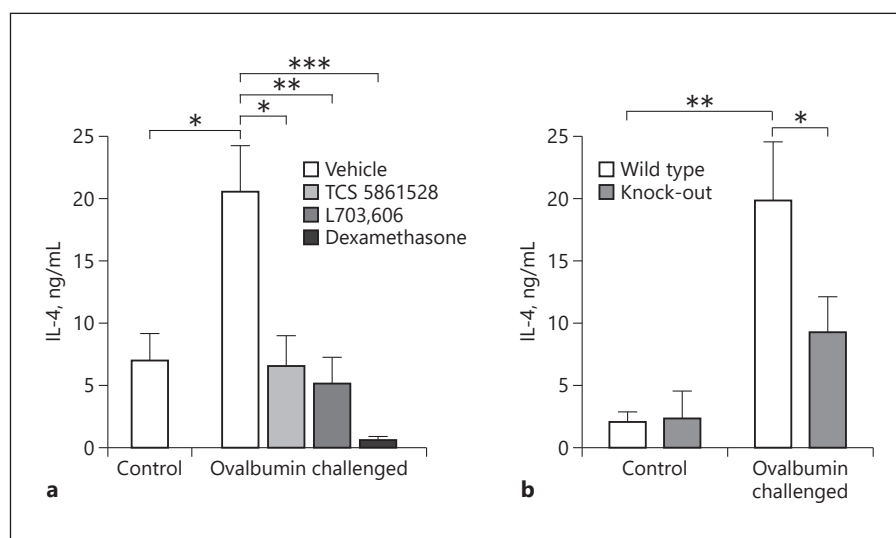
Subsequent to the release of vasoactive substances from the mast cells and the development of tissue edema, early allergic inflammation includes the production of proinflammatory and chemotactic cytokines such as IL-4 [17, 23]. To study the initial phase of allergic inflammation, we challenged the sensitized mice with an s.c. injection of ovalbumin into the hind paw and measured the formation of acute paw edema. Interestingly, treatment with the TRPA1 blocker TCS 5861528 inhibited this reaction, similar to effect of the control compound dexamethasone. Furthermore, blockade of the action of substance

P, the secondary effector of TRPA1, by antagonizing its receptor NK1R with L703,606, also resulted in attenuated paw edema in response to ovalbumin. Correspondingly, TRPA1-deficient mice developed reduced paw edema in response to ovalbumin injection when compared to their wild-type counterparts (Table 1).

The paw tissue injected with ovalbumin was collected for IL-4 analysis. The level of IL-4 was markedly elevated in the paw challenged with ovalbumin when compared to the control paw injected with saline only. Interestingly, treatment with the TRPA1 blocker TCS 5861528 was effective in inhibiting the production of IL-4 comparably to the control compound dexamethasone. TRPA1 deficiency also resulted in the attenuated production of IL-4 when compared to the corresponding wild-type mice (Fig. 3a, b).

Allergen-specific T_H2 -type sensitization is crucial to allergic hypersensitivity and inflammation [17]. It can be studied by isolating splenocytes from sensitized animals and testing the production of T_H2 -associated cytokines in response to the presentation of the allergen. To study if attenuated allergic reactions in TRPA1-deficient mice are due to milder allergic sensitization, we studied the degree of allergic sensitization in both wild-type and TRPA1-deficient mice. Unlike the splenocytes derived from non-sensitized mice, those from mice sensitized to ovalbumin produced IL-13 when exposed to ovalbumin, indicating a successful sensitization process. However, the splenocytes from TRPA1-deficient and corresponding wild-type mice did not differ in their production of IL-13. The splenocytes from these 2 genotypes produced equal amounts of IL-13 when stimulation with ovalbumin was compared to stimulation with a combination of T cell-

Fig. 3. The inhibitory effects of systemic TRPA1 blocker TCS 5861528, NK1 receptor blocker L703,606, control compound dexamethasone (**a**) and genetic TRPA1 deficiency (**b**) on ovalbumin-induced IL-4 production in the inflamed paw tissue in mice previously sensitized to ovalbumin. Time point 6 h, mean $\pm$ SEM, $n = 6$ –8 mice/group.



activating CD3 and CD28 antibodies, which induced a robust production of IL-13 (Fig. 4).

As TRPA1 was found to play a role in ovalbumin-induced hypersensitivity, we wondered whether ovalbumin is a direct activator of TRPA1. To determine this, ovalbumin was introduced to HEK 293 cells transfected with TRPA1. It was clear that ovalbumin did not induce Ca^{2+} influx into the cells, but the known TRPA1 agonist, AITC, induced an intense Ca^{2+} influx which was inhibited by the TRPA1 blocker HC-030031 (Fig. 5). Hence, ovalbumin is not a direct TRPA1 agonist but most likely induces a release of endogenous TRPA1 activators, such as ROS, RNS, or their metabolites, which activate the ion channel that drove the TRPA1-mediated effects in ovalbumin-induced allergic inflammation revealed in this study.

Discussion and Conclusion

This study describes a hitherto unknown role for TRPA1 activation in the early phase of allergic inflammatory reaction and the recruitment of eosinophils into the target tissue, as seen in the acute allergic paw inflammation and allergic conjunctivitis tests, respectively. Pharmacological inhibition and genetic deletion of TRPA1 were both effective in attenuating the ovalbumin-induced immediate formation of paw edema and IL-4 production as well as eosinophil accumulation into the inflamed conjunctiva. This was further supported by the finding that blockade of a secondary effector of TRPA1, namely substance P, also attenuated the development of acute paw

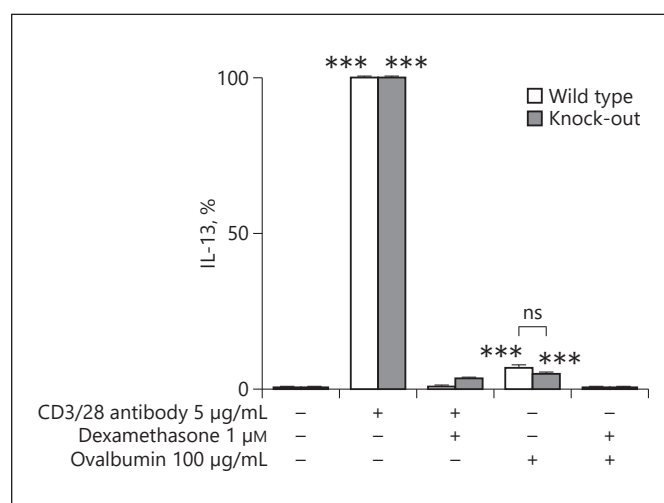


Fig. 4. Cultured splenocytes derived from ovalbumin-sensitized wild-type and TRPA1-deficient mice responded similarly to ovalbumin or T cell-activating CD3 and CD28 antibodies when measured as IL-13 production. Time point 42 h, comparison against the untreated control, mean $\pm$ SEM, $n = 6$ mice/group.

edema in response to s.c. challenge with ovalbumin. We also observed that the sensitization towards ovalbumin in TRPA1-deficient and wild-type mice was similar, as measured by the allergen-induced production of the proinflammatory cytokine IL-13 in splenocytes. Finally, ovalbumin did not present a direct activating effect on the TRPA1 ion channel.

TRPA1 was originally described in 1999 [24] and has since been recognized as a chemosensor for potentially

Fig. 5. Ovalbumin does not induce Ca^{2+} influx into cultured HEK 293 cells transfected with TRPA1 as measured by fluorescence in Fluo-3 AM assay. The TRPA1 opener AITC induces a Ca^{2+} influx that is totally abolished with TRPA1 blocker HC-030031. Dark gray indicates mean and light gray indicates SEM ($n = 5-7$).

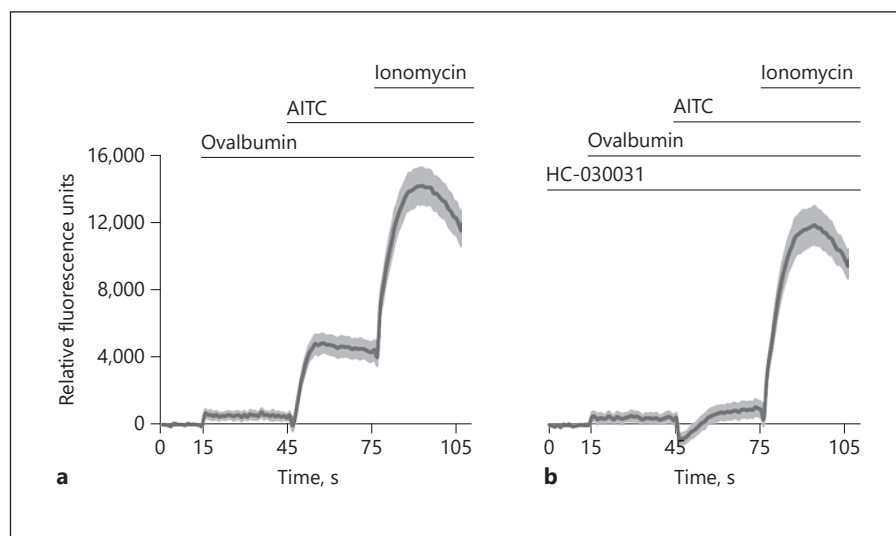


Table 1. The inhibitory effects of systemic TRPA1 blocker TCS 5861528, NK1 receptor blocker L703,606, control compound dexamethasone (A) and genetic TRPA1 deficiency (B) on ovalbumin-induced acute inflammatory paw edema in mice previously sensitized to ovalbumin

Treatment	Paw volume increase from baseline at different time points, μL						AUC, a.u.
	1 h	2 h	3 h	4 h	5 h	6 h	
Vehicle	57.5 $\pm$ 4.9	48.8 $\pm$ 3.7	36.3 $\pm$ 1.8	36.3 $\pm$ 4.2	27.5 $\pm$ 1.6	17.5 $\pm$ 2.6	215.0 $\pm$ 12.9
TCS 5861528	40.0 $\pm$ 5.8	33.3 $\pm$ 3.0	28.3 $\pm$ 2.8	18.3 $\pm$ 3.7	15.0 $\pm$ 3.1	8.3 $\pm$ 4.4	140.8 $\pm$ 14.6*
L703,606	35.0 $\pm$ 3.9	33.3 $\pm$ 3.0	16.7 $\pm$ 5.6	10.0 $\pm$ 2.4	5.0 $\pm$ 3.1	6.7 $\pm$ 3.0	103.3 $\pm$ 11.2**
Dexamethasone	38.8 $\pm$ 3.4	25.0 $\pm$ 3.4	16.7 $\pm$ 3.5	18.3 $\pm$ 6.1	11.7 $\pm$ 6.7	6.7 $\pm$ 1.8	113.3 $\pm$ 23.7**

TRPA1 genotype	Paw volume increase from baseline at different time points, μL						AUC, a.u.
	1 h	2 h	3 h	4 h	5 h	6 h	
Wild-type	66.7 $\pm$ 5.1	90.0 $\pm$ 6.3	38.3 $\pm$ 6.4	41.7 $\pm$ 6.4	30.0 $\pm$ 5.2	15.0 $\pm$ 2.0	274.2 $\pm$ 13.6
Knock-out	37.5 $\pm$ 3.4	31.3 $\pm$ 4.8	30.0 $\pm$ 5.8	18.8 $\pm$ 2.1	10.0 $\pm$ 4.0	8.8 $\pm$ 3.3	131.9 $\pm$ 13.3***

The area under the curve (AUC) represents the entire experiment (0–6 h) and compared to the vehicle treated or wild-type mice, $n = 6-8$. a.u., arbitrary units. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

harmful environmental compounds [25, 26]. In addition to its physiological function, TRPA1 is also involved crucially in pathological conditions such as pain, hypersensitivity, and inflammation [4, 5, 27, 28]. Antagonizing TRPA1 as a therapeutic modality in such conditions is therefore under intensive research. TRPA1 is a membrane-associated cation channel expressed mainly in peripheral-nerve terminals but also in other cell types rel-

evant in allergic inflammation, such as mast cells [29], endothelial and epithelial cells [8, 9], and fibroblasts [30, 31]. The list of exogenous activators of TRPA1 is long, but also some endogenously formed substances, especially those induced by inflammatory conditions, have been identified as TRPA1 activators. Interestingly, many of them are ROS and RNS such as hydrogen peroxide [32] and nitric oxide [33], or their reactive metabolites

such as 4-hydroxynonenal [34] and nitro-oleic acid [35], suggesting that TRPA1 plays a key role also in endogenously triggered inflammatory processes [5, 6]. After activation, a release of the proinflammatory neuropeptides substance P, calcitonin gene-related peptide, and neurokinin A are frequently described as secondary effectors of TRPA1 [5, 10]. Other proinflammatory properties of TRPA1 have also been identified. For example, inflammatory edema caused by TRPA1 activation is dependent on classical inflammatory mechanisms such as mast cell degranulation, neutrophil migration, the release of histamine, serotonin, and adrenalin, and the production of prostaglandins [6, 36]. Activation of TRPA1 has also been shown to enhance the expression of some inflammatory genes such as the prostaglandin-producing enzyme COX-2, myeloperoxidase, IL-6, and IL-1 β [6, 9, 10, 21, 37, 38].

Previously, the role of TRPA1 in enhancing allergic inflammation was demonstrated in studies conducted in models of ovalbumin-induced experimental murine asthma [15, 16], IL-13-induced itch [39], and oxazolone-induced allergic contact dermatitis [11]. Interestingly, a previously known powerful contact sensitizer, 2,4-dinitrochlorobenzene, has also proven to be a direct activator of TRPA1 [40]. However, the previous studies focusing on the airway inflammation induced by ovalbumin, or ovalbumin and formaldehyde challenge, analyzed the outcome of a repeated/continuous exposure to the allergen lasting from several days to weeks [15, 16]. Interestingly, the results presented clear evidence that pharmacological blockade and genetic deletion of TRPA1 results in diminished amounts of proinflammatory cytokines and leukocytes in the bronchoalveolar lavage fluid, in addition to reduced airway hyperresponsiveness [15, 16]. TRPA1 expression has also been noted to increase as a result of ovalbumin-induced airway inflammation [41]. The sensitization against ovalbumin was similar in the TRPA1-deficient and the wild-type mice, as measured by the ovalbumin-specific IgE in the serum, but the amounts of proinflammatory neuropeptides substance P, calcitonin gene-related peptide, and neurokinin A were reduced in the inflamed airways in TRPA1-deficient animals [15, 16]. Our study expands on the previous data on the role and mechanisms of TRPA1 in allergic conjunctivitis and acute allergic s.c. inflammation.

In this study, we demonstrated that ovalbumin-induced allergic conjunctivitis was reduced in mice treated with TRPA1 blocker and also in TRPA1-deficient mice, as seen by an attenuated eosinophil accumulation

into the target tissue. We also obtained clear results that the early responses in acute allergic s.c. inflammation are crucially dependent on the activation of TRPA1 and the function of neuropeptides. Pharmacological blockade of TRPA1 and neurokinin-1 receptor, in addition to the genetic depletion of TRPA1, resulted in significantly diminished edema formation and production of the allergy-associated proinflammatory cytokine IL-4. Based on the theory of the classical allergic inflammatory reaction, blood-derived eosinophils play a key role in the late phase of the allergic inflammatory reaction which usually takes >6 h to spring into action [42]. The recruitment of eosinophils to the site of allergic inflammation is mediated by a combination of adhesion molecule interactions and chemokines induced by the earlier-produced cytokines, such as IL-4, and lipid mediators, such as prostaglandin D₂ and leukotrienes [17]. If we take another step backwards in the cascade of acute allergic reaction, the production of lipid mediators and cytokines is preceded by the activation of mast cells, with the immediate degranulation and release of vasoactive factors and the formation of local edema can be observed. It is obvious that the inhibition of degranulation or function of the released factors dramatically decreases the formation of the early phase of the acute inflammatory reaction, as seen in experimental models of ovalbumin-induced acute paw edema [43] and ovalbumin-induced conjunctivitis [44].

So, what is the role of TRPA1 in the complexity of allergic inflammation? As we observed no evidence of direct TRPA1 activation by ovalbumin, it is highly likely that TRPA1 is activated by endogenous factors released or synthesized following exposure to the allergen. Interestingly, mast cell activation and the early phase of the allergic reaction involve endogenously formed substances, such as ROS and RNS, with potency to activate TRPA1 in neuronal and nonneuronal cells [45]. Furthermore, the expression of TRPA1 in mast cells has been proposed, and, interestingly, this has been shown to increase due to stimulation by IL-13 and leukotriene B₄, both produced in allergic inflammation [39, 46]. TRPA1 could therefore also be involved in the activation and operation of mast cells. However, mast cells express both substance P and its target neurokinin-1 receptor [23], meaning that, in theory, these cells could play a role as a target and/or a source of neuropeptides. Endothelial cells have also been shown to express TRPA1 [8], and a direct TRPA1-mediated vasodilatation may contribute to the allergen-induced local edema. Nonetheless, it is known that the neuronal activity of TRPA1 and the re-

sulting release of substance P are crucial for many inflammatory conditions [5, 27], and so the neurogenic component of TRPA1 activity in allergic inflammation is highly likely. TRPA1 is also positioned in the central nervous system, and its inhibition by intrathecally administered drugs has been shown to elicit antinociceptive effects [47]. However, our results showed that treatment with TRPA1 blocker, both systemically in the paw edema test and locally at the site of the allergic inflammation in the conjunctivitis test, was able to inhibit the reaction, suggesting that the effect is due to peripherally distributed TRPA1 ion channels.

In this study, we also investigated the role of TRPA1 in the longevity of cultured eosinophils as a possible mechanism for the effects of TRPA1 in allergy. In allergic inflammation, eosinophil survival is prolonged because of delayed apoptosis induced by cytokines such as IL-5, and the spontaneous apoptosis of eosinophils has been shown to be a physiological, restrictive mechanism in allergic inflammation [22]. Furthermore, the antiallergic activity of glucocorticoids lies partly in their ability to enhance eosinophil apoptosis [22]. We noticed no alteration in eosinophil apoptosis in response to TRPA1 activator or blocker in conditions mimicking allergic inflammation, although glucocorticoid dexamethasone enhanced eosinophil apoptosis as expected. It should be noted that human and murine eosinophils have some species-related differences, which is a limitation of the eosinophil experiments in our study and also their applicability. However, human and murine eosinophils share many functional and structural similarities and, although no direct comparative studies on the species differences in eosinophil apoptosis are available, they also share many apoptosis signaling features [48]. As described earlier [15], we observed a similar degree of sensitization towards the allergen ovalbumin in the wild-type and TRPA1-deficient mice, deduced from the experiments conducted in the splenocytes derived from the sensitized mice.

Taken together, our results strongly suggest that the activation of TRPA1 plays an enhancing role in allergic inflammation. TRPA1 did not seem to affect the process of allergic sensitization, but we found a clear inhibitory effect of blockade of TRPA1 on the early vascular events and eosinophil accumulation in allergic reactions. As there is also previous evidence on the role of TRPA1 in ovalbumin-induced airway inflammation [15, 16], it is highly possible that the inhibition of TRPA1 will turn out to be an effective means to treat allergic conditions.

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Statement of Ethics

Animal experiments were carried out in accordance with the legislation for the protection of animals used for scientific purposes (Directive 2010/63/EU) and approved by The National Animal Experiment Board (permit Nos. ESAVI/2139/04.10.03/2012 and ESAVI/3280/04.10.07/2015).

Disclosure Statement

The authors have no conflicts of interest to declare.

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Author Contributions

L.J.M., M.H., P.I., and R.M.N. made substantial contributions to the conception and design of the work and on the acquisition, analysis and interpretation of data for the work. H.K., E.M., and L.L. made substantial contributions to the conception and design of the work and on the analysis and interpretation of data for the work. L.J.M., E.M., and L.L. made substantial contributions to drafting the work and all authors revised it critically for important intellectual content. All authors approved the final version to be published and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

References

- Ramallo R, Almeida J, Beltrão M, Pirraco A, Costa R, Sokhatska O, et al. Substance P antagonist improves both obesity and asthma in a mouse model. *Allergy*. 2013 Jan;68(1):48–54.
- Yamaji M, Takada M, Fujiwara R, Ohishi H, Izushi K, Sugimoto Y, et al. Role of substance P in experimental allergic conjunctivitis in guinea pigs. *Methods Find Exp Clin Pharmacol*. 1997 Nov;19(9):637–43.
- O'Connor TM, O'Connell J, O'Brien DI, Goode T, Bredin CP, Shanahan F. The role of substance P in inflammatory disease. *J Cell Physiol*. 2004 Nov;201(2):167–80.
- Nilius B, Appendino G, Owsianik G. The transient receptor potential channel TRPA1: from gene to pathophysiology. *Pflugers Arch*. 2012 Nov;464(5):425–58.
- Zygmunt PM, Högestätt ED. TRPA1. *Handb Exp Pharmacol*. 2014;222:583–630.
- Moilanen LJ, Laavola M, Kukkonen M, Korhonen R, Leppänen T, Högestätt ED, et al. TRPA1 contributes to the acute inflammatory response and mediates carrageenan-induced paw edema in the mouse. *Sci Rep*. 2012;2(1):380.
- Koivisto A, Chapman H, Jalava N, Korjamo T, Saarnilehto M, Lindstedt K, et al. TRPA1: a transducer and amplifier of pain and inflammation. *Basic Clin Pharmacol Toxicol*. 2014 Jan;114(1):50–5.
- Earley S, Gonzales AL, Crnich R. Endothelium-dependent cerebral artery dilation mediated by TRPA1 and Ca²⁺-Activated K⁺ channels. *Circ Res*. 2009 Apr;104(8):987–94.
- Atoyán R, Shander D, Botchkareva NV. Non-neuronal expression of transient receptor potential type A1 (TRPA1) in human skin. *J Invest Dermatol*. 2009 Sep;129(9):2312–5.
- Moilanen LJ, Hämäläinen M, Nummenmaa E, Ilmarinen P, Vuolteenaho K, Nieminen RM, et al. Monosodium iodoacetate-induced inflammation and joint pain are reduced in TRPA1 deficient mice—potential role of TRPA1 in osteoarthritis. *Osteoarthritis Cartilage*. 2015 Nov;23(11):2017–26.
- Liu B, Escalera J, Balakrishna S, Fan L, Caceres AI, Robinson E, et al. TRPA1 controls inflammation and pruritogen responses in allergic contact dermatitis. *FASEB J*. 2013 Sep;27(9):3549–63.
- Engel MA, Leffler A, Niedermirtl F, Babes A, Zimmermann K, Filipović MR, et al. TRPA1 and substance P mediate colitis in mice. *Gastroenterology*. 2011 Oct;141(4):1346–58.
- Trevisan G, Hoffmeister C, Rossato MF, Oliveira SM, Silva MA, Ineu RP, et al. Transient receptor potential ankyrin 1 receptor stimulation by hydrogen peroxide is critical to trigger pain during monosodium urate-induced inflammation in rodents. *Arthritis Rheum*. 2013 Nov;65(11):2984–95.
- Moilanen LJ, Hämäläinen M, Lehtimäki L, Nieminen RM, Moilanen E. Urate crystal induced inflammation and joint pain are reduced in transient receptor potential ankyrin 1 deficient mice—potential role for transient receptor potential ankyrin 1 in gout. *PLoS One*. 2015 Feb;10(2):e0117770.
- Caceres AI, Brackmann M, Elia MD, Bessac BF, del Camino D, D'Amours M, et al. A sensory neuronal ion channel essential for airway inflammation and hyperreactivity in asthma. *Proc Natl Acad Sci USA*. 2009 Jun;106(22):9099–104.
- Wu Y, You H, Ma P, Li L, Yuan Y, Li J, et al. Role of transient receptor potential ion channels and evoked levels of neuropeptides in a formaldehyde-induced model of asthma in BALB/c mice. *PLoS One*. 2013 May;8(5):e62827.
- Abbas AK, Lichtman AH, Pillai S. *Allergy. Cellular and Molecular Immunology*. 8th ed. Elsevier Health Sciences; 2014. pp. 417–35.
- Kankaanranta H, Lindsay MA, Gienbycz MA, Zhang X, Moilanen E, Barnes PJ. Delayed eosinophil apoptosis in asthma. *J Allergy Clin Immunol*. 2000 Jul;106(1 Pt 1):77–83.
- Cascieri MA, Ber E, Fong TM, Sadowski S, Bansal A, Swain C, et al. Characterization of the binding of a potent, selective, radioiodinated antagonist to the human neurokinin-1 receptor. *Mol Pharmacol*. 1992 Sep;42(3):458–63.
- AfCS Procedure Protocol. Assay of Intracellular Free Calcium in RAW 264.7 Cells Loaded with Fluo-3. 2003; Available from: http://www.signaling-gateway.org/data/cgi-bin/ProtocolFile.cgi/afcs_PP00000210.pdf?pid=PP00000210
- Moilanen LJ, Hamalainen M, Lehtimäki L, Nieminen RM, Muraki K, Moilanen E. Pinosylvin Inhibits TRPA1-Induced Calcium Influx In Vitro and TRPA1-Mediated Acute Paw Inflammation In Vivo. *Basic Clin Pharmacol Toxicol*. 2016 Mar;118(3):238–42.
- Ilmarinen P, Moilanen E, Kankaanranta H. Regulation of spontaneous eosinophil apoptosis—a neglected area of importance. *J Cell Death*. 2014 Feb;7:1–9.
- Theoharides TC, Alysandratos KD, Angelidou A, Delivanis DA, Sismanopoulos N, Zhang B, et al. Mast cells and inflammation. *Biochim Biophys Acta*. 2012 Jan;1822(1):21–33.
- Jaquemar D, Schenker T, Trueb B. An ankyrin-like protein with transmembrane domains is specifically lost after oncogenic transformation of human fibroblasts. *J Biol Chem*. 1999 Mar;274(11):7325–33.
- Jordt SE, Bautista DM, Chuang HH, McKemy DD, Zygmunt PM, Högestätt ED, et al. Mustard oils and cannabinoids excite sensory nerve fibres through the TRP channel ANKTM1. *Nature*. 2004 Jan;427(6971):260–5.
- Bautista DM, Movahed P, Hinman A, Axelsson HE, Sterner O, Högestätt ED, et al. Pun- gent products from garlic activate the sensory ion channel TRPA1. *Proc Natl Acad Sci USA*. 2005 Aug;102(34):12248–52.
- Bautista DM, Pellegrino M, Tsunozaki M. TRPA1: A gatekeeper for inflammation. *Annu Rev Physiol*. 2013;75(1):181–200.
- McNamara CR, Mandel-Brehm J, Bautista DM, Siemens J, Deranian KL, Zhao M, et al. TRPA1 mediates formalin-induced pain. *Proc Natl Acad Sci USA*. 2007 Aug;104(33):13525–30.
- Prasad P, Yanagihara AA, Small-Howard AL, Turner H, Stokes AJ. Secretogranin III directs secretory vesicle biogenesis in mast cells in a manner dependent upon interaction with chromogranin A. *J Immunol*. 2008 Oct;181(7):5024–34.
- Hu H, Tian J, Zhu Y, Wang C, Xiao R, Herz JM, et al. Activation of TRPA1 channels by fenamate nonsteroidal anti-inflammatory drugs. *Pflugers Arch*. 2010 Mar;459(4):579–92.
- Mukhopadhyay I, Gomes P, Aranake S, Shetty M, Karnik P, Damle M, et al. Expression of functional TRPA1 receptor on human lung fibroblast and epithelial cells. *J Recept Signal Transduct Res*. 2011 Oct;31(5):350–8.
- Bessac BF, Sivula M, von Hehn CA, Escalera J, Cohn L, Jordt SE. TRPA1 is a major oxidant sensor in murine airway sensory neurons. *J Clin Invest*. 2008 May;118(5):1899–910.
- Yoshida T, Inoue R, Morii T, Takahashi N, Yamamoto S, Hara Y, et al. Nitric oxide activates TRP channels by cysteine S-nitrosylation. *Nat Chem Biol*. 2006 Nov;2(11):596–607.
- Andersson DA, Gentry C, Moss S, Bevan S. Transient receptor potential A1 is a sensory receptor for multiple products of oxidative stress. *J Neurosci*. 2008 Mar;28(10):2485–94.
- Taylor-Clark TE, Ghatta S, Bettner W, Urdem BJ. Nitrooleic acid, an endogenous product of nitrative stress, activates nociceptive sensory nerves via the direct activation of TRPA1. *Mol Pharmacol*. 2009 Apr;75(4):820–9.
- Perin-Martins A, Teixeira JM, Tambeli CH, Parada CA, Fischer L. Mechanisms underlying transient receptor potential ankyrin 1 (TRPA1)-mediated hyperalgesia and edema. *J Peripher Nerv Syst*. 2013 Mar;18(1):62–74.
- Trevisan G, Hoffmeister C, Rossato MF, Oliveira SM, Silva MA, Silva CR, et al. TRPA1 receptor stimulation by hydrogen peroxide is critical to trigger hyperalgesia and inflammation in a model of acute gout. *Free Radic Biol Med*. 2014 Jul;72:200–9.

- 38 Nummenmaa E, Hamalainen M, Moilanen LJ, Paukeri EL, Nieminen RM, Moilanen T, et al. Transient receptor potential ankyrin 1 (TRPA1) is functionally expressed in primary human osteoarthritic chondrocytes. *Arthritis Res Ther*. 2016 Aug;18(1):185.
- 39 Oh MH, Oh SY, Lu J, Lou H, Myers AC, Zhu Z, et al. TRPA1-dependent pruritus in IL-13-induced chronic atopic dermatitis. *J Immunol*. 2013 Dec;191(11):5371–82.
- 40 Saarnilehto M, Chapman H, Savinko T, Lindstedt K, Lauerma AI, Koivisto A. Contact sensitizer 2,4-dinitrochlorobenzene is a highly potent human TRPA1 agonist. *Allergy*. 2014 Oct;69(10):1424–7.
- 41 Liu H, Fan X, Wang N, Zhang Y, Yu J. Exacerbating effects of PM2.5 in OVA-sensitized and challenged mice and the expression of TRPA1 and TRPV1 proteins in lungs. *J Asthma*. 2017 Oct;54(8):807–17.
- 42 Choi SH, Bielory L. Late-phase reaction in ocular allergy. *Curr Opin Allergy Clin Immunol*. 2008 Oct;8(5):438–44.
- 43 Feitosa RF, Melciades GB, Assreuy AM, Rocha MF, Ribeiro RA, Lima AA. The pharmacological profile of ovalbumin-induced paw oedema in rats. *Mediators Inflamm*. 2002 Jun;11(3):155–63.
- 44 Calonge M, Montero JA, Herreras JM, Ramón JJ, Pastor JC. Efficacy of nedocromil sodium and cromolyn sodium in an experimental model of ocular allergy. *Ann Allergy Asthma Immunol*. 1996 Aug;77(2):124–30.
- 45 Swindle EJ, Metcalfe DD. The role of reactive oxygen species and nitric oxide in mast cell-dependent inflammatory processes. *Immunol Rev*. 2007 Jun;217(1):186–205.
- 46 Fernandes ES, Vong CT, Quek S, Cheong J, Awal S, Gentry C, et al. Superoxide generation and leukocyte accumulation: key elements in the mediation of leukotriene B₄-induced itch by transient receptor potential ankyrin 1 and transient receptor potential vanilloid 1. *FASEB J*. 2013 Apr;27(4):1664–73.
- 47 da Costa DS, Meotti FC, Andrade EL, Leal PC, Motta EM, Calixto JB. The involvement of the transient receptor potential A1 (TRPA1) in the maintenance of mechanical and cold hyperalgesia in persistent inflammation. *Pain*. 2010 Mar;148(3):431–7.
- 48 Lee JJ, Jacobsen EA, Ochkur SI, McGarry MP, Condjella RM, Doyle AD, et al. Human versus mouse eosinophils: “that which we call an eosinophil, by any other name would stain as red”. *J Allergy Clin Immunol*. 2012 Sep;130(3):572–84.