The Journal of Immunology

Extracellular Vesicles Derived from Gram-Negative Bacteria, such as *Escherichia coli* , Induce Emphysema Mainly via IL-17A–Mediated Neutrophilic Inflammation

You-Sun Kim, Won-Hee Lee, Eun-Jeong Choi, Jun-Pyo Choi, Young Joo Heo, Yong Song Gho, Young-Koo Jee, Yeon-Mok Oh and Yoon-Keun Kim

J Immunol 2015; 194:3361-3368; Prepublished online 25 February 2015;
doi: 10.4049/jimmunol.1402268
http://www.jimmunol.org/content/194/7/3361

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

Extracellular Vesicles Derived from Gram-Negative Bacteria, such as *Escherichia coli*, Induce Emphysema Mainly via IL-17A–Mediated Neutrophilic Inflammation

You-Sun Kim,*,†,[1] Won-Hee Lee,‡,[1] Eun-Jeong Choi,‡,[1] Jun-Pyo Choi,§,[1] Young Joo Heo,* Yong Song Gho,‡ Young-Koo Jee,¶ Yeon-Mok Oh,*,† and Yoon-Keun Kim§

Recent evidence indicates that Gram-negative bacteria–derived extracellular vesicles (EVs) in indoor dust can evoke neutrophilic pulmonary inflammation, which is a key pathology of chronic obstructive pulmonary disease (COPD). *Escherichia coli* is a ubiquitous bacterium present in indoor dust and secretes nanometer-sized vesicles into the extracellular milieu. In the current study, we evaluated the role of *E. coli*–derived EVs on the development of COPD, such as emphysema. *E. coli* EVs were prepared by sequential ultrafiltration and ultracentrifugation. COPD phenotypes and immune responses were evaluated in C57BL/6 wild-type (WT), IFN-γ–deficient, or IL-17A–deficient mice after airway exposure to *E. coli* EVs. The present study showed that indoor dust from a bed mattress harbors *E. coli* EVs. Airway exposure to *E. coli* EVs increased the production of proinflammatory cytokines, such as TNF-α and IL-6. In addition, the repeated inhalation of *E. coli* EVs for 4 wk induced neutrophilic inflammation and emphysema, which are associated with enhanced elastase activity. Emphysema and elastase activity enhanced by *E. coli* EVs were reversed by the absence of IFN-γ or IL-17A genes. In addition, during the early period, lung inflammation is dependent on IL-17A and TNF-α, but not on IFN-γ, and also on TLR4. Moreover, the production of IFN-γ is eliminated by the absence of IL-17A, whereas IL-17A production is not abolished by IFN-γ absence. Taken together, the present data suggest that *E. coli*–derived EVs induce IL-17A–dependent neutrophilic inflammation and thereby emphysema, possibly via upregulation of elastase activity. *The Journal of Immunology*, 2015, 194: 3361–3368.

Biological contaminants in indoor air can induce immune dysregulation in the lung, resulting in inflammatory pulmonary disorders, such as asthma and chronic obstructive pulmonary disease (COPD) (1). In terms of the immunopathogenesis of airway inflammation, the eosinophilic subtype represents inflammation induced by IL-4– and IL-13–secreting Th2 cells, whereas the neutrophilic subtype is related to both IFN-γ– and IL-17–dependent Th1 and Th17 inflammation (2–4). Neutrophilic inflammation is known to be important in the COPD pathogenesis (5). Emphysema (a key phenotype of COPD) is an important cause of irreversible airflow limitation, because the destruction of lung tissue around small airways seen in emphysema patients prevents the airways from holding their functional shape upon exhalation (6, 7).

Extracellular vesicles (EVs) are spherical bilayered phospholipids ranging in size from 20 to 200 nm in diameter, so-called nanovesicles, that are produced ubiquitously from all Gram-negative bacteria and some Gram-positive bacteria investigated to date (8, 9). Previous biochemical and proteomic studies have revealed that Gram-negative bacteria–derived EVs are composed of outer membrane proteins, LPS, outer membrane lipids, periplasmic proteins, DNA, RNA, and other factors associated with virulence (8). The fact that bacteria-derived EVs harbor pathogen-associated molecular patterns (PAMPs), such as LPS, peptidoglycan (PG), lipoteichoic acid (LTA), that induce innate immunity, and also harbor proteins that induce T cell response, led to us the notion that the inhalation of bacterial EVs can evoke immune dysregulation and inflammation in the lung.

Escherichia coli is one of the most important model organisms of Enterobacteriaceae. Our previous work has shown that *E. coli*–derived EVs induce systemic inflammation mimicking sepsis after entering the bloodstream (10) and that EVs from indoor dust and also from *Staphylococcus aureus* can induce neutrophilic pulmonary inflammation (11, 12). In the current study, we hypothesized that EVs from Gram-negative bacteria, especially *E. coli*, are an important cause of neutrophilic inflammation and thereby emphysema in the lung. To test this, we aimed to evaluate whether *E. coli* EVs are causally related to the pathogenesis of emphysema. We also tried to determine the immunologic mechanisms of emphysema induced by *E. coli* EVs.

*Asan Institute for Life Science, Seoul 138-736, Republic of Korea; †Ulsan University College of Medicine, Seoul 138-736, Republic of Korea; ‡Department of Life Sciences, Pohang University of Science and Technology, Pohang 790-784, Republic of Korea; §Institute of Convergence Medicine, Ewha Womans University School of Medicine, Seoul 158-710, Republic of Korea; and ¶Department of Internal Medicine, Dankook University College of Medicine, Cheonan 330-714, Republic of Korea

[1]Y.-S.K., W.-H.L., E.-J.C., and J.-P.C. contributed equally to this work.

Received for publication September 9, 2014. Accepted for publication January 25, 2015.

This work was supported by National Research Foundation of Korea Grant 2010-0028684 funded by the Korea government (to Y.-K.K.).

Y.-S.K., W.-H.L., and E.-J.C. designed and did experiments, analyzed and interpreted results, and wrote the manuscript; J.-P.C. and Y.J.H. performed animal experiments; Y.S.G. and Y.-K.J. designed experiments and wrote the manuscript; and Y.-M.O. and Y.-K.K. directed the study and wrote the manuscript.

Address correspondence and reprint requests to Dr. Yoon-Keun Kim or Dr. Yeon-Mok Oh, Institute of Convergence Medicine, Ewha Womans University Medical Center, 1071, Anyancheon-ro, Yangcheon-gu, Seoul 158-710, Republic of Korea (Y.-K.K.) or Asan Institute for Life Science, 88 Olympic-ro 43-gil, Songpa-gu, Seoul 138-736, Republic of Korea (Y.-M.O.). E-mail addresses: juinea@ewha.ac.kr (Y.-K.K.) or ymoh55@amc.seoul.kr (Y.-M.O.)

Abbreviations used in this article: BAL, bronchoalveolar lavage; COPD, chronic obstructive pulmonary disease; EV, extracellular vesicle; MMP, matrix metalloproteinase; PAMP, pathogen-associated molecular pattern; PMB, polymyxin B; POSTECH, Pohang University of Science and Technology; WT, wild-type.

www.jimmunol.org/cgi/doi/10.4049/jimmunol.1402268

Materials and Methods

Mice and cell cultures

Wild-type (WT), IFN-γ–deficient, and TNF-α–deficient mice (12, 13) (all C57BL/6 background) were purchased from The Jackson Laboratory (Bar Harbor, ME). IL-17A–deficient mice (C57BL/6 background) were gifted from Y.-C. Sung (Pohang University of Science and Technology [POSTECH], Pohang, Republic of Korea) (12). TLR2-deficient and TLR4-deficient mice (12) (both of C57BL/6 background) were purchased from Oriental BioService (Kyoto, Japan). These mice were bred in pathogen-free facilities at POSTECH. The animal experimental protocols were approved by the Institutional Animal Care and Use Committee at POSTECH (permit number 2011-01-0021). MH-S cells (mouse alveolar macrophages originated from BALB/c; American Type Culture Collection) were cultured in RPMI 1640 medium supplemented with 10% FBS and grown in the presence of 100 U/ml penicillin and 100 μg/ml streptomycin.

The collection of indoor dust

Indoor dust was collected from a bed mattress in an apartment using a vacuum cleaner (Samsung, Seoul, Republic of Korea). The dust was stored at room temperature until further use (11).

The preparation of EVs from indoor dust and E. coli

EVs were isolated from indoor dust and from the culture supernatants of *E. coli* isolated from the peritoneal lavage fluid of mice with cecal ligation and puncture, as described previously (10, 11). Briefly, the dust samples were incubated in PBS for 12 h at 4˚C with stirring and centrifuged twice at 10,000 × *g* for 15 min. *E. coli* in lysogeny broth was cultured at 37˚C and centrifuged twice at 10,000 × *g* for 15 min. Supernatants were filtered with a 0.22-μm vacuum filter to remove any remaining cells. EVs were prepared by centrifugation in a 45 Ti rotor (Beckman Instruments, Fullerton, CA) at 150,000 × *g* for 3 h at 4˚C. EVs were diluted in PBS and stored at −80˚C.

The in vitro analysis of innate immune response

To confirm the role of LPS in *E. coli*–derived EV-induced cytokine production, MH-S cells were incubated with *E. coli*–derived EVs (100 ng/ml) in the presence or the absence of polymyxin B (PMB; 1 μg/ml). To examine in vitro innate immune responses induced by *E. coli*–derived EVs in WT, TLR2-deficient, and TLR4-deficient mice, peritoneal macrophages were isolated as described previously (12). Briefly, thioglycollate-elicited peritoneal macrophages were seeded in 24-well plates, incubated with *E. coli*–derived EVs, TLR2 agonist (heat-killed *Listeria monocytogenes*, 10^7 cells/ml), or TLR4 agonist (LPS from *E. coli* K12), and the culture supernatants were collected 16 h after the application. The levels of TNF-α and IL-6 in culture supernatants were measured by ELISA.

In vivo protocol for E. coli*–derived EVs on the development of lung inflammation*

To evaluate the effects of *E. coli*–derived EVs on the development of lung inflammation, *E. coli*–derived EVs were administered intranasally to WT C57BL/6, IFN-γ–deficient, IL-17A–deficient, or TNF-α–deficient mice. Lung inflammation was evaluated 24 h after the final application. PBS was applied as a negative control. To inhibit LPS activity, PMB (1 μg) was instilled intranasally with 100 ng *E. coli*–derived EVs.

The evaluation of COPD phenotypes

Cellularity in bronchoalveolar lavage (BAL) fluid was analyzed, as described previously (1). Briefly, after counting the total number of cells in the BAL samples, the cell were stained by Diff-Quik solution and were classified as macrophages, lymphocytes, neutrophils, or eosinophils. To detect emphysema, lungs were fixed in buffered 4% formaldehyde and embedded in paraffin. Sections were stained with H&E. Chord length was measured using the Computer Assisted Stereological Toolbox system (Olympus, Tokyo, Japan). In H&E-stained lung sections, the average

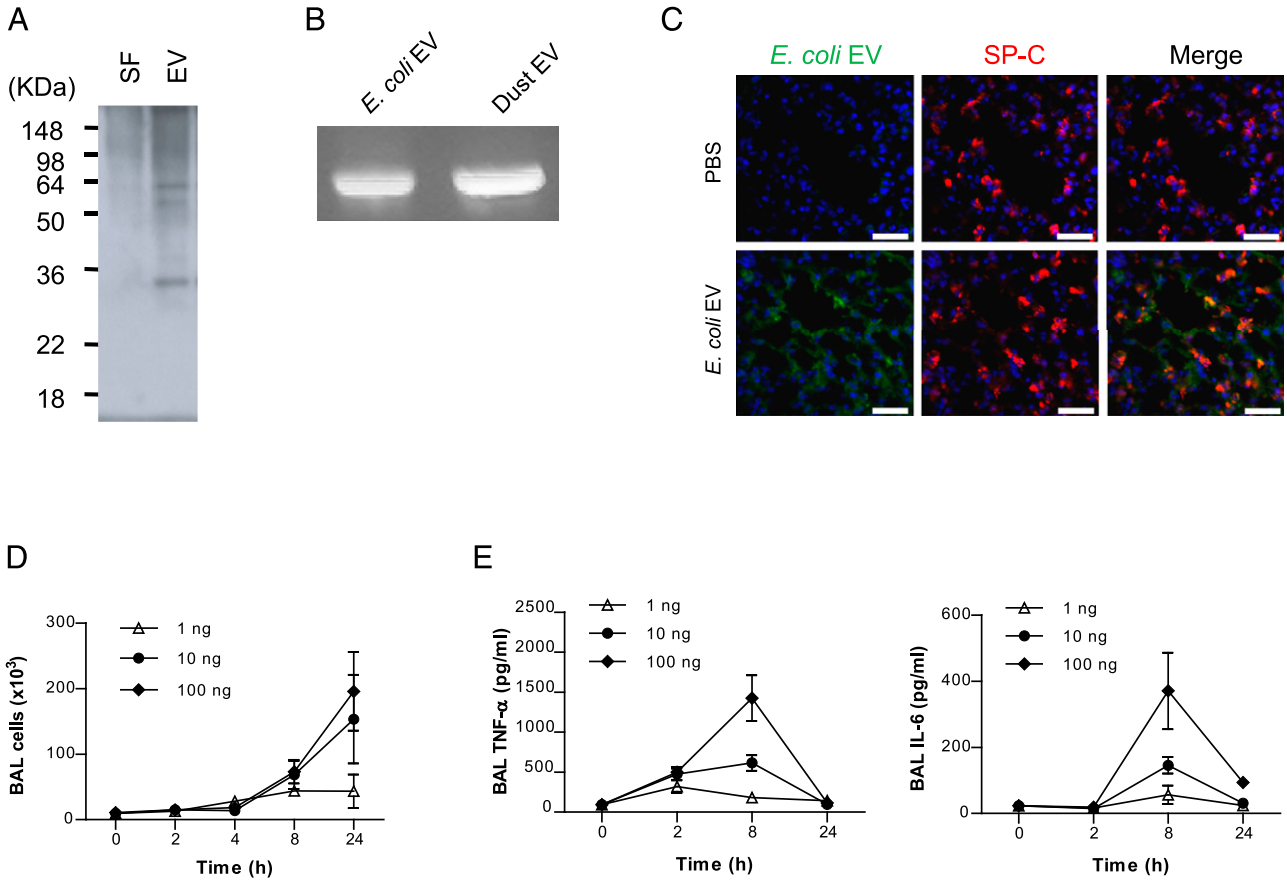

FIGURE 1. Indoor dust harbors *E. coli* EVs that induce pulmonary inflammation. (**A**) Western blotting of soluble factors (SF, 5 μg) and EVs (5 μg) in indoor dust using *E. coli* EV–specific polyclonal Abs. (**B**) PCR of dust EVs using *E. coli*–specific primer. (**C**) Whole-mount imaging of lung tissues using *E. coli* EV-specific polyclonal Abs (green) and SP-C (red) 1 h after airway administration of *E. coli* EVs (1 μg) and PBS. The nuclei were counterstained by Hoechst (blue). Scale bars, 30 μm. (**D**) Total cells in BAL fluid 2, 4, 8, and 24 h after the airway application of different doses of *E. coli* EVs ($n = 4$/group). (**E**) Cytokine levels in BAL fluid after the airway administration of *E. coli* EVs (1-, 10-, and 100-ng doses, respectively).

interalveolar septal wall distance (mean linear intercept) was measured separately by two people in a blinded fashion (14).

Whole-mount imaging

One microgram of *E. coli* EVs was administered intranasally to C57BL/6 mice. After 1 h, mice were sacrificed, and lungs were removed. Lungs were fixed with 1% paraformaldehyde in PBS containing 1% sucrose, blocked with 5% horse serum in TBST (0.3% Triton X-100 in TBS), and incubated with anti-*E. coli* EV polyclonal Ab, which had been developed in our laboratory by immunizing rabbits with the *E. coli* EVs, as previously described with minor modifications (15), and anti–SP-C Ab (Santa Cruz Biotechnology, Santa Cruz, CA). After treatment with Alexa-Fluor 488–conjugated donkey anti-rabbit IgG and Alexa-Fluor 594–conjugated donkey anti-goat IgG (Invitrogen, Carlsbad, CA), lungs were counterstained with Hoechst (Sigma-Aldrich, St. Louis, MO) and analyzed using an FV1000 laser scanning confocal microscope (Olympus).

Immunocytochemistry

MH-S cells were incubated with 1 μg/ml DiO (Invitrogen)-labeled *E. coli* EVs in the presence or the absence of 10 μg/ml PMB for 6 h. Cells were then washed with PBS, fixed with 4% paraformaldehyde in PBS containing 4% sucrose, permeabilized with 0.2% Triton X-100 in PBS, stained with Hoechst (Sigma-Aldrich), and analyzed using an FV1000 laser scanning confocal microscope (Olympus). To examine the role of TLRs in the uptake of *E. coli*–derived EVs, the above protocol was applied to peritoneal macrophages isolated from WT, TLR2-deficient, and TLR4-deficient mice.

Elastase activity

The elastase activity from lung lysates without detergent was measured using an Elastase assay kit according to the manufacturer's instructions (Invitrogen).

Western blotting

Proteins from soluble and EV fractions isolated from indoor dust were separated using SDS–PAGE and transferred to polyvinylidene difluoride membranes. Blocked membranes were incubated with an *E. coli* EV polyclonal Ab.

mRNA expression

A method of RT-PCR using dust EVs to detect *E. coli* EV 16S rRNA has been described previously (16). The primers for *E. coli* EV 16S rRNA were 5′-GGAAGAAGCTTGCTTCTTTGCTG-3′ and 5′-AGCCCGGGGATTTCACATCTGACTTA-3′. The measurement of matrix metalloproteinases (MMPs) mRNA expression were performed by RT-PCR described previously (16, 17). The real-time quantitative analysis of MMPs was performed using the LightCycler 480 system (Roche Diagnostics, Indianapolis, IN) with LightCycler 480 SYBR Green I master (Roche Diagnostics).

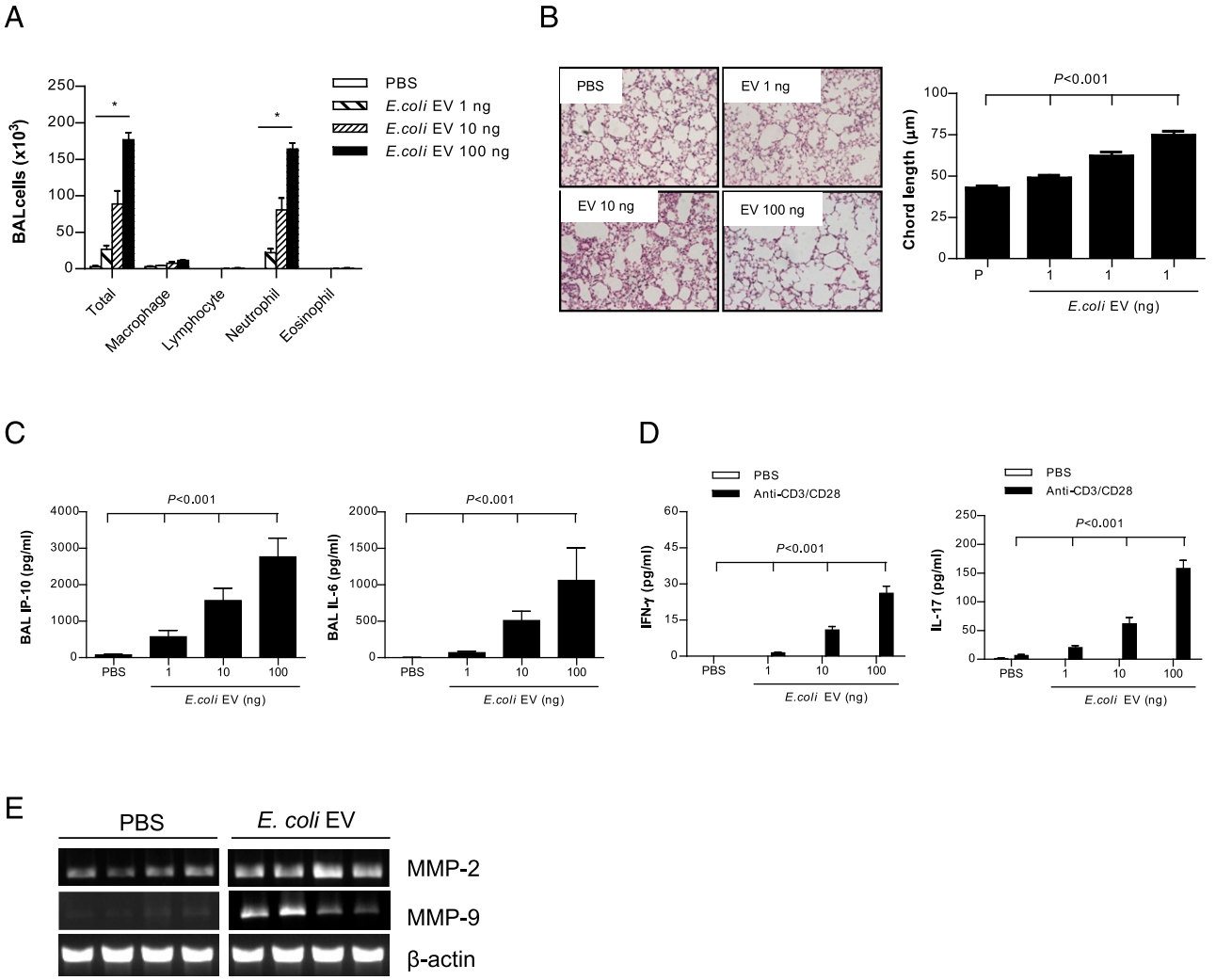

FIGURE 2. The repeated airway application of *E. coli* EVs induces neutrophilic inflammation and thereby emphysema. (**A**–**C**) The different doses (1, 10, and100 ng) of *E. coli* EVs were administered into the mouse airways (n = 5/group) twice weekly for 4 wk, and analyses were performed 24 h after the final application. (A) Cellularity in BAL fluid; *$p < 0.05$ using two-way ANOVA. (B) Chord length (*right panel*) and representative lung histology (*left panel*). H&E staining. Original magnification ×100. (C) The levels of IFN-γ–inducible protein (IP)-10 and IL-6 in BAL fluid. (**D**) IFN-γ and IL-17 levels in the culture supernatant of lung T cells 6 h after stimulation with anti-CD3 and anti-CD28 Abs (each 1 μg/ml). (**E**) mRNA expression of MMP-9 and MMP-2 were measured in lung tissue from mice administered *E. coli* EVs (100 ng) twice weekly for 4 wk (n = 4/group). *$p < 0.05$ using ANOVA linearity testing.

The measurement of cytokine levels

Cells isolated from regional lymph nodes were restimulated with plate-bound anti-CD3 and CD28 Abs for 6 h. The culture supernatant was collected and examined by ELISA. Cytokine levels from BAL fluid were measured by ELISA, according to the manufacturer's instructions (R&D Systems, Minneapolis, MN).

Statistical analysis

For multiple comparisons among groups, ANOVA was initially used followed by unpaired *t* or nonparametric Mann–Whitney *U* test between two groups. To test for trends, the ANOVA linearity test was used. For statistical analysis, we used GraphPad Prism 5, and the statistical significance was set a priori at $p < 0.05$.

Results

The presence of E. coli*–derived EVs in indoor dust and their uptake by lung resident cells*

E. coli is a Gram-negative bacterium that is not confined to the intestine, and its ability to survive for brief periods outside the body makes it an ideal indicator organism to test indoor dust samples for fecal contamination (18). In our previous report, indoor dust harbors lots of EVs (11). Thus, we evaluated whether indoor dust contains *E. coli*–derived EVs. The presence of *E. coli* EVs in indoor dust was assessed using an anti-*E. coli* EV polyclonal Ab. The present data showed that the Ab reacted with EVs in indoor dust but not soluble factors derived from indoor dust (Fig. 1A). In addition, genotyping using an *E. coli*–specific 16S rRNA primer showed that *E. coli* EVs were present in indoor dust (Fig. 1B). Furthermore, it was determined that *E. coli* EVs are taken up by entering the airways via resident lung cells. After the airway administration of *E. coli*–derived EVs, whole-mount imaging of lung tissue labeled with an *E. coli* EV Ab indicated the uptake of *E. coli* EVs into resident lung cells, including alveolar type II cells (Fig. 1C). To evaluate innate immune response induced by *E. coli* EVs, EVs were administered intranasally into the mouse airways in differing doses of 1, 10, and 100 ng. Following the administration of *E. coli* EVs, total cell number in BAL fluid was increased by EVs in a dose-dependent manner (Fig. 1D). In mice receiving 10 and 100 ng *E. coli* EVs, the production of TNF-α and IL-6 in BAL fluid also increased within 8 h after the administration (Fig. 1E).

The repeated inhalation of E. coli *EVs induces neutrophilic pulmonary inflammation and emphysema*

To evaluate whether the repeated inhalation of *E. coli* EVs induces neutrophilic inflammation and thereby structural changes in the lung, *E. coli* EVs (1, 10, and 100 ng) were administered into the mouse airways twice per week for 4 wk. The lung infiltration of neutrophils was enhanced by the inhalation of *E. coli* EVs in a dose-dependent manner (Fig. 2A). As for structural changes, the inhalation of *E. coli* EVs induced emphysema in a dose-dependent manner (Fig. 2B). In addition, the production of IFN-γ–inducible protein-10 and IL-6 (downstream mediators of IFN-γ and IL-17, respectively) was also significantly increased by airway exposure to *E. coli* EVs in a dose-dependent manner (Fig. 2C). The airway administration of *E. coli* EVs also dose-dependently enhanced the production of both IFN-γ and IL-17A by lung T cells (Fig. 2D). Moreover, *E. coli* EVs enhanced the expression of MMP-2 and MMP-9 mRNA in lung tissues (Fig 2E). Taken together, these observations indicate that *E. coli* EVs can induce neutrophilic inflammation and emphysema in a dose-dependent manner.

The role of IFN-γ and IL-17A in the development of COPD phenotypes induced by E. coli *EVs*

We evaluated the role of IFN-γ and IL-17A in the development of emphysema after airway exposure to *E. coli* EVs (100 ng) twice

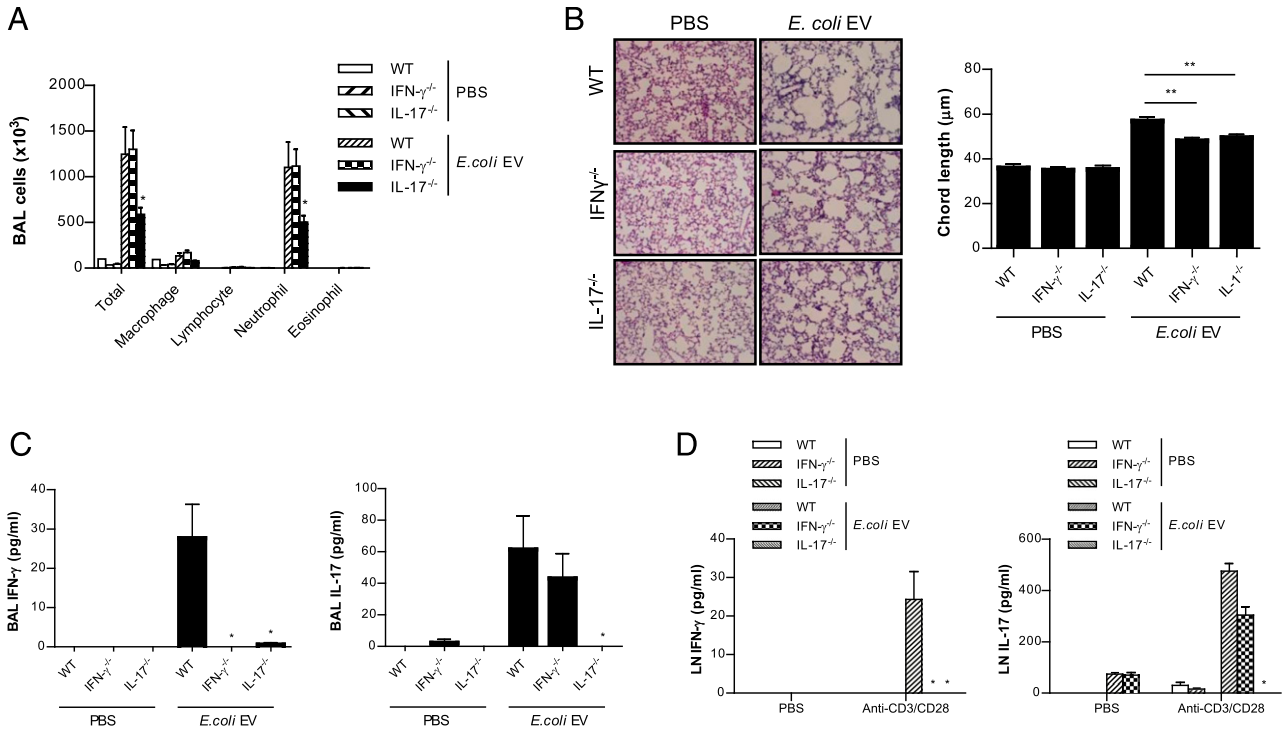

FIGURE 3. Neutrophilic inflammation and emphysema induced by *E. coli*–derived EVs depend on both IFN-γ and IL-17A. (**A**–**C**) *E. coli* EVs (100 ng) were administered into the airways of WT, IFN-γ–deficient, and IL-17A–deficient mice ($n = 5$/group) twice weekly for 4 wk, and analyses were performed 24 h after the final application. (A) Cellularity in BAL fluid. (B) Chord length (*right panel*) and representative lung histology (*left panel*). H&E staining. Original magnification ×100. (C) The levels of IFN-γ and IL-17A in BAL fluid. (**D**) The levels of IFN-γ and IL-17A in lymph nodes restimulated with anti-CD3 and CD28 Abs for 6 h. $^{*}p < 0.05$, $^{**}p < 0.01$ using ANOVA.

per week for 4 wk in IFN-γ–deficient and IL-17A–deficient mice, and WT control mice. The present study showed that the lung infiltration of neutrophils induced by *E. coli* EVs was significantly inhibited by the absence of IL-17A but not by the absence of IFN-γ (Fig. 3A). In contrast, emphysematous changes induced by *E. coli* EVs were partially abolished by the absence of IFN-γ or IL-17 (Fig. 3B). Remarkably, the protein amount of IFN-γ in BAL fluid were absent in IL-17–deficient mice, whereas the BAL IL-17A levels were not inhibited by the absence of IFN-γ (Fig. 3C). In addition, the production of IFN-γ from regional lymph node cells restimulated with anti-CD3 and CD28 Abs was also inhibited by the absence of IL-17A, but IL-17A production was not diminished by the IFN-γ absence (Fig. 4D). These findings suggest that IL-17A is a key mediator in the development of emphysema induced by exposure to *E. coli*–derived EVs and that the production of IFN-γ is dependent on IL-17A.

The early-phase effects of exposure to E. coli *EVs*

To evaluate the early-phase effects of *E. coli* EVs on the emphysema pathogenesis, 100 ng *E. coli* EVs were administered into the mouse airways and then evaluated 18 h after this application. This experiment showed that neutrophilic inflammation and emphysema were induced by *E. coli* EVs (Fig. 4A, 4B). Elastase activity was enhanced in *E. coli* EV–treated mice in comparison with PBS-treated mice (Fig. 4C). In addition, the mRNA expression of MMP-9, but not MMP-2, in the lung was increased by the application of *E. coli* EVs (Fig. 4D). Taken together, these observations indicate that *E. coli* EVs can induce emphysema, possibly via protease and elastase activity of neutrophils recruited by *E. coli* EVs.

The role of proinflammatory cytokines in early-stage development of E. coli *EV–induced emphysema*

To evaluate the role of proinflammatory cytokines (IFN-γ, IL-17A, and TNF-α) on the emphysema pathogenesis causally related to *E. coli* EVs, 100 ng EVs was administered into the airways of mice in which the candidate genes had been disrupted, and then phenotypes were evaluated 18 h after the application. This study showed that lung inflammation induced by *E. coli* EVs was reversed by the absence of IL-17A or TNF-α (Fig. 5A). The elastase activity enhanced by *E. coli* EVs was reversed by the absence of IFN-γ, IL-17A, or TNF-α (Fig. 5B). The mRNA expression of MMP-9 enhanced by *E. coli* EVs was reversed by the absence of IL-17A or TNF-α (Fig. 5C). Collectively, these findings suggest that early immune responses, such as elaboration of proinflammatory cytokines, are important in the development of emphysema via protein degradation by inflammatory cells.

The role of TLR signaling in the early stage effects induced by E. coli *EVs*

E. coli EVs harbor various PAMPs, including LPS and outer membrane proteins, which can be recognized by pattern recognition receptors, such as TLRs. We tried to elucidate the role of TLR signaling in the development of lung inflammation induced by *E. coli* EVs. The production of cytokine by *E. coli* EVs was evaluated in TLR2-deficient and TLR4-deficient mice. Peritoneal macrophages were isolated from WT, TLR2-deficient, and TLR4-deficient mice and stimulated with *E. coli* EVs. The production of TNF-α and IL-6 was abolished completely by the absence of TLR4 but only partially by the absence of TLR2 (Fig. 6A). In addition, we evaluated the role of TLRs on the uptake of *E. coli*

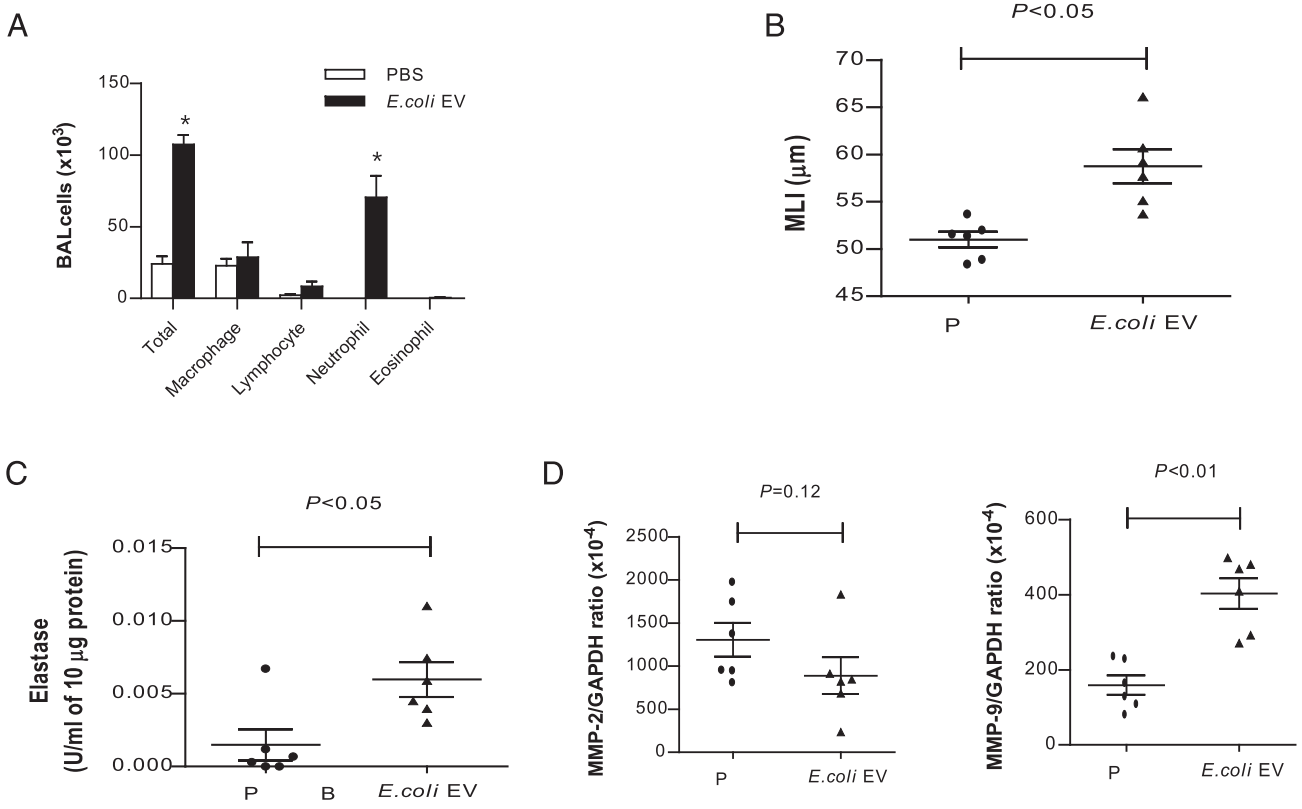

FIGURE 4. The early-phase effects of *E. coli* EVs on the development of emphysema. (**A**–**D**) *E. coli* EVs (100 ng) were administered once into the airways of C57BL/6 mice (n = 6/group), and analyses were performed 18 h after the administration. (A) Cellularity in BAL fluid. (B) Mean linear intercept (MLI). (C) Elastase activity using assay kit from lung lysate without detergent. (D) Quantitative analysis of the mRNA expression of MMP-2 and MMP-9 with real-time RT-PCR. The p values in (B)–(D) were analyzed with nonparametric Mann–Whitney U test between two groups. *$p < 0.05$ using ANOVA.

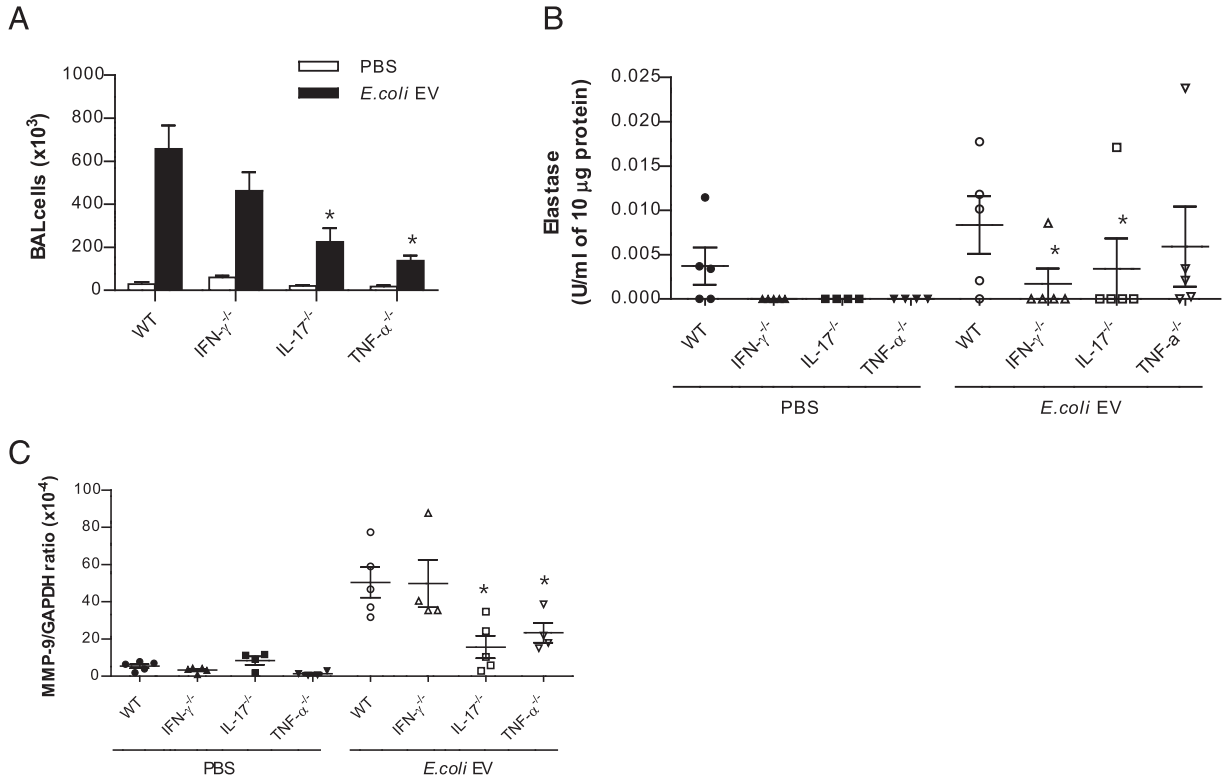

FIGURE 5. The role of IFN-γ, IL-17A, and TNF-α on the development of early-phase phenotypes induced by *E. coli* EVs. (**A**–**C**) *E. coli* EVs (100 ng) were administered into the airways of C57BL/6 mice, IFN-γ–deficient, IL-17A–deficient, and TNF-α–deficient mice (n = 5/group), and analyses were performed 18 h after the administration. (A) Cellularity in BAL fluid. (B) Elastase activity using assay kit from lung lysate without detergent. (C) The quantitative analysis of MMP-9 mRNA expression with real-time RT-PCR. *$p < 0.05$ using ANOVA.

EVs by inflammatory cells. *E. coli* EVs were taken up by peritoneal macrophages isolated from WT mice but not from TLR4-deficient mice (Fig. 6B). Taken together, these findings suggest that TLR4 signaling is important in the uptake of *E. coli* EVs and the production of proinflammatory cytokines induced by *E. coli* EVs.

The role of LPS in E. coli *EVs in the early-stage effects induced by* E. coli *EVs*

To examine the role of LPS in *E. coli* EVs in the development of emphysema, PMB, LPS antagonist, was treated with *E. coli* EVs. The uptake of *E. coli* EVs by MH-S cells (mouse alveolar macrophages) was inhibited by PMB treatment compared with PBS treatment (Fig. 6C). The production of TNF-α and IL-6 induced by *E. coli* EVs was partly abolished by PMB treatment (Fig. 6D). To assess the in vivo effect of LPS on phenotypes induced by *E. coli* EVs, EVs were administered into the mouse airways with or without PMB. This experiment showed that the number of inflammatory cells in BAL fluid was partly decreased by PMB treatment compared with PBS treatment (Fig. 6E). In addition, the production of IL-6 and TNF-α induced by *E. coli* EVs was also partly inhibited by PMB treatment (Fig. 6F). Taken together, these results suggest that LPS in *E. coli* EVs plays an important role in the uptake of EVs by host cells.

Discussion

The role of infectious agents in the etiology of diseases once believed to be noninfectious is increasingly being recognized (19). Noxious or biological contaminants in indoor air can induce chronic inflammatory pulmonary disorders, such as asthma and COPD (20). Common biological contaminants in indoor dust include viruses, bacteria, fungi, dust mites, and pet dander (21, 22). Previous reports indicate that organic dust can induce airway inflammation related with Th1/Th17 cell responses or TLR2 signaling (23, 24). Our previous findings showed that indoor dust collected from bed mattress induces neutrophilic pulmonary inflammation and that EVs in indoor dust also induce neutrophilic pulmonary inflammation, which is related with Th1 and Th17 cell responses (25). To our knowledge, this is the first report demonstrating that EVs derived from Gram-negative bacteria, especially *E. coli*, can induce neutrophilic inflammation and thereby emphysema mainly in an IL-17A–dependent manner. We also demonstrate that the TLR4-signaling pathway is critical in the development of *E. coli* EV–induced phenotypes.

Gram-negative bacteria, including *E. coli*, produce EVs during normal growth (26). Gram-negative bacteria–derived EVs contain a wide variety of PAMPs, which can be capable of inducing innate immune responses and include LPS, outer membrane lipids, outer membrane proteins, periplasmic proteins, cytoplasmic proteins, DNA, RNA, and other virulence-associated factors (8, 27). Our previous study demonstrated that the entry of *E. coli* EVs into the bloodstream induces systemic inflammation mimicking sepsis (10). In addition, our previous work indicates that EVs in indoor dust are important in the development of neutrophilic inflammation in the lung (11). Previously, we also found that the Gram-positive bacterium *S. aureus* produces EVs (9), which may be causally related to atopic dermatitis in the skin (28) and also neutrophilic inflammation in the lung (12). The present study showed that the repeated inhalation of *E. coli* EVs induced neutrophilic inflammation and thereby emphysema mainly via an IL-17A–dependent mechanism. These findings suggest that bacteria-derived

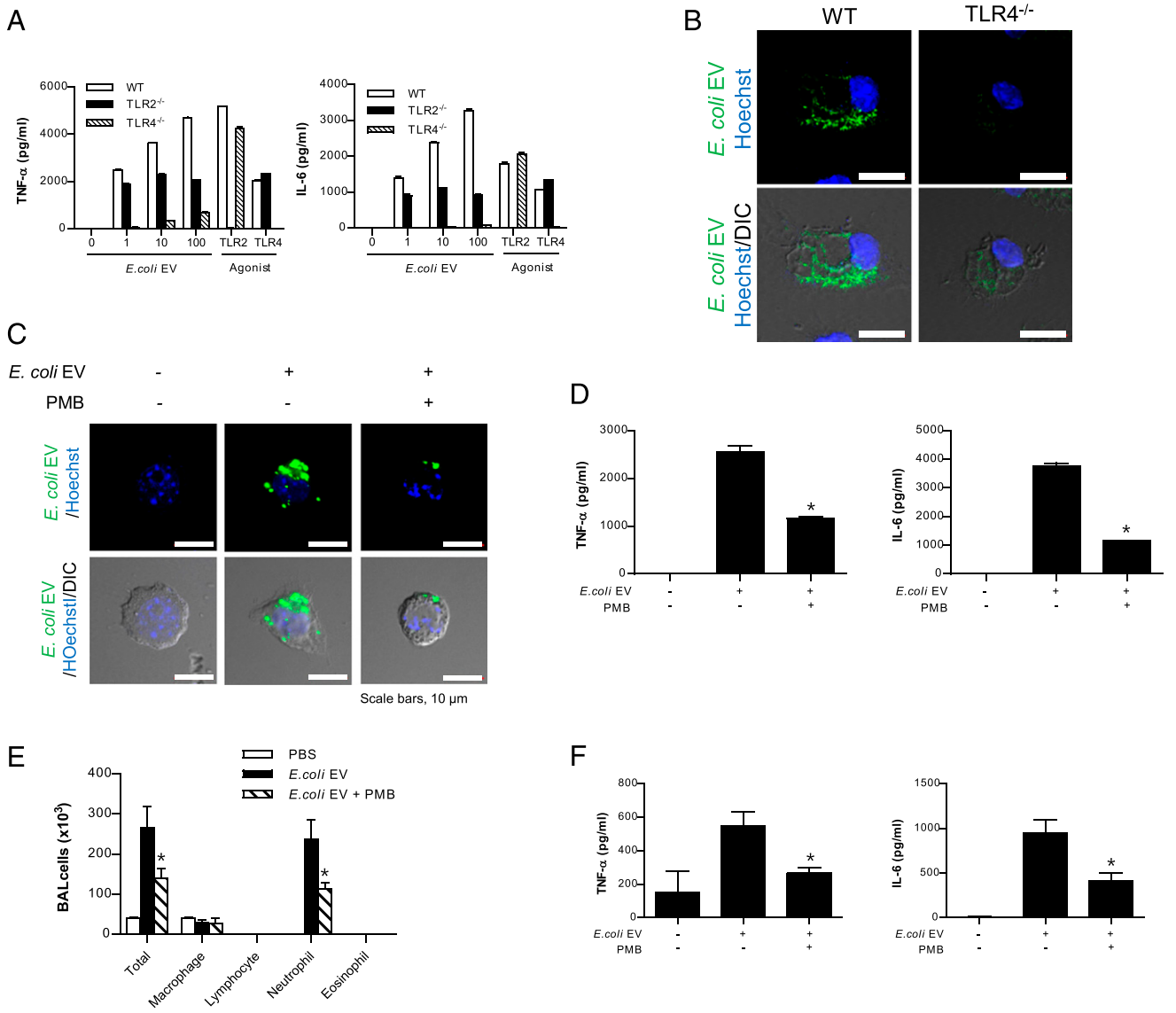

FIGURE 6. The role of TRL receptors and LPS on the development of lung inflammation induced by *E. coli* EVs. (**A**) The levels of TNF-α and IL-6 in the culture supernatant of peritoneal macrophages (derived from WT, TLR2-deficient, and TLR4-deficient mice) incubated for 16 h with *E. coli*–derived EVs (1, 10, and 100 ng/ml), TLR2 agonist (heat-killed *Listeria monocytogenes*, 10^7 cells/ml), or TLR4 agonist (LPS from *E. coli* K12, 100 ng/ml). (**B**) The confocal microscopic images of peritoneal macrophages (derived from WT and TLR4-deficient mice) stimulated with DiO-labeled *E. coli*–derived EVs. (**C**) The confocal microscopic images of MH-S cells stimulated with DiO-labeled *E. coli* EVs in the presence or the absence of PMB. In (B) and (C), nuclei were counterstained with Hoechst (blue). Scale bars, 10 μm. (**D**) The levels of TNF-α and IL-6 in the culture supernatant of MH-S cells after stimulation with *E. coli*–derived EVs (100 ng/ml) in the presence or the absence of PMB (1 μg/ml). For (**E**) and (**F**), mice were sensitized once with 100 ng *E. coli*–derived EVs with or without PMB, and evaluation was performed 18 h after the administration. (E) BAL cellularity. (F) The levels of TNF-α and IL-6 in BAL fluid. $*p < 0.05$ using nonparametric Mann–Whitney *U* test between *E. coli* EV–only treated group and *E.coli* EV– and PMB-treated group. DIC, differential interference contrast.

EVs may be a novel causative agent of inflammatory diseases whose agent(s) are unknown.

Our previous data demonstrated that the repeated inhalation of indoor dust can induce neutrophilic inflammation associated with enhanced infiltration of IFN-γ–positive and IL-17A–positive T cells in the lung (11). Much clinical evidence indicates that neutrophilic inflammation in the airways is related to asthma severity (1). Neutrophilic inflammation is also important in the pathogenesis of COPD (5). Most animal models of emphysema are provoked by cigarette smoke, and IFN-γ is also a key mediator of cigarette-induced emphysema (29, 30). In addition, transgenic studies have shown that high levels of IFN-γ in the airways induce not only noneosinophilic asthma but also emphysema (31, 32). The present study showed that IFN-γ is important in the development of emphysema, but not neutrophilic inflammation, induced by airway exposure to *E. coli* EVs and also in the elastase activity enhanced by *E. coli* EVs. To sum up, these findings suggest that IFN-γ is involved in the development of emphysema, possibly via elastin degradation by inflammatory cells.

Although recent evidence indicates that IL-17A contributes to the recruitment of neutrophils in the lungs (33) and to the development of asthma (3, 33, 34), the role of IL-17A in the emphysema pathogenesis remains to be determined. The present data show that both neutrophilic inflammation and emphysema induced by *E. coli* EVs were abolished by the absence of IL-17A. In addition, elastase activity enhanced by *E. coli* EVs was found to be absent in IL-17A–deficient mice. Recent data have shown that Th17 cells can also produce IFN-γ, in addition to IL-17A (35).

Interestingly, the current study showed that IFN-γ production was completely abolished by the absence of IL-17A, whereas IL-17A production was unaffected by the absence of IFN-γ. Taken together, these findings imply that IL-17A is a key mediator in the development of neutrophilic inflammation and emphysema induced by *E. coli* EVs, in which IFN-γ appears to be produced by Th17 cells rather than Th1 cells.

LPS, a component of the outer membrane of Gram-negative bacteria, is ubiquitously present in the indoor environment and induces the production of proinflammatory and immune modulating mediators via TLR4 (36, 37). The present study showed that *E. coli* EV uptake by macrophages is dependent on both LPS and TLR4 signaling. In addition, lung inflammation and the production of proinflammatory mediators induced by *E. coli* EVs are markedly inhibited by the absence of TLR-4 and also partly inhibited by the absence of TLR-2. In addition, the uptake of *E. coli* EVs by macrophages was completely abolished by the absence of TLR-4 or by PMB treatment. Collectively, these findings suggest that signals other than LPS are also involved in the development of emphysema phenotypes induced by *E. coli* EVs; however, the interaction of LPS in *E. coli* EVs and the TLR4 receptor is critical in the uptake of EVs by inflammatory cells.

In summary, our present data indicate that airway exposure to EVs derived from *E. coli*, the most important model organism of enteric Gram-negative bacteria, can induce neutrophilic inflammation and emphysema mainly in an IL-17A–dependent manner. Moreover, the present in vitro and in vivo data show that the TLR4 receptor is important in the development of immune and pathologic phenomena induced by *E. coli* EVs mainly via initial interaction between LPS in *E. coli* EVs and the TLR4 receptor. Thus, EVs derived from Gram-negative bacteria, including *E. coli*, represent a novel target for the control of COPD.

Disclosures

The authors have no financial conflicts of interest.

References

1. Jeon, S. G., S. Y. Oh, H. K. Park, Y. S. Kim, E. J. Shim, H. S. Lee, M. H. Oh, B. Bang, E. Y. Chun, S. H. Kim, et al. 2007. TH2 and TH1 lung inflammation induced by airway allergen sensitization with low and high doses of double-stranded RNA. *J. Allergy Clin. Immunol.* 120: 803–812.
2. Kim, Y. K., S. Y. Oh, S. G. Jeon, H. W. Park, S. Y. Lee, E. Y. Chun, B. Bang, H. S. Lee, M. H. Oh, Y. S. Kim, et al. 2007. Airway exposure levels of lipopolysaccharide determine type 1 versus type 2 experimental asthma. *J. Immunol.* 178: 5375–5382.
3. Choi, J. P., Y. S. Kim, Y. M. Tae, E. J. Choi, B. S. Hong, S. G. Jeon, Y. S. Gho, Z. Zhu, and Y. K. Kim. 2010. A viral PAMP double-stranded RNA induces allergen-specific Th17 cell response in the airways which is dependent on VEGF and IL-6. *Allergy* 65: 1322–1330.
4. Cosmi, L., F. Liotta, E. Maggi, S. Romagnani, and F. Annunziato. 2011. Th17 cells: new players in asthma pathogenesis. *Allergy* 66: 989–998.
5. Tetley, T. D. 2005. Inflammatory cells and chronic obstructive pulmonary disease. *Curr. Drug Targets Inflamm. Allergy* 4: 607–618.
6. Elias, J. 2004. The relationship between asthma and COPD: lessons from transgenic mice. *Chest* 126: 111S–116S; discussion 159S–161S.
7. Hogg, J. C. 2004. Pathophysiology of airflow limitation in chronic obstructive pulmonary disease. *Lancet* 364: 709–721.
8. Lee, E. Y., J. Y. Bang, G. W. Park, D. S. Choi, J. S. Kang, H. J. Kim, K. S. Park, J. O. Lee, Y. K. Kim, K. H. Kwon, et al. 2007. Global proteomic profiling of native outer membrane vesicles derived from *Escherichia coli. Proteomics* 7: 3143–3153.
9. Lee, E. Y., D. Y. Choi, D. K. Kim, J. W. Kim, J. O. Park, S. Kim, S. H. Kim, D. M. Desiderio, Y. K. Kim, K. P. Kim, and Y. S. Gho. 2009. Gram-positive bacteria produce membrane vesicles: proteomics-based characterization of *Staphylococcus aureus*-derived membrane vesicles. *Proteomics* 9: 5425–5436.
10. Park, K. S., K. H. Choi, Y. S. Kim, B. S. Hong, O. Y. Kim, J. H. Kim, C. M. Yoon, G. Y. Koh, Y. K. Kim, and Y. S. Gho. 2010. Outer membrane vesicles derived from *Escherichia coli* induce systemic inflammatory response syndrome. *PLoS One* 5: e11334.
11. Kim, D. K., B. Kang, O. Y. Kim, D. S. Choi, J. Lee, S. R. Kim, G. Go, Y. J. Yoon, J. H. Kim, and S. C. Jang, et al. 2013. EVpedia: an integrated database of high-throughput data for systemic analyses of extracellular vesicles. *J. Extracell. Vesicles* 2: 20384. http://dx.doi.org/10.3402/jev.v2i0.20384.
12. Kim, M. R., S. W. Hong, E. B. Choi, W. H. Lee, Y. S. Kim, S. G. Jeon, M. H. Jang, Y. S. Gho, and Y. K. Kim. 2012. *Staphylococcus aureus*-derived extracellular vesicles induce neutrophilic pulmonary inflammation via both Th1 and Th17 cell responses. *Allergy* 67: 1271–1281.
13. Choi, J. P., Y. S. Kim, O. Y. Kim, Y. M. Kim, S. G. Jeon, T. Y. Roh, J. S. Park, Y. S. Gho, and Y. K. Kim. 2012. TNF-alpha is a key mediator in the development of Th2 cell response to inhaled allergens induced by a viral PAMP double-stranded RNA. *Allergy* 67: 1138–1148.
14. Huh, J. W., S. Y. Kim, J. H. Lee, J. S. Lee, Q. Van Ta, M. Kim, Y. M. Oh, Y. S. Lee, and S. D. Lee. 2011. Bone marrow cells repair cigarette smoke-induced emphysema in rats. *Am. J. Physiol. Lung Cell. Mol. Physiol.* 301: L255–L266.
15. Lee, H. M., E. J. Choi, J. H. Kim, T. D. Kim, Y. K. Kim, C. Kang, and Y. S. Gho. 2010. A membranous form of ICAM-1 on exosomes efficiently blocks leukocyte adhesion to activated endothelial cells. *Biochem. Biophys. Res. Commun.* 397: 251–256.
16. Sabat, G., P. Rose, W. J. Hickey, and J. M. Harkin. 2000. Selective and sensitive method for PCR amplification of *Escherichia coli* 16S rRNA genes in soil. *Appl. Environ. Microbiol.* 66: 844–849.
17. Asanuma, K., I. Shirato, K. Ishidoh, E. Kominami, and Y. Tomino. 2002. Selective modulation of the secretion of proteinases and their inhibitors by growth factors in cultured differentiated podocytes. *Kidney Int.* 62: 822–831.
18. Rosas, I., E. Salinas, A. Yela, E. Calva, C. Eslava, and A. Cravioto. 1997. *Escherichia coli* in settled-dust and air samples collected in residential environments in Mexico City. *Appl. Environ. Microbiol.* 63: 4093–4095.
19. Wu, S., K. J. Rhee, E. Albesiano, S. Rabizadeh, X. Wu, H. R. Yen, D. L. Huso, F. L. Brancati, E. Wick, F. McAllister, et al. 2009. A human colonic commensal promotes colon tumorigenesis via activation of T helper type 17 T cell responses. *Nat. Med.* 15: 1016–1022.
20. Seltzer, J. M. 1994. Biological contaminants. *J. Allergy Clin. Immunol.* 94: 318–326.
21. Husman, T. 1996. Health effects of indoor-air microorganisms. *Scand. J. Work Environ. Health* 22: 5–13.
22. Camara, A. A., J. M. Silva, V. P. Ferriani, K. R. Tobias, I. S. Macedo, M. A. Padovani, C. M. Harsi, M. R. Cardoso, M. D. Chapman, E. Arruda, et al. 2004. Risk factors for wheezing in a subtropical environment: role of respiratory viruses and allergen sensitization. *J. Allergy Clin. Immunol.* 113: 551–557.
23. Poole, J. A., A. M. Gleason, C. Bauer, W. W. West, N. Alexis, S. J. Reynolds, D. J. Romberger, and T. Kielian. 2012. αβ T cells and a mixed Th1/Th17 response are important in organic dust-induced airway disease. *Ann. Allergy. Asthma. Immunol.* 109: 266–273 e262.
24. Poole, J. A., T. A. Wyatt, T. Kielian, P. Oldenburg, A. M. Gleason, A. Bauer, G. Golden, W. W. West, J. H. Sisson, and D. J. Romberger. 2011. Toll-like receptor 2 regulates organic dust-induced airway inflammation. *Am. J. Respir. Cell Mol. Biol.* 45: 711–719.
25. Kim, Y. S., E. J. Choi, W. H. Lee, S. J. Choi, T. Y. Roh, J. Park, Y. K. Jee, Z. Zhu, Y. Y. Koh, Y. S. Gho, and Y. K. Kim. 2013. Extracellular vesicles, especially derived from Gram-negative bacteria, in indoor dust induce neutrophilic pulmonary inflammation associated with both Th1 and Th17 cell responses. *Clin. Exp. Allergy* 43: 443–454.
26. Mashburn-Warren, L. M., and M. Whiteley. 2006. Special delivery: vesicle trafficking in prokaryotes. *Mol. Microbiol.* 61: 839–846.
27. Lee, E. Y., D. S. Choi, K. P. Kim, and Y. S. Gho. 2008. Proteomics in Gram-negative bacterial outer membrane vesicles. *Mass Spectrom. Rev.* 27: 535–555.
28. Hong, S. W., M. R. Kim, E. Y. Lee, J. H. Kim, Y. S. Kim, S. G. Jeon, J. M. Yang, B. J. Lee, B. Y. Pyun, Y. S. Gho, and Y. K. Kim. 2011. Extracellular vesicles derived from *Staphylococcus aureus* induce atopic dermatitis-like skin inflammation. *Allergy* 66: 351–359.
29. Taraseviciene-Stewart, L., and N. F. Voelkel. 2008. Molecular pathogenesis of emphysema. *J. Clin. Invest.* 118: 394–402.
30. Kang, MJ, CG Lee, SJ Cho, and RJ Homer, and a. E. JA. 2006. IFN-γ-dependent DNA injury and/or apoptosis are critical in cigarette smoke-induced murine emphysema. *Proc. Am. Thorac. Soc.* 3: 517–518.
31. Lee, B. J., H. G. Moon, T. S. Shin, S. G. Jeon, E. Y. Lee, Y. S. Gho, C. G. Lee, Z. Zhu, J. A. Elias, and Y. K. Kim. 2011. Protective effects of basic fibroblast growth factor in the development of emphysema induced by interferon-γ. *Exp. Mol. Med.* 43: 169–178.
32. Wang, Z., T. Zheng, Z. Zhu, R. J. Homer, R. J. Riese, H. A. Chapman, Jr., S. D. Shapiro, and J. A. Elias. 2000. Interferon gamma induction of pulmonary emphysema in the adult murine lung. *J. Exp. Med.* 192: 1587–1600.
33. Kim, Y. S., S. W. Hong, J. P. Choi, T. S. Shin, H. G. Moon, E. J. Choi, S. G. Jeon, S. Y. Oh, Y. S. Gho, Z. Zhu, and Y. K. Kim. 2009. Vascular endothelial growth factor is a key mediator in the development of T cell priming and its polarization to type 1 and type 17 T helper cells in the airways. *J. Immunol.* 183: 5113–5120.
34. Moon, H. G., Y. M. Tae, Y. S. Kim, S. Gyu Jeon, S. Y. Oh, Y. Song Gho, Z. Zhu, and Y. K. Kim. 2010. Conversion of Th17-type into Th2-type inflammation by acetyl salicylic acid via the adenosine and uric acid pathway in the lung. *Allergy* 65: 1093–1103.
35. Zielinski, C. E., F. Mele, D. Aschenbrenner, D. Jarrossay, F. Ronchi, M. Gattorno, S. Monticelli, A. Lanzavecchia, and F. Sallusto. 2012. Pathogen-induced human TH17 cells produce IFN-γ or IL-10 and are regulated by IL-1β. *Nature* 484: 514–518.
36. Michel, O., R. Ginanni, J. Duchateau, F. Vertongen, B. Le Bon, and R. Sergysels. 1991. Domestic endotoxin exposure and clinical severity of asthma. *Clin. Exp. Allergy* 21: 441–448.
37. Gereda, J. E., D. Y. Leung, A. Thatayatikom, J. E. Streib, M. R. Price, M. D. Klinnert, and A. H. Liu. 2000. Relation between house-dust endotoxin exposure, type 1 T-cell development, and allergen sensitisation in infants at high risk of asthma. *Lancet* 355: 1680–1683.

Corrections

Kim, Y.-S., W.-H. Lee, E.-J. Choi, J.-P. Choi, Y. J. Heo, Y. S. Gho, Y.-K. Jee, Y.-M. Oh, and Y.-K. Kim. 2015. Extracellular vesicles derived from Gram-negative bacteria, such as *Escherichia coli*, induce emphysema mainly via IL-17A–mediated neutrophilic inflammation. *J. Immunol.* 194: 3361–3368.

There was an error in the funding information in the published article. Some important information for Yeon-Mok Oh was omitted.

In the footnotes, the sentence "This work was supported by National Research Foundation of Korea Grant 2010-0028684 funded by the Korea government (to Y.-K.K.)" should read "This work was supported by National Research Foundation of Korea Grant funded by the Korea government 2010-0028684 (to Y.-K.K.) and Asan Institute for Life Sciences Grant 14-306 (to Y.-M.O.)."

www.jimmunol.org/cgi/doi/10.4049/jimmunol.1500973