Original article

Alveolar macrophage phagocytosis is impaired in children with poorly controlled asthma

Anne M. Fitzpatrick, PhD,[a] Fernando Holguin, MD,[b] W. Gerald Teague, MD,[a] and Lou Ann S. Brown, PhD[a] *Atlanta, Ga*

Background: Lower respiratory tract infection is a differentiating feature of children with poorly controlled asthma.
Objective: Given the role of alveolar macrophages (AMs) in innate immunity, we hypothesized that AM phagocytosis might be impaired in poorly controlled asthma.
Methods: Bronchoalveolar lavage fluid AMs were isolated from 28 asthmatic children (moderate asthma, n = 12; severe asthma, n = 16), 10 nonasthmatic children with chronic cough treated with inhaled corticosteroids, and 10 healthy adult control subjects. AMs were stimulated with LPS and exposed to fluorescein isothiocyanate–conjugated *Staphylococcus aureus* for 2 hours. Phagocytosis was quantified by using a phagocytic index (PI) calculated from the percentage of phagocytic cells multiplied by the relative fluorescence (RFU) units of *S aureus* per cell. Apoptosis was determined from the percentage of cells positive for poly (adenosine diphosphate–ribose) polymerase.
Results: Phagocytosis as measured by using the unstimulated PI was decreased in subjects with poorly controlled asthma (healthy control subjects, 9330 ± 3992 RFU; chronic cough, 9042 ± 5976 RFU; moderate asthma, 4361 ± 2536 RFU; severe asthma, 3153 ± 1886 RFU; $P < .001$) and remained unchanged with LPS stimulation. Children with severe asthma also had increased AM apoptosis, both the unstimulated and LPS-simulated states ($P < .001$), which correlated with the PI.
Conclusions: AM function is compromised in children with poorly controlled asthma and is characterized by decreased phagocytosis and increased apoptosis. (J Allergy Clin Immunol ■■■■;■■■:■■■-■■■.)

Key words: *Asthma, children, innate immunity, alveolar macrophage, respiratory infection*

Abbreviations used

AM:	Alveolar macrophage
BAL:	Bronchoalveolar lavage
FE_{NO}:	Fraction of exhaled nitric oxide
FVC:	Forced vital capacity
ICS:	Inhaled corticosteroid
PARP:	Poly (adenosine diphosphate–ribose) polymerase
PI:	Phagocytic index
RFU:	Relative fluorescence unit

Children with poorly controlled asthma remain symptomatic despite treatment with inhaled corticosteroids (ICSs).[1] Symptoms are thought to result from persistent airway inflammation, with viral respiratory tract infection as a major trigger.[2] In children atypical bacteria and viruses have been implicated in more than 50% of acute exacerbations and hospitalizations.[2-4] Thus respiratory tract infection can be an important risk factor for poor symptom control. The mechanisms linking asthma and respiratory tract infection are not well defined.

Alveolar macrophages (AMs) are the primary airway cells responsible for innate immune defense, including phagocytosis of respiratory pathogens. Although other inflammatory disorders, such as chronic alcoholism[5] and particulate matter inhalation,[6] have been linked with AM dysfunction, AM function has not been extensively evaluated in asthma. Recently, increased AM activation and decreased phagocytosis of apoptotic cells were reported in asthmatic adults.[7] Although that study provided insight into decreased clearance of eosinophils and neutrophils, no study to date has examined phagocytosis of infectious particles in asthmatic children. The purpose of this study was to characterize unstimulated and stimulated phagocytosis in children with moderate versus severe poorly controlled asthma.

METHODS

Sample

Children 5 to 17 years of age undergoing flexible bronchoscopy with bronchoalveolar lavage (BAL) for clinical indications were recruited from the outpatient pulmonary clinic at Emory University. Indications for bronchoscopy included (1) poor asthma control despite maximum ICS doses, (2) suspected aspiration, (3) suspected atypical infection, or (4) confirmation of habitual cough or vocal cord dysfunction. This study was approved by the Emory University Institutional Review Board. Informed consent and assent were obtained from all caregivers and their children, respectively.

Poorly controlled asthma was diagnosed by a pediatric pulmonologist and was defined by 12% or greater FEV_1 reversibility after short-acting β_2-agonist administration and daily asthma symptoms (minimum of 5 of 7 days) requiring bronchodilator use. Asthmatic children were further classified as having severe disease if they had a baseline FEV_1 of less than 80% of predicted value and an asthma-related hospitalization within the previous year. All asthmatic subjects received ICSs for at least 8 weeks before bronchoscopy. Children with immunodeficiency or other pulmonary morbidities, such as cystic fibrosis or bronchopulmonary dysplasia, were excluded.

From the Departments of [a]Pediatrics and [b]Medicine, Emory University School of Medicine.

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Reprint requests: Anne M. Fitzpatrick, PhD, 2015 Uppergate Dr, Atlanta, GA 30322. E-mail: anne.fitzpatrick@emory.edu.

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Control subjects for this study were recruited from 2 populations: (1) children with suspected habitual cough or vocal cord dysfunction undergoing clinical bronchoscopy for definitive diagnosis and (2) healthy nonsmoking adults undergoing bronchoscopy for research purposes. Children and adults serving as control subjects had no historical evidence of 12% or greater FEV_1 reversibility[8] and exhaled offline nitric oxide (FE_{NO}) concentrations of less than 10 ppb.[9] Adult control subjects were asymptomatic and were not taking ICSs. By contrast, pediatric control subjects were symptomatic and were empirically treated with ICSs.

Procedures

Spirometry was performed with a portable spirometer (KoKo Legend; Ferraris, Louisville, Colo) according to American Thoracic Society criteria for reproducibility,[10] and results were interpreted by using reference standards.[11] FE_{NO} was collected with a reservoir bag at a fixed exhaled flow rate of 0.35 L/s[12] and analyzed offline by means of chemiluminescence (Sievers NOATM 280-I; Ionic Instruments, Boulder, Colo).

Clinical bronchoscopy was performed in children by pediatric pulmonologists using a laryngeal airway mask (n = 37) or endotracheal tube (n = 3). All subjects received inhaled general anesthesia (fluothane or sevoflurane). BAL fluid was collected from the right middle lobe through three 10-mL saline lavages (37°C, pH 6.8–7.0) flushed through the suction channel of a flexible bronchoscope (Olympus BF-3C160 [3.7 mm] or BF-P160 [4.9 mm]; Olympus America, Inc, Melville, NY) by using hand pressure on a syringe. BAL fluid was collected and pooled under continuous low-pressure suction. Half of the return volume was used for this research.

Research bronchoscopy was performed in healthy adults by a pulmonary specialist with a flexible bronchoscope (Olympus BF-1T20D; Olympus American, Inc) passed transnasally into the right middle lobe. Subjects received intravenous sedation for the procedure (midazolam and fentanyl). Three 50-mL saline aliquots were instilled and immediately aspirated into 50-mL suction traps under continuous low-pressure suction. BAL fluid was pooled for analysis.

BAL fluid was centrifuged at 1200 rpm for 7 minutes for recovery of the cell pellet. Bacterial cultures and RT-PCR for *Mycoplasma pneumoniae* and *Chlamydia pneumoniae* were performed on the supernatant by a clinical microbiology laboratory (Children's Healthcare of Atlanta, Atlanta, Ga). Manual cell counts were performed with a hemocytometer, and differentials were obtained from 300 consecutive cells after Diff-Quik staining (Andwin Scientific, Addison, Ill). The cell pellet was resuspended in 10 mL of 1:1 Dulbecco's modified Eagle's medium/Ham's F-12 solution containing 2% FBS, L-glutamine, 15 mmol/L HEPES, penicillin (10,000 U), streptomycin (10,000 mg/mL), amphotericin (25 mg/mL), and gentamicin (4 μg/mL). AMs (100,000 cells) were added to glass-chamber slides containing 100 μL of medium and 20 μL of PBS and were assessed before and after the addition of 20 μL of 1 mg/mL LPS. AMs were incubated at 37°C with 10% CO_2 for 15 hours, after which 10×10^5 particles of fluorescein isothiocyanate–conjugated inactivated *Staphylococcus aureus* (Invitrogen, Carlsbad, Calif) were added (10:1 ratio of *S aureus*/AMs) to the cultures and incubated for 2 hours. Cells were fixed with 3.7% paraformaldehyde and stored at 4°C until analysis.

Bacterial phagocytosis was assessed with an Olympus confocal microscope (model BX51; Olympus America, Inc) containing an argon/krypton laser. AMs were analyzed from 10 experimental fields per set by using quantitative digital fluorescence imaging software (Olympus FluoView 300, Version 4.3). Laser confocal microscopy was performed at 50% of the AM depth by using identical background and gain settings to ensure internalization of *S aureus*. AMs with bacterial inclusions at 50% of the cell depth, regardless of the number, were considered positive for phagocytosis. AMs without inclusions that had nonspecific binding of *S aureus* to the plasma membrane were considered positive for external binding. The remaining AMs were classified as unresponsive to *S aureus* particles. The relative fluorescence units (RFU) of *S aureus* were determined for each phagocytic cell at 50% of the cell depth to further quantify phagocytic internalization. A phagocytic index (PI) was calculated as follows:

$$PI = \%\ \text{AMs undergoing phagocytosis} \times \text{Average RFUs per positive AM}.$$ [13]

Apoptosis was determined by means of immunostaining for cleavage of poly (adenosine diphosphate–ribose) polymerase (PARP) by using a 1:100 dilution of PARP-1 primary antibody (Santa Cruz Biotechnology, Santa Cruz, Calif) and a 1:200 dilution of secondary antibody (TRITC F[ab′]2, Santa Cruz Biotechnology).[5] Cleavage of PARP was assessed in AMs immediately after isolation from the BAL fluid and after 15 hours of culture with or without 20 μg/mL LPS. Cellular fluorescence was determined by means of quantitative digital analysis with Image-Pro Plus for Windows (Version 3.1; Media Cybernetics, Inc, Silver Spring, Md). Data were expressed as the percentage of AMs fluorescently positive from 10 experimental fields per set.

Data analysis

Data analysis was performed with SPSS software (Version 15; SPSS, Inc, Chicago, Ill). FE_{NO} measurements were logarithmically transformed. Kruskal-Wallis tests and Wilcoxon signed-rank tests were performed for all between-group and within-group comparisons, respectively. Pearson correlation coefficients were calculated between the unstimulated and LPS-stimulated PI and all subject features. Stepwise forward linear regression analysis was then performed with the PI (unstimulated and LPS stimulated) as the dependent variables and severity, age, history of asthma-related hospitalization, FEV_1/forced vital capacity ratio, FE_{NO} (log value), ICS dose, and history of abnormal chest radiograph as predictors. Multicolinearity between predictors was assessed with tolerance statistics. Two-tailed tests were used for all analyses, with significance determined by using an α value of less than .05.

RESULTS

Thirty-four children with poorly controlled asthma (severe asthma, n = 18) were recruited for the study. Four children (severe asthma, n = 2) had BAL fluid colonization with *Streptococcus pneumoniae*, *Moraxella catarrhalis*, or both and were excluded from data analyses. Ten children with chronic cough treated with ICSs served as control subjects. All children in this group had been receiving ICSs for at least 16 weeks before bronchoscopy (median, 30 weeks; range, 16-52 weeks). Postbronchoscopy diagnoses in this control group included habitual cough (n = 5), gastroesophageal reflux aspiration (n = 3), and vocal cord dysfunction (n = 2). Given the symptomatic nature of this group, 10 nonsmoking healthy adults were also recruited for comparison. Adults in this control group were free of asthma symptoms and medication use; however, they were significantly older (Table I). Features of the groups appear in Table I.

Flexible bronchoscopy with BAL was well tolerated. Prolonged bronchospasm was observed in 1 child with severe asthma, which normalized with intraoperative albuterol and positive airway pressure. Postoperative cough was a common finding in more than 75% of participants and improved after airway clearance measures, albuterol administration, or both. There was no incidence of postoperative fever, and no participant required prolonged observation.

BAL fluid cellularity is presented in Table II. Cultures for fungus and viruses (adenovirus, influenza A/B, parainfluenza, and respiratory syncytial virus) were negative for all subjects. *M pneumoniae* and *C pneumoniae* were also undetectable by means of RT-PCR. Cellular composition was similar between groups, with AMs comprising at least 85% of the total cell count.

Unstimulated phagocytosis

Children with poorly controlled asthma had impaired phagocytosis evidenced by fewer AMs with cytoplasmic *S aureus* inclusions at 50% of the AM depth (control, 88% ± 9%; chronic cough, 89% ± 10%; moderate asthma, 71% ± 11%; severe asthma, 58% ± 18%; $P < .001$). This was accompanied by decreased overall *S aureus* uptake as measured by the RFUs for each phagocytic

TABLE I. Baseline characterization

	Healthy control subjects, no ICS (n = 10)	Chronic cough, ICS (n = 10)	Moderate asthma, ICS (n = 14)	Severe asthma, ICS (n = 16)
Age (y)*	37 ± 7	11 ± 4	9 ± 4	10 ± 5
Male (%)	5 (50)	5 (50)	10 (71)	9 (56)
Ethnicity				
White	5 (50)	10 (100)	10 (71)	8 (50)
African American§	5 (50)	0	4 (29)	8 (50)
Daily medications				
Budesonide†	0	2 (20)	5 (36)	4 (25)
Fluticasone/salmeterol*	0	7 (70)	6 (43)	11 (69)
Fluticasone	0	0	2 (14)	1 (6)
Beclomethasone	0	1 (10)	1 (7)	0
Montelukast*	0	6 (60)	10 (71)	16 (100)
Prednisone‖	0	0	0	7 (44)
Daily ICS dose*‖ (μg fluticasone equivalents/d)	0	440 ± 357	515 ± 289	890 ± 329
Pulmonary function				
FVC (% predicted)‖	96 ± 18	102 ± 10	105 ± 21	78 ± 14
FEV_1 (% predicted)‖	104 ± 20	99 ± 6	100 ± 21	65 ± 12
FEV_1/FVC ratio‖	0.90 ± 0.06	0.85 ± 0.05	0.86 ± 0.08	0.74 ± 0.13
FEF_{25-75} (% predicted)‖	115 ± 27	93 ± 14	90 ± 27	47 ± 26
FEV_1 reversibility (%)‖	2 ± 4	3 ± 4	13 ± 11	23 ± 13
FE_{NO} (offline, ppb)‖	5 ± 2	7 ± 2	6 ± 4	16 ± 13
Abnormal chest radiograph#				
Peribronchial thickening*‡	0	0	7 (50)	5 (31)
Lobar consolidation†§	0	0	4 (29)	4 (25)
Atelectasis†	0	1 (10)	4 (29)	7 (44)
Hyperinflation†§	0	0	1 (7)	4 (25)
Asthma-related hospitalization‡#	0	0	4 (29)	16 (100)

Data represent the mean ± SD or frequency (percentage).
FVC, forced vital capacity; FEF_{25-75}, forced expiratory flow.
*$P < .01$, healthy control subjects versus other groups.
†$P < .05$, healthy control subjects versus other groups.
‡$P < .01$, chronic cough versus other groups.
§$P < .05$, chronic cough versus other groups.
‖$P < .01$, severe asthma versus other groups.
#Within the previous 12 months.

cell (control, 9742 ± 4547 RFU/cell; chronic cough, 9789 ± 5947 RFU/cell; moderate asthma, 6235 ± 3820 RFU/cell; severe asthma, 5813 ± 3356 RFU/cell; $P = .045$). The resulting PI was also decreased in asthmatic children and was most impaired in children with severe asthma (Fig 1). External *S aureus* binding without internalization was increased in subjects with severe asthma compared with that seen in the other groups (Fig 2).

LPS-stimulated phagocytosis

With LPS stimulation, the PI remained lower in children with severe asthma (Fig 1). Whereas LPS stimulation did not alter phagocytosis in control subjects (percentage of phagocytosis for unstimulated vs LPS stimulated: control, 88% ± 9% vs 86% ± 10%, P = not significant; chronic cough, 89% ± 10% vs 85% ± 13%, P = not significant), it further decreased phagocytosis in subjects with poorly controlled asthma (moderate asthma, 71% ± 11% vs 65% ± 17%, $P = .028$; severe asthma, 58% ± 18% vs 48% ± 21%, $P = .003$). AMs from asthmatic subjects were also characterized by fewer *S aureus* RFUs than control subjects after LPS treatment (control, 10,583 ± 4116 RFU/cell; chronic cough, 10,022 ± 6068 RFU/cell; moderate asthma, 6550 ± 5273 RFU/cell; severe asthma, 6321 ± 4306 RFU/cell; $P = .05$), but these were not different from the unstimulated values (P = not significant). Although LPS increased the extent of nonspecific external binding of *S aureus* in subjects with moderate asthma (Fig 2), no differences were observed in the other groups with LPS stimulation. Representative images of AM phagocytosis are presented in Fig 3.

AM apoptosis

Compared with control subjects, subjects with severe asthma had greater AM apoptosis at isolation (severe asthma, 15% ± 6%; moderate asthma, 8% ± 4%; chronic cough, 7% ± 4%; control, 5% ± 3%; $p = .05$) and after culture with and without LPS (Fig 4). Apoptotic indices from freshly isolated and cultured AMs were associated ($r = 0.55$, $P = .034$), suggesting that the cell culture conditions were not sufficient to influence the results. In the combined sample apoptosis was correlated with phagocytosis in the unstimulated state ($r = -0.45$, $P = .003$) and after LPS stimulation ($r = -0.43$, $P = .008$; Fig E1) and further accounted for approximately 20% of the variance in phagocytosis (unstimulated R^2 = 0.205; LPS stimulated R^2 = 0.183). Isolation of the apoptotic AMs from each subject in the unstimulated state revealed that the majority of the apoptotic cells underwent phagocytosis regardless of severity classification (percentage phagocytic: control, 86% ± 16%; chronic cough, 89% ± 10%; moderate asthma, 83% ± 12%; severe asthma, 78% ± 9%; P = not significant). Furthermore, no differences in the ability of apoptotic cells to ingest *S aureus* were observed after LPS stimulation (percentage

TABLE II. BAL fluid composition

	Healthy control subjects (n = 10)	Chronic cough, ICS (n = 10)	Moderate asthma (n = 14)	Severe asthma (n = 16)
BAL return (% of volume instilled)	50 ± 16	41 ± 16	46 ± 11	38 ± 14
BAL cell count (cells/mL, × 10^6)*	8.66 ± 5.67	4.03 ± 2.42	4.16 ± 3.20	3.77 ± 2.47
BAL cellularity (%)				
Macrophages/monocytes	90.8 ± 3.8	91.5 ± 7.8	91.6 ± 4.2	88.5 ± 6.2
Neutrophils	3.0 ± 2.6	5.2 ± 6.2	4.7 ± 1.9	5.2 ± 4.5
Eosinophils	0.7 ± 0.5	0.4 ± 0.8	0.9 ± 1.6	1.9 ± 3.9
Lymphocytes	4.5 ± 2.2	2.6 ± 2.3	2.5 ± 2.7	4.2 ± 2.4
Basophils	0.2 ± 0.5	0.2 ± 0.4	0.2 ± 0.5	0.1 ± 0.3
BAL total protein (μg/mL)	138 ± 76	234 ± 155	224 ± 110	197 ± 55

Data represent the mean ± SD.
*$P < .01$, healthy control subjects versus other groups.

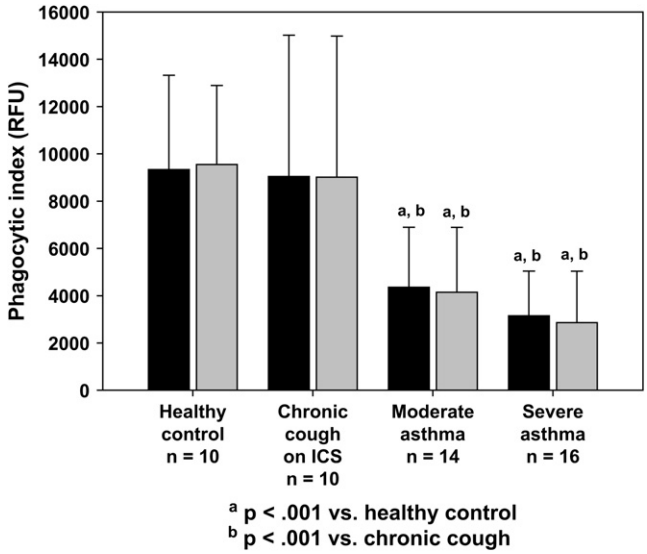

FIG 1. Percentage of AMs with bacterial inclusions.

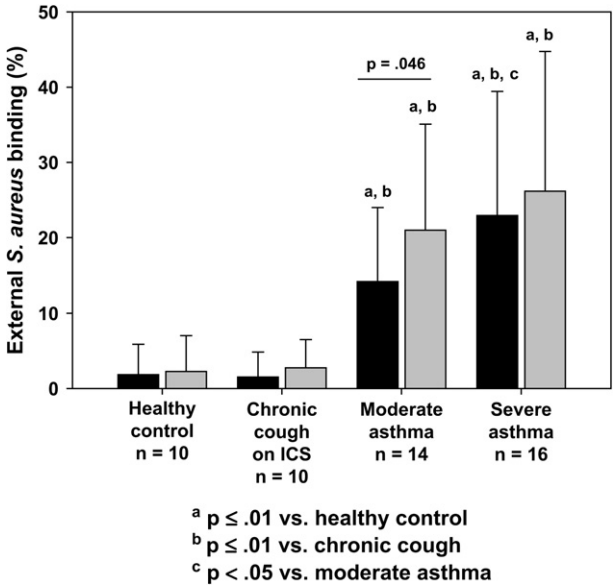

FIG 2. Percentage of AMs with nonspecific external *S aureus* binding (no internalization).

phagocytic: control, 84% ± 15%; chronic cough, 81% ± 8%; moderate asthma, 83% ± 12%; severe asthma, 81% ± 11%; P = not significant). Representative images of apoptotic AMs are provided in Fig E2.

Effect of corticosteroid treatment and other potential confounders

Given the clinical heterogeneity of children with severe asthma, a subanalysis was undertaken to determine whether corticosteroid treatment and other clinical features might account for impaired phagocytosis. We first compared subjects with severe asthma receiving daily oral corticosteroids and high-dose ICSs (n = 7) with subjects with severe asthma receiving high-dose ICSs alone (n = 9). Sex, ethnicity, pulmonary function, chest radiographic abnormalities, baseline FE_{NO}, and daily ICS dose (fluticasone equivalents) did not differ between groups, although subjects with severe asthma treated with oral corticosteroids were significantly older (median age, 15 vs 7 years; $P < .001$). Unstimulated phagocytosis and LPS-stimulated phagocytosis, as measured by the median PI, did not differ between groups (unstimulated: oral corticosteroids vs ICS only, 2991 vs 3044 RFU; LPS stimulated, 1981 vs 1767 RFU; P = not significant), although there was a trend toward decreased apoptosis in children treated with oral corticosteroids (unstimulated: oral corticosteroid vs ICS alone, 25% vs 40%, $P = .05$; LPS stimulated, 25% vs 45%, $P = .05$).

Stepwise forward linear regression analysis was performed by using the PI (unstimulated and LPS stimulated) as the dependent variable and severity (unstimulated: LPS stimulated, $r = -0.58$, -0.61), age ($r = 0.31, 0.36$), history of asthma-related hospitalization ($r = -0.41, -0.47$), FEV_1/forced vital capacity ratio ($r = 0.30, 0.35$), log FE_{NO} ($r = -0.36, -0.35$), ICS dose ($r = -0.41, -0.37$), and history of abnormal chest radiograph ($r = -0.43, -0.39$) as predictors to control for the differing clinical features that might have influenced phagocytosis. Severity alone showed the most significant slope (unstimulated, $P = .001$; LPS stimulated, $P = .001$). Addition of the other predictors to the model was not statistically significant (Table E1), suggesting that corticosteroid use and other clinical features could not sufficiently explain the impaired phagocytosis observed in asthmatic children.

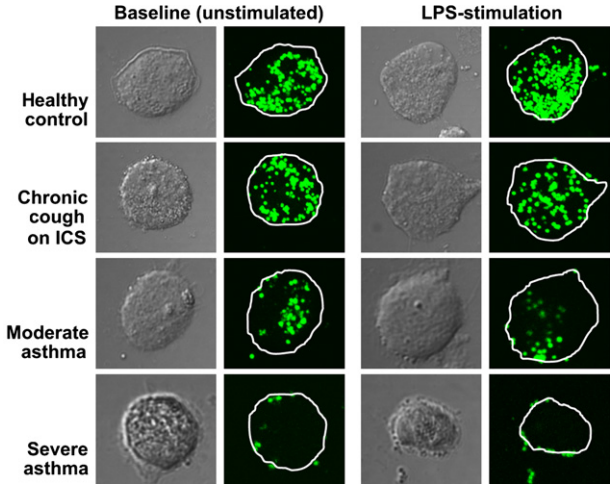

FIG 3. Representative cross-sectional confocal images of AMs at 50% of the cell depth depicting internalization of *S aureus* (green particles).

DISCUSSION

This is the first study to demonstrate impairment of AM phagocytosis in children with poorly controlled asthma. In subjects with moderate and severe asthma, phagocytosis was decreased by more than 50% compared with that seen in adult and pediatric control subjects. AM apoptosis was also greater in asthmatic subjects and increased further with LPS stimulation. These data suggest that the airway innate immune response might be impaired in children with poorly controlled asthma, a finding that might account for the aberrant response to respiratory tract infection commonly observed in this population.[14,15]

AMs internalize foreign airway particles through a variety of mechanisms, including pinocytosis, receptor-mediated endocytosis, and phagocytosis. Whereas pinocytosis refers to the nonspecific uptake of fluid and solutes, receptor-mediated endocytosis is a specific process in which small particles (typically <0.5 μm) enter cells. In the presence of larger particles (>0.5 μm), phagocytosis occurs either through opsonization of the antigen or unopsonized nonspecific uptake.[16] In this study phagocytosis was assessed by adding inactivated *S aureus* (particle size, 0.8-1.1 μm) to AMs in culture media containing 2% FBS. Therefore our results do not permit conclusion as to the specific type of phagocytosis observed. Our findings might reflect impairment in both opsonized and unopsonized phagocytosis in children with poorly controlled asthma, although further studies are needed to define the specific factors responsible for our observations.

AM phagocytosis is a complex process triggered by a variety of activation pathways.[17] Here we focused on phagocytosis resulting from innate activation of the AM after a bacterial microbial stimulus *(S aureus)*. With innate activation, microbes are recognized by pattern-recognition receptors, which induce proinflammatory cytokine production and promote phagocytosis.[17] Although it is true that the majority of asthma exacerbations in young children and older school-age children are triggered by respiratory tract viruses and not by acute bacterial infections,[2,18] innate AM activation is also important for respiratory viral clearance. Huber et al[19] recently observed phagocytosis of influenza virus resulting from direct binding of the opsonized virus to Fc scavenger receptors on the AM surface. Furthermore, adenovirus and respiratory syncytial virus infection result in increased AM Fcγ scavenger receptor expression, suggesting that opsonization and phagocytic engulfment are important for viral clearance.[20,21] Direct binding of rhinovirus to the AM cell surface has also been observed,[22] although the associated receptors and mechanisms of engulfment remain unclear.[22,23] Recently, a 3-fold increase in AM expression of the pattern-recognition Toll-like receptor 4 was observed in rhinovirus-infected children,[24] which might contribute to AM engulfment of the virus. Although these studies highlight the importance of innate AM activation in respiratory tract virus clearance, the precise mechanisms involved with this process are far from understood and can vary between viral strains. Further studies of AM phagocytosis as it relates to viral respiratory tract infection in asthmatic children are needed.

Our findings of decreased AM phagocytosis in children with poorly controlled asthma are similar to those previously observed in other chronic airway disorders. In patients with cystic fibrosis[25] and chronic obstructive pulmonary disease,[26] phagocytosis of bacteria and apoptotic cells is reduced to half of the levels of healthy control subjects. In patients with chronic obstructive pulmonary disease, *ex vivo* treatment with broad anti-inflammatory agents, such as lovastatin and azithromycin, improves phagocytic activity by 50% or more.[27,28] Although the precise mechanisms responsible for AM dysfunction can vary between disease states, these findings suggest that underlying inflammation might have an important effect on AM function in the human airway.

There are a limited number of studies on AM phagocytosis in patients with asthma, and few have targeted patients with poor asthma control. In one study phagocytosis did not differ between control subjects and subjects with mild intermittent asthma with good symptom control, although the total number of opsonized particles was lower in asthmatic subjects with airway eosinophilia compared with those without.[29] In a similar sample the number of opsonized particles in airway macrophages was reduced by approximately 50% six hours after endotoxin (LPS) inhalation.[30] These data suggest that even despite good symptom control, asthmatic subjects might be more susceptible to a secondary airway insult. This effect is more pronounced in those with severe disease. Recently, Huynh et al[7] observed no phagocytic differences between adults with mild-to-moderate asthma and control subjects, but phagocytosis was impaired by nearly 50% in subjects with severe asthma. LPS stimulation further decreased phagocytosis in subjects with severe asthma, a finding that was reversed with *ex vivo* dexamethasone treatment.[7] These data suggest that asthmatic AMs might be functionally modulated by airway inflammation, thus rendering the asthmatic patient more susceptible to a secondary airway insult. However, the focus of that study was to determine whether asthma compromised phagocytosis of apoptotic cells, a process critical for the clearance of neutrophils or eosinophils recruited to the airspace. In contrast, the goal of the present study was to assess phagocytosis in response to innate immune activation of the AM and clearance of infectious particles.

Although few studies have examined the capacity of asthmatic AMs to phagocytose foreign particles, previous studies have observed increased activation of AMs from asthmatic patients. Compared with control subjects, asthmatic subjects have increased basal spontaneous generation of superoxide anion,[31] proinflammatory cytokines,[32] and regulators of inflammatory gene expression, such as histone acetyltransferase.[33] These alterations are further increased with antigen stimulation[34] and are accompanied by decreased production of anti-inflammatory cytokines, such as IL-10.[35] Taken together, these findings provide

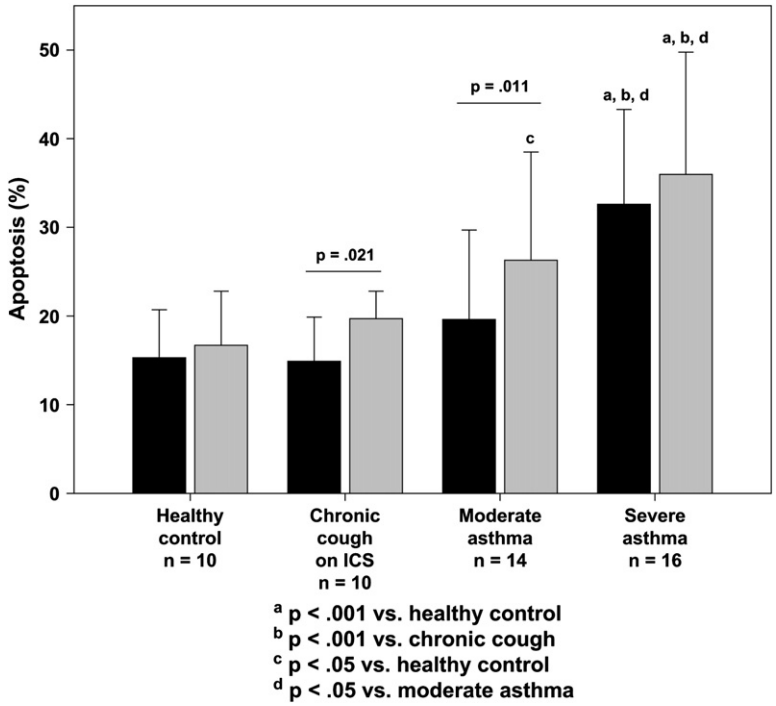

FIG 4. Percentage of apoptotic AMs, as indicated by positive staining for cleaved PARP.

evidence that the respiratory burst of asthmatic AMs might be impaired, thus inhibiting microbe killing. However, it is important to note that the AM respiratory burst and phagocytosis are regulated by different cellular mechanisms and might not necessarily occur in parallel.[35] Further study of the dynamic relationships between the respiratory burst and phagocytosis is warranted in asthmatic subjects to better define the mechanisms associated with respiratory infection in this population.

This study has a number of limitations. Because bronchoscopy cannot be performed on otherwise healthy children solely for research purposes, our pediatric control group was limited to children with symptomatic respiratory tract illnesses undergoing bronchoscopy for diagnostic purposes. Although this sample was sufficient to detect differences in AM phagocytosis and apoptosis between groups, the AMs isolated from these children might be phenotypically different from those of true pediatric control subjects. The fact that our healthy adult control subjects were significantly older than our pediatric sample also raises the question as to whether age is a determinant of AM function across the lifespan. Because AM function was similar between our adult and pediatric control subjects, it is unlikely that age contributed to our findings, particularly because age was treated as a potential confounder in multivariate analyses. However, additional studies are needed to more adequately address the effect of age on AM function in children.

Because pediatric bronchoscopies were not performed for research purposes, BAL samples from children were pooled before analysis. This practice is common at our institution and provides an increased sample yield for clinical laboratory analysis. Because pooling of the BAL fluid intermixes the bronchial and alveolar airway constituents, it is possible that the macrophages obtained from children for this study were not purely alveolar (or bronchial) in origin. It is also possible that bronchial macrophages and AMs have distinct functional abilities. The BAL samples from adult control subjects were similarly pooled to minimize this potential effect on our results. However, given the differences between adults and children with regard to airway structure and the lavage volumes used, we cannot exclude the possibility that the samples from healthy adults contained more alveolar cells. Further studies are needed to characterize the differences between the bronchial and alveolar cellular constituents, particularly in asthmatic subjects.

It is also possible the differences in AM function that we observed could be attributed to the confounding effects of asthma treatment or other unmeasured clinical variables. Although the effects of unmeasured clinical variables remain unknown, we do not believe that corticosteroid use sufficiently explains the discrepancies in AM function between asthmatic children and control subjects. Because our pediatric control subjects were symptomatic, all children in this group were treated empirically with ICSs for a minimum of 16 weeks, yet AM phagocytosis in this group was similar to that of healthy adult control subjects. A subgroup analysis of children with severe asthma receiving oral corticosteroids further revealed no differences in AM function compared with subjects with severe asthma treated with ICSs alone. Finally, statistical control of ICS dose and other potential confounding variables revealed that asthma severity alone, as defined by this study, was the most significant predictor of AM function. Although this evidence suggests a limited association between corticosteroid use and AM function, additional studies are needed to thoroughly examine this relationship in children with poorly controlled asthma.

Our findings of impaired AM phagocytosis and increased AM apoptosis warrant further study. Although apoptosis correlated with phagocytosis, the association was modest and did not sufficiently account for the differences in phagocytosis observed between groups. Furthermore, despite increased apoptosis in children with severe asthma, the apoptotic cells isolated from these children were

not less likely to undergo phagocytosis compared with the other groups. These data suggest that other factors aside from apoptosis are related to the impairment in AM function that we observed.

In conclusion, the mechanisms associated with respiratory tact infection in asthmatic children are not well understood. These data support the hypothesis that AMs are functionally impaired in children with poorly controlled asthma and are characterized by increased apoptosis and decreased phagocytosis of pathogenic bacteria. Although there are limited studies on the epidemiology of bacterial infection in asthma, a recent study found that asthma was a risk factor for invasive pneumococcal disease.[36] These findings, when taken into account with those demonstrating an increased prevalence of atypical mycobacteria in subjects with acute asthma,[3] suggest that bacterial infection might play a role in asthma morbidity. However, the relationship between bacterial and respiratory tract viral infection in children with asthma is not clear. Further studies are needed to define how impairments in AM phagocytosis relate to bacterial phagocytosis and viral clearance in patients with poorly controlled asthma.

Clinical implications: These findings might account for the increased severity of lower respiratory tract infections in children with poorly controlled asthma.

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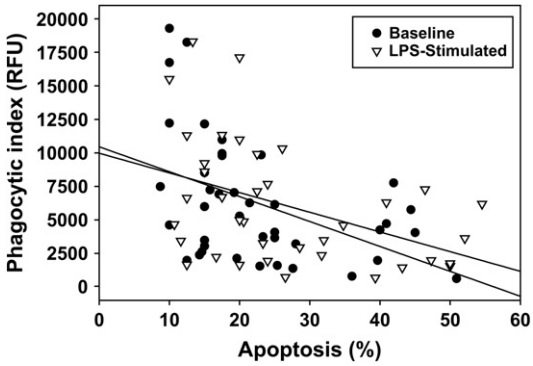

FIG E1. Scatterplot depicting the relationship between apoptosis and phagocytosis in the combined sample.

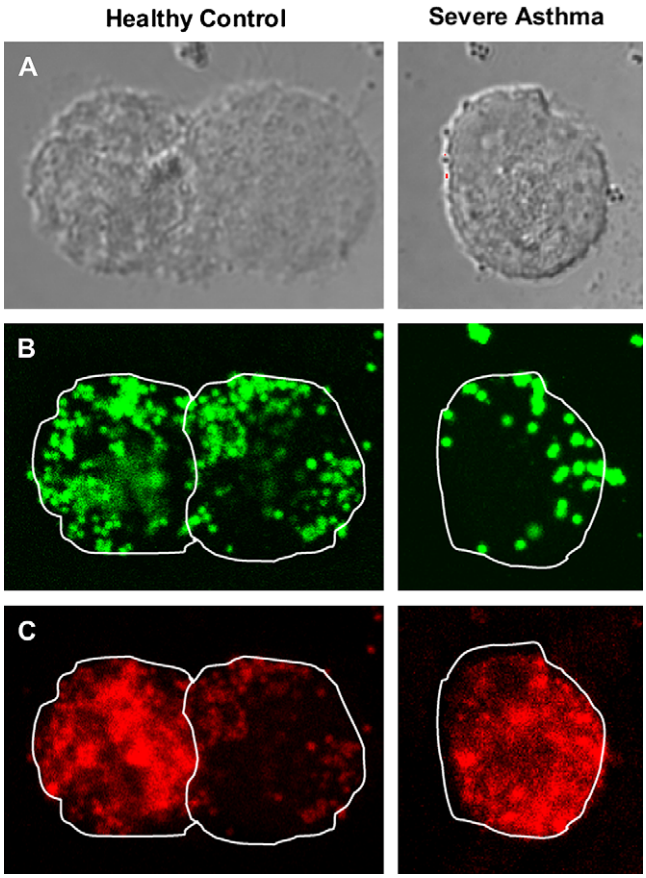

FIG E2. Representative cross-sectional confocal images of AMs from a child with severe asthma and a healthy adult control subject depicting phagocytosis of *S aureus* (*green particles*) despite cellular apoptosis (*red staining*). Images were taken at 50% of the cell depth.

TABLE E1. Results of the stepwise forward linear regression of the PI in the unstimulated and stimulated states on selected clinical features

Model	Regression coefficient	SE	*t*	*P* value
Unstimulated PI				
Included variables				
Constant	9805.02	1216.33	8.061	.000
Severity*	−2154.29	590.61	−3.648	.001
Excluded variables				
Age	−0.37		−1.623	.117
Hospitalization	0.04		0.154	.879
FEV_1/FVC ratio	−0.03		−0.126	.901
Log FE_{NO}	−0.19		−1.078	.291
Daily ICS dose	−0.14		−0.462	.648
Abnormal chest radiograph	−0.01		−0.029	.977
LPS-stimulated phagocytosis				
Included variables				
Constant	9956.42	1272.54	7.824	.000
Severity†	−2365.03	605.08	−3.909	.001
Excluded variables				
Age	−0.14		−0.609	.548
Hospitalization	−0.03		−0.091	.929
FEV_1/FVC ratio	0.08		0.410	.685
Log FE_{NO}	−0.17		−0.871	.393
Daily ICS dose	−0.21		−0.718	.480
Abnormal chest radiograph	0.25		0.705	.488

FVC, Forced vital capacity.
*Sum of squares for severity = $1.9 \times 10^8/3.8 \times 10^8$, $R^2 = 0.330$; 95% CI for the regression coefficient = −3366 to −942.
†Sum of squares for severity = $2.1 \times 10^8/3.3 \times 10^8$, $R^2 = 0.389$; 95% CI for the regression coefficient = −3614 to −1116.