Immunology
The Journal of cells, molecules, systems and technologies

British Society for immunology

IMMUNOLOGY ORIGINAL ARTICLE

Recognition of the microbiota by Nod2 contributes to the oral adjuvant activity of cholera toxin through the induction of interleukin-1β

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doi:10.1111/imm.13105
Received 29 April 2018; revised 30 June 2019; accepted 25 July 2019.
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Summary

The role of symbiotic bacteria in the development of antigen-specific immunity remains poorly understood. Previous studies showed that sensing of symbiotic bacteria by nucleotide-binding oligomerization domain-containing protein 2 (Nod2) regulates antibody responses in response to nasal immunization with antigen and cholera toxin (CT). In this study, we examined the role of the microbiota in the adjuvant activity of CT induced after oral immunization with antigen. Germ-free (GF) mice showed impaired production of antibody responses and T-cell-specific cytokines after oral immunization when compared with that observed in conventionally raised mice. Similar to GF mice, Nod2-deficient mice showed reduced humoral responses upon oral immunization with antigen and CT. Treatment with CT enhanced the production of interleukin-1β (IL-1β), but not tumor necrosis factor-α or IL-12p40, induced by stimulation of dendritic cells with muramyl dipeptide, the Nod2 ligand. Mechanistically, the enhanced production of IL-1β induced by muramyl dipeptide and CT stimulation required Nod2 and was mediated by both increased synthesis of pro-IL-1β and caspase-1 activation. Furthermore, antigen-specific antibody and cytokine responses induced by CT were impaired in orally immunized IL-1β-deficient mice. Collectively, our results indicate that Nod2 stimulation by symbiotic bacteria contributes to optimal CT-mediated antigen-specific oral vaccination through the induction of IL-1β production.

Keywords: adjuvant; cholera toxin; interleukin-1β; microbiota; Nod2; symbiotic bacteria.

Introduction

Cholera toxin (CT), an enterotoxin secreted by the bacterium *Vibrio cholerae*, is responsible for the massive diarrhea that is characteristic of cholera infection.[1] The CT protein complex is composed of one A subunit and five B subunits. The A subunit exhibits adenosine diphosphate-ribosyltransferase activity, which activates the guanine nucleotide-binding protein Gsα and, in turn, adenylate cyclase, thereby increasing intracellular levels of cyclic adenosine monophosphate (cAMP). In contrast, the B subunit plays a role in the binding of the toxin to GM1 ganglioside receptors found on the host cell surfaces.[1–4] CT is known to exhibit a potent adjuvant activity, mainly linked to the activity of its monomeric A subunit.[5] However, CT holotoxin is too toxic for human use.[1] Despite extensive studies on the mechanism underlying the adjuvant activity of CT, the lack of sufficient understanding

has hampered the development of non-toxic but effective adjuvants based on this toxin.

Cholera toxin is known to promote mucosal and systemic immune responses. Treatment with CT drives naive T cells toward T helper type 2 (Th2) type responses.[6,7] However, other reports have shown that the toxin also promotes Th1 and Th17 differentiation through a cAMP-dependent pathway, which might be required for its adjuvant activity.[8,9] CT activates T cells by inducing the production of cytokines from antigen-presenting cells and co-stimulatory molecules on the cell surface.[10] In addition, the toxin enhances the antigen presentation capacity of antigen-presenting cells, an initial step in the adaptive immune response, through a cAMP-dependent pathway.[11,12] Collectively these studies show that the effect of CT on antigen-presenting cells might be critical to its ability to promote adaptive immunity.

The microbiota plays an important role in the induction of immune cell populations, development of gut-associated lymphoid tissues, and protection against viral and bacterial infections.[13,14] Moreover, the microbiota enhances immune responses induced by unadjuvanted and inactivated influenza vaccines through stimulation of the Toll-like receptor 5 (TLR5) receptor.[15] In addition, symbiotic bacteria located in the nasal cavity enhance antibody responses induced by nasal immunization with antigen and CT.[16] Although the gastrointestinal tract is where most symbiotic bacteria reside, the contribution of the gut microbiota to oral vaccination remains unclear.

Members of the nucleotide-binding oligomerization domain (Nod)-like receptor (NLR) family function as intracellular pattern recognition receptors to activate host immunity in response to microbial products and damage-associated signals.[17] The NLR family member Nod2 recognizes the muramyl dipeptide (MDP) motif, which is conserved in peptidoglycan produced by both Gram-negative and Gram-positive bacteria.[18–20] Upon stimulation, Nod2 activates nuclear factor-κB and mitogen-activated protein kinases to induce expression of pro-inflammatory and antimicrobial molecules.[18–21] MDP exhibits adjuvant activity and induces predominant Th2 responses and, in combination with TLR agonists, promotes the production of a high level of antigen-specific antibody in mice.[22,23] In addition, Nod2-mediated recognition of the microbiota plays a critical role in the mucosal adjuvant activity of CT induced via the nasal route.[16] Here, we examined the role of the microbiota and Nod2 in antibody responses induced by oral immunization with antigen and CT.

Material and methods

Mice

Wild-type (WT), *Nod2*$^{-/-}$, and *Ilb1*$^{-/-}$ mice on a C57BL/6 background were bred and maintained under specific pathogen-free (SPF) conditions at the University of Michigan Animal Facility. C57BL/6 mice purchased from Orient Bio Inc. (Seongnam, Korea) were held under SPF conditions in an animal facility at Wide River Institute of Immunology of Seoul National University College of Medicine. *Nod2*$^{-/-}$ mice were bred and held under SPF conditions in an animal facility at Wide River Institute of Immunology of Seoul National University College of Medicine. Germ-free (GF) C57BL/6 mice were bred and maintained at the GF Animal Core Facility of the University of Michigan. Mice were allocated randomly into experimental groups. We performed all experimental procedures in accordance with protocols approved by the University Committee on Use and Care of Animals at the University of Michigan and by the Institutional Animal Care and Use Committee at the Seoul National University Hospital.

Reagents

Cholera toxin (List Biological Laboratories, Campbell, CA), human serum albumin (HSA; Talecris Biotherapeutics, Research Triangle Park, NC, or Sigma-Aldrich, St Louis, MO), MDP (Bachem, Saint Helen's, UK), Ultrapure lipopolysaccharide (LPS) (InvivoGen, San Diego, CA), Pam3 (Pam3CSK4; InvivoGen), lipoteichoic acid (InvivoGen), PolyI:C (PolyI:C HMW; InvivoGen), CpG (ODN 1826; InvivoGen), CT A subunit (List Biological Laboratories), 8-Br-cAMP (Sigma-Aldrich), *N*6-benzoyl-cAMP (BioLog Life Science Institute, Bremen, Germany), 8-CPT-2′-*O*-Me-cAMP (Enzo Life Sciences, Farmingdale, NY), and H-89 (InvivoGen) were purchased from the indicated commercial sources. Interleukin-1β (IL-1β) antibody was purchased from R&D Systems (Minneapolis, MN) and antibodies for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and β-actin were obtained from Santa Cruz Biotechnology (Dallas, TX); antibody against caspase-1 was generated in our laboratory.

Immunization protocol

We orogastrically immunized 7- to 10-week-old age- and sex-matched mice with 200 μl of distilled water containing 10 mg of HSA and 10 μg of CT by gavage. Mice were killed and then blood and spleen were obtained for analysis at 2 weeks after immunization or at the indicated time-points. For the GF experiments, all reagents were autoclaved or passed through 0·2-μm filters (Corning, Corning, NY) before immunization. No animals or samples used in the mouse experiments were excluded from the analyses.

Measurement of HSA-specific antibodies

Mouse blood samples were collected in plasma separator tubes with lithium heparin (BD Biosciences, Franklin

Lakes, NJ), and plasma was separated by centrifugation. Mouse feces were suspended in phosphate-buffered saline (100 mg/ml) by vigorous vortexing. ELISA plates were coated with HSA and levels of HSA-specific total IgG, IgA, IgG1, IgG2b and IgM were measured using an indirect ELISA method based on the manufacturer's instructions (SBA Clonotyping™ System/AP; SouthernBiotech, Birmingham, AL).

Re-stimulation of splenocytes

Splenocytes were isolated from spleens on day 14 after immunization as previously described.[24] Briefly, spleens were mashed through a cell strainer (Falcon, Thermo Fisher Scientific, Waltham, MA), and then the cells were spun down and resuspended in red blood cell lysis buffer (eBioscience, San Diego, CA). After 5 min, the cells were rinsed with complete RPMI-1640 medium containing 10% heat-inactivated fetal bovine serum, 2-β-mercaptoethanol (50 μM), L-glutamine (2 mM), sodium pyruvate (1 mM), minimum essential medium non-essential amino acids, and penicillin–streptomycin (Gibco, Waltham, MA) and pushed through the cell strainer. Isolated splenocytes (2×10^6 cells in 200 μl inoculated into each well in a 48-well plate) were resuspended in complete RPMI-1640 medium and re-stimulated with 500 μg/ml of HSA. After 4 days, culture supernatants were harvested and analyzed for cytokines by ELISA.

Bone-marrow-derived dendritic cell culture conditions and in vitro stimulation

Bone-marrow-derived dendritic cells (DCs) were generated by differentiating bone marrow progenitors isolated from femurs and tibiae of a mouse in RPMI-1640 medium containing 10% heat-inactivated fetal bovine serum, 2-mercaptoethanol (50 μM), penicillin–streptomycin and 20 ng/ml of granulocyte–macrophage colony-stimulating factor (GM-CSF; PeproTech, Rocky Hill, NJ) and were supplemented with fresh medium on days 3 and 5. On day 7 after bone marrow isolation, non-adherent cells were collected as differentiated DCs by vigorous aspiration. Dendritic cells (4×10^5 cells in 200 μl inoculated into each well in a 48-well plate) were stimulated with MDP (10 μg/ml) or LPS (100 ng/ml) and, 30 min later, CT (500 ng/ml), CT A subunit (1 μg/ml) and cAMP derivatives [8-Br-cAMP (100 μM), 6-Bnz-cAMP (50 μM) and 8-CPT-2′-O-Me-cAMP (50 μM)] were added. For selective inhibition of the protein kinase A (PKA) signaling pathway, DCs were pretreated with H-89 (10 μM) before being treated with MDP for 30 min. Culture supernatants were harvested for ELISA and immunoblotting and cell lysates were collected for immunoblotting and real-time quantitative PCR (qPCR).

Cytokine measurements

Cytokines were measured using ELISA kits according to the manufacturer's instructions (R&D Systems).

Immunoblotting

Cells were lyzed in RIPA buffer supplemented with complete protease inhibitor cocktail (Roche, Basel, Switzerland). Supernatants were mixed with loading buffer and then separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes (Millipore, Billerica, MA). Membranes were incubated with antibodies against all forms of IL-1β or caspase-1 (1 : 1000 dilution). Protein bands were detected using an ECL kit (Thermo Fisher Scientific). Membranes were removed using restore stripping buffer (Thermo Scientific) and re-probed with antibody against GAPDH (1 : 5000) or β-actin (1 : 5000) as a loading control. The intensities of blots were measured by using the IMAGEJ program (National Institutes of Health, Bethesda, MD). Intensities of all samples were normalized by those of their loading control and then the final numerical values were calculated as relative values against the mean value of Nod2-deficient samples.

Real-time qPCR

RNA was extracted using an E.Z.N.A. Total RNA Kit I (Omega bio-tek, Norcross, GA) and cDNA was then generated from the isolated RNA using a High-capacity RNA-to-cDNA Kit (Applied Biosystems, Foster City, CA). The cDNA was used for real-time qPCR with specific primer sets and SYBR Green PCR Master Mix (Applied Biosystems) according to the manufacturer's instruction for StepOnePlus Real-Time PCR systems (Applied Biosystems). The real-time qPCR primers (Invitrogen, Carlsbad, CA) were as follows: IL-1β (5′-CAACCAACAAGTGATATTCTCCATG-3′ and 5′-GATCCACACTCTCCAGCTGCA-3′) and GAPDH (5′-TGCGACTTCAACAGCAACTC-3′ and 5′-GCCTCTCTTGCTCAGTGTCC-3′). The real-time qPCR conditions for mRNA quantification were 95° for 10 min, followed by 40 cycles of denaturation at 95° for 15 seconds and annealing and extension at 60° for 1 min. The cycle threshold (C_t) values of respective samples were normalized internally using the average C_t value of GAPDH.

Statistical analyses

Statistical analyses were performed using GRAPHPAD PRISM software (GraphPad Software, San Diego, CA). For cytokine comparisons, linear regression with a 95% confidence interval, and unpaired, two-tailed Student's *t*-test were used. We examined differences in the results

between groups of individual animals using the non-parametric Mann–Whitney test. No samples or animals were excluded from the analyses. Differences with $P < 0{\cdot}05$ were considered statistically significant.

Results

Symbiotic bacteria contribute to the efficacy of oral immunization with antigen and cholera toxin

We previously showed that antibiotic treatment reduces the efficacy of oral immunization with the model antigen, HSA and CT as an adjuvant.[16] Because treatment with antibiotics does not completely deplete bacteria in the gastrointestinal tract,[25] we compared immune responses between SPF and GF mice after oral immunization with HSA and CT. Consistent with previous results following antibiotic treatment,[12,16] the amounts of HSA-specific IgG in the plasma of GF mice were reduced when compared with those observed in conventionally raised SPF mice (Fig. 1a). In addition, when mice were given HSA or CT alone, the SPF mice failed to produce HSA-specific IgG (see Supplementary material, Fig. S1). These data confirm that commensal bacteria play an important role in the CT-mediated adjuvant effect on orally injected antigen. Moreover, *ex vivo* re-stimulation of splenocytes derived from SPF mice with HSA showed increased production of the Th1 cytokine interferon-γ, the Th2 cytokine IL-5 and the Th17 cytokine IL-17 in splenocytes, whereas the induction of T-cell cytokines was significantly suppressed in splenocytes from GF mice (Fig. 1b). These results indicate that symbiotic bacteria play an important role in promoting the adjuvant activity of CT after oral immunization.

Nod2 receptor contributes to the adjuvant activity of cholera toxin

To investigate the contribution of Nod2 to oral immunization with antigen and CT, WT and *Nod2*$^{-/-}$ mice were orogastrically immunized with HSA and CT. The analysis showed impaired antigen-specific antibody production in plasma from *Nod2*$^{-/-}$ mice when compared with that in WT mice (Fig. 2a, and see Supplementary material, Fig. S2). In addition, the amounts of antigen-specific IgA in the feces of *Nod2*$^{-/-}$ mice were less than in the feces of WT mice (Fig. 2b). In line with these antibody responses, the production of IL-5 and IL-17, induced by antigen re-stimulation, was lower in splenocytes from *Nod2*$^{-/-}$ mice than in splenocytes from WT

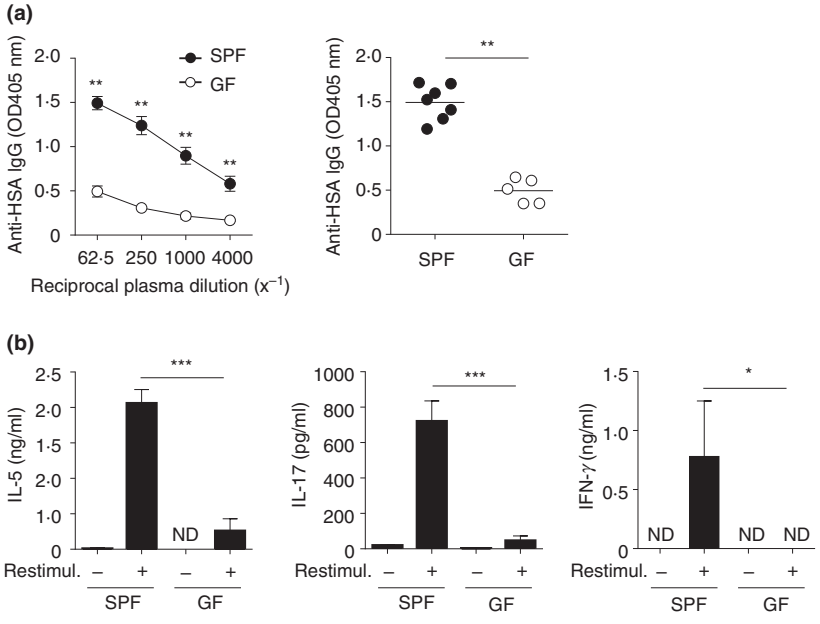

Figure 1. Symbiotic bacteria are critical for the oral adjuvant activity of cholera toxin. (a) The levels of human serum albumin (HSA) -specific IgG were analyzed in plasma obtained from germ-free (GF) ($n = 7$) and conventionally raised (specific pathogen-free; SPF) ($n = 5$) mice on day 14 after oral immunization with 10 mg of HSA and 10 μg of cholera toxin (CT). The left panel shows the relative amounts of antigen-specific IgG in the serially diluted plasma as means ± SEM of the optical density at 405 nm (OD405 nm). The right panel displays HSA-specific IgG in the plasma diluted 62·5-fold. Each dot in the right panel represents an individual mouse and the means are displayed as a line. (b) Splenocytes isolated from immunized GF and SPF mice on day 14 after immunization were re-stimulated with 500 μg/ml of HSA. Interleukin-5 (IL-5), IL-17 and interferon-γ (IFN-γ) were measured in triplicate supernatant samples on day 4 after stimulation; values represent means ± SD. The results are representative of two independent experiments. $^{*}P < 0{\cdot}05$, $^{**}P < 0{\cdot}01$ and $^{***}P < 0{\cdot}001$ by Mann–Whitney test (a) and two-tailed Student's *t*-test (b). ND, not detected.

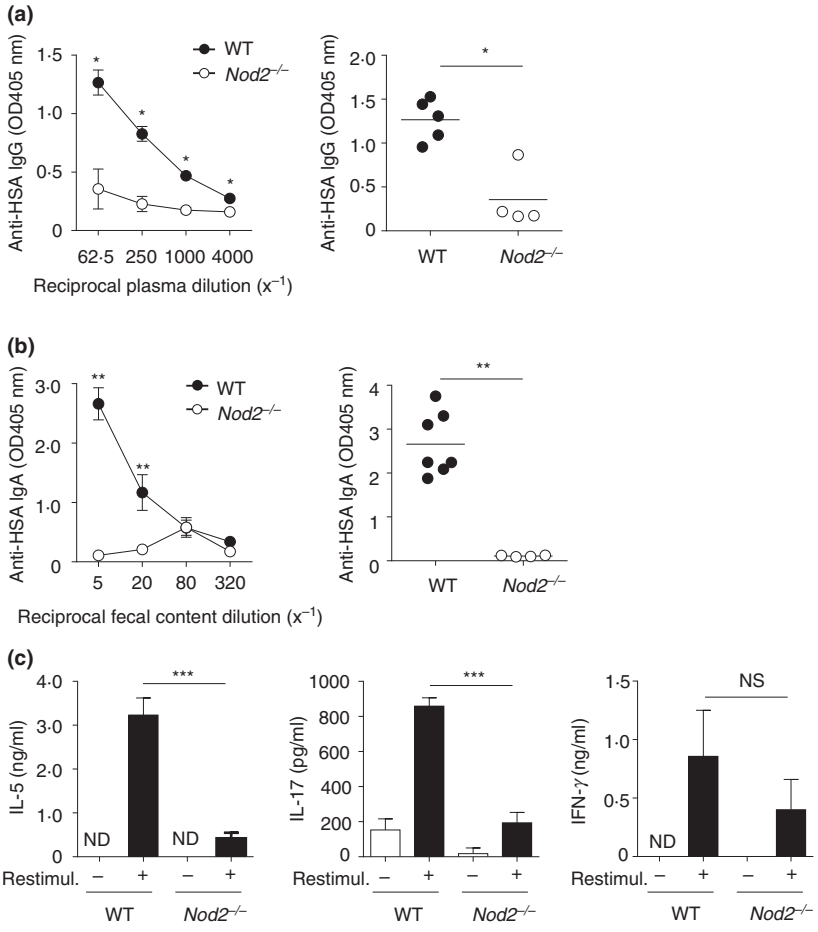

Figure 2. Nod2 plays a crucial role in promoting the adjuvant activity of cholera toxin. (a, b) Wild-type (WT) (n = 5 or 7) and *Nod2*$^{-/-}$ (n = 4) mice were orogastrically immunized with 10 mg of human serum albumin (HSA) and 10 μg of cholera toxin (CT), and plasma and feces were collected on day 14 after oral immunization. The relative amounts of antigen-specific IgG (a) and IgA (b) were measured in serially diluted plasma and feces (100 mg/ml in phosphate-buffered saline), respectively, and the results are displayed in the left panels. Right panels show HSA-specific IgG (a) and IgA (b) in the plasma diluted 62·5-fold and 5-fold, respectively. Each dot in the right panels represents an individual mouse, and the means are displayed as a line. (c) Splenocytes were isolated from WT and *Nod2*$^{-/-}$ mice on day 14 after immunization and then re-stimulated with or without HSA for 4 days. The amounts of interleukin-5 (IL-5), IL-17 and interferon-γ (IFN-γ) in triplicate supernatant samples were measured by ELISA. The results are representative of two (b) or three (a, c) independent experiments. Data are shown as means ± SEM (a, b) or ±SD (c). *$P < 0{\cdot}05$, **$P < 0{\cdot}01$ and ***$P < 0{\cdot}001$ by Mann–Whitney test (a, b) and by two-tailed t-test (c). ND, not detected. NS, not significant.

mice (Fig. 2c). The amount of interferon-γ produced after antigen re-stimulation was also reduced in Nod2-deficient splenocytes when compared with that in WT splenocytes, although the difference was not statistically significant (Fig. 2c). These data suggest that Nod2 recognition of the symbiotic bacteria is important for the adjuvant activity induced by oral immunization with CT.

Nod2 ligand and cholera toxin act synergistically to enhance IL-1β production in DCs

Because Nod2 in CD11c^{+} cells plays an important role in the adjuvant activity of CT administered via the nasal route,[16] we examined the regulation of cytokine production by the Nod2 ligand MDP and CT in *in vitro* experiments using bone-marrow-derived DCs. To mimic conditions in the intestinal tract after oral immunization, we pretreated DCs with microbe-associated molecular patterns for 30 min and then stimulated the cells with CT. Sequential treatment with MDP and CT led to a synergistic induction of IL-1β secretion, but not of tumor necrosis factor-α (Fig. 3a). Unlike IL-1β, which was not induced by treatment of MDP alone, IL-12p40 was induced by single treatment with both MDP and CT. Increased secretion of IL-12p40 from cells sequentially treated with MDP and CT can be ascribed to cumulative stimulation by MDP and CT, rather than to synergistic enhancement (Fig. 3a). In contrast to MDP, the ability of multiple TLR agonists including those for TLR1/2, TLR2/6, TLR3, TLR4 and TLR9 to stimulate the production of IL-1β was minimally or not enhanced by CT (see Supplementary material, Fig. S3a,b). To examine the kinetics and Nod2-dependence of IL-1β production by MDP and CT stimulation, we pretreated DCs from WT and *Nod2*$^{-/-}$ mice with MDP or medium and then treated the cells with CT or control. Treatment with MDP and CT induced IL-1β production in a synergistic manner compared with treatment with MDP or CT alone in WT DCs (Fig. 3b). In contrast, the enhancement of IL-1β production by MDP and CT was not observed in DCs from *Nod2*$^{-/-}$ mice (Fig. 3b).

CT enhances IL-1β production by increasing pro-IL-1β expression and caspase-1 activation

To understand the mechanism by which CT enhances the secretion of IL-1β in MDP-stimulated DCs, we first assessed the production of pro-IL-1β in DCs pre-

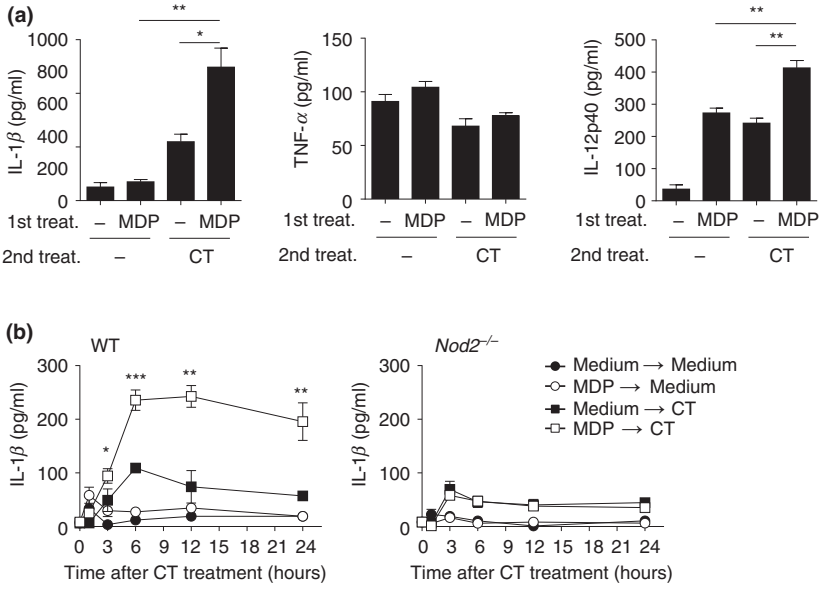

Figure 3. Nod2 ligand and cholera toxin act synergistically to enhance IL-1β production in dendritic cells (DCs). (a, b) Bone-marrow-derived DCs from wild-type (WT) (a, b) and *Nod2*$^{-/-}$ (b) mice were first treated with muramyl dipeptide (MDP) (10 μg/ml) or left untreated, and, 30 min later, the cells were either stimulated with cholera toxin (CT0 (0·5 μg/ml) or left unstimulated. The supernatants were collected at 24 hr (a) or at the indicated time-points (b) after CT addition. The indicated cytokines in triplicate supernatant samples were measured by ELISA. Results are representative of two (b) or three (a) independent experiments. Data are shown as means ± SD. *$P < 0{\cdot}05$, **$P < 0{\cdot}01$ and ***$P < 0{\cdot}001$ by two-tailed *t*-test for comparisons between all samples (a) and between sequential treatment with MDP and CT and treatment with CT alone (b). ND, not detected.

stimulated with MDP or LPS followed by treatment with CT by immunoblotting. In the absence of CT stimulation, there was little or no induction of pro-IL-1β by MDP whereas LPS induced pro-IL-1β (Fig. 4a). In contrast, CT treatment induced robust production of pro-IL-1β in DCs pretreated with MDP compared with DCs treated with CT alone (Fig. 4a). Importantly, the enhancement of pro-IL-1β production in MDP-stimulated DCs by CT was not observed in the absence of Nod2 (Fig. 4a), indicating that the synergism between MDP and CT requires the expression of Nod2 in DCs. In contrast to MDP, treatment with CT did not enhance the production of pro-IL-1β induced by LPS (Fig. 4a), consistent with previous results (see Supplementary material, Fig. S2). To determine whether CT enhances the expression of *Il1b* mRNA, we pretreated DCs from WT and *Nod2*$^{-/-}$ mice with MDP or medium and then treated the cells with CT or not. Sequential treatment with MDP and CT induced *Il1b* mRNA in a synergistic manner compared with treatment with MDP or CT alone in WT DCs (Fig. 4b), which parallels that observed with IL-1β protein release (Fig. 3b). As expected, the enhancement of *Il1b* mRNA production by MDP and CT was not observed in DCs from *Nod2*$^{-/-}$ mice (Fig. 4b).

The secretion of mature IL-1β is regulated through a two-step process that includes the induction of pro-IL-1β and the proteolytic activation of caspase-1, which cleaves pro-IL-1β.[26] To determine whether MDP and CT also regulate the activation of caspase-1, we assessed the processing of caspase-1 and production of pro-IL-1β in DCs in the presence and absence of MDP and/or CT stimulation by immunoblotting. Consistent with results shown in Fig. 4(a), CT enhanced the production of pro-IL-1β in DCs pre-stimulated with MDP (Fig. 4c). Importantly, treatment with CT induced the proteolytic activation of caspase-1 in untreated and MDP-treated DCs (Fig. 4c). These results indicate that CT regulates the release of IL-1β by enhancing both the production of pro-IL-1β and inducing the activation of caspase-1 in MDP-stimulated DCs.

CT enhances the production of IL-1β via cAMP/PKA signaling

Cholera toxin stimulates adenylate cyclase to increase the intracellular cAMP concentration, which has been linked to its adjuvant activity.[1,4] Elevated intracellular cAMP in turn stimulates PKA or Rap guanine nucleotide exchange factors [also known as exchange protein directly activated by cAMP (Epac) 1 and 2], which regulate specific cellular functions.[9,27] To determine whether cAMP, PKA and/or Epac are involved in MDP and CT-mediated IL-1β induction, we treated DCs with cell-permeable cAMP analog (8-bromo-cAMP), PKA activator (N^6-benzoyl-

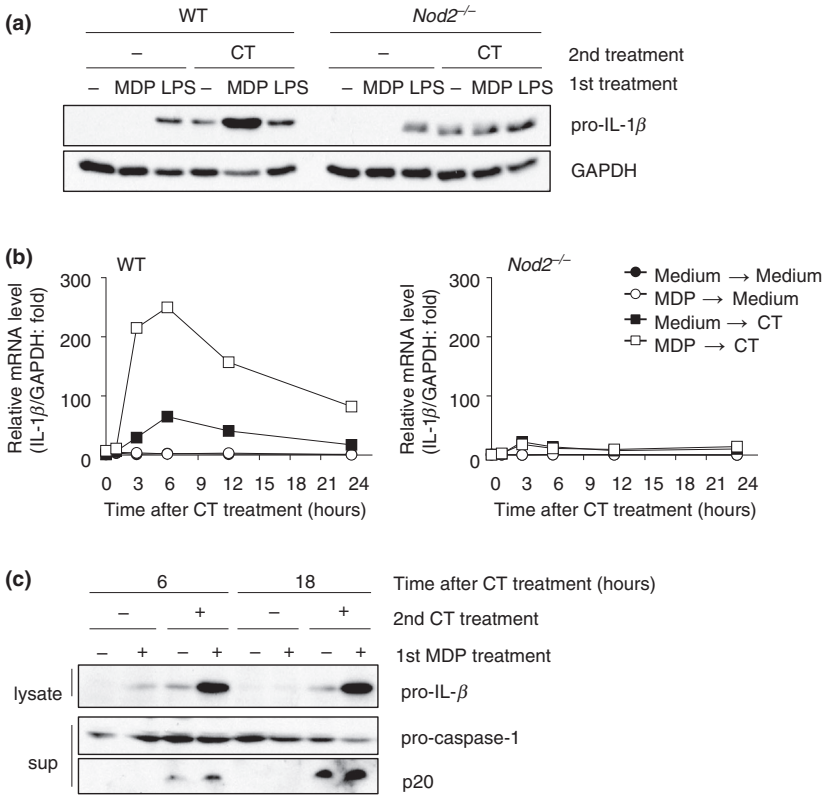

Figure 4. Cholera toxin (CT) enhances interleukin-1β (IL-1β) production by increasing pro-IL-1β expression and caspase-1 activation. (a–c). Bone-marrow-derived dendritic cells from wild-type (WT) (a–c) and *Nod2*$^{-/-}$ (a,b) mice were treated with muramyl dipeptide (MDP) (10 μg/ml), lipopolysaccharide (LPS) (100 ng/ml) or left untreated, and, 30 min later, the cells were either stimulated with CT (0·5 μg/ml) or left unstimulated. Samples were collected at 18 hr (a) or at the indicated time-points (b, c) after CT addition. (a, c) Pro-IL-1β and GAPDH were detected in cell lysates and pro-caspase-1 and p20 (the active form of caspase-1) were analyzed in supernatants by immunoblotting. GAPDH was used as a loading control. (b) The amount of *Il1b* mRNA was determined by real-time quantitative PCR and normalized relative to *Gapdh* expression. Data are shown as means ± SD. Results are representative of two independent experiments.

cAMP), or Epac activator (8-CPT-2′-*O*-Me-cAMP) after pretreatment with MDP. Like CT, treatment with 8-bromo-cAMP or N^6-benzoyl-cAMP enhanced the ability of MDP to induce the secretion of IL-1β (Fig. 5a). In contrast, treatment with 8-CPT-2′-*O*-Me-cAMP did not enhance the secretion of IL-1β (Fig. 5b). These results suggest that CT enhances pro-IL-1β production via cAMP and PKA, but not Epac activation. Consistent with these observations, treatment with 8-bromo-cAMP or N^6-benzoyl-cAMP, but not 8-CPT-2′-*O*-Me-cAMP, enhanced the ability of MDP to induce the production of pro-IL-1β (Fig. 5b). To confirm the role of the PKA signaling pathway in IL-1β induction by MDP and CT, DCs were treated with a selective inhibitor of PKA, H-89, before sequential stimulation with MDP and CT. Consistent with results obtained with the use of an activator of PKA, the ability of CT to synergize with MDP in the production of IL-1β in DCs was suppressed by pretreatment with H-89 (Fig. 5c). Collectively, these results indicate that CT enhances the production of IL-1β in MDP-stimulated DCs through cAMP/PKA signaling.

Nod2 is required for IL-1β induction after oral immunization with antigen and cholera toxin

To determine whether the production of IL-1β is induced in response to oral immunization *in vivo*, we orogastrically immunized mice with HSA and CT, and then the spleens were isolated at the indicated time-point to examine the levels of *Il1b* mRNA. The amounts of *Il1b* mRNA in the spleens increased by 3 days following oral immunization and declined to basal levels by 9 days following oral immunization (Fig. 6a). To examine whether Nod2 is involved in the induction of IL-1β, the expression of pro-IL-1β was examined in the spleens from WT and *Nod2*$^{-/-}$ mice on day 3 after oral immunization with antigen and CT. As shown in Fig. 6(b), pro-IL-1β expression was markedly reduced in the spleens of *Nod2*$^{-/-}$

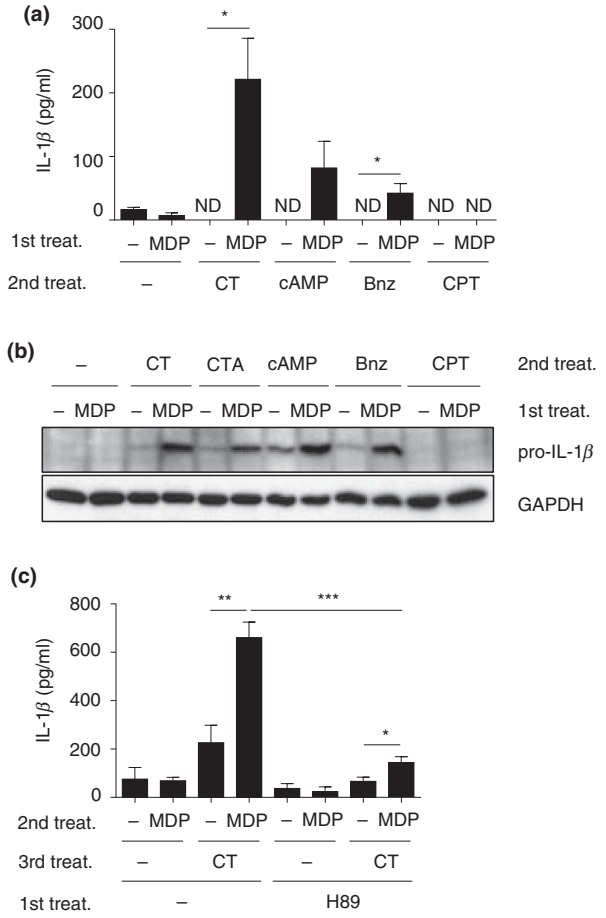

Figure 5. Cholera toxin (CT) enhances the production of interleukin-1β (IL-1β) in muramyl dipeptide (MDP) -stimulated dendritic cells (DCs) via cAMP/protein kinase A (PKA) signaling. (a–c) Bone-marrow-derived dendritic cells were treated with MDP (10 μg/ml) or left untreated, and, 30 min later, the cells were either stimulated with CT (0·5 μg/ml), CT A subunit (CTA; 1 μg/ml), 8-Br-cAMP (cAMP; 100 μM), 6-Bnz-cAMP (Bnz; 50 μM), 8-CPT-2′-*O*-Me-cAMP (CPT; 50 μM) or left unstimulated. (c) Bone-marrow-derived dendritic cells were pretreated with the PKA inhibitor H-89 (10 μM) at 30 min before sequential addition of MDP and CT. Cell lysates and supernatants were collected at 18 hr (b) or 24 hr (a, c) after CT addition, respectively. (a, c) IL-1β in triplicate supernatant samples was measured by ELISA. (b) Pro-IL-1β and GAPDH were detected in cell lysates by immunoblotting. GAPDH was used as a loading control. (a, c) Data are shown as means ± SD. ND, not detected. *$P < 0{\cdot}05$, **$P < 0{\cdot}01$ and *** $P < 0{\cdot}001$ by two-tailed Student's *t*-test for comparisons between sequential treatment with MDP and CT and treatment with CT alone or between sequential treatments with MDP and CT.

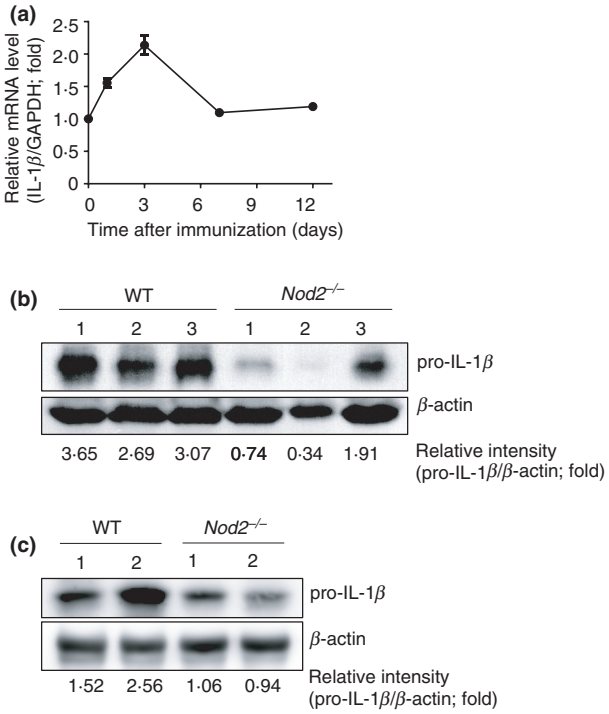

Figure 6. Interleukin-1β (IL-1β) expression induced by oral immunization with antigen and cholera toxin (CT) depends on *Nod2*. (a–c) Wild-type (WT) ($n = 5$, $n = 3$, or $n = 2$) (b, c) and *Nod2*$^{-/-}$ ($n = 3$ or $n = 2$) mice were orogastrically immunized with human serum albumin (HSA) and CT, and spleens were collected at the indicated time-points (a) and on day 3 (b) after oral immunization. (c) Peyer's patches were isolated from ileum on day 1 after oral immunization. (a) *Il1b* mRNA expression was analyzed by real-time quantitative PCR. *Gapdh* expression was used to normalize data. (b, c) Pro-IL-1β protein was detected by immunoblotting, with β-actin as an internal control. Each lane represents an individual mouse. The relative intensities of pro-IL-1β blots indicated below each lane were normalized by their β-actin amounts and then were displayed as relative values against the mean value of *Nod2*-deficient samples. The results are representative of two independent experiments.

mice compared with those of control WT mice. Likewise, when we investigated Peyer's patches isolated from ileum on day 1 after oral immunization, deficiency of Nod2 reduced the pro-IL-1β expression compared with that of control Peyer's patches (Fig. 6c). These results suggest that oral immunization with antigen and CT increases IL-1β expression in the spleen and Peyer's patches via Nod2.

IL-1β is important for the adjuvant activity of CT

To determine the role of IL-1β in the adjuvant activity of CT, we orogastrically immunized WT and *Il1b*$^{-/-}$ mice with HSA and CT. Similar to what was observed in *Nod2*$^{-/-}$ mice, the production of antigen-specific IgG was impaired in *Il1b*$^{-/-}$ mice compared with that detected in WT mice (Fig. 7a). Moreover, the production of T-cell cytokines, interferon-γ, IL-5 and IL-17, which was increased by *ex vivo* antigen re-stimulation, was markedly reduced in splenocytes from IL-1β-deficient mice when compared with splenocytes from WT mice (Fig. 7b).

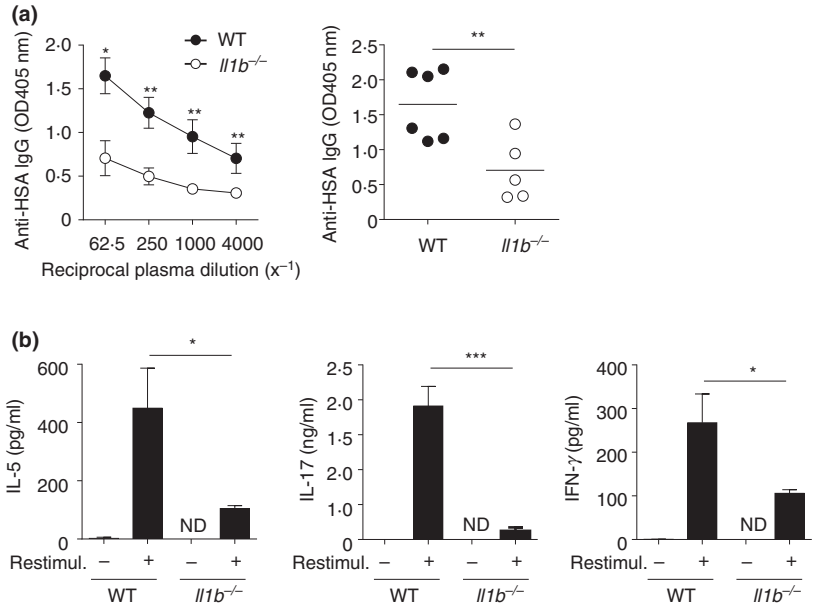

Figure 7. Interleukin-1 β (IL-1β) plays a crucial role in promoting the adjuvant activity of cholera toxin (CT). (a) The relative amounts of human serum albumin (HSA) -specific IgG were measured in the plasma of wild-type (WT) ($n = 6$) and *Il1b*$^{-/-}$ ($n = 5$) mice on day 14 after oral immunization with HSA and CT. The left panel shows the relative amounts of antigen-specific IgG in the serially diluted plasma as means ± SEM. The right panel displays antigen-specific IgG in the plasma diluted 62·5-fold. Each dot in the right panel represents an individual mouse, and the means are displayed as a line. (b) Splenocytes were isolated from WT and *Il1b*$^{-/-}$ mice on day 14 after immunization and then re-stimulated with HSA or left unstimulated for 4 days. Interleukin-5 (IL-5), IL-17 and interferon-γ (IFN-γ) in triplicate supernatant samples were measured by ELISA. Data are shown as means ± SD. The results are representative of three independent experiments. $^{*}P < 0{\cdot}05$, $^{**}P < 0{\cdot}01$ and $^{***}P < 0{\cdot}001$ by Mann–Whitney test (a) and by two-tailed Student's *t*-test (b). ND, not detected.

These results indicate that IL-1β plays an important role in the adjuvant activity of CT administered orally.

Discussion

The efficacy of mucosal vaccines differs in various geographical regions.[28,29] There have been several hypotheses to explain the variation in immune responses after mucosal vaccination. For example, malnutrition may contribute to oral vaccination in developing countries.[30] In addition, an unsanitary environment in developing countries could lead to persistent exposure of people to various enteric pathogens, which may be associated with reduced efficacy of mucosal vaccines.[31] A major finding of this study is that the microbiota plays a crucial role in oral immunization with antigen and CT. Hence, our results raise the possibility that differences in the composition of the microbiota among individuals may affect the efficacy of oral vaccination. The composition of the gut microbiota varies widely among individuals and can be affected by various environmental factors, including dietary habits, pathogen infections and geographical location.[32] Well-designed epidemiological studies are needed to examine the effects of the microbiota on vaccination efficacy and whether the microbiota plays a role; it will be important to design appropriate vaccine protocols to overcome any limitations to elicit protective immune responses due to specific microbiota profiles.

Successful vaccination is the result of orchestrated innate and adaptive immune responses.[33] Given that innate immune signals modulate the magnitude, quality and duration of adaptive responses,[33] many microbe-associated molecular patterns, which promote innate immune signals through host pattern recognition receptors,[34] have been studied as adjuvant candidates and, among them, a TLR ligand adjuvant has been approved for clinical use with a human papillomavirus vaccine.[33,35] MDP, a Nod2 ligand, was originally identified as the active component in the adjuvanticity of complete Freund's adjuvant and several studies have reported its potential for use as a non-mucosal adjuvant.[22,36] We recently showed that, upon immunization via the nasal route, MDP enhanced the adjuvant activity of CT, even though MDP itself has no mucosal adjuvant activity.[16] Likewise, the significance of the Nod2 receptor in the adjuvant activity of CT via the oral route was confirmed in this study, which suggests that MDP delivered in concert with CT may exhibit potent mucosal adjuvant activity. Hence, the development of less pyrogenic and more potent adjuvants based on derivatives of CT and MDP should be considered. For example, like CTA1-DD, a fusion protein of CT A subunit and the cell-binding domain of *Staphylococcus*

aureus,[37] a molecule that combines the CT A subunit and MDP derivatives might be effective in providing enhanced adjuvant activity.

Dendritic cells and macrophages are important mediators of the adjuvant activity of CT.[38,39] Consistently, expression of Nod2 receptor in $CD11c^+$ cells, including DCs, is required for optimal elicitation of an adaptive immune response by nasal immunization with antigen and CT.[16] In the current work, we show that Nod2 is also important for the induction of antibody responses in response to oral immunization with antigen and CT. Furthermore, we found that IL-1β is a critical mediator of the adjuvant activity of CT induced via the oral route. Mechanistically, the production of IL-1β was induced synergistically by stimulation of DCs with the Nod2 agonist and CT via induction of pro-IL-1β and caspase-1 activation. These observations suggest that CT induces its adjuvant activity, at least in part, by enhancing the Nod2-stimulatory activity of the microbiota to induce IL-1β in intestinal DCs. IL-1β is a pleiotropic cytokine that has a pivotal role in the onset and development of immune responses.[40] In line with our observations, administration of recombinant IL-1β induces adjuvant activity in both mucosal and systemic immunization protocols.[41–43] Even recombinant *Lactobacillus casei*, which was engineered to produce biologically active IL-1β, can function as an adjuvant in oral immunization.[44] However, the use of IL-1β as an adjuvant has never been pursued because of its overwhelming inflammatory effects.[42] CT induced IL-1β production at least in part by enhancing the expression of pro-IL-1β in MDP-stimulated DCs via a cAMP–PKA-dependent pathway. Many other adjuvants originating from bacterial toxins, such as heat-labile toxin from *Escherichia coli*, and pertussis toxin and adenylate cyclase toxin from *Bordetella pertussis*, either directly or indirectly increase the concentration of intracellular cAMP.[5,45–47] Although correlation between the increased cAMP and their adjuvanticity is controversial,[46,47] our observations with CT suggest that Nod2 ligand or bacteria having high Nod2 activity could also improve the adjuvant activity of others toxins through induction of IL-1β production.

Author Contributions

DK and GN conceived this study. DK performed most of the experiments. YMK and SUS helped with the experiments. WUK and JHP helped in the design of several experiments and provided critical advice. DK, SUS and GN wrote the manuscript, with contributions from all of the authors.

Funding

This work was supported by National Institutes of Health grants (Grants R01AI063331 and R01DK091191 to GN), National Research Foundation of Korea grants funded by the Ministry of Science, ICT and Future Planning (Grants 2015R1A3A2032927 to WUK and 2016R1C1B2008089 to SUS), a National Research Foundation of Korea grant funded by the Ministry of Education (Grant 2017R1D1A1B04033009 to DK), and Creative-Pioneering Researchers Program through Seoul National University (DK).

Acknowledgements

We thank Lisa Haynes and Jong-Hyoek Jung for animal husbandry and the University of Michigan GF Animal Core Facility and Host Microbiome Initiative for support.

Disclosures

The authors have no conflicting interests to declare.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure. S1. Production of antigen-specific IgG is induced by oral immunization with both human serum albumin (HSA) and cholera toxin (CT), but not CT or HSA alone or mock control.

Figure. S2. Productions of antigen-specific IgG1, IgG2b and IgM induced by oral immunization with human serum albumin (HSA) and cholera toxin are suppressed in Nod2-deficient mice.

Figure. S3. Nod1 or Toll-like receptor (TLR) agonists have no synergistic effect with cholera toxin on interleukin-1β (IL-1β) production in dendritic cells.