

Allergic Airway Inflammation Inhibits Pulmonary Antibacterial Host Defense¹

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The innate immune system of the lung is a multicomponent host defense system and in addition has an instructing role in regulating the quality and quantity of the adaptive immune response. When the interaction between innate and adaptive immunity is disturbed, pathological conditions such as asthma can develop. It was the aim of the study to investigate the effect of the allergic inflammation of the lung on the innate host defense during bacterial infection. Human bronchial epithelial cells were preincubated with Th2 cytokines and infected with *Pseudomonas aeruginosa*. The effect of the Th2 cytokines on the mRNA levels of antimicrobial peptides and the antimicrobial activity of HBEC was determined. To investigate the influence of an allergic inflammation on pulmonary host defense in vivo, mice sensitized and challenged with OVA were infected with *P. aeruginosa*, and the number of viable bacteria in the lungs was determined together with markers of inflammation like cytokines and antimicrobial peptides. Exposure of airway epithelial cells to Th2 cytokines resulted in a significantly decreased antimicrobial activity of the cells and in suppressed mRNA levels of the antimicrobial peptide human β -defensin 2. Furthermore, mice with allergic airway inflammation had significantly more viable bacteria in their lungs after infection. This was consistent with reduced levels of proinflammatory cytokines and of the antimicrobial peptide cathelin-related antimicrobial peptide. These results show that an allergic airway inflammation suppresses the innate antimicrobial host defense. The adaptive immune system modulates the functions of the pulmonary innate immune system. *The Journal of Immunology*, 2006, 177: 1833–1837.

The pulmonary mucosal immune system is constantly faced with the challenge of generating a potent defense against pathogens while maintaining tolerance to both self and innocuous environmental Ags. When this balance is lost, pathological conditions such as autoimmunity, allergy, or impaired resistance can develop. Allergic asthma is characterized by the overwhelming activation of the adaptive immune system against environmental Ags. The specific pathophysiology is mediated by CD4⁺ T lymphocytes that produce a type 2 cytokine profile with IL-4, IL-5, and IL-13 as prototypical cytokines (1). The amount of these cytokines is increased in the airway tissues of patients with asthma and in animals with experimental allergic airway inflammation.

The innate immune system has an instructing role in regulating the quality and quantity of the adaptive immune response (2). The innate immune system of the lung is a multicomponent host defense system that involves structural, physical, and functional mechanisms. In addition to classical immune cells like macrophages and neutrophils, airway epithelial cells are an active part of the pulmonary innate immune system and are capable of recog-

nizing microorganisms (3). Airway epithelium can mount a direct antimicrobial host defense, attract immune and inflammatory cells, and educate these cells when they reside in the proximity of the airway epithelium. Antimicrobial peptides (AMPs)⁴ are effector molecules that are secreted by epithelial and other cells (4). These peptides have direct antimicrobial activity and a variety of other functions. Airway epithelium is functional in antimicrobial host defense and likely also in the pathogenesis of asthma (5).

The innate and the adaptive immune systems represent two distinct parts of the protection system of tissues and organs and interact in many ways. These interactions are critical in the pathogenesis of atopic diseases. A central link between innate and adaptive immunity is the dendritic cell. Dendritic cells can drive the differentiation of T cells toward a Th1, Th2, or T regulatory response (6, 7). There is evidence that pathogen-associated molecular patterns can promote either Th1 or Th2 responses depending on the strength of the microbial signal and modulating factors such as the genetic or epigenetic background. Genetic deletion studies in animals showed that receptors of the innate immune system significantly modulate the phenotypes of allergic airway inflammation. For instance, bacterial LPS signaling through TLR4 suppresses asthma-like responses via NO synthase 2 activity (8). Furthermore, CpG oligodeoxynucleotides inhibit the allergic lung inflammation in a mouse model of asthma (9). The deletion of MyD88 results in a blunted TLR4-dependent Th2 response to intranasal Ag in a murine model (10).

These data provide clear evidence that the innate immunity influences the quality and quantity of the allergic inflammation. In contrast, it is not known whether an allergic airway inflammation in response to aeroallergens has a reciprocal impact on the innate immune system and the antibacterial host defense.

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⁴ Abbreviations used in this paper: AMP, antimicrobial peptide; HBEC, human bronchial epithelial cell; ALI, air-liquid interface; LB, Luria-Bertani; BAL, bronchoalveolar lavage; BALF, bronchoalveolar lavage fluid; CRAMP, cathelin-related antimicrobial peptide; hBD-1, human β -defensin 1; hBD-2, human β -defensin 2.

The aim of the present study was to investigate the relationship between an allergic airway inflammation and the pulmonary antibacterial host defense. Experiments *in vitro* and in a murine asthma model showed that the atopic milieu inhibits the production of AMPs and decreases the clearance of a pulmonary infection.

Materials and Methods

Cell culture, medium, and reagents

Human bronchial epithelial cells (HBEC) were isolated from large airways resected during surgery and cultivated as submersed or air-liquid interface (ALI) cultures as described previously (11). The protocol was approved by the Institutional Review Board (ethics committee) of the University of Marburg, and informed consent was obtained from the patients. Cells were cultivated using airway epithelial cell growth medium (PromoCell). All cultures were incubated at 37°C in humidified 5% CO₂ air atmosphere in a tissue culture incubator. *Pseudomonas aeruginosa* PAO 1 bacteria were grown to an OD₆₀₀ of 0.8 in Luria-Bertani (LB) medium and heat inactivated for 30 min at 96°C. Recombinant human IL-4 and IL-13 were purchased from R&D Systems.

Stimulation of HBEC and harvest of the supernatants and cellular RNA

HBEC cultivated as submersed cultures were preincubated for 1 h with IL-4 (50 ng/ml) or IL-13 (50 ng/ml). Heat-inactivated *P. aeruginosa* (5 × 10⁷ CFU/ml) were added, and the cells were incubated for another 6 h. Total RNA was isolated by using the RNeasy Mini kit (Qiagen). RNA isolation included DNA digestion using RNase free DNase Set (Qiagen). Experiments were repeated at least twice and run in triplicates.

Bacterial survival assay

To determine the effect of IL-4 and IL-13 on the antimicrobial activity of human airway epithelial cells cultured as ALI cultures, cells were preincubated with IL-4 and IL-13 (each 50 ng/ml) for 3 days using culture medium without antibiotics. On day 4, the cells were infected with viable *P. aeruginosa*. To determine the bacterial loads of the ALI cultures 24 h after infection, the apical side of the cultures were washed with 100 µl of PBS. The numbers of bacteria attached to or taken up by the cells was determined by lysing the cells in 0.1% Triton X-100 that has no effect on bacterial viability. Serial dilutions of washings or lysates were plated onto LB agar plates.

Determination of cytokine concentrations

Levels of murine IL-1, IL-4, IL-6, and IL-13 in cell-free bronchoalveolar lavage fluids (BALFs) were determined by commercially available sandwich-type ELISAs, according to the manufacturer's instructions (R&D Systems).

Real-time RT-PCR

A total of 1.5 µg of total RNA preparation was reverse transcribed using a cDNA synthesis kit (MBI Fermentas) applying oligo(dT)₁₈ primers. cDNA was diluted 1/5, and 5 µl was used as template in a 25 µl of SYBR-Green-PCR mix, according to the manufacturer's protocol (AB-gene). GAPDH primers (sense, 5'-GAAGGTGAAGGTCGGAGTC-3'; antisense, 5'-GAAGATGGTGATGGGATTTC-3') and hBD-2 primers (sense, 5'-TCAGCTCCTGGTGAAGTC-3'; antisense, 5'-GGGCAAAA GACTGGATGACA-3') were purchased from TIB Molbiol. Specificity of RT-PCR was controlled by omission of the template or the reverse transcription. Quantitative PCR results were obtained using the $\Delta\Delta C_T$ method. Because PCR efficiencies for all four reactions were similar, threshold values were normalized to GAPDH.

Animals

Female BALB/c mice (6–8 wk old) were obtained from Charles River. Animals were housed in a pathogen-free facility with single ventilated cages and received OVA-free diet. All animal experiments were approved by the local authorities (Regierungspraesidium Giessen).

Allergic sensitization, allergen challenge, and administration of bacteria

Mice were sensitized by three i.p. injections of 10 µg of OVA grade VI (Sigma-Aldrich) adsorbed to 1.5 mg of AL(OH)₃ (Inject Alum; Pierce) diluted in 200 µl of PBS on days 1, 14, and 21. Nonsensitized animals

(control) received adjuvant 1.5 mg of AL(OH)₃ diluted in PBS alone. The animals received three local allergen challenges performed by an exposure to aerosolized OVA (1% in PBS) for 20 min on days 21, 22, and 23. Twenty-four hours after the third exposure to OVA, mice were slightly anesthetized by i.p. injection of 2.6 mg of ketaminhydrochloride (Ketanest; Parke Davis) and 0.18 mg of xylazinhydrochloride (Rompun; Bayer) per mouse and infected intranasally with *P. aeruginosa* (300,000 CFU) in PBS or PBS alone as control. The animals were monitored by registration of mobility and appearance of the fur. Twenty-four hours after bacterial infection, the animals were euthanized, the tracheae were cannulated and a bronchoalveolar lavage (BAL) was performed (five times with 1 ml of PBS). The BALF was centrifuged at 300 × g for 10 min at 4°C to obtain alveolar cells and cell-free lavage fluid. The cell-free lavage fluids were kept at -80°C. Alveolar cells were suspended in 1 ml of PBS, total cell numbers were determined, and cytopspins were prepared. The cytopspins were dried and stained with May-Grünwald Giemsa stain. Macrophages, neutrophils, lymphocytes, and eosinophils were differentiated by light microscopy. To determine the bacterial load of the lungs 24 h after infection, whole lungs were homogenized in 1 ml of PBS, serial dilutions were plated onto LB agar, and colonies were counted after incubation overnight.

Gel electrophoresis and Western blotting

BALFs were analyzed by gel electrophoresis and Western blotting to determine differences in the cathelin-related antimicrobial peptide (CRAMP) levels. BALFs were first concentrated to 1/10 of their original volume by vacuum centrifugation and then subjected to SDS-PAGE on a 10–20% Tris-HCl precast gel (Bio-Rad). Dissolved proteins were transferred to a nitrocellulose membrane. Nonspecific binding sites were blocked with 10% (w/v) nonfat milk in PBS, and the membrane was incubated with a rabbit polyclonal antiserum against CRAMP (Pineda-Antikörper-Service), and a HRP-conjugated anti-rabbit Ab (Amersham Biosciences). The SuperSignal West Pico detection system (Pierce) was used to reveal immunoreactivity.

Immunohistochemistry

Formalin-fixed and paraffin-embedded lung tissue was sectioned and incubated with polyclonal rabbit antiserum to CRAMP or control serum. Bound Abs were visualized using a biotin-labeled secondary Ab, a streptavidin-peroxidase conjugate, and AEC Single Solution (Zymed). Nuclei were counterstained with hemalaun. CellF software (Olympus) was used to quantify numbers of neutrophils. For each animal, five randomly selected areas adjacent to an airway were analyzed. The size of the area of interest and the number of stained neutrophils were measured. The number of neutrophils per square millimeter was calculated. This analysis was performed by a blinded investigator.

Statistical analysis

Values are displayed as mean ± SEM. Comparisons between groups were analyzed by *t* test (two-sided), or ANOVA for experiments with more than two subgroups. Post hoc range tests were performed with the *t* test (two-sided) with Bonferroni adjustment. Results were considered statistically significant for *p* < 0.05.

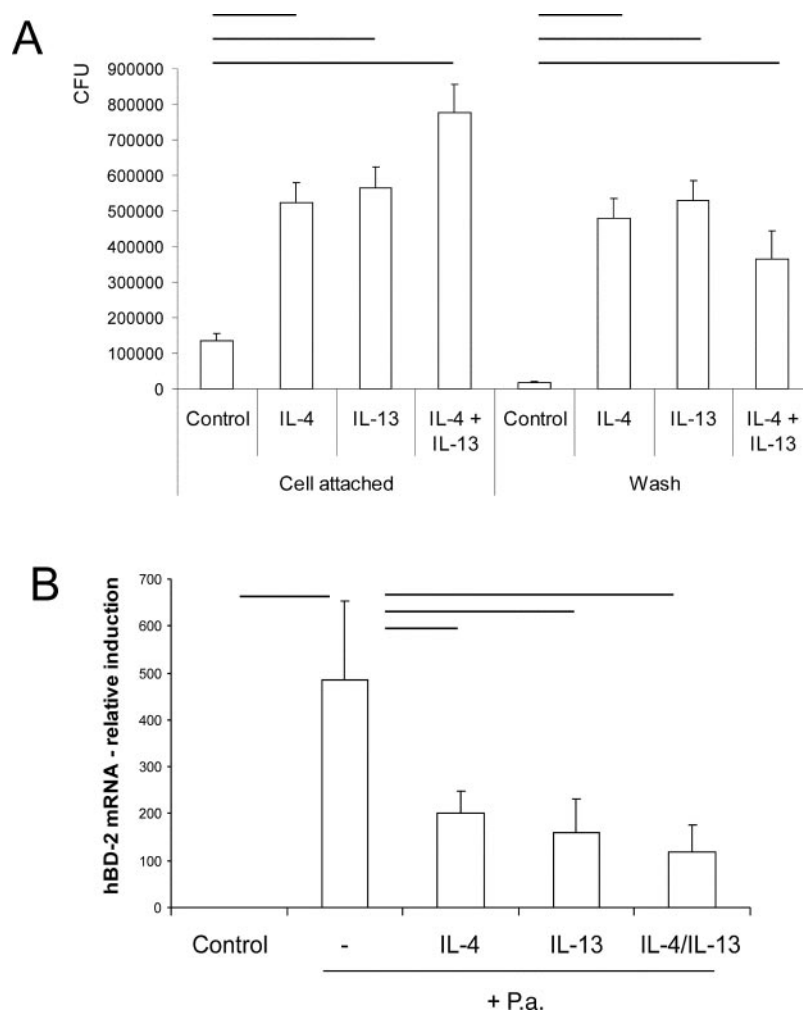
Results

Th2 cytokines suppress the antimicrobial host defense of airway epithelium

Airway epithelium is a first line host defense tissue and secretes a variety of antimicrobial factors. We tested whether Th2 cytokines affect the response of airway epithelium to a bacterial challenge. Airway epithelial cells grown in ALI culture were exposed to IL-4 and IL-13, infected with *P. aeruginosa* PAO1 from the apical surface, and tested for viable bacteria after 24 h. The preincubation of the cell cultures with the Th2 cytokines IL-4 and IL-13 resulted in a significant increase of viable bacteria in washings from the apical surface of the cells and in cell lysates (Fig. 1A) showing that Th2 cytokines compromise the antibacterial host defense of the airway epithelium.

We next investigated whether Th2 cytokines influence the induction of AMPs triggered by the exposure to heat-inactivated bacteria. The incubation with IL-4 or IL-13 suppressed the *P. aeruginosa*-induced expression of the AMP human β -defensin 2 (hBD-2) (Fig. 1B). The combination of both interleukins did not result in significantly decreased mRNA levels compared with the application of the individual cytokines. The mRNA levels of two

FIGURE 1. Th2 cytokines inhibit the epithelial host defense. *A*, The Th2 cytokines IL-4 and IL-13 inhibit the antimicrobial activity of airway epithelial cells grown as ALI culture. Epithelial cells were preincubated with IL-4 and IL-13 and infected with *P. aeruginosa*. The bacterial survival was determined after 24 h by plating of apical washings (Wash) and cell lysates (Cell attached) to agar plates. The bar indicates a significant difference of $p < 0.05$ ($n = 4$). *B*, IL-4 and IL-13 suppress the induction of hBD-2 by bacteria. Expression of hBD-2 was measured by quantitative RT-PCR. Bars indicate significant differences of $p < 0.05$ ($n = 4$).



other AMPs, LL-37 and human β -defensin 1 (hBD-1), were not significantly changed by exposure to bacteria (data not shown).

These data show that Th2 cytokines suppress the antibacterial host defense of airway epithelial cells and inhibit the induction of the epithelial AMP hBD-2 by bacteria.

Allergic inflammation modulates the antimicrobial host defense in vivo

In a next step, we characterized the impact of an allergic airway inflammation on different aspects of antimicrobial host defense in vivo. Conventional murine models of allergic airway inflammation and pneumonia were combined. Mice were immunized by i.p. injections of OVA and challenged by inhalation of the allergen. They were infected with *P. aeruginosa* 24 h before euthanasia. All animals survived until euthanasia. Overall, the sensitized and challenged animals appeared much less compromised by the infection compared with nonsensitized mice. They were more active and their fur was smooth. Analysis of the cells in the BAL showed that after infection the total number of cells was significantly increased in the group of the sensitized and challenged animals compared with the control group (Fig. 2A). The infection of nonsensitized animals resulted in an inflammatory reaction dominated by neutrophils (Fig. 2B). In contrast, the BAL of the control group of sensitized, but noninfected animals showed a strong influx of eosinophils, which is characteristic for the allergic inflammation of the lung (Fig. 2B). In the infected mice, the number of neutrophils was significantly reduced in OVA-treated animals compared with

nonsensitized animals. The two groups also differed with respect to the cytokine profile in the BALF. Infected animals with allergic airway inflammation showed decreased concentrations of the proinflammatory cytokines IL-1 β and IL-6 compared with the control group (Fig. 2, C and D). They had elevated levels of the Th2 cytokines IL-4 and IL-13 (data not shown).

To investigate the influence of an allergic pulmonary inflammation on the clearance of microorganisms, the number of viable bacteria in the lungs was determined 24 h after infection. Intriguingly, the number of CFU recovered from the OVA-sensitized animals was significantly higher compared with the nonsensitized animals (Fig. 3A). These data show that Th2-based allergic inflammation of the airways results in a delayed clearance of bacteria and in suppression of antibacterial host defense.

We then addressed the question whether the levels of AMPs are decreased in the infected sensitized animals. We analyzed the presence of the murine AMP CRAMP in the lavage fluids by Western blotting. Although the peptide or its precursor were clearly present in the BALFs from infected nonsensitized mice, the peptide was absent in the samples from the infected sensitized animals (three representative BALF samples per group is shown) (Fig. 3B). Immunohistochemistry using an antiserum directed to CRAMP showed decreased staining for the peptide and a lower number of CRAMP-positive neutrophils in the infected animals with allergic airway inflammation (40 ± 25 neutrophils/mm²) compared with the infected animals without allergic inflammation (121 ± 24 neutrophils/mm²) (Fig. 3, C–E).

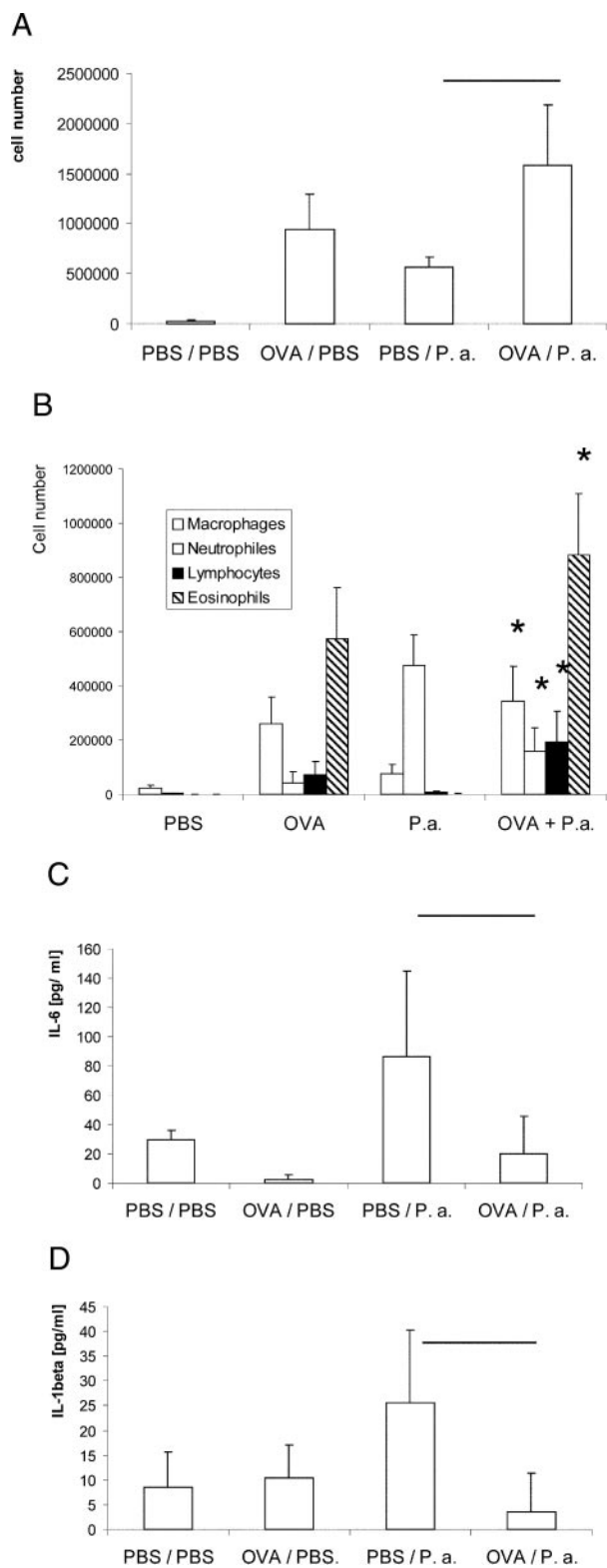


FIGURE 2. The allergic airway inflammation modulates the host defense reaction against bacteria. **A**, OVA-sensitized and -challenged animals showed significantly increased total cell numbers in the BALs after infection with *P. aeruginosa* (P.a.). The bars indicate a significant difference of $p < 0.05$ ($n = 10$). **B**, Differential counts showed that infected sensitized animals (OVA/P.a.) had increased numbers of eosinophils in their lavages. In contrast, the numbers of neutrophils were significantly decreased. Asterisks indicate significant differences of $p < 0.05$ compared with the PBS/P.a. group ($n = 10$). **C** and **D**, Infected OVA-sensitized mice had significantly less IL-6 (**C**) and IL-1 β (**D**) in their lavages. Bars indicate significant differences of $p < 0.05$ ($n = 10$).

These data show that the allergic airway inflammation modulates the host defense of the lung and inhibits innate immune responses.

Discussion

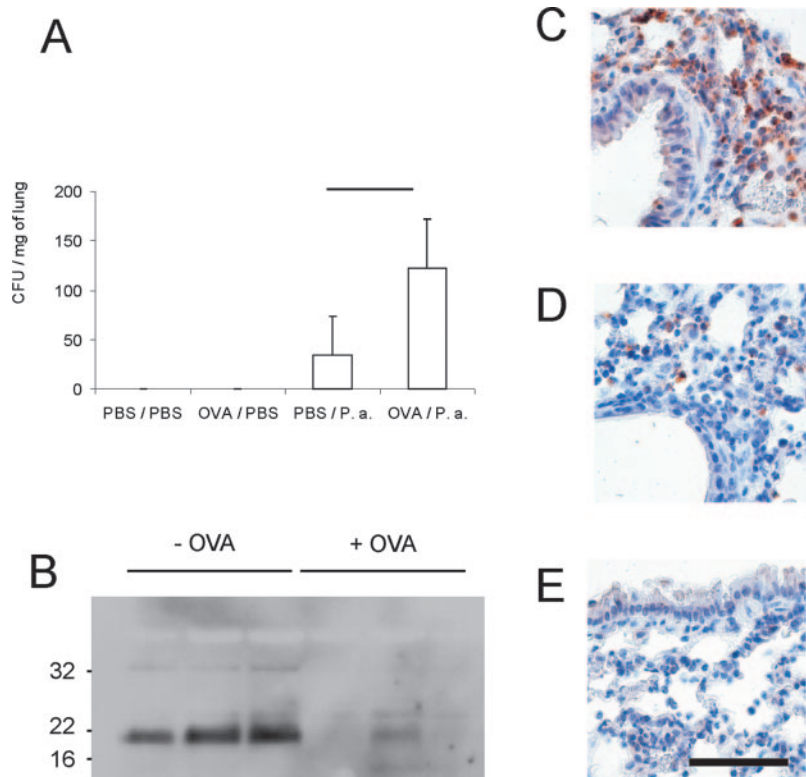
The main finding of the present study is that an allergic inflammation of the airways inhibits the antibacterial host defense. In an animal model of allergic airway disease, Th2-type inflammation in the lung results in suppressed antibacterial host defense that is associated with decreased production of antimicrobial factors. Furthermore, the antimicrobial activity of airway epithelium and the induction of antimicrobial factors are inhibited by Th2 cytokines in vitro.

These data provide further evidence that the innate immune system closely interacts with the adaptive immune system. We specifically show that the adaptive immune response influences the innate host defense. A large body of evidence exists that shows that the innate immunity regulates the quality and the quantity of the adaptive immune response as outlined in the introduction. It is likely that this interaction is disturbed in patients with asthma. In contrast, there is limited evidence that the adaptive immune system influences the innate host defense. It has been described for the skin that Th2-based inflammation results in suppression of the host defense and inhibited expression of AMPs in the skin (12, 13). The exact mechanism how the Th2 milieu inhibits the antimicrobial host defense is not yet clear. It has been demonstrated that Th2 cytokines inhibit the TNF- α /NF- κ B system through activation of STAT6 acting as a transcriptional inhibitor of NF- κ B-dependent gene expression (14). This mechanism could also account for the inhibitory effect of IL-4 on the activation of neutrophils by LPS (15). A complete understanding of how the Th2 cytokines suppress antimicrobial activity of the airway epithelial cells remains to be determined.

Here, we show that the antimicrobial activity of the airway epithelium is inhibited by Th2 cytokines. Airway epithelial cells were unable to kill bacteria when incubated with Th2 cytokines. Specifically, the expression of the AMP hBD-2 based on mRNA levels was suppressed. Airway epithelial cells secrete a variety of antimicrobial factors and represent a first-line host defense system (3, 16). AMPs are one family of antimicrobial factors and are effector molecules of the innate immune system of the lung. AMPs have a broad antimicrobial spectrum and lyse microbial cells by interaction with their biomembranes (17, 18). In addition to their direct antimicrobial function, they have multiple roles as mediators of inflammation with impact on epithelial and inflammatory cells influencing diverse processes such as cytokine release, cell proliferation, angiogenesis, wound healing, chemotaxis, immune induction, and protease-antiprotease balance. The defensins and the cathelicidins are the principal families of AMPs expressed in the lung and are secreted by airway epithelial or classical host defense cells such as macrophages and neutrophils. The expression of individual AMPs is tightly regulated and can be induced by microorganisms or some of their specific structural components (19). hBD-2 is an AMP that is classically found regulated in tissue culture experiments (20). In contrast, no stimuli have been identified that induce the expression of hBD-1 and LL-37/hCAP-18 in vitro in airway epithelial cells. The results of the present study are concordant with these data from the literature. It is currently unclear how much the epithelium contributes to the in vivo effects described in this study. Likely, the inhibitory effects of allergic inflammation on host defense involve many other cell types in addition to airway epithelial cells.

The data of the present study help to explain observations in patients with asthma. It has been recognized that these patients are more likely to develop pneumonia. Asthma was found to be a risk factor in several epidemiologic studies (21–23). Also, patients with atopic dermatitis develop more infections compared with nonatopic individuals. This is also associated with suppressed induction of AMPs (12, 13).

FIGURE 3. Antibacterial host defense is decreased in mice with allergic airway inflammation. **A**, The number of viable bacteria recovered from sensitized (OVA/P.a.) animals 24 h after infection was significantly increased compared with the control group (PBS/P.a.). Bars indicate a significant difference of $p < 0.05$ ($n = 10$). **B**, Western blotting of BAL fluids using an anti-serum against CRAMP. Sensitized animals (+OVA) had almost undetectable CRAMP in their airway secretions, the infected animals without OVA-challenge (−OVA) showed the presence of the CRAMP. **C–E**, Immunohistochemical analysis showed decreased numbers of CRAMP-positive neutrophils in the lungs of OVA-sensitized animals (**D**) compared with the infected, nonsensitized animals (**C**). **E**, Control using a nonspecific primary Ab on sections from an infected, nonsensitized animal; counterstain with hematoxylin; bar, 100 μm .



The findings of the present study also stimulate speculations about the role of a suppressed host defense and chronic infection in the development of asthma. Asthma is a chronic inflammatory disease that has been connected to dysregulated reactions of the adaptive immune system. Patients with asthma are often chronically colonized by *Mycoplasma* or *Chlamydia* species (24). Chronic colonization of the airways of patients with asthma could be a factor in the development of asthmatic lung disease. The Th2-dependent suppression of the pulmonary innate immune system could result in increased susceptibility to colonization and infection.

In conclusion, the present study shows that the allergic airway inflammation modulates the innate antimicrobial host defense. The adaptive immune system modulates the innate host defense.

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Disclosures

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