

ORIGINAL ARTICLE

EXPERIMENTAL ALLERGY AND IMMUNOLOGY

***Staphylococcus aureus*-derived extracellular vesicles induce neutrophilic pulmonary inflammation via both Th1 and Th17 cell responses**

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Abstract

Background: Recent evidence indicates that *Staphylococcus aureus*, one of the most important human pathogens, secretes vesicles into the extracellular milieu.

Objective: To evaluate whether inhalation of *S. aureus*-derived extracellular vesicles (EV) is causally related to the pathogenesis of inflammatory pulmonary diseases.

Methods: *Staphylococcus aureus* EV were prepared by sequential ultrafiltration and ultracentrifugation. The innate immune response was evaluated *in vitro* after the application of EV to airway epithelial cells and alveolar macrophages. *In vivo* innate and adaptive immune responses were evaluated after airway exposure to EV. Adjuvant effects of EV on the development of hypersensitivity to inhaled allergens were also evaluated after airway sensitization with *S. aureus* EV and ovalbumin (OVA).

Results: *Staphylococcus aureus* and *S. aureus* EV were detected in house dust. Alveolar macrophages produced both tumor necrosis α (TNF- α) and interleukin 6 (IL-6) after *in vitro* stimulation with *S. aureus* EV, whereas airway epithelial cells produced only IL-6. Repeated airway exposure to *S. aureus* EV induced both Th1 and Th17 cell responses and neutrophilic pulmonary inflammation, mainly via a Toll-like receptor 2 (TLR2)-dependent mechanism. In terms of adjuvant effects, airway sensitization with *S. aureus* EV and OVA resulted in neutrophilic pulmonary inflammation after OVA challenge alone. This phenotype was partly reversed by the absence of interferon γ (IFN- γ) or IL-17.

Conclusion: *Staphylococcus aureus* EV can induce Th1 and Th17 neutrophilic pulmonary inflammation, mainly in a TLR2-dependent manner. Additionally, *S. aureus* EV enhance the development of airway hypersensitivity to inhaled allergens.

Asthma is a chronic inflammatory disease characterized by inflammation in the airways and airway hyper-responsiveness (1). Asthma is caused by overwhelming activation of the adaptive immune system against inhaled allergens (2). The Th2 hypothesis may explain asthma pathogenesis because the asthma phenotype, which includes eosinophilic inflammation and increased IgE, is in accordance with the phenotype of the Th2 cell response. However, much evidence indicates that a significant proportion of asthmatic and chronic obstructive pulmonary disease (COPD) patients are characterized by

neutrophilic inflammation and increased interferon γ (IFN- γ) and interleukin 17 (IL-17) levels (3, 4). Although these phenotypes are obvious in severe and corticosteroid-resistant patients, the pathogenesis of Th1 and Th17 neutrophilic inflammation in asthma and COPD patients is unclear.

Airborne allergens are important causative agents of asthma. House dust mite-derived allergens demonstrate protease activity and induce a Th2 cytokine-mediated immune response. For example, Der p1, a house dust mite-derived allergen, can stimulate mast cells and eosinophils and elicit a

Th2-type immune response (5, 6). However, many allergens are immunologically inert. For example, the body maintains tolerance to inhaled innocuous antigens (7). Nevertheless, airway inflammation can be caused by such antigens, and it remains unclear why some individuals develop airway inflammation to inhaled allergens, while others do not. One possible explanation for hypersensitivity to inhaled allergens is disruption of airway tolerance by pathogen-associated molecular patterns (PAMP). Studies have shown that airway exposure to allergens contaminated with low-dose lipopolysaccharide (LPS) induces Th2 immune responses, whereas high-dose LPS induces both Th1 and Th17 immune responses (3, 8). Viral PAMP also induce hypersensitivity to inhaled allergens; allergen plus high-dose double-stranded RNA (dsRNA) induce a Th1 cell response, while low-dose dsRNA elicits both Th2 and Th17 cell responses (9, 10).

Staphylococcus aureus is a Gram-positive bacterium and an important pathogen that mediates both community-acquired and nosocomial infections (11, 12). Many reports have demonstrated interesting links between *S. aureus* and allergic diseases, such as asthma and atopic dermatitis. For example, *S. aureus* colonized skin lesions in 70–90% of atopic dermatitis patients (13). Asthma severity is associated with *S. aureus* (14), and *S. aureus*-derived molecules, such as lipoteichoic acid (LTA) and peptidoglycan (PGN), can induce airway inflammation (15, 16). Recently, we demonstrated that *S. aureus* secretes vesicles into the extracellular milieu; these are termed 'extracellular vesicles' (EV) (17). We also showed that *S. aureus*-derived EV can induce atopic dermatitis-like skin inflammation (18).

Here, we hypothesized that *S. aureus*-derived EV in house dust are an important causative agent underlying the development of inflammation pulmonary diseases, such as asthma and/or COPD. To test this, we evaluated pulmonary inflammation and associated immunologic mechanisms induced by airway exposure to *S. aureus*-derived EV. We also evaluated whether *S. aureus*-derived EV enhance the development of airway hypersensitivity to inhaled allergens.

Methods

Mice

Wild-type (WT) C57BL/6 and BALB/c mice and IL-4R^{-/-} and IFN- γ ^{-/-} mice (BALB/c background) were purchased from Jackson Laboratories (Bar Harbor, ME, USA). IL-17^{-/-} (BALB/c background) mice were gifted from Y-C Sung (POSTECH, Pohang, Korea). Toll-like receptor 2 (TLR2)^{-/-} mice (C57BL/6 background) were purchased from Oriental BioService (Kyoto, Japan). Mice were bred in specific pathogen-free facilities at POSTECH, and all live animal experiments were approved by the Pohang University Science and Technology (POSTECH) Ethics Committee.

Reagents

The innocuous allergen ovalbumin (OVA) grade V was purchased from Sigma-Aldrich (St. Louis, MO, USA).

Endotoxin in OVA was removed by DetoxiGel Endotoxin Removing Gel (Thermo, Rockford, IL, USA). LTA and PGN were purchased from InvivoGen (San Diego, CA, USA).

Isolation of *S. aureus* EV

Staphylococcus aureus-derived EV were obtained as described previously (17). Briefly, *S. aureus* (ATCC14458) was cultured in nutrient broth (Merck, Darmstadt, Germany) at 37°C to an OD₆₀₀ of 1.0. Bacteria were harvested by centrifugation and filtration. The filtrate was concentrated by ultrafiltration using a 100-kD hollow-fiber membrane (Amersham Biosciences, Uppsala, Sweden). The resulting concentrated filtrate was filtered to remove any remaining cells, and extracellular vesicles were isolated by ultracentrifugation at 150 000 g for 3 h. Protein concentrations of the *S. aureus*-derived vesicles were measured using the Bradford assay (Bio-Rad Laboratories, Hercules, CA, USA). Hereafter, the *S. aureus*-derived vesicle dose refers to the quantity of *S. aureus*-derived vesicle proteins. Silkworm Larva Plasma assay (Wako Pure Chemical Industries Ltd., Osaka, Japan) to determine the PGN amount in EV was performed according to manufacturer's instruction.

Evaluation of *in vitro* innate immune response by *S. aureus*-derived EV

An airway epithelial cell line (A549 cells) and alveolar macrophage cell line (MH-S cells) were seeded (1×10^5 /well) onto 24-well plates at 37°C. These cells were stimulated with various amounts of LTA (1, 10 μ g/ml), PGN (3, 30 μ g/ml), and *S. aureus* EV (0.1, 1, and 10 μ g/ml). After 24 h (for A549) or 15 h (for MH-S) of stimulation, culture supernatants were collected and cytokine levels were measured by enzyme-linked immunosorbent assay (ELISA).

To evaluate *in vitro* innate immune responses induced by *S. aureus*-derived EV in TLR2^{-/-} mice, peritoneal macrophages were isolated as described previously (19). Briefly, mice were injected intraperitoneally with thioglycollate medium. Four days later, peritoneal fluid was collected and cells were isolated. Cells were seeded (2×10^5 /well) onto 24-well plates and incubated for 2 h. Plates were then washed twice to remove nonadherent cells. After an overnight incubation, peritoneal macrophages were incubated with stimulants and supernatants were collected after 15 h.

Confocal microscopy imaging

A549 and MH-S cells were seeded onto a gelatin-coated coverslip and administered 5 μ g/ml of Vybrant DiO (Invitrogen, Eugene, OR, USA)-labeled *S. aureus* EV. After 5 h of this procedure, coverslips were washed three times with phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde. Then, to label cell membrane, coverslips were stained with Vybrant DiI (Invitrogen). Coverslips were then stained with Hoechst and mounted on a glass slide. Images were obtained using FluoView 1000 (Olympus).

Analysis of house dust

House dust was collected from a westernized house, dissolved in sterile PBS, and inoculated on Luria–Bertani (LB) agar spread plates. Plates were incubated at 37°C for 24 h, and colonies were picked and analyzed by biochemical assessment (Samkwang Medical Laboratories, Seoul, Korea). To detect EV in house dust, bacteria were removed from dust-dissolved PBS by centrifugation and the supernatants were ultracentrifuged at 150 000 *g* for 3 h. Pellets were collected as the dust EV fraction. Protein levels in dust EV fractions were measured by Bradford assay. The levels of *S. aureus* EV-related proteins in house dust were determined using a polyclonal anti-*S. aureus* EV antibody, as described previously (18).

In vivo evaluation of the effects of *S. aureus* EV on the development of innate and adaptive immune responses

To evaluate the innate immune response to *S. aureus* EV, 0.1 or 1 µg of EV in 30 µl PBS was administered intranasally to mice once or twice, and then inflammation over time was measured. To evaluate the adaptive immune response, *S. aureus* EV were applied intranasally to mouse airways twice per week for three weeks. Immunologic parameters and inflammation were evaluated 6 and 24 h after the last application, respectively.

In vivo evaluation of the adjuvant effects of *S. aureus* EV on the development of hypersensitivity to inhaled allergens

A noneosinophilic asthma model induced by sensitization with LPS-contaminated allergens was generated, as described previously (3). In the present study, *S. aureus* EV were used as a substitute for LPS. Briefly, 6-week-old mice were sensitized intranasally with 75 µg LPS-depleted OVA plus *S. aureus* EV (0.1 or 1 µg) on days 0, 1, 2, and 7 and then challenged with 50 µg OVA alone on days 14, 15, 21, and 22. Immunologic analysis was conducted 6 h after the allergen challenge on day 21. Lung inflammation was evaluated 24 h after the last allergen challenge on day 22.

Evaluation of lung inflammation

Cellularity in bronchoalveolar lavage (BAL) fluid was analyzed as described previously. (3) Briefly, after counting the total number of cells in BAL samples, the cell pellet was diluted with 200 µl PBS. In total, 300 inflammatory cells were counted and the BAL differential cell count was determined by Diff-Quik staining. Inflammatory cells were classified as macrophages, lymphocytes, neutrophils, or eosinophils.

Evaluation of T-cell responses

Single cells were isolated from lung tissues. For the re-stimulation response, lung cells were incubated (4×10^6 /ml) in 96-well plates at 37°C with *S. aureus* EV (0.1 µg/ml) or

medium only. After 72-h incubation, culture supernatant was collected and cytokine levels therein were determined. For CD3 and CD28 antibody stimulation, isolated lung cells were incubated in plates coated with anti-CD3 and anti-CD28 antibodies (1 µg/ml each; eBioscience, San Diego, CA) at 37°C. After 12-h incubation, culture supernatant was collected and cytokine levels were determined.

Measurement of cytokine and antibody levels

Cytokine levels from BAL fluids or culture supernatants were measured by ELISA, according to the manufacturer's instructions (R&D Systems, Minneapolis, MN, USA). Serum levels of total IgG1, IgG2a, and IgE antibodies were measured by ELISA according to the manufacturer's instructions (Bethyl Laboratory Inc., Montgomery, TX, USA).

Statistical analysis

For multiple comparisons among groups, an analysis of variance (ANOVA) was used, followed by unpaired *t*-tests or Wilcoxon rank sum tests for pairwise comparisons. An ANOVA linearity test was used to test for trends. The statistical significance was set *a priori* at $P < 0.05$.

Results

S. aureus-derived EV in house dust

To evaluate the presence of *S. aureus*, house dust was cultured on LB agar plates. After biochemical analysis, *S. aureus*, *S. hominis*, *Micrococcus lylae*, *Pseudomonas stutzeri*, and three unidentified bacilli were identified in house dust (Fig. 1A). Next, we evaluated the presence of *S. aureus*-derived EV in house dust. The levels of *S. aureus*-derived EV were measured by ELISA using polyclonal antibodies to *S. aureus* EV (18). Our present data indicated that 25 and 4 ng of *S. aureus* EV were present in 1 µg of the dust EV fraction and 1 µg of dust, respectively (Fig. 1B). Taken together, these results suggest that *S. aureus*-derived EV are present in dust from a westernized house.

Induction of pro-inflammatory mediator production from airway epithelial cells and alveolar macrophages by *S. aureus* EV *in vitro*

Airway epithelial cells and alveolar macrophages are the first line of defense against pathogenic nanoparticles. We examined whether *S. aureus* EV were internalized by these cells and whether this induced pro-inflammatory mediator production. Using confocal microscopy, *S. aureus* EV were detected in the cytoplasm of both airway epithelial cells (A549 cells) and alveolar macrophages (MH-S cells) (Fig. 2A). The production of both tumor necrosis factor α (TNF- α) and IL-6 by alveolar macrophages was enhanced by stimulation with *S. aureus* EV as well as with LTA and PGN in a dose-dependent manner; however, TNF- α was not produced by airway epithelial cells after stimulation with *S. aureus* EV, LTA, or

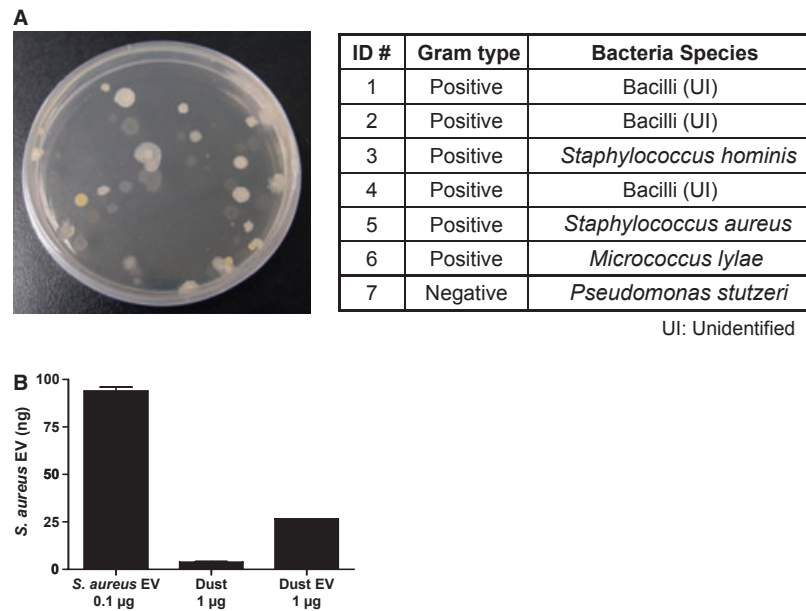


Figure 1 *Staphylococcus aureus* and *S. aureus* extracellular vesicles (EV) in house dust. (A) House dust from a westernized house was inoculated on a Luria–Bertani (LB) agar plate. After incubation for 24 h, bacterial colonies were obtained and then biochemically

analyzed. (B) *S. aureus* EV-related proteins in house dust and the isolated EV fraction were quantified by enzyme-linked immunosorbent assay (ELISA).

PGN, whereas IL-6 was enhanced by only *S. aureus* EV, but not by LTA and PGN (Fig. 2B). Staphylococcal enterotoxin B is a well-known exotoxin produced by *S. aureus*. We evaluated the production of TNF- α and IL-6 after the stimulation with this toxin. This study showed that the production of these cytokines from alveolar macrophages (MH-S cells) was not enhanced by the stimulation with staphylococcal enterotoxin B (Fig. S1). Taken together, these findings suggest that *S. aureus* EV can be internalized into both airway epithelial cells and alveolar macrophages and that the production of pro-inflammatory mediators by these two cells is distinct after the stimulation with *S. aureus* EV.

Innate immune responses in the lung induced by airway exposure to *S. aureus* EV

To evaluate *in vivo* innate immune responses by *S. aureus* EV, different doses of *S. aureus* EV were applied to mouse airways. The number of inflammatory cells in BAL fluid was enhanced after exposure to EV; inflammatory cells were significantly increased by exposure to 1 μ g EV as compared with 0.1 μ g EV (Fig. 2C). Similarly, the production of pro-inflammatory and immunomodulatory mediators, such as TNF- α , IL-12 (a Th1-polarizing cytokine), and IL-6 (Th17-polarizing), was significantly enhanced after the application of *S. aureus* EV in a dose-dependent manner (Fig. 2D). However, the production of IL-4 (a Th2-polarizing cytokine) was not enhanced by the inhalation of *S. aureus* EV (data not shown). These results show that short-term inhalation of *S. aureus* EV induces the production of pro-inflammatory mediators, such as TNF- α and Th1- and Th17-polarizing cytokines, in a dose-dependent manner.

Pulmonary inflammation caused by repeated inhalation of *S. aureus* EV

To evaluate whether repeated airway exposure to *S. aureus* EV induces pulmonary inflammation, *S. aureus* EV were administered intranasally to mouse airways twice per week for three weeks, and phenotypes were evaluated 24 h after the last application (Fig. S3A). Lung infiltration of inflammatory cells, especially neutrophils, was enhanced by *S. aureus* EV to a significantly greater degree than by PBS, in a dose-dependent manner (Fig. 3A). Histological analysis of lung tissues also showed that both peribronchiolar and perivascular infiltration of inflammatory cells was enhanced by exposure to *S. aureus* EV (Fig. 3B). In terms of antibody production, repeated exposure to EV increased IgG1 production in a dose-dependent manner, whereas it did not enhance IgG2a or IgE production (Fig. S3B). Taken together, these findings suggest that repeated airway exposure to *S. aureus* EV induces neutrophilic pulmonary inflammation in a dose-dependent manner.

Production of T-cell subset cytokines by repeated inhalation of *S. aureus* EV

To characterize T-cell responses induced by repeated inhalation of *S. aureus* EV, immunologic parameters were measured 6 h after the final *S. aureus* EV inhalation (Fig. S3A). IFN- γ (Th1 cytokine) and IL-17 (Th17 cytokine) levels were increased by the inhalation of *S. aureus* EV in a dose-dependent manner, whereas BAL IL-4 levels were not (Fig. 3C). To evaluate T-cell-related cytokine production in the lungs induced by *S. aureus* EV inhalation, isolated lung cells were

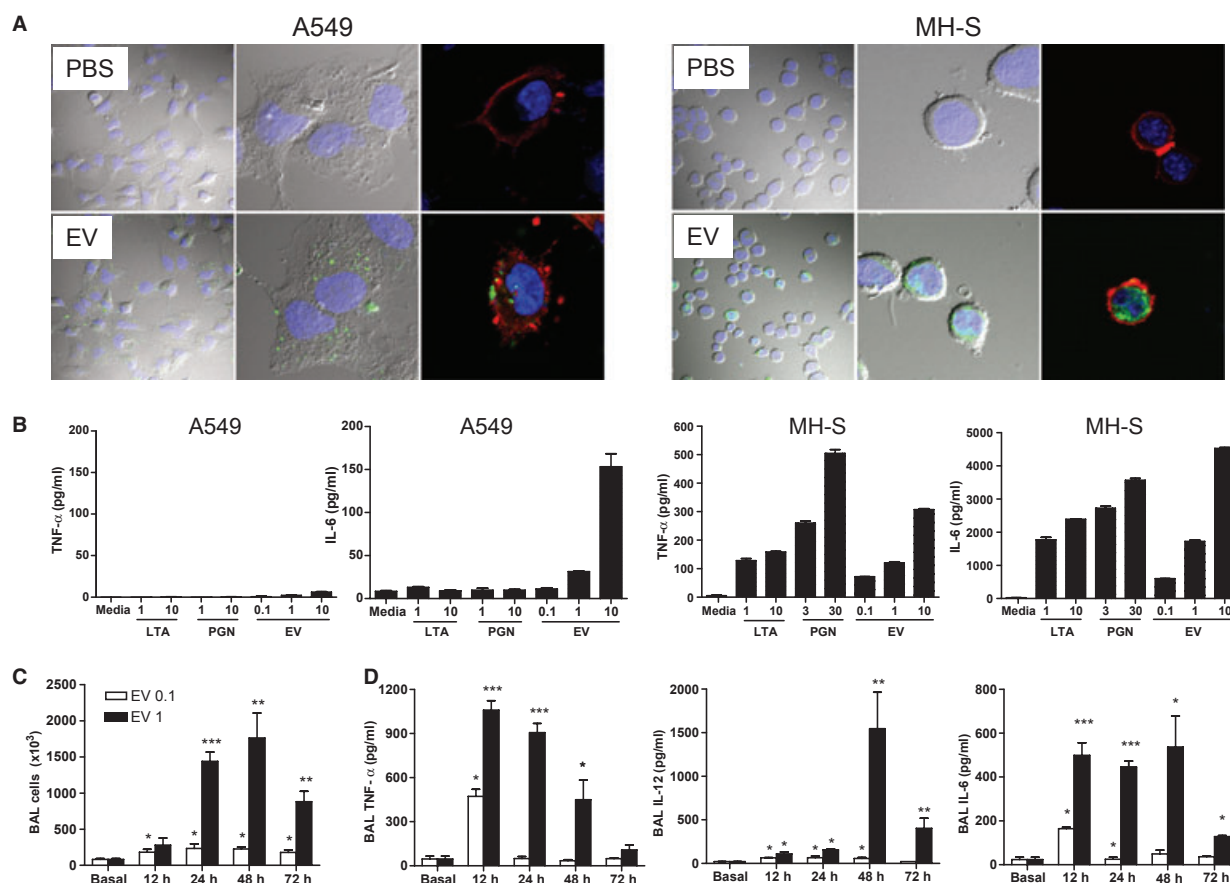


Figure 2 *In vitro* and *in vivo* innate immune response induced by *Staphylococcus aureus* extracellular vesicles (EV). (A) Confocal microscopic imaging showed that *S. aureus* EV is internalized by airway epithelial cells (A549) and alveolar macrophages (MH-S). Left and middle panels show green-colored EV on DIC images. Right panel shows merged image (green: *S. aureus* EV, blue: nucleus, red: cell membrane). *S. aureus* EV were detected in cytoplasm. (B) Levels of pro-inflammatory cytokines (tumor necrosis factor α [TNF- α] and interleukin 6 [IL-6]) in supernatants of A549

cells and MH-S cells after stimulation with lipoteichoic acid (LTA), peptidoglycan (PGN), and *S. aureus* EV. (C) The number of inflammatory cells in bronchoalveolar lavage (BAL) fluids at different time points after the inhalation of *S. aureus* EV. (D) TNF- α , IL-12, and IL-6 levels in BAL fluids at different time points after the inhalation of *S. aureus* EV. Number means concentration (μ g/ml). Data are expressed as mean $\pm$ SEM; * P < 0.05; ** P < 0.01; *** P < 0.001 vs the basal level.

stimulated with *S. aureus* EV (0.1 μ g/ml) and then the levels of T-cell-derived cytokines were determined. Consistent with BAL cytokine levels, the production of both Th1 (IFN- γ) and Th17 (IL-17) cytokines was enhanced in mice exposed to *S. aureus* EV as compared with those exposed to PBS, in a dose-dependent manner; however, BAL IL-4 levels were not (Fig. 3D). Taken together, these results suggest that repeated inhalation of *S. aureus* EV induces both Th1 and Th17 immune responses.

Adjuvant effects of *S. aureus* EV on the development of airway hypersensitivity to inhaled allergens

Inhalation of allergens contaminated with PAMP breaks airway tolerance to inhaled allergens (3, 9, 10). Because *S. aureus* EV and allergens can easily be inhaled simultaneously, we evaluated the adjuvant effects of

S. aureus EV on the development of immune responses to inhaled allergens. To test this, mice were sensitized intranasally with an allergen (OVA) and *S. aureus* EV and then challenged with OVA alone as shown in Fig. S4A. BAL cellularity 24 h after the last OVA challenge showed that lung infiltration of inflammatory cells, especially macrophages and neutrophils, was significantly increased in mice sensitized with OVA plus 0.1 or 1 μ g EV as compared with those sensitized with PBS or OVA alone (Fig. 4A). Similar to BAL cellularity, histological analysis of lung tissues showed that both peribronchiolar and perivascular infiltration of inflammatory cells was enhanced in OVA + EV (0.1 or 1 μ g)-sensitized mice compared to OVA- or PBS-sensitized mice (Fig. 4B). Serum IgG1 levels were also significantly increased in the former as compared with the latter groups, whereas IgG2a and IgE levels were similar in all groups (Fig. S4B). When cytokine levels in BAL fluids were

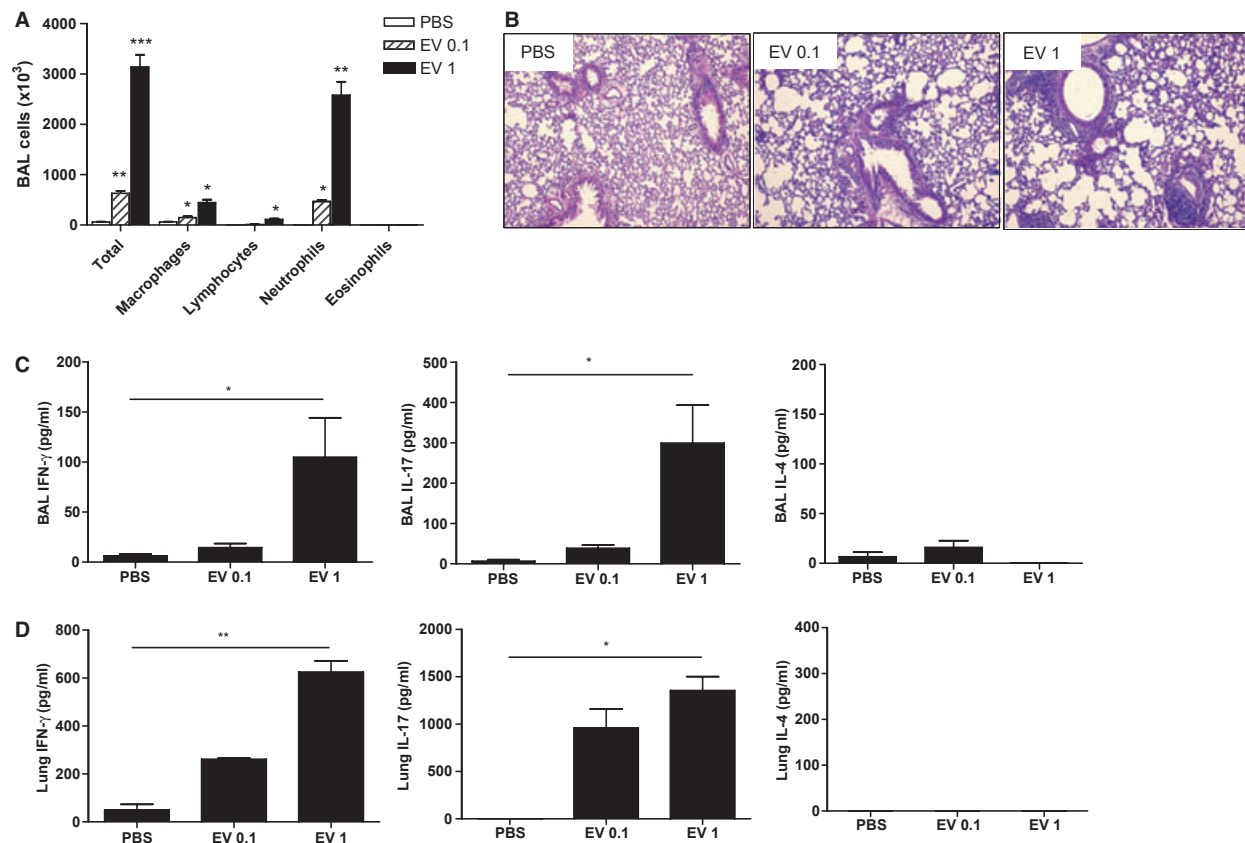


Figure 3 Both Th1 and Th17 cell responses are induced by repeated inhalation of *Staphylococcus aureus* extracellular vesicles (EV). BALB/c wild-type mice ($n = 5$ per group) were exposed to *S. aureus* EV twice per week for 3 weeks. (A) Bronchoalveolar lavage (BAL) cellularity 24 h after the final *S. aureus* EV exposure on day 15. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ vs the phosphate-buffered saline (PBS) group. (B) Representative lung histology 24 h after the final *S. aureus* EV exposure on day 15. (hematoxylin &

eosin [H&E] stain, $\times 100$). (C) Interferon γ (IFN- γ), interleukin 17 (IL-17), and IL-4 levels in BAL fluids 6 h after the final EV application. * $P < 0.05$ by an analysis of variance (ANOVA) linearity test. (D) IFN- γ , IL-17, and IL-4 levels in culture supernatants 72 h after the stimulation of lung cells with *S. aureus* EV (0.1 $\mu\text{g}/\text{ml}$). * $P < 0.05$; ** $P < 0.05$ by ANOVA linearity test. Data are expressed as mean $\pm$ SEM.

evaluated 6 h after OVA challenge, IL-17 levels were significantly increased in OVA + EV (0.1 or 1 μg)-sensitized mice as compared with OVA- or PBS-sensitized mice, IFN- γ levels were enhanced in only OVA + EV (1 μg)-exposed mice as compared with the other groups, and IL-4 levels were similar among all groups (Fig. 4C). Additionally, to evaluate cytokine production by lung T cells, isolated lung cells were stimulated with anti-CD3 and anti-CD28 antibodies. IL-17 levels in culture supernatants were enhanced in OVA + EV (0.1 or 1 μg)-sensitized mice to a greater degree than in OVA- or PBS-sensitized mice. IFN- γ levels were enhanced only in the OVA + EV (1 μg)-sensitized mice vs the other groups. Lastly, IL-4 levels were similar among all groups (Fig. 4D). Collectively, these results suggest that inhalation of *S. aureus* EV with allergens disrupts airway tolerance to inhaled allergens, which is characterized by Th17 and/or Th1 cell responses and noneosinophilic inflammation.

Role of IFN- γ , IL-4, and IL-17 in the development of allergic inflammation induced by sensitization with allergen plus *S. aureus* EV

To delineate the roles of Th1, Th2, and Th17 cytokines in the development of allergic inflammation induced by sensitization with *S. aureus* EV, IFN- γ -, IL-4R α -, and IL-17-deficient mice were sensitized intranasally with OVA plus EV (1 μg) and then challenged with OVA alone (Fig. S4A). When we evaluated phenotypes 24 h after the last OVA challenge, lung infiltration of inflammatory cells was partly reversed by the absence of IFN- γ or IL-17, but was unaffected by the absence of IL-4R α (Fig. 5A). Histological analysis showed that both peribronchiolar and perivascular infiltration of inflammatory cells was decreased in OVA + EV-exposed IFN- γ ^{-/-} and IL-17^{-/-} mice compared to WT and IL-4R α ^{-/-} mice sensitized in the same manner (Fig. 5B). In terms of antibody production, serum OVA-

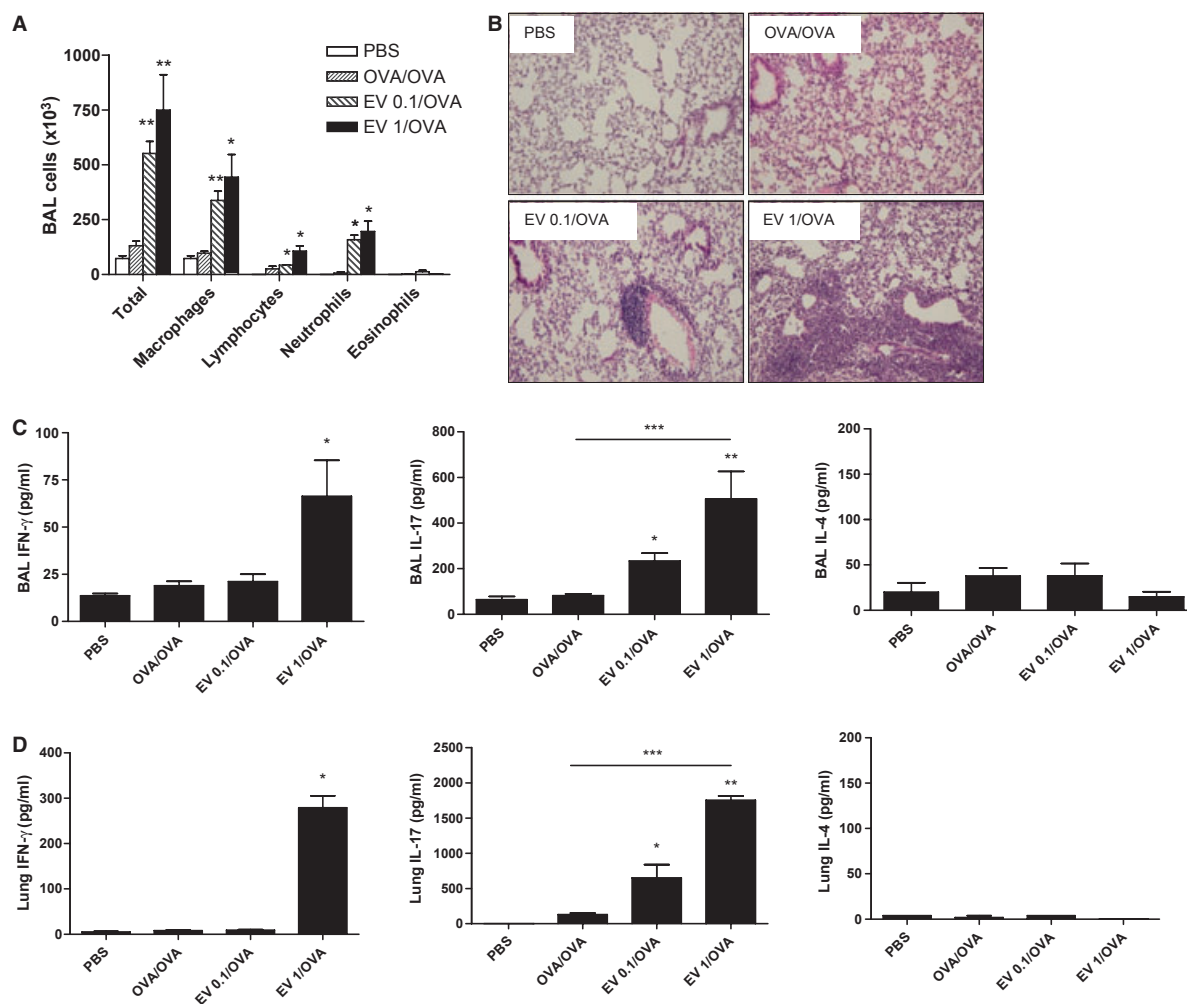


Figure 4 Sensitization with *Staphylococcus aureus* extracellular vesicles (EV) and allergens induces both Th1 and Th17 cell responses and noneosinophilic pulmonary inflammation. BALB/c wild-type mice ($n = 5$ each group) were sensitized intranasally with ovalbumin (OVA, 75 μ g) plus *S. aureus* EV (0.1 or 1 μ g) on days 0, 1, and 7 and then challenged with OVA (50 μ g) on days 14, 15, 21, and 22. (A) Bronchoalveolar lavage (BAL) cellularity 24 h after the final OVA challenge. OVA/OVA: sensitized with OVA alone, EV0.1/OVA and EV1/OVA: sensitized with OVA plus 0.1 and 1 μ g

S. aureus EV, respectively. (B) Representative lung histology (hematoxylin & eosin [H&E] stain, $\times 200$). (C) Interferon γ (IFN- γ), interleukin 17 (IL-17), and IL-4 levels in BAL fluids 6 h after OVA challenge on day 21. (D) IFN- γ , IL-17, and IL-4 levels in culture supernatants 12 h after the stimulation of lung cells with anti-CD3 and anti-CD28 antibodies. Data are expressed as mean $\pm$ SEM; * $P < 0.05$; ** $P < 0.01$ vs OVA/OVA group; *** $P < 0.05$ by an analysis of variance (ANOVA) linearity test.

specific IgG1 levels were significantly decreased in OVA + EV-sensitized IFN- $\gamma^{-/-}$, IL-4 $\alpha^{-/-}$, and IL-17 $^{-/-}$ mice compared to WT mice sensitized in the same manner; however, OVA-specific IgG2a levels were significantly increased in OVA + EV-sensitized IL-4 $\alpha^{-/-}$ mice compared to IFN- $\gamma^{-/-}$, IL-17 $^{-/-}$, and WT mice sensitized in the same manner (Fig. 5C). Taken together, these results suggest that pulmonary inflammation induced by allergens plus *S. aureus* EV is dependent on both the Th1 and Th17 cell responses, irrespective of allergen-specific antibody production.

TLR2-dependency of *S. aureus* EV-induced innate immune responses

Staphylococcus aureus is recognized by TLR2 in host cells (20). *S. aureus* EV were found to harbor LTA, which is a TLR2 ligand (21) (Fig. S2). Thus, we examined the role of TLR2 in the recognition of *S. aureus* EV using TLR2 $^{-/-}$ mice. Peritoneal macrophages were isolated from TLR2 $^{-/-}$ and WT mice and then stimulated with *S. aureus* EV and LTA. IL-6 and TNF- α production by peritoneal macrophages after the stimulation with *S. aureus* EV was almost completely abolished by

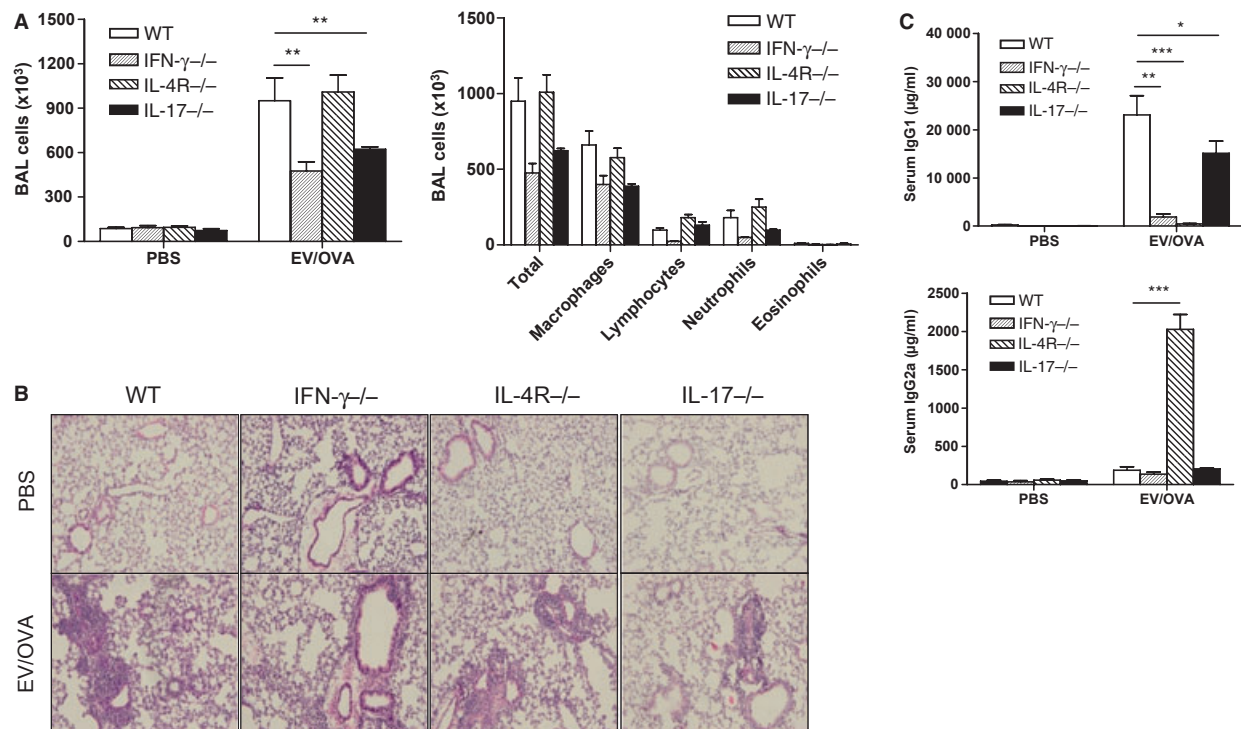


Figure 5 Pulmonary inflammation induced by sensitization with allergens and *Staphylococcus aureus* extracellular vesicles (EV) is dependent on both interferon γ (IFN- γ) and interleukin 17 (IL-17). IFN- γ ^{-/-}, IL4R α ^{-/-}, IL-17^{-/-}, and wild-type (WT) (all BALB/c background) mice ($n = 5$ each) were sensitized intranasally with ovalbumin (OVA, 75 μ g) plus *S. aureus* EV (1 μ g) on days 0, 1, and 7 and

then challenged with OVA (50 μ g) on days 14, 15, 21, and 22. (A) Bronchoalveolar lavage (BAL) cellularity 24 h after the final OVA challenge. (B) Representative lung histology (hematoxylin & eosin [H&E] stain, $\times 200$). (C) Serum OVA-specific IgG1 and IgG2a levels. Data are expressed as mean $\pm$ SEM; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

the absence of TLR2. In addition, IL-6 and TNF- α production after the stimulation with LTA was completely blocked in the absence of TLR2 (Fig. 6A). Next, to evaluate *in vivo* innate immune responses after airway exposure to *S. aureus* EV in TLR2^{-/-} and WT mice, *S. aureus* EV were twice administered intranasally to mouse airways. Lung infiltration of inflammatory cells, especially macrophages and neutrophils, was significantly lower in *S. aureus* EV-exposed TLR2^{-/-} mice than in WT mice exposed in the same manner (Fig. 6B). Additionally, the production of pro-inflammatory and immunomodulatory mediators, such as TNF- α and IL-6, was significantly enhanced after *S. aureus* EV inhalation in WT mice, whereas this enhanced production was abolished by the absence of TLR2 (Fig. 6C). Collectively, these results suggest that inhalation of *S. aureus* EV induces pulmonary inflammation and the production of pro-inflammatory and immunomodulatory mediators in a principally TLR2-dependent manner.

Discussion

The role of infectious agents in the etiology of many inflammatory diseases is increasingly being recognized. *S. aureus* colonizes the skin and nasopharynx and is important in the development or aggravation of allergic diseases, such as

atopic dermatitis and/or asthma. Recent evidence demonstrates that *S. aureus* produces EV that contain many pathogenesis-related molecules (17). Here, we aimed to elucidate the relationship between *S. aureus*-derived EV and the development of inflammatory pulmonary diseases. Our data suggest that *S. aureus* EV in an indoor environment can induce neutrophilic pulmonary inflammation and enhance airway hypersensitivity to inhaled allergens.

Many environmental factors and pathogens are associated with allergic diseases (12, 22). *S. aureus* colonization of the skin and nasopharynx has been suggested to be a major risk factor for many diseases (23, 24). However, the relationship between nasopharyngeal colonization by *S. aureus* and asthma has been reported not to be significant (14, 25). The current study showed that *S. aureus* and *S. aureus* EV are present in indoor dust, indicating that *S. aureus* in house dust produces EV. These findings led us to speculate that EV derived from *S. aureus* that is present in the indoor environment, rather than *S. aureus* colonization of the nasopharynx, is important in the development of pulmonary inflammation.

Recent evidence has demonstrated that bacteria-derived EV can act as a pathogenic agent for the development of inflammatory diseases (18, 26, 27). In addition, many reports have shown that EV from many pathogenic bacteria modulate the

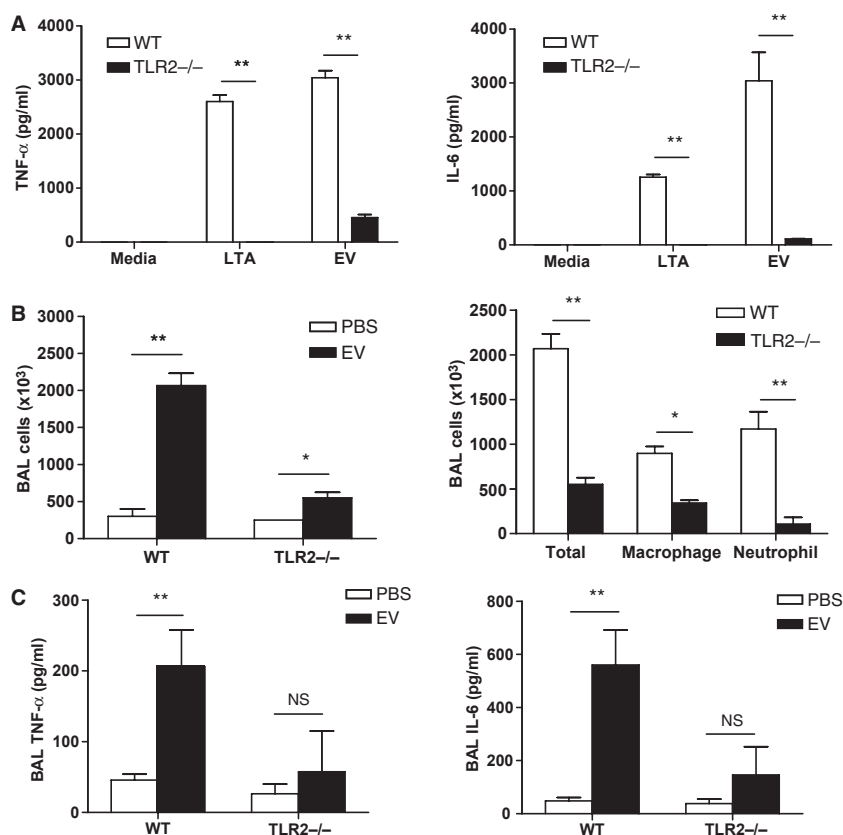


Figure 6 Pulmonary inflammation and production of pro-inflammatory and/or immune modulating mediators induced by *Staphylococcus aureus* extracellular vesicles (EV) are dependent on Toll-like receptor 2 (TLR2). (A) Peritoneal macrophages isolated from TLR2^{-/-} mice were stimulated with 1 μ g of lipoteichoic acid (LTA) and *S. aureus* EV. After the stimulation for 15 h, culture supernatants were collected and tumor necrosis factor α (TNF- α) and interleukin

6 (IL-6) levels were measured by enzyme-linked immunosorbent assay (ELISA). For (B) and (C), TLR2^{-/-} and wild-type (WT) (all C57BL/6 background) mice ($n = 5$ each) were exposed intranasally to *S. aureus* EV (1 μ g) twice and their phenotypes were evaluated 24 h after the final application. (B) Bronchoalveolar lavage (BAL) cellularity. (C) The levels of TNF- α and IL-6 in BAL fluids. Data are expressed as mean $\pm$ SEM; * $P < 0.05$; ** $P < 0.01$.

host immune response and induce inflammation (28–30). The first line of defense against inhaled pathogenic particles is airway epithelial cells and alveolar macrophages. The present study showed that *S. aureus* EV can be internalized by these cells, leading to the production of pro-inflammatory mediators, including TNF- α , and pulmonary inflammation after airway exposure to *S. aureus* EV. Moreover, the production of these pro-inflammatory mediators and pulmonary inflammation were found to be dependent on TLR2 signaling. Collectively, these findings suggest that airway exposure to *S. aureus* EV induces innate immune dysfunction, mainly via a TLR2-dependent mechanism.

Adaptive immune responses play an important role in the pathogenesis of allergic diseases. Growing evidence indicates that Th1 and Th17 as well as Th2 cell responses are important for the development of asthma (31–34). Both Th1 and Th17 cell responses have been found to be related to the development of neutrophilic inflammation (3, 35). The present study showed that repeated inhalation of *S. aureus* EV

induced neutrophilic pulmonary inflammation, which was associated with enhanced production of both Th1- and Th17-related cytokines. IL-12 and IL-6 are well-known mediators of Th1 and Th17 polarization, respectively (36, 37). The current study showed that inhalation of *S. aureus* EV enhanced the production of both IL-12 and IL-6. Together, these findings suggest that inhalation of *S. aureus* EV induces both Th1 and Th17 cell responses, partly via up-regulation of Th1- and Th17-polarizing cytokines.

Much evidence has been shown that the production of IgG1 and IgE antibodies is related to IL-4 and that IgG2a production is up-regulated by IFN- γ , whereas down-regulated by IL-4 (38, 39). However, some evidence suggests that the production of IgG1 is dependent on IFN- γ and IL-17 (40, 41). The present study showed that the production of IgG1 enhanced by *S. aureus* EV exposure was reversed by the absence of IFN- γ or IL-17. In contrast, the production of IgG2a after exposure to *S. aureus* EV was enhanced in the

absence of IL-4R, although this antibody production was not enhanced in WT mice. To sum up, these findings suggest that the production of IgG1 enhanced by *S. aureus* EV is dependent on both Th1 and Th17 cell responses.

The immune system maintains tolerance to inert antigens. However, much evidence suggests that simultaneous inhalation of innocent antigens (allergens) and adjuvants, such as bacteria- or virus-derived PAMP, induces airway hypersensitivity to those allergens (3, 10). Our data suggest that sensitization by allergens plus *S. aureus* EV induced allergen-induced neutrophilic pulmonary inflammation as well as the infiltration of mononuclear cells, which was associated with both Th1 and Th17 cell responses. Moreover, the infiltration of neutrophils and mononuclear cells into airways was found to be dependent on both IFN- γ and IL-17. These results suggest that Th1 and Th17 cell responses induced by *S. aureus* EV are important in chemotaxis of inflammatory cells. Given the presence of *S. aureus* EV in indoor environments, these data suggest that inhalation of allergens contaminated with *S. aureus* EV may be an important mechanism in the development of airway hypersensitivity to inhaled allergens.

In summary, this is to our knowledge the first report that inhalation of *S. aureus* EV induces both Th1 and Th17 cell responses and neutrophilic pulmonary inflammation. In addition, we found that inhalation of *S. aureus* EV enhances airway immune responses to inhaled allergens, characterized by both Th1 and Th17 cell responses and noneosinophilic pulmonary inflammation. Moreover, our data suggest that immune responses induced by *S. aureus* EV are principally dependent on TLR2 signaling. Thus, we suggest that *S. aureus* EV in indoor environments may be an important causative agent of pulmonary inflammatory diseases.

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References

- Adcock IM, Caramori G, Chung KF. New targets for drug development in asthma. *Lancet* 2008;**372**:1073–1087.
- Asher MI, Montefort S, Björkstén B, Lai CK, Strachan DP, Weiland SK et al. Worldwide time trends in the prevalence of symptoms of asthma, allergic rhinoconjunctivitis, and eczema in childhood: ISAAC Phases One and Three repeat multicountry cross-sectional surveys. *Lancet* 2006;**368**:733–743.
- Kim YK, Oh SY, Jeon SG, Park HW, Lee SY, Chun EY et al. Airway exposure levels of lipopolysaccharide determine type 1 versus type 2 experimental asthma. *J Immunol* 2007;**178**:5375–5382.
- Cosmi L, Liotta F, Maggi E, Romagnani S, Annunziato F. Th17 cells: new players in asthma pathogenesis. *Allergy* 2011;**66**:989–998.
- Wong CK, Li ML, Wang CB, Ip WK, Tian YP, Lam CW. House dust mite allergen Der p 1 elevates the release of inflammatory cytokines and expression of adhesion molecules in co-culture of human eosinophils and bronchial epithelial cells. *Int Immunol* 2006;**18**:1327–1335.
- Huang HJ, Lin YL, Liu CF, Kao HF, Wang JY. Mite allergen decreases DC-SIGN expression and modulates human dendritic cell differentiation and function in allergic asthma. *Mucosal Immunol* 2011;**4**:519–527.
- Akbari O, Freeman GJ, Meyer EH, Greenfield EA, Chang TT, Sharpe AH et al. Antigen-specific regulatory T cells develop via the ICOS-ICOS-ligand pathway and inhibit

Conflict of interest

The authors declare that there are no conflicts of interest.

Author contributions

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

Supporting Information

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

Figure S1. Cytokine production after *in vitro* stimulation with Staphylococcal enterotoxin B and *Staphylococcus aureus*-derived extracellular vesicles. Levels of pro-inflammatory cytokines in supernatants of MH-S cells after the stimulation with staphylococcal enterotoxin B and *S. aureus* EV. Numbers in X-axis are expressed as concentration ($\mu\text{g/ml}$).

Figure S2. The presence of lipoteichoic acid (LTA) in *Staphylococcus aureus*-derived extracellular vesicles. LTA were detected in *S. aureus* EV (10 μg) by western blot.

Figure S3. Production of *Staphylococcus aureus* extracellular vesicles (EV)-reactive IgG1 is enhanced by repeated airway exposure to *S. aureus* EV. (A) Study protocol. (B) Serum IgG1, IgG2a, and IgE levels 24 h after the final application. Data are expressed as mean $\pm$ SEM; * P < 0.05 by an analysis of variance (ANOVA) linearity test.

Figure S4. Production of allergen-specific IgG1 levels is enhanced by sensitization with an allergen (ovalbumin, OVA) plus *Staphylococcus aureus* extracellular vesicles (EV). (A) Study protocol. (B) Serum IgG1, IgG2a, and IgE levels 24 h after the final OVA challenge on day 22. Data are expressed as mean $\pm$ SEM; * P < 0.05; ** P < 0.01 vs OVA/OVA group; *** P < 0.05 by an analysis of variance (ANOVA) linearity test.

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- allergen-induced airway hyperreactivity. *Nat Med* 2002;**8**:1024–1032.
8. Kim YS, Choi SJ, Tae YM, Lee BJ, Jeon SG, Oh SY et al. Distinct roles of vascular endothelial growth factor receptor-1- and receptor-2-mediated signaling in T cell priming and Th17 polarization to lipopolysaccharide-containing allergens in the lung. *J Immunol* 2010;**185**:5648–5655.
 9. Choi JP, Kim YS, Tae YM, Choi EJ, Hong BS, Jeon SG et al. A viral PAMP double-stranded RNA induces allergen-specific Th17 cell response in the airways which is dependent on VEGF and IL-6. *Allergy* 2010;**65**:1322–1330.
 10. Jeon SG, Oh SY, Park HK, Kim YS, Shim EJ, Lee HS et al. TH2 and TH1 lung inflammation induced by airway allergen sensitization with low and high doses of double-stranded RNA. *J Allergy Clin Immunol* 2007;**120**:803–812.
 11. Greiner W, Rasch A, Kohler D, Salzberger B, Fatkenheuer G, Leidig M. Clinical outcome and costs of nosocomial and community-acquired Staphylococcus aureus bloodstream infection in haemodialysis patients. *Clin Microbiol Infect* 2007;**13**:264–268.
 12. Sakwinska O, Blanc DS, Lazor-Blanchet C, Moreillon M, Giddey M, Moreillon P. Ecological temporal stability of Staphylococcus aureus nasal carriage. *J Clin Microbiol* 2010;**48**:2724–2728.
 13. Huang JT, Abrams M, Tloutan B, Rademaker A, Paller AS. Treatment of Staphylococcus aureus colonization in atopic dermatitis decreases disease severity. *Pediatrics* 2009;**123**:e808–e814.
 14. Pastacaldi C, Lewis P, Howarth P. Staphylococci and staphylococcal superantigens in asthma and rhinitis: a systematic review and meta-analysis. *Allergy* 2011;**66**:549–555.
 15. Leemans JC, Heikens M, van Kessel KP, Florquin S, van der Poll T. Lipoteichoic acid and peptidoglycan from Staphylococcus aureus synergistically induce neutrophil influx into the lungs of mice. *Clin Diagn Lab Immunol* 2003;**10**:950–953.
 16. Leemans JC, Vervoordeldonk M, Florquin S, Van Kessel KP, van der Poll T. Differential role of interleukin-6 in lung inflammation induced by lipoteichoic acid and peptidoglycan from Staphylococcus aureus. *Am J Respir Crit Care Med* 2002;**165**:1445–1450.
 17. Lee EY, Choi DY, Kim DK, Kim JW, Park JO, Kim S et al. Gram-positive bacteria produce membrane vesicles: proteomics-based characterization of staphylococcus aureus-derived membrane vesicles. *Proteomics* 2009;**9**:5425–5436.
 18. Hong SW, Kim MR, Lee EY, Kim JH, Kim YS, Jeon SG et al. Extracellular vesicles derived from staphylococcus aureus induce atopic dermatitis-like skin inflammation. *Allergy* 2011;**66**:351–359.
 19. Kim HS, Armstrong D, Hamilton TA, Tebo JM. IL-10 suppresses LPS-induced kc mRNA expression via a translation-dependent decrease in mRNA stability. *J Leukoc Biol* 1998;**64**:33–39.
 20. Takeuchi O, Hoshino K, Akira S. Cutting edge: TLR2-deficient and MyD88-deficient mice are highly susceptible to Staphylococcus aureus infection. *J Immunol* 2000;**165**:5392–5396.
 21. Schwandner R, Dziarski R, Wesche H, Rothe M, Kirschning CJ. Peptidoglycan- and lipoteichoic acid-induced cell activation is mediated by toll-like receptor 2. *J Biol Chem* 1999;**274**:17406–17409.
 22. Williams RE. Healthy carriage of Staphylococcus aureus: its prevalence and importance. *Bacteriol Rev* 1963;**27**:56–71.
 23. Peacock SJ, de Silva I, Lowy FD. What determines nasal carriage of Staphylococcus aureus? *Trends Microbiol* 2001;**9**:605–610.
 24. Wertheim HF, Melles DC, Vos MC, van Leeuwen W, van Belkum A, Verbrugh HA et al. The role of nasal carriage in Staphylococcus aureus infections. *Lancet Infect Dis* 2005;**5**:751–762.
 25. Bisgaard H, Hermansen MN, Buchvald F, Loland L, Halkjaer LB, Bonnelykke K et al. Childhood asthma after bacterial colonization of the airway in neonates. *N Engl J Med* 2007;**357**:1487–1495.
 26. Amano A, Takeuchi H, Furuta N. Outer membrane vesicles function as offensive weapons in host-parasite interactions. *Microbes Infect* 2010;**12**:791–798.
 27. Park KS, Choi KH, Kim YS, Hong BS, Kim OY, Kim JH et al. Outer membrane vesicles derived from Escherichia coli induce systemic inflammatory response syndrome. *PLoS ONE* 2010;**5**:e11334.
 28. Bomberger JM, Ye S, Maceachran DP, Koeppen K, Barnaby RL, O'Toole GA et al. A Pseudomonas aeruginosa toxin that hijacks the host ubiquitin proteolytic system. *PLoS Pathog* 2011;**7**:e1001325.
 29. Nakao R, Hasegawa H, Ochiai K, Takashiba S, Aina A, Ohnishi M et al. Outer membrane vesicles of Porphyromonas gingivalis elicit a mucosal immune response. *PLoS ONE* 2011;**6**:e26163.
 30. Schaar V, de Vries SP, Perez Vidakovic ML, Bootsma HJ, Larsson L, Hermans PW et al. Multicomponent Moraxella catarrhalis outer membrane vesicles induce an inflammatory response and are internalized by human epithelial cells. *Cell Microbiol* 2011;**13**:432–449.
 31. Magnan AO, Mely LG, Camilla CA, Badier MM, Montero-Julian FA, Guillot CM et al. Assessment of the Th1/Th2 paradigm in whole blood in atopy and asthma. Increased IFN-gamma-producing CD8(+) T cells in asthma. *Am J Respir Crit Care Med* 2000;**161**:1790–1796.
 32. Cho SH, Stancia LA, Holgate ST, Johnston SL. Increased interleukin-4, interleukin-5, and interferon-gamma in airway CD4+ and CD8+ T cells in atopic asthma. *Am J Respir Crit Care Med* 2005;**171**:224–230.
 33. Molet S, Hamid Q, Davoine F, Nutku E, Taha R, Page N et al. IL-17 is increased in asthmatic airways and induces human bronchial fibroblasts to produce cytokines. *J Allergy Clin Immunol* 2001;**108**:430–438.
 34. Zhao Y, Yang J, Gao YD, Guo W. Th17 immunity in patients with allergic asthma. *Int Arch Allergy Immunol* 2010;**151**:297–307.
 35. Kim YS, Hong SW, Choi JP, Shin TS, Moon HG, Choi EJ et al. 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* 2009;**183**:5113–5120.
 36. Kimura A, Kishimoto T. IL-6: regulator of Treg/Th17 balance. *Eur J Immunol* 2010;**40**:1830–1835.
 37. Macatonia SE, Hosken NA, Litton M, Vieira P, Hsieh CS, Culpepper JA et al. Dendritic cells produce IL-12 and direct the development of Th1 cells from naive CD4+ T cells. *J Immunol* 1995;**154**:5071–5079.
 38. Takeda K, Tanaka T, Shi W, Matsumoto M, Minami M, Kashiwamura S et al. Essential role of Stat6 in IL-4 signalling. *Nature* 1996;**380**:627–630.
 39. Takahashi S, Fossati L, Iwamoto M, Merino R, Motta R, Kobayakawa T et al. Imbalance towards Th1 predominance is associated with acceleration of lupus-like autoimmune syndrome in MRL mice. *J Clin Invest* 1996;**97**:1597–1604.
 40. Mitsdoerffer M, Lee Y, Jager A, Kim HJ, Korn T, Kolls JK et al. Proinflammatory T helper type 17 cells are effective B-cell helpers. *Proc Natl Acad Sci U S A* 2010;**107**:14292–14297.
 41. Mattos AM, Almeida Cde S, Franken KL, Alves CC, Abramo C, de Souza MA et al. Increased IgG1, IFN-gamma, TNF-alpha and IL-6 responses to Mycobacterium tuberculosis antigens in patients with tuberculosis are lower after chemotherapy. *Int Immunol* 2010;**22**:775–782.

Fig. S1

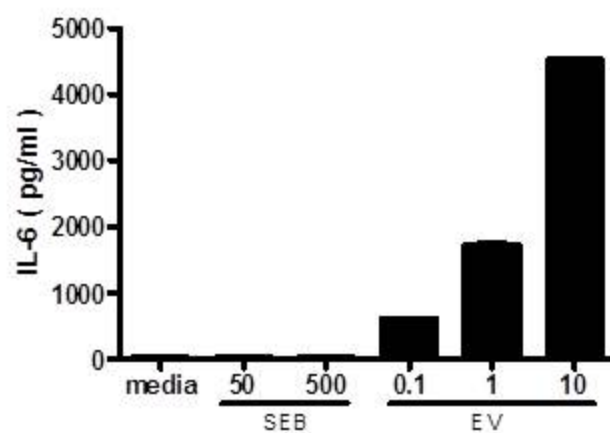
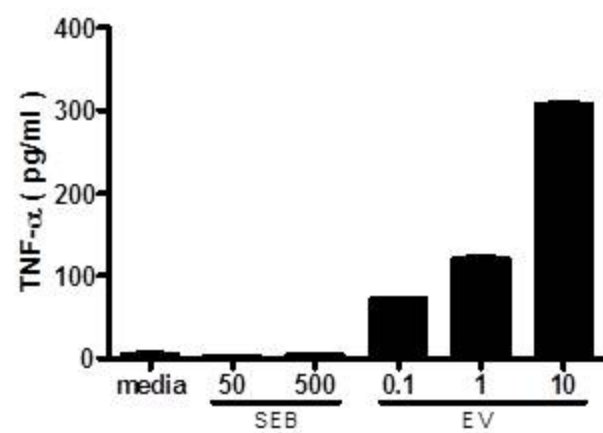


Fig. S2

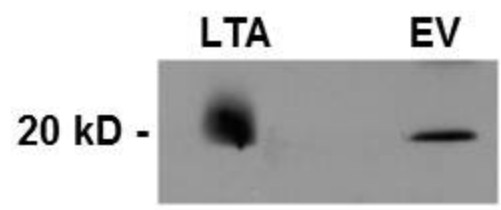


Fig. S3

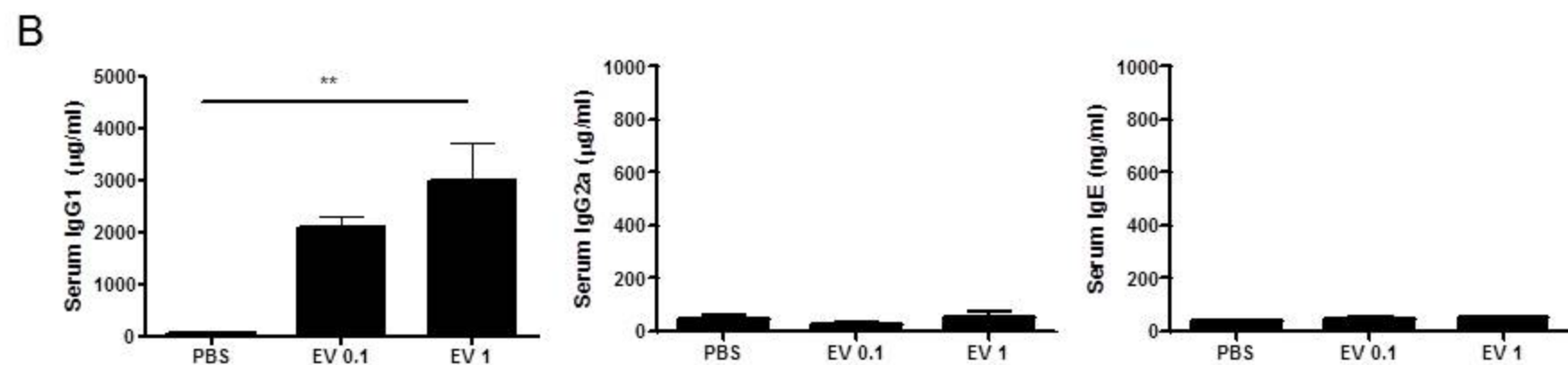
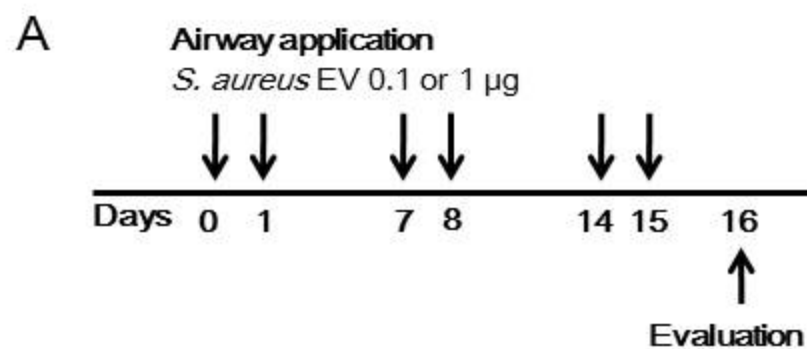


Fig. S4

