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Probiotics promote endocytic allergen degradation in gut epithelial cells

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ABSTRACT

Background and aims: Epithelial barrier dysfunction plays a critical role in the pathogenesis of allergic diseases; the mechanism is to be further understood. The ubiquitin E3 ligase A20 (A20) plays a role in the endocytic protein degradation in the cells. This study aims to elucidate the role of A20 in the maintenance of gut epithelial barrier function.
Methods: Gut epithelial cell line, HT-29 cell, was cultured into monolayers to evaluate the barrier function in transwells. RNA interference was employed to knock down the A20 gene in HT-29 cells to test the role of A20 in the maintenance of epithelial barrier function. Probiotic derived proteins were extracted from the culture supernatants using to enhance the expression of A20 in HT-29 cells.
Results: The results showed that the knockdown of A20 compromised the epithelial barrier function in HT-29 monolayers, mainly increased the intracellular permeability. The fusion of endosome/lysosome was disturbed in the A20-deficient HT-29 cells. Allergens collected from the transwell basal chambers of A20-deficient HT-29 monolayers still conserved functional antigenicity. Treating with probiotic derived proteins increased the expression of A20 in HT-29 cells and promote the barrier function.
Conclusion: A20 plays an important role in the maintenance of epithelial barrier function as shown by HT-29 monolayer. Probiotic derived protein increases the expression of A20 and promote the HT-29 monolayer barrier function.

1. Introduction

Epithelial cells form a barrier with the tight junction; the barrier covers on the surface of gastrointestinal lumen, airway and urogenital tract. One of the functions of the epithelial barrier is to restrict proteins with antigenicity to be absorbed into the deep tissue to avoid inducing skewed immune responses in the body [4]. However, some proteins with antigenicity are actually absorbed into the subepithelial region to contact immune cells and induce skewed immune responses [9,18]. The procedures by which epithelial cells handle the endocytic allergens are not fully elucidated yet.

The endocytic proteins are wrapped by the plasma membrane in the cell to form small vesicles; the vesicles can fuse each other to form endosomes [2]. The allergen-carrying endosomes can then fuse with the lysosomes. The carried allergens are to be degraded by the acid hydrolase enzymes in the lysosomes [14]. Thus, the fusion of endosome/lysosome is a critical step in the endocytic allergen degradation in the cell.

It is proposed that the ubiquitination plays a role in the fusion of endosome/lysosome [11]. The ubiquitin E3 ligase A20 (A20, in short) is one of the ligase proteins in the polyubiquitinations that marks proteins for degradation by the proteasome [15]. Recent reports indicate that A20 plays a critical role in the maintenance of the homeostasis in the body [6,10]. Whether A20 is associated with the processing of endocytic allergens in epithelial cells has not been investigated. In this study, we observed the effect of A20 on the endocytic allergen degradation in the gut epithelial cell line, HT-29 cells. The results indicate that A20 plays an important role in the processing the endocytic allergen in HT-29 cells.

2. Materials and methods

2.1. Reagents

The peanut allergen, Ara h II (Ara, in short) was a gift from Dr. Zhigang Liu (Shenzhen University, China). Anti-Ara antibody was obtained from AbBioTec (Guangzhou, China). The probiotics, Clostridium butyridium CGMCC0313-1, was a gift from Dr. Xun He (Shandong Kexing Bioproducts Co. Ltd., China). The antibodies of A20, EEA1, LAMP2 were purchased from Santa Cruz Biotech (Shanghai, China). The real time RT-PCR reagents were purchased from Invitrogen (Shanghai, China).

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2.2. Cell culture

Human colon epithelial cell line, HT-29 cell (passages 35–45), was purchased from ATCC (Mannassas, VA) and cultured in McCoy's media with 2.2 g/l sodium bicarbonate; supplemented with 10% fetal bovine serum (FBS) and antibiotics (50 U/ml penicillin and 50 μg/ml streptomycin). Cells were seeded at a density of 5×10^5 cells/well on 12 well Costar Transwell™ permeable inserts (0.4 μm pore polycarbonate membrane).

2.3. Transepithelial resistance (TER)

The HT-29 monolayer integrity was assessed by recording TER at the beginning and the end of the experiment using the Millicell ERS apparatus (Millipore, Bedford, Massachusetts, USA). TER measurements were expressed as ohm/cm^2.

2.4. Allergen permeability study

Permeability studies were performed using confluent HT-29 monolayers (TER $\geqslant$ 650 ohm/cm^2) 14 days after seeding. The allergen, Ara, was added to the apical chambers at 10 μg/ml. Samples were taken from the basal chambers 24 h later. The levels of Ara in the samples were determined by enzyme-linked immunoassay (ELISA).

2.5. ELISA

Samples (or diluted standard proteins) were added to 96-well plates at 20 μg/ml and incubated at 4 °C overnight. The plates were blocked by 5% skim milk for 1 h; the first antibodies (20–40 ng/ml; 0.1 ml) were added and incubated at room temperature for 1 h, and followed by adding the HRP-labeled secondary antibodies (5–10 ng/ml; 0.1 ml) and incubated for 1 h at room temperature. 0.1 ml 3,3′,5,5′-Tetramethylbenzidine was added and incubated for 15 min; the reaction was stopped by addition 25 μl H_2SO_4. The plate was read with a microplate reader (BioTek; Shanghai, China).

2.6. Concentrating proteins from culture supernatants

The probiotic proteins in culture supernatant and the transported Ara through HT-29 cell monolayers in the culture supernatant at the transwell basal chamber were concentrated by the ammonium sulfate precipitation. The culture supernatants were collected from the basal chambers to concentrate the proteins by saturated ammonium sulfate precipitation following the standard operating procedures in our laboratory that was also published elsewhere [5].

2.7. Probiotics culture

The probiotics were cultured anaerobically in de Man–Rogosa–Sharpe (MRS) broth (Difco Laboratories, Shanghai, China) at 37 °C.

2.8. Immunocytochemistry

HT-29 cells were collected from transwell inserts with trypsin–EDTA; the cells were fixed with 2% freshly prepared paraformaldehyde for 2 h. Following the standard operating procedure in our laboratory [3] with modifications, the cells were stained with antibodies (1 μg/ml) against A20, Ara, EEA1 (an endosome marker) and LAMP2 (a lysosome marker) in an Eppendorf tube for 1 h; then, fluorescence labeled second antibodies (1 μg/ml) was added and incubated for 1 h. Washing with PBS for three times was performed after incubation with antibodies. The cells were smeared onto a slide, mounted with a cover slip with anti-fade media. The slides were observed under a confocal microscope with the ×630 objective. The positive staining part was further enlarged with the built-in "enlarge feature" of the microscope to observe the fine structure of the cells and photographed when the image was appropriate.

2.9. Image analysis

In each image, the positive staining particles of Ara, A20, EEA1 and LAMP2 in each cell were counted. The merged colors were sorted respectively and counted. Thirty cells were analyzed for each group. The slides and photographs were coded. The observer was not aware of the code to avoid the observer bias.

2.10. RNA interference

To observe the effect of A20 in maintaining the barrier function, some HT-29 cells were transduced with shRNA of A20 (or control shRNA using as controls) to knock down the A20 gene following the manufacturer's instruction.

2.11. Western blotting

The effect of RNA interference on silencing the A20 gene in HT-29 cells was demonstrated by Western blotting. The cellular extracts were fractioned in SDS–PAGE and transferred onto nitrocellulose membrane. The membrane was blocked with 5% skim milk, incubated with anti-A20 antibody (100 ng/ml) and followed by the HRP-labeled secondary antibody (50 ng/ml). The immune complexes were revealed with the enhanced ECL kit. The results were recorded with X-ray film.

2.12. Real time RT-PCR (qRT-PCR)

The expression of the A20 gene in HT-29 cells was assessed by qRT-PCR. Total RNA was extracted from HT-29 cells with the Trizol reagents and was converted to cDNA with the reverse transcriptase with the primer of A20 (forward: gagagcacaatggctgaaca; reverse: tccagtgtgtatcggtgcat; NCBI: NM_006290.2). The qPCR was performed with SYBR Green Master Mix (Qiagen, Shanghai, China) in a Bio-Rad thermocycler (Bio-Rad Biotech, Shanghai, China). The results were expressed as percentage of the housekeeping gene β-actin).

2.13. Assessment of antigen-specific T cell activation

Eight patients (4 males, 4 females, age: 33–45 years old) with Ara allergy history, serum Ara-specific IgE was greater than 30 ng/ml, were recruited to this study. The using human tissue in the study was approved by the Human Study Ethic Committee at Zhengzhou University. Forty milliliter blood was obtained from each patient. The peripheral blood mononuclear cells were isolated by the gradient density centrifugation; $CD3^+$, $CD4^+$, $CD25^-$ T cells and dendritic cells (DC) were purified with purchased reagent kits. The purity of the cells was greater than 98% as checked by flow cytometry. The T cells were labeled with CFSE (carboxyfluorescein succinimidyl ester) and cultured with DC (T cell:DC = 10:1; 10^5/ml) in the presence of the specific allergen, Ara (10 μg/ml), or BSA (control) for 3 days. The CFSE-dilution was assessed by flow cytometry.

2.14. Cellular protein extraction

HT-29 cells were harvested from the inserts by 0.05% trypsin–EDTA. Total proteins were extracted from the cells. Briefly, 0.2 ml cell lysis buffer [20 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium

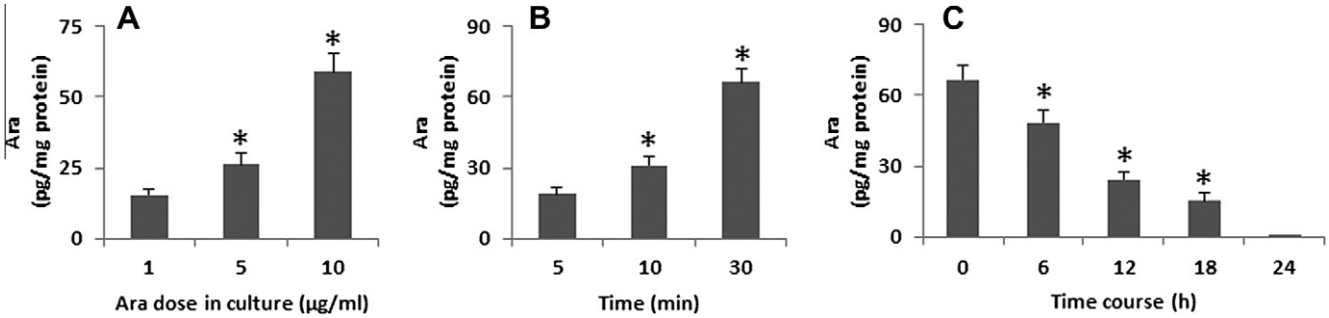

Fig. 1. Gut epithelial cells endocytose allergens. HT-29 cells were cultured into monolayers in transwells. Peanut allergen, Ara, was added to the apical chambers. The monolayers were harvested; the cell extracts were prepared to analyse the contents of Ara by ELISA. (A) and (B) The bars indicate the levels of Ara in the cell extracts. (C) After exposing to Ara in culture for 30 min, HT-29 cells were washed and continued culturing in fresh medium. The cells were collected at the indicated time points; the Ara residue in the cell extracts was assessed by ELISA. The bars indicate the levels of Ara. The data were expressed as mean ± SD. *$p < 0.01$, compared with group "1 μg/" (A), or group "5 min" (B), or the time point "0" (C). The data represent three separate experiments.

pyrophosphate, 1 mM beta-glycerophosphate, 1 mM Na_3VO_4, 10 mM NaF, 1 mM PMSF, 1 μg/ml leupeptin, and 0.3 μM aprotinin, 130 μM bestatin, 14 μM E-64] was added to 10^7 cells in an Eppendorf's tube. Placing the tube on ice, the cells were passed through an 18 gauge needle for 10 times; the tube remained on ice for 30 min. The tube was centrifuged (×14,000*g*) for 20 min at 4 °C. The supernatants were collected; the concentration of protein was determined by Bio-Rad protein assay.

2.15. Statistics

Data are expressed as means ± SD. Statistical analysis was performed by using Student's *t*-test for data between two groups and ANOVA for three and more groups. $p < 0.05$ was considered significant.

3. Results

3.1. HT-29 cells endocytose peanut allergen

Human colon epithelial cell line, HT-29 cells, was used in the present study. Since colon epithelial cells are not the professional absorbing cells, we firstly tested if HT-29 cells could absorb the major peanut allergen, Ara h II (Ara). HT-29 cells were cultured into a monolayer in the inserts of transwells to confluence. Ara was added to the apical chambers at graded concentrations. The cells were harvested 30 min after the addition of Ara. In separate experiments, HT-29 cells were exposed to Ara at 10 μg/ml; the cells were harvested at several time points from 1 to 30 min. The cellular extracts were prepared with the harvested HT-29 cells. As shown by ELISA, Ara was detected in the cellular extracts of HT-29 cells in an Ara dose- (Fig. 1A) and exposure time (Fig. 1B)-dependent manner. The data indicate that HT-29 cells can endocytose Ara.

3.2. A20. is required in the degradation of peanut allergen in HT-29 monolayers

It is accepted that intestinal epithelial cells can degrade the endocytic cargo. We next observed the time course that gut epithelial cells degraded the endocytic peanut allergens. We exposed HT-29 monolayers to Ara in culture for 30 min; the non-absorbed Ara was washed out with fresh medium. The cells were cultured with fresh medium. The cells were collected at time points of 0, 6, 12, 18 and 24 h respectively. The cellular extracts of HT-29 cells were prepared and analyzed by ELISA to determine the levels of residue Ara. The results showed that the contents of Ara in the extracts were gradually reduced; that were below the detectable levels in the samples collected at the 24 h time point (Fig. 1). The results indicate that the endocytic Ara is either degraded in the polarized HT-29 cells, or converted to the basal compartment of transwell. To this end, we assessed the levels of Ara in the medium taken from the basal chambers of transwell. The results showed that low levels of Ara were converted to the basal chambers by medium treated HT-29 monolayers within 24 h. Thus, at this time point, the data have not clarified whether the reduction of Ara contents in HT-29 cells is resulted from the degradation within the cells.

It is proposed that the ubiquitination plays a role in the endocytic cargo degradation [1]. Thus, we knocked down the ubiquitin E3 ligase A20 gene from HT-29 cells (Fig. 2A); these cells were cultured into monolayers to confluence (the TER in A20-deficient HT-29 monolayers was similar to the A20-sufficient HT-29 monolayers; data not shown). The Ara flux experiments were performed with the A20-deficient HT-29 monolayers. Abundant amounts of Ara were detected in the basal chambers in the course of 24 h (Fig. 2B). To elucidate whether the Ara was transported across the HT-29 monolayers via the paracellular pathway, we recorded the TER. The results showed that TER was not decreased in the A20-deficient HT-29 monolayers; instead, it was increased about 10% above the baseline after the addition of Ara in the apical chamber for 24 h. There were no significant differences between the A20-sufficient group and A20-deficient group (Fig. 3C). The results indicate that the knockdown of A20 in HT-29 does not affect the paracellular permeability; the increased Ara flux to the basal chambers was via the intracellular pathway.

3.3. Probiotics increase the expression of A20 in HT-29 cells to strengthen the barrier function

Administration with probiotics can ameliorate the clinical symptoms of mucosal inflammation, such as food allergy [16] and allergic dermatitis [20]. The epithelial barrier dysfunction, such as the hyperpermeability, is involved in the pathogenesis of these disorders [7,21]. We wondered if the promotion of endocytic allergen degradation in epithelial cells was one of the mechanisms by which probiotics ameliorate mucosal immune inflammation. To this end, we added the extracted proteins from the probiotic culture supernatants (PCS) to the HT-29 monolayer culture; the Ara flux was performed subsequently. The results showed that a small amount of Ara still passed the naïve HT-29 monolayer to the basal chambers, which was below the detectable levels in the HT-29 monolayers pretreated with PCS. Pretreatment with control PCS (the cPCS) did not show any effect on strengthening the barrier function (Fig. 3A). Since the knockdown of A20 in HT-29 cells resulted in the dysfunction of the barrier function, we next tested

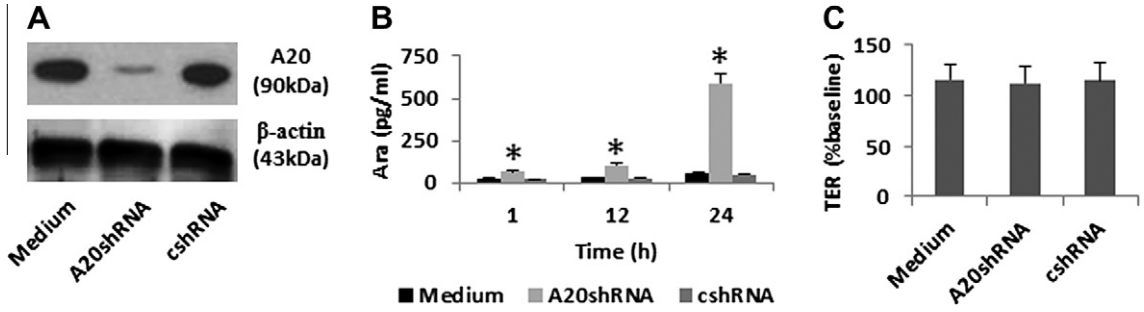

Fig. 2. A20-deficient HT-29 monolayers increase the transport of endocytic allergens to transwell basal chambers. (A) The immune blots show the A20 gene knockdown results. (B) The bars indicate the levels of Ara in the supernatants of basal chambers of transwells. (C) The bars indicate the TER recorded from HT-29 monolayers at 24 h time point after the addition of Ara to the culture medium of apical chambers. Medium: HT-29 cells were treated with medium using as controls. A20shRNA: HT-29 cells were transduced with A20 shRNA. cshRNA: HT-29 cells were transduced with control shRNA. The data in (B) and (C) were presented as mean ± SD. $^{*}p < 0.01$, compared with the medium group. The data represent three separate experiments.

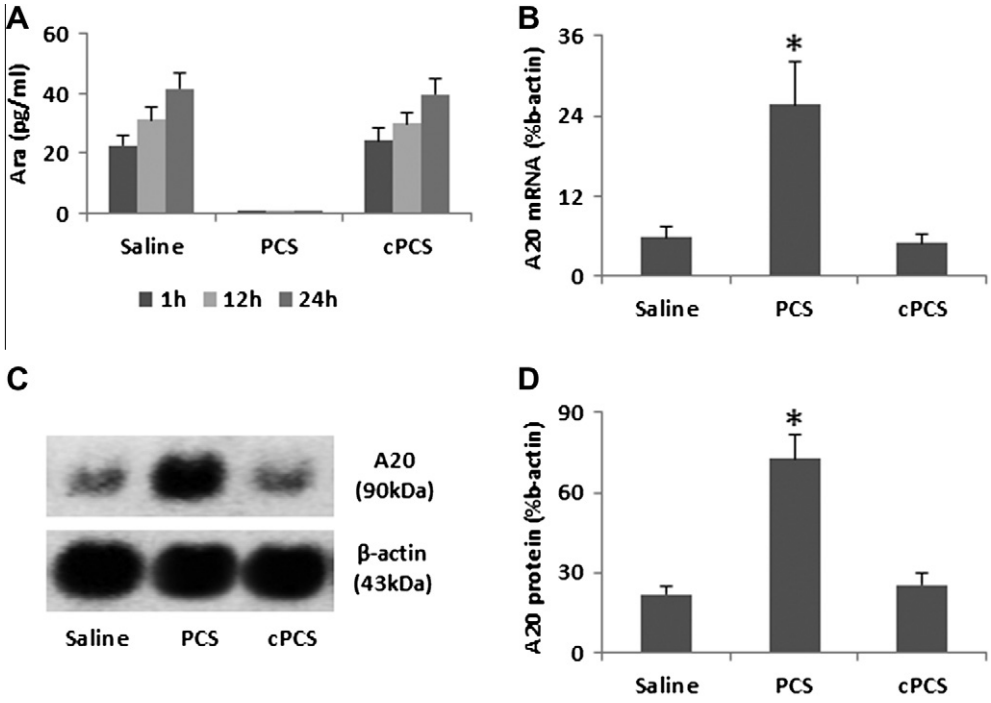

Fig. 3. Probiotics increase A20 expression to suppress allergen flux across the HT-29 monolayer. (A) The bars indicate the levels of Ara flux in the supernatant of the transwell basal chamber. (B) The bars indicate the A20 mRNA levels in HT-29 cellular extracts. (C) The immune blots indicate the levels of A20 protein in HT-29 cellular extracts. (D) The bars indicate the summarized data of the integrated density of the immune blots in (C). The data in bar graphs were expressed as mean ± SD. $^{*}p < 0.01$, compared with the medium group. The data represent three separate experiments. Saline (or PCS, or BSA): cells were cultured in the presence of saline (or PCS, or cPCS). PCS: proteins extracted from probiotics culture supernatants. cPCS: proteins extracted from the same culture medium (broth) without probiotics using as a control.

if the treatment with PCS increased the expression of A20 in HT-29 cells. As shown by Fig. 3B–D), exposure to PCS, but not cPCS, did increase the expression of A20 in HT-29 cells.

3.4. Probiotics promote A20 expression to promote tethering endosome and lysosome in HT-29 cells

By immunocytochemistry, we observed Ara-carrying endosomes in HT-29 cells; the Ara-carrying endosomes were fused with lysosomes. After exposure to PCS, the expression of A20 increased markedly and the amounts of endosome/lysosome fusion were also increased. In addition, in A20-deficient HT-29 cells, the amounts of Ara-carrying endosome were increased; the positive staining of Ara/endosome/lysosome was decreased (Fig. 4; Table 1). The results implicate that PCS promotes the expression of A20 to promote the endosome/lysosome fusion.

3.5. Probiotics facilitate HT-29 cells to quench the antigenicity of endocytic allergens

To clarify if the allergens converted to the basal transwell chambers by HT-29 monolayers still conserved the antigenicity, we collected immune cells from patients with Ara allergy. The cells were cultured for 3 days in the presence of the specific antigen, Ara, or the proteins of culture supernatant (PCS) obtained from the experiments of Fig. 2. As shown by CFSE-dilution assay, the Ara specific $CD4^{+}$ T cells proliferated markedly in response to the exposure to Ara or PCS from A20-deficient group, which was significantly reduced in the cells from the A20-sufficient group; the proliferation

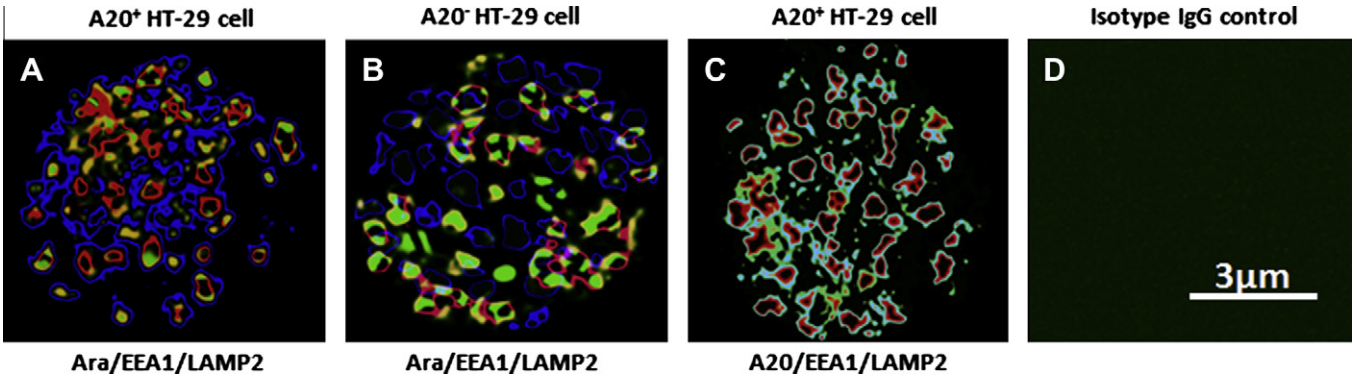

Fig. 4. A20 facilitates endosome/lysosome fusion. A20-sufficient (A and C) or A20-deficient (B) HT-29 cells were exposed to Ara for 30 min and processed for immunocytochemistry. The staining was specified below each confocal image. Each image shows one cell with the staining of endocytic Ara (green), EEA1 (red; an endosome marker), LAMP2 (blue; a lysosome marker) or A20 (green). The yellow color is merged by green and red colors. The light blue color is merged by green, red and blue colors. The original magnification was ×630 that was further enlarged with the built-in software in a confocal microscope. The experiment was repeated for three times. The image analysis data were presented in Table 1. The split color images were presented in supplemental materials (Fig. S2). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1
Image analysis data on immune positively stained particles.

Cells	Analyzed cells	Particle number	$Ara^+/EEA1^+$	$Ara^+/EEA1^+/LAMP2^+$
$A20^+$ cells	30	1088	78.54 (8.2)%	87.69 (9.5)%
$A20^-$ cells	30	1218	91.58 (9.5)%*	3.87 (1.8)%*
			A20/EEA1	A20/EEA1/LAMP2
$A20^+$ cells	30	1138	96.84 (8.6)%	91.45 (9.3)%

Particle: immune positively stained particle in the cells.
* $p < 0.01$, compared with $A20^+$cells.

was abolished in the T cells exposed to the PCS from probiotics-treated group (Fig. S1 in Supplemental materials).

4. Discussion

The present study has revealed that gut epithelial cell line, HT-29 cells, expresses A20; the latter plays a critical role in the degradation of endocytic allergens via a mechanism to facilitate the endosome/lysosome fusion. Knockdown of the A20 gene results in the epithelial barrier dysfunction manifesting a hyperpermeability of the epithelial barrier via an intracellular pathway.

The tight junction disintegrate plays a critical role in the epithelial barrier dysfunction. Our previous work noted that a number of factors could "open" the paracellular space to increase the epithelial barrier permeability; for example, rats treated with chronic psychological stress showed marked hyperpermeability to the macromolecular protein tracer, horseradish peroxidase as well as increase the conductance of the epithelial layer [19]. Microbe-derived factors can significantly down regulate the transepithelial resistance (TER) indicating the tight junction disintegrate is induced, which can be antagonized by the presence of probiotics [22]. In the present data, however, we observed an increase in the hyperpermeability in the A20-deficient HT-29 monolayer, but the TER was not affected, indicating the intracellular permeability is enhanced. This phenomenon was observed previously in allergic animal model studies; significantly more allergen tracers were observed in the intestinal epithelial cells of sensitized rats; more specific allergens were transported across the epithelial layer [17].

The data indicate that A20 facilitates the endocytic allergen degradation in HT-29 cells by a mechanism of promoting the endosome/lysosome fusion. This is in line with previous studies that ubiquitin E3 ligase functions to promote protein degradation [15]. Li et al. reported that A20 was capable of targeting the TRAF2 (TNF receptor-associated factor 2) to promote its degradation in the lysosome [8]. Our results are consistent with this finding by showing that the knockdown of A20 markedly enhanced the intracellular permeability; allergens with antigenicity were converted across the HT-29 monolayers. The underlying mechanism is that A20 is required in the tethering of endosome to lysosome as shown by the present data. Others also indicate the role of A20 in the endosome/lysosome fusion [8].

Although probiotics have been used in ameliorating immune disorders in clinic, the mechanisms are to be further investigated. It is proposed that treating with probiotics results in the increase in the expression of Th1 cytokines such as IFN-γ, and immune suppression cytokines such as IL-10 [12]. The present data provide further information on probiotics' function by showing that treating HT-29 cells with probiotics-derived proteins promotes the expression of A20. The increase in A20 in HT-29 cell contributes to maintain the epithelial barrier function. Knockdown of A20 compromised the barrier function by showing hyperpermeability in HT-29 monolayers. On the other hand, treated with probiotic-derived protein, increased the expression of A20 in HT-29 cells, which resulted in much less antigen with antigenicity to be converted to the transwell basal chambers. Others also noted that administration with probiotics improved the epithelial barrier function by enhancing the expression of tight junction associated protein claudin 3 [13].

In summary, the present data indicate that A20 plays an important role in the maintenance of the barrier function in gut epithelial cells, the HT-29 cells. Administration of probiotic derived proteins increases the expression of A20 in HT-29 cells that further improve the epithelial barrier function.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.08.051.

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