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Establishment of immortalized mouse intestinal epithelial cells line and study of effects of Arg-Arg on inflammatory response

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Abstract Primary mouse intestinal epithelial cells (MIEs) are not ideal models for long-term culture in vitro and a limited amount of approximate three generations. In addition, the mechanism that arginine-arginine dipeptide (Arg-Arg) regulates mouse intestinal inflammatory response remains unknown. Therefore, the aim of this study was to establish immortal MIEs and study the effects of Arg-Arg on inflammatory response after challenging the MIEs with lipopolysaccharide (LPS) or staphylococcal enterotoxin C (rSEC). Our data showed that immortalized MIEs could be cultured over 100 generations. The immortalized MIEs showed positive reaction against cytokeratine 18 antigen, E-cadherin, and peptide transporters (Pept1) using indirect immunofluorescence. Cytokeratine 18 and Pept1 can be expressed in immortalized MIEs by immunoblotting. Fatty acid-binding proteins (FABPs) and villin known as intestinal epithelial cell functional protein were constitutively expressed in immortalized MIEs. For inflammatory response, these results showed that Arg-Arg can decrease the LPS-induced expression of IL-1β and the rSEC-induced expression of TNF-α; however, it can upregulate the LPS-induced expression of IL-6 and TNF-α and the rSEC-induced expression level of IL-1β. In addition, in the MAPK signaling pathway, pSAPK/JNK and p-Erk1/2 in LPS with Arg-Arg treatment were upregulated than that in LPS treatment. p-p38 in LPS with Arg-Arg treatment was attenuated than that in LPS treatment. pSAPK/JNK and p-p38 in rSEC with Arg-Arg treatment were enhanced than that in rSEC treatment. Conversely, p-Erk1/2 in rSEC with Arg-Arg treatment was attenuated than that in rSEC treatment. These novel findings suggest that Arg-Arg dipeptide plays an important role for regulation of the immunologic balance in mouse intestinal inflammatory response.

Keywords Mouse intestinal epithelial cells · Immortalize · Cytokeratine 18 · Villin · Arg-Arg

Introduction

Enterocytes isolated in vitro can be an important tool to investigate absorption and transportation of nutrients of small peptide, intestinal mucosal immunity, and the relationship between enterocytes and enteric microorganisms (Miyazawa et al. 2010). Unfortunately, in vitro studies of how to transport dipeptide in intestinal epithelial cells have been limited by the lack of immortalized intestinal epithelial cell lines. Although immortalized murine intestinal epithelial cell lines have been established, these immortalized murine intestinal epithelial cell lines have not identified some physiological functions of murine intestinal epithelial cells, e.g., uptake or transport of some substances (Macartney et al. 2000; Iwamoto et al. 2011). In addition, the successful establishment of a primary intestinal cell isolation method is hampered in approximately three generations due to upregulation of cyclin-dependent kinase inhibitor (CDKI) levels (Fridman and Tainsky 2008) and shortening of telomeres, resulting in the high rate of cell death when isolating them from the intestinal crypt. Therefore, it is difficult to achieve the amount of intestinal cells to study intestinal cells transporting the nutrient substance in vitro.

Mammalian peptide transporters (PEPT1) in intestinal epithelial cells play a key role in the absorption of dipeptide and tripeptide (Ingersoll et al. 2012). With regard to the transport

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of small peptide nutrient substance in the physiological function system in intestinal epithelial cells, we will focus on the effect of dipeptide on inflammatory response in intestinal epithelial cells. *Escherichia coli* lipopolysaccharide (LPS) gut infections often result in intestinal inflammation; however, symptoms induced by *Staphylococcus aureus* enterotoxin infection can produce a large amount of toxins including staphylococcal enterotoxin C (SEC) which demonstrated emetic activity (Hu and Nakane 2014). In addition, different immune inflammation responses between *E. coli* and *S. aureus* were demonstrated in vivo and vitro (Jensen et al. 2013). Once the gut is in contact with LPS and rSEC, proinflammatory cytokines IL-1β, IL-6, and TNF-α can be excessively activated. It is widely known that arginine is considered to be a functional amino acid (Wu 2013). Apart from the role of protein synthesis, arginine plays an essential role in the immunity regulation. Arginine gives play to its immunologic function by regulating the functional activity, proliferation, and apoptosis of immune cells (Coleman 2001). More importantly, L-arginine stimulates cell proliferation and inhibits endotoxin-induced death of intestinal cells (Tan et al. 2010). Moreover, L-arginine can decrease the expression level of proinflammatory cytokines in broiler chickens which are fed with diet supplementation at 1.42% (Tan et al. 2014). Arg can attenuate the inflammatory response in bovine mammary epithelial cells with LPS induction (Wu et al. 2016a, b). However, Arg-Arg dipeptide regulating mouse intestinal inflammatory response remains not reported.

The cell cytokine inflammatory network is related to inhibition and activation of ERK, JNK, and p38 MAPK in the MAPK signaling pathway. SAPK/JNK, Erk1/2, and p38 MAPK function in protein kinase cascades that play an important role in the control of cellular responses to cytokines and stress (Wu et al. 2016a, b). Although SAPK/JNK and p38 MAPK are mainly responsible for cell inflammatory response, the role of Erk1/2 in regulating inflammatory response remains unknown. Importantly, we found that our recent studies are not consistent with the above research. Arg-Arg dipeptide cannot enhance and attenuate simultaneously the inflammatory expression level in LPS-induced or rSEC-induced mouse intestinal epithelial cells (MIEs). Therefore, the aim of this study is to focus the effects of Arg-Arg on the expression level of inflammatory cytokines and MAPK signaling pathway Erk1/2, SAPK/JNK, and p38 family expression levels in immortalized MIEs induced by LPS and rSEC.

Materials and Methods

Isolation primary mouse intestinal epithelial cells The use of 5-wk-old male c57 mice used in this study complied with the guidelines of the Institutional Animal Care and Use Committee of Yang Zhou University. Mouse duodenum, jejunum, and ileum tissue fragments were provided by the Comparative Medical Center of Yangzhou University. These intestinal fragments were used to obtain primary MIEs. Mice was slaughtered in a biological safety cabinet. These duodenum, jejunum, and ileum fragments were put into 20 mL cold DMEM/F12 (Invitrogen, Shanghai, China) complete medium containing 500 U/mL penicillin, 500 μg/mL streptomycin, and 12.5 μg/mL amphotericin B (Sigma-Aldrich, Shanghai, China). All the following steps were performed in condition of a sterile environment. Briefly, these tissue fragments were washed repeatedly with cold phosphate-buffered saline containing 500 U/mL penicillin, 500 μg/mL streptomycin, and 12.5 μg/mL amphotericin B. These tissue pieces removed the mesentery carefully. Then, these intestinal fragments were incubated with PBS digestive solution containing 100 U/mL collagenase (Invitrogen, Shanghai, China) and 0.05 mg/mL dispase (Sigma-Aldrich) for 30 min at 37°C in a shaking water bath. The digestive solutions were discarded carefully. These remaining intestinal tissues were again incubated with the same digestive solution for 45 min in the same conditions. Then, the digestive solutions were carefully transferred to another sterile 50-mL centrifuge tube, and any tissues were not aspirated. Cells were pelleted by centrifugation at 200×*g* at 4°C for 5 min, and the supernatant was discarded. The cell pellets were resuspended in DMEM/F12 complete medium containing 10% fetal bovine serum, 1% non-essential amino acids, 4 mm/L glutamine, 1× ITS (Invitrogen, Shanghai, China), 5 ng/mL EGF (PeproTech, Shanghai, China), 100 U/mL penicillin, 100 μg/mL streptomycin, and 1 μg/mL hydrocortisone (Sigma-Aldrich) and seeded in a six-well plate. Subsequently, each intestinal segment was longitudinally opened wide and the digested mucosa was scraped with a sterile scalpel blade. The scrapings were incubated with PBS supplemented with 0.05 mg/mL dispase for 15 min at 37°C in a shaking water bath. The digestive solution was filtered through a nylon mesh (37 μm) to remove the intestinal tissues. Cells were pelleted by centrifugation at 140×*g* for 3 min. The cell pellet was resuspended using DMEM/F12 medium containing 2% of sorbitol (Sigma-Aldrich) and centrifuged at 50×*g* for 3 min. The supernatant was then discarded. The pellet wash was repeated for about five times to separate as much single lymphocyte. The final pellets were resuspended in the same complete medium. These cells were seeded in six-well plates at 37°C with 5% CO_2. Complete attachment of newly digested mucosa may require up to 48 h. Therefore, when we examine the cells under a microscope, we must be careful and not shake the plate. When cell aggregates were cultured for 48 h, these cell aggregates are well-attached. At day 7 of culture, intestinal epithelial cells were markedly proliferated accompanied by fibroblast contamination. However, at day 10 of culture, intestinal epithelial cells

appear to show marked cell growth arrest; interestingly, some fibroblast cells have an apparent proliferation. Therefore, we must immortalize the MIEs in the stage.

Retroviral vector constructs and infections The pLXSN vector containing SV40T construction and infection protocol has been described (Zhan et al. 2016b). Following the step, cells infected continue to culture approximately three generations. Then, the cells were diluted to five cells per milliliter using complete DMEM/F12 medium, and 200 μL cell suspension was aliquoted into each well of 96-well plates to obtain the single clone-immortalized MIEs. In this way, immortalized MIE clones were established. The immortalized MIEs were collected in Institute of Animal Culture Collection and Application (IACCA), Yangzhou University.

Cell proliferation assays These cells were cultured in 24-well plates at 0.5×10^4 cells/well to obtain the growth curves. Cells were counted every day in triplicate wells from day 1 to day 7. According to cell number means, the growth curves were depicted.

Immunocytochemistry MIEs were seeded in an eight-well Lab-Tek chamber slide (Nunc, Shanghai, China) at a cell density of 0.5×10^4 cells/cm^2 for 2 d. These cells were washed with cold PBS for three times and then fixed with 4% paraformaldehyde for 30 min at 4°C and washed in PBS for three times. Then, cells were incubated with 3% normal horse serum (Invitrogen) for 1 h at room temperature. Cells were washed with PBS three times and then incubated with anti-mouse cytokeratin 18 mouse antibody, anti-mouse E-cadherin goat antibody, and anti-mouse Pept1 goat antibody (1:250; Santa Cruz Biotechnology, Santa Cruz) for overnight at 4°C. Following washing three times with PBS, cells were incubated with secondary fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (1: 400; Santa Cruz Biotechnology) and Alexa Fluor 594 chicken anti-goat IgG secondary antibody (1: 400; Molecular Probes, Shanghai, China) for 1 h at room temperature and washed gently three times. Cell nuclei were stained with DAPI for 5 min, and slides were washed three times. Finally, the coverslip was mounted on the chamber slide using PermaFluor-mounting medium (Thermo Scientific, Shanghai, China), and the sample was observed using a confocal laser scanning microscope (Olympus, Tokyo, Japan).

Effects of Arg-Arg on inflammatory response The immortalized MIEs were seeded in six-well culture plates at the density of 2×10^5 per well for gene expression analysis and seeded in six-well culture plates at 4×10^5 cells for protein expression analysis. After 18 h incubation, cells were divided into six experimental groups and the medium was added with a different material. The control was treated with normal medium. Arg-Arg treatment was added with medium supplemented with 0.5 mM Arg-Arg. LPS treatment was added in medium supplemented with 1 μg/mL LPS from *E. coli* 055:B5 (Sigma-Aldrich, cat. L6529). LPS with Arg-Arg treatment was added with medium supplemented with both 1 μg/mL LPS and 0.5 mM Arg-Arg. rSEC treatment was added in medium supplemented with 1 μg/mL rSEC (kindly provided by Prof. Hu DL, School of Veterinary Medicine, Kitasato University, Japan). rSEC with Arg-Arg treatment was added with medium supplemented with both 1 μg/mL rSEC and 0.5 mM Arg-Arg.

Cytotoxicity The cytotoxicity of Arg-Arg on MIEs was determined using the Cell Counting Kit-8 (CCK-8; Dojindo, Shanghai, China) according to the manufacturer's protocol. MIEs (1×10^4) were seeded into each of 96-well tissue culture plate. These cells were incubated for 12 h with 10 μL of Arg-Arg (0, 0.5, 2, 4, 8 mM), respectively. After incubation, 10 μL CCK-8 was added in these cells, and the cells were then incubated for 2 h. To investigate cytotoxicity of Arg-Arg on MIEs, absorbance was measured for each well at 450 nm wavelength using an auto-microplate reader (Thermo Scientific).

RNA extraction and analysis After incubation for 12 h, cell total RNA was isolated using TRIzol reagent (Invitrogen). The RT reaction was performed containing 1 μg total RNA, 2.5 μM anchored-oligo $(dT)_{18}$ primer, 60 μM random hexamer, 1× transcriptor reverse transcriptase reaction buffer, 20 U protector RNase inhibitor, 1 mM dNTP, and 10 U transcriptor reverse transcriptase (Roach). The annealing reaction was carried out for 30 min at 55°C, and cDNA synthesis was performed for 10 min at 25°C, followed by incubation for 30 min at 55°C. The transcriptor reverse transcriptase was inactivated by heating to 85°C for 5 min. The RT product was diluted to 20 ng/μL cDNA with free DNase H_2O. The following primers were used for PCR amplification in Table 1. The PCR products were separated on 1% agarose gel containing GoldView and were visualized under UV light.

Western blot analysis MIEs were lysed in a radio immunoprecipitation assay buffer containing a 1× protease inhibitor buffer. Equal amounts of protein lysates were fractionated by SDS-PAGE and transferred to nitrocellulose membranes (PALL, Shanghai, China). The membrane was incubated with the following antibodies: anti-mouse GAPDH rabbit antibody, anti-mouse p-p38 rabbit antibody, anti-mouse Erk1/2 rabbit antibody, anti-mouse p-Erk1/2 rabbit antibody, anti-mouse SNAK/JNK rabbit antibody, anti-mouse p-SAPK/JNK rabbit antibody (1:1000; CST, Shanghai, China), anti-mouse p38 mouse antibody, anti-mouse cytokeratin 18 mouse (1:1000; Abcam, Shanghai, China), and anti-mouse Pept1 goat antibody (1:250; Santa Cruz, Shanghai, China), which are primary antibodies, as well as donkey anti-goat-HRP (1:3000; Santa

Table 1. Primers for RT-PCR analyses

Name (GenBank accession no.)	Sequence	Length (bp)
SV40 Large T	F: GACTCAGGGCATGAAACAGG	
(YP_003708382.1)	R: ACTGAGGGGCCTGAAATGA	61
Fatty acid binding protein 2	F: CACACAGCTGAGATCATGGC	
(NM_007980.3)	R: CACCGAGCTCAAACACAACA	214
Villin	F: CGCATGGTGGTTTATCAGGG	
(NM_009509.2)	R: ATCTCCCTCTCATCCCCACT	224
IL-1β	F: ACCTTCCAGGATGAGGACATGA	
NM_008361.4	R: CTAATGGGAACGTCACACACCA	116
IL-6	F: CACATGTTCTCTGGGAAATCG	
NM_001314054.1	R: TTGTATCTCTGGAAGTTTCAGATTGTT	116
TNF-α	F: ACCCTCACACTCACAAACCA	
NM_001278601.1	R: GGCAGAGAGGAGGTTGACTT	246

F forward, *R* reverse

Cruz Biotechnology), goat anti-mouse-HRP, and goat anti-rabbit-HRP (1:5000; CST, Shanghai, China) which are secondary antibodies. The target bands were detected using SuperSignal West Substrate (Thermo Scientific).

Statistical analysis The statistical analysis was performed by one-way analysis of variance (ANOVA) followed by the least significant difference (LSD) test for post hoc multiple comparisons of treatment means, using SPSS 16.0 software (SPSS Inc., Chicago, IL). p Values were represented on figures as follows: $*p < 0.05$ and $**p < 0.01$. p Values <0.05 were considered significant. Data are shown in column bars representing the mean ± SEM of at least three independent experiments.

Results

Establishment and characterization of immortalized MIEs As shown in Fig. 1, most of them were mixed to grow together. Fibroblast cells show fusiform and elongated morphology (Fig. 1*A*); conversely, intestinal epithelial cells show the morphology of cobblestone and pave stone (Fig. 1*B*). These cells were further cultured approximately for three generations, and uninfected cells will stop to proliferate, and these cells were senescent and dead after five generations. Then, we use a limiting dilution clone method to obtain the immortalized MIE clone. The immortalized MIEs were further analyzed for SV40T expression by RT-PCR (Fig. 1*C*).

Proliferation characterization of immortalized MIEs As shown in Fig. 2, immortalized MIEs still maintain a marked proliferation between the 1st and 6th days in vitro. In addition, immortalized MIE proliferation was highest at 6 d of culture than at other times ($p < 0.05$).

Immunocytochemistry analyses Immunofluorescence staining against cytokeratin 18 was used to distinguish cells of epithelial origin from contaminating elongated or stellate-like cells and muscle cells (Fig. 3*A*). Moreover, we also used immunostaining of another epithelial marker E-cadherin, which plays a key role for cell-cell junctions. E-cadherin can be observed in the cytoplasm of MIEs (Fig. 3*B*). More importantly, we also detect functional marker protein Pept1 of intestinal epithelial cells. All cells were strongly immunopositive for anti-Pept1antibodies, which plays a critical role in the transport and absorption of small peptides from the intestine (Fig. 3*C*). The result indicated that cultured cells are from intestinal epithelial cells.

Gene and protein expression analyses of MIE markers FABP and villin are known as function marker proteins of the intestinal epithelial cell. Immunoblotting and RT-PCR analyses allowed us to detect the expression of MIE markers, namely, FABP, villin, cytokeratin 18, and Pept1. The RT-PCR results demonstrate that FABP and villin can be expressed in isolated cells (Fig. 4*A*). In addition, the western blot results also suggested that cytokeratin 18 and Pept1 can be expressed in isolated cells (Fig. 4*B*, *C*). Taken together, RT-PCR and western blot results strongly suggested that the isolated cells are from mouse intestinal epithelial cells.

The cytotoxicity of Arg-Arg on MIEs As shown in Fig. 5, the result demonstrated that 0.5 mM of Arg-Arg was not toxic, whereas 2, 4, and 8 mM of Arg-Arg was toxic than 0.5 mM ($P < 0.05$). Therefore, 0.5 mM of Arg-Arg was selected for further analysis.

Effects of Arg-Arg on inflammatory cytokine expression level The expression of cytokines for IL-1β, IL-6, and TNF-α were detected by qPCR. In comparison with LPS treatment, Arg-Arg decreased the LPS-induced expression of IL-1β

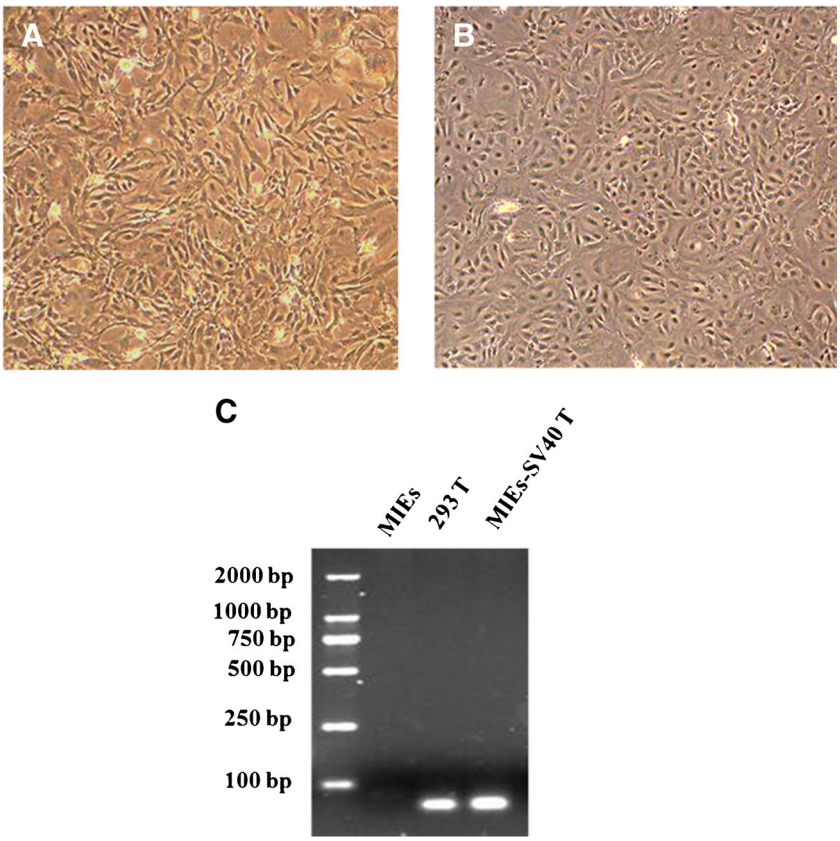

Figure 1. The morphology and characterization of immortalized mouse intestinal epithelial cells (MIEs). A typical picture of primary MIEs and mouse fibroblast cells is shown (*A*: primary mouse fibroblast cells, *B*: primary MIEs). ×40. (*C*) immortalized MIEs were achieved by infection of retroviral containing SV40T genes to bypass the cell senescence. The SV40T mRNA expression was detected by RT-PCR in 293T and immortalized MIEs. MIEs: not infected, as negative control, 293T: infected cells as positive control, MIEs-SV40T: the infected cells used in the present study.

($P < 0.01$; Fig. 6*A*). Interestingly, it enhanced the expression of IL-6 and TNF-α in LPS induction ($P < 0.01$; Fig. 6*B*, *C*). However, the TNF-α expression level was reduced in rSEC induced with Arg-Arg treatment than that in rSEC induction ($P < 0.01$; Fig. 6*C*). The expression of IL-1β was upregulated in rSEC induced with Arg-Arg treatment than that in rSEC induction ($P < 0.01$; Fig. 6*A*).

Effects of Arg-Arg on MAPK signaling pathway As shown in Fig. 7, pSAPK/JNK and p-Erk1/2 in LPS induced with Arg-Arg treatment were enhanced than that in LPS treatment.

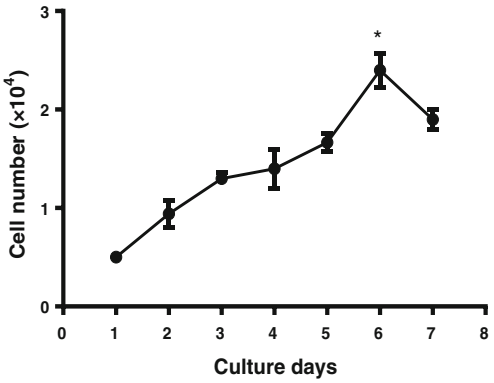

Figure 2. Proliferation characterization of immortalized MIEs. Values shown are the average ± SE of at least three independent experiments (*$p < 0.05$ compared with other culture time).

p-p38 in LPS with Arg-Arg treatment was markedly attenuated than that in LPS treatment. However, p-p38 and pSAPK/JNK in rSEC with Arg-Arg treatment were markedly enhanced than that in rSEC treatment. p-Erk1/2 in rSEC with Arg-Arg treatment was reduced than that in rSEC treatment.

Discussion

In the present study, we have established the immortalized MIEs from the small intestine. Primary MIEs underwent significant senescence after passage 3, and large amounts of cells were subjected to die irreversibly. Therefore, the first aim of the study was to establish immortalized MIEs using a retrovirus containing SV40T. In the previous study, we have isolated bovine intestinal epithelial cells (BIEs) and mammary epithelial cells (BMECs) in vitro (Zhan et al. 2016a; Zhan et al. 2016b). The primary BIEs can undergo approximately 10 passages; however, the primary BMECs can be cultured for 40 passages at least in vitro. Although there are similarities among primary epithelial cell senescent mechanisms, the process that cells were subjected to senesce is likely to depend on cell type-specific and species of origin of the cell (Carnero et al. 2000). Mouse embryo fibroblasts may mainly depend on the $p19^{ARF}$/p53 pathway (Carnero et al. 2000); conversely, human keratinocytes are preferentially associated with the

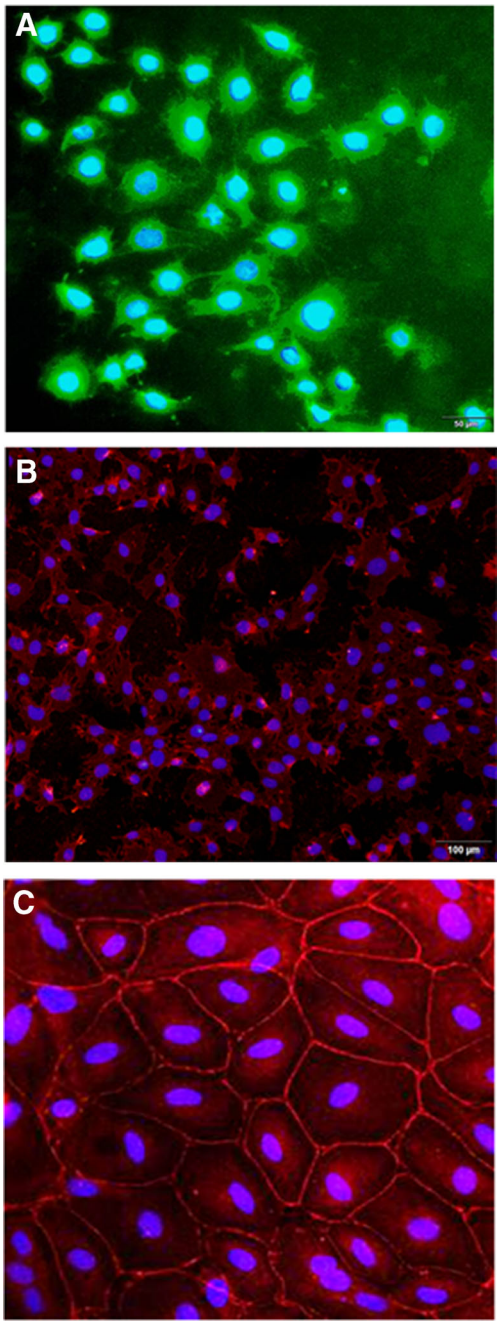

Figure 3. Immunofluorescense staining of epithelial marker in MIEs. (*A*) cytokeratin 18 was stained with FITC in conjunction with anti-mouse secondary antibody (*green*). (*B*) E-cadherin and (*C*) Pept1 were stained with anti-goat Alexa Fluor-594 secondary antibody (*red*). *Bars* represent 50 μm (*A*), 100 μm (*B*), and 200× (*C*). All of the nuclei (*blue*) were counterstained with DAPI.

p16^{INK4A}/pRB pathway to enhance the cell senescence process (Kiyono et al. 1998). p53 and pRB can interact with SV40T, and the function of p53 and pRB was further disrupted to make primary cells escape from the cell senescent cycle. Therefore, the cells can be immortal using SV40T due to the disruption of the p19^{ARF}/p53 and p16^{INK4A}/pRB pathway.

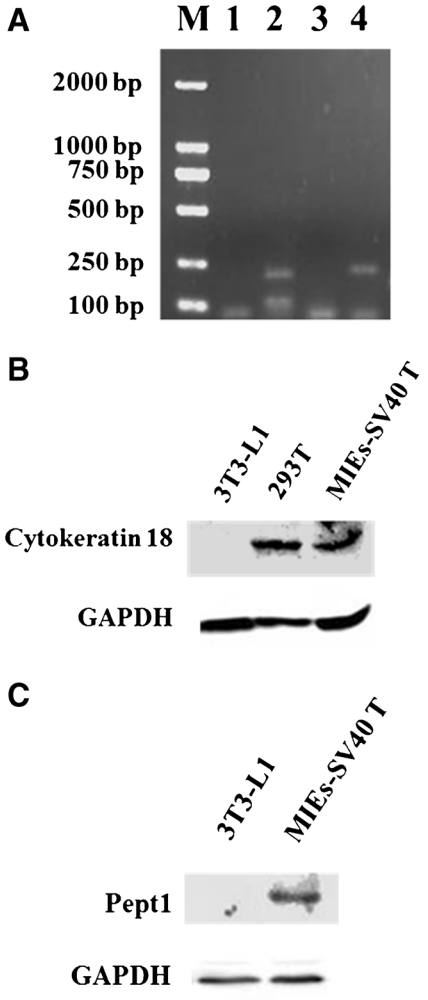

Figure 4. Western blot and RT-PCR analysis of cell markers in MIEs. (*A*) RT-PCR analysis of FABP and villin; amplifications of MIE specific markers were obtained using primers directed against (*1–2 lanes*) FABP, 214 bp (*3–4 lanes*), and villin, 224 bp. *Lanes 1*, *3* 3T3-L1 fibroblast cells; *2*, *4* immortalized MIEs. *M*, molecular weight standard in bp. (*B*, *C*) Immunoblotting analysis of cytokeratin-18 and Pept1; 3T3-L1 fibroblast cells were used as a negative control; 293T cells were used as a positive control to demonstrate the epithelial origin; MIEs-SV40T represents the infected cells used in the present study. The data were based on triplicate experiments.

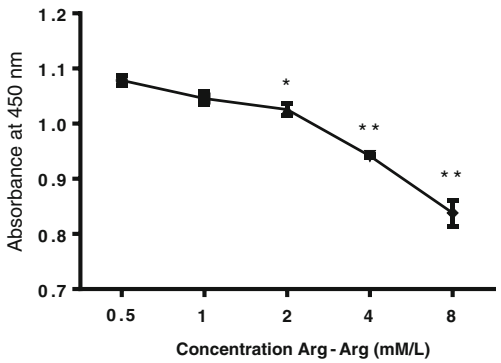

Figure 5. The cytotoxicity of Arg-Arg (0, 0.5, 2, 4, 8 mM) on MIEs. Data shown are means ± SEM of three independent experiments.

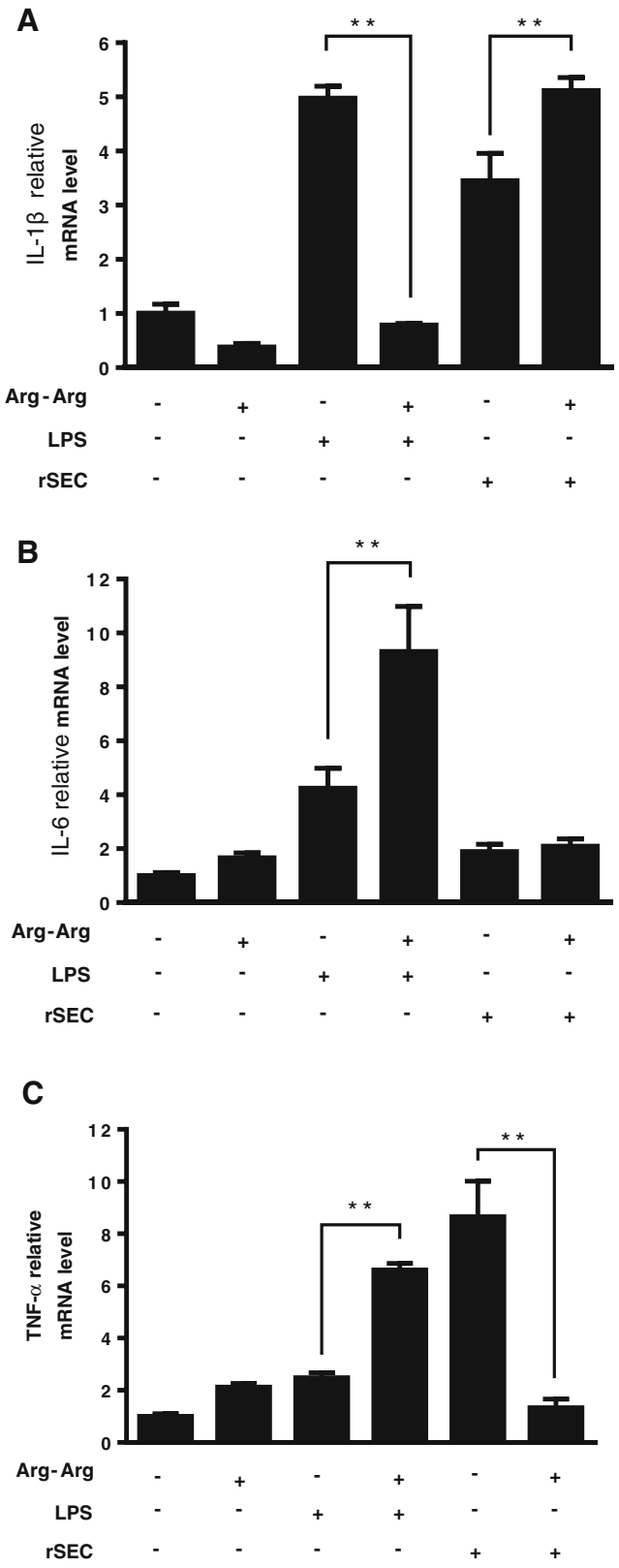

Figure 6. Effects of Arg-Arg on inflammatory cytokine mRNA expression. The immortalized MIEs were cultured for 12 h to detect gene expression mRNA levels of IL-1β, IL-6, and TNF-α. β-Actin was used as an internal reference gene. Data shown are means ± SEM of three independent experiments.

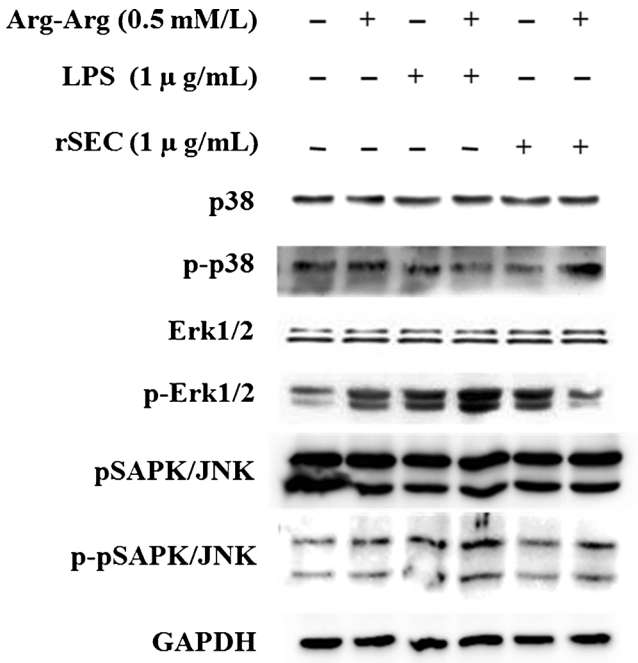

Figure 7. Effects of Arg-Arg on the MAPK signaling pathway. The immortalized MIEs were cultured for 12 h to extract total protein. Western blot analysis for p38, p-p38, Erk1/2, p-Erk1/2, SAPK/JNK, and p-SAPK/JNK. The data were based on triplicate experiments.

In order to demonstrate whether isolated cells were originated from epithelial style cells, we performed immunoblotting and immunocytochemical analysis to detect cytokeratin 18 and E-cadherin. The isolated cells have strong immunopositive reaction for the anti-cytokeratin 18 and E-cadherin antibody. Cytokeratin 18 only exhibited the specific characterization of epithelial cells (Kanaya et al. 2008). In addition, the cytokeratin 18 band can be observed in isolated cells. The result further indicated that isolated cells showed characterization of epithelial origin cells. However, previous studies suggest that cytokeratin 18 was expressed in both epithelial and myoepithelial cells from the goat mammary gland (Li et al. 1999). Furthermore, cytokeratin 18 cannot be found in bovine myoepithelial cells (Zavizion et al. 1996). This is not consistent with the above study. Therefore, we will further investigate intestinal epithelial cell transporters to evaluate whether the isolated cells are from intestinal epithelial cells.

Intestinal epithelial cells play a critical role for the transport and absorption of nutrients. The solute carrier 15 family (SLC15), particularly Pept1 transporter, was well known for its role in the cellular uptake of dipeptide and tripeptide. The Pept1 transporter is mainly localized in the brush-border membranes of enterocytes and dependent on the transmembrane H^+ gradient to execute transport of small peptides. In the present study, the isolated cells were strongly positive to the anti-Pept1 antibody; moreover, the immunoblotting band can be detectable in the isolated cells. Furthermore, we also detect whether isolated cells can express FABP and villin which is a

specific marker for intestinal epithelial cells. FABP is mainly located in the cytoplasm, which is expressed in a highly tissue-specific manner and plays an integral role in the balance between lipid and carbohydrate metabolism. FABP can be expressed in the adipocyte, liver, brain, and intestine; however, the FABP mRNA sequence is different in the adipocyte, liver, brain, and intestine. Villin is a tissue-specific actin-binding protein and is detected only in the microvilli of some epithelial cells from the gastrointestinal, urogenital, and respiratory tracts (Golaz et al. 2007). Our result showed that the isolated cells can express FABP and villin. These data further demonstrate that isolated cells were derived from intestinal epithelial cells, and they can be polarized in vitro.

Dipeptide and tripeptide are actively transported across membranes as an efficient route for dietary protein absorption via Pept1 (Solcan et al. 2012). However, the effect of Arg-Arg dipeptide on intestinal epithelial cell inflammatory response remains unknown. To investigate the effects of Arg-Arg on the inflammatory response and the MAPK signaling pathway in LPS-induced or rSEC-induced cells, IL-1β, IL-6, and TNF-α were performed by qRT-PCR. In addition, p38, Erk1/2, and SAPK/JNK were also determined by Western blot. It is well known that cells induced by LPS can elicit an amount of proinflammatory cytokines IL-1β, IL-6, and TNF-α in macrophages and epithelial cells (Günther et al. 2011). These proinflammatory cytokines play a key role in regulating the immune system response to defend against bacteria invaders (Lapointe et al. 2010). IL-1β and TNF-α are early proinflammatory cytokines (Dinarello 2000). IL-6 is the third master proinflammatory cytokine. It is one of the key mediators of the "acute-phase response" in inflammation (Heinrich et al. 2003). Arginine relieves the IL-1β, IL-6, and TNF-α expression level in bovine mammary epithelial cells caused by LPS (Wu et al. 2016a, b). The intestine of carp with 100–300 μg/mL arginine treatment can completely inhibit mRNA expression of IL-1β, IL-6, and TNF-α (Jiang et al. 2015). However, our study showed that Arg-Arg decreased the LPS-induced expression of IL-1β in MIEs induced by LPS; interestingly, it enhanced the expression of IL-6 and TNF-α. Accordingly, pSAPK/JNK and p-Erk1/2 in LPS induced with Arg-Arg treatment were enhanced. p-p38 in LPS with Arg-Arg treatment was markedly attenuated than that in LPS treatment. It appears that the pSAPK/JNK and p-Erk1/2 signaling pathway was responsible for the expression of IL-6 and TNF-α, and p-p38 controls the expression of IL-1β. The result indicated that Arg-Arg and arginine have different mechanisms which regulate the inflammatory response caused by LPS. Infections from *S. aureus* often cause mild, subclinical inflammation and persistent infections. Furthermore, *S. aureus* elicited a slow response and failed to induce bactericidal factor expression (Bannerman et al. 2004). The avoidance to quickly induce the inflammatory factor release can elicit the persistent survival of *S. aureus* (Günther et al. 2011). In our study, Arg-Arg supplementation in rSEC induction contributed to p-p38 and pSAPK/JNK activation to enhance the IL-1β expression level, which swiftly and strongly induced inflammatory response. However, p-Erk1/2 was markedly downregulated in rSEC with Arg-Arg treatment to reduce the expression of TNF-α. These results indicated that Arg-Arg meanwhile enhanced and attenuated the bactericidal factor release, which may support the balance of the immune system.

Conclusions

MIEs can be immortal using a retrovirus containing SV40T and passaged 100 generations at least without any senescent sign. In addition, immortal MIEs also express Pept1 transporter and villin. We also find that Arg-Arg cannot meanwhile enhance or decrease the expression of early proinflammatory cytokines (IL-1β and TNF-α). Therefore, Arg-Arg plays an essential role for the balance of the immune inflammatory response in MIEs caused by LPS or rSEC.

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