Cellular & Molecular Immunology (2016) 13, 110–118

www.nature.com/cmi

RESEARCH ARTICLE

Regulation of TWIK-related potassium channel-1 (Trek1) restitutes intestinal epithelial barrier function

Huang Huang[1,5], Jiang-Qi Liu[2,3,4,5], Yong Yu[1], Li-Hua Mo[2], Rong-Ti Ge[4], Huan-Ping Zhang[4], Zhi-Gang Liu[2], Peng-Yuan Zheng[1] and Ping-Chang Yang[2,4]

The disruption of epithelial barrier integrity is an important factor in the pathogenesis of various immune disorders. However, the restitution of the compromised barrier functions is difficult. This study investigates the regulation of TWIK-related potassium channel-1 (Trek1) in the restitution of intestinal epithelial barrier functions. The human colon epithelial cell line T84 was cultured in monolayers and used to observe epithelial barrier functions *in vitro*. An intestinal allergy mouse model was created. Cytokine levels were determined by enzyme-linked immunosorbent assay and western blotting. The results showed that Trek1 deficiency induced T84 monolayer barrier disruption. Allergic responses markedly suppressed the expression of Trek1 in the intestinal epithelia *via* activating the mitogen-activated protein kinase pathways and increasing the expression of histone deacetylase-1. The inhibition of histone deacetylase-1 by sodium butyrate or the administration of a butyrate-producing probiotic (*Clostridium butyricum*) restored the intestinal epithelial barrier functions and markedly enhanced the effect of antigen-specific immunotherapy. The data suggest that Trek1 is required for the maintenance of intestinal epithelial barrier integrity. Allergic responses induce an insufficiency of Trek1 expression in the intestinal epithelia. Trek1 expression facilitates the restoration of intestinal epithelial barrier functions in an allergic environment.
Cellular & Molecular Immunology (2016) **13,** 110–118; doi:10.1038/cmi.2014.137; published online 16 February 2015

Keywords: allergy; epithelial barrier, intestine; probiotics; Trek1

INTRODUCTION

Epithelial barrier integrity has a critical role in the maintenance of homeostasis in the body. A large number of disorders are associated with epithelial barrier disruption, such as intestinal allergies,[1] inflammatory bowel disease,[2] allergic asthma[3] and allergic dermatitis.[4] However, the pathogenesis of epithelial barrier disruption has not yet been fully elucidated, and the remedies for restoring epithelial barrier function are currently limited.

A number of factors have been associated with epithelial barrier disruption, including the pro-inflammatory cytokines interleukin (IL)-4, IL-13,[5] interferon (IFN)-γ[6] and tumor necrosis factor (TNF)-α.[7] The status of the paracellular space reflects the function of the epithelial barrier and is dependent on the tight junction-associated proteins that seal the tight junctions. Hyperpermeability to macromolecular substances (such as protein antigens) is the major feature of epithelial barrier disruption and may allow antigens to cross the epithelial barrier *via* the paracellular pathway or the intracellular pathway.[8] Multiple factors have been reported to induce epithelial barrier disruption. Thus, in an allergic or inflammatory environment, the restitution of the barrier functions by blocking only one or some of the causative factors is unlikely.

The TWIK-related potassium channel-1 (Trek1) is located in epithelial cells,[9] endothelial cells and the cardiovascular system,[10] and has also been identified in the intestine.[11] Recent reports have suggested that Trek1 regulates barrier function.[9,12] Based on these findings, we hypothesized that Trek1 regulates intestinal epithelial barrier functions. The results of this study showed that intestinal epithelial cells expressed Trek1, and that Trek1 inhibition markedly compromised epithelial barrier functions. The restoration of Trek1 expression facilitated the

[1]Department of Gastroenterology, the Second Hospital, Zhengzhou University, Zhengzhou, China; [2]ENT Institute, Shenzhen Key Laboratory of Allergy & Immunology, Shenzhen University, Shenzhen, China; [3]ENT Hospital, Longgang Central Hospital, Shenzhen, China and [4]Brain Body Institute, McMaster University, Hamilton, ON, Canada
[5]These authors equally contributed to this work.
Correspondence: Dr PC Yang, Dr PY Zheng and Dr ZG Liu, Room 722 of the Medical School Building; 3688 Nanhai Ave., Shenzhen, 518060, China.
E-mail: pcy2356@szu.edu.cn
Received: 27 October 2014; Revised: 26 December 2014; Accepted: 27 December 2014

recovery of epithelial barrier function and enhanced the effect of specific immunotherapies on inhibiting allergic responses in the intestine.

MATERIALS AND METHODS

Reagents

Clostridium butyricum (#1987252: 285–289) was a gift from Shenzhen Kexing Biotech Co., Ltd (Shenzhen, China). Sodium butyrate, p38 inhibitor (PD169316) and horseradish peroxidase (HRP) were purchased from Sigma-Aldrich (Shanghai, China). The Trek1 (C-20) and HDAC1 (C-19) antibodies were purchased from Santa Cruz Biotech (Shanghai, China). The IL-4, IL-5 and IL-13 ELISA kits were purchased from R&D Systems (Shanghai, China). The ovalbumin (OVA)-specific IgE ELISA kit was purchased from AbD Sertec (Shenzhen, China). The IL-4, IL-5, IL-13, mouse mast cell protease-1 and TNF-α recombinant proteins were purchased from Biomart (Shenzhen, China).

Mice

BALB/c mice (6–8 months old, 20–25 g) obtained from the Guangzhou Experimental Animal Center were used in this study. The mice were housed in a pathogen-free environment and allowed access to food and water *ad libitum*. The cages were kept in a 12-h light/dark cycle. The experimental procedures were approved by the Animal Ethic Committee at Shenzhen University; the procedures were performed in accordance with the guidelines.

Cell culture

Human intestinal epithelial cell line T84 cells (ATCC, USA Millipore (Shanghai, China)) were seeded onto the inserts with collagen supports (0.4 μm) in the Transwell system. The cells were cultured with Dulbecco's Modified Eagle Medium supplemented with 10% fetal calf serum, 100 U/ml penicillin, 0.1 mg/ml streptomycin and 2 mM *L*-glutamine. The medium was changed daily. Upon reaching confluence, the cells were collected and seeded into the inserts of Transwells at a density of 10^6 cells/ml. After transepithelial resistance (TER) reached or surpassed 1000 Ω cm^2 (recorded with an ohmmeter), the cells were used for further experiments.

Assessment of T84 monolayer barrier function

TER and permeability to HRP (MW=45 000 Dalton) were evaluated as the indicators of T84 monolayer barrier function. TER was recorded at time points as specified in Figure 1. HRP flux was performed with established procedures that were published elsewhere.[13] In separate experiments, FITC-dextran (10 mg/ml; 1, MW=4000; 2, MW=70 000) was added to the

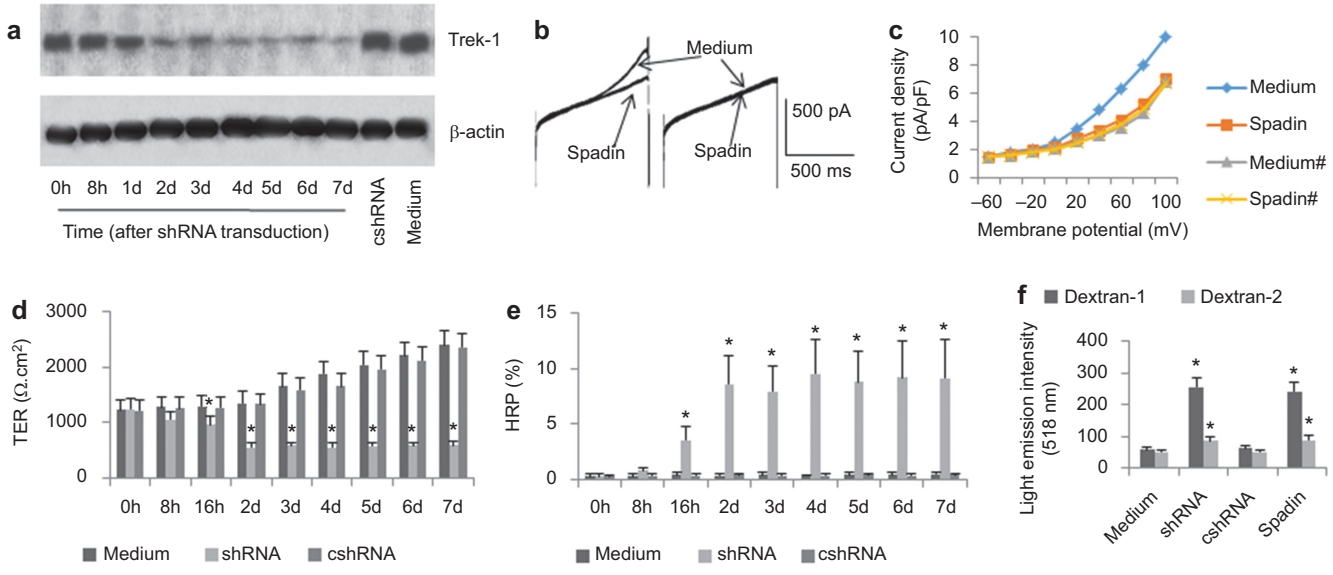

Figure 1 Trek1 maintains T84 monolayer barrier functions. (**a**) T84 cells were treated with medium, Trek1 shRNA (shRNA), or control shRNA (cshRNA). The immune blots indicate the levels of the Trek1 protein in the T84 cell extracts 0 h–7 days after shRNA transduction. (**b**) Electrophysiological assessment of the membrane currents of single T84 cells (10 cells/group) from wild-type and Trek1-null (Trek1-n) T84 cells in the presence or absence of the Trek1 inhibitor spadin (1 μM). (**c**) The curves show the summarized current density. (**d** and **e**) T84 cells were cultured in Transwells. Upon reaching confluence, the T84 cells were treated with medium, Trek1 shRNA, or control shRNA. (**d**) The TER was recorded from 0 h to 7 days as denoted on the *X* axis. The bars indicate the TER. (**e**) HRP flux was performed with the T84 monolayers at time points as denoted on the *X* axis of the bar graphs. Each Transwell was only used for one permeability experiment. The test period ran for 2 h. (**f**) Confluent T84 monolayers were treated in Transwells as denoted on the *X* axis. FITC-dextran (10 mg/ml; 1, MW=4000; 2, MW=70 000) was added to the apical chambers. Samples were collected from the basal chambers 6 h later and analyzed by a fluorometer at 518 nm. The bars indicate the fluorescence intensity. The data are presented as mean±s.d. **P*<0.01, compared with the medium group. The data are representative of three independent experiments. HRP, horseradish peroxidase; TER, transepithelial resistance; Trek1, TWIK-related potassium channel-1.

apical chambers. Samples were collected from the basal chambers 6 h later and analyzed by a fluorometer at 518 nm.

Gene silencing of Trek1

The Trek1 gene was knocked down in T84 cells with a shRNA kit according to the manufacturer's instructions as well as reported procedures.[14] Briefly, the Trek1 shRNA lentiviral packing plasmid (GeneChem, Shanghai, China) was transducted into 293 T packaging cells using Lipofectamine 2000 (Invitrogen, Shanghai, China); after 48 h, the lentivirus-containing supernatant was harvested and used for the infection of human T84 cells in the presence of 4 μg/ml polybrene (Sigma-Aldrich).The effect of gene silencing was assessed by western blotting.

Real-time RT-PCR (qRT-PCR) analysis

Total RNA was extracted from cells using the TRIzol reagent. The reverse transcription reactions were performed using a cDNA reverse transcription reagent kit. Real time PCR was performed using a MiniOpticon Real-Time PCR System (Bio-Rad, Shanghai, China) with the SYBR Green Master Mix. Fold changes in gene expression were calculated using the $\Delta\Delta C_t$ method with β-actin as an internal control. All of the reactions were performed in triplicate. The primers used in this study included: Trek1, forward, caattcgacggagctggatg and reverse, cttctgtgcgtggtgagatg; HDAC1, forward, attatggacaaggccaccca and reverse, gcttgctgtactccgacatg; andβ-actin, forward cgcaaagacctgtatgccaa and reverse, cacacagagtacttgcgctc.

Methylation analysis of the Trek1 gene in T84 cells

DNA was extracted from T84 cells with a reagent kit following the manufacturer's instructions. The genomic DNA was treated with sodium bisulfite as described by Herman *et al.*[15] Briefly, the DNA was treated with 2 M NaOH at 37 °C for 10 min, followed by incubation with 3 M sodium bisulphite (pH 5.0) at 50 °C overnight (covered with mineral oil). The samples were treated with 3 M NaOH for 5 min, and then concentrated by ammonium precipitation and 100% ethanol, washed with 70% ethanol and resuspended in 20 μl distilled water. Methylation-specific PCR was performed with the samples to determine the amount of demethylated DNA of the Trek1 gene. The primer sequences for the methylated Trek1 gene were as follows: sense, tatttttattaagcggaagttttcg and antisense, tctcttcaccaacacaatataacgt. The primer sequences for the unmethylated Trek1 gene were: sense, atttttattaagtggaagtttttgg and antisense, ctcttcaccaacacaatataacatc. Expression was normalized to the housekeeping gene β-actin.

Western blot

Total proteins were extracted from the cells, fractionated with SDS–PAGE and transferred onto a PVDF membrane. After blocking with 5% skim milk for 30 min, the membrane was incubated with the primary antibodies (100–500 ng/ml) for 1 h at room temperature, followed by incubation with the HRP-conjugated secondary antibodies. Washes with TBST (Tris-buffered saline-Tween 20) were performed after the incubation. The membrane was developed using an ECL system. The results were photographed with a Kodak Image Station 4000 mm Pro (Kodak, Shanghai, China). The integrated density of the immune blots was measured with PhotoShop and expressed as a percentage of the internal loading control β-actin.

A mouse model of intestinal allergy

BALB/c mice were fed with ovalbumin (OVA; 0.1 mg/mouse) mixed with cholera toxin (20 μg/mouse) in 0.3 ml saline weekly for 5 consecutive weeks. The allergic response of the mice was assessed by evaluating the allergic parameters, including serum levels of Th2 cytokines and specific IgE, frequency of mast cells and eosinophils in the intestinal mucosa and the antigen-specific $CD4^+$ T cell proliferation by the methods below.

Enzyme-linked immunosorbent assay (ELISA)

The levels of cytokines and IgE were determined by ELISA with commercial reagent kits following the manufacturer's instructions.

Mast cell and eosinophil counts in the intestinal mucosa

Two pieces of jejunal segments were excised at the time of sacrifice and snap frozen in liquid nitrogen. The cryosections were fixed with acetone and stained with anti-major basic protein (1 : 200; for eosinophils) or anti-mouse mast cell protease-1 (1 : 100; for mast cells). Mast cells and eosinophils were counted in 20 fields/mouse on the sections under a light microscope; the results were converted to cell number/mm^2. The slides were coded; the observers were not aware of the codes to avoid observer bias.

Isolation of immune cells

The spleens were excised from the mice at the time of killing and single spleen cell suspensions were prepared. $CD4^+CD25^-$ T cells and dendritic cells (DCs) were isolated from the single spleen cell suspensions using commercial reagent kits following the manufacturer's instructions. Briefly, the single spleen cell suspensions were labeled with MACS CD11c and MHC II microbeads. $CD11c^+MHC\ II^+$ DCs were separated by passing the antibody-coated cell suspension through a VS^+ column on a SuperMACS magnetic cell separator. Positive cells were collected by removing the column from the magnetic field and then flushing it with phosphate-buffered saline (PBS) containing 0.5% FCS. For $CD4^+CD25^-$ T-cell isolation, the single spleen cell suspensions were first labeled with MACS CD3 and CD4 microbeads, and then the $CD3^+CD4^+$ T cells were isolated using the same procedure as that described above for DC isolation. The cells were then labeled with MACS CD25 microbeads and isolated as described above. Finally, the negatively selected $CD4^+CD25^-$ T cells were collected. The purity of the cells was >98% as determined by flow cytometry (Supplementary Figure 1).

Antigen-specific $CD4^+$ T-cell proliferation assay

The T cells (labeled with 5 μM CFSE) and DCs were cultured at a ratio of 5 : 1 (T cell/DC) in the presence of a specific antigen

(OVA, 10 μg/ml) for 3 days. Then, the cells were analyzed by flow cytometry for the CFSE-dilution assay.

Immunohistochemistry

A piece of small intestine was excised and processed for cryosections. The sections were fixed with cold acetone for 20 min, blocked with 1% bovine serum albumin (BSA) for 30 min, incubated with the primary antibodies (1 : 100) for 1 h at room temperature and incubated with the secondary antibodies (labeled with fluorochrome) for 1 h. The nuclei were stained with propidium iodide for morphological viewing. The samples were washed with PBS after each incubation. The slides were mounted with cover slips and observed under a confocal microscope.

Antigen-specific immunotherapy (SIT)

The allergic mice were treated with SIT following published procedures with a minor modification. Briefly, an increasing dose of OVA (the specific antigen) was administered to the mice by gavage in 0.3 ml saline daily for 14 days as follows: 10 μg (days 1 and 2), 50 μg (days 3 and 4), 0.1 mg (days 5–7), 0.25 mg (days 8 and 9) and 0.5 mg (days 10–14).

Culture of *Clostridium butyricum* (*C. butyricum*)

C. butyricum was cultured anaerobically at 37 °C overnight in a medium consisting of (per liter; pH 6.5) 10 g glucose, 10 g polypeptone, 2 g KH_2PO_4, 0.5 g yeast extract, 0.5 g $MgSO_4.7H_2O$ and 1 g *L*-cysteinehydrochloride monohydrate. The culture broth was centrifuged at 8500*g* for 10 min; the harvested cells were washed three times with PBS. The cells were suspended in saline to obtain a cell suspension. Mice were fed 1×10^9 CFU/day for a total of 15 days beginning 1 day before the SIT.

Electrophysiological measurements of T84 cells

T84 cells were cultured in DMEM. The membrane current was measured using the patch-clamp technique following published procedures[16] with minor modifications. Whole-cell recording pipettes were fabricated from borosilicate glass and filled with an intracellular solution containing (in mM): 95 K-gluconate; 20 K3-citrate; 10 NaCl; 10 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; 1 MgCl2; 0.5 CaCl2; 3 BAPTA; 3 Mg-ATP; and 0.5 Na2-GTP. Membrane currents were recorded using an EPC-10 amplifier (HEKA Elektronik). Outward currents were elicited by repeated >500-ms ramps to 40 mV starting from a holding potential of −80 mV or from −60 to +100 mV applied at 30-s intervals. The Clampex software was used to analyze the data.

Collection of intestinal epithelial samples

After sacrifice, a piece of the jejunal segment was opened longitudinally. Epithelial samples were collected by gently scraping with a glass slide. The proteins were extracted and analyzed by western blotting.

Statistical analysis

The data are expressed as mean±s.d. The difference between groups was determined by Student's t test or ANOVA test followed by Bonferroni post-tests if more than two groups were compared. A P value <0.05 was considered significant.

RESULTS

Trek1 deficiency compromises intestinal epithelial barrier function

In the first approach, the Trek1 gene was knocked down in T84 cells by gene silencing (Figure 1a). The TER of the T84 monolayers was recorded daily from 0 h to 7 days. The results showed that after Trek1 gene silencing, the TER dropped significantly at 16 h and further dropped at 48 h. To show that Trek1 was present in the plasma membrane of the T84 cells, whole-cell patch clamp measurements were performed. A Trek1-specific antagonist(spadin[17]) markedly reduced the outward currents of single T84 cells. The outward rectification in Trek1-null T84 cells was attenuated significantly (Figure 1b and c). The TER remained at low levels over the entire observation period (Figure 1d). Next, the permeability of the T84 monolayers was assessed. The results showed that the knockdown of the Trek1 gene also increased the permeability of the T84 monolayers, which was inversely associated with the changes in TER (Figure 1e and f). The addition of the Trek1 inhibitor spadin also markedly inhibited the expression of Trek1 in T84 cells (Supplementary Figure 2) and increased T84 monolayer permeability (Figure 1f). The results suggest that Trek1 is associated withT84 monolayer barrier function integrity.

Allergic responses suppress Trek1 expression in intestinal epithelial cells

Because allergic responses are one of the causative factors of intestinal epithelial barrier disruption[1,18] and the data shown in Figure 1 suggest that Trek1 is involved in maintaining intestinal epithelial barrier integrity, we inferred that allergic mediators might interfere with Trek1 expression and compromise barrier functions. To this end, we created an intestinal allergy mouse model. The mice exhibited intestinal allergic inflammation, including high levels of serum IL-4, IL-5 and IL-13 (Figure 2a) and antigen-specific IgE (Figure 2B), high frequencies of eosinophils and mast cells in the intestinal mucosa (Figure 2c and Supplementary Figure 3), and antigen-specific $CD4^+$ T-cell proliferation in response to stimulation by specific antigens in culture (Figure 2d). Both western blotting (Figure 2e) and immunohistochemistry (Figure 2f–h) demonstrated that the levels of Trek1 in the intestinal epithelial layers were significantly reduced in allergic mice compared with control mice. The results indicate that allergic responses are associated with the decreased expression of Trek1 in mouse intestinal epithelial cells.

Allergic mediators inhibit Trek1 in the intestinal epithelia *via* the p38 MAPK pathway

Next, we investigated the mechanism by which the allergic responses suppressed the expression of Trek1 in intestinal epithelial cells. Based on the data shown in Figures 1 and 2, we inferred that the factors suppressing the expression of Trek1 were one (or several) of the Th2 cytokines or mediators produced by the pro-inflammatory cells. To test this hypothesis,

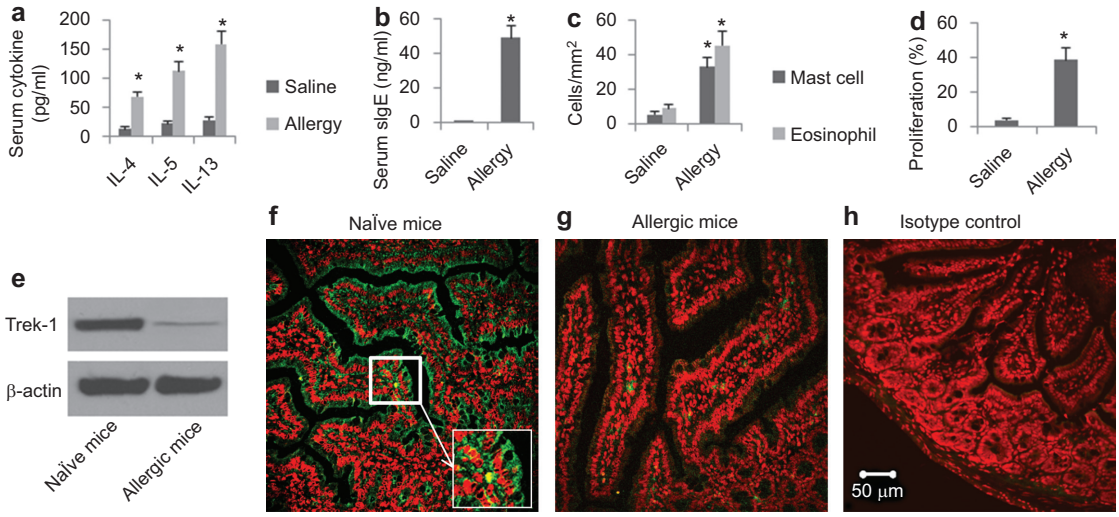

Figure 2 Allergic response suppresses Trek1 production by intestinal epithelial cells. BALB/c mice were sensitized to OVA. The serum and intestinal segments were collected and analyzed for the allergic response in the intestine. A-b, the bars indicate the serum levels of Th2 cytokines (a) and specific IgE (sIgE; b). c, the bars indicate the frequency of mast cells (MC) and eosinophils (Eo) in the intestinal tissue. d, the bars indicate the frequency of $CD4^{\times}$ T cell proliferation in response to exposure to specific antigens in the culture. e, the immune blots indicate the protein levels of Trek1 in the intestinal mucosa. f-h, the representative confocal images show the expression of Trek1 (in green) in the intestinal epithelia (the insert is an enlarged image field showing Trek1 staining). The nuclei were stained with propidiumidodide (in red) for morphological view. The data of bars are presented as mean ± SD. *, $p<0.01$, compared with saline group. Each group consists of 6 mice. Specimens from individual mice were processed separately. The data are a representative of 6 independent experiments.

T84 cells were exposed to IL-4, IL-5, IL-13, mouse mast cell protease-1 and TNF-α for 72 h. The cells were analyzed by qRT-PCR and western blotting. As shown in Figure 3, exposure to any one of the five cytokines markedly suppressed the expression of Trek1 in T84 cells. All of the mediators are capable of activating p38 MAPK, suggesting that this pathway might be involved in the suppression of Trek1 by these cytokines. To test this inference, we used the same experimental procedures as described above but with an inhibitor of p38 added to the culture and found thatthe suppression of Trek1 was abolished. To strengthen the results, we analyzed Trek1 gene DNA methylation. The results showed that the unmethylated Trek1 gene DNA levels were in line with the Trek1 protein levels (Figure 3b) in T84 cells (Figure 3c).

HDAC1 is involved in allergic mediator-suppressed Trek1 expression in T84 cells

Based on published data demonstrating that HDAC1 affects epithelial barrier functions,[19] we inferred that HDAC1 might be involved in the allergic mediator-induced suppression of Trek1 and the resulting epithelial barrier disruption. To test this hypothesis, we assessed the expression of HDAC1 in T84 cells after exposure to the allergic mediators. As assessed by qRT-PCR and western blotting, the exposure to allergic mediators significantly increased the expression of HDAC1 in T84 cells (Figure 4a and b). The results imply that allergic mediators upregulate the expression of HDAC1 in T84 cells. Moreover, the addition of the HDAC inhibitor Trichostatin A to the cultures abolished the allergic mediator-suppressed expression of Trek1 in T84 cells (Figure 4c and d).

Recovery of Trek1 expression restitutes the intestinal epithelial barrier functions and enhances the effect of specific immunotherapy

The present data indicate that allergic responses compromise the expression of Trek1 in the intestinal epithelial cells (as depicted in Figure 2). Epithelial barrier disruption is one of the pathological features of intestinal allergies. Thus, the restoration of the expression of Trek1 has the potential to facilitate recovery from intestinal allergies. To test this hypothesis, we used the intestinal allergy mouse model described above. To mimic the desensitization observed in clinical allergy patients, the mice were treated with SIT following published procedures[20] with or without the concomitant administration of sodium butyrate or *C. butyricum*. The results in Figure 5 showed that SIT only weakly inhibited the allergic responses ($P<0.01$) in the allergic mice. By contrast, the combination of SIT and sodium butyrate or SIT and *C. butyricum* significantly suppressed the allergic responses in the mice.

To further investigate the mechanism by which the administration of *C. butyricum* improves the allergic response, in separate experiments the allergic mice were treated with the Trek1 inhibitor spadin in combination with SIT or *C. butyricum*. The results showed that the inhibitory effect on allergic intestinal inflammation was abolished, and implicated the butyrate-producing *C. butyricum*in the upregulation of Trek1 expression to facilitate the inhibition of allergic responses. Next, we assessed the expression of Trek1 in the mouse intestinal epithelia. The results showed that treatment with *C. butyricum* significantly increased the expression of Trek1 and suppressed the levels of HDAC1 in the intestinal epithelia compared with

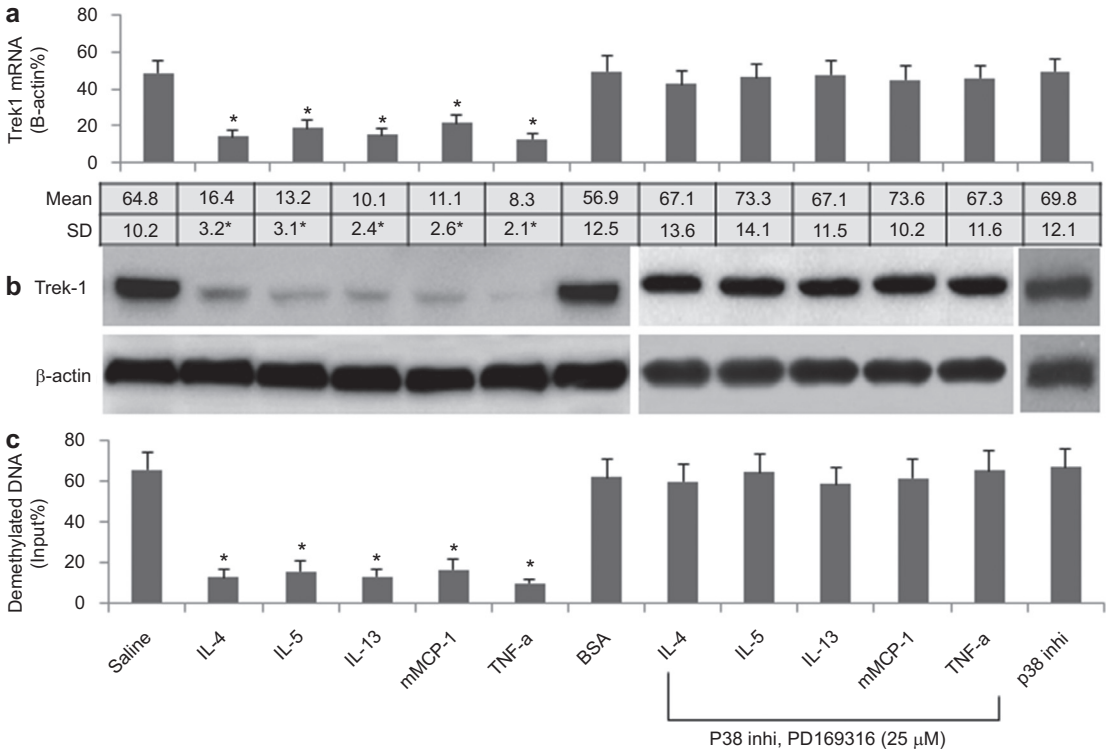

Figure 3 Allergic mediators suppress Trek1 expression in T84 cells. T84 cells were exposed to agents as denoted on the *X* axis of C (p38 inhi=p38 inhibitor). The cells were collected 72 h later and analyzed by qRT-PCR, western blotting and methylation-specific PCR. (**a**) The bars indicate the mRNA levels of Trek1. (**b**) The immune blots indicate the protein levels of Trek1. The table above the blots shows the integrated density of the immune blots. (**c**) The bars indicate the unmethylated Trek1 gene DNA levels. Concentrations of reagents: IL-4=20 ng/ml, IL-5=30 ng/ml, IL-13=50 ng/ml, mMCP-1=20 ng/ml and TNF-α=10 ng/ml. **P*<0.01, compared with the saline group. The data are presented as mean±s.d., and are representative of three independent experiments. mMCP-1, mouse mast cell protease-1; TNF, tumor necrosis factor; Trek1, TWIK-related potassium channel-1.

the controls. The epithelial barrier functions of the small intestine were also assessed by measuring the *Isc* (Figure 6a), G (Figure 6b) and HRP flux (Figure 6c), which were in agreement with the suppression of the allergic responses depicted in Figure 5.

DISCUSSION

Epithelial barrier integrity is critical for the maintenance of homeostasis in the body. A number of factors can affect epithelial barrier functions, and epithelial barrier deficiency is involved in a broad array of disorders. Thus, finding remedies capable of restoring barrier functions is expected to contribute to the treatment of these disorders. The present study revealed that intestinal epithelial cells expressed Trek1, and that the knockdown of Trek1 resulted in epithelial barrier disruption. The expression of Trek1 by intestinal epithelial cells was inhibited by a number of allergic mediators *via* increasing HDAC1 expression; in turn, HDAC1 expression could be suppressed by sodium butyrate or the administration of *C. butyricum*, which significantly enhanced the therapeutic effect of SIT, as well as restituted the intestinal epithelial barrier functions.

Although Trek1 is a potassium channel, it is also involved in many other cell activities. Roan *et al.*[9] reported that TREK-1 regulates the stretch-induced detachment of alveolar epithelial cells. La and Gebhart[21] demonstrated that the dorsal root ganglion neurons of the mouse colon express mechanosensitive Trek1 channels. The authors suggested that a decrease in the activities of Trek1 contributed to the enhancement of colon mechanosensitivity, which in turn contributed to intestinal inflammation. The present data revealed that several allergic mediators individually induce epithelial barrier disruption *via* suppressing the expression of Trek1. The results imply that Trek1 may serve as a potential checkpoint for epithelial barrier disruption. This inference is in line with a recent report in which Bittner *et al.*[12] demonstrate that Trek1 is critical for brain–blood barrier function. Our experimental data also support this inference, because knocking down the Trek1 gene significantly compromise depithelial barrier function.

A great deal of effort has been spent on restituting epithelial barrier functions. Some promising results on this topic have been reported. Fischer *et al.*[22] reported that the anti-TNF-α antibody adalimumab blocked TNF-α-induced T84 and Caco-2 monolayer barrier disruption by preventing the increased phosphorylation of the myosin light chain and the activation of p38 MAPK and NF-κB. Colgan *et al.*[23] found that exposure of epithelial monolayers to IL-4 attenuated epithelial

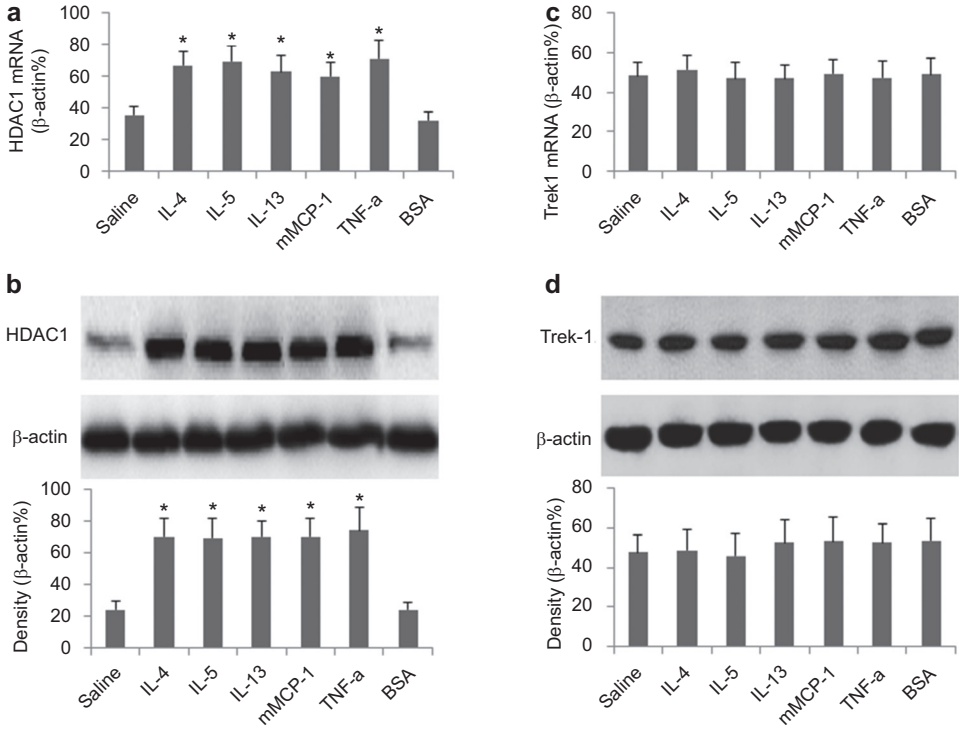

Figure 4 Increases in HDAC1 are associated with allergic mediator-suppressed Trek1 expression in T84 cells. T84 cells were treated with allergic mediators in the same procedures described in Figure 3. (**a** and **b**) The data show the levels of HDAC1 expression. (**c** and **d**) In the presence of HDAC inhibitor Trichostatin A (1 μM), the data show the effect of the allergic mediators on the levels of Trek1. The data are presented as mean ± s.d. *$P<0.01$, compared with saline group. The data are representative of three independent experiments. Trek1, TWIK-related potassium channel-1.

barrier functions in a manner that was inhibited by neutralizing anti-IL-4 and anti-IL-4R antibodies. However, in allergic diseases, a number of pro-inflammatory cytokines are increased that have the potential to affect epithelial barrier function. Thus, it is necessary to find a common checkpoint in epithelial barrier disruption. The present data indicate that Trek1 is a common target of the allergic mediators because the disruption of the intestinal epithelial barrier was associated with the suppression of Trek1 in these cells.

HDAC is involved in the upregulation or downregulation of gene expression. Jeong *et al.*[24] indicated that HDAC1, 2 and 3 deacetylate MAPK-phosphatase-1, thereby increasing MAPK signaling and innate immune signaling and resulting in inflammatory responses; thus, these factors may be potential therapeutic targets for inflammatory diseases. Our results showing that, upon exposure to allergic mediators, the levels of HDAC1 increased are in line with the reports of Jeong *et al.* Based on the notion that butyrate is an HDAC inhibitor and the fact that *C. butyricum* produces butyrate, we treated allergic mice with *C. butyricum* together with SIT. This treatment restored the epithelial barrier function and facilitated the therapeutic effect of SIT on allergic responses in the intestine. The beneficial effect of probiotics has been recognized for use as a therapeutic supplement in a number of diseases;[25,26] the present data are in agreement with those from previous reports. It is noteworthy that using probiotics alone induced significant improvement in the barrier function and attenuated the allergic responses. Other studies have also reported similar observations.[26] However, although treating allergic mice with SIT improved the epithelial barrier functions and the allergic responses, they were not satisfactorily inhibited; this is in agreement with the studies by Dr Berin's group.[20] One of the novel findings of this study is that the combination of SIT and *C. butyricum* (or sodium butyrate) significantly restituted the epithelial barrier functions and suppressed the allergic responses in the intestine.

However, Roan *et al.*[9] suggested that Trek1 may enforce stretch-induced injuries of airway epithelial cells because they observed that Trek1-deficient cells contained less F-actin, thereby making them more resistant to stretch-induced injury. Thus, Roan's study revealed a disagreeable aspect of Trek1 on epithelial layers. Our results show that Trek1 is involved in maintaining the barrier function that is beneficial to the epithelium. Thus, the present data have revealed another aspect of Trek1 on epithelial cells. Additionally, it is possible that a causative connection exists between HDAC1 and TREK1, although the characterization of this possible connection needs to be further investigated.

The present data show that inhibiting HDAC1 with sodium butyrate or *C. butyricum* decreased the markers for intestinal allergic responses, and that this decrease required 'functional' TREK-1. Moreover, we showed that spadin alone induced T84

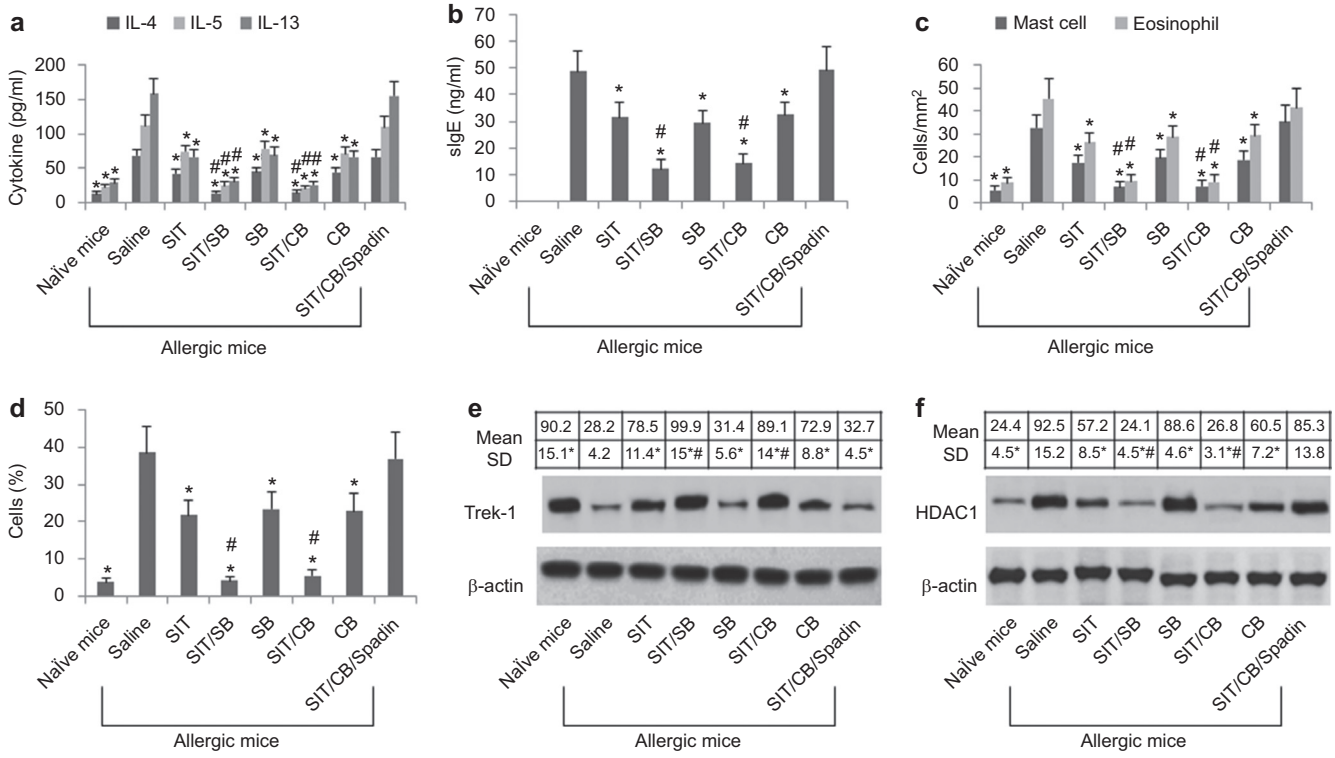

Figure 5 Recovery of Trek1 facilitates the inhibitory effect of SIT on allergic responses. The treatment of allergic mice is denoted on the *X* axis. The bars indicate the serum Th2 cytokines (**a**), serum-specific IgE (**b**), frequency of mast cells and eosinophils in the intestinal mucosa (**c**) and the frequency of $CD4^+$ T-cell proliferation after exposure to a specific antigen in the culture (**d**). (**e** and **f**) The epithelial specimens were collected from the mouse small intestine by scraping. The protein extracts were analyzed by western blot. The immune blots indicate the protein levels of Trek1 (**e**) and HDAC1 (**f**). SB, or CB or spadin was given i.p. prior to the exposure to specific antigens. Each group consists of six mice. Samples from individual mice were processed separately. The data are presented as mean±s.d. $^*P<0.01$, compared with the saline group (**a**–**d**) or naive mice (**e** and **f**). $^{\#}P<0.05$, compared with the SIT group. The data are representative of six independent experiments. CB, *C. Butyricum* (0.3 ml of 10^9 CB/ml); SB, sodium butyrate (0.5 ml of a 50 mM sodium butyrate in PBS); PBS, phosphate-buffered saline; SIT, specific immunotherapy; Spadin, Trek1 inhibitor (0.1 ml of 10 μM); Trek1, TWIK-related potassium channel-1.

monolayer barrier dysfunction. These results suggest that spadin may potentially initiate disease processes in the intestine, although this possibility requires further study.

According to the data presented in Figure 1b and c, spadin had no effect on currents when the membrane potential was in the −70 to −10 mV range, implying that there may be no resting activity of TREK-1 in cells that are at rest. Consequently, the deleterious effect of spadin on epithelial barrier function (Figure 1f) may not be due to blocking TREK-1 background currents unless the channels are activated

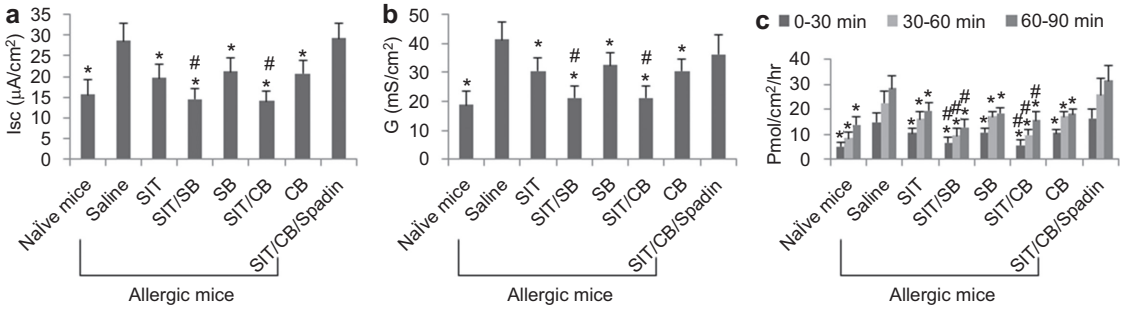

Figure 6 Recovery of Trek1 restores intestinal epithelial barrier function. Mice were treated with the same procedures as those described in Figure 5. The small intestine segments were excised at the time of sacrifice and assessed in Ussing chambers. The bars indicate the levels of Isc (**a**), G (**b**) and HRP flux (**c**). The data are presented as mean±s.d. $^*P<0.01$, compared with the saline group. $^{\#}P<0.05$, compared with the SIT group. The data are representative of six independent experiments. HRP, horseradish peroxidase; SIT, specific immunotherapy; Trek1, TWIK-related potassium channel-1.

by other types of stimulation (e.g., by mechanical force) when the cells are at rest in their Transwell cultures.

In summary, the present study revealed that mouse intestinal epithelial cells express Trek1, and that the inhibition of Trek1 induced epithelial barrier disruption. Exposure to allergic mediators increased the levels of HDAC1 leading to the suppression of Trek1 in intestinal epithelial cells. Administration of sodium butyrate or *C. butyricum* and SIT inhibited the levels of HDAC1 and upregulated the expression of Trek1 in intestinal epithelial cells, resulting in the restitution of barrier functions and the inhibition of intestinal allergic responses.

AUTHORS' CONTRIBUTION

HH, JQL, YY, LHM, RTG and HPZ performed experiments, analyzed data and revised the manuscript. PCY, PYZ and ZGL organized and supervised the experiments. PCY designed the project and wrote the paper.

CONFLICT OF INTEREST

The authors have no conflicts of interest regarding this paper.

ACKNOWLEDGEMENTS

This study was supported by grants from the 973 Program (#2011CB512006) of China, The Natural Science Foundation of SZU (No. 000004), the Innovation of Science and Technology Commission of Shenzhen Municipality (JCYJ20120613172559904) and The Natural Science Foundation of China (Nos. 30871752 and 81373176).

Supplementary Information accompanies the paper on *Cellular & Molecular Immunology*'s website. (http://www.nature.com/cmi).

1 Groschwitz KR, Hogan SP. Intestinal barrier function: molecular regulation and disease pathogenesis. *J Allergy Clin Immunol* 2009; **124**: 3–20.
2 Fries W, Belvedere A, Vetrano S. Sealing the broken barrier in IBD: intestinal permeability, epithelial cells and junctions. *Curr Drug Targets* 2013; **14**: 1460–1470.
3 Swindle EJ, Collins JE, Davies DE. Breakdown in epithelial barrier function in patients with asthma: identification of novel therapeutic approaches. *J Allergy Clin Immunol* 2009; **124**: 23–34.
4 Leung DY. New insights into atopic dermatitis: role of skin barrier and immune dysregulation. *Allergol Int* 2013; **62**: 151–161.
5 Saatian B, Rezaee F, Desando S, Emo J, Chapman T, Knowlden S *et al*. Interleukin-4 and interleukin-13 cause barrier dysfunction in human airway epithelial cells. *Tissue Barriers* 2013; **1**: e24333.
6 Wang WS, Liang HY, Cai YJ, Yang H. DMOG ameliorates IFN-gamma-induced intestinal barrier dysfunction by suppressing PHD2-dependent HIF-1alpha degradation. *J Interferon Cytokine Res* 2014; **34**: 60–69.
7 Al-Sadi R, Guo S, Ye D, Ma TY. TNF-alpha modulation of intestinal epithelial tight junction barrier is regulated by ERK1/2 activation of Elk-1. *Am J Pathol* 2013; **183**: 1871–1884.
8 Weber CR. Dynamic properties of the tight junction barrier. *Ann NY Acad Sci* 2012; **1257**: 77–84.
9 Roan E, Waters CM, Teng B, Ghosh M, Schwingshackl A. The 2-pore domain potassium channel TREK-1 regulates stretch-induced detachment of alveolar epithelial cells. *PLoS ONE* 2014; **9**: e89429.
10 Hund TJ, Snyder JS, Wu X, Glynn P, Koval OM, Onal B *et al*. Beta IV-Spectrin regulates TREK-1 membrane targeting in the heart. *Cardiovasc Res* 2014; **102**: 166–175.
11 Gil V, Gallego D, Moha Ou Maati H, Peyronnet R, Martínez-Cutillas M, Heurteaux C *et al*. Relative contribution of SKCa and TREK1 channels in purinergic and nitrergic neuromuscular transmission in the rat colon. *Am J Physiol Gastrointest Liver Physiol* 2012; **303**: G412–G423.
12 Bittner S, Ruck T, Schuhmann MK, Herrmann AM, Moha ou Maati H, Bobak N *et al*. Endothelial TWIK-related potassium channel-1 (TREK1) regulates immune-cell trafficking into the CNS. *Nat Med* 2013; **19**: 1161–1165.
13 Yang PC, Jury J, Söerholm JD, Sherman PM, McKay DM, Perdue MH. Chronic psychological stress in rats induces intestinal sensitization to luminal antigens. *Am J Pathol* 2006; **168**: 104–114.
14 Zhang HP, Wu Y, Liu J, Jiang J, Geng XR, Yang G *et al*. TSP1-producing B cells show immune regulatory property and suppress allergy-related mucosal inflammation. *Sci Rep* 2013; **3**: 3345.
15 Herman JG, Graff JR, Myöhänen S, Nelkin BD, Baylin SB. Methylation-specific PCR: a novel PCR assay for methylation status of CpG islands. *Proc Natl Acad Sci USA* 1996; **93**: 9821–9826.
16 Lolicato M, Bucchi A, Arrigoni C, Zucca S, Nardini M, Schroeder I *et al*. Cyclic dinucleotides bind the C-linker of HCN4 to control channel cAMP responsiveness. *Nat Chem Biol* 2014; **10**: 457–462.
17 Mazella J, Pérault O, Lucas G, Deval E, Béraud-Dufour S, Gandin C *et al*. Spadin, a sortilin-derived peptide, targeting rodent TREK-1 channels: a new concept in the antidepressant drug design. *PLoS Biol* 2010; **8**: e1000355.
18 Yu LC. Intestinal epithelial barrier dysfunction in food hypersensitivity. *J Allergy (Cairo)* 2012; **2012**: 596081.
19 Bordin M, D'Atri F, Guillemot L, Citi S. Histone deacetylase inhibitors up-regulate the expression of tight junction proteins. *Mol Cancer Res* 2004; **2**: 692–701.
20 Leonard SA, Martos G, Wang W, Nowak-Węgrzyn A, Berin MC. Oral immunotherapy induces local protective mechanisms in the gastrointestinal mucosa. *J Allergy Clin Immunol* 2012; **129**: 1579–1587.
21 La JH, Gebhart GF. Colitis decreases mechanosensitive K2P channel expression and function in mouse colon sensory neurons. *Am J Physiol Gastrointest Liver Physiol* 2011; **301**: G165–G174.
22 Fischer A, Gluth M, Pape UF, Wiedenmann B, Theuring F, Baumgart DC *et al*. Adalimumab prevents barrier dysfunction and antagonizes distinct effects of TNF-alpha on tight junction proteins and signaling pathways in intestinal epithelial cells. *Am J Physiol Gastrointest Liver Physiol* 2013; **304**: G970–G979.
23 Colgan SP, Resnick MB, Parkos CA, Delp-Archer C, McGuirk D, Bacarra AE *et al*. IL-4 directly modulates function of a model human intestinal epithelium. *J Immunol* 1994; **153**: 2122–2129.
24 Jeong Y, Du R, Zhu X, Yin S, Wang J, Cui H *et al*. Histone deacetylase isoforms regulate innate immune responses by deacetylating mitogen-activated protein kinase phosphatase-1. *J Leukoc Biol* 2014; **95**: 651–659.
25 Kim HJ, Kim HY, Lee SY, Seo JH, Lee E, Hong SJ. Clinical efficacy and mechanism of probiotics in allergic diseases. *Korean J Pediatr* 2013; **56**: 369–376.
26 Nermes M, Salminen S, Isolauri E. Is there a role for probiotics in the prevention or treatment of food allergy? *Curr Allergy Asthma Rep* 2013; **13**: 622–630.