The Effects of Airway Microbiome on Corticosteroid Responsiveness in Asthma

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Rationale: **The role of airway microbiome in corticosteroid response in asthma is unknown.**

Objectives: **To examine airway microbiome composition in patients with corticosteroid-resistant (CR) asthma and compare it with patients with corticosteroid-sensitive (CS) asthma and normal control subjects and explore whether bacteria in the airways of subjects with asthma may direct alterations in cellular responses to corticosteroids.**

Methods: **16S rRNA gene sequencing was performed on bronchoalveolar lavage (BAL) samples of 39 subjects with asthma and 12 healthy control subjects. In subjects with asthma, corticosteroid responsiveness was characterized, BAL macrophages were stimulated with pathogenic versus commensal microorganisms, and analyzed by real-time polymerase chain reaction for the expression of corticosteroid-regulated genes and cellular p38 mitogen-activated protein kinase (MAPK) activation.**

Measurements and Main Results: **Of the 39 subjects with asthma, 29 were CR and 10 were CS. BAL microbiome from subjects with CR and CS asthma did not differ in richness, evenness, diversity, and community composition at the phylum level, but did differ at the genus level, with distinct genus expansions in 14 subjects with CR asthma. Preincubation of asthmatic airway macrophages with *Haemophilus parainfluenzae*, a uniquely expanded potential pathogen found only in CR asthma airways, resulted in p38 MAPK activation, increased IL-8 ($P < 0.01$), mitogen-activated kinase phosphatase 1 mRNA ($P < 0.01$) expression, and inhibition of corticosteroid responses ($P < 0.05$). This was not observed after exposure to commensal bacterium *Prevotella melaninogenica*. Inhibition of transforming growth factor-β–associated kinase-1 (TAK1), upstream activator of MAPK, but not p38 MAPK restored cellular sensitivity to corticosteroids.**

Conclusions: **A subset of subjects with CR asthma demonstrates airway expansion of specific gram-negative bacteria, which trigger TAK1/MAPK activation and induce corticosteroid resistance. TAK1 inhibition restored cellular sensitivity to corticosteroids.**

Keywords: microbiome; asthma; corticosteroids

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

Culture-independent microbiota profiling based on sequence polymorphisms in the 16S rRNA present in all bacteria is widely applied in human microbiome studies. To date, limited knowledge about the airway microbiome and its role in health and disease is available.

What This Study Adds to the Field

This study provides evidence that a subset of subjects with corticosteroid-resistant asthma demonstrate expansion of specific gram-negative bacteria in the airways. Moreover, airway cell stimulation by bacteria in the airway microbiome can inhibit corticosteroid responses, potentially influencing the efficacy of corticosteroid treatment.

Current guidelines (1, 2) recommend the use of corticosteroids to control airway inflammation in persistent asthma. Clinical studies, however, demonstrate highly variable responses to corticosteroid therapy, with up to 45% of patients responding suboptimally to inhaled corticosteroids (3–5), and up to 25% of patients do not respond to oral corticosteroids (6). These corticosteroid-resistant (CR) patients demonstrate airway inflammation (6, 7) and remodeling (8), which contribute to the severe asthma phenotype often observed in these patients. For those patients with variable corticosteroid response, alternative treatment strategies are needed to improve asthma control (9).

Recently, 16S rRNA gene sequence analyses of airway specimens have demonstrated diversified microbial communities in the airways of subjects with asthma (10, 11). When compared with healthy patients, subjects with asthma manifest significantly higher bacterial burden and diversity caused by expansion of pathogenic bacteria. Moreover, airway microbiome composition and diversity correlate with bronchial hyperresponsiveness (11). The functional and pathobiologic impact of the airway microbiome in asthma remains unknown. In the current study, we hypothesized that expanded pathogenic microbial communities residing in the airways of subjects with CR asthma may inhibit cellular responses to corticosteroids.

Some of the results of these studies have been previously reported in the form of an abstract (12, 13).

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Author Contributions: E.G. aided in designing the research project; acquisition, analysis, and interpretation of data; and drafting and revising the article. L.P.J. performed *in vitro* experiments with bacteria, corticosteroids, and inhibitors. J.K.H. performed bacterial DNA sequencing analysis from bronchoalveolar lavage samples collected and assisted with sequencing data analysis. C.E.R. developed Explicet software used for microbiome data analysis in this manuscript and assisted with the sequencing data informatics. C.F.H. assisted with bacterial cultures. E.R.S., J.T.G., and R.J.M. performed bronchoscopies and assisted with clinical evaluation of subjects with asthma. E.W.G. assisted with patient characterization and revising of this manuscript. D.Y.M.L. aided in designing the research, interpretation of data, and drafting and revising the article.

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METHODS

Study Subjects

Thirty-nine adult subjects with asthma and 12 healthy control participants were enrolled. All subjects with asthma had baseline FEV_1 percent predicted less than or equal to 85% with either airway hyperresponsiveness (PC_{20} methacholine, <10 mg/ml) or bronchodilator responsiveness (>12% improvement in FEV_1% predicted after inhalation of 180 μg albuterol), as previously described (8, 14–16). The Juniper Asthma Control Questionnaire was used to assess asthma control. All subjects with asthma underwent bronchoscopy, followed by prednisone, 20 mg by mouth twice daily for 7 days. Subjects with asthma were categorized as corticosteroid sensitive (CS) if the FEV_1% predicted value improved greater than or equal to 15%, and as CR if the FEV_1% predicted value improved less than 10%. Healthy participants underwent bronchoscopy but did not receive prednisone. Smokers were excluded from participation in this study. Informed consent was obtained from all study participants before enrollment in this study. The Institutional Review Board at National Jewish Health approved this study. Details of patient characteristics are presented in Table 1.

Specimen Collection

Fiberoptic bronchoscopies with bronchoalveolar lavage (BAL) were performed according to the guidelines of the American Thoracic Society (17–19). BAL cells were prepared as described (14). BAL cell suspensions (250 μl containing 1×10^6 cells/ml) were preserved at $-80^{\circ}C$ before DNA extraction. Presence of LPS in the BAL fluid samples was analyzed by chromogenic limulus amebocyte lysate test as previously described (20).

BAL Differential Analysis

BAL differentials were obtained on cytospin preparations using a Diff-Quick (Scientific Products, McGraw Park, IL) stain, counting a minimum of 500 cells.

TABLE 1. PATIENT CHARACTERISTICS

	Normal Control Subjects (*n* = 12)	CR Asthma (*n* = 29)	CS Asthma (*n* = 10)
Age, yr (mean ± SD)	31.1 ± 9.2	34.2 ± 11.1	37.9 ± 10.8
Sex, male/female	4/8	14/15	2/8
Race, white/black/other	11/0/1	19/4/6	9/0/1
Body mass index, kg/m^2 (mean ± SD)	24.1 ± 4.7	26.3 ± 6.4	32.2 ± 8.5
IgE, U/ml (mean ± SD)	75 ± 108	253 ± 289	177 ± 211
Number of positive skin tests	0.1 ± 0.3	6 ± 4	6 ± 4
PC_{20}, methacholine, mg/ml (mean ± SD)	>25	1.5 ± 2.2	0.5 ± 0.6
ACQ score (mean ± SD)	NA	1.5 ± 0.8	1.9 ± 0.6
eNO, ppm (mean ± SD)	NA	34.9 ± 24.8	47.3 ± 29.2
Baseline FEV_1% predicted (mean ± SD)	98.7 ± 11.7	76.3 ± 10.3	61.9 ± 16.1
FEV_1% reversal with albuterol (mean ± SD)	5.1 ± 2.4	15.0 ± 9.4	37.2 ± 24.7
FEV_1% change after prednisone burst (mean ± SD)	NA	−0.2 ± 5.9*	31.0 ± 23.5
Corticosteroid medications†			
ICS/LABA	NA	5	4
ICS		7	0
None		17	6

Definition of abbreviations: ACQ = Asthma Control Questionnaire; CR = corticosteroid resistant; CS = corticosteroid sensitive; eNO = exhaled nitric oxide; ICS = inhaled corticosteroids; LABA = long-acting β-agonists.

* $P < 0.0001$ as compared with subjects with CS asthma.

† For the subjects with CR and CS asthma that received ICS/LABA or ICS, the mean ± SD of the ICS dose in budesonide equivalents was 837 ± 713 μg and 1,450 ± 1,034 μg, respectively.

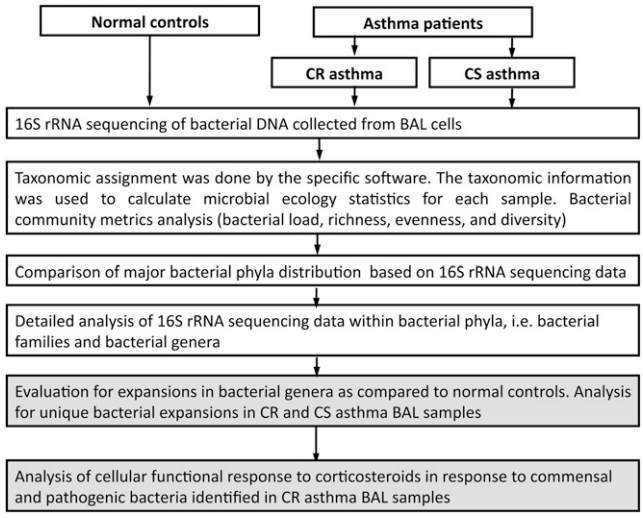

Figure 1. A schematic diagram of the analysis approaches for the bronchoalveolar lavage (BAL) 16S rRNA sequencing data to characterize airway microbiome of corticosteroid-resistant (CR) and corticosteroid-sensitive (CS) subjects with asthma.

Bacterial DNA Extraction, Quantitative Polymerase Chain Reaction, and Sequencing

Details of bacterial DNA preparation from BAL samples, sequencing, and sequencing data analysis are presented in the online supplement.

Bacterial Cultures

Haemophilus parainfluenzae (ATCC number 9796) and *Prevotella melaninogenica* (ATCC number 25845) were obtained from ATCC (Manassas, VA) and cultured according to ATCC instructions (*see* online supplement).

Cell Cultures

In a separate group of subjects with asthma (n = 7) (*see* Table E1 in the online supplement) BAL and peripheral blood monocytes were collected to examine functional interaction with bacteria. Details of the cells culture conditions and bacterial stimulation are shown in the online supplement.

The effect of bacteria on p38 mitogen-activated protein kinase (MAPK) activation and cellular response to corticosteroids and the influence of selected pathway inhibitors on corticosteroid responses were assessed (*see* online supplement). Statistical data analyses are described in the METHODS section of the online supplement.

RESULTS

Clinical Characteristics

In the current study, we recruited 39 subjects with asthma and 12 healthy control subjects for lung microbiome evaluation. Study subject characteristics are summarized in Table 1. Subjects with asthma were divided into CR and CS groups based on FEV_1% predicted responses after a 1-week burst with oral prednisone. Both groups had comparable baseline FEV_1% predicted. Patients in the CR group did not show any improvement in FEV_1 after exposure to prednisone; in contrast, patients in the CS group showed significant improvement in lung function after steroid burst (ΔFEV_1% after oral prednisone burst was −0.2 ± 5.9% and 31.0 ± 23.5% for subjects with CR and CS asthma, respectively; $P < 0.0001$) (Table 1).

BAL sample differentials are presented in Table E2. BAL samples consisted mostly of macrophages (>80%), with significantly

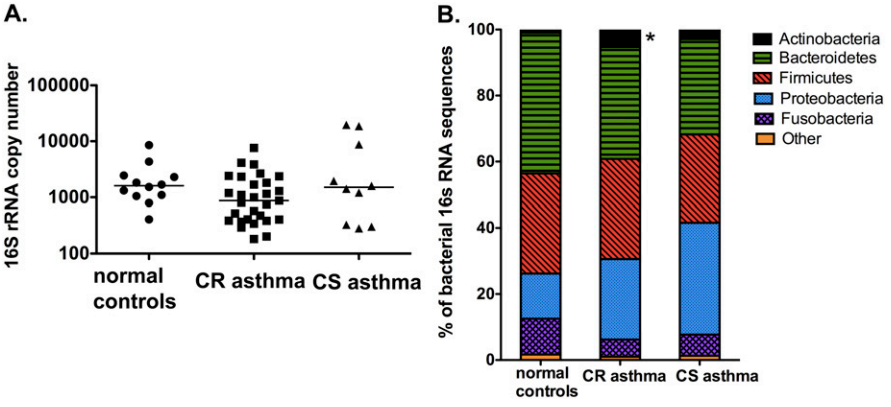

Figure 2. Bacterial load and composition of the airway microbiome based on 16S rRNA sequencing of the bacterial DNA isolated from bronchoalveolar lavage samples from normal control subjects, and corticosteroid-resistant (CR) and corticosteroid-sensitive (CS) subjects with asthma. (*A*) Bacterial load based on 16S RNA copy number in bronchoalveolar lavage samples from normal control subjects, and patients with CR and CS asthma. (*B*) The taxonomic composition of the airway microbiome in normal control subjects, and subjects with CR and CS asthma. A mean % of sequences for the major bacterial taxonomic groups is presented. The following phyla are shown: Actinobacteria, Bacteroidetes, Firmicutes, Proteobacteria, and Fusobacteria. The remaining microorganisms that do not belong to these phyla are presented as "Other." *$P < 0.05$ as compared to normal control.

higher levels of eosinophils in subjects with asthma as compared with normal control subjects ($P < 0.01$) (*see* Table E2). No significant differences were noted in the number of macrophages, lymphocytes, and neutrophils in BAL samples between groups (*see* Table E2).

Airway Microbiome in Subjects with Asthma

Bacterial 16S rRNA sequencing of BAL from 29 subjects with CR asthma, 10 subjects with CS asthma, and 12 control subjects was performed. Figure 1 summarizes the strategic approach for analysis of the BAL microbiome, which involved (1) bacterial community metrics analysis (bacterial load, richness, evenness, and diversity); (2) overall comparison of major bacterial phylum distribution between subjects with CR asthma, subjects with CS asthma, and normal control subjects; (3) detailed analysis of 16S rRNA sequencing data within bacterial phyla (i.e., bacterial families and bacterial genera); and (4) evaluation for expansions in bacterial genera as compared with normal control subjects and analysis for unique bacterial expansions in CR and CS asthma. Step 4 was introduced into analysis, because significant variations in the percentage of sequences for different bacterial genera within CR and CS asthma groups were noted. 16S rRNA sequencing data obtained from BAL samples from normal control subjects were used to establish the ranges for normal distribution of the bacterial genera in the airways. Microbial genera were considered as expanded in asthmatic airway microbiome if they represented more than 5% of the total 16S rRNA sequences and the percentage of sequences was increased at least twofold over control subjects for the genera present both in the airways of subjects with asthma and normal control subjects; for the genera found only in subjects with asthma they were considered as expanded if they represented more than 5% of the total 16S rRNA sequences. Bacterial expansion was considered as unique if the bacterial expansion was only found in one group of patients with asthma (only subjects with CR asthma or only subjects with CS asthma), but not the other group of subjects with asthma.

No significant differences in airway bacterial load were observed between subjects with asthma and normal control subjects as assessed by 16S rRNA copy number (Figure 2A). The medium (range) number of sequences was 727 (335–941), 1,127 (167–2,821), and 1,475.5 (397–2,777) for BAL samples from normal control subjects and patients with CR and CS asthma, respectively, represented by 24 (18–40), 36 (11–47), and 28 (19–49) bacterial genera, respectively. No significant differences in the microbiome richness, evenness, and diversity between normal control subjects and subjects with CR and CS asthma were observed (Table 2).

We first compared main bacterial phylum composition of BAL samples of normal control subjects and patients with CR and CS asthma. No significant difference in bacterial phylum composition was noted between normal control subjects and subjects with CR and CS asthma (Figure 2B; *see* Table E3). Patients with CR asthma had significantly greater number of sequences of phylum Actinobacteria as compared with normal control subjects ($P < 0.05$). Differences in Proteobacteria phylum composition in subjects with CR asthma as compared with normal control subjects were observed (*see* Figure E1). The summaries of BAL samples bacterial composition at bacterial family and bacterial genus levels are shown in Tables E4 and E5, respectively. Again, no significant differences in BAL bacterial composition between subjects with CR and CS asthma were observed at bacterial family and bacterial genus level.

However, significant variations in the percentage of sequences for different bacterial genera within CR and CS asthma groups were noted, with some patients demonstrating either expansions or reductions in the number of sequences. This prompted us to further subgroup subjects with asthma based on bacterial expansions or lack of bacterial expansions. A total of 33 out of 39 subjects with asthma studied had expansions of specific groups of microorganisms. Subjects with CR asthma with microbial expansions (24 of 29 patients) had significantly increased proportion of sequences of microorganisms in phyla Actinobacteria ($P < 0.001$) and Proteobacteria ($P < 0.05$), and significantly reduced number of sequences for genera *Prevotella* ($P < 0.05$) and *Veillonella* ($P < 0.05$) and phylum

TABLE 2. BACTERIAL DIVERSITY IN BAL SAMPLES FROM NORMAL CONTROL SUBJECTS, AND SUBJECTS WITH CR AND CS ASTHMA

Alpha diversity metric	Normal Control Subjects ($n = 12$)	CR Asthma ($n = 29$)	CS Asthma ($n = 10$)
Good's coverage, %	97.4 (95.1–98.6)	95.8 (93.1–98.6)	97.0 (93.0–98.4)
Richness (Sobs)	14.7 (99.5–25.3)	16.8 (12.3–28.4)	16.8 (12.3–28.4)
Estimated richness (Chao1)	19.2 (12.3–34.1)	27.4 (11.0–42.0)	21.8 (14.6–41.4)
Shannon diversity index	2.8 (1.7–3.8)	2.7 (1.7–3.9)	2.9 (2.3–3.7)
Shannon evenness	0.71 (0.44–0.83)	0.68 (0.51–0.80)	0.74 (0.52–0.80)

Definition of abbreviations: BAL = bronchoalveolar lavage; CR = corticosteroid resistant; CS = corticosteroid sensitive; Sobs = sequences observed.

Data are given as median (range).

Fusobacteria ($P < 0.05$) as compared with normal control subjects (Figure 3). Subjects with CS asthma with bacterial expansions (9 of 10 patients) had significant increased proportion of sequences for bacteria from phylum Proteobacteria ($P < 0.05$) and significantly reduced number of sequences for genera *Prevotella* ($P < 0.05$) and *Veillonella* ($P < 0.001$) (Figure 3).

Subjects with asthma without bacterial expansion (n = 6; five patients with CR asthma and one patient with CS asthma) did not vary significantly from normal control subjects based on bacterial phylum distribution, but had a significantly reduced bacterial diversity and bacteria evenness compared with normal control subjects ($P < 0.05$ and $P < 0.05$) and subjects with asthma with bacterial expansions ($P < 0.001$ and $P < 0.01$) (data not shown). No difference in bacterial evenness was observed between normal control subjects and subjects with asthma with bacterial expansions (data not shown).

Unique Bacterial Genus Expansions in the Airways of CR and CS Subjects with Asthma

Fourteen of 29 subjects with CR asthma had unique bacterial expansions in the airways that were not expanded in subjects with CS asthma (genera *Neisseria*, *Haemophilus*, *Simonsiella*, and *Campylobacter*, all phylum Proteobacteria; genus *Leptotrichia* [phylum Fusobacteria]; genus *Tropheryma* [phylum Actinobacteria]; and genera *Leuconostoc* and *Megasphaera* [phylum Firmicutes]) ($P < 0.01$ as compared with patients with CS asthma) (Table 3). Four out of 10 patients with CS asthma had bacterial expansions not present in subjects with CR asthma (genera *Bradyrhizobium*, *Aquabacterium*, *Limnobacter*, and *Pasteurella*, all phylum Proteobacteria; genus *Fusobacterium* [phylum Fusobacteria]; and genus *Streptophyta* [phylum Cyanobacteria]) (Table 4) ($P < 0.01$ as compared with patients with CR asthma).

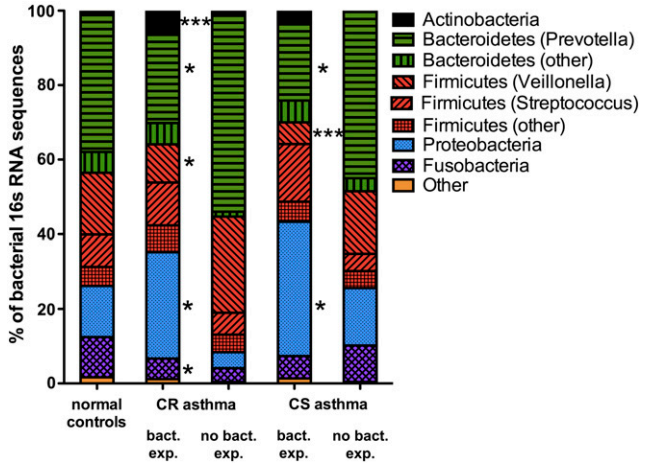

Figure 3. The taxonomic composition of the airway microbiome in corticosteroid-resistant (CR) and corticosteroid-sensitive (CS) subjects with asthma with (bact. exp.) and without (no bact. exp.) bacterial expansions. Microbial genera were considered as expanded in asthmatic airway microbiome if they represented more than 5% of the total 16S rRNA sequences and the % of sequences was increased at least twofold over control subjects for the genera present both in the airways of subjects with asthma and normal control subjects; for the genera found only in subjects with asthma they were considered as expanded if they represented more than 5% of the total 16S rRNA sequences. Twenty-four subjects with CR asthma and nine subjects with CS asthma were found to have bacterial expansions as compared with normal control subjects; five CR subjects with asthma and one CS subject with asthma did not have bacterial expansions. A mean % sequences of major bacterial phyla per group is shown. *$P < 0.05$, ***$P < 0.001$ as compared to normal control.

TABLE 3. UNIQUE MICROORGANISMS EXPANDED IN THE AIRWAYS OF PATIENTS WITH CR ASTHMA*

Types of organisms		Number of Patients with Bacterial Expansions (*n*)		Expanded Microorganisms Present in the Airways of Normal Control Subjects, Y/N (*Mean % Sequences*)
		CR Asthma†‡	CS Asthma	
Phylum	Genus			
Actinobacteria		**1**	**0**	
	Tropheryma	1	0	N
Firmicutes		**2**	**0**	
	Leuconostoc	1	0	N
	Megasphaera	1	0	Y (1.2%)
Fusobacteria		**4**	**0**	
	Leptotrichia	4	0	Y (4.2%)
Proteobacteria		**9**	**0**	
β-Proteobacteria	*Neisseria*	5	0	Y (6.0%)
	Simonsiella	1	0	Y (0.5%)
γ-Proteobacteria	*Haemophilus*	2	0	Y (2.9%)
ε-Proteobacteria	*Campylobacter*	1	0	Y (3.2%)

Definition of abbreviations: CR = corticosteroid resistant; CS = corticosteroid sensitive; N = no; Y = yes.

* Genera were considered as expanded in the asthma airway microbiome if they represented more than 5% of the total 16S rRNA sequences in the sample and the % of sequences representing these genera were increased at least twofold over mean % of sequences for the corresponding genera in normal control subjects; for the genera found only in patients with asthma they were considered expanded if they represented more than 5% of the total 16S rRNA sequences.

† Number of patients with CR asthma with unique bacterial expansions for each bacterial phylum is shown in bold font. Some subjects with asthma had expansions of several bacteria simultaneously; therefore, the sum for the individual expanded bacterial genera (n = 16) does not sum up to the number of patients with expansions (n = 14).

‡ Fourteen CR patients had unique bacterial expansions not found in patients with CS asthma ($P < 0.01$).

The distinct bacteria expanded in the airways of these subjects with CR asthma were mainly gram-negative organisms (phyla Proteobacteria and Fusobacteria and genus *Tropheryma* [phylum Actinobacteria]) (Table 3). Significantly higher levels of LPS ($P < 0.05$) were detected in the BAL fluid of these patients (*see* Figure E2A). BAL cells from these participants expressed significantly higher IL-8 mRNA levels ($P < 0.05$ compared with subjects with CR asthma with no bacterial expansions or subjects with CR asthma with gram-negative bacteria expansions that were common between subjects with CR and CS asthma) (*see* Figure E2B).

Effects of Bacteria from the Airways on Cell Activation and Response to Corticosteroids in Airway Macrophages

Haemophilus parainfluenzae (phylum Proteobacteria), one of the organisms identified as uniquely expanded in the airways of subjects with CR asthma, was used to examine the effects of this microorganism on cellular responses to corticosteroids. The effects of *H. parainfluenzae* were compared with the airway commensal organism, *P. melaninogenica*. For these experiments, a separate group of subjects with asthma (*see* Table E1) was recruited to collect BAL and peripheral blood monocytes to examine functional interaction with bacteria. Because

TABLE 4. UNIQUE MICROORGANISMS EXPANDED IN THE AIRWAYS OF PATIENTS WITH CS ASTHMA*

Types of organisms		Number of Patients with Bacterial Expansions (*n*)		Expanded Microorganisms Present in the Airways of Normal Control Subjects, Y/N (*% Sequences*)
		CR Asthma	CS Asthma†‡	
Phylum	Genus			
Cyanobacteria		**0**	**1**	
	Streptophyta	0	1	Y (0.4%)
Fusobacteria		**0**	**1**	
	Fusobacterium	0	1	Y (6.3%)
Proteobacteria		**0**	**4**	
α-Proteobacteria	*Bradyrhizobium*	0	1	N
β-Proteobacteria	*Aquabacterium*	0	1	N
	Limnobacter	0	1	N
γ-Proteobacteria	*Pasteurella*	0	1	Y (0.6%)

Definition of abbreviations: CR = corticosteroid resistant; CS = corticosteroid sensitive; N = no; Y = yes.

*Genera were considered as expanded in the asthma airway microbiome if they represented more than 5% of the total 16S rRNA sequences in the sample and the % of sequences representing these genera were increased at least twofold over mean % of sequences for the corresponding genera in normal control subjects; for the genera found only in patients with asthma they were considered expanded if they represented more than 5% of the total 16S rRNA sequences.

† Number of patients with CS asthma with unique bacterial expansions for each bacterial phylum is shown in bold font. Some subjects with asthma had expansions of several bacteria simultaneously; therefore, the sum for the individual expanded bacterial genera (n = 6) does not sum up to the number of patients with expansions (n = 4).

‡ Four patients with CS asthma had unique bacterial expansions not found in patients with CS asthma ($P < 0.01$).

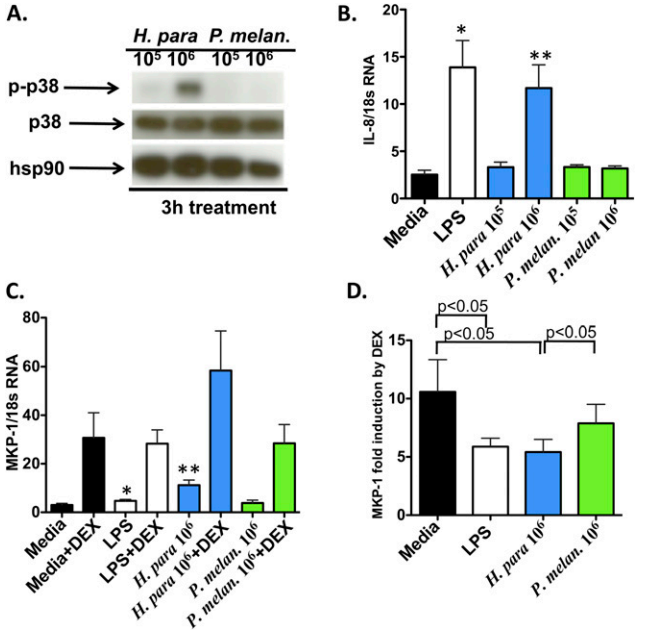

Figure 4. Effects of bacteria from the airways of subjects with asthma on bronchoalveolar lavage (BAL) macrophages activation and response to corticosteroids *in vitro*. Incubation of asthmatic BAL macrophages with *Haemophilus parainfluenzae* (*H. para*) results in p38 mitogen-activated protein kinase activation in the cells as detected by Western blot (*A*), up-regulation of IL-8 (*B*) and mitogen-activated kinase phosphatase 1 (MKP-1) mRNA production (*C*), and reduced responsiveness to corticosteroids *in vitro* (*D*) as shown by real-time polymerase chain reaction. Cells cultured with airway commensal organism *Prevotella melaninogenica* (*P. melan*) do not activate p38, do not up-regulate IL-8 mRNA and MKP-1 mRNA expression, and remain sensitive to corticosteroid treatment. (*B–D*) For IL-8 mRNA and MKP-1 mRNA production the cells were cultured overnight in X-Vivo 15 medium, incubated with bacteria for 15 minutes followed by 3 hours of treatment with 10^{-6}M dexamethasone (DEX) or medium, and analyzed by real-time polymerase chain reaction. 0.25×10^6 cells was used per condition (bacterium to cell ratio 0.1:1 and 1:1). The amount of bacteria shown was calculated per 1×10^6 cells/ml per condition. The responses of BAL macrophages from five subjects with asthma were examined. *$P < 0.05$, **$P < 0.01$ as compared with medium-treated cells.

the numbers of BAL macrophages from subjects with asthma were limited, we initially assessed interactions of these microbes with peripheral blood monocytes (*see* online supplement), and then used established protocols to assess BAL macrophages interactions with these bacteria. As shown in Figure E3, *H. parainfluenzae*, but not *P. melaninogenica*, induced a dose-dependent and time-dependent activation of the p38 MAPK pathway, which persisted even after 3 hours since addition of *H. parainfluenzae* (*see* Figure E3). Cells cultured with *H. parainfluenzae* had significantly reduced responses to corticosteroids (*see* Figure E3).

P38 MAPK activation in asthmatic BAL macrophages was only observed in response to *H. parainfluenzae*, but not *P. melaninogenica*, after 3 hours of stimulation (Figure 4A). *H. parainfluenzae*, but not *P. melaninogenica*, significantly up-regulated IL-8 mRNA (Figure 4B) and mitogen-activated kinase phosphatase 1 (MKP-1) mRNA (Figure 4C) expression in BAL macrophages. In the presence of *H. parainfluenzae* MKP-1 induction by dexamethasone (DEX) was significantly reduced (Figure 4D) ($P < 0.05$). BAL cells remained steroid sensitive in the presence of *P. melaninogenica* (Figure 4D).

Effects of MAPK and Transforming Growth Factor-β–associated Kinase-1 Inhibitors on Cellular Responses to Corticosteroids in the Presence of *Haemophilus parainfluenzae*

Toll-like receptor (TLR) engagement by bacteria is a major pathway of cell activation by bacteria (21). Transforming growth factor-β–associated kinase-1 (TAK1) phosphorylation represents a key downstream TLR signaling branching point that control subjects' MAPK and nuclear factor-kB pathway activation in the cells (22). We examined the role of MAPK and TAK1 activation in the alteration of cellular response to corticosteroids by *H. parainfluenzae*.

Peripheral blood monocytes from subjects with asthma cultured in the presence of *H. parainfluenzae* had significant up-regulation of MKP-1 mRNA ($P < 0.05$) (Figure 5A) and IL-8 mRNA ($P < 0.05$) (Figure 5B) expression. Cells cultured with *H. parainfluenzae* had reduced responses to corticosteroids as shown by significant inhibition of MKP-1 mRNA induction by DEX (Figure 5C). Pretreatment of monocytes with the TAK1 inhibitor resulted in significant inhibition of MKP-1 mRNA (Figure 5A) and IL-8 mRNA (Figure 5B) induction by *H. parainfluenzae* and restoration of cellular sensitivity to corticosteroids *in vitro* (Figure 5C). P38 MAPK inhibitor and the combination of p38 MAPK/ERK/JNK inhibitors also reduced MKP-1 mRNA and IL-8 mRNA induction by *H. parainfluenzae*, but not as effectively as in the presence of the TAK1 inhibitor (Figures 5A and 5B). P38 and the combination of MAPK inhibitors failed to restore cellular sensitivity to corticosteroids in the

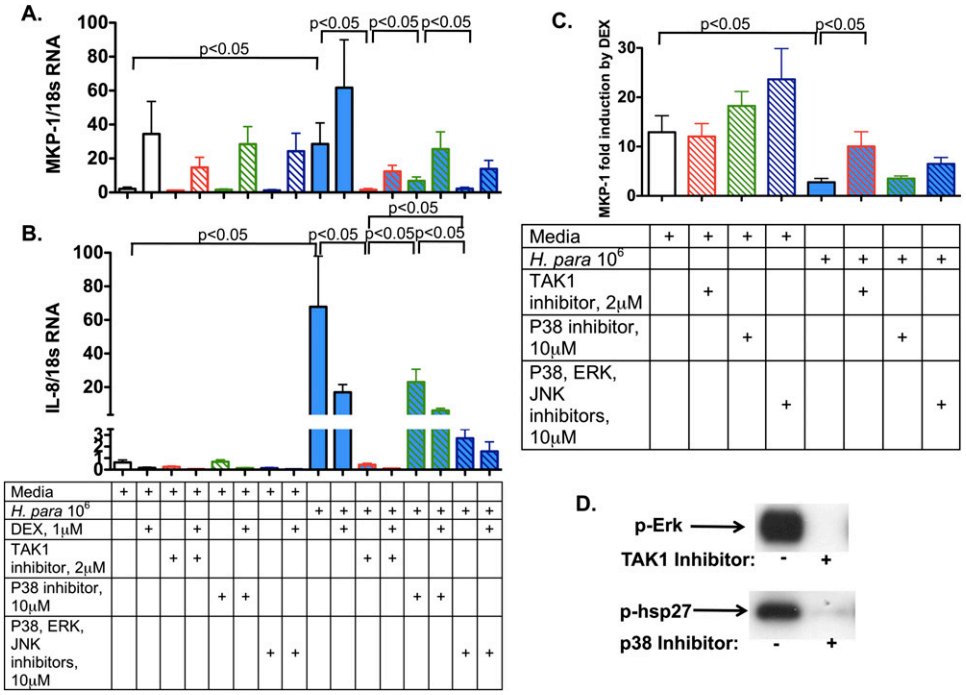

Figure 5. Influence of Toll-like receptor pathway inhibitors on cellular response to corticosteroids in the presence of bacteria. Pretreatment of asthmatic peripheral blood monocytes with transforming growth factor-β–associated kinase-1 (TAK1) but not p38 mitogen-activated protein kinase (MAPK) or p38 MAPK/ERK/JNK inhibitors results in significant inhibition of mitogen-activated kinase phosphatase 1 (MKP-1) mRNA (*A*) and IL-8 mRNA (*B*) induction by *Haemophilus parainfluenzae* (*H. para*) and restoration of cellular sensitivity to corticosteroids *in vitro* (*C*) as shown by real-time polymerase chain reaction. (*D*) A total of 2 μM TAK1 and 10 μM p38 inhibitors fully inhibited activation of downstream signaling targets in response *H. para*. Phosphorylation of ERK and hsp27 as a downstream read out targets for TAK1 and p38 MAPK activation, respectively, in response to 15 minutes of treatment of monocytes from subjects with asthma with *H. para* (bacterium to cell ratio 1:1) with and without corresponding inhibitor is shown by Western blot. For IL-8 mRNA and MKP-1 mRNA production the cells were cultured overnight in X-Vivo 15 medium, incubated with inhibitors for 1 hour, stimulated with bacteria for 15 minutes followed by 3 hours of treatment with 10^{-6} M dexamethasone (DEX) or medium, and analyzed by real-time polymerase chain reaction. Bacteria were added to 0.5×10^6 cells per condition (bacterium to cell ratio 1:1). The responses of monocytes from four subjects with asthma were examined.

presence of *H. parainfluenzae* (Figure 5C). As shown in Western blots the doses of the inhibitors used were sufficient to inhibit their respective targets as 2 μM TAK1 inhibitor and 10 μM p38 MAPK inhibitor fully suppressed activation of ERK and hsp27 phosphorylation by *H. parainfluenzae* as downstream read out targets for TAK1 and p38 MAPK activation, respectively (Figure 5D).

DISCUSSION

In the current study, we evaluated the lung microbiome in 39 subjects with asthma and 12 healthy control subjects. In our study, all subjects with asthma were defined clinically as CR or CS based on the change in lung function after 1 week of an oral prednisone burst. The two main objectives of the study were comparison of the airway microbiome composition in subjects with CR versus CS asthma, and examination of the effects of bacteria found in BAL from subjects with CR asthma on cellular response to corticosteroids.

Based on BAL microbiome data distribution in the major bacterial phyla, subjects with CR and CS asthma were divided into subgroups with bacterial expansions or lack of bacterial expansions. Most subjects with asthma in both CR and CS groups (24 out of 29 subjects with CR asthma and 9 out of 10 subjects with CS asthma) had bacterial expansions. Subjects with CR and CS asthma with bacterial expansions had significant alteration in their airway microbiome composition as compared with normal control subjects, but not compared with each other. The differences were only observed at the bacterial genus level, with distinct bacteria found expanded in 14 subjects with CR asthma, and these were not present in subjects with CS asthma ($P < 0.01$ as compared with subjects with CS asthma) (Table 3). Four subjects with CS asthma had unique bacterial genera expanded not found in patients with CR asthma ($P < 0.01$ as compared with subjects with CR asthma) (Table 4).

As compared with normal control subjects, subjects with CR and CS asthma with bacterial expansions had alterations in bacterial community structure as manifested by reduction of the airway commensals, genera *Prevotella* and *Veillonella*, reduction in phylum Fusobacteria and expansions of phyla Actinobacteria and Proteobacteria. The airway microbiome of subjects with asthma without bacterial expansion was also abnormal with less diversified microbial communities as compared with normal control subjects. We did not find any unique clinical features for this group that would distinguish them from other patients. Contrary to prior reports (11), we did not observe differences in bacterial load between samples from subjects with asthma and control subjects, suggesting that the microbial expansions in subjects with asthma reflected changes in the qualitative structure of the bacterial communities that reside in the airways of subjects with asthma. Also in contrast to previous reports (10, 11) no significant differences in the microbiome diversity between the subjects with asthma and control subjects were observed. Microbial expansions in subjects with asthma were distinct from previously reported alterations in airway microbiome in patients with chronic obstructive pulmonary disease (23–25).

At the genus level 14 out of 29 subjects with CR asthma had expansion of microbial genera that were expanded in the airways of none of the subjects with CS asthma. These microorganisms were mainly gram-negative LPS-producing bacteria with the short acyl chains lipid A LPS structures with known high endotoxic activity (Table 3) (26, 27). These patients had significantly higher IL-8 expression by BAL cells and significantly elevated levels of LPS in their BAL fluid, suggesting microbial stimulation. These data are in agreement with a previous report demonstrating induction of LPS signaling pathways in BAL cells from subjects with CR asthma (20).

Four out of 10 subjects with CS asthma had distinct microbial genera expanded not present in subjects with CR asthma. Among

five gram-negative organisms identified as uniquely expanded in subjects with CS asthma, *Bradyrhizobium* (28, 29), *Aquabacterium* (30), *Limnobacter* (31), and *Fusobacterium* (32) have long acyl chain lipid A and low endotoxic activity. We were unable to locate information about the structure of the LPS produced by *Pasteurella*.

In this study we used limulus amebocyte lysate assay to examine LPS levels in BAL fluid. However, limulus amebocyte lysate assay cannot distinguish the differences in LPS structures among various gram-negative microorganisms. Additional methods, like mass spectrometry (33, 34), are more suited for these purposes to further characterize the differences in LPS structure. Our laboratory previously used mass spectrometry for LPS characterization in BAL fluid and reported higher levels of LPS in CR than CS subjects with asthma (20).

To date, functional interactions of the microbiome with BAL macrophages, and its effects on cellular response to corticosteroids have not been investigated. We explored whether bacteria in the airway of subjects with CR asthma direct alterations in cellular responsiveness to corticosteroids. The effects of bacteria found to be uniquely expanded in CS asthma airways were not studied further. We chose one of the microorganisms from the Proteobacteria phylum that was uniquely expanded in the airways of subjects with CR asthma (*H. parainfluenzae*) and a commensal microorganism (*P. melaninogenica*) to examine functional responses to these bacteria in the presence of DEX. We introduced MKP-1 and IL-8 as gene targets to assess cellular responsiveness to corticosteroids in the presence of bacteria *in vitro*. Alterations in DEX suppression of these targets in the presence of bacteria were used as a read-out of cellular sensitivity to corticosteroids. High levels of p38 MAPK activation and reduced cellular responses to corticosteroids were observed in both peripheral blood monocytes and BAL macrophages in the presence of *H. parainfluenzae*, but not *P. melaninogenica*. The absolute increase in MKP-1 mRNA was much greater in cells incubated with *H. parainfluenzae* than with media or *P. melaninogenica*. An increase in MKP-1 mRNA expression is one of the cellular regulatory mechanisms that inhibits p38 MAPK activation, and reduces the inflammatory response (35, 36). However, this MKP-1 induction seemed to be insufficient. As shown in Figure E3, p38 MAPK activation persisted after 3 hours of treatment with *H. parainfluenzae*, and at the same time the cells expressed high IL-8 mRNA levels. Based on the data on the cellular responses to *H. parainfluenzae* versus *P. melaninogenica*, we suggest that the regulation of these two targets by DEX should be assessed concurrently, as likely both absolute levels of induction of these targets by bacteria and a proportional increase (for MKP-1) or decrease (for IL-8) by DEX are critical to assess the efficacy of corticosteroid responses in each setting.

The experiments suggest intrinsic differences in cell-stimulating properties between commensal versus expanded pathogenic bacteria associated with CR asthma airway microbiota. We hypothesize that the difference in LPS composition between LPS-producing commensal and pathogenic microorganisms in the airways (27, 37–39) accounts for airway cell activation only by pathogenic bacteria and results in altered cellular response to corticosteroids. Of note, microorganisms found to be uniquely expanded in CS asthmatic airways were also long acyl chain lipid A producers. We have studied the effects of commercial long acyl chain bacterial LPS on monocyte responses to corticosteroids and found that they are not immunostimulatory and do not subvert cellular responses

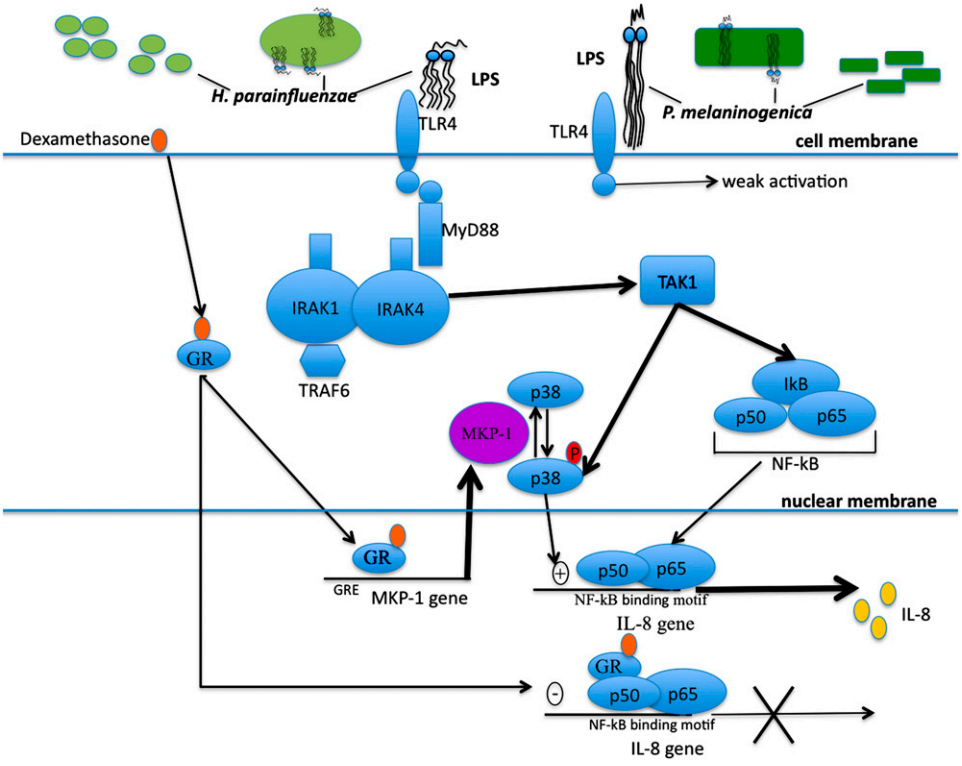

Figure 6. Proposed schematic diagram of monocyte-macrophage activation and cellular response to corticosteroids in the presence of *Haemophilus parainfluenzae* and *Prevotella melaninogenica*. *H. parainfluenzae* short length acyl chains lipid A LPS interacts with Toll-like receptor (TLR) 4 and activates transforming growth factor-β–associated kinase-1 (TAK1) by MyD88 pathway, resulting in p38 mitogen-activated protein kinase phosphorylation and nuclear factor-κB (NF-κB) activation, which activate transcription of the proinflammatory cytokines like IL-8 (21, 26, 27, 53). On the contrary, *P. melaninogenica* long acyl chains lipid A LPS is a poor agonist for TLR4 (54). On interaction cytoplasmic glucocorticoid receptor (GR) with dexamethasone receptor translocates to the cell nuclei and activates mitogen-activated kinase phosphatase 1 (MKP-1) mRNA production. MKP-1 dephosphorylates activated p38 mitogen-activated protein kinase. GR interacts with NF-κB and inhibits IL-8 transcription. Monocyte-macrophage activation by *H. parainfluenzae* results in reduced cellular responses to corticosteroids. TAK1 activation by *H. parainfluenzae* inhibits GR-mediated MKP-1 production and suppresses GR inhibition of NF-κB–induced IL-8 production. The cells remain corticosteroid sensitive in the presence of *P. melaninogenica*.

to corticosteroids (data not shown). Figure 6 presents the proposed schematic diagram of monocyte/macrophage activation and cellular response to corticosteroids in the presence of *H. parainfluenzae* versus *P. melaninogenica*.

Bacterial alteration in airway macrophage responses to corticosteroids is of great importance, because macrophages are the major cell type in the airway lumen, and produce an array of proinflammatory mediators that control responses of various cell types, including T cells and airway epithelium that are involved in host defense, airway inflammation, tissue remodeling, and repair (40–42). Lack of responses of pulmonary macrophages to corticosteroids leads to persistent airway inflammation in CR asthma (42). Corticosteroids are the most effective antiinflammatory drugs used in the treatment of eosinophilic disorders, including asthma (43). Failure of steroids to suppress macrophage responses in the presence of bacteria may result in failure to propagate apoptotic signals for eosinophils and may result in the deficiency of eosinophil apoptotic bodies clearance by macrophages (7, 43).

It is unclear whether expansions in Actinobacteria and Proteobacteria in BAL from subjects with CR and CS asthma resulted in reciprocal reductions in commensal organisms like *Prevotella* and *Veillonella*. It is possible that initial reduction in airway commensals resulted in overexpansion of other bacterial phyla. Recent literature suggests that commensal microbiota maintains and shapes normal mucosal immunity in the gut (44, 45). Similarly, it has been reported that *Staphylococcus epidermidis*, a commensal organism in the skin, can protect the host from development of injurious inflammation by tolerizing the response by TLR (46). By analogy, it is possible that commensal microbiota in the airways is protective from development of inflammatory responses, and loss of commensal organisms allows cellular inflammatory response. Restoration of the commensal microbiota in the airways of subjects with asthma should be evaluated for its protective role and alleviation of cellular steroid response in the airways of subjects with asthma.

Persistent infection and colonization with pathogenic bacteria may be difficult to eradicate with antibiotic treatment (47), therefore alternative approaches are needed. In this study, we evaluated whether inhibition of TAK1 and MAPK activation by bacteria via TLR by selective inhibitors would restore cellular responses to corticosteroids in the presence of pathogenic bacteria. We found that TAK1 inhibitor–treated cells were steroid sensitive despite incubation with *H. parainfluenzae*. MAPK inhibitors reduced IL-8 and MKP-1 mRNA induction by *H. parainfluenzae* but did not restore cellular sensitivity to corticosteroids in the presence of *H. parainfluenzae*. The data suggest that activation of TAK1 by bacteria is essential in alteration of cellular responses to corticosteroids.

Heterogeneity in asthma is well described, and prior studies have reported a wide variety of other factors that can contribute to corticosteroid insensitivity, such as obesity (48), allergen exposure (6, 15), vitamin D deficiency (49, 50), sex (51), and race (52). The novelty of our study is the identification of a distinct microbial trigger for CR asthma caused by unique mainly gram-negative expansions in the airways, and demonstration that these microorganisms can alter responses of airway macrophages to corticosteroids *ex vivo*.

In summary, we propose that airway cell stimulation by bacteria as a result of alterations in the airway microbiome composition and microbial expansions reduce cellular responses to corticosteroids and influences efficacy of corticosteroid treatment. TAK1 inhibition should be explored as a potential therapeutic pathway for modulation of corticosteroid responses in subjects with asthma with poor responses to corticosteroids as an alternative approach to their management.

Author disclosures are available with the text of this article at www.atsjournals.org.

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