



Post-translational suppression of the high affinity IgE receptor expression on mast cells by an intestinal bacterium

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ABSTRACT

Mast cells, which express the high-affinity IgE receptor (FcεRI) on their surface, play a crucial role in inducing allergic inflammation. Since mast cells are activated by crosslinking of FcεRI with IgE and allergens, the cell surface expression level of FcεRI is an important factor in determining the sensitivity to allergens. Recently, the involvement of gut microbiota in the prevalence and regulation of allergy has attracted attention but the precise underlying mechanisms are not fully understood. In this study, the effect of intestinal bacteria on cell surface expression of FcεRI was examined. *Bacteroides acidifaciens* type A 43 specifically suppressed cell surface expression of FcεRI on mouse bone marrow-derived mast cells (BMMCs) without reduction in FcεRI α and β-chain mRNA and total protein expression. The suppressive effect required sustained exposure to this bacterium, with a corresponding reduction in Erk activation. Inhibition of Erk decreased cell surface distribution of FcεRI in BMMCs, at least in part, through facilitated endocytosis of FcεRI. These results indicate that *B. acidifaciens* type A 43 suppresses cell surface expression of FcεRI on mast cells in a post-translational manner via inhibition of Erk. The suppression of FcεRI expression on mast cells by specific bacteria might be the underlying mechanism involved in the regulation of allergy by gut microbiota.

1. Introduction

In recent years, the incidence of allergic diseases has increased rapidly. However, although symptomatic treatment is currently the main approach, several efforts have been made to establish fundamental therapeutic methods. Some environmental risk factors are known to contribute to the development of allergies. For example, the use of antibiotics, which causes an imbalance in gut microbiota, increases the risk of allergic diseases (Bashir et al., 2004; Kim et al., 2014; Alhasan et al., 2020), suggesting that commensal bacteria may regulate allergic

reactions. Approximately 4×10^{13} bacteria inhabit the mammalian intestinal tract (Sender et al., 2016) and establish a mutual relationship with the host. In fact, many studies have reported the relationship between composition of the gut microbiota and the morbidity of allergic disease. Particularly, it has been suggested that gut microbial composition just after birth plays an important role in determining the risk of allergy later in life. Arrieta et al. reported that the relative abundance of some specific genera was transiently reduced at three months after birth in fecal samples of Canadian children with a high risk of asthma. However, the difference was not evident at one year of age (Arrieta

Abbreviations: Ab, antibody; BA, *Bacteroides acidifaciens* type A 43; BMMC, bone-marrow-derived mast cell; BSA, bovine serum albumin; FcεRI, the high affinity IgE receptor (FcεRI); HRP, horseradish peroxidase; SDS-PAGE, sodium dodecyl sulphate–polyacrylamide gel electrophoresis; PBS, phosphate buffered saline; SCFA, short chain fatty acid; TLR, Toll-like receptor.

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et al., 2015). They subsequently reported that dysbiosis of gut microbiota, which was different from that observed in Canadian children, was also identified at three months after birth in Ecuadorian children who went on to develop atopic wheeze at five years of age (Arrieta et al., 2018). In addition, a neonatal group characterized by a lower relative abundance of *Bifidobacterium*, *Akkermansia*, and *Faecalibacterium* was shown to be at the highest risk of developing allergy at a later point in life by a cohort study in the United States (Fujimura et al., 2016). Recently, it was reported that the difference in allergy sensitization at three years of age between Finnish and Estonian children could be due to different abundance of *Acinetobacter* at six months after birth (Ruokolainen et al., 2020).

The regulatory actions of gut microbiota are mediated by both bacterial components and their metabolites. For example, butyrate, one of the short chain fatty acids (SCFAs), well-known metabolites produced by gut microbiota, plays a crucial role in inducing colonic Treg to suppress intestinal inflammation (Furusawa et al., 2013). Consistent with this, a decrease in the production of SCFAs by antibiotic-induced dysbiosis in early life facilitated the development of atopic dermatitis in mice through regulating the number of Treg and ILC3 (Kim et al., 2020). It has been considered that cross-talk with the host's immune system is vital for the regulation of allergic diseases by gut microbiota. Indeed, gut microbiota contributes to the modulation of Th1-Th2 balance (Sudo et al., 1997; Gaboriau-Routhiau et al., 2003). Several studies have shown that the composition of gut microbiota affects the development and function of T cells, which results in the modulation of allergy. Ohnmacht et al. found the presence of a gut microbiota-responsive Treg subset which suppresses Th2 immune responses (Ohnmacht et al., 2015). Another report indicated that early change in gut microbiota from one rich in *Bifidobacterium longum* to that with increased butyrate-producing bacteria causes early maturation of Treg population characterized by a decrease in their frequency accompanied by an increase in the highly activated Treg cells, which leads to the prevention of subsequent atopic sensitization (Ruotula et al., 2019). Moreover, defined microbial transfer protected germ-free mice from food allergy by inducing ROR γ T Treg in a MyD88-dependent manner (Abdel-Gadir et al., 2019). These effects on T cells are thought to be mainly mediated by the action of commensal bacteria on antigen-presenting cells. In addition to such effects on T cells, it has been shown that invariant natural killer cells, which induce Th2 differentiation and IgE class switching through the release of IL-4 and IL-13 (Akbari et al., 2003), are reduced by early exposure to microbiota. In contrast, dysbiosis may exacerbate allergy due to an impairment in these mechanisms (Salameh et al., 2020).

However, unidentified mechanisms underlying the regulation of allergy by gut microbiota, specifically those that do not act through the effects on T cells, remain to be elucidated. Mast cells, which act as key players in inducing allergic inflammation, express the high-affinity IgE receptor, Fc ϵ RI. This receptor consists of α , β , and γ subunits, of which the α -chain binds IgE through its extracellular domain, while the β and γ -chains mediate intracellular signals. When Fc ϵ RI is bound by allergen-specific IgE and further crosslinked by allergens, mast cells are activated through intracellular signaling pathways and then secrete various chemical mediators, including histamine, stored in intracellular granules. This phenomenon is known as degranulation, which occurs as a threshold reaction; it is only triggered by antigens (allergens) with concentrations higher than the threshold. Since the density of cell surface Fc ϵ RI determines antigen sensitivity, i.e. the threshold value of antigen concentration, cell surface expression levels of Fc ϵ RI on mast cells is an important factor influencing the strength of allergic responses.

Mast cells are present mainly in the mucosa and skin. Among the mucosal tissues, the intestine has the largest area, therefore, the intestinal mucosa is equipped with a relatively large number of mast cells in the body. Intestinal mast cells exist in the lamina propria under the epithelium. However, they are likely to interact with commensal bacteria inhabiting the intestinal tract, considering that luminal bacteria are translocated to the lamina propria through villus M cells and dendritic

cells (Mabbott et al., 2013; Rescigno et al., 2001). Additionally, bacteria could be transported across the epithelium through transcytosis (Nazli et al., 2006), indicating that these bacterial components also act on intestinal mast cells. In particular, the increase in mast cells accumulated close to the epithelium observed during intestinal inflammation may result in more susceptible conditions to commensals. Since mast cells have been reported to express several Toll-like receptors (TLRs) (Sandig and Bulfone-Paus, 2012) and exert phagocytic activity (Wesolowski et al., 2012), they can interact with intestinal bacteria through both cell surface receptors such as TLRs and intracellular receptors.

In this study, to clarify the novel mechanism underlying the regulation of allergy by gut microbiota, the effect of intestinal bacteria on the expression of Fc ϵ RI on mast cells was investigated.

2. Materials and methods

2.1. Mice

C57BL/6 mice (female, 6 weeks old) were purchased from CLEA Japan (Tokyo, Japan). All animal experiments were approved by the Nihon University Animal Care and Use Committee.

2.2. Preparation of bone marrow-derived mast cells (BMMCs)

BMMCs were prepared as described in the previous report (Kasakura et al., 2014a). Briefly, bone marrow cells were prepared from femurs of mice and cultured in RPMI medium supplemented with 10% fetal bovine serum, 100 μ g/mL streptomycin, 100 U/mL penicillin, 0.1 mM nonessential amino acids, 100 μ g/mL sodium pyruvate, 5×10^{-5} M 2-mercaptoethanol, and 5 ng/mL recombinant mouse IL-3 (Pepro Tech, Rocky Hill, NJ, or Bio Legend, San Diego, CA) at 37 °C in a humidified incubator with 5% CO $_2$. Cells after 5–8 weeks of culture were used for the experiments.

2.3. Preparation of heat-killed bacteria

Strains of *Bacteroides* (*B. vulgatus* JCM 5826^T, *B. ovatus* JCM 5824^T, *B. fragilis* DSM 2151^T, *B. theta* JCM 5827^T, *B. vulgatus* Toma-1), *E. coli* (E-17 and JCM 1649^T), *Lactobacillus* (*L. casei* JCM 1134^T), *Bifidobacterium* (*B. pseudocatenulatum* JCM 7041), and *Enterococcus* (*E. faecalis* JCM 8726^T) were purchased from Japan Collection of Microorganisms (RIKEN BRC, Ibaraki, Japan). *Bacteroides acidifaciens* type A43 (BA) and *Lactobacillus johnsonii* 129 were isolated from the murine intestine. *Bacteroides* strains were cultured in Eggerth-Gagnon broth supplemented with 0.0001% hemin and 5 μ g/mL menadione under anaerobic conditions. *E. coli* strains were cultured in nutrient broth under aerobic conditions. *Lactobacillus* and *Enterococcus* were cultured in MRS broth under anaerobic conditions. *Bifidobacterium* was cultured in GAM broth under anaerobic conditions. These bacteria were cultured for 24 or 48 h with 1/100 inoculation of the preculture. After washing three times with sterile ultrapure water, bacterial cells were suspended in ultrapure water, heated at 80 °C for 50 min, and then lyophilized.

2.4. Flow cytometry

BMMCs were cultured with 100 μ g/mL of heat-killed bacteria for 24–96 h. Alternatively, BMMCs were cultured in the presence of 10 μ M of U0126 (Cell Signaling Technology, Danvers, MA) for 24 h. For treatment with both BA and U0126, U0126 was added to BMMCs after stimulation with BA for 48 h and cells were cultured for an additional 24 h. In experiments using cycloheximide, BMMCs were cultured for 6–24 h in the presence of 10 μ M of U0126 for 30 min after the addition of 0.2 μ g/mL cycloheximide (Wako, Osaka, Japan). Cells were then stained with PE-labeled anti-Fc ϵ RI α antibody (Ab) (clone MAR-1, Bio Legend or Bay bioscience, Hyogo, Japan) or PE-labeled Armenian hamster IgG as isotype control (Bio Legend or e-Bioscience-Thermo Fisher Scientific,

Carlsbad, CA) in FACS buffer (DMEM pH7.2 containing 1 mM EDTA, 10 mM HEPES, 2 mM sodium pyruvate, 0.5% bovine serum albumin (BSA), and 0.02% sodium azide) on ice for 30 min. After washing with FACS buffer, cells were analyzed by FACSCanto (BD Biosciences, San Jose, CA). For evaluation of cell viability, cells were stained with FITC Annexin V Apoptosis Detection Kit with 7-AAD (Bio Legend) according to the manufacturer's instructions and analyzed by FACSCanto.

2.5. Degranulation assay

Degranulation assay was performed as described previously (Kasakura et al., 2014b). Briefly, BMMCs were sensitized with 0.2 µg/mL of anti-trinitrophenyl IgE (BD Biosciences) for 2 h and then stimulated with 3 ng/mL of TNP-BSA (LSL; Tokyo, Japan) for 30 min. Degranulation was determined by measuring the enzymatic activity of released β-hexosaminidase using p-nitrophenyl-N-acetyl-β-D-glucosaminide as a substrate. The ratio of released β-hexosaminidase to total intracellular content was calculated by dividing the obtained value by that of cells treated with 0.1% Triton-X 100. TNP-BSA-induced β-hexosaminidase release was determined by subtracting the percentage of β-hexosaminidase release in the absence of TNP-BSA from that in the presence of TNP-BSA.

2.6. qRT-PCR

BMMCs were cultured with 100 µg/mL of heat-killed bacteria for 6–96 h. After isolating total RNA from cells using the High Pure RNA isolation kit (Roche, Basel, Switzerland), cDNA was synthesized using SuperScript IV reverse transcriptase (Invitrogen-Thermo Fisher Scientific) and oligo (dT)₁₈ primers (Invitrogen-Thermo Fisher Scientific). Messenger RNA expression was quantified by real-time PCR using SYBR Green I Master reagent (Roche) and performed on a LightCycler 480 system (Roche). The following oligonucleotide pairs were used as PCR primers:

FcεRIα	forward, 5'-TGACAGCCACTGAGAAATCTGTA-3' reverse, 5'-TCACAATGACCAATGTGAAGAG-3'
FcεRIβ	forward, 5'-AGTGCTGTTGTTTGTCTGGAT-3' reverse, 5'-CGAAGTTATTGGTCAGATTGAGG-3'
Gapdh	forward, 5'-TGAACGGGAAGCTCACTGG-3' reverse, 5'-TCCACCACCTGTTGCTGTA-3'

The relative expression of each gene was quantified using the calibration curve generated by serial dilutions of standard samples. Expression levels of FcεRI α and β-chains were calculated by normalizing to the levels of *Gapdh*. The specificity of PCR amplification for each gene was confirmed by melting curve analysis.

2.7. Western blotting and immunoprecipitation

BMMCs were cultured with 100 µg/mL heat-killed bacteria for 24–96 h. Cells were then washed with ice-cold phosphate buffered saline (PBS) and lysed for 30 min on ice using lysis buffer (20 mM Tris, pH 7.6, 1% Nonidet P-40, 60 mM octyl-β-glucoside, 1 mM EDTA, protease inhibitor cocktail (Nacalai Tesque, Kyoto, Japan). For analysis of Erk phosphorylation, phosphatase inhibitor cocktail (Nacalai Tesque) was also added to lysis buffer. The cell lysates were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride membranes. To detect FcεRI α-chain, the lysate was immunoprecipitated with anti-FcεRIα Ab (MAR-1, eBioscience) coupled with Protein G Sepharose 4 Fast Flow (GE Healthcare, Chicago, IL), before performing SDS-PAGE. Biotin-labeled anti-FcεRIα Ab (MAR-1, eBioscience) and avidin-horseradish peroxidase (HRP), and anti-FcεRIβ Ab (N-18, Santa Cruz Biotechnology, Dallas, TX) and HRP-linked anti-goat IgG Ab (Santa Cruz Biotechnology) were used to detect the expression of FcεRI α and β-chains, respectively. To analyze phosphorylation of Erk, anti-phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) and anti-p44/42 MAPK (Erk1/2) Abs (9101 and 9102, Cell

Signaling Technology, Danvers, MA), and HRP-linked anti-rabbit IgG Ab (Cell Signaling Technology) were used as primary and secondary antibodies, respectively. HRP-linked anti-β-actin Ab (AC-15, Abcam, Cambridge, UK) was used for immunoblotting of β-actin.

2.8. Fluorescence microscopy analysis

BMMCs were cultured in the presence or absence of 10 µM U0126 for 48 h and then fixed with phosphate buffered 4% paraformaldehyde. After treatment with 0.5% Triton-X100 in PBS and blocking with 3% BSA in PBS, cells were stained with anti-FcεRIα Ab (MAR-1, Bio Legend) in PBS containing 1% BSA and 0.3% Triton X-100 followed by staining with secondary Ab, Alexa Fluor 647-labeled goat anti-Armenian hamster IgG in the same buffer. For staining of the Golgi apparatus, Alexa Fluor 488-labeled lectin *Helix pomatia* agglutinin (HPA) (Thermo Fisher Scientific) was added to the cells simultaneously with the addition of secondary Ab. After mounting using ProLong Gold antifade reagent with DAPI (Thermo Fisher Scientific), cells were observed under a confocal laser scanning fluorescence microscope (FV1000, Olympus, Tokyo, Japan).

2.9. Statistics

Differences between two or more groups were analyzed by two-tailed Student's *t*-test or one-way ANOVA followed by Tukey's test, respectively. Results with a *p*-value < 0.05 were considered as statistically significant.

3. Results

3.1. *Bacteroides acidifaciens* type A43 suppresses cell surface expression of FcεRI on mast cells

The effects of intestinal bacteria on the expression of FcεRI on mast cells were analyzed by adding various heat-killed intestinal bacteria including *Bacteroides*, *Bifidobacterium*, *Lactobacillus*, *Enterococcus*, and *Escherichia coli* to BMMCs (Fig. 1). Among the intestinal bacteria used for the experiments, *Bacteroides acidifaciens* type A 43 (BA), which was isolated from the murine intestine, solely suppressed the cell surface expression of FcεRI. None of the other bacteria affected the expression of FcεRI on BMMCs. Notably, several species belonging to the genus *Bacteroides* other than BA, such as *B. thetaiotaomicron* and *B. fragilis*, did not suppress FcεRI expression, indicating that the suppressive effect is species or strain-specific.

Furthermore, the cell surface expression of FcεRI on BMMCs was analyzed after 24 and 72 h of stimulation with BA. Stimulation with BA for 24 h did not affect cell surface FcεRI expression on BMMCs (Fig. 2A), whereas stimulation for 72 h suppressed its expression (Fig. 2B), indicating that prolonged exposure to BA results in decreased cell surface expression of FcεRI on mast cells. BA treatment for 72 h did not increase the ratios of early apoptotic (Annexin V⁺7-AAD⁻) or dead (Annexin V⁺7-AAD⁺) cells (Fig. 2C), indicating that it had no cytotoxic effect. In addition, since BA did not affect cell surface expression of c-kit (Supplementary Fig. S1), it was thought to specifically suppress FcεRI expression, although we cannot rule out the possibility that some receptors other than c-kit are also influenced by BA in addition to FcεRI. The effect of suppressed FcεRI expression by prolonged exposure to BA on IgE/antigen-induced degranulation of BMMCs was analyzed next. As shown in Fig. 2D, pretreatment with BA for 72 h significantly decreased degranulation.

3.2. Suppression of FcεRI expression on mast cells by BA is mediated by post-translational regulation

FcεRI is composed of multiple subunits, α, β, and γ-chains, of which the α and β-chains are specific to FcεRI, while the γ-chain is a common

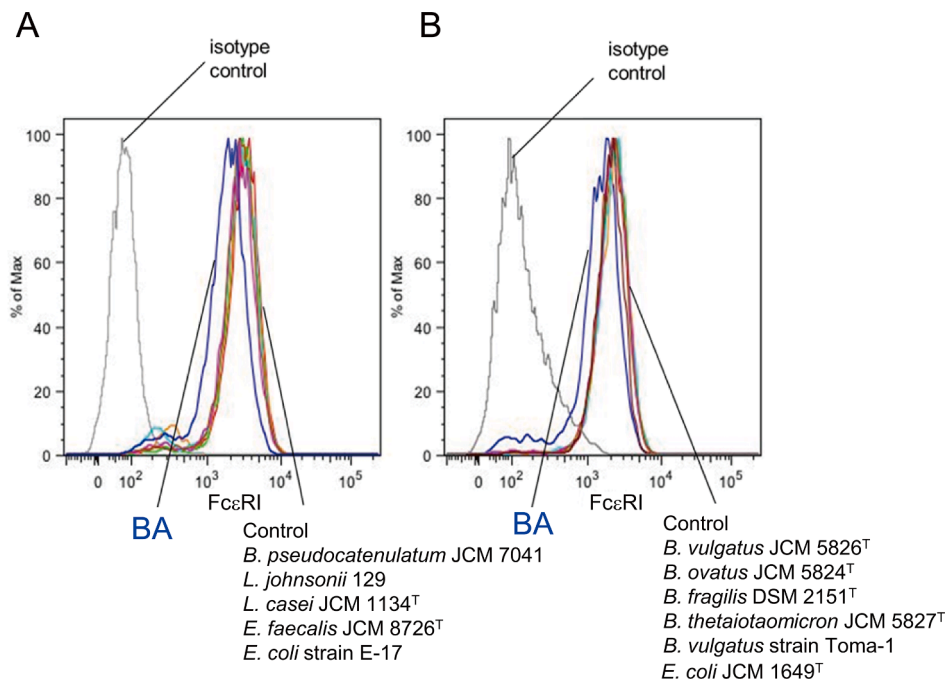


Fig. 1. Effect of various intestinal bacteria on the cell surface expression of FcεRI on mast cells. BMMCs were stimulated with various heat-killed intestinal bacteria for 96 h. Cell surface expression of FcεRI was then analyzed by flow cytometry using PE-labeled anti-FcεRIα Ab. Representative histogram data of 2–4 independent experiments are shown. Control, in the absence of bacteria.

subunit constituting other receptors such as IgG receptor, FcγR, in addition to FcεRI. Thus, to determine the effects of BA on the expression of FcεRI α and β-chain mRNA, BMMCs were stimulated with BA for 6, 24, 48, 72, and 96 h to quantify the mRNA expression of α and β-chain by qRT-PCR. As shown in Fig. 3, neither α or β-chain mRNA expression was significantly affected by BA treatment at any of the indicated time points. Although the α-chain mRNA expression seemed to decrease transiently at 24 and 48 h, there was no statistical difference when compared with the expression in the absence of BA stimulation at each time point. Next, total protein expression of FcεRI α and β-chains including both cell surface and intracellular expression was analyzed by western blotting using whole cell lysates. For detection of the α-chain, immunoprecipitation followed by western blotting was performed. The results showed that the total protein expression of α and β-chains was not affected by BA stimulation (Fig. 4). These results indicate that BA does not affect the transcriptional or translational levels of α and β-chain genes but it suppresses cell surface FcεRI expression via post-translational regulation.

3.3. Suppression of FcεRI expression on mast cells by BA is dependent on inhibition of Erk

To clarify the underlying mechanism by which BA suppresses the cell surface expression of FcεRI on mast cells, the effect of BA stimulation on intracellular signaling was analyzed next. Since it has been suggested that inhibition of Erk suppresses FcεRI expression (Shim et al., 2017), we examined the effect of BA on the activation of Erk by western blotting. As shown in Fig. 5, stimulation with BA for 24 h did not affect the phosphorylation of Erk. However, stimulation for 72 or 96 h decreased it, indicating that prolonged, but not transient, exposure to BA leads to decreased phosphorylation of Erk. Furthermore, inhibition of Erk by the addition of U0126, an inhibitor of the upstream molecule MEK1/2, suppressed cell surface expression of FcεRI on BMMCs (Fig. 6A). In addition, treatment with both BA and U0126 showed suppressive effects similar to treatment with BA or U0126 alone (Fig. 6B), indicating that the effect of BA was dependent on Erk inhibition. Collectively, these results showed that prolonged exposure to BA suppressed cell surface

expression of FcεRI on mast cells by inhibiting activation of Erk. Since phosphorylation of Erk was inhibited only upon stimulation with BA for more than 72 h, this is consistent with the finding that decrease in cell surface expression of FcεRI occurred after more than 72 h of exposure to BA. Moreover, stimulation with *L. johnsonii* 129, which did not affect FcεRI expression (Fig. 1), did not suppress phosphorylation of Erk (Supplementary Fig. S2). Taken together, it was shown that sustained stimulation with BA for more than 72 h inhibits Erk activation and thus suppresses the cell surface expression of FcεRI at the post-translational level.

3.4. Facilitated endocytosis of FcεRI by Erk inhibition is one of the mechanisms causing post-translational suppression of cell surface expression

Next, intracellular distribution of FcεRI in BMMCs in the presence or absence of U0126 was analyzed by fluorescence confocal laser microscopy (Fig. 6C). While FcεRI was mainly distributed on the cell surface in the absence of U0126, it was detected inside the cell excluding the nucleus in the presence of U0126, indicating that inhibition of Erk impairs cell surface distribution of FcεRI.

Cell surface expression levels of receptors are determined by a balance between the transport of *de novo* synthesized proteins to the cell surface and uptake of cell surface receptors into the cells through endocytosis. We next examined whether BA influenced the endocytosis of cell surface FcεRI using a protein synthesis inhibitor, cycloheximide. BMMCs were treated with cycloheximide and then further treated with U0126 to analyze the expression of FcεRI by flow cytometry. As shown in Fig. 7, cycloheximide treatment reduced the cell surface expression of FcεRI after 18 and 24 h, which reflected the inhibition of *de novo* protein synthesis. The cell surface expression of FcεRI in the presence of cycloheximide was lower in BMMCs treated with U0126 than in those without U0126 treatment at 18 and 24 h, indicating that addition of U0126 enhanced endocytosis of FcεRI. Therefore, these results suggested that the post-translational suppression of cell surface FcεRI expression by Erk inhibition is, at least in part, dependent on increased FcεRI endocytosis.

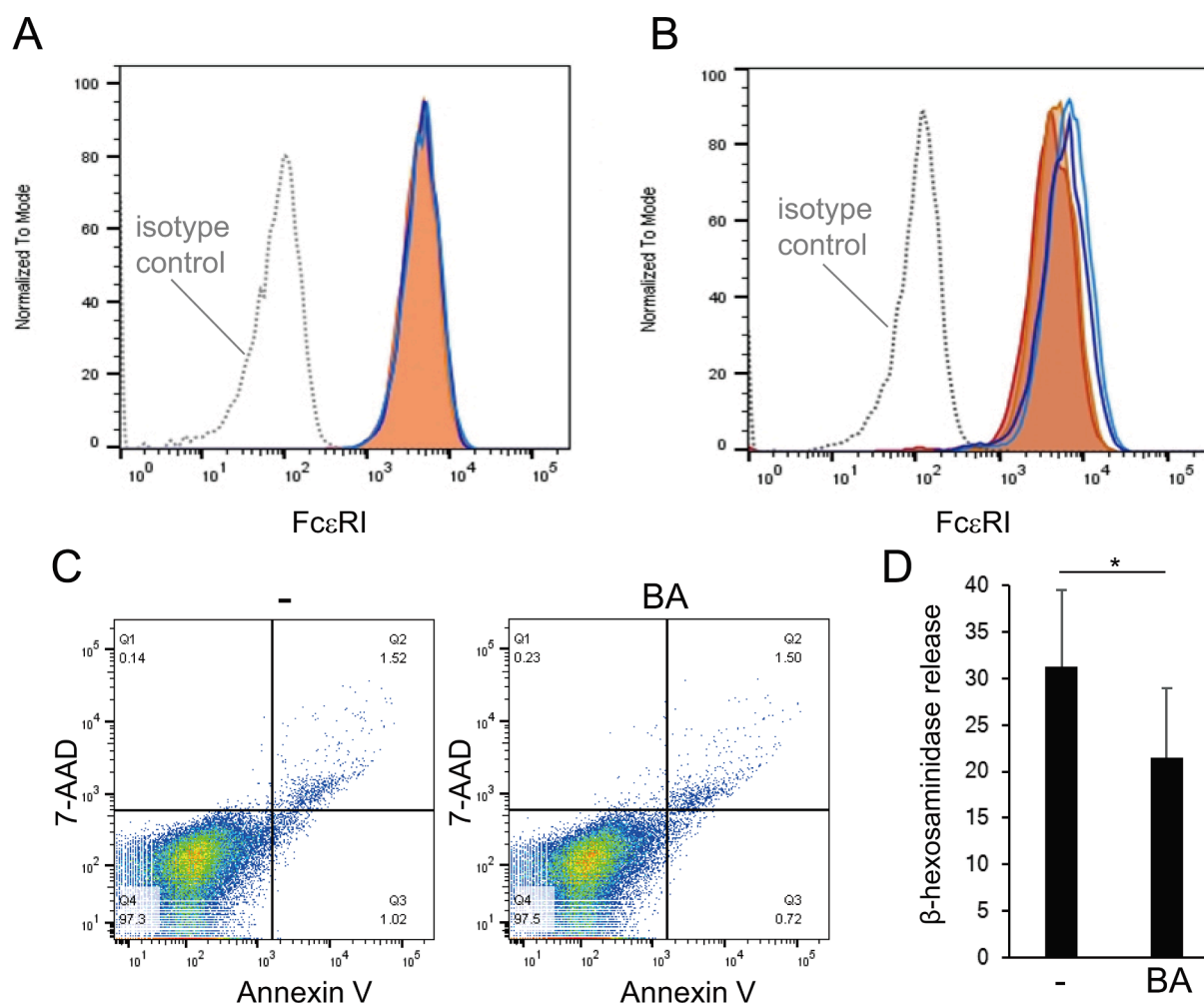


Fig. 2. Prolonged exposure to BA suppresses the cell surface expression of FcεRI on mast cells. (A, B) BMMCs were stimulated with heat-killed BA for 24 h (A) or 72 h (B) and cell surface expression of FcεRI was analyzed by flow cytometry. Representative histograms of 3 independent experiments, each performed in duplicate, are shown. Opened blue and light blue histograms, without BA; filled red and orange histograms, with BA. (C) After culturing in the absence (left) or presence (right) of BA for 72 h, BMMCs were stained with FITC-Annexin V and 7-AAD to evaluate cell viability. Representative results of 3 independent experiments are shown. (D) After treatment of BMMCs with BA for 72 h, degranulation upon IgE and antigen stimulation was measured using a β-hexosaminidase release assay. Results are shown as mean ± SD of 3 independent experiments. *Significantly different ($p < 0.01$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

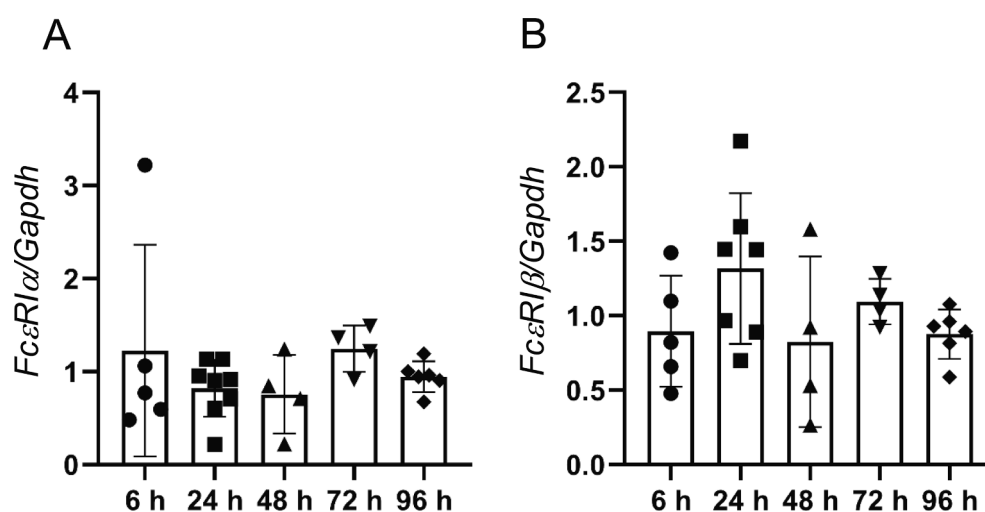


Fig. 3. BA does not suppress the mRNA expression of FcεRI α and β-chains. BMMCs were cultured in the presence or absence of heat-killed BA for 6–96 h and mRNA expression of FcεRI α and β-chains (A and B, respectively) was analyzed using qRT-PCR. Values are normalized to Gapdh mRNA levels and expressed relative to those in the absence of BA at each time point. Results are shown as mean ± SD of 4–8 independent experiments.

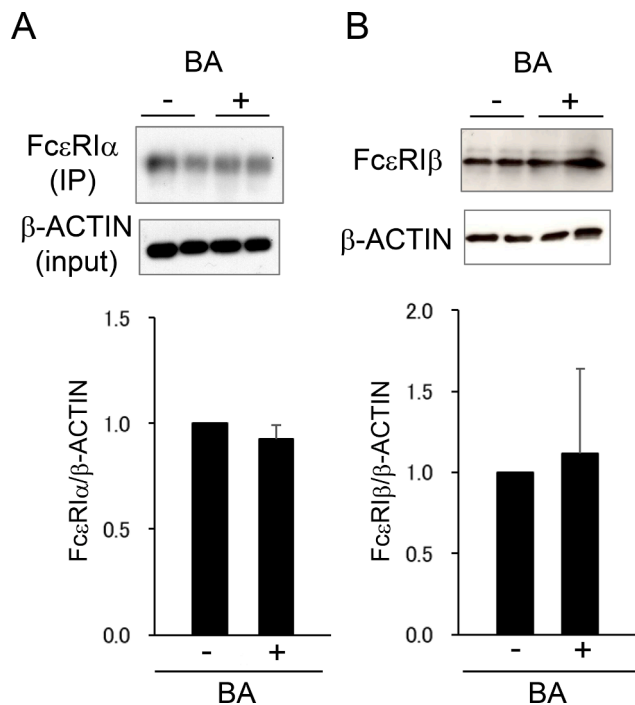


Fig. 4. BA does not suppress total protein expression of FcεRI α and β-chains. BMMCs were cultured in the presence or absence of heat-killed BA for 96 h and protein expression of FcεRI α and β-chains (A and B, respectively) was analyzed by western blotting. For detection of the α-chain, immunoprecipitation followed by western blotting was performed. Representative blots (top) and the mean $\pm$ SD of relative band intensities to those in the absence of BA (bottom) from 5 (α-chain) or 7 (β-chain) independent experiments are shown. Band intensities were calculated by normalizing to those of β-actin.

4. Discussion

We have previously examined the effect of gut microbiota on mast cells and reported that gut microbiota increases migration of mast cells from blood to the intestine (Kunii et al., 2011). Moreover, specific intestinal bacteria reduce granule formation in mast cells (Kasakura et al., 2014a) or suppress degranulation of mast cells (Kasakura et al., 2014b). These findings suggest that gut microbiota regulates allergy, at least in part, via its effects on mast cells. In this study, we showed that specific intestinal bacteria suppressed the expression of FcεRI on mast cells. Since cell surface densities of FcεRI determine the sensitivity of mast

cells to allergens, this may act as a mechanism through which gut microbiota exerts anti-allergic activities. In fact, degranulation upon IgE and antigen stimulation was decreased by BA (Fig. 2D) although there remains a possibility that additional mechanisms other than suppression of FcεRI expression were also involved. Pretreatment with BA for 72 h significantly suppressed degranulation in this study, whereas 2 h of treatment showed only a slight effect in our previous report (Kasakura et al., 2014b), which is consistent with the finding that prolonged exposure to BA was required to suppress FcεRI expression.

The regulatory effects of gut microbiota on mast cells are possibly driven by both bacterial components and their metabolites. Recently, some metabolites produced by gut microbiota have attracted attention for their physiological effects on the host (Song et al., 2020; Nemet et al., 2020; Sharon et al., 2019; Kikuchi et al., 2019; Koh et al., 2018; Legoux et al., 2019). However, in the case of BA shown in this study, the suppression of FcεRI expression is mediated by cell components of BA and not by metabolites because heat-killed bacteria were used for the experiments. Identification of the active component is an important issue that needs to be addressed. The suppressive activity was observed only for BA and not for other bacteria examined, suggesting that a component specific for BA or present abundantly in BA is the active ingredient. Considering that other species belonging to genus *Bacteroides* other than BA did not suppress cell surface FcεRI expression, the active component might be some molecule with specific modifications such as a sugar chain rather than a specific constituent. For example, *Bacteroides* is known to have a variety of capsular polysaccharides and lipoproteins, and it has been reported recently that such phase-variable capsular polysaccharides and lipoproteins of *B. thetaiotaomicron* are involved in the interaction with bacteriophages (Porter et al., 2020). A comparison of such structures among different isolates of *B. acidifaciens* would be helpful in identifying the active components. In addition, since BA was isolated from the murine intestine, whereas many of the other *Bacteroides* used in the experiments were from human feces, elucidation of the relationship between bacterial origin and appearance of the suppressive effect may provide another clue to clarify the species specificity of suppressive activity. Analysis using human mast cells and these bacteria would also be helpful for this purpose. Mast cells are known to express pattern recognition receptors including TLRs (Sandig and Bulfone-Paus, 2012), suggesting that bacterial components are recognized by these receptors. In fact, several reports have shown that stimulation through TLRs affects the activities of mast cells (Lee et al., 2017; Xu et al., 2017; Saluja et al., 2012). In addition, it has been reported that mast cells uptake some materials into cells via phagocytic activity (Wesolowski et al., 2012), which leads to recognition of bacterial components by intracellular pattern recognition receptors such as Nod-like

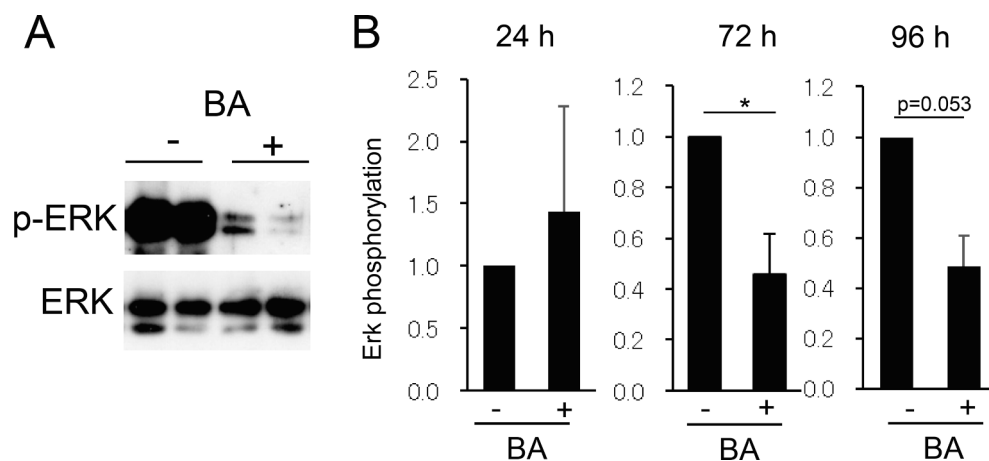


Fig. 5. Sustained exposure to BA suppresses phosphorylation of Erk in mast cells. BMMCs were stimulated with heat-killed BA for 24–96 h and phosphorylation of Erk was analyzed by western blotting. (A) Representative blots indicating 72 h stimulation. (B) Band intensities of phosphorylated Erk normalized to total Erk are expressed as relative values to those without BA stimulation. The mean $\pm$ SD of 3–6 independent experiments are shown. *Significantly different ($p < 0.05$).

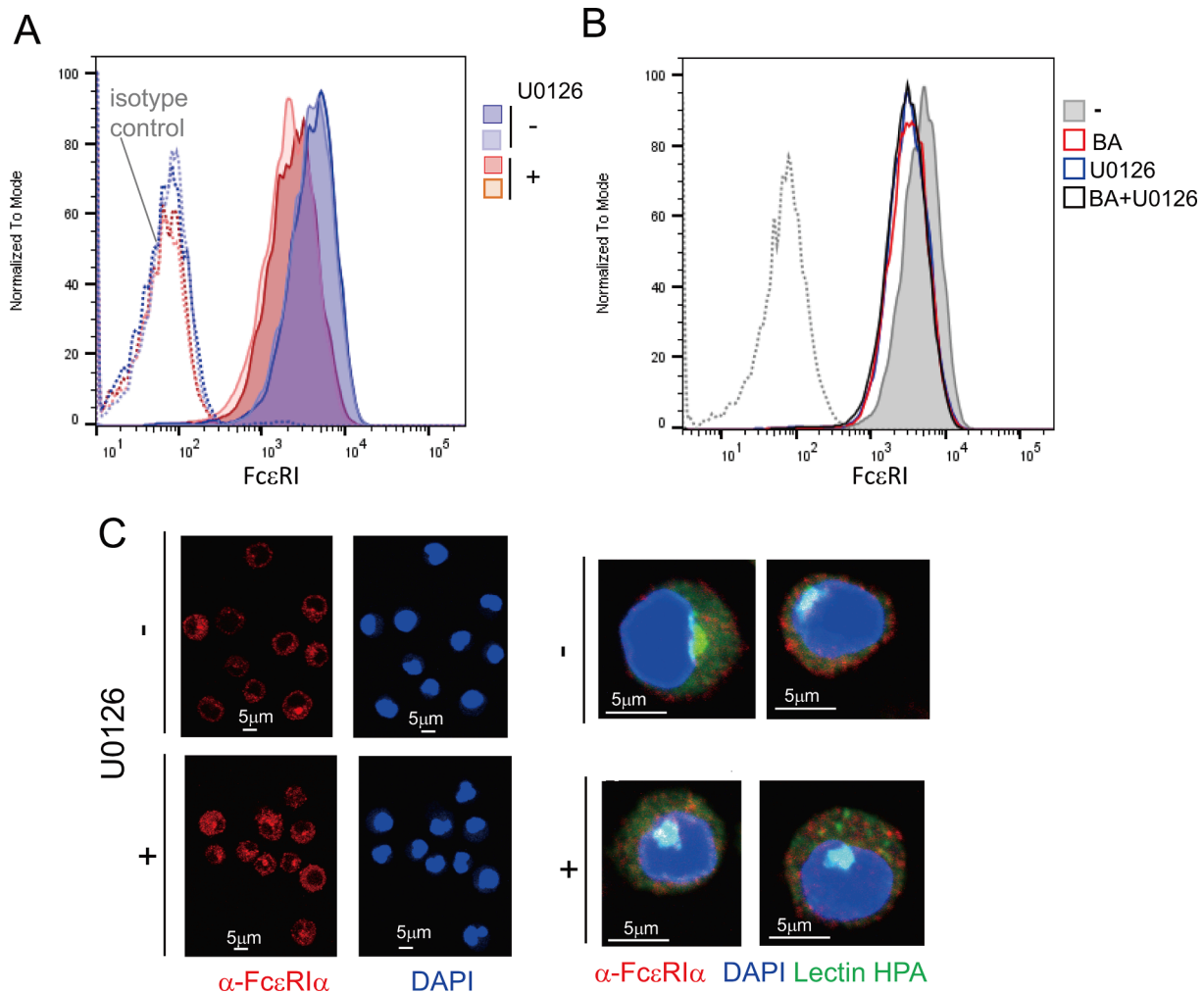


Fig. 6. Inhibition of Erk suppresses cell surface distribution of FcεRI in mast cells. (A) BMMCs were treated with U0126 to analyze cell surface expression of FcεRI by flow cytometry. Representative histograms of 4 independent experiments, each performed in duplicate, are shown. (B) BMMCs were treated with BA alone, U0126 alone, or both BA and U0126 to analyze cell surface expression of FcεRI by flow cytometry. Representative histograms of 3 independent experiments are shown. (C) BMMCs were treated with U0126, stained with Alexa 647-labeled anti-FcεRIα Ab and DAPI (left) or anti-FcεRIα Ab, Alexa 488-labeled lectin HPA, and DAPI (right), and observed under a fluorescence confocal microscope. Representative images of 4 (left) or 3 (right) independent experiments are shown.

receptors.

In this study, it was shown that prolonged exposure to BA causes suppression of cell surface FcεRI expression via reduction of Erk phosphorylation in mast cells. Since BA did not influence the activation status of NF-κB at 24, 72, or 96 h (Supplementary Fig. S3), the effect of BA on signaling molecules in mast cells is thought to be specific to certain targets. The reason for such sustained stimulation of BA resulting in reduction of Erk phosphorylation remains to be elucidated. BA stimulation might inhibit Erk phosphorylation through induction of gene expression followed by *de novo* synthesis of protein or through multi-step processes. Shim et al. suggested that suppression of Erk phosphorylation led to the reduction of FcεRI expression via decreased mRNA expression of FcεRI α-chain gene (Shim et al., 2017). However, mRNA expression of FcεRIα did not significantly decrease in our study; instead, the reduction of cell surface FcεRI expression was shown to be regulated at the post-translational level. Although FcεRIα mRNA expression decreased slightly 24–48 h after stimulation, the decrease was not statistically significant. This may also be involved in the reduction in cell surface FcεRI expression in addition to post-translational regulation. Cell surface expression of FcεRI is regulated at the post-translational level by various processes, such as association of subunit proteins constituting FcεRI, transport to cell membrane, recycling, and internalization. In our study, facilitated endocytosis of FcεRI

by Erk inhibition was shown to be one of the mechanisms, but we cannot deny the possibility that other post-translational mechanisms might also be involved. It is well known that IgE acts as a critical regulator of FcεRI, which upregulates cell surface FcεRI expression through stabilization and accumulation of the receptor (Kubo et al., 2001), and thus analysis of the effect of BA in the presence of IgE may also be important.

Cell wall components of Gram-positive bacteria and probiotic *Lactobacillus* have been reported to suppress cell surface expression of FcεRI on mast cells (Yoshioka et al., 2007; Oksaharju et al., 2011). Lipoteichoic acid and peptide glycan downregulated FcεRI expression on mast cells, and particularly, the effect of lipoteichoic acid was shown to be dependent on TLR2 (Yoshioka et al., 2007). Their study reported that suppression of FcεRI expression was observed starting at 24 h after stimulation, reached a maximum at 48 h, and then recovered. In addition, this was associated with the decrease in mRNA expression of FcεRI α-chain gene. Phosphorylation of Erk is transiently induced immediately after stimulation with these ligands. Therefore, the underlying mechanism might differ between their study and our study. Since lipoteichoic acid and peptide glycan are major cell wall components of Gram-positive bacteria, some other constituent of BA, a Gram-negative bacterium, may exert a suppressive effect through post-translational mechanism associated with Erk inhibition. Probiotic *Lactobacillus*, *L. rhamnosus* GG and *L. rhamnosus* Lc705, suppressed mRNA expression

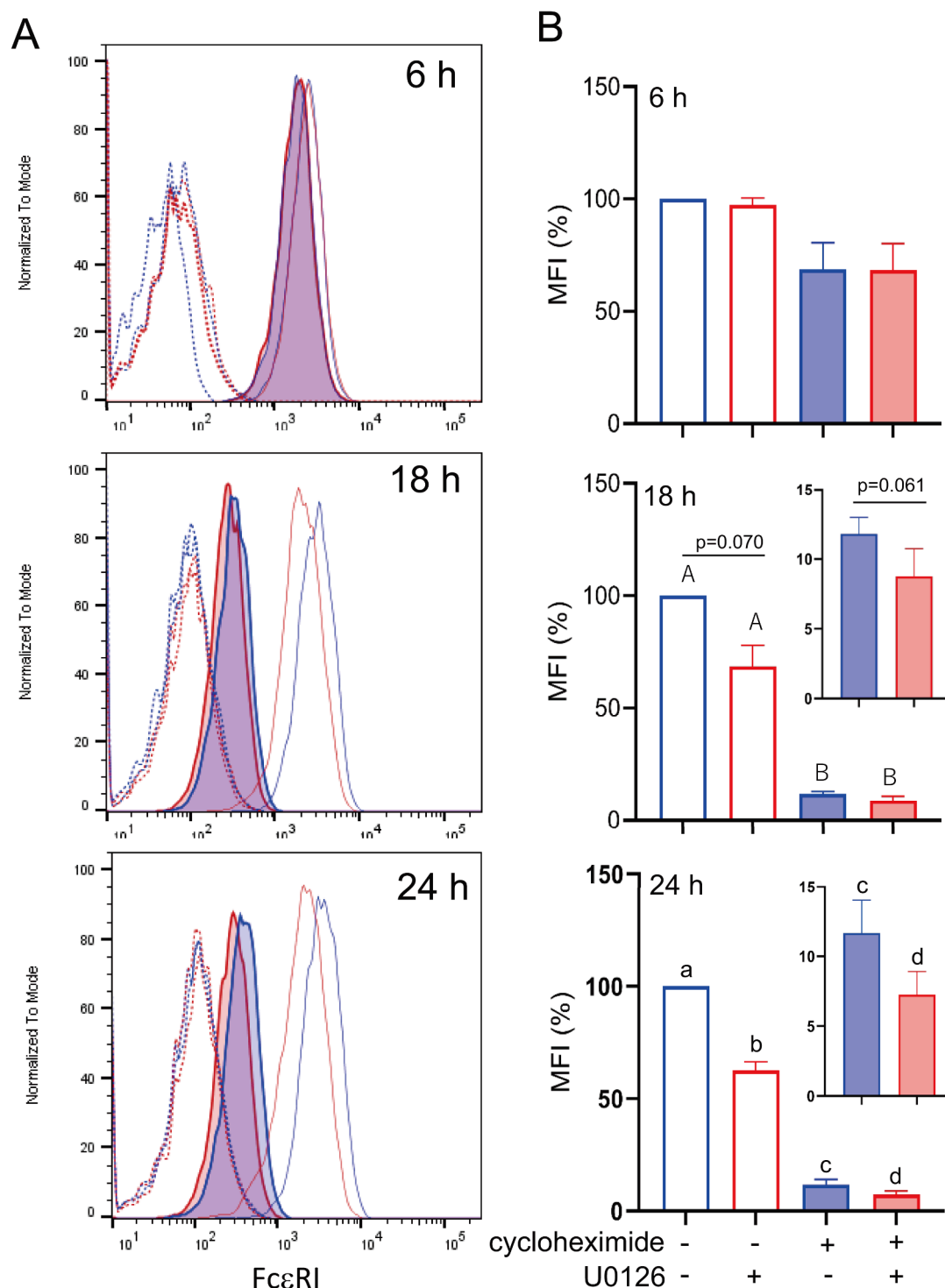


Fig. 7. Suppression of cell surface FcεRI expression is, at least in part, dependent on its facilitated endocytosis by Erk inhibition. BMMCs were treated with or without cycloheximide prior to the addition of U0126. After 6–24 h, cell surface expression of FcεRI was analyzed by flow cytometry. (A) Representative histograms of 3–6 independent experiments are shown. Dotted line, isotype control; thin line with open area, in the absence of cycloheximide; bold line with filled area, in the presence of cycloheximide. Blue, without U0126 treatment; red, with U0126 treatment. (B) Expression of FcεRI are represented as mean fluorescent intensities $\pm$ SD. Values are percentages to those of control without cycloheximide and U0126. Different letters mean statistically significant differences (A–B, $p < 0.05$; a–d, $p < 0.005$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

of FcεRI α -chain gene in cultured human mast cells (Oksaharju et al., 2011). The underlying mechanism might also be different from our study in which the mRNA expression of FcεRI α -chain gene did not significantly decrease. Since live bacteria were used in the above-cited study, the decrease in FcεRI α -chain mRNA expression could be mediated by both bacterial components and metabolites.

Emerging evidence including ours supports the interaction between mast cells and the microbiota in the intestine. Although it is not well known how often mast cells, which are frequently associated with tissues close to epithelial surfaces, directly interact with intact bacteria in the intestine, they might interact with intestinal bacterial components, such as those derived from dead bacteria, in addition to low molecular

weight metabolites. The intestinal mucosa serves as a reservoir of mast cells in the body due to its vast surface area. Hence, the effect of gut microbiota on mast cells in the intestine might have a systemic influence by migration of mast cells, whose functions are modulated by gut microbiota from the intestine to other peripheral tissues. This suggests that gut microbiota might possibly affect allergic reactions other than those in the intestine such as atopic eczema and asthma in addition to intestinal allergic reactions like food allergy. Moreover, various physiological functions of mast cells, other than induction of allergy, such as innate immune defense are attracting attention as the original physiological roles of these cells. Therefore, the effect of gut microbiota on intestinal mast cells might affect such functions throughout the body. Further studies are required to elucidate how communication with gut microbiota regulates the function of mast cells and thereby contributes to intestinal and systemic homeostasis.

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CRediT authorship contribution statement

Sakino Fukatsu: Investigation, Formal analysis. **Hikari Hori-nouchi:** Investigation, Formal analysis. **Shiho Nagata:** Investigation, Formal analysis. **Risa Kamei:** Investigation, Formal analysis. **Daichi Tanaka:** Investigation. **Wonki Hong:** Investigation. **Yui Kazami:** Investigation. **Minami Fujimori:** Investigation. **Kikuji Itoh:** Resources, Writing - review & editing. **Yoshika Momose:** Resources, Writing - review & editing. **Kazumi Kasakura:** Conceptualization, Methodology. **Akira Hosono:** Writing - review & editing. **Shuichi Kaminogawa:** Supervision, Writing - review & editing. **Shigemasa Hanazawa:** Supervision, Writing - review & editing. **Yusuke Nakanishi:** Conceptualization, Methodology. **Kyoko Takahashi:** Conceptualization, Methodology, Investigation, Formal analysis, Writing - original draft, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

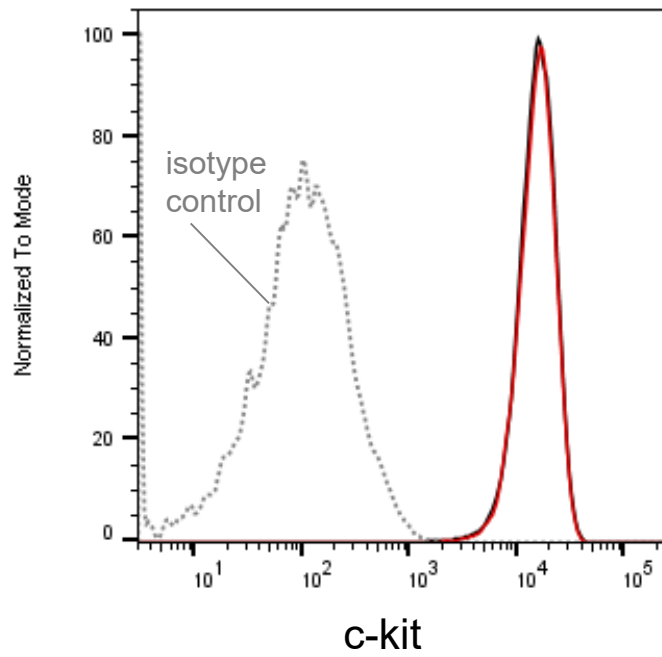
Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.imbio.2021.152056>.

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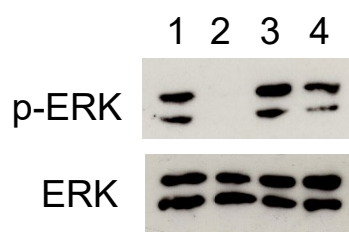
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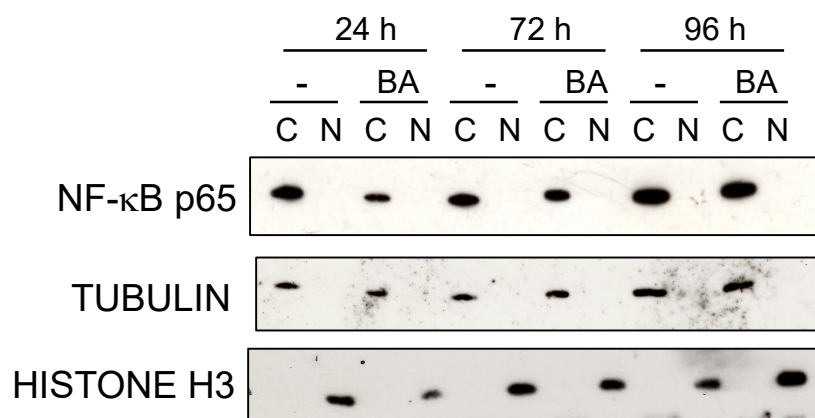
Supplementary Fig. S1

After culturing in the absence (black line) or presence (red line) of BA for 72 h, BMMCs were stained with PE-Cy7-labeled anti CD117(c-kit) antibody (Bio Legend) and analyzed by flow cytometry. Representative histogram of 3 independent experiments is shown.



Supplementary Fig. S2

BMMCs were treated with U0126, *L. johnsonii* 129, or BA for 72 h to analyze phosphorylation of Erk by western blotting. Lane 1, no treatment; lane 2, U0126; lane 3, *L. johnsonii* 129; lane 4, BA. Representative blots of 3 independent experiments are shown.



Supplementary Fig. S3

BMMCs were stimulated with BA for 24, 72, 96 h and nuclear translocation of NF- κ B was evaluated by analyzing cytoplasmic and nuclear fractions by western blotting. Representative blots of 3 independent experiments are shown.