

Citrobacter koseri stimulates dendritic cells to induce IL-33 expression via abundant ATP production

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Abstract

Introduction. Food allergies (FAs) occur due to intestinal immune dysfunction elicited by dysbiotic conditions. It was previously determined by us that *Citrobacter* species propagate in the faeces of mice with FAs and worsen allergic symptoms by inducing the allergenic cytokine IL-33. Dendritic cells can play important roles in regulation of FA responses.

Hypothesis. *Citrobacter* species propagating in intestines of mice worsen allergic symptoms by stimulating dendritic cells to induce IL-33 expression.

Aim. The aim of the present study was to analyse whether *C. koseri* stimulates dendritic cells to induce IL-33 expression.

Methodology. IL-33 expression was evaluated in a DC2.4 mouse dendritic cell line stimulated by live or heat-inactivated *C. koseri* JCM1658, ATP, LPS extracted from *C. koseri* JCM1658 or other enterobacteria by real-time PCR. The ATP concentration and number of live bacteria in the culture supernatant were measured simultaneously.

Results. Live *C. koseri* JCM1658 induced higher levels of IL-33 expression than other enterobacteria tested, but such a response was not elicited by heat-inactivated *C. koseri* JCM1658. LPS extracted from *C. koseri* JCM1658 did not induce IL-33 expression and suppressed live *C. koseri* JCM1658-induced IL-33 expression via the activation of Toll-like receptor 4 signalling. Furthermore, ATP produced by *C. koseri* JCM1658 stimulated dendritic cells to induce IL-33 expression by stimulating the P2X₇ receptor, and LPS attenuated extracellular ATP-induced IL-33 expression. *C. koseri* JCM1658 was observed to proliferate more vigorously and produce more ATP than other enterobacteria.

Conclusion. *C. koseri* acts as an allergenic bacterium through ATP production, stimulating dendritic cells to induce IL-33 expression, while LPS released from inactivated *C. koseri* JCM1658 attenuates this allergenicity.

INTRODUCTION

Food allergies (FAs) have dramatically increased in prevalence in recent decades [1]. However, their underlying cause has yet to be fully elucidated. FAs occur via disruption of the immune tolerance network in the intestine, the latter of which consists of the microbiome, mucosal epithelial cells, dendritic cells, regulatory T (Treg) cells, IgA antibodies and dietary factors [2]. Recent findings have indicated that this network can be disrupted by dysbiosis, which is a pathological alteration of the bacterial levels and/or diversity in the microbiome [3]. Dysbiosis in the intestinal microbiome alters either the effector response or the tolerant response of helper T (Th) cells, which polarize the Th1/Th2 balance to Th2 and control the balance of other Th subsets, including Th17 and Treg

in the intestine, leading to the onset of allergic responses [4]. Despite the significance of this process, the mechanism underlying the disruption of immune tolerance by dysbiotic enterobacteria is poorly understood.

The profiles of the intestinal microbiome in patients with FAs have revealed unique differences compared to the microbiome profile in healthy patients. Multiple bacterial orders of multiple bacteria, most commonly *Clostridiales* and *Lactobacillales* as well as *Bacteroidales* and *Enterobacterales*, are thought to be implicated in the development of FAs [3, 5]. The intestinal microbiome of a murine model of FAs identified several bacterial characteristics of FAs [6]. Importantly, *Citrobacter* species, Gram-negative facultative anaerobes of

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Abbreviations: ATP, adenosine triphosphate; FA, food allergy; LPS, lipopolysaccharide; Th, helper T; TLR, Toll-like receptor; Treg, regulatory T.

Enterobacteriaceae that utilize citrate as their sole carbon source [7], were found to be especially abundant in FA mice, and could stimulate expression of the allergenic cytokine IL-33 in intestinal epithelial cells. Moreover, orally administered *Citrobacter koseri* was found to exacerbate allergic symptoms by reducing the number of intestinal Th17 cells [6]. Therefore, the dysbiotic abundance of *Citrobacter* species in the intestinal microbiome is believed to be associated with the development of FAs. However, how *Citrobacter* species induce allergic responses remains largely unclear.

IL-33 is a member of the IL-1 family of cytokines. It is released from the nuclei of damaged or necrotic cells and acts as a crucial factor for the initiation and perpetuation of allergic responses via its recognition by a cell surface receptor complex consisting of ST2 and IL-1RAcP [8, 9]. IL-33 is known to be an essential cytokine not only for sensitization to allergens, in addition to the onset of allergic symptoms and their exacerbation [9, 10]. The release of IL-33 stimulates the production of the type 2 cytokines IL-5 and IL-13 from various immune cells, including ILC2s, Th2 cells, NKT cells and NK cells, and stimulates the allergenic degranulation of mast cells and basophils [8]. IL-33 expression is constitutively expressed in epithelial cells and fibroblasts, and is released in response to cellular or tissue damage or injury. Additionally, macrophages and dendritic cells are an important source of inducible IL-33 during allergic inflammation and infection [8, 9]. This inducible production is thought to be dependent on cellular activation through the recognition of mucosal surface microbes by pattern-recognition receptors, such as Toll-like receptors (TLRs) [11–13]. However, under dysbiotic conditions, the regulatory mechanism underlying the expression of this cytokine is not yet fully understood.

Dendritic cells in the mucosal lamina propria sometimes recognize microbes on the mucosal surface, allowing them to mediate allergic responses via the production of cytokines [14]. The quantity of dendritic cell-derived IL-12 determines the Th1/Th2 balance, wherein polarization to Th2 leads to the onset of allergic responses [2]. Dendritic cells also control the balance of other helper T subsets, including Th17 and Treg cells, in the intestine [15–17]. However, the relationship between dendritic cells and enterobacteria in the induction of allergic responses has yet to be elucidated. In this study, we attempted to determine whether enterobacteria can induce IL-33 expression in dendritic cells. Our results suggest strongly that *C. koseri* serves as an allergenic enterobacterium that stimulates dendritic cells via the production of plentiful amounts of extracellular ATP.

METHODS

Cell culture and reagents

A C57BL/6 mouse-derived dendritic cell line, DC2.4, was obtained from Merck-Millipore and cultured in RPMI 1640 medium (FUJIFILM Wako) supplemented with 10% heat-inactivated foetal bovine serum (FBS), 1×10^{-6} M L-glutamine, 1×10^{-6} M non-essential amino acids, 20 mM HEPES, and 0.0054×10^{-6} M β -mercaptoethanol at 37 °C in a humidified 5% CO₂

atmosphere. TAK-242, a cell-permeable compound that selectively binds to Cys747 of TLR4 and selectively disrupts its interaction with the signalling adapter molecules TIRAP and TRAM [18], was purchased from ChemScience. A-438079, a selective and competitive P2X₇ purinergic receptor antagonist [19], was purchased from Abcam. ATP (018-16911) was purchased from FUJIFILM Wako.

Bacterial strains and LPS extraction

C. koseri JCM1658 was obtained from the Cell Engineering Division of RIKEN BioResource Center. A strain of *Enterococcus gallinarum* isolated from mouse faeces was cultured as described previously [6]. A common *Escherichia coli* strain, K-12, was used. These bacterial strains were cultured at 37 °C under aerobic conditions in brain heart infusion (BHI) broth (BD). Bacterial growth was monitored by measuring the optical density at 600 nm (OD₆₀₀). Bacterial cell numbers at OD₆₀₀ were estimated by determining the number of colonies formed on BHI agar plates. Logarithmic-phase bacterial cells were collected by centrifugation at 20000 g for 2 min and washed with PBS, followed by the stimulation of DC2.4 cells at an m.o.i. of 5. LPS was extracted from the collected *C. koseri* using an LPS extraction kit (iNTRON BIOTECHNOLOGY). The concentration of LPS was measured using the ToxinSensor Chromogenic LAL Endotoxin Assay Kit (GenScript).

Extracellular ATP quantification

DC2.4 cells were inoculated in six-well plates at a density of 1.0×10^6 cells per well and were stimulated with each of the bacterial cells at an m.o.i. of 5 (5.0×10^6 c.f.u. of bacterial cells suspended in 50 μ l PBS were dropped into each well) for 24 h. The culture supernatants were then collected to determine the ATP concentration by ATP-driven luminescence using the luciferin substrate and luciferase enzyme of the BacTiter Glo Microbial Cell Viability Assay Kit (Promega), as previously described [20].

Real-time RT-PCR for IL-33 gene expression

DC2.4 cells were inoculated in six-well plates at a density of 1.0×10^6 cells per well. Pretreatment of LPS extracted from *C. koseri* (10 ng ml^{-1}), TAK-242 as an inhibitor of TLR signalling ($1 \mu\text{M}$) and A-438079 as an P2X₇ receptor antagonist ($1 \mu\text{M}$) was done for 1 h. Stimulation with bacterial cells was carried out at an m.o.i. of 5 as described above, or with ATP (1 mM) for 24 h. Non-stimulated cells served as a negative control. After the pretreatment and stimulation, cell viabilities were estimated by staining with 0.4% Trypan Blue (Thermo Fisher Scientific) and counting by using a Countess automated cell counter (Thermo Fisher Scientific). The total RNA of DC2.4 cells was isolated using TRIzol reagent (Thermo Fisher Scientific), and spectrophotometrically quantified. Subsequently, 200 ng of total RNA was retrotranscribed into cDNA using a ReverTra Ace qPCR RT kit (TOYOBO). The primer pairs for mouse *Il33* (sense 5'-GATGGGAAGAAGGTGA-3' and anti-sense 5'-TTGTGAAGGACGAAGA-3') and *Gapdh* (sense 5'-GCACAGTCAAGGCCGAGAAT-3' and anti-sense

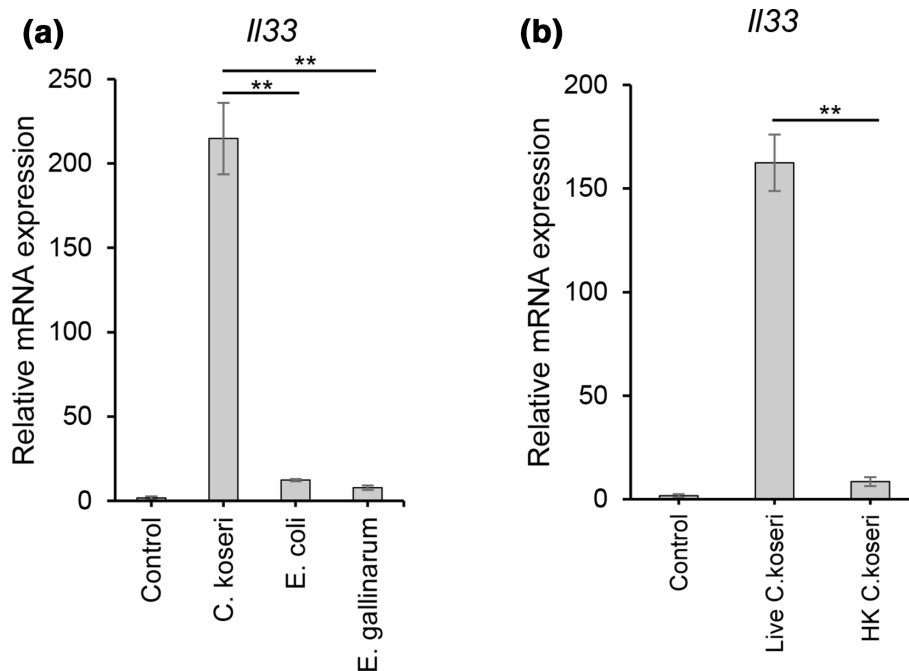


Fig. 1. Live bacterial *C. koseri* stimulates IL-33 gene expression in dendritic cells. DC2.4 cells were either stimulated with live enterobacterial cells of *C. koseri*, *Escherichia coli* and *Enterococcus gallinarum* (a), or treated with live or heat-inactivated *C. koseri* (b) at an m.o.i. of 5 for 24 h. Relative *IL33* mRNA expression levels were determined by qRT-PCR. Each value is expressed as relative mRNA expression and represents the mean $\pm$ SD ($n=3$). ** $P<0.01$.

5'-GCCTTCTCCATGGTGGTGAA-3') were used for real-time PCR. Amplification was performed using a Fast SYBR Green Master Mix (Thermo Fisher Scientific) and the Step One Plus Real-Time PCR System (Thermo Fisher Scientific). The level of *IL33* expression in each sample was normalized to that of *Gapdh* (as an internal control) and non-stimulated cell sample (as calibrator) using the comparative *Ct* method.

Statistical analysis

In all the experiments conducted, three independent experiments were performed in triplicate. All experimental data were confirmed to be normally distributed. Statistical differences were assessed using Student's *t*-test. Multiple groups were compared using a two-tailed one-way ANOVA with Dunnett's test (Figs 3b and 4a–c). *P*-values <0.01 were considered statistically significant. The analysis tool applied was Ekuseru-Toukei 2012 (version 1.0, Social Survey Research Information).

RESULTS

Live *C. koseri* induced IL-33 gene expression in dendritic cells

First, we tested the ability of enterobacteria to induce IL-33 using a cell-based infection model. Equivalent numbers of logarithmic-phase bacterial cells of three enterobacterial species (*C. koseri*, *Escherichia coli* and *Enterococcus gallinarum*) were prepared and used to stimulate a mouse dendritic cell line, DC2.4. IL-33 gene expression was found to be strongly induced

by *C. koseri*, while *Escherichia coli* and *Enterococcus gallinarum* affected IL-33 expression to a negligible degree (Fig. 1a). This result was consistent with the IL-33 expression profile of *C. koseri* reported in a previous study using an intestinal epithelial cell line [6]. Next, whether the IL-33-inducing ability of *C. koseri* involves bacterial viability was tested. As shown in Fig. 1b, the IL-33-induction ability of heat-inactivated *C. koseri* was almost completely disabled, indicating that bacterial viability, but not the recognition of bacterial antigens by dendritic cells, is essential for IL-33 expression.

Effect of *C. koseri*-derived LPS on IL-33 gene expression

Next, we examined whether LPS extracted from *C. koseri* exerted any influence on the induction of IL-33 expression. Extracted *C. koseri* LPS demonstrated a similar biological activity profile to *Escherichia coli* LPS, which was assessed using *Limulus* amoebocyte lysate tests and proinflammatory cytokine-inducing activity in RAW264.7 murine macrophages (data not shown). *C. koseri* LPS did not affect cell viability or IL-33 induction in DC2.4 cells (Fig. 2a, b). However, the pretreatment of cells with *C. koseri* LPS significantly diminished live *C. koseri*-induced IL-33 expression (Fig. 2a), indicating that LPS exerts a suppressive effect on IL-33 expression. The effect of the pretreatment of cells with a specific inhibitor of TLR4 signalling, TAK-242, was tested further. As shown in Fig. 2c, TAK-242 pretreatment significantly augmented IL-33 expression in cells stimulated with live *C. koseri*, indicating that LPS recognition of TLR4 attenuates IL-33 expression. To

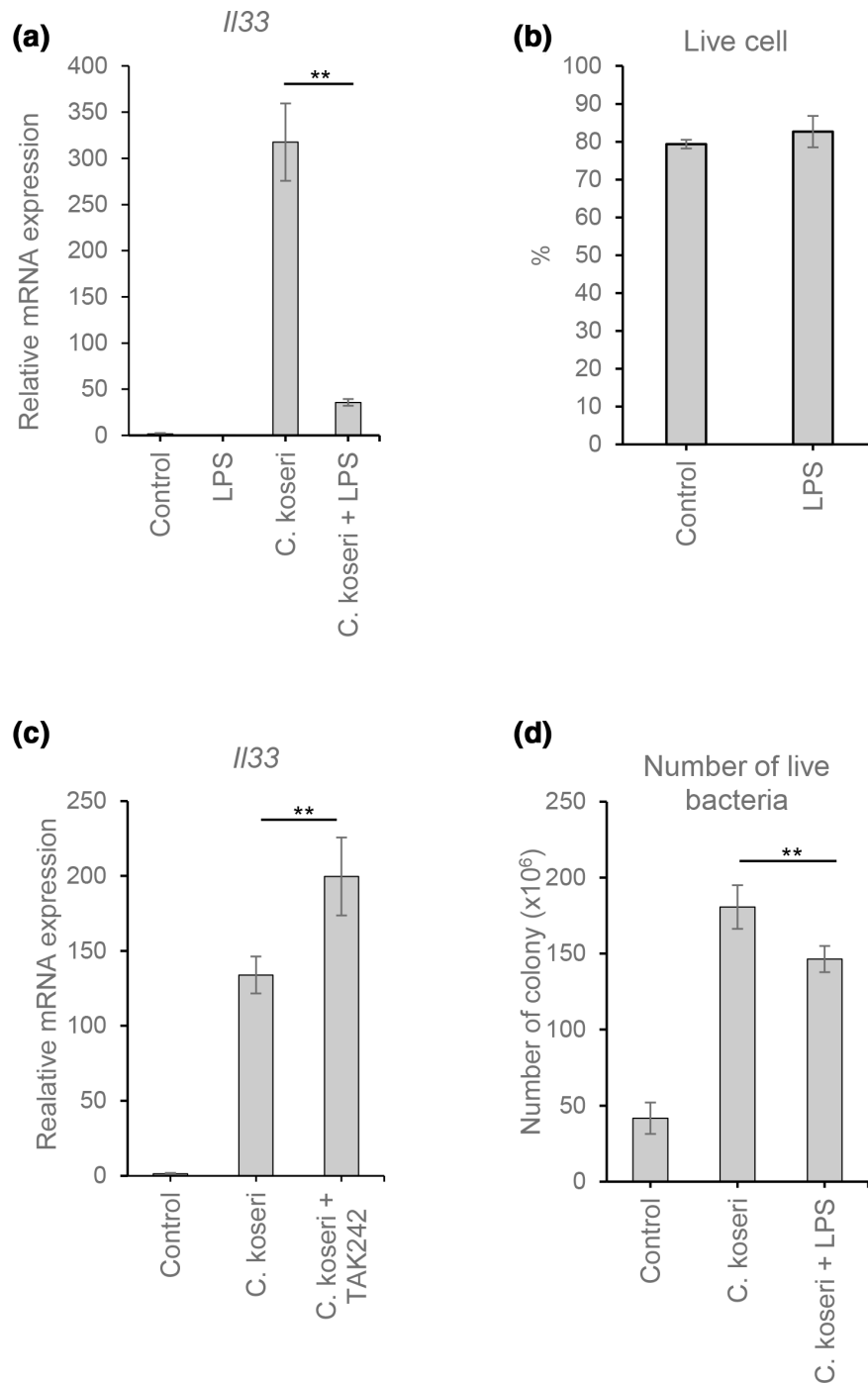


Fig. 2. TLR4 recognition of *C. koseri*-derived LPS suppresses IL-33 gene expression induced by *C. koseri*. (a) DC2.4 cells were pretreated with or without 10 ng ml⁻¹ of *C. koseri* LPS for 1 h, followed by stimulation with live *C. koseri* at an m.o.i. of 5 for 24 h. The relative expression levels of *IL33* were determined by qRT-PCR. Each value is expressed as relative mRNA expression and represents the mean $\pm$ SD ($n=3$). ** $P<0.01$. (b) DC2.4 cells were stained with 0.4% Trypan Blue and counted using the Countess automated cell counter, following LPS stimulation for 24 h. Each value is expressed as the percentage of live cells and represents the mean $\pm$ SD ($n=3$). (c) DC2.4 cells were pretreated with or without 1 mM of the TLR4 inhibitor TAK-242 for 1 h, followed by stimulation with live *C. koseri* at an m.o.i. of 5 for 24 h. The relative expression levels of *IL33* were determined by qRT-PCR. Each value is expressed as relative mRNA expression and represents the mean $\pm$ SD ($n=3$). ** $P<0.01$. (d) DC2.4 cells were pretreated with or without 10 ng ml⁻¹ of *C. koseri* LPS for 1 h, followed by stimulation with live *C. koseri* at an m.o.i. of 5 for 24 h. Thereafter, the culture supernatants were collected and diluted for inoculation and each 50 μ l of the supernatant was inoculated into BHI agar plates. After 24 h of incubation, the number of colonies formed on the plate was counted. Each value is expressed as the number of colonies and represents the mean $\pm$ SD ($n=3$). ** $P<0.01$.

further investigate the effect of LPS, we quantified the number of live bacterial cells in the culture supernatant of DC2.4 cells. LPS treatment significantly suppressed the number of *C. koseri* (Fig. 2d), implying that *C. koseri* LPS activates cellular bactericidal activity, thereby resulting in a decrease in the number of *C. koseri*, in addition to a partial reduction in their IL-33-inducing ability.

ATP production in *C. koseri* stimulated IL-33 expression

To clarify the detailed mechanism by which live *C. koseri* induces IL-33 expression in DC2.4 cells, the involvement of ATP, as well as its recognition by the P2X₇ extracellular ATP receptor, was examined. A previous study indicated that apoptotic intestinal epithelial cell-derived ATP activates mast cells to induce IL-33 expression via the P2X₇ receptor [21]. A selective P2X₇ receptor antagonist, A-438079, was employed and it was found that the pretreatment of DC2.4 cells with A-438079 suppressed IL-33 expression in a dose-dependent manner (Fig. 3a). Additionally, live *C. koseri*, but not inactivated cells, actually generated ATP in the culture supernatant of DC2.4 cells (Fig. 3b), indicating that extracellular ATP produced by live *C. koseri* induces IL-33 expression through the P2X₇ receptor. Furthermore, the effect of *C. koseri* LPS on ATP production and the induction of IL-33 expression was examined. Consistent with the results shown in Fig. 2c, the LPS treatment of cells inhibited ATP production in live *C. koseri* (Fig. 3c). Moreover, it was found that extracellularly added ATP induced IL-33 expression in DC2.4 cells, and that the LPS pretreatment of cells attenuated ATP-induced IL-33 expression (Fig. 3d). These results indicated that *C. koseri* LPS exerts an inhibitory effect on P2X₇ receptor-driven IL-33 expression.

C. koseri demonstrated a higher proliferative ability than other enterobacteria to produce more ATP

Because an ATP-producing feature is not unique to *C. koseri*, we investigated why this species alone induced IL-33 expression among the three enterobacteria tested, as shown in Fig. 1b. The concentration of extracellular ATP and the number of live bacterial cells in the culture were evaluated against each other. In agreement with the results shown in Fig. 1a, *C. koseri* was found to produce more ATP than the other enterobacteria (Fig. 4a). The number of live *C. koseri* was also significantly higher than that of the other enterobacteria (Fig. 4b). These results indicated that the vigorous proliferative activity of *C. koseri* results in the plentiful production of ATP, leading to a higher ability to induce IL-33 expression than the other enterobacteria.

DISCUSSION

Whether the relationship between *Citrobacter* species and allergic responses directly causes dysbiosis, and thereby allergies, or whether the allergic response indirectly induces dysbiosis to augment *Citrobacter* species, has yet to be fully elucidated. In the present study, it was demonstrated that large amounts of ATP produced by live *C. koseri* stimulated

dendritic cells to induce IL-33 expression. Our findings indicated that abundant production of ATP by vigorous proliferative enterobacteria exacerbates allergic responses. Moreover, vigorous proliferative bacteria may be a causative agent of dysbiosis, directly facilitating allergic responses via IL-33 production. This is supported by our previous study, in which a *Citrobacter* species was found to a predominantly proliferative enterobacteria in an FA mouse model, and the administration of *C. koseri* exacerbated allergic symptoms [5].

Citrobacter species are Gram-negative, facultative anaerobes that occasionally inhabit the gastrointestinal tract of animals and humans [7, 22]. They have been reported to be opportunistic pathogens in immunocompromised hosts [7, 23]. Importantly, *C. koseri* has been associated with the majority of *Citrobacter* infectious diseases, including neonatal meningitis, brain abscesses and pneumonia underlying pulmonary adenocarcinoma [24–26]. Moreover, *C. koseri* is believed to be a pathogen of microbial substitution disease because it carries a unique chromosomal β -lactamase, termed CKO [22, 27, 28]. Thus, *C. koseri* may act as an important indigenous pathogen, but the mechanism of virulence of *C. koseri* in allergic diseases, especially FAs, remains unclear. In the present study, *C. koseri* was found to proliferate more vigorously than other enterobacteria (*Escherichia coli* and *Enterococcus gallinarum*), which led to a higher ability to induce IL-33 expression compared to the other enterobacteria. Our results demonstrated the novel virulence of *C. koseri* to exacerbate allergic responses in the intestine. Furthermore, these results also indicated that the allergenic virulence of enterobacteria can be elicited not only through their cytotoxic activity to the epithelial cells, in addition to the regulation of allergenic cytokine production in dendritic cells.

It was demonstrated here that *C. koseri*-derived extracellular ATP directly induces IL-33 expression via the P2X₇ receptor in DC2.4 cells. Previous studies have shown that elevated IL-33 levels are released by an increase in the level of intracellular calcium, which is mediated through stimulation of the P2X₇ receptor by extracellular ATP [29, 30]. Furthermore, Shimokawa *et al.* found that mast cells can induce IL-33 production in response to extracellular ATP released from intestinal epithelial cells infected with the nematode *Heligmosomoides polygyrus* [21]. These observations imply strongly that extracellular ATP acts as a crucial factor for stimulating IL-33 induction in the intestine, although the source of ATP in these studies is host cells. It is possible that other factors of *C. koseri* are involved in IL-33 induction as co-stimuli, as the IL-33-inducing activity of extracellularly added ATP was not very strong. This represents a potential topic for future investigation.

To date, several studies have revealed the effects of extracellular ATP on dendritic cell maturation and function. Extracellular ATP released from injured cells is known to serve as a danger signal to promote the migration of dendritic cells to lymph nodes via recognition by the P2X₇ receptor [31]. With regard to the effects on Th2 responses, Sala *et al.* reported that T cells co-cultured with dendritic cells that were matured in the

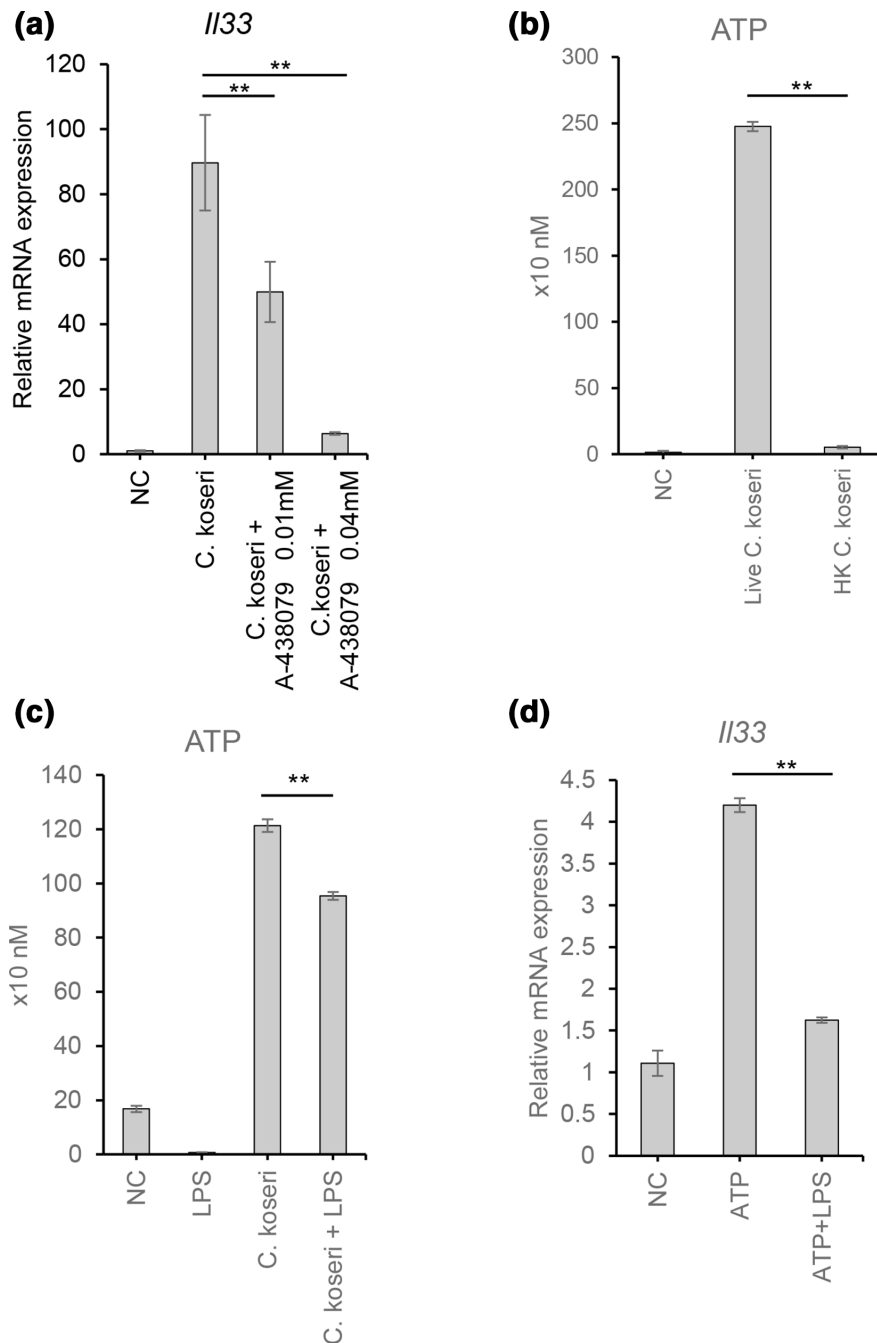


Fig. 3. ATP production from live *C. koseri* stimulates IL-33 gene expression. (a) DC2.4 cells were pretreated with or without the P2X₇ receptor antagonist A-438079 (at 10 or 40 μ M) for 1 h, followed by stimulation with live *C. koseri* at an m.o.i. of 5 for 24 h. The relative expression levels of IL33 were determined by qRT-PCR. Each value is expressed as relative mRNA expression and represents the mean $\pm$ SD ($n=3$). ** $P<0.01$. (b) DC2.4 cells were stimulated with live or heat-inactivated *C. koseri* at an m.o.i. of 5 for 24 h. Thereafter, the culture supernatants were collected to determine the ATP concentrations. Each value is expressed as $\times 10$ concentration (nM) and represents the mean $\pm$ SD ($n=3$). ** $P<0.01$. (c) DC2.4 cells were pretreated with or without 10 ng ml⁻¹ of *C. koseri* LPS for 1 h, followed by stimulation with live *C. koseri* at an m.o.i. of 5 for 24 h. Following this, the culture supernatants were collected to determine the ATP concentrations. Each value is expressed as $\times 10$ concentration (nM) and represents the mean $\pm$ SD ($n=3$). ** $P<0.01$. (d) DC2.4 cells were pretreated with or without 10 ng ml⁻¹ of *C. koseri* LPS for 1 h, followed by stimulation with 1 mM ATP for 24 h. The relative expression levels of IL33 were determined by qRT-PCR. Each value is expressed as relative mRNA expression and represents the mean $\pm$ SD ($n=3$). ** $P<0.01$. Live bacterial *C. koseri* stimulate IL-33 gene expression in dendritic cells. DC2.4 cells were either stimulated with live enterobacterial cells of *C. koseri*, *E. coli*, and *E. gallinarum* (A), or treated with live or heat-inactivated *C. koseri* (B) at MOI:5 for 24 h. The relative IL33 mRNA expression levels of IL33 were determined by qRT-PCR. Each value is expressed as relative mRNA expression and represents the mean $\pm$ SD ($n = 3$). **, $p < 0.01$.

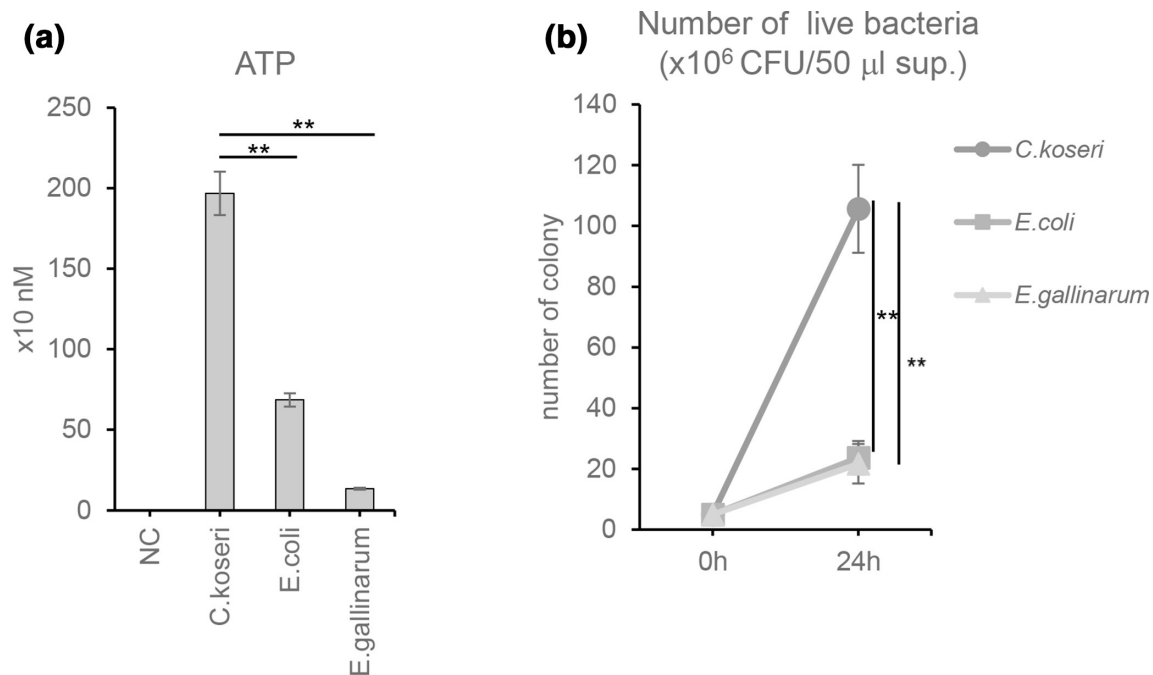


Fig. 4. *C. koseri* exhibits a vigorous proliferative activity, producing larger amounts of ATP than other enterobacteria. DC2.4 cells were stimulated with live enterobacterial cells of *C. koseri*, *Escherichia coli* and *Enterococcus gallinarum* at an m.o.i. of 5 for 24 h. (a) Culture supernatants were collected to determine the ATP concentrations. Each value is expressed as $\times 10$ concentration (nM) and represents the mean $\pm$ SD ($n=3$). $**P<0.01$. (b) Culture supernatants were collected from the DC2.4 culture before and after stimulation (0 and 24 h, respectively), and diluted for inoculation on BHI agar plates. After 24 h of incubation, the number of colonies formed on the plate was counted. Each value is expressed as the number of colonies and represents the mean $\pm$ SD ($n=3$). Circle, *C. koseri*; square, *Escherichia coli*; triangle, *Enterococcus gallinarum*; $**P<0.01$.

presence of LPS express more IL-4 and less IFN- γ than those co-cultured with LPS alone [32]. They also revealed that dendritic cells matured by LPS stimulation released high levels of IL-12, while those stimulated by ATP released low levels of IL-12 [33]. Furthermore, dendritic cells matured by ATP in addition to LPS produced higher levels of Th2 response-facilitating chemokines, MDC (macrophage-derived chemokine) and TARC (thymus- and activation-regulated chemokine), compared to those stimulated by LPS [33]. These findings indicated that the stimulation of dendritic cells by ATP facilitates Th2 responses. Conversely, extracellular ATP was reported to promote Th17 differentiation by inducing IL-6 and tumour growth factor- β in dendritic cells [34]. However, Englezou *et al.* found that the levels of extracellular ATP augmented IL-1 α , a member of the IL-1 family similar to IL-33, and decreased IL-6 from LPS-primed dendritic cells [35]. This finding implied that extracellular ATP facilitates allergic responses by regulating Th differentiation and by promoting Th2 response-inducing cytokines or chemokines.

In the present study, TAK-242 was used to inhibit TLR4 signalling [18]. TAK-242 pretreatment significantly boosted IL-33 expression in live *C. koseri*. Additionally, *C. koseri* LPS pretreatment attenuated IL-33 expression induced by ATP. These results indicated that LPS-triggered TLR4 signalling inhibits IL-33 expression, consistent with the two preceding studies. Furthermore, heat-inactivated *C. koseri* did not induce IL-33 expression. The cause of this phenomenon is ATP production

by heat-inactivated *C. koseri* as well as the release of LPS from the dead bacterial cells, which inhibited the expression induced by whole *C. koseri*. LPS has been previously reported to suppress IL-33 expression and production. Lin *et al.* reported that LPS produced by *Escherichia coli* attenuates polyI:C and human parechovirus-induced IL-33 and TSLP (thymic stromal lymphopoietin) in respiratory epithelial cells [36]. They proposed that LPS recognition by TLR4 inhibits NF- κ B and IRF3, the activation of which is known to mediate IL-33 expression. Additionally, Tada *et al.* demonstrated that TLR4-agonistic LPS derived from *Porphyromonas gingivalis* did not induce IL-33 expression in dendritic cells, but live *P. gingivalis* cells induced IL-33 [37], suggesting that *P. gingivalis* LPS inhibits IL-33-inducing activity in *P. gingivalis* cells. These observations suggest that TLR4 signalling inhibits IL-33 expression.

The limitations of the present study include the fact that a comparison of all enterobacteria to *C. koseri* with regard to IL-33 induction ability and ATP production is difficult to execute. As an immense number of enterobacterial species are anticipated to inhabit the intestinal microenvironment, this comparison is virtually unfeasible. However, comparisons with specific species of bacteria that were selected and identified in our previous study [6] would provide accurate data to assess bacterial pathogenicity in the intestines of individuals suffering from FAs. Furthermore, the intestinal environment is too complex to recreate in *in vitro* experiments. Thus, there was no other choice

except to adopt data from cell-culture experiments under the same culture conditions as in the intestinal environment. This is also a limitation of the present study.

Our results indicated that the allergenic bacterium *C. koseri* induces IL-33 expression in dendritic cells through the abundant production of extracellular ATP. Although other energetically proliferating enterobacteria may be the causative agents of FAs, our findings elucidate the mechanism by which dysbiotic enterobacteria exacerbate FAs. The effective suppression of such bacterial activity may be used in the development of effective treatments and therapeutic strategies for FAs.

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Conflicts of interest

The authors declare that there are no conflicts of interest.

Ethical statement

The present study did not include any animal experiments and clinical trials. Additionally, the study was not designed for the involvement of any genetic recombination experiments. Therefore, the present study is not governed by the regulations pertaining to experimental ethic codes.

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