

# IL-6 Deficiency Exacerbates Allergic Asthma and Abrogates the Protective Effect of Allergic Inflammation against *Streptococcus pneumoniae* Pathogenesis

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Allergic asthma (AA) is characterized as a Th2-biased airway inflammation that can develop lung inflammation and remodeling of the respiratory tract. *Streptococcus pneumoniae* is a major respiratory pathogen, causing noninvasive (otitis media and pneumonia) and invasive diseases (sepsis) in humans. We sought to determine the role of IL-6 in the regulation of lung inflammation in murine AA caused by *Aspergillus fumigatus* as well as its consequence on the regulation of airway barrier integrity and *S. pneumoniae* disease. In an AA model, IL-6 deficiency led to increased lung inflammation, eosinophil recruitment, tissue pathology, and collagen deposition. Additionally, IL-6-deficient asthmatic mice exhibited reduced goblet cell hyperplasia and increased TGF- $\beta$  production. These key changes in the lungs of IL-6-deficient asthmatic mice resulted in dysregulated tight junction proteins and increased lung permeability. Whereas the host response to AA protected against *S. pneumoniae* lung disease, the IL-6 deficiency abrogated the protective effect of allergic inflammation against *S. pneumoniae* pathogenesis. Consistent with in vivo data, IL-6 knockdown by small interfering RNA or the blockade of IL-6R signaling exacerbated the TGF- $\beta$ -induced dysregulation of tight junction proteins, E-cadherin and N-cadherin expression, and STAT3 phosphorylation in MLE-12 epithelial cells. Our findings demonstrate a previously unrecognized role of host IL-6 response in the regulation of lung inflammation during AA and the control of *S. pneumoniae* bacterial disease. A better understanding of the interactions between lung inflammation and barrier framework could lead to the development of therapies to control asthma inflammation and preserve barrier integrity. *The Journal of Immunology*, 2020, 205: 469–479.

Allergic asthma (AA) is characterized as a Th2-biased airway inflammation with the potential to cause lung pathology and remodeling of the respiratory tract (1–3). The most common trigger for asthma is the continuous exposure to allergens, of which fungal agents are important factors (4, 5) as there is evidence for the presence of fungal sensitization in patients with asthma (6). Eventually, this would result in the physiological dysfunction of breathing, often requiring medical attention (7). Inflammation manifested by AA consists of diverse immune phenotypes and exhibits differential lung pathology ranging from mild to severe airway inflammation (8). Several

factors, including age, genetics, immune disorders, and differential exposure to allergens, collectively contribute to the development of these diverse immune phenotypes observed in asthma airway pathology (9). *Streptococcus pneumoniae* colonizes the human nasopharynx, causing a variety of diseases, including life-threatening pneumonia, sepsis, and meningitis (10). Although severe asthmatics are shown to be at risk for airway infections, there is a lack of data showing that all asthmatics are at risk for *S. pneumoniae* or other airway infections (11). Eosinophils, a dominant effector cell type in asthma, have been shown to exacerbate inflammation in viral-infected hosts (12). However, other reports have also demonstrated an antiviral effect of pulmonary eosinophils in a murine model of allergic fungal inflammation (13). Therefore, the host immune mechanisms implicated in the exacerbation or control of airway infections during asthma remain incompletely understood.

IL-6 is a soluble inflammatory mediator produced by immune (myeloid/lymphoid) and nonimmune cells (epithelial/endothelial cells/fibroblasts) in response to an antigenic stimulus (1). IL-6 acts as a crucial immune mediator in maintaining the barrier integrity during airway bacterial and viral infections by promoting lung repair, epithelial cell survival, and reduced fibroblast accumulation (5). A protective role of IL-6 has been shown in a number of murine infection models (3–5). Therefore, IL-6 acts as a critical regulator of the host immune response during infection and inflammation. The homeostatic cross-talk between host inflammation and barrier framework is essential to maintain the barrier integrity at mucosal surfaces in the lung (6–9). However, the dysregulated host response results in damage-associated structural changes to the barrier. These changes then lead to the development of altered epithelial and/or endothelial phenotypes during inflammatory conditions. There is a lack of data on the role

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Abbreviations used in this article: AA, allergic asthma; AF, Alexa Fluor; ATCC, American Type Culture Collection; BAL, bronchiolar lavage; BV, Brilliant Violet; EMT, epithelial to mesenchymal transition; IH1, first inhalational challenge; MLE-12, mouse lung epithelial cell; PAS, periodic acid–Schiff; siRNA, small interfering RNA; TJ, tight junction; Treg, T regulatory; WT, wild-type.

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of IL-6 response in the regulation of lung inflammation during AA as well as its consequence on the regulation of airway barrier integrity and *S. pneumoniae* diseases.

In current transmission, we used *Aspergillus fumigatus* to develop an AA model. We infected asthmatic mice with *S. pneumoniae* to develop a murine model of asthma and *S. pneumoniae* coinfection. IL-6 deficiency exacerbated lung inflammation in response to AA. Whereas the host response to AA protected against *S. pneumoniae* lung pathogenesis, the IL-6 deficiency abrogated the protective effect of allergic inflammation against *S. pneumoniae*, leading to the permissiveness of asthmatic IL-6<sup>-/-</sup> mice for *S. pneumoniae*. Mechanistically, the loss of IL-6 response led to increased lung inflammation, eosinophil recruitment, tissue pathology, and collagen deposition. Additionally, IL-6-deficient asthmatic mice exhibited reduced goblet cell hyperplasia and increased TGF- $\beta$  production. The key inflammatory and physiological changes in the lungs of IL-6-deficient asthmatic mice resulted in dysregulated tight junction (TJ) proteins claudin-4 and ZO-1 and increased lung permeability. Therefore, IL-6 is required to regulate pulmonary inflammation and maintain epithelial barrier integrity during AA, leading to the control of *S. pneumoniae* disease.

## Materials and Methods

### Animals, cells, and microbial strains

Wild-type (WT) C57BL/6 and IL-6<sup>-/-</sup> mice (6–8 wk old) were purchased from The Jackson Laboratory and bred in-house. An equal proportion of age-matched male and female mice were included in the study. Mice were used in compliance with the National Institutes of Health recommendations for the use of the mice published in the guide for the care and use of laboratory animals. The animal protocol detailing the procedures and techniques used was reviewed and approved by the University of North Dakota Institutional Animal Care and Use Committee under the protocol 1807-9. Mouse lung epithelial cells (MLE-12) were purchased from American Type Culture Collection (ATCC) (identifier: ATCC CRL-2110) and were cultured and maintained according to the manufacturer's instructions. *A. fumigatus* was purchased from ATCC (Manassas, VA). *A. fumigatus* extract was purchased from Greer Laboratories (Lenoir, NC). The *S. pneumoniae* serotype 6A strain (BG7322) was obtained from Rochester General Hospital Research Institute and has been used by us as well as others in the past (10, 14).

### *A. fumigatus* AA, asthma–*S. pneumoniae* coinfection, and IL-6 reconstitution models

The animals were sensitized as previously described (11). WT or IL-6<sup>-/-</sup> mice were sensitized using 10  $\mu$ g of *A. fumigatus* Ag extract in 0.1 ml PBS mixed with 0.1 ml of Alum (Pierce Biotechnology, Rockford, IL), which was injected both s.c. (0.1 ml) and i.p. (0.1 ml). After 2 wk, the mice were given three intranasal 20- $\mu$ g doses of *A. fumigatus* Ag in 20  $\mu$ l of PBS at 1-wk intervals. One week after the final dose, the animals were challenged by being exposed to a nasal aerosol of live *A. fumigatus* conidia as the first inhalational challenge (IH1). For the inhalational challenge, each anesthetized mouse was placed supine with its nose in an inoculation port to inhale the live fungal conidia for 10 min. This challenge was performed once week 7 (IH1) and repeated at week 8 (second inhalational challenge). To develop asthma–*S. pneumoniae* coinfection, 2 d post-second fungal inhalational challenge, the mice were inoculated with 2000 CFUs of *S. pneumoniae* serotype 6A in 50  $\mu$ l of sterile PBS. Two days (48 h) after *S. pneumoniae* inoculation, mice were bled and euthanized by CO<sub>2</sub> exposure. The trachea was cannulated, and retrograde nasal lavage was obtained in 200  $\mu$ l of sterile PBS (10). The lungs were aseptically isolated and processed accordingly for downstream applications. To determine nasal and lung *S. pneumoniae* bacterial burden, nasal lavage and homogenized lungs were serially diluted and plated on blood agar plates. Bacterial CFUs were enumerated the next day. For IL-6 reconstitution studies, asthma–*S. pneumoniae* coinfection models were performed as stated above. One day prior to IH1 and every alternative day until euthanasia, 1  $\mu$ g murine rIL-6 (BioLegend, San Diego, CA) was injected i.p. (0.1 ml in PBS).

### Lung immune phenotyping and cytokine analysis

The lungs were aseptically excised and digested with collagenase (25 U/ml). RBCs were removed by treating lung digests with ammonium–potassium–chloride lysis buffer (Life Technologies), and a single-cell suspension was

prepared by passing lung digests through a 70- $\mu$ m cell strainer. Then,  $5 \times 10^5$  cells were stained with Abs against Ghost Dye (Brilliant Violet [BV] 421), CD45 (PerCP-Cy5.5), CD11b (allophycocyanin/Cy-7), Ly-6C (BV711), Ly-6G (FITC), CD19 (allophycocyanin), F4/80 (BV605), CD11c (PE/Cy5), and Siglec F (Alexa Fluor [AF] 647), according to the manufacturer's recommendations. For intracellular staining of Foxp3,  $1 \times 10^6$  cells were surface stained with Ghost Dye (BV421), CD3 (allophycocyanin/Cy7), CD4 (BV786), CD25 (BV605), and CD19 (allophycocyanin). Following surface staining, the cells were permeabilized and stained intracellularly using the Foxp3/Transcription Factor Staining Buffer Kit (Tonbo Biosciences) for Foxp3 (BV711), following the manufacturer's instructions. All fluorochrome-labeled Abs used for staining were purchased from BioLegend unless mentioned otherwise. A BD FACSsymphony cytometer was used to acquire 100,000 events, and the data were analyzed using FlowJo (Tree Star). For cytokine analysis, the lungs were homogenized and centrifuged, and supernatants were collected. Cytokine analysis was performed using the LEGENDplex Mouse Multi-analyte Inflammation Panel (BioLegend), following the manufacturer's recommendation. Samples were run on BD FACSsymphony, and data were analyzed using LEGENDplex V8.0 Data Analysis Software (BioLegend).

### FACS eosinophil cell sorting

The lungs were prepared as mentioned above. One hundred million cells were stained with Siglec F (PE), and eosinophils were sorted on a FACSARIA, according to SSC-A high and Siglec F expression. Six million eosinophils were sorted, washed once with PBS, and homogenized in a tissue protein extraction reagent containing protease and phosphatase inhibitors. Homogenates were spun at 12,000 rpm for 15 min, and the supernatant was stored at  $-80^\circ\text{C}$  until used for a Western blot experiment. For epithelial cell sorting, the lungs from three to four asthma–*S. pneumoniae*-coinfecting WT and IL-6<sup>-/-</sup> mice were individually stained for CD45 (allophycocyanin/Cy-7) and Epcam (allophycocyanin) and sorted on FACSARIA, according to CD45<sup>+</sup> Epcam<sup>+</sup> expression. Five hundred thousand cells from each mouse were washed in PBS, lysed in RNA lysis buffer (Zymo Research), and stored at  $-80^\circ\text{C}$ .

### Lung histology

The lung sections were prepared, processed, and stained with the assistance of the histology core (Department of Biomedical Sciences, University of North Dakota). Whole lungs were perfused and fixed with 10% neutral buffered formalin for 24 h at room temperature. Tissues were embedded in paraffin and sliced into 5- $\mu$ m sections to reveal the maximum longitudinal view of the main intrapulmonary bronchus of the left lobe. The lung sections were stained with H&E, periodic acid–Schiff (PAS), and Masson trichrome (Abcam). H&E slides were coded, and the inflammation (H&E) in each lung section was evaluated by three pathologists in a blinded fashion. Scoring for each section was evaluated on a scale of 0–4 with increments of 0.5 (15). PAS slides were quantified by calculating a ratio of goblet cells to total epithelial cells in 100- $\mu$ m increments along the large airway. This was repeated 10 times for each sample. Masson trichrome slides were quantified by measuring the thickness of the collagen matrix along 10 randomly selected airways per slide. Representative histological images were acquired using a NanoZoomer 2.0-HT Brightfield Fluorescence Slide Scanning System (Hamamatsu Photonics, Japan) and analyzed using NDP.view2 Viewing Software (Hamamatsu) for H&E, PAS, and Masson trichrome slides.

### Quantitation of serum IgE and bronchiolar lavage protein assays

Total IgE in sera was quantified using the Mouse IgE ELISA Quantitation Set (Bethyl Laboratories, Montgomery, TX), following the manufacturer's recommendations. Sera was diluted in PBS containing 1% BSA and 0.05% Tween in a 1:50 ratio and quantified within the range of 3.9–250 ng/ml. Total protein in bronchiolar lavage (BAL) samples was determined using the Pierce Rapid Gold BCA Protein Assay Kit (Thermo Fisher Scientific), following the manufacturer's instructions. Albumin concentration in BAL samples was determined using a Mouse Albumin ELISA (ALPCO, Salem, NH) at a 1:10,000 dilution, following the manufacturer's instructions. All samples were read on Synergy HT spectrophotometer, and data were analyzed using KC4 Data Analysis Software (BioTek Instruments).

### Quantitative PCR

Total RNA was extracted from lung tissues using the RNeasy Plus Mini Kit (QIAGEN, Valencia, CA). cDNA was synthesized using SensiFast cDNA Synthesis Kit (Bioline USA, Taunton, MA), and quantitative PCR was carried out using PowerUp SYBR Green Master Mix (Life Technologies,

Carlsbad, CA). Changes in specific gene expression were normalized to housekeeping genes from each sample, and fold differences were calculated using the  $2(-\Delta\Delta Ct)$  values (16). The primers used to analyze the gene expression are listed in Supplemental Table I.

#### Western blot

Lung tissues were homogenized in T-PER lysis buffer with a protease and phosphatase inhibitor mixture (Thermo Fisher Scientific). Samples were centrifuged at  $14,000 \times g$  for 10 min at  $4^{\circ}\text{C}$ , and supernatants were aliquoted and preserved at  $-80^{\circ}\text{C}$ . Thirty micrograms of total protein from lung homogenates and 15  $\mu\text{g}$  of eosinophil protein lysates were used for electrophoresis. Western blotting was conducted as previously described (17). Densitometric analysis was performed using Quantity One 1-D software (Bio-Rad Laboratories, Hercules, CA) to determine the relative expression of specific proteins to actin.

#### Effect of IL-6 and TGF- $\beta$ response on epithelial integrity in an *in vitro* model

Mouse type II alveolar epithelial cells (MLE-12) were cultured in a DMEM–Ham Nutrient Mixture F12 (1:1) supplemented with 2% FBS, 10 mM HEPES, 10 nM estradiol, 10 nM hydrocortisone, 2 mM L-glutamine, 0.005 mg/ml insulin, 0.01 ng/ml transferrin, and 30 nM sodium selenite (full-nutrient medium). MLE-12 cells were incubated with a combination of 0, 2.5, 5, 10, or 20 ng/ml TGF- $\beta$  (R&D Systems), IL-6 small interfering RNA (siRNA) (100 nm; Thermo Fisher Scientific), and anti-IL-6R (100 ng/ml; R&D Systems) on cover glass-bottom dishes for 24, 48, 72, or 96 h. Following incubation, the cells on the cover glasses were used for immunofluorescence, and the cells on the plates were lysed in protein lysis buffer for Western blot experiments.

#### Immunofluorescence

Cells were fixed in 4% formaldehyde for 10 min, washed three times in PBS for 5 min each, and permeabilized with 0.1% Triton-X 100. Cells were blocked for 60 min in UltraCruz Blocking Reagent (Santa Cruz Biotechnology, Dallas, TX) and incubated with E-cadherin or N-cadherin (Cell Signaling Technology) at  $4^{\circ}\text{C}$  overnight. The next day, cells were incubated with AF488- or AF546-conjugated secondary Abs for 60 min and mounted with ProLong Diamond Antifade Mountant with DAPI. Tissues were deparaffinized and rinsed, and Ag retrieval was performed in a microwaveable vegetable steamer containing sodium citrate buffer (pH 6.0) for 15 min (high power) and brought to room temperature in the buffer for 60 min. Tissues were blocked in 7% goat serum/PBS for 2 h and stained overnight at  $4^{\circ}\text{C}$  with mouse anti-mouse claudin-4 (1:50; Santa Cruz Biotechnology) and goat anti-rabbit ZO-1 (1:100; Cell Signaling Technology) in 5% goat serum. The next day, tissues were incubated with anti-mouse AF488 (1:1000; Thermo Fisher Scientific) and anti-rabbit AF546 (1:1000; Thermo Fisher Scientific) in 5% goat serum. Cells and tissues were visualized by an Olympus Total Internal Reflection Fluorescence microscope containing Hamamatsu ORCA-Flash4.0 camera.

#### Statistical analysis

The data are presented as mean  $\pm$  SEM. A multigroup analysis was performed using one-way ANOVA, followed by Tukey post hoc analysis to compare the two groups. The comparison between just two groups was made using the Mann–Whitney nonparametric test. Statistical analysis was performed with Prism 5.0 (GraphPad Software, La Jolla, CA). Survival data were analyzed by the Mantel–Cox log-rank test. A  $p$  value  $<0.05$  was considered statistically significant.

## Results

#### Murine *A. fumigatus* AA model

C57BL/6 (WT) mice were globally sensitized with an adjuvanted (alum) mix of *A. fumigatus* fungal Ag extract, followed by repeated intranasal exposures with Ag extract and live *A. fumigatus* conidia spores (Supplemental Fig. 1A) (18). Consistent with previously published reports, several fundamental immune and physiological changes in the lung were used as a measure of asthma development at the peak of acute inflammation (day 3 post-conidia inhalation challenge). Compared with mock-infected (WT) mice, asthmatic mice had significantly higher bronchoalveolar infiltration of immune cells (inflammation) and lung

tissue pathology, demonstrated by the H&E histopathology score (Supplemental Fig. 1B). PAS stain was performed to measure the number of goblet cells in PAS-stained cells. Goblet cell metaplasia and higher mucus production (Muc5Ac expression) were observed in the airways of asthmatic mice following live conidia challenge (Supplemental Fig. 1C). Lung tissue pathology correlated with significantly higher levels of eosinophils in the lungs of asthmatic mice (Supplemental Fig. 1D). We did not observe any difference in the level of collagen deposition (Masson trichrome staining) between mock and asthmatic mice (data not shown). Asthmatic mice also had higher levels of B cells (lungs) (Supplemental Fig. 1E) and serum IgE (Supplemental Fig. 1F). Additionally, the upregulation of lung IL-6 response was detected in WT asthmatic mice, both at transcript and protein levels (Supplemental Fig. 1G). Together, our data suggest that a typical asthmatic model was induced by challenge with *A. fumigatus* Ag extract and live *A. fumigatus*.

#### IL-6 deficiency exacerbates lung inflammation and induces airway remodeling in a murine asthma model

Next, we sought to determine the role of IL-6 in our *A. fumigatus*-induced murine asthma model. WT and IL-6 $^{-/-}$  mice were globally sensitized, followed by intranasal Ag exposure and fungal conidia inhalation, as described above (Fig. 1A). Additionally, to establish the differences in response to infection versus allergic influences, the WT and IL-6 $^{-/-}$  mice were infected with *A. fumigatus* conidia in the absence of sensitization, and a number of parameters associated with lung inflammation were determined. Compared with mock infection, *A. fumigatus* infection alone (no sensitization) resulted in only a marginal nonsignificant increase in lung inflammation (H&E) (Fig. 1B) and goblet cells (PAS) (Fig. 1C) in WT and IL-6 $^{-/-}$  mice. No differences in baseline inflammation were observed between mock WT and IL-6 $^{-/-}$  mice (Fig. 1B). Compared with mock or single *A. fumigatus* infection (no sensitization), WT asthmatic mice exhibited increased lung inflammation (H&E score) (Fig. 1B). Additionally, a lack of IL-6 response was associated with an exacerbation of asthma-induced inflammation and pathology in the lungs as demonstrated by increased infiltration of immune cells and a higher H&E pathological score (Fig. 1B). However, compared with asthmatic WT, IL-6 $^{-/-}$  mice exhibited significantly lower goblet cells in the epithelium, which correlated with decreased Muc5Ac transcript expression in lung homogenates (Fig. 1C). The reduced mucus production in IL-6 $^{-/-}$  mice is consistent with previous reports about the context-dependent role of IL-6 in mucus production in the lung (19). Trichrome staining was performed to assess the subepithelial collagen deposition (airway remodeling) in the lungs of single *AF* infection versus AA. No changes in subepithelial fibrosis (collagen thickness) were observed in WT versus IL-6 $^{-/-}$  mice in response to single *A. fumigatus* infection (Fig. 1D). Additionally, compared with respective mock or single *A. fumigatus* infection, WT asthmatic mice did not exhibit subepithelial fibrotic changes (Fig. 1D). However, an IL-6 deficiency promoted subepithelial fibrosis demonstrated by increased collagen thickness around the large and distal airways of asthmatic IL-6 $^{-/-}$  mice (Fig. 1D). Compared with mock infection, single infection (non-sensitized) with *A. fumigatus* resulted in a higher level of lung eosinophils in both groups (WT and IL-6 $^{-/-}$ ), and a further increase was observed in asthmatic mice (Fig. 1E). However, compared with WT asthmatics, lung eosinophils measured significantly higher in IL-6 $^{-/-}$  asthmatic mice (Fig. 1E), which correlated with increased lung tissue pathology (Fig. 1B). Compared with their respective mock controls, both asthmatic WT and IL-6 $^{-/-}$  mice had significantly higher levels of serum IgE, but no difference was detected in

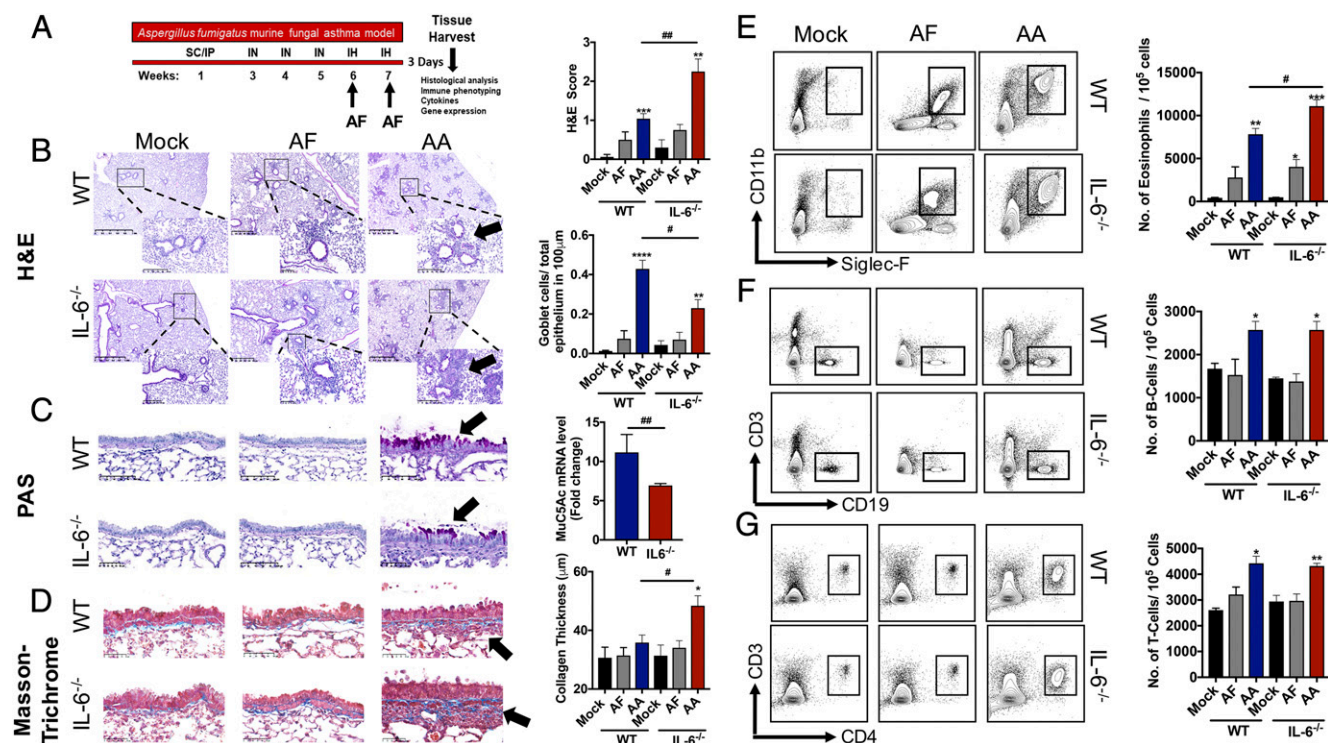


**FIGURE 1.** Inflammation in WT and IL-6<sup>-/-</sup> asthmatic mice. **(A)** Schematic outline of *A. fumigatus* AA model. After 5-wk sensitization, the mice received two inhalational challenges of live conidia (AF) 1 wk apart. The mice were euthanized 72 h after the second inhalational challenge. **(B)** Lung H&E stain of WT and IL-6<sup>-/-</sup> mock, *A. fumigatus* only (AF), and AA mice. **(C)** PAS stain of the large airway in lungs of WT and IL-6<sup>-/-</sup> mock, AF, and AA mice shown at original magnification  $\times 40$  and Muc5Ac mRNA expression of lung lysate of WT and IL-6<sup>-/-</sup> AA mice. **(D)** Masson-trichrome stain of the large airway in lungs of WT and IL-6<sup>-/-</sup> mock, AF, and AA mice with asthma shown at original magnification  $\times 40$ . Number of eosinophils **(E)**, B cells **(F)**, and CD3<sup>+</sup>CD4<sup>+</sup> T cells **(G)** recruited to lungs of WT and IL-6<sup>-/-</sup> mock, AF, and AA mice 72 h after second inhalational challenge. Real-time PCR was used to quantify the mRNA expression levels. Data are expressed as fold changes compared with uninfected WT or IL-6<sup>-/-</sup> groups. Data are representative of three independent experiments ( $n = 5-12$ ) expressed as SEM. Data was analyzed by a nonparametric Mann-Whitney  $U$  test.  $^{\#}p < 0.05$ ,  $^{\#\#}p < 0.01$  represent comparisons made between groups.  $^*p < 0.05$ ,  $^{**}p < 0.01$  represent comparisons made from respective mock controls.

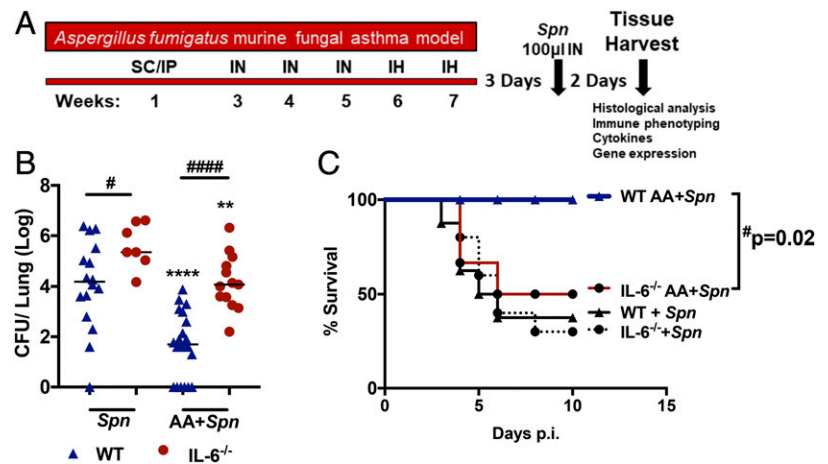
serum IgE levels between WT and IL-6<sup>-/-</sup> asthmatic mice (data not shown). Compared with mock or single *A. fumigatus* infection (no sensitization), both WT and IL-6<sup>-/-</sup> asthmatic mice exhibited significantly higher levels of B and T cells in the lung (Fig. 1F, 1G). However, asthmatic WT and IL-6<sup>-/-</sup> mice did not have any difference in B cells (Fig. 1F) or T cell frequencies in the lungs (Fig. 1G). Despite the higher inflammation and pathology, no apparent sign of sickness, weight loss, or mortality was observed in asthmatic IL-6<sup>-/-</sup> mice. Together, these data suggest that IL-6 deficiency is dispensable to the control of *A. fumigatus* in a single infection model and that the IL-6 response is significant in regulating inflammation during AA.

#### AA confers IL-6-dependent protection against *S. pneumoniae* infection

Allergic lung inflammation has been shown to have a protective effect against infections in the respiratory tract. WT and IL-6<sup>-/-</sup> asthmatic mice were challenged with a *S. pneumoniae* infection inocula (with 50–60% mortality in a single infection) three days after the second *A. fumigatus* inhalation challenge (Fig. 2A). A three-day post-fungal inhalation time point was chosen explicitly because of the peak of inflammation being observed at this time point. Additionally, *S. pneumoniae* inocula, with ~50–60% mortality in single infection, was mainly chosen to study the protective or deleterious role of allergic inflammation on *S. pneumoniae* pathogenesis. Consistent with prior reports, in our model, WT mice with AA effectively cleared *S. pneumoniae* infection, and all of the coinfecting mice with AA and

*S. pneumoniae* survived compared with 40–50% survival observed in single *S. pneumoniae* infection (Fig. 2C). Because the IL-6 response was involved in the regulation of inflammation in our asthma model, we postulated the IL-6 as a protective component of host response in AA, leading to the control of *S. pneumoniae* pathogenesis. Compared with single *S. pneumoniae* infection, WT mice with AA exhibited significantly lower *S. pneumoniae* lung burden (Fig. 2B), and all coinfecting mice (AA + *S. pneumoniae*) survived as compared with over 50% mortality in a single *S. pneumoniae* infection (Fig. 2C). The IL-6 deficiency (IL-6<sup>-/-</sup>) abrogated the protective phenotype of *S. pneumoniae* control in asthmatic mice, leading to higher *S. pneumoniae* lung burden (Fig. 2B) and around 50% of mortality in the IL-6<sup>-/-</sup> asthmatic mice coinfecting with *S. pneumoniae* (Fig. 2C). Despite higher lung *S. pneumoniae* burden in IL-6<sup>-/-</sup> mice, no difference in survival was observed between WT and IL-6<sup>-/-</sup> mice single infected (no sensitization) for *S. pneumoniae* (Fig. 2C). Additionally, IL-6<sup>-/-</sup> mice did not exhibit any difference in the mortality between single *S. pneumoniae* infection and coinfecting mice (AA + *S. pneumoniae*). These data highlight the crucial immunoregulatory role of IL-6 in the control of lung inflammation during AA, leading to protection against *S. pneumoniae* infection. Because the central focus of this transmission is to determine the role of AA and IL-6 response in the pathogenesis of *S. pneumoniae*, the data in this study will compare the coinfection of *S. pneumoniae* in asthmatic WT and IL-6<sup>-/-</sup> mice. Moreover, IL-6 deficiency did not significantly contribute to the control of *S. pneumoniae* disease

**FIGURE 2.** Asthma-*S. pneumoniae* (*Spn*) coinfection model. **(A)** WT and IL-6<sup>-/-</sup> mock and AA mice were intranasally inoculated with 2000 CFUs of *Spn* 6A 72 h after the second inhalational challenge. **(B)** Bacterial burden in lung homogenate of WT and IL-6<sup>-/-</sup> *Spn* and AA + *Spn* mice 48 h after *Spn* infection. The data are representative of three independent experiments ( $n = 9$ –16) expressed as medians. The data were analyzed by a nonparametric Mann-Whitney  $U$  test. **(C)** Survival data were analyzed using the Mantel-Cox log-rank test. The data are representative of two independent experiments ( $n = 10$ –19). #  $p < 0.05$ , ####  $p < 0.0001$  represent comparisons made between groups. \*\* $p < 0.01$ , \*\*\*\* $p < 0.0001$  represent comparisons made from respective mock controls.



in a single infection model (Fig. 2C), and *S. pneumoniae* infection did not exacerbate lung inflammation and pathology in asthmatic mice (data not shown).

#### Asthmatic IL-6<sup>-/-</sup> mice exhibit increased lung inflammatory burden and pathology following *S. pneumoniae* coinfection

Because of the protective role of IL-6 response against *S. pneumoniae* disease in mice with AA, we compared the pulmonary inflammation of asthmatic WT and IL-6<sup>-/-</sup> mice coinfecting with *S. pneumoniae*. It is so because inflammation acts as a double-edged sword in the control of pulmonary infections (20). Compared with mock infection, both WT and IL-6<sup>-/-</sup> coinfecting mice

exhibited significantly higher levels of lung inflammation (H&E) (Fig. 3A), eosinophils (Fig. 3B), and serum IgE (Fig. 3D). However, compared with coinfecting WT mice, IL-6<sup>-/-</sup> mice showed higher bronchointerstitial infiltration of immune cells and lung damage (Fig. 3A). The lung damage in coinfecting IL-6<sup>-/-</sup> mice correlated with significantly higher levels of pulmonary eosinophils (Fig. 3B), neutrophils (Fig. 3C), and serum IgE levels (Fig. 3D). We also profiled cytokines and chemokine levels in the lungs of WT and IL-6<sup>-/-</sup> coinfecting mice. Both WT and coinfecting IL-6<sup>-/-</sup> mice exhibited significantly higher levels of cytokines, MCP-1, IFN- $\gamma$ , IL-1 $\alpha$ , TNF- $\alpha$ , IL-4, and IL-5 than their respective mock-infected mice (Fig. 3E). However, coinfecting

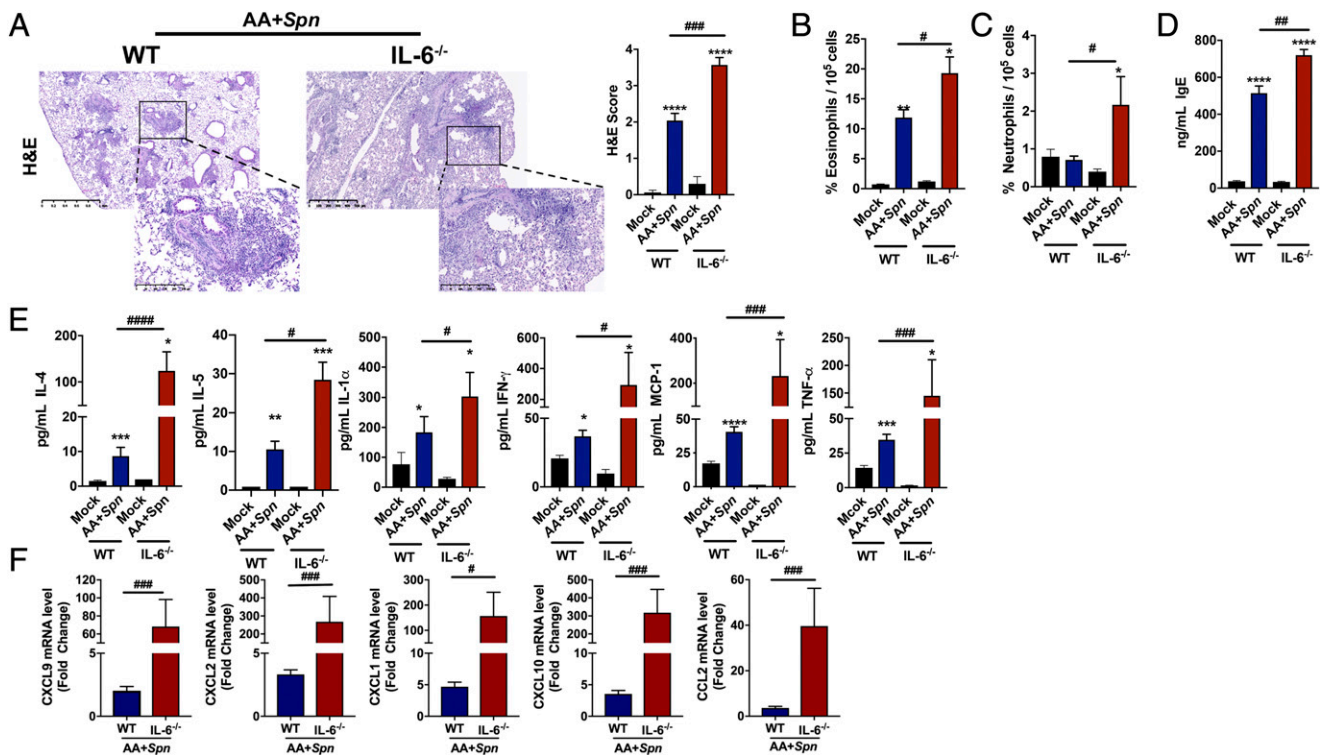


**FIGURE 3.** Inflammatory burden in WT and IL-6<sup>-/-</sup> AA mice 48 h postinfection with 2000 CFU *S. pneumoniae* (*Spn*). **(A)** H&E scoring and representative image of WT and IL-6<sup>-/-</sup> AA + *Spn* mice. Percentage of eosinophils **(B)** and neutrophils **(C)** recruited to the lungs of WT and IL-6<sup>-/-</sup> AA + *Spn* mice. **(D)** Total IgE sera levels of WT and IL-6<sup>-/-</sup> AA mice infected with *Spn*. **(E)** Cytokine levels in lung homogenates of WT and IL-6<sup>-/-</sup> AA + *Spn* mice. **(F)** Chemokine mRNA expression levels of lung lysate of WT and IL-6<sup>-/-</sup> AA + *Spn* mice. The data are expressed as fold changes compared with uninfected WT or IL-6<sup>-/-</sup> controls. The data are representative of three independent experiments ( $n = 9$ –16) expressed as SEM. In a multigroup analysis, the data were analyzed by ANOVA followed by Tukey post hoc test for differences between two groups. In a two-group analysis, the data were analyzed by a nonparametric Mann-Whitney  $U$  test. #  $p < 0.05$ , ##  $p < 0.01$ , ###  $p < 0.001$ , \*\*\*\*  $p < 0.0001$  represent comparisons made between groups. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , \*\*\*\* $p < 0.0001$  represent comparisons made from respective mock controls.

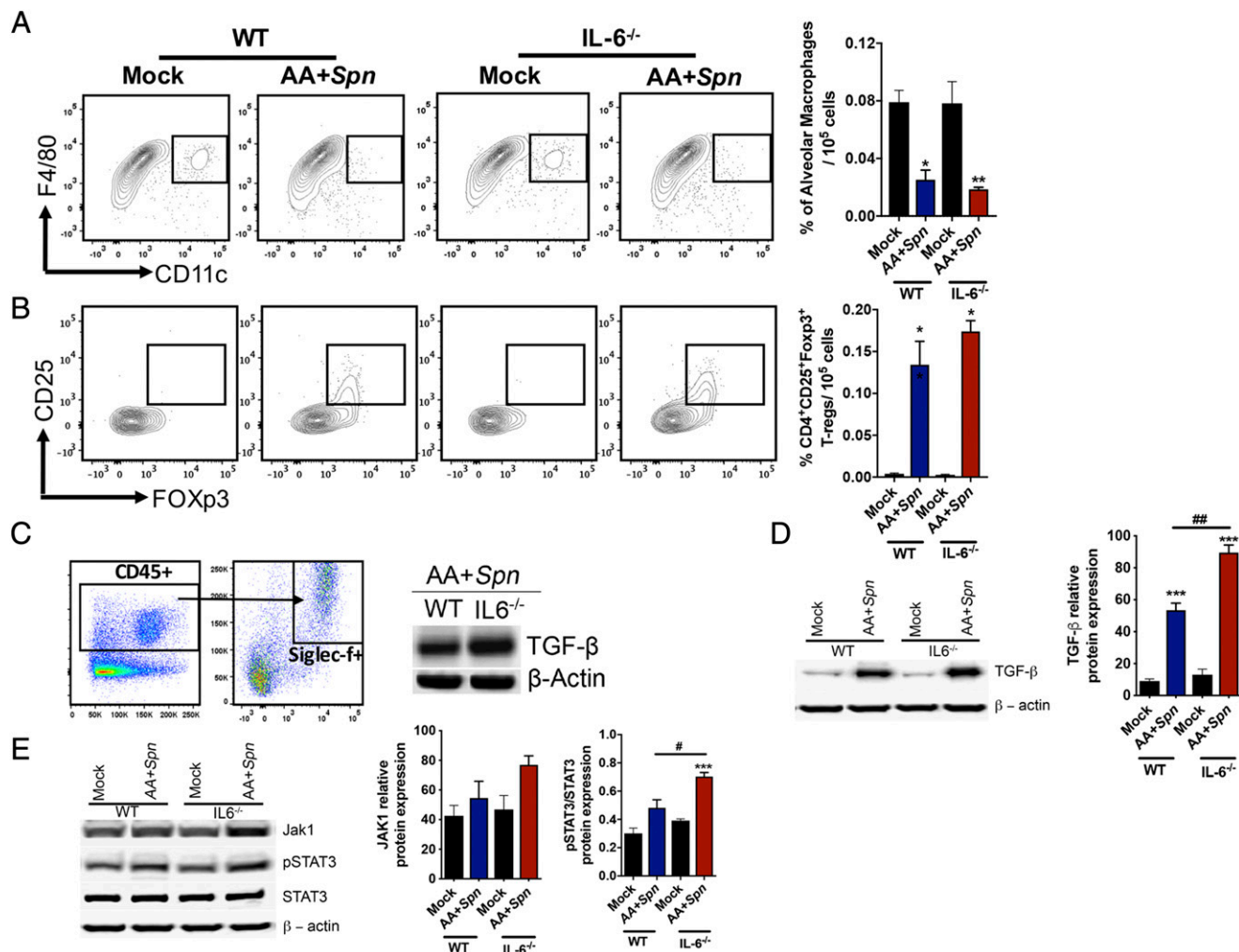


**FIGURE 4.** TGF- $\beta$  and STAT3 expression in WT and IL-6<sup>-/-</sup> AA mice 48 h after *S. pneumoniae* (Spn) infection. Percentage of alveolar macrophages (A) and Treg cells (B) in the lungs of WT and IL-6<sup>-/-</sup> AA mice challenged with 2000 CFU Spn 6A. (C) Eosinophil cell sorting and Western blot data of TGF- $\beta$  protein expression from the eosinophils of WT and IL-6<sup>-/-</sup> AA mice coinfecting with Spn. Protein levels of TGF- $\beta$  (D), JAK1, STAT3, and pSTAT3 (E) in the lung homogenates of WT and IL-6<sup>-/-</sup> AA mice coinfecting with Spn. Western blots are shown as representative images, and densitometry quantification of the target proteins are normalized with  $\beta$ -actin. The data are representative of two independent experiments ( $n = 6-8$ ). The data were analyzed using nonparametric Mann-Whitney  $U$  test and represented as SEM. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$  represent comparisons made between groups. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$  represent comparisons made from respective mock controls.

IL-6<sup>-/-</sup> mice had significantly higher levels of MCP-1, IFN- $\gamma$ , IL-1 $\alpha$ , TNF- $\alpha$ , IL-4, and IL-5 when compared with the coinfecting WT mice (Fig. 3E). Similarly, compared with coinfecting WT mice, a more robust increase in the levels of chemokines (CCL2, CCL-9, CXCL-1, CXCL-2, CXCL-9) was also observed in coinfecting IL-6<sup>-/-</sup> mice (Fig. 3F). These data demonstrate that although WT mice develop an eosinophilic response and lung inflammation, an IL-6 deficiency dysregulates the lung inflammation and pathology, leading to the abrogation of an allergic inflammation-induced protective effect against *S. pneumoniae* infection.

#### IL-6<sup>-/-</sup> coinfecting mice exhibit increased TGF- $\beta$ and pSTAT3 signaling

IL-6 performs an essential immune-regulatory function in maintaining a cellular balance of IL-6 and TGF- $\beta$  during an inflammatory condition (2). T regulatory (Treg) cells, alveolar macrophages, and eosinophils have been shown to be the significant producers of TGF- $\beta$  response in murine asthma models (21-23). Alveolar macrophages were depleted in both asthmatic WT and IL-6<sup>-/-</sup> mice infected with *S. pneumoniae* (Fig. 4A), and no

quantitative difference was observed in Treg cells (Fig. 4B) between these two groups. As eosinophils are considered hallmarks of AA, we sought to determine the manifestation of IL-6 deficiency on the effector response of eosinophils, a critical inflammatory cell type in asthma. Eosinophils were sorted (FACS) from asthmatic WT and IL-6<sup>-/-</sup> mice coinfecting with *S. pneumoniae*, and Western blot was performed to investigate the levels of TGF- $\beta$ . Compared with WT mice, eosinophils from IL-6<sup>-/-</sup> mice displayed a marked increase in TGF- $\beta$  protein expression (Fig. 4C). To corroborate the findings from purified eosinophils, we determined the protein levels of TGF- $\beta$  in total lung homogenates of coinfecting WT and IL-6<sup>-/-</sup> mice. Compared with respective mock-infected mice, WT and IL-6<sup>-/-</sup> coinfecting mice exhibited higher TGF- $\beta$  levels in the lung (Fig. 4D). However, compared with WT coinfecting lungs, a higher TGF- $\beta$  level was detected in lung homogenates from IL-6<sup>-/-</sup> mice (Fig. 4D). Increased inflammation and TGF- $\beta$  protein expression in IL-6-deficient mice were associated with elevated levels of JAK1 and pSTAT3 (Fig. 4E) but not total STAT3. We also detected a marginal increase in the expression of pSmad 2/3 in the lung homogenates of IL-6-deficient mice (Supplemental Fig. 2A).

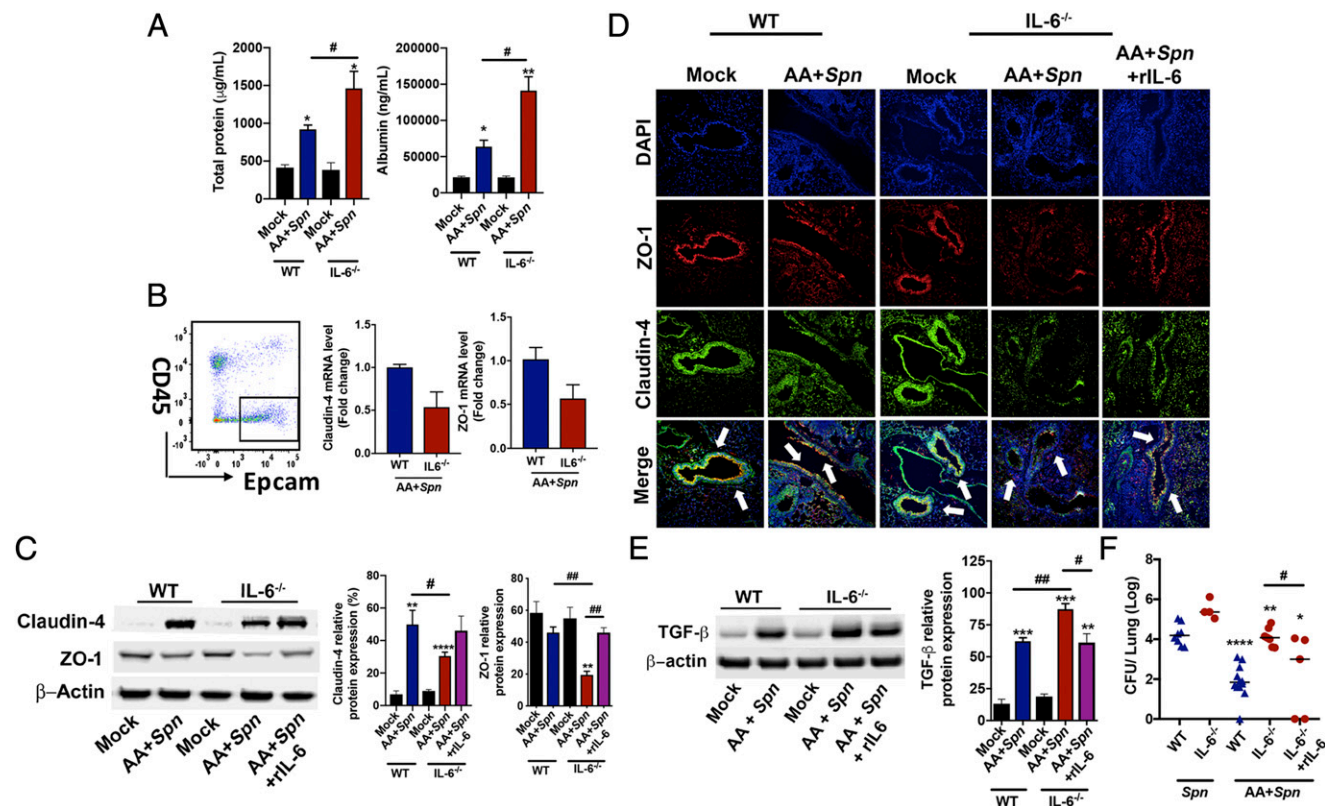


**FIGURE 5.** Lung barrier response in WT and IL-6<sup>-/-</sup> AA mice 48 h after *S. pneumoniae* (Spn) infection. **(A)** Total protein and albumin concentration in the BAL of WT and IL-6<sup>-/-</sup> AA mice challenged with Spn. **(B)** Cell sorting strategy and mRNA expression levels of TJ proteins claudin-4 and ZO-1 in sorted cells of WT and IL-6<sup>-/-</sup> AA + Spn mice. **(C–F)** Additional IL-6<sup>-/-</sup> AA + Spn mice were injected with exogenous murine rIL-6 prior to inhalational challenges (see Materials and Methods). **(C)** Protein expression levels and quantification of TJ proteins in lung homogenates. **(D)** Immunofluorescent images of TJ proteins claudin-4 and ZO-1 around pulmonary airways of the lungs. **(E)** Protein expression level of TGF-β in lung homogenates. **(F)** Groups were challenged 72 h after second inhalational challenge with 2000 CFU Spn 6A and euthanized for bacterial burden 48 h later. The data are representative of three independent experiments ( $n = 8–16$ ) expressed as SEM. The data were analyzed by a nonparametric Mann–Whitney  $U$  test. # $p < 0.05$ , ## $p < 0.01$  represent comparisons made between groups. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , \*\*\*\* $p < 0.0001$  represent comparisons made from respective mock controls.

However, no difference in Smad 4 level was observed between coinfecting WT and IL-6<sup>-/-</sup> mice (Supplemental Fig. 2A).

#### IL-6 deficiency compromises lung barrier integrity and reconstitution of IL-6<sup>-/-</sup> mice with rIL-6 restores TJ proteins and resistance against *S. pneumoniae*

Heretofore, our data demonstrate that an IL-6 deficiency exacerbates lung inflammation and pathology, leading to the abrogation of IL-6-dependent control of *S. pneumoniae* disease during AA. Hyperinflammation causes tissue damage and disintegration of barrier integrity (24, 25). Additionally, increased TGF-β response could dysregulate barrier integrity by promoting epithelial to mesenchymal transition (EMT) (26). TJs form the continuous intercellular barrier between epithelial cells, which regulates the selective movement of solutes across the epithelium (27). TJs also determine epithelial cell polarity and form a crucial barrier framework for the prevention of bacterial invasion (28). To determine the lung barrier permeability, we measured the total protein and albumin in the BAL. Compared with mock, both WT and IL-6<sup>-/-</sup> coinfecting mice measured higher levels of total protein and albumin in the BAL (Fig. 5A). The coinfecting IL-6<sup>-/-</sup> mice had higher levels of total protein and albumin than WT mice, suggesting increased lung permeability in IL-6<sup>-/-</sup> mice (Fig. 5A). Furthermore, we FACS sorted the epithelial cells (CD45<sup>-</sup>Epcam<sup>+</sup>) from the lungs of coinfecting WT and IL-6<sup>-/-</sup> mice and determined the expression of TJ genes by quantitative PCR. Epithelial cells from IL-6<sup>-/-</sup> mice had the reduced mRNA expression of claudin-4 and ZO-1 (Fig. 4B). Next, we reconstituted the

coinfecting IL-6<sup>-/-</sup> mice with exogenous rIL-6 as described earlier (29, 30) and investigated the effect of IL-6 treatment on TJ proteins and *S. pneumoniae* infection in the lung. Consistent with epithelial TJ data, IL-6<sup>-/-</sup> mice exhibited the lower expression of TJ proteins claudin-4 and ZO-1 in the lung, as evidenced by Western blot and immunofluorescence (Fig. 5C, 5D). The reconstitution of coinfecting IL-6<sup>-/-</sup> mice with rIL-6 led to a significant increase in the expression of TJ protein ZO-1 (Fig. 5C, 5D). The level of claudin-4 protein was also increased in IL-6<sup>-/-</sup> mice supplemented with rIL-6 (Fig. 5C, 5D). Interestingly, the reconstitution of coinfecting IL-6<sup>-/-</sup> mice by rIL-6 resulted in the reduction of TGF-β levels in the lung (Fig. 5E), further corroborating the protective role of IL-6 on the lung barrier and IL-6-dependent regulation of TGF-β in our model. Additionally, the reconstitution of IL-6<sup>-/-</sup> mice with rIL-6 also resulted in significantly less bacterial burden in the lung (Fig. 5F). Coinfecting IL-6<sup>-/-</sup> mice also manifested higher expression of EMT-related proteins N-cadherin, Zeb-1, and α-SMA (Supplemental Fig. 2B), which correlated with increased TGF-β level in IL-6<sup>-/-</sup> mice coinfecting with AA and *S. pneumoniae*. Our data demonstrates the importance of the IL-6 response in the regulation of TJ proteins and lung permeability, leading to the control of *S. pneumoniae* disease during AA.

#### IL-6 silencing or the blockade of IL-6R exacerbates the effect of TGF-β on barrier dysregulation in alveolar epithelial cells

We sought to determine the role of homeostatic IL-6 response in TGF-β-induced dysregulation of TJs in MLE-12 alveolar epithelial cells in vitro. Western blot was performed to determine the

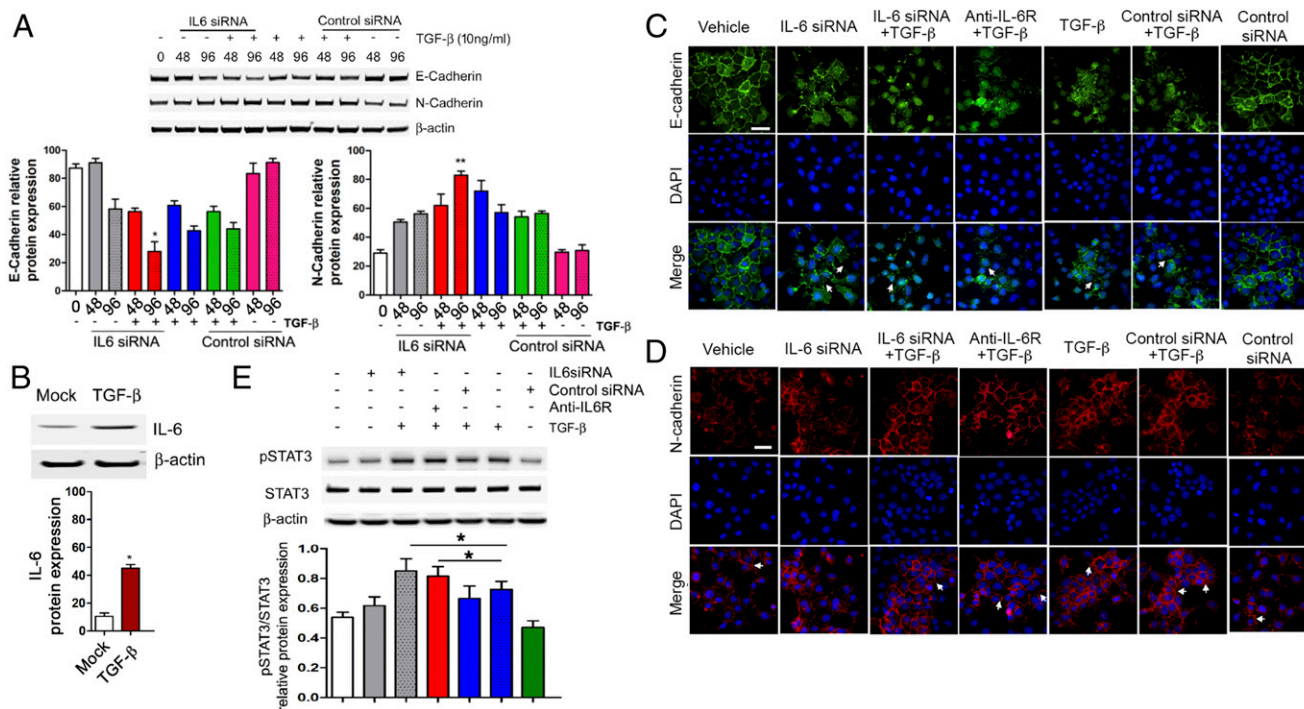


**FIGURE 6.** siRNA-mediated knockdown of IL-6 response in TGF- $\beta$ -treated MLE-12 epithelial cells. **(A)** Time and dose optimization of TGF- $\beta$ -induced protein expression of EMT-related proteins E-cadherin and N-cadherin in MLE-12 cells. **(B)** IL-6 relative protein expression in cell lysate of MLE-12 cells treated with 10 ng/ml TGF- $\beta$ . **(C and D)** E-cadherin and N-cadherin protein fluorescence of MLE-12 cells treated with 10 ng/ml TGF- $\beta$ , IL-6 siRNA/Anti-IL-6R, both, or neither at 96-h posttreatment. Fluorescent images are representative of Western blot data (B). **(E)** Protein expression levels of STAT3 and pSTAT3 in the cell lysates of MLE-12 cells treated with 10 ng/ml TGF- $\beta$  with or without IL-6 siRNA, control siRNA, and anti-IL-6R at 96 h posttreatment. The data are representative of three independent experiments and expressed as SEM. The data were analyzed by a nonparametric Mann-Whitney  $U$  test. \* $p < 0.05$ , \*\* $p < 0.01$  represent comparisons made from the respective mock controls or groups indicated.

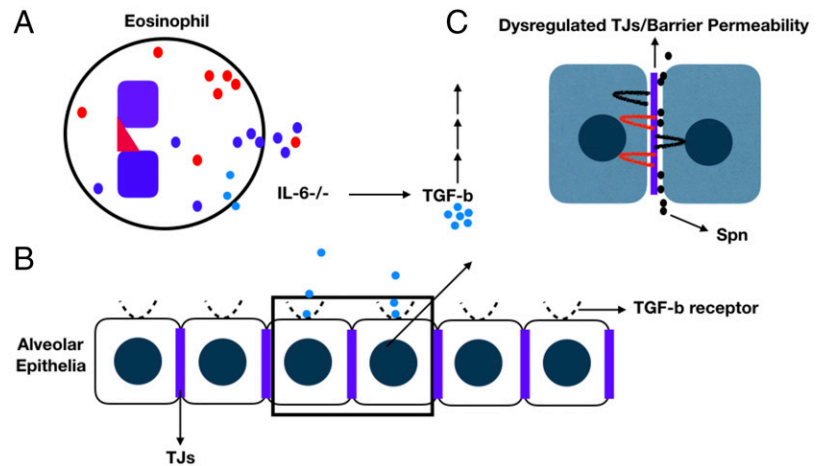
TGF- $\beta$  concentration and stimulation time windows required to induce the disruption of TJ proteins, E-cadherin, and N-cadherin in MLE-12 epithelial cells. The 96-h window (TGF- $\beta$ : 10 ng/ml) induced an optimal EMT phenotype in MLE-12 epithelial cells, demonstrated by a significant downregulation and upregulation of E-cadherin and N-cadherin protein expression, respectively (Fig. 6A). The differential expression of E-cadherin and N-cadherin proteins has been used as markers for cell junction integrity as well as EMT phenotype (31, 32). To determine the effect of IL-6 silencing on TGF- $\beta$ -induced dysregulation of epithelial TJ proteins, Western blots and immunofluorescence were performed to assess the expression of epithelial E-cadherin and N-cadherin proteins. Western blot was performed to detect the IL-6 in mock and TGF- $\beta$ -treated MLE-12 epithelial cells. The IL-6 expression was detected in MLE-12 cells at a homeostatic level (no stimulation), and TGF- $\beta$  treatment significantly increased the IL-6 level (Fig. 6B). IL-6 knockdown by siRNA exacerbated the effect of TGF- $\beta$ -induced dysregulation of epithelial E-cadherin and N-cadherin expression, as shown by Western blot and immunofluorescence microscopy (Fig. 6A, 6C, 6D). Similarly, the blockade of IL-6R signaling by anti-IL-6RA Ab resulted in a similar phenotype as IL-6 knockdown vis-à-vis dysregulation of epithelial E-cadherin and N-cadherin expression (immunofluorescence) (Fig. 6C, 6D). These data are in sync with in vivo data above vis-à-vis the impact of an IL-6 deficiency on epithelial TJ proteins in the presence of increased TGF- $\beta$  response (Fig. 7). We detected a mild (nonsignificant) increase in pSTAT3 level in TGF- $\beta$ -treated epithelial cells (Fig. 6E). However, consistent with the lung data, we detected a significant increase in the expression of pSTAT3 in TGF- $\beta$ -treated epithelial cells, deficient of IL-6 or IL-6R signaling (Fig. 6E). No difference in the levels of normal or

phospho-Smad was observed (data not shown) between mock or TGF- $\beta$ -treated cells (data not shown). These data show that TGF- $\beta$  has the propensity to activate pSTAT3 signaling, the evidence of which has been presented in the past (33).

## Discussion

In the current study, we examined the role of IL-6 response in murine asthma and asthma-*S. pneumoniae* coinfection models. We used fungal allergen *A. fumigatus* to develop a murine asthma model as exposure to fungal allergens causes allergic sensitization in patients with asthma (34). Therefore, this model mimics the natural pathogenesis of asthma disease in humans. IL-6, an important cytokine produced by both immune and nonimmune cells, plays a critical role in the regulation of inflammation and tissue repair (35, 36). Additionally, IL-6 is important for the maintenance of barrier integrity in airway infection models (2, 5). Therefore, we hypothesized that the IL-6 response is crucial in the control of pulmonary inflammation during AA and, consequently, the control of *S. pneumoniae* bacterial disease. AA develops a Th2 response that drives the recruitment of eosinophils to the lungs (37) to function as an effector arm of Th2 immunity (38). Although IL-6 is considered an important determinant of Th1/Th2 balance, Th2 differentiation and response development in vivo is not dependent on IL-6 (39). Our data show that IL-6 deficiency drives increased eosinophil recruitment (lung) and lung pathology in asthmatic IL-6<sup>-/-</sup> mice. The IL-6 response has been shown to induce the production of mucus (19), and the decreased goblet cell hyperplasia and Muc5 expression in asthmatic IL-6<sup>-/-</sup> mice are in agreement with the previously published literature (40). The increased collagen deposition in the airways of asthmatic IL-6<sup>-/-</sup> mice suggests the protective role of IL-6 response against

**FIGURE 7.** Schematic representation of the immunoregulatory role of IL-6 response in asthma pathogenesis. An IL-6 deficiency leads to hyperinflammation and increased TGF- $\beta$  response, resulting in the dysregulation of epithelial barrier integrity and *Spn* disease. **(A)** Eosinophils as producers of inflammatory cytokines and TGF- $\beta$ . **(B)** TGF- $\beta$  interacts with lung epithelial cells via TGF- $\beta$  receptor 1. **(C)** Binding of TGF- $\beta$  to the receptor leads to dysregulated pSTAT3 and TJ proteins resulting in barrier permeability.



subepithelial fibrosis and airway remodeling, physiological events associated with the severity of asthma. IL-6-deficient eosinophils dysregulated the lung inflammation by upregulating TGF- $\beta$  and STAT3 phosphorylation. Higher inflammation and TGF- $\beta$  levels in the lungs of asthmatic IL-6 $^{-/-}$  mice correlated with compromised lung barrier integrity, leading to an increased permissiveness for *S. pneumoniae* disease.

Inflammation acts as a double-edged sword; although it is necessary for the resolution of infection, an overly exuberant inflammatory response causes tissue damage and promotes infection (41). The host response developed to allergic airway inflammation is protective against respiratory infections (42, 43). However, a better understanding of protective inflammation in airway allergic models is required. Consistent with the published data, we observed a protective role of AA in the control of *S. pneumoniae* disease in our model, as all WT coinfectd (AA + *S. pneumoniae*) mice survived the *S. pneumoniae* infection as opposed to over 50% mortality in the *S. pneumoniae* single infection. However, the protective allergic response to *S. pneumoniae* was abrogated in the absence of IL-6 (IL-6 $^{-/-}$  mice). The WT and IL-6 $^{-/-}$  mice did not significantly differ in survival in single *S. pneumoniae* infection models, suggesting that IL-6 plays a more significant role in protecting *S. pneumoniae* disease during AA. The phagocytic response and barrier integrity are crucial factors in the control of *S. pneumoniae* in the lung (44). Our data show that IL-6 is critical to a balanced eosinophil response as well as regulating the lung barrier. The IL-6 deficiency dysregulated eosinophilia and barrier integrity, leading to an exacerbated inflammatory response and loss of protection against *S. pneumoniae*. Eosinophils are shown to be involved in the development of antiviral effector T cell response and clearance of influenza viral infection in murine AA model (13, 45). Other reports have shown the role of the IL-6 response in bacterial phagocytosis in vitro (46). However, there is a lack of data on the role of the IL-6 response in murine models of asthma and *S. pneumoniae* coinfection. Our data show that the IL-6 response plays an immunoregulatory role in adjusting lung inflammation during AA and therefore acts as a protective component of the host response against *S. pneumoniae* during allergic inflammation.

A regulated IL-6 response is central to maintaining the homeostatic balance of IL-6 and TGF- $\beta$  in myeloid or nonimmune cells, such as epithelial cells and fibroblasts (47, 48). However, an IL-6 deficiency dysregulates inflammation by overexpressing the TGF- $\beta$ , which could manifest fundamental changes in lung barrier framework (2). Eosinophils, Treg cells, and alveolar macrophages are all shown to be associated with TGF- $\beta$  production in murine

asthma models (21–23). However, in our *A. fumigatus* asthma model, we did not see any change in the quantitative number of Treg cells between WT and IL-6 $^{-/-}$  coinfectd mice. Additionally, alveolar macrophages were depleted in our asthma model. Therefore, we investigated the role of IL-6 response in the regulation of TGF- $\beta$  response and eosinophilic inflammation in our murine model of asthma and *S. pneumoniae* coinfection. IL-6 deficiency produced an increased TGF- $\beta$  response in eosinophils, and this elevation was associated with increased phosphorylation of STAT3. IL-6 regulates STAT3 phosphorylation by activating JAK1, which contributes to the activation of the transcription of genes containing STAT3 response elements (49). Prior reports showed the significance of TGF- $\beta$ R signaling in STAT3 phosphorylation (33, 50). Our data show that in the absence of an IL-6 response, increased lung inflammation and TGF- $\beta$  responses could phosphorylate the transcriptional factor STAT3. However, further investigation is required to understand the TGF- $\beta$ -induced STAT3 activation and regulation of STAT3 response elements in eosinophilic asthma.

An apical part of respiratory epithelia expresses TJ proteins, which are central in maintaining airway barrier integrity against bacterial pathogens (27). The data from several models of acute and chronic inflammatory diseases suggest that hyperinflammation can cause tissue damage and dysregulation of barrier integrity (51). Additionally, increased TGF- $\beta$  production in response to hyperinflammation and tissue damage could activate TGF- $\beta$ R signaling in epithelial cells, leading to the development of an EMT phenotype with decreased cell adhesion and compromised epithelial TJs. The expression of TJ proteins, claudin-4, and ZO-1 was downregulated in IL-6 $^{-/-}$  mice. Furthermore, a higher albumin level in the BAL demonstrates the compromised lung barrier in IL-6 $^{-/-}$  coinfectd mice. Importantly, the reconstitution of IL-6 $^{-/-}$  mice with rIL-6 partially restored the protective phenotype, leading to an enhanced expression of TJ proteins and reduced *S. pneumoniae* lung burden in IL-6 $^{-/-}$  coinfectd mice. The IL-6 response has been shown as a crucial regulator of lung repair and barrier integrity in airway infection models of acute inflammation (52, 53). Our findings are consistent with previously published reports regarding the role of IL-6 response in the regulation of airway barrier integrity during an inflammatory response, leading to the control of *S. pneumoniae* bacterial disease.

We sought to determine the protective role of epithelial IL-6 response in an in vitro TGF- $\beta$ -induced EMT model in lung MLE-12 epithelial cells, leading to the dysregulation of TJ proteins, E-cadherin, and N-cadherin. The TGF- $\beta$  treatment of MLE-12 cells led to the downregulation of TJ proteins, E-cadherin, and

N-cadherin. The siRNA knockdown or homeostatic IL-6 response or blockade of IL-6R signaling exacerbated TGF- $\beta$ -mediated dysregulation of E-cadherin and N-cadherin expression, suggesting that not only does IL-6 response play a crucial immunoregulatory role in eosinophilic inflammation but IL-6 response is also important to resist TGF- $\beta$ -mediated changes in epithelial barrier integrity. Consistent with the lung data, IL-6 deficiency or blockade of IL-6R signaling led to an increased STAT3 in MLE-12 treated with TGF- $\beta$ . Epithelial STAT3 signaling has been shown to mediate changes to barrier integrity in murine models of chronic inflammation, including asthma (54, 55). However, we recognize that further investigation is required to establish the association of IL-6 and TGF- $\beta$  responses vis-à-vis epithelial STAT3 signaling in our asthma model. When combined, our findings demonstrate a previously unrecognized role of IL-6 in the regulation of lung inflammation during AA, leading to the protection against *S. pneumoniae* bacterial disease. Further investigations should focus on better understanding the relationship between the IL-6 response in the regulation of inflammation and epithelial barrier integrity. These questions are under investigation in our laboratory.

## Disclosures

The authors have no financial conflicts of interest.

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