

Up-regulation of Orai1 in murine allergic rhinitis

Lin Lin · Chunquan Zheng · Luping Zhang ·
Cuidi Da · Keqing Zhao

Accepted: 29 May 2010
© Springer-Verlag 2010

Abstract Orai1 is an essential pore-forming subunit of the Ca^{2+} release-activated Ca^{2+} channels and plays a key role in the store-operated Ca^{2+} entry. However, little is known about the function of this pathway in allergic airway diseases. In this study, we evaluated Orai1 expression in normal and allergic rhinitis (AR) mice airway and spleen. AR models were established by repetitive intraperitoneal sensitization followed by intranasal challenge with ovalbumin. Sneezing was counted, and eosinophils infiltration was analyzed through Luna stain. We performed the analysis of Orai1 protein in airway and spleen by immunohistochemical staining, Western blotting and enzyme-linked immunosorbent assay, and quantitatively examined Orai1 mRNA in the above tissues by real-time reverse transcription-polymerase chain reaction. Sneezes and eosinophil counts in the AR group were increased in comparison to those in the normal group. Orai1 protein was expressed in mucosal epithelium and submucosal glands epithelium of airway, and in immune cells of spleen. The immunostaining appeared stronger in AR mice than that in normal ones. Both the Orai1 protein and mRNA levels showed up-regulation in the AR group compared with those in the normal one. Our results indicate that Orai1 is up-regulated in the airway and spleen in allergic inflammation and may participate in the pathogenesis of allergic rhinitis.

Keywords Orai1 · Allergic rhinitis · Mouse model · Airway · Spleen

Abbreviations

AR	Allergic rhinitis
Th2	T helper 2
IL	Interleukin
CRAC	Ca^{2+} release-activated Ca^{2+}
STIM1	Stromal interaction molecule 1
SOCE	Store-operated Ca^{2+} entry
NFAT	Nuclear factor of activated T cells
RT-PCR	Reverse transcription-polymerase chain reaction
ELISA	Enzyme-linked immunosorbent assay
NIH	National Institutes of Health
i.p.	Intraperitoneal
i.n.	Intranasal
PBS	Phosphate-buffered saline
DAB	3,3'-Diaminobenzidine chromogene
TBS	Tris-buffered saline
TBS-T	Tris-buffered saline with Tween-20

Introduction

Allergic rhinitis (AR) is one of type I allergic disorders characterized by an aberrant immune response to harmless environmental antigens. In allergic states, the differentiation of CD4^{+} T cells to effectors that produce a T helper 2 (Th2) profile of cytokines, such as interleukin (IL)-4, IL-5, IL-9 and IL-13, is the initial event and the key factor for the development of allergic responses (Kay 2001). Also, eosinophilic infiltration contributes to persistent airway inflammation and remodeling and/or airway hyperresponsiveness in patients with AR (Kay 2001).

Although antiallergic and antihistamine agents are commonly used for the clinical treatment, the effect is transient and can suppress only the terminal branches of allergic responses. A novel therapeutic strategy for AR that strikes

L. Lin · C. Zheng (✉) · L. Zhang · C. Da · K. Zhao
Department of Otorhinolaryngology, Head and Neck Surgery,
Eye and ENT Hospital of Fudan University,
No. 83 Fenyang Road, Shanghai 200031, China
e-mail: CqZheng@online.sh.cn

at the root of its pathogenesis is desired. It can be assumed that some drugs may be designed to block certain pathways in the development of AR, such as Ca^{2+} signaling pathway that regulates a diverse array of cellular physiological processes including secretion, muscle contraction, cell growth and gene expression (Berridge et al. 2003).

There are growing evidences that Ca^{2+} is an essential second messenger in almost all cell types. Cytoplasmic Ca^{2+} concentration changes are evoked by altered influx across the plasma membrane, and by release from intracellular compartments, such as the endoplasmic reticulum (ER) which is a major cellular Ca^{2+} store. Store-operated Ca^{2+} entry (SOCE) is activated by depletion of ER Ca^{2+} stores, and is a widely observed mechanism of plasma membrane Ca^{2+} influx (Parekh and Putney 2005; Venkatachalam et al. 2002). Store-operated Ca^{2+} channel (SOC) activity was first identified in 1992 by patch-clamp electrophysiology (Hoth and Penner 1992) and was termed the Ca^{2+} release-activated Ca^{2+} (CRAC) channel. This SOC is the most extensively characterized SOCE pathway (Parekh and Putney 2005). The mechanisms that regulate SOCE and the molecular identity of the CRAC channel have been the subjects of intense investigation for more than 17 years. An elegant study made use of linkage analysis and a *Drosophila* S2 cell genome-wide RNA interference (RNAi) screen to complete the identification of Orai1 as an elemental component of the CRAC channel (Feske et al. 2006). Mammalian cells have two additional Orai1 isoforms, Orai2 and Orai3, but their Ca^{2+} currents were two- to three-fold smaller compared to Orai1 (Mercer et al. 2006). These reduced currents may reflect differences in their expression levels, efficiency in coupling to STIM1 and distinct single channel properties (Lis et al. 2007). Co-expression of Orai1 with stromal interaction molecule 1 (STIM1), an identified molecule in ER by means of RNAi screening in *Drosophila* S2 cells, dramatically increases SOCE and CRAC channel activity (Varnai et al. 2009). Sustained Ca^{2+} influx results in cytoplasmic nuclear factor of activated T cells (NFAT) family of transcription factors dephosphorylating through the calmodulin-dependent protein phosphatase calcineurin, and also promotes NFAT translocation to the nucleus in immune system cells. These always emerge as important processes in the induction and development of allergic reactions (Rao et al. 1997).

It is well known that immune system cells activation, proliferation and degranulation dependent on Orai1, the pore-forming subunit of CRAC channels (Gwack et al. 2007), are essential for the pathogenesis of AR. However, the association between Orai1 protein and AR pathogenesis is not clear. In addition, it has been reported that Orai1 protein exists in multiple mammalian cell types including endothelial cells (Abdullaev et al. 2008), airway smooth muscle cells (Peel et al. 2008), platelets, and megakaryo-

cytes (Tolhurst et al. 2008), besides immune system cells, and plays a role in their various activities. Based on the above evidences, we hypothesized that Orai1 molecule, CRAC channels and SOCE pathway may play a role in the development of AR. We also assumed that there were other cell types with Orai1 pathway which participated in the development of this allergic disorder, as well as immune system cells which underwent activation and effector functions after specific antigen stimulation.

In this present study, we examined the expression and distribution of Orai1 protein in nasal mucosa and spleen of normal and AR mice using immunohistochemistry method. As the relationship between AR and allergic asthma is often summarized as “united airways disease”, which means that allergic inflammation is not a disease confined to a specific target organ, but rather a disorder of the whole respiratory tract with a broad spectrum of clinical manifestations (Passalacqua et al. 2001), we also investigated Orai1 in the trachea of these two groups of mice by immunohistochemical staining. To further elucidate the possible expression of Orai1 molecule in a mouse model of AR, we performed the analysis of Orai1 protein in the airway and spleen by Western blotting and enzyme-linked immunosorbent assay (ELISA), and examined Orai1 mRNA in the above tissues using quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR). To our knowledge, we are the first to describe the expression of Orai1 molecule in a murine model of AR.

Materials and methods

Mice

Female BALB/c mice (6–8 weeks) were purchased from the Chinese Academy of Sciences Shanghai Laboratory Animal Center. These mice were maintained in horizontal laminar flow cabinets and provided sterile food and water in a specific pathogen-free facility. All animals were handled in accordance with the National Institutes of Health (NIH) Guidelines for the Care and Use of Laboratory Animals. The mice were randomly divided into two experimental groups ($n = 12$ for each group).

AR models

According to published procedures (Ikeda et al. 2002), a group of mice was administered 0.5 mg/ml ovalbumin (Grade V, Sigma-Aldrich, St. Louis, MO, USA) and 20 mg/ml aluminum hydroxide (Sinopharm Chemical Reagent Co., Ltds, China) in saline at a dosage of 0.2 ml/mouse by intraperitoneal (i.p.) injection (AR mice). The sensitization was repeated three times at weekly intervals (priming, days 0, 7 and 14) followed by daily injections of ovalbumin solution

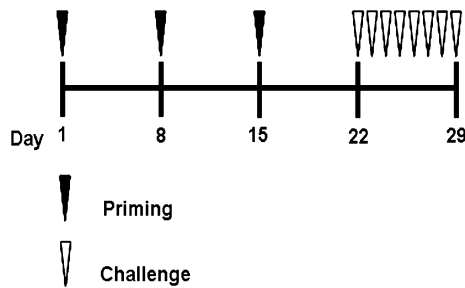


Fig. 1 Protocols for sensitization and challenge. AR mice were prepared by i.p. immunization with ovalbumin plus aluminum hydroxide on days 1, 8, and 15 (priming) followed by daily i.n. challenge with ovalbumin solution on days 22–29. Normal (nonallergic) mice were given only the last i.n. challenge on day 29

(40 mg/ml in saline) into the nostrils (0.02 ml/mouse) on days 21–29 (challenge) (Fig. 1). As a negative control, another group of mice received neither sensitization nor challenge treatment, except for the last challenge on day 29 (normal mice). Nasal symptoms were evaluated by counting the number of sneezes during 10 min immediately after the last ovalbumin intranasal (i.n.) provocation on day 29.

Antibody

We used Orai1-L1 rabbit polyclonal antibody in our study. This antibody was raised against a peptide from the extracellular loop 1 region of human Orai1 and could be used for the detection of mouse Orai1 by Western blotting at 1,000–1:2,000 dilution, immunohistochemistry at 1:100–1:200 dilution and ELISA at 1:1,000–1:5,000 according to the manufacturer's instructions. It might also recognize Orai2 and Orai3, which meant that this polyclonal antibody was often not isoform specific. This broad reactivity of polyclonal antibodies may be of advantage in many cases. For example, they can be used in most instances for both immunoblotting and for immunostaining of chemically fixed cells and tissues (Drenckhahn et al. 1993). Monoclonal antibodies are often specific for isoforms and spliced variants, and their cross-reactivity is rare. However, their cross-reactivity cannot be eliminated by absorption or affinity purification (Drenckhahn et al. 1993). In this context, we did not perform pre-incubation experiments and affinity purification, since Orai1-L1 rabbit polyclonal antibody cross-reacted to a lesser extent with Orai2 and Orai3 due to their weaker expression levels in plasma membrane compared to Orai1 (Lis et al. 2007).

Sample preparation

After exsanguinations of one set of mice, the head, trachea and spleen of each mouse were excised and immersed in 10% neutral buffered formalin. After fixation, the heads

were decalcified in 8.8% formic acid for 6 days, embedded in paraffin and coronally sectioned at a thickness of 3 μ m. The tracheas and spleens were also embedded in paraffin and sectioned at a thickness of 3 μ m. Sections were placed on aminosilane-coated slides, in a multisection format (up to 6 different specimens on the same slide) to facilitate concomitant immunostaining of the whole experiment and eliminate inter-experimental variability.

The other set of mice was also killed, and nasal mucosa, trachea and spleen of each mouse were obtained. These tissues were cut into two parts: one was dissected for protein isolation and measurement, and the other for RNA extraction. All of these were frozen at -70°C .

Luna stain and eosinophils infiltration

For the Luna stain, sections were immersed in working Hematoxylin-Biebrich (Sigma-Aldrich, St. Louis, MO, USA and Acros, Geel, B, Belgium, respectively) scarlet solution for 5 min, then dipped in 1% acid alcohol about eight times and rinsed in tap water. Then, the sections were dipped in lithium carbonate solution about five times until sections turned blue and then washed in running tap water for 2 min. Luna stain is specific for eosinophils and renders their cytoplasm red-brown on a blue background (van de Rijn et al. 1998). The number of infiltrating eosinophils was determined microscopically in a blinded manner at a high-power field of $400\times$ magnification.

Immunohistochemistry

One set of specimens received immunohistochemical staining. Sections were deparaffinized and rehydrated with water. Endogenous peroxidase was blocked with 3% hydrogen peroxide for 10 min. Normal goat serum (Vector Lab, Burlingame, CA, USA) was used to block non-specific binding sites and a previously optimized concentration (100 μ g/150 μ l) of primary antibody: Orai1-L1 rabbit polyclonal antibody (NewEast Biosciences, Malvern, USA) in antibody diluent (Dako, Via Real Carpinteria, CA, USA) at 1:100 dilution was immediately applied to the sections and incubated for 45 min. Unbound antibody was removed by three washes in phosphate-buffered saline (PBS) solution. Biotinylated anti-rabbit polyclonal IgG (Vector Lab, Burlingame, CA, USA), secondary antibody was applied to each section for 30 min, followed by 20 min incubation with horseradish peroxidase streptavidin (Vector Lab, Burlingame, CA, USA). Slides were washed in PBS, stained with 3,3'-diaminobenzidine chromogene (DAB) (Sigma-Aldrich, St. Louis, MO, USA) for 5 min and counterstained with hematoxylin. Sections were dehydrated through an alcohol series and xylene and mounted in neutral balsam mounting medium. Antibody localization was

detected microscopically by brown staining. Microscopic examination was performed by two independent observers using different magnifications (200, 400 or 1,000 $\times$). Immunoreactivity for Orai1 proteins was evaluated from the intensity of staining. The results were scored for the absence of positivity or the presence of weakly positive, definitely positive or strongly positive intensity. The degree and distribution of immunostaining were assessed under light microscopy by two independent observers who were blinded to the sample groups.

Western blotting

A portion of frozen tissues was sliced into small pieces and homogenized, then lysed in Mouse Tissue Extract Protein Array Kit (Sigma-Aldrich, St. Louis, MO, USA) and stored at -70°C . Proteins were separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane (Sigma-Aldrich, St. Louis, MO, USA). The membrane was then blocked for 30 min in Tris buffered saline (TBS) with 0.2% (vol/vol) Tween-20 (TBS-T) and 5% (wt/vol) nonfat dry milk and incubated at 4°C overnight with primary antibody: Orai1-L1 rabbit polyclonal antibody (NewEast Biosciences, Malvern, USA) at a dilution of 1:1,000. After incubation, the membrane was washed three times for 5 min in TBS-T buffer and then treated with peroxidase-conjugated secondary anti-mouse IgG antibody for 1 h at room temperature. The membrane was washed, and the antigen–antibody complexes were detected using chemiluminescent substrate (Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer's instructions and exposed to X-ray film for 10 s. β -actin expression was analyzed using the β -actin antibody (Sigma-Aldrich, St. Louis, MO, USA).

ELISA

Another portion of frozen tissues was sliced into small pieces and homogenized, and then cells were lysed in RIPA buffer [$1\times$ PBS, 1% NP40, 0.5% sodium deoxycolate, 0.1% SDS, protease inhibitor cocktail for mammalian tissue (Sigma-Aldrich, St. Louis, MO, USA), 1 mM sodium orthovalbuminnadate]. Tissue lysates were centrifuged at 12,000g for 15 min at 4°C to clarify and stored at -70°C . Tissue Orai1 protein from nasal mucosa, trachea and spleen was measured using a 96-well microplate (Corning Inc., New York, USA) coated with 1 $\mu\text{g}/\text{ml}$ Orai1 rabbit polyclonal antibody (NewEast Biosciences, Malvern, USA) and incubated overnight at 4°C . After washing, the microplate was incubated in 3% bovine serum albumin (Calbiochem, La Jolla, CA) at 37°C for 1 h. Samples at 1:10 dilution were then added followed by incubation at 37°C for 1 h. After washing, Orai1 rabbit polyclonal antibody that had been biotinylated using a

biotinylation kit (American Qualex International Inc., San Clemente, CA, USA) was then added at 1 $\mu\text{g}/\text{ml}$ and allowed to incubate at 25°C for 1 h. After washing, 1.5 $\mu\text{g}/\text{ml}$ of streptavidin peroxidase was added followed by incubation at 25°C for 1 h. After washing, tetramethylbenzidine (TMB) substrate [12.5 ml citric phosphate buffer, 200 μl of TMB stock solution (6 mg/ml in DMSO), 100 μl 1% H_2O_2 , Fluka Chemical Co., Ronkonkoma, NY, USA] was added to produce a color reaction. The reaction was terminated by the addition of 6 N H_2SO_4 . Optical density was determined at 450 nm using a microplate reader (Microplate Reader MTP-32, Corona Electric, Ibaraki, Japan).

Real-time RT-PCR

Total cellular RNA of a portion of specimens was extracted with Trizol (Invitrogen, Carlsbad, CA, USA) and treated with RNase-free DNase I (Ambion, Austin, TX, USA) twice at 37°C for 30 min to eliminate contaminating genomic DNA. Total RNA concentrations were determined for each sample. Absorbance at 280 nm (A280 and A260 readings) was taken on a UNICO UV-2000 ultraviolet spectrophotometer (Unico Instruments Co. Ltd., Shanghai, China) for the diluted sample. To estimate the total RNA concentration of the sample, the reading was multiplied by the dilution factor of the sample and a constant of 40, assuming that 40 $\mu\text{g}/\text{ml}$ sample has an absorbance of 1.0. To assess the purity of the RNA, a ratio of the A260 to the A280 was taken. The ratios from all our samples fell within the range of 1.9–2.1. For reverse transcription, 2 μg of total cellular RNA was reversely transcribed with random hexamers (Invitrogen, Carlsbad, CA, USA) and cDNA was amplified according to the manufacturer's instructions. Primers were designed using Primer Express software (ABI) from sequence available from Genbank and synthesized (Geneland Biotech, Shanghai, China). Orai1 primers were: forward primer 5'-TTTGCCCTCATGATCAGCAC-3' and reverse primer 5'-TTGGCGACGATGACTGATTC-3'. The intron-spanning GAPDH primers were: forward primer 5'-ACCACAGTCCATGCCATCAC-3' and reverse primer 5'-TCCACCACCTGTTGCTGTA-3'. After initial denaturation at 95°C for 10 min, the amplification profile was 15 s of denaturation at 95°C , 1 min of annealing and extension at 60°C for 45 cycles. Negative control RT reaction mixtures contained no reverse transcriptase and no cDNA in the PCR amplification mixtures. For real-time PCR, 2 μl of diluted cDNA was amplified in a total reaction volume of 20 μl using a 7500 real-time PCR System (Applied Biosystems, Foster City, CA, USA) with 20 $\times$ SYBR Green mixture (Invitrogen, Carlsbad, CA, USA). Specificity of PCR products was evaluated by melting curve analysis and by size in agarose gels. Using three dilutions of cDNA, linearity of PCR amplification was controlled. Evaluation

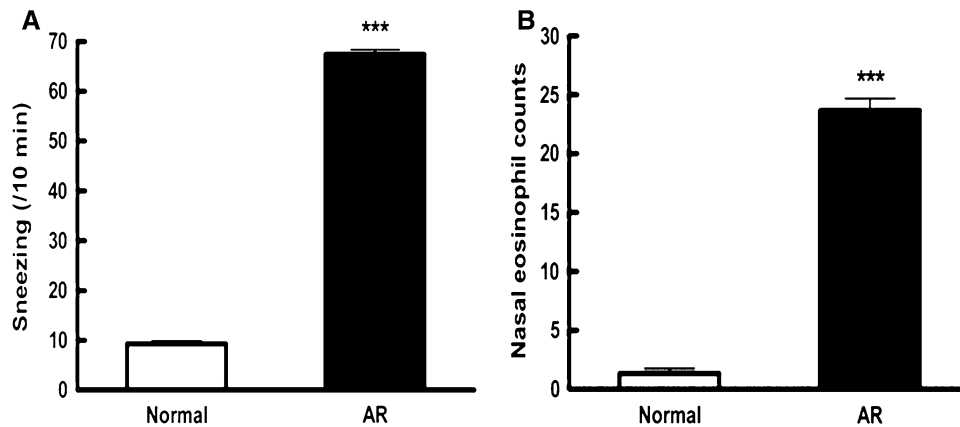
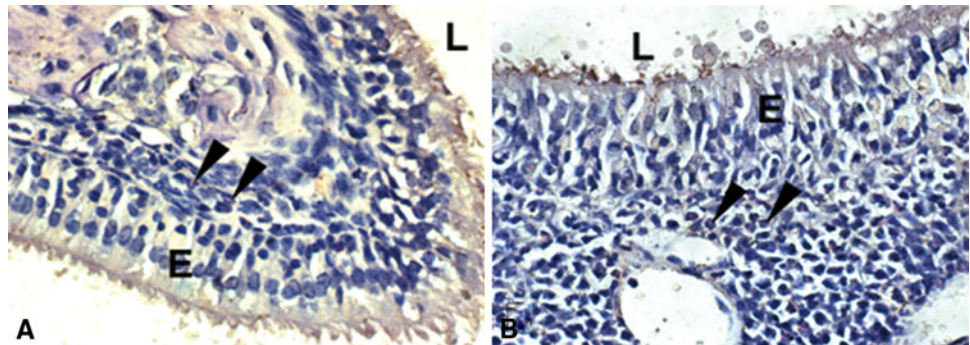


Fig. 2 Ovalbumin-induced allergic reactions. Mice were sensitized and challenged with the specific allergen as in Fig. 1. Sneezes were counted for 10 min after the last challenge (a). The bar indicates the mean $\pm$ SEM of 12 mice in each group. The number of eosinophils

infiltrating the nasal mucosa was counted based on the Luna-stained sections as in Fig. 3 ($n = 5$ fields at a magnification of $\times 400$) (b). The bar indicates the mean $\pm$ SEM of six mice in each group. *** $P < 0.001$ compared to normal group. AR allergic rhinitis

Fig. 3 Histological examination and eosinophil infiltration. Luna stain is specific for eosinophils and renders their cytoplasm red-brown on a blue background. **a** Normal mice; **b** AR mice. The filled arrowheads indicate the infiltration of eosinophils (original magnification, $\times 400$). E epithelium of nasal mucosa, L lumen of nasal cavity



of data was performed using the ΔC_T method with GAPDH as internal standard.

Statistical analysis

We used the unpaired Student's t test $\pm$ Welch's correction to compare sneezes, eosinophil counts, Orai1 protein concentrations and Orai1 mRNA expression levels between the normal and AR groups. Significance of a difference was accepted at the 5% level of confidence. Statistical analysis was performed using a commercially available statistical software prism 4.0 (GraphPad Software Inc., San Diego, CA, USA). Data are presented as mean $\pm$ SEM (standard error of the mean). P values of <0.05 were considered to be statistically significant.

Results

Ovalbumin-induced allergic reactions in AR mice

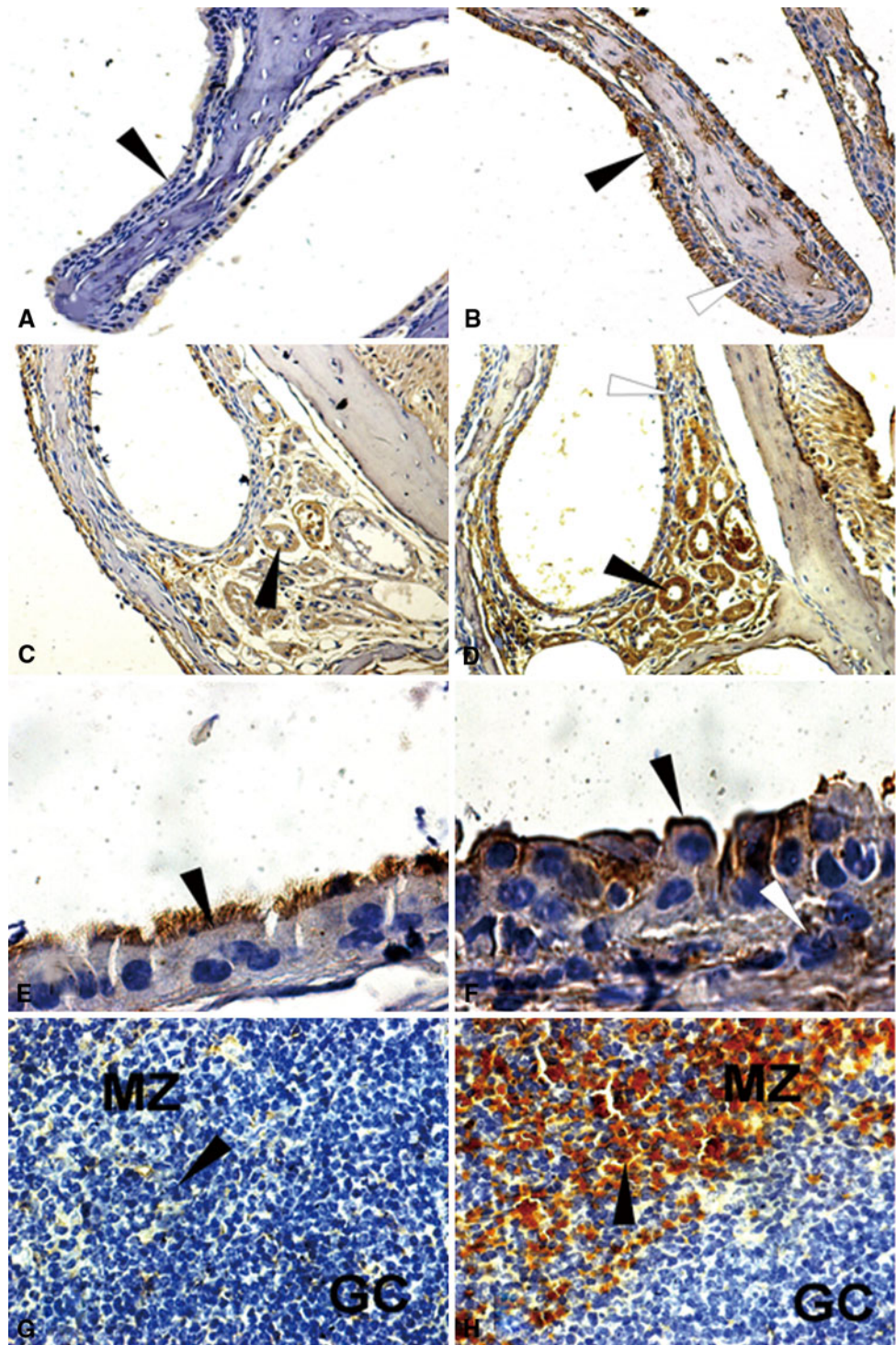
To examine the effects of ovalbumin-induced allergic reactions, sneezes were counted after the last antigen challenge with ovalbumin. AR mice showed an increase in the num-

ber of sneezes (67.42 ± 0.9000 counts/10 min, $P < 0.001$) compared with that of normal mice that had received only the last challenge (9.333 ± 0.5817 counts/10 min) (Fig. 2a). After the last challenge with ovalbumin, the nasal mucosa of allergic mice revealed changes, including the conspicuous infiltration of eosinophils and other inflammatory cells, as well as thickening of mucosal tissues (Fig. 3). The number of eosinophils in the AR group (23.67 ± 1.0220 , $P < 0.001$) displays an increase compared to that in normal mice (1.333 ± 0.4216) (Fig. 2b). The results clearly indicated that the AR mouse model was established successfully.

Immunoreactivity of Orai1 protein in AR mice nasal and tracheal mucosa and spleen

Immunohistochemical staining of Orai1 showed that this protein was distributed in mice nasal and tracheal mucosa and spleen, and intense immunoreactivity was found only in AR mice. Orai1 was localized primarily in the plasma membrane of these tissue cells. Positively immunostained cells appeared brown. In nasal mucosa, Orai1 protein was located at the surface of the epithelium of the nasal mucosa (Fig. 4b) and ductal epithelium of the submucosal glands

Fig. 4 Immunoreactivity for Orai1 in normal (a, c, e, g) and AR (b, d, f, h) mice nasal and tracheal mucosa and spleen. *Filled arrowheads* immunohistochemical staining of epithelial cells in nasal mucosa, ductal epithelium in submucosal gland, epithelial cells in tracheal mucosa and immune cells in spleen, respectively, *open arrowheads* the infiltration of inflammatory cells. *GC* germinal center, *MZ* marginal zone (original magnifications, a–d $\times 200$; e, f $\times 1,000$; g, h $\times 400$)



(Fig. 4d) in the AR mice, but was less detectable in normal mice (Fig. 4a, c). Furthermore, it was also shown that Orai1 protein was localized to the plasma membrane of allergic mice tracheal mucosa epithelium (Fig. 4f), and its immunostaining was intense in the allergic trachea compared with that in normal trachea (Fig. 4e). Additionally, there apparently existed Orai1 in the plasma membrane of immune

cells residing in the marginal zones of AR mice spleen according to immunohistochemistry, but Orai1 positive staining was less detected in the germinal centers (Fig. 4h), and immunoreactivity of Orai1 in normal group showed a decrease (Fig. 4g) in comparison with that in the AR group. The detailed distribution and expression of Orai1 immunopositive cells are shown in Table 1.

Table 1 Immunoreactivity for Orai1 in nasal and tracheal mucosa and spleen

No.	Nasal mucosa		Tracheal mucosa	Spleen
	NE	SE	TE	IC
Normal group				
1	—	+	—	+
2	—	—	—	—
3	+	+	+	+
4	—	—	—	—
5	+	+	+	+
6	—	—	—	—
AR group				
7	+++	+++	+++	+++
8	++	+++	++	++
9	+++	+++	+++	+++
10	+++	++	++	++
11	++	+++	++	+++
12	+++	++	++	+++

Numbers 1–12 stand for individual mice

NE, nasal mucosa epithelial cells; SE, submucosal glands epithelium; TE, tracheal mucosa epithelial cells; IC, immune cells; AR, allergic rhinitis; —, absence of positivity; +, weakly positive; ++, definitely positive; +++, strongly positive

Western blotting analysis of Orai1 protein production in AR mice

The extracted proteins from nasal mucosa, trachea and spleen of normal and AR mice were examined by Western blotting. The results showed an apparent immunoreactive band with a molecular mass of approximately 35 kDa reacting with Orai1 antibody in AR mice nasal mucosa, trachea and spleen. A less apparent band of the same molecular mass was detected in normal mice (Fig. 5). The result indicated that the relative Orai1 production was increased in allergic conditions in contrast to that in the normal one.

ELISA determination of Orai1 protein production in AR mice

It has been reported that mRNA expression does not directly correspond to protein expression due to post-translational processes (Greenbaum et al. 2003). We quantified Orai1 content at the protein level in frozen tissue samples using ELISA. As shown in Fig. 6, the expression of Orai1 protein increased in allergic mice nasal mucosa and trachea compared with that in nonallergic mice ($P < 0.001$ and $P < 0.001$, respectively, Fig. 6a, b). Similarly, the quantitative analysis of the protein level in spleen also indicated an enhancement of Orai1 in AR group compared to that in the

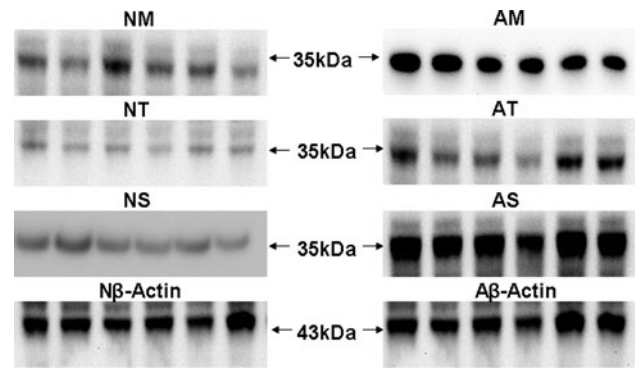


Fig. 5 Western blotting analysis of Orai1 protein in normal and AR mice nasal and tracheal mucosa and spleen. NM normal mice nasal mucosa, AM AR mice nasal mucosa, NT normal mice trachea, AT AR mice trachea, NS normal mice spleen, AS AR mice spleen; N β -Actin normal mice β -Actin, A β -Actin AR mice β -Actin. The number indicates the size of immunodetected proteins

normal group ($P < 0.001$, Fig. 6c). The result revealed an up-regulation of Orai1 at the protein level in this mouse model system after allergen provocation.

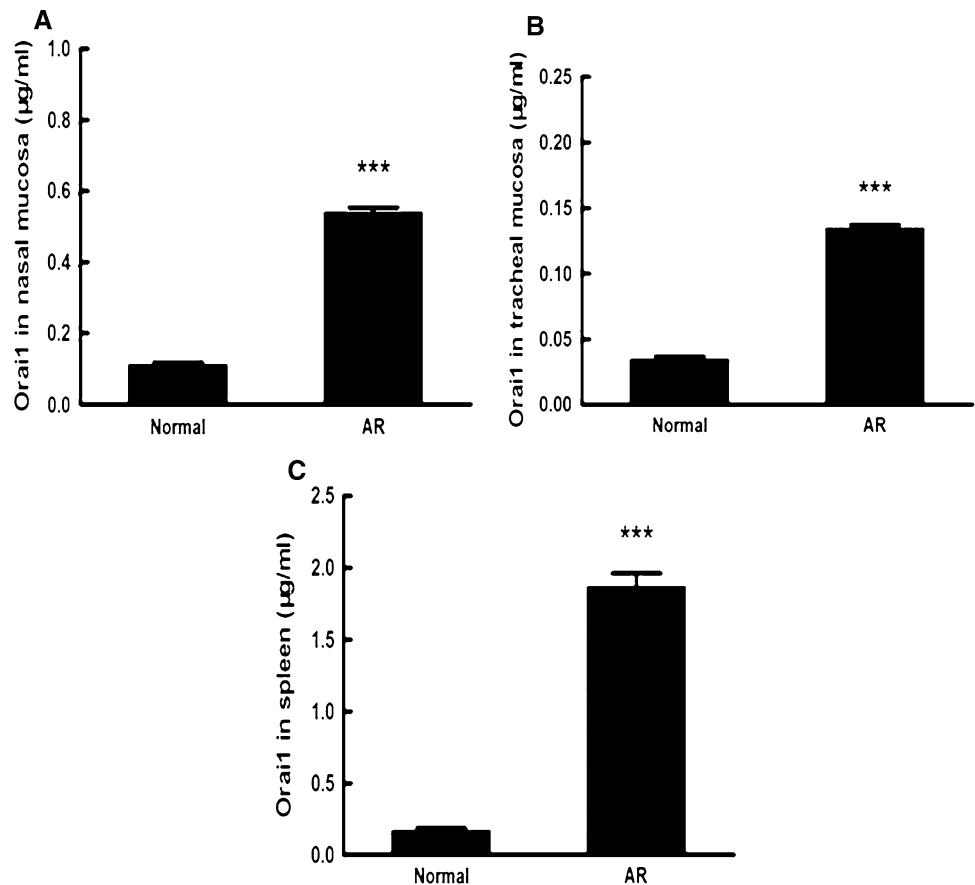
Real-time RT-PCR examination of Orai1 mRNA in AR mice

We performed real-time RT-PCR to detect the presence of Orai1 mRNA in mice nasal mucosa, trachea and spleen. Total RNA was extracted from the tissues of AR and normal group. The PCR products were detected in normal and AR mice samples. Our results revealed that Orai1 mRNA was up-regulated in AR mice nasal mucosa (Fig. 7a) ($P < 0.01$), trachea mucosa (Fig. 7b) ($P < 0.01$) and spleen (Fig. 7c) ($P < 0.05$) in contrast to that in normal mice. The results indicated that Orai1 transcription was increased in allergic inflammation in comparison with that in normal state.

Discussion

The nasal mucosa plays a crucial role in the etiology of AR. Recent studies demonstrated that nasal epithelial cells had a much wider range of activities, including release of cytokines and chemokines in human nasal epithelial cells (Kenney et al. 1994; Daffern et al. 1999; Nonaka et al. 1996), which contributed to the enhancement of allergic reactions and presentation of antigens to T cells through increased expression of HLA-DR and CD86 molecules in patients with AR (Takizawa et al. 2007). However, the performances of these cellular functions such as secretion, degranulation and proliferation are dependent on Ca^{2+} signaling process. Orai1 protein, crucial to CRAC channels which master SOCE through depletion of Ca^{2+} from

Fig. 6 ELISA analysis of Orai1 protein in normal and AR mice nasal and tracheal mucosa and spleen. **a** Orai1 in nasal mucosa, **b** Orai1 in tracheal mucosa, **c** Orai1 in spleen. The bar indicates the mean $\pm$ SEM of six mice in each group. *** $P < 0.001$ compared to normal group. AR allergic rhinitis



endoplasmic reticulum stores, may modify the concentration of cytoplasmic Ca^{2+} so as to control these cellular activities. We characterized the elevation of Orai1 protein and up-regulation of Orai1 mRNA in AR mice nasal mucosa compared to those in normal mice in our animal experiment (Figs. 4b, 5, 6a, 7a). The results may demonstrate the activation, proliferation and gene transcription of nasal epithelial cells. These cells may exert their functions to induce and enhance allergic responses through various processes.

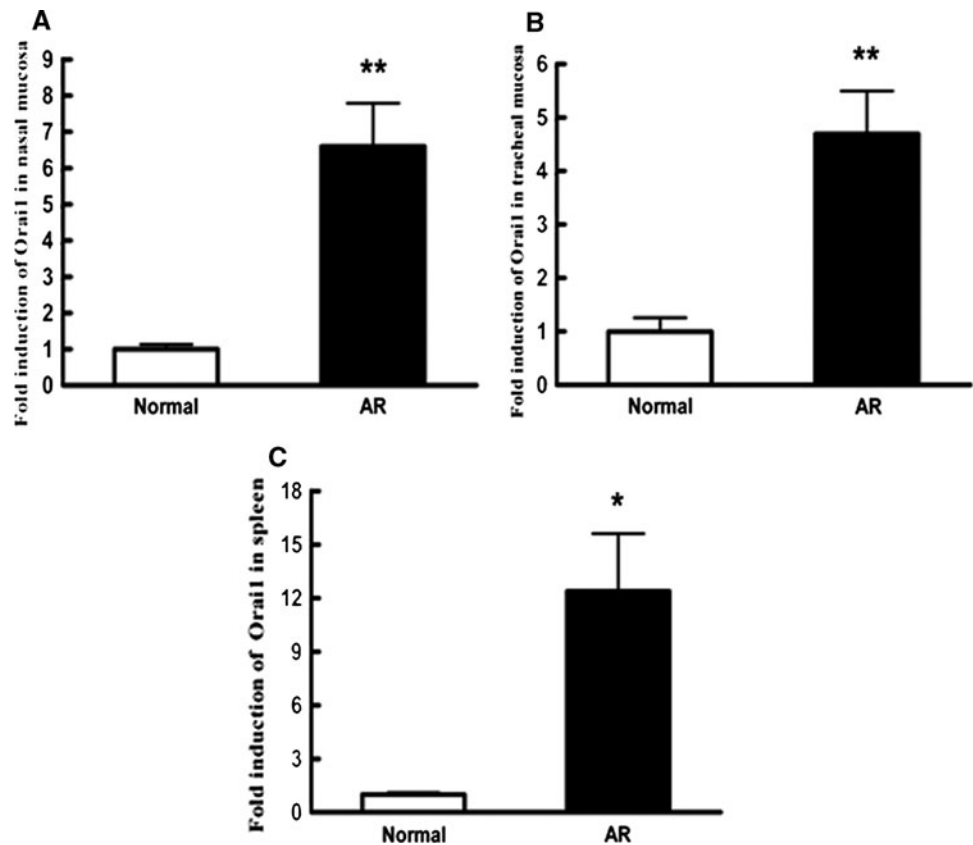
In allergic conditions, submucosal glands discharge more secretions with structural alterations such as hyperplasia and distention, which lead to the increase in the number and activity of glandular tissue in response to inflammatory mediators like leukotrienes and IL-13 (Shinya et al. 2008). Excessive serous and mucous secretion from submucosal glands leads to watery rhinorrhea in allergic nasal mucosa. Secretory function is always accompanied by the changing Ca^{2+} concentration in cytoplasm. The expression of Orai1 protein relatively increased in the epithelium of submucosal glands in allergic mice according to the immunohistochemical staining (Fig. 4d). The result indicates that the CRAC channels enhance its activity in the allergic state and may participate in the pathological actions of submucosal glands, such as facilitation of fluid and

macromolecule secretion. However, the detailed description of contribution of Orai1 to submucosal glands deserves to be further investigated.

We did not find positive staining of eosinophils, which governed major infiltrating inflammatory cells in the late phase of AR (Fig. 4b, d, f), although they can release granule-associated cationic proteins, pro-inflammatory mediators and cytokines (Trivedi and Lloyd 2007). Eosinophils perform cellular functions through highly regulated mechanisms such as classical exocytosis, compound exocytosis and piecemeal degranulation, which are related to cytoplasmic concentration of Ca^{2+} . However, our data may not support the activation of CRAC channels in eosinophils. It is likely that other Ca^{2+} signaling pathways may be responsible for their physiological and pathological actions. The mechanisms need to be further investigated.

SOCE is an important route of Ca^{2+} influx in mammalian immune cells (Salido et al. 2009). Stimulation of these cells causes depletion of Ca^{2+} from endoplasmic reticulum stores, thereby triggering sustained Ca^{2+} transmembrane entry through CRAC channels. This is an essential process for lymphocytes activation and proliferation, which involves a long-term response in allergic conditions (Salido et al. 2009; Macian 2005). Peripheral immune cells of rodents primarily reside in spleen, which may be a major

Fig. 7 Real-time RT-PCR analysis of Orai1 mRNA in normal and AR mice nasal and tracheal mucosa and spleen. Total RNA was extracted from these samples, and real-time RT-PCR was performed to detect mRNA for the indicated Orai1 in normal and AR mice nasal mucosa (**a**), tracheal mucosa (**b**) and spleen (**c**). The bar indicates the mean $\pm$ SEM of six mice in each group. * $P < 0.05$, ** $P < 0.01$ compared to the normal group. AR allergic rhinitis



manufacturer of various immunoglobulins. There was an increase of Orai1 protein concentration and Orai1 mRNA level in AR mice spleen samples compared to those in normal mice in the context (Figs. 4h, 5, 6c, 7c), which may reveal the activation of splenic immune cells in response to the stimulation of specific antigens. The regional immunity and immune cells trafficking may disclose in part the mechanism of Orai1 up-regulation in the spleen. In addition, Orai1 protein was expressed and distributed primarily in the splenic marginal zone, which consisted of specialized macrophages, dendritic cells and non-recirculating B cells in murine species (Kumaratne et al. 1981), but not in the germinal center (Fig. 4). However, the mechanisms are not clear.

The fundamental relationship between AR and asthma is that these two conditions represent one entity in two different parts of the respiratory tract (Passalacqua et al. 2001). Epidemiological data also attest to the strong clinical link between them. The exact mechanisms underlying this association are not known. In our work, the analysis of Orai1 protein by Western blotting and ELISA, and the quantitative determination of Orai1 mRNA through real-time RT-PCR showed the protein and mRNA up-regulation of Orai1 in AR mice trachea (Figs. 4f, 5, 6b, 7b) and that the protein existed in the tracheal mucosa epithelium (Fig. 4f). Challenging by specific allergen in nasal mucosa induces

responses in lower airway, which may demonstrate the functional relationship between AR and allergic asthma. However, the mechanisms by which the Orai1 pathway located in the upper airway influences the common signaling pathway in the lower airway is not clear. It is hypothesized that trafficking of lymphocytes in the airway mucosa may partly be an answer to this phenomenon. This is also required to be further studied.

In summary, we performed an analysis of Orai1 protein in the airway and spleen using immunohistochemical staining, Western blotting and ELISA, and quantitatively analyzed Orai1 mRNA in these tissue samples with real-time RT-PCR. Orai1 is expressed in mice nasal and tracheal mucosa and spleen, and the Orai1 protein and mRNA contents are up-regulated in the AR group compared with those in the normal group. We suggest a possible contribution of Orai1 pathway to the pathogenic mechanisms of AR. Furthermore, it should be emphasized that some intervention measures may offer novel and effective prophylactic and/or therapeutic means to control AR by directly interfering with Orai1 molecule, CRAC channels and SOCE pathway. As a result, the symptoms of AR may be improved effectively.

Conflict of interest statement The authors have no financial conflicts of interest.

References

- Abdullaev IF, Bisaillon JM, Potier M, Gonzalez JC, Motiani RK, Trebak M (2008) Stim1 and Orai1 mediate CRAC currents and store-operated calcium entry important for endothelial cell proliferation. *Circ Res* 103:1289–1299
- Berridge MJ, Bootman MD, Roderick HL (2003) Calcium signaling: dynamics, homeostasis and remodelling. *Nat Rev Mol Cell Biol* 4:517–529
- Daffern PJ, Jagels MA, Saad JJ, Fischer W, Hugli TE (1999) Upper airway epithelial cells support eosinophil survival in vitro through production of GM-CSF and prostaglandin E2: regulation by glucocorticoids and TNF- α . *Allergy Asthma Proc* 20:243–253
- Drenckhahn D, Jöns T, Schmitz F (1993) Production of polyclonal antibodies against proteins and peptides. *Methods Cell Biol* 37:7–56
- Feske S, Gwack Y, Prakriya M, Srikanth S, Puppel SH, Tanasa B, Hogan PG, Lewis RS, Daly M, Rao A (2006) A mutation in Orai1 causes immune deficiency by abrogating CRAC channel function. *Nature* 441:179–185
- Greenbaum D, Colangelo C, Williams K, Gerstein M (2003) Comparing protein abundance and mRNA expression levels on a genomic scale. *Genome Biol* 4:117
- Gwack Y, Feske S, Srikanth S, Hogan PG, Rao A (2007) Signaling to transcription: store-operated Ca^{2+} entry and NFAT activation in lymphocytes. *Cell Calcium* 42:145–156
- Hoth M, Penner R (1992) Depletion of intracellular calcium stores activates a calcium current in mast cells. *Nature* 355:353–356
- Ikeda Y, Kaneko A, Yamamoto M, Ishige A, Sasaki H (2002) Possible involvement of suppression of Th2 differentiation in the anti-allergic effect of Sho-seiryu-to in mice. *Jpn J Pharmacol* 90:328–336
- Kay AB (2001) Allergy and allergic diseases. *N Engl J Med* 344:30–37
- Kenney JS, Baker C, Welch MR, Altman LC (1994) Synthesis of interleukin-1 α , interleukin-6, and interleukin-8 by cultured human nasal epithelial cells. *J Allergy Clin Immunol* 93:1060–1067
- Kumararatne DS, Bazin H, MacLennan IC (1981) Marginal zones: the major B cell compartment of rat spleens. *Eur J Immunol* 11:858–864
- Lis A, Peinelt C, Beck A, Parvez S, Monteilh-Zoller M, Fleig A, Penner R (2007) CRACM1, CRACM2, and CRACM3 are store-operated Ca^{2+} channels with distinct functional properties. *Curr Biol* 17:794–800
- Macian F (2005) NFAT proteins: key regulators of T-cell development and function. *Nat Rev Immunol* 5:472–484
- Mercer JC, Dehaven WI, Smyth JT, Wedel B, Boyles RR, Bird GS, Putney JW Jr (2006) Large store-operated calcium-selective currents due to co-expression of Orai1 or Orai2 with the intracellular calcium sensor, Stim1. *J Biol Chem* 281:24979–24990
- Nonaka M, Nonaka R, Jordana M, Dolovich J (1996) GM-CSF, IL-8, IL-1R, TNF- α R, and HLA-DR in nasal epithelial cells in allergic rhinitis. *Am J Respir Crit Care Med* 153:1675–1681
- Parekh AB, Putney JW (2005) Store-operated calcium channels. *Physiol Rev* 85:757–810
- Passalacqua G, Ciprandi G, Canonica GW (2001) The nose–lung interaction in allergic rhinitis and asthma: united airways disease. *Curr Opin Allergy Clin Immunol* 1:7–13
- Peel SE, Liu B, Hall IP (2008) ORAI and store-operated calcium influx in human airway smooth muscle cells. *Am J Respir Cell Mol Biol* 38:744–749
- Rao A, Luo C, Hogan PG (1997) Transcription factors of the NFAT family: regulation and function. *Annu Rev Immunol* 15:707–747
- Salido GM, Sage SO, Rosado JA (2009) Biochemical and functional properties of the store-operated Ca^{2+} channels. *Cell Signal* 21:457–461
- Shinya S, Kazuhiko T, Hajime I, Carol B, Yuichi M (2008) Leukotriene D4 upregulates MUC2 gene transcription in human epithelial cells. *Pharmacology* 81:221–228
- Takizawa R, Pawankar R, Yamagishi S, Takenaka H, Yagi T (2007) Increased expression of HLA-DR and CD86 in nasal epithelial cells in allergic rhinitis: antigen presentation to T cells and up-regulation by diesel exhaust particles. *Clin Exp Allergy* 37:420–433
- Tolhurst G, Carter RN, Amisten S, Holdich JP, Erlinge D, Mahaut-Smith MP (2008) Expression profiling and electrophysiological studies suggest a major role for Orai1 in the store-operated Ca^{2+} influx pathway of platelets and megakaryocytes. *Platelets* 19:308–313
- Trivedi SG, Lloyd CM (2007) Eosinophils in the pathogenesis of allergic airways disease. *Cell Mol Life Sci* 64:1269–1289
- van de Rijin M, Mehlhop PD, Judkins A, Rothenberg ME, Luster AD, Oettgen HC (1998) A murine model of allergic rhinitis: studies on the role of IgE in pathogenesis and analysis of the eosinophil influx elicited by allergen and eotaxin. *J Allergy Clin Immunol* 102:65–74
- Varnai P, Hunyady L, Balla T (2009) STIM and Orai: the long-awaited constituents of store-operated calcium entry. *Trends Pharmacol Sci* 30:118–128
- Venkatachalam K, van Rossum DB, Patterson RL, Ma HT, Gill DL (2002) The cellular and molecular basis of store-operated calcium entry. *Nat Cell Biol* 4:E263–E272