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SHP-1 As a Critical Regulator of Mycoplasma pneumoniae-Induced Inflammation in Human Asthmatic Airway Epithelial Cells

Ying Wang,* Zhou Zhu,† Tony D. Church,* Njira L. Lugogo,* Loretta G. Que,* Dave Francisco,* Jennifer L. Ingram,* Molly Huggins,* Denise M. Beaver,* Jo Rae Wright,‡ and Monica Kraft*

Asthma is a chronic inflammatory disease in which airway epithelial cells are the first line of defense against exposure of the airway to infectious agents. Src homology protein (SHP)-1, a protein tyrosine phosphatase, is a negative regulator of signaling pathways that are critical to the development of asthma and host defense. We hypothesize that SHP-1 function is defective in asthma, contributing to the increased inflammatory response induced by Mycoplasma pneumoniae, a pathogen known to exacerbate asthma. M. pneumoniae significantly activated SHP-1 in airway epithelial cells collected from nonasthmatic subjects by bronchoscopy with airway brushing but not in cells from asthmatic subjects. In asthmatic airway epithelial cells, M. pneumoniae induced significant PI3K/Akt phosphorylation, NF-κB activation, and IL-8 production compared with nonasthmatic cells, which were reversed by SHP-1 overexpression. Conversely, SHP-1 knockdown significantly increased IL-8 production and PI3K/Akt and NF-κB activation in the setting of M. pneumoniae infection in nonasthmatic cells, but it did not exacerbate these three parameters already activated in asthmatic cells. Thus, SHP-1 plays a critical role in abrogating M. pneumoniae-induced IL-8 production in nonasthmatic airway epithelial cells through inhibition of PI3K/Akt and NF-κB activity, but it is defective in asthma, resulting in an enhanced inflammatory response to infection. The Journal of Immunology, 2012, 188: 3371–3381.

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Abbreviations used in this article: FEV₁, forced expiratory volume in 1 s; FVC, forced vital capacity; P-SHP-1, tyrosine phosphorylated Src homology protein 1; PVDF, polyvinylidene difluoride; rAAV, recombinant adeno-associated virus; SHP, Src homology protein; siRNA, small interfering RNA.

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We demonstrate that via dysregulation of TLR2-mediated proinflammatory pathways. M. pneumoniae associated with reduced ability to modulate inflammation following from well-characterized asthmatic subjects, dysfunction of SHP-1, production in asthmatic airway epithelial cells compared with non- mechanisms by which SHP-1 regulates TLR2-mediated proinflammatory cytokines in heterozygous SHP-1-deficient mice after OVA sensitization and challenge. After challenge with OVA, eosinophilic inflammation, mucus secretion, and airway hyperresponsiveness were enhanced compared with treatment with saline. Spontaneous Th2-like inflammatory responses in the lung were further shown in SHP-1-deficient mice, along with increased airway resistance (37).

SHP-1 was shown to play an important role in regulating oxidative stress, which has also been demonstrated in asthma (37). Increased intracellular oxidative stress and lack of SHP-1 in the presence of Th2-prone cellular activation may lead to the development of allergic airway inflammation. However, there is no report that links SHP-1 deficiency or dysfunction in cells or tissues directly with allergic airway disease in humans. Although the mechanism of SHP-1 in modulating inflammation has been studied in hematopoietic cells of mice, little is known about the molecular mechanisms of SHP-1 in the regulation of innate immunity of airway epithelial cells in human asthma, especially the mechanisms by which SHP-1 regulates TLR2-mediated proinflammatory cytokine production after M. pneumoniae infection.

In the current study, we hypothesize that, in airway epithelial cells from well-characterized asthmatic subjects, dysfunction of SHP-1, due to quantitative and functional deficiencies in the protein, is associated with reduced ability to modulate inflammation following M. pneumoniae infection. We propose that this dysfunction occurs via dysregulation of TLR2-mediated proinflammatory pathways. We demonstrate that M. pneumoniae significantly induced IL-8 production in asthmatic airway epithelial cells compared with nonasthmatic cells and that SHP-1 is critical in the regulation of this process. Defective activation of SHP-1 in asthma resulted in increased Akt and NF-κB activity, which dramatically increased IL-8 production. Thus, SHP-1 is a critical regulator of M. pneumoniae-induced inflammation in human asthmatic airway epithelial cells.

Materials and Methods

Subjects

Subjects were recruited via newspaper and radio advertisements from the general Research Triangle, North Carolina community. The asthmatic subjects fulfilled criteria for asthma (38), exhibiting a provocative concentration of methacholine resulting in a 20% decrease in the forced expiratory volume in 1 s (FEV1) < 80 ml/min per liter, as demonstrated by ≥12% increase in FEV1 or the forced vital capacity (FVC) with inhaled albuterol. Asthmatic subjects recruited used short-acting β2-agonists, as needed, and were not on controller medications. Exclusion criteria included the use of any controller therapy within the previous 4 wk, in-patient status, upper or lower respiratory tract infection within 3 mo of study, smoking history >5 pack-years or any cigarette use within the previous 2 y, significant nonasthma pulmonary disease, or other medical problems. Nonasthmatic subjects had no clinical history of atopy, were skin test negative, and took no medications. All subjects provided consent for this Institutional Review Board-approved protocol.

Bronchoscopy

Subjects underwent bronchoscopy with endobronchial-protected brushing and bronchoalveolar lavage, as previously described (11). The brushing of the proximal airways was performed under direct visualization using a separate protected cytologic brush for each pass, for a total of five passes. Bronchoalveolar lavage was performed via instillation of warm sterile saline in 60-ml aliquots, with return via gentle hand suction, for a total of 300 ml. Subjects were discharged when their FEV1 achieved 90% of their prebronchoscopy, postalbuterol value.

Airway epithelial cell culture

Freshly isolated airway epithelial cells from endobronchial brushing were cultured with BEGM (Lonza, Walkersville, MD), as previously described (11). After reaching confluence, cells were trypsinized and seeded onto collagen-coated polyether Transwell insert membranes of 12-mm diameter, at a concentration of 4 × 10^5/well. Then the cells were cultured at air-liquid interface and cultured for 2 wk to allow for differentiation.

M. pneumoniae culture and infection of cultured airway epithelial cells

M. pneumoniae strain 15531 (American Type Culture Collection, Manassas, VA) was inoculated in SP4 broth (Remel, Lenexa, KS) at 35°C until adherent. The concentration was determined by plating serial dilutions of M. pneumoniae on pleomorphic pneumonia-like organisms agar plates (Remel). CFU were counted after incubation for 14 d. Differentiated airway epithelial cells were infected on the apical surface by M. pneumoniae with a titer of 50 CFU/cell and incubated for 48 h. The concentration of 50 CFU/cell was chosen based on our previous work, in which a dose-response experiment was performed (11). Supernatant was collected for IL-8 measurement by ELISA (R&D Systems, Minneapolis, MN), and cytokines were collected and quantified by bead-based Luminex.

To determine M. pneumoniae amounts in the cultured airway epithelial cells from nonasthmatic and asthmatic subjects, cells infected or not with M. pneumoniae for 48 h were rinsed with PBS three times, and total RNA was extracted using TRIzol reagent (Sigma, St. Louis, MO). Reverse transcription was performed using 1 μg total RNA and random hexamers in a 50-μl reaction, according to the manufacturer’s protocol (Applied Biosystems, Branchburg, NJ). M. pneumoniae was quantified by RT-PCR with TaqMan gene-expression assays (Applied Biosystems) specifically for the mpn372 (Community Acquired Respiratory Distress Syndrome toxin) gene in mycoplasma (GenBank accession number DQ447750; http://www.ncbi.nlm.nih.gov/genbank/). Real-time PCR was performed on the Mx3005 sequence-detection system (Stratagene). Relative amounts of mycoplasma mpn372 present in airway epithelial cells from nonasthmatic and asthmatic subjects were determined based on values obtained from a standard curve for mycoplasma mpn372 and was normalized to the housekeeping gene GAPDH that was present in the airway epithelial cells.

Small interfering RNA-mediated SHP-1 gene silencing

The oligonucleotides encoding the specific small interfering RNA (siRNA) for SHP-1 (sense, 5'-CGGTTTCATGAAACCGCTTTTCCGCCGAGTATTCAACATGAACTCGGAGCT-3' and antisense 5'-CCAGTTGACCTGTTCCATGACTT-3') were delivered into primary cultured human airway epithelial cells by double-strand recombinant adeno-associated virus (rAAV) (39) serotype 6. Basic local alignment search tool searches confirmed that the selected oligonucleotide sequences were not homologous to any other genes. A scramble sequence of 5'-TC-GTCCAGTGTCACGTTCGAGGAGCTGCTGAAATGAC-3' was used to construct control rAAV as a negative control. Serotype 6 double-strand rAAVs were produced by the triple plasmid cotransfection method and purified by ammonium sulfate precipitation on cesium chloride gradients (40, 41). For rAAV transduction, airway epithelial cells from asthmatic and nonasthmatic subjects were seeded at a concentration of 4 × 10^5 cells/well in 12-well Transwell plates. Approximately 18 h after seeding, airway epithelial cells were transduced with rAAV carrying the SHP-1-siRNA-silencing cassette or full-length SHP-1 cDNA with a concentration of 0.3 multiplicity of infection. After reaching 100% confluence, the cells were cultured at an air-liquid interface for 2 wk. The culture medium was changed every other day.

Immunofluorescence staining and confocal laser scanning

Airway epithelial cells, which had been cultured at an air-liquid interface for 2 wk, were fixed with 3% paraformaldehyde, permeabilized with buffer containing 20 mM HEPES (pH 7.4), 0.3 M sucrose, 50 mM NaCl, 3 mM MgCl2, and 0.5% Triton X-100, and then incubated with mouse anti-human SHP-1 Ab (Santa Cruz Biotechnology, Santa Cruz, CA), followed by incubation with Dylight 488-labeled goat anti-mouse IgG (Jackson ImmunoResearch, West Grove, PA). Cell nuclei were detected by
Ab (IMGENEX) for 1 h at 4˚C, followed by incubation with 20 μl TLR2 Ab (IMGENEX) to determine the amount of TLR2 in the immunoprecipitated PVDF membrane was then stripped and reprobed with mouse anti-human SHP-1 to determine the amount of SHP-1 coimmunoprecipitated with TLR2 in the samples. The ratio of densitometry of P-SHP-1/SHP-1 was determined at each time point (0, 2, and 4 h) following mycoplasma infection.

Immunoblotting analysis

Following a time-course treatment of mycoplasma, airway epithelial cell lysates were lysed in ice-cold Pierce IP lysis buffer (Thermo Scientific, Rockford, IL). Lysates (500 μg/500 μl) were cleared with 1.0 μg normal rabbit IgG (Santa Cruz Biotechnology), together with 20 μl resuspended volume of Protein A/G PLUS-Agarose (Santa Cruz Biotechnology) for 30 min at 4˚C. Then the supernatants were incubated with 2 μg rabbit anti-human TLR2 Ab (IMGENEX) for 1 h at 4˚C, followed by incubation with 20 μl Protein A/G PLUS-Agarose overnight at 4˚C. The beads were precipitated by centrifugation steps (1000 × g for 5 min) and washed four times in PBS buffer. The immunoprecipitates were analyzed by SDS-PAGE and immunoblotting using mouse anti-human TLR2 Ab to confirm equal protein loading and quantified by densitometry. The ratio of densitometry of P-SHP-1/SHP-1 was determined at each time point (0, 2, and 4 h) following mycoplasma infection.

Immunoprecipitation analysis

Following a time-course treatment of mycoplasma, airway epithelial cell lysates were lysed in ice-cold Pierce IP lysis buffer (Thermo Scientific, Rockford, IL). Lysates (500 μg/500 μl) were cleared with 1.0 μg normal rabbit IgG (Santa Cruz Biotechnology), together with 20 μl resuspended volume of Protein A/G PLUS-Agarose (Santa Cruz Biotechnology) for 30 min at 4˚C. Then the supernatants were incubated with 2 μg rabbit anti-human TLR2 Ab (IMGENEX) overnight at 4˚C. After washes, the membranes were incubated with HRP-conjugated anti-IgG and developed by ECL, documented using the Kodak Image Station 4000 MM PRO, and quantified using Carestream Molecular Image software (version 5.0).

SHP-1 immunoprecipitation and Western blot

Airway epithelial cell lysates (500 μg) were immunoprecipitated with 2 μg mouse anti-human SHP-1 Ab (Santa Cruz Biotechnology). Immunoprecipitates were run on a 10% gel, transferred, and probed with HRP-conjugated mouse anti-phosphotyrosine Ab (4G10, Millipore, Temecula, CA) overnight to detect tyrosine phosphorylated SHP-1 (P-SHP-1). Immunoblots were stripped and reprobed with anti–SHP-1 Ab to confirm equal protein loading and quantified by densitometry. The ratio of densitometry of P-SHP-1/SHP-1 was determined at each time point (0, 2, and 4 h) following mycoplasma infection.

Coimmunoprecipitation analysis

Following a time-course treatment of mycoplasma, airway epithelial cell lysates were lysed in ice-cold Pierce IP lysis buffer (Thermo Scientific, Rockford, IL). Lysates (500 μg/500 μl) were cleared with 1.0 μg normal rabbit IgG (Santa Cruz Biotechnology), together with 20 μl resuspended volume of Protein A/G PLUS-Agarose (Santa Cruz Biotechnology) for 30 min at 4˚C. Then the supernatants were incubated with 2 μg rabbit anti-human TLR2 Ab (IMGENEX) overnight at 4˚C. The beads were precipitated by centrifugation steps (1000 × g for 5 min) and washed four times in PBS buffer. The immunoprecipitates were analyzed by SDS-PAGE and immunoblotting using mouse anti-human TLR2 Ab to determine the total amount of SHP-1 coimmunoprecipitated with TLR2 in the samples. The PVDF membrane was then stripped and reprobed with mouse anti-human TLR2 Ab (IMGENEX) to determine the amount of TLR2 in the immunoprecipitates and quantified by densitometry.

SHP-1 protein phosphatase activity assay

SHP-1 tyrosine phosphatase activity was measured with the use of the Tyrosine-Phosphatase Assay kit (Promega, Madison, WI), following the manufacturer’s protocol. Briefly, SHP-1 in airway epithelial cell lysate was immunoprecipitated from 500 μg protein, as described above. The immunoprecipitates were incubated with tyrosine phosphopeptides as substrate. Total phosphate content was determined by phosphate standards run in parallel. Phosphopeptide-specific activity was calculated by subtracting the nonspecific activity.

NF-κB DNA-binding activity assay and Western blot

NF-κB DNA-binding activity was determined using the TransAM NF-κB p65 transcription factor assay kit (Active Motif, Carlsbad, CA), following the manufacturer’s instructions, and as previously described (42). Briefly, nuclear extracts were prepared (43) from nonasthmatic and asthmatic airway epithelial cells that were infected with mycoplasma, as described above. Twenty micrograms of nuclear protein was incubated in a 96-well plate coated with oligonucleotide containing the NF-κB consensus-binding sequence 5′-GGGACTTTCC-3′. Bound NF-κB was then detected by a p65-specific primary Ab. An HRP-conjugated secondary Ab was then applied to detect the bound primary Ab, and colorimetric quantification was performed at 450 nm. Serial dilutions of purified p65 recombinant protein (20–0.16 ng) were used as a standard. To detect the cytoplasmic and nuclear NF-κB by Western blot, the cytoplasmic and nuclear proteins were extracted from airway epithelial cells from nonasthmatic and asthmatic subjects and resolved on 10% SDS-PAGE, followed by transfer to a PVDF membrane. The membranes were probed with a p65-specific primary Ab (Cell Signaling) overnight at 4˚C. The immunoblots were then incubated with HRP-conjugated secondary Abs, developed by ECL, and quantified by densitometry, as described above.

Statistical analysis

Statistical evaluation was performed using JMP statistical software (SAS, Cary, NC). Data are expressed as mean ± SE, because data were distributed normally. The Student t test or Wilcoxon test was used to evaluate group differences, depending on distribution of the data. Differences between groups with a p value < 0.05 were considered statistically significant.

Results

Subject characteristics

Subject characteristics are shown in Table I. The asthmatic subjects were considered to have mild disease by the National Asthma Education and Prevention Panel criteria and used short-acting β agonists as needed only (38). The nonasthmatic subjects had no history of atopy, smoking, or significant medical illness and were not on medications.

Localization of endogenous SHP-1 in human airway epithelial cells

SHP-1 is expressed predominantly in hematopoietic cells, although lower levels of expression have been observed in nonhematopoietic cells, such as HeLa cervical adenocarcinoma, A549 lung carcinoma, and MCF-7 mammary adenocarcinoma cell lines (44, 23). Previous studies demonstrated that SHP-1 localization differs dramatically between the nonhematopoietic and hematopoietic cell lines (45), with the SHP-1 protein being present almost entirely within the cytoplasm of hematopoietic cells and the nucleus of nonhematopoietic cells. To our knowledge, the localization of SHP-1 in human airway epithelial cells has not been reported. Immunostaining using an SHP-1–specific Ab revealed that endogenous SHP-1 in our primary cultured airway epithelial cells from nonasthmatic and asthmatic subjects was distributed mainly within the nucleus, with mild cytoplasm expression also present. There was no difference in the SHP-1 subcellular distribution between nonasthmatic and asthmatic cells (Fig. 1).

SHP-1 plays a critical role in regulating M. pneumoniae–induced inflammation in human airway epithelial cells

To determine whether SHP-1 plays any role in regulating M. pneumoniae–induced IL-8 production by airway epithelial cells, we performed siRNA-mediated gene silencing experiments to knock down SHP-1. In addition, airway epithelial cells were transduced with rAAV vector that contained full-length SHP-1 cDNA to induce SHP-1 overexpression. The efficacy of knockdown and overexpression were confirmed by immunoblot analyses (Fig. 2B). The level of SHP-2 protein was not affected in the epithelial cells in which SHP-1 was knocked down (data not shown). In addition, rAAV alone did not cause a nonspecific regulatory effect on IL-8 levels in either nonasthmatic or asthmatic airway epithelial cells (Supplemental Fig. 1).

Table I. Subject characteristics

<table>
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<th>Asthma (n = 12)</th>
<th>Normal (n = 10)</th>
<th>p Value</th>
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<td>4/6</td>
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<tr>
<td>Age (y)</td>
<td>31 ± 3</td>
<td>28 ± 4</td>
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<td>Albuterol</td>
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<td>None</td>
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<tr>
<td>FEV1 (l)</td>
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<td>3.33 ± 0.2</td>
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<td>FEV1 (% predicted)</td>
<td>88 ± 4.23</td>
<td>102 ± 4.81</td>
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<tr>
<td>FVC (l)</td>
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<td>4.51 ± 0.39</td>
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<tr>
<td>FVC (% predicted)</td>
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<td>105 ± 5</td>
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<td>FEV1/FVC</td>
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<td>79 ± 2.12</td>
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<td>Methacholine PC20mg/ml</td>
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<td>&gt;16</td>
<td>0.0001</td>
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*The provocative concentration of methacholine resulting in a 20% decrease in FEV1.*
within the nucleus (gene in mycoplasma, as described in Materials and Methods) formed real-time RT-PCR using primers specific for the epithelial cells from nonasthmatic and asthmatic subjects, we performed real-time RT-PCR using primers specific for the mnn 372 gene in mycoplasma, as described in Materials and Methods. Expression of the mnn372 gene was not detectable in either nonasthmatic or asthmatic airway epithelial cells, which had been cultured at an air–liquid interface for 2 wk prior to mycoplasma treatment. Forty-eight hours after M. pneumoniae infection, the mnn372 gene was detected, but there was no significant difference in its expression between nonasthmatic and asthmatic airway epithelial cells (mnn372/GAPDH: 0.61 ± 0.05 versus 0.53 ± 0.04 in the asthmatic and nonasthmatic groups, respectively, p = 0.45; n = 10 for nonasthma, n = 12 for asthma). However, following infection with M. pneumoniae, asthmatic airway epithelial cells produced significantly more IL-8 compared with the cells from nonasthmatic subjects (Fig. 2C), consistent with our previous study by Slevogt et al. (46). Interestingly, the amount of SHP-1 associating with TLR2 at 2 h after M. pneumoniae infection in nonasthmatic airway epithelial cells was significantly higher than that of asthmatic cells (Fig. 2B). No difference was observed in TLR2 protein levels between nonasthmatic and asthmatic cells at baseline (Fig. 2A) and after time-course treatment with mycoplasma (Fig. 2B, left panel). These data support the role of SHP-1 in regulating M. pneumoniae-induced IL-8 production, possibly through interaction with the TLR2-signaling pathway.

SHP-1 inhibits M. pneumoniae-induced IL-8 production in airway epithelial cells through PI3K and Akt

Phosphorylation of the kinase Akt at the serine residue of position 473 (Ser473) is absolutely dependent on PI3K activity (47). Thus, evaluation of phosphorylation of Akt Ser473 provides a surrogate measure of PI3K activity in intact cells. It is also well established that Akt is integrated into the activation process of NF-kB by multiple mechanisms (48–50). To further explore the molecular mechanism of SHP-1 in regulating M. pneumoniae-activated TLR2-signaling pathway in airway epithelial cells, we pretreated the asthmatic airway epithelial cells with an NF-kB inhibitor, a PI3K inhibitor (LY294002), or an Akt inhibitor for 30 min and then infected the cells with M. pneumoniae. PI3K/Akt and NF-kB inhibitors significantly decreased M. pneumoniae-induced IL-8 production in nonasthmatic and asthmatic airway epithelial cells with or without SHP-1 knockdown (Fig. 5A, 5C), suggesting that activation of the PI3K/Akt pathway contributes to M. pneumoniae-induced IL-8 production.

To further determine the role of SHP-1 in the regulation of mycoplasma-induced PI3K/Akt activation in airway epithelial cells, phosphorylated Akt was measured by Western blot. There was no difference in the baseline phosphorylated Akt (Fig. 5B, left panel) between nonasthmatic and asthmatic airway epithelial cells prior to mycoplasma infection. However, significant phosphorylation of Akt Ser473 after exposure to M. pneumoniae for 2 h was detected in asthmatic airway epithelial cells, which was reversed by pretreatment with LY294002 or by SHP-1 overexpression (Fig. 5B). M. pneumoniae-induced phosphorylation of Akt Ser473 was also detected in nonasthmatic cells, but it was less than that in asthmatic cells, and phosphorylation was enhanced by SHP-1 knockdown and reversed by pretreatment with LY294002 (Fig. 5B). AAV alone did not cause nonspecific effects upon Akt phosphorylation (Supple-
mental Fig. 2). In addition, SHP-1 knockdown enhanced IL-8 production in the setting of mycoplasma infection in nonasthmatic cells and was significantly inhibited by NF-κB and PI3K/Akt inhibitors (Fig. 5C). The data representing the percentage of densitometry values of nonasthmatic cells are expressed as mean ± SEM (right panel). The data representing the percentage of densitometry values of untreated cells are expressed as mean ± SEM (right panel). *p < 0.01 compared with untreated and control (Ctrl) in the same group. (C) Measurement of IL-8 production induced by M. pneumoniae in nonasthmatic and asthmatic airway epithelial cells with or without SHP-1 knockdown and overexpression. Data representing the fold change of IL-8 from control rAAV-transduced cells from the same group without mycoplasma infection are presented as mean ± SEM (n = 10 for nonasthma; n = 12 for asthma). Baseline SHP-1 expression was not different between asthmatic and nonasthmatic cells, and SHP-1 was knocked down and overexpressed similarly in nonasthmatic and asthmatic cells. IL-8 levels were higher in asthmatic cells infected with M. pneumoniae. SHP-1 overexpression reduced IL-8 production in asthmatic epithelial cells following mycoplasma infection. In the setting of SHP-1 knockdown, mycoplasma infection augmented IL-8 production in nonasthmatic, but not in asthmatic, cells. *p < 0.01 compared with Ctr&M of nonasthmatic cells; †p < 0.01 compared with Ctr&M or S- &M of asthmatic cells. Ctr&M, control rAAV-transduced airway epithelial cells treated with M. pneumoniae; S-, airway epithelial cells transduced with SHP-1-specific siRNA carrying rAAV to induce SHP-1 knockdown; S+, airway epithelial cells transduced with full-length SHP-1 cDNA carrying rAAV to induce SHP-1 overexpression; S- &M, airway epithelial cells with SHP-1 knocked down and infected by M. pneumoniae; S+ &M, airway epithelial cells with SHP-1 overexpressed and infected by M. pneumoniae.

SHP-1 inhibits NF-κB activation induced by M. pneumoniae in airway epithelial cells

It was reported that M. pneumoniae induces IL-8 (19) and MUC5AC production through activation of NF-κB (11). SHP-1 also negatively regulates NF-κB activation (51–53). Consistent with these previous studies, our results demonstrate that inhibition of NF-κB significantly attenuated M. pneumoniae-induced IL-8 production in asthmatic and nonasthmatic airway epithelial cells with or without SHP-1 knockdown (Fig. 5A, 5C). To further determine the molecular mechanism of SHP-1 in regulating M. pneumoniae-induced IL-8 production by asthmatic airway epithelial cells, we assessed the effect of SHP-1 on M. pneumoniae-induced NF-κB nuclear translocation and activation by immunoblot and p65 DNA binding assay (Fig. 6). Immunoblot analysis showed that M. pneumoniae induced significant NF-κB nuclear translocation in asthmatic airway epithelial cells 2 h after infection (Fig. 6A). However, M. pneumoniae infection induced less NF-κB nuclear translocation in nonasthmatic airway epithelial cells compared with asthmatic cells (Fig. 6A, 6B). SHP-1 knockdown dramatically accentuated M. pneumoniae-in-
duced NF-κB nuclear translocation in nonasthmatic cells (Fig. 6B). In asthmatic cells, NF-κB nuclear translocation following mycoplasma infection was unchanged with SHP-1 knockdown (Fig. 6B). However, SHP-1 overexpression significantly inhibited *M. pneumoniae*-induced NF-κB nuclear translocation in asthmatic and nonasthmatic airway epithelial cells (Fig. 6B).

To further confirm NF-κB nuclear translocation results, we performed a binding activity assay of NF-κB DNA. Nuclear extracts

FIGURE 3. *M. pneumoniae* infection in airway epithelial cells activates SHP-1. (A) *M. pneumoniae* infection of airway epithelial cells from nonasthmatic and asthmatic subjects resulted in time-course–dependent SHP-1 tyrosine phosphorylation. Airway epithelial cells without mycoplasma treatment (0 h) or infected with *Mycoplasma* for 2, 4, and 8 h were immunoprecipitated for SHP-1 and probed with anti-phosphotyrosine Ab 4G10 to detect P–SHP-1. Data representing P–SHP-1/SHP-1 of each time point compared with 0 h in nonasthmatic group are expressed as mean ± SEM, n = 5 (right panel). (B) Tyrosine phosphatase activity of immunoprecipitated SHP-1 from nonasthmatic and asthmatic airway epithelial cells without mycoplasma treatment (0 h) or infected with mycoplasma for 1, 2, or 4 h. *p < 0.01, †p < 0.05, compared with 0 h in the same group; ‡p < 0.05 between nonasthmatic and asthmatic cells at the same time point. The data are presented as mean ± SEM (n = 10 for nonasthmatic subjects; n = 12 for asthmatic subjects). Although *M. pneumoniae* infection induced SHP-1 phosphorylation in asthmatic airway epithelial cells, it was significantly less than in nonasthmatic cells at 2 and 4 h following *M. pneumoniae* infection.

FIGURE 4. *M. pneumoniae* infection induced TLR2–SHP-1 dynamic association. (A) Immunoblot analysis of baseline TLR2 expression in airway epithelial cells from nonasthmatic and asthmatic subjects. The data representing the percentage of densitometry values of nonasthmatic cells are expressed as mean ± SEM (right panel). (B) TLR2-associated SHP-1 levels in airway epithelial cells was measured by coimmunoprecipitation. TLR2 was immunoprecipitated from airway epithelial cell lysates of nonasthmatic and asthmatic subjects without mycoplasma treatment (0 h), infected with mycoplasma for 1 and 2 h. TLR2 immunoprecipitates were blotted with anti-TLR2 (top left panel) and anti–SHP-1 (bottom left panel) Ab. TLR2-associated SHP-1 at 2 h after *M. pneumoniae* infection is expressed as fold change of densitometry values from nonasthmatic cells without mycoplasma treatment (0 h) and was increased in nonasthmatic cells compared with asthmatic cells. The data are presented as mean ± SEM (n = 5). *p < 0.01 compared with asthmatic group.
SHP-1 regulates *M. pneumoniae*-induced IL-8 production through PI3K/Akt in airway epithelial cells from nonasthmatic and asthmatic subjects. (A) Fold change in IL-8 production in nonasthmatic and asthmatic airway epithelial cells with or without pretreatment with NF-κB inhibitor II (30 μM), LY294002 (25 μM), or Akt inhibitor (50 nM) for 30 min, followed by mycoplasma infection for 48 h. IL-8 levels are expressed as fold change from cells in the same group without mycoplasma infection. *p < 0.01, †p < 0.05, compared with mycoplasma-infected cells without inhibitor pretreatment in the same group. The data are presented as mean ± SEM (n = 10 for nonasthma, n = 12 for asthma). (B) Immunoblot analysis of Akt phosphorylation (P-Akt) in total cell lysates of nonasthmatic and asthmatic cells with or without mycoplasma infection for 2 h (left panel). The levels of P-Akt 2 h after *M. pneumoniae* infection in asthmatic and nonasthmatic cells, with or without SHP-1 knockdown or overexpression, are expressed as fold change of densitometry from cells in the same group transduced with control rAAV only (right panel). *p < 0.01, compared with control rAAV-transduced cells treated with mycoplasma in the same group; †p < 0.01, compared with control rAAV-transduced nonasthmatic cells treated with mycoplasma only; ‡p < 0.01, compared with SHP-1 knockdown cells with mycoplasma infection only in the same group. Data are presented as mean ± SEM (n = 5). Mycoplasma-induced phosphorylation of Akt in asthmatic cells, which was significantly higher than that in nonasthmatic cells, was reversed by pretreatment with LY294002 and attenuated by SHP-1 overexpression. SHP-1 knockdown dramatically increased phosphorylation of Akt in nonasthmatic cells, but not in asthmatic cells, which was reversed by pretreatment with LY294002. (C) IL-8 fold change in SHP-1 knockdown nonasthmatic and asthmatic airway epithelial cells that were pretreated or not with NF-κB inhibitor II (30 μM), LY294002 (25 μM), or Akt inhibitor (50 nM) for 30 min and then infected with mycoplasma for 48 h. SHP-1 knockdown dramatically accentuated IL-8 production in nonasthmatic cells, but not in asthmatic cells, which were all significantly inhibited by these three inhibitors. *p < 0.01 compared with mycoplasma-infected “S-” cells in the same group, without pretreatment of inhibitors; †p < 0.01, compared with “Ctr” cells of nonasthma, without pretreatment of inhibitors. The data are presented as mean ± SEM (n = 10 for nonasthma; n = 12 for asthma). Ctr, control rAAV-transduced airway epithelial cells; S-, airway epithelial cells with SHP-1 knockdown; S+, airway epithelial cells with SHP-1 overexpression.

from freshly isolated airway epithelial cells were allowed to bind to an NF-κB consensus oligonucleotide sequence. Bound NF-κB was then detected by a p65 (RelA)-specific Ab and quantified based on a standard curve using purified p65 recombinant protein. NF-κB DNA binding activity was significantly increased in asthmatic airway epithelial cells 2 h after *M. pneumoniae* infection compared with nonasthmatic cells (Fig. 6C). Baseline NF-κB DNA binding activity was not significantly different between the asthmatic and nonasthmatic groups. In the cells in which SHP-1 was knocked down, *M. pneumoniae* induced further increased NF-κB activity in nonasthmatic cells, without a significant change in the asthmatic cells. SHP-1 overexpression significantly attenuated *M. pneumoniae*-induced NF-κB activation in asthmatic and nonasthmatic airway epithelial cells (Fig. 6C). rAAV alone did not induce nonspecific regulatory effects on NF-κB activity (Supplemental Fig. 3). These results collectively demonstrate that SHP-1 plays an important role in inhibiting *M. pneumoniae*-induced NF-κB activation, which was significantly higher in asthmatic airway epithelial cells compared with nonasthmatic cells (Fig. 7).

**Discussion**

*M. pneumoniae* is an atypical bacterium that causes asthma exacerbations through increased airway inflammation and mucus hypersecretion. Clinical investigation and our ex vivo study show that asthmatic patients are more susceptible to *M. pneumoniae* infection (8), and asthmatic airway epithelial cells produced significantly higher levels of mucin (11) and IL-8 (54) compared with normal epithelial cells. The mechanisms by which SHP-1 regulates the TLR2-mediated proinflammatory signaling pathway activated by *M. pneumoniae* in asthmatic airway epithelial cells have not been defined. In this study, the role of SHP-1 in regulating *M. pneumoniae*-induced inflammation in asthmatic airway epithelial cells, the molecular mechanisms, and the relevant signaling pathways were investigated. Our study demonstrates that SHP-1 plays a critical role in inhibiting *M. pneumoniae*-induced IL-8 production in human airway epithelial cells. SHP-1 activation was defective in asthmatic cells, which subsequently resulted in overactivation of PI3K/Akt and NF-κB, leading to significantly increased IL-8 production in the setting of *M. pneumoniae* infection.
In the current study, we showed that *M. pneumoniae* induced significantly increased IL-8 production in asthmatic airway epithelial cells compared with nonasthmatic cells, which indicates that defective innate immune responses exist in asthmatic cells. Previous studies showed that asthmatic patients are more susceptible to mycoplasma infection (8), but this is not due to increased *M. pneumoniae* amounts, because we demonstrated that there were no differences in *mpn372* gene expression between ex vivo-cultured nonasthmatic and asthmatic airway epithelial cells before or following mycoplasma infection.

Furthermore, SHP-1 overexpression significantly decreased *M. pneumoniae*-induced IL-8 in asthmatic cells. Conversely, SHP-1 knockdown significantly increased IL-8 production in the setting of mycoplasma infection in nonasthmatic cells, but it did not change the increased IL-8 already produced by asthmatic cells following mycoplasma infection (Fig. 2C). Although one possible explanation for the difference in response to *M. pneumoniae* between asthmatic and nonasthmatic airway epithelial cells is significantly lower expression levels of baseline SHP-1 in asthmatic cells, we demonstrated that the baseline SHP-1 protein levels were not significantly different between nonasthmatic and asthmatic airway epithelial cells (Fig. 2A). An alternative explanation is that the function or activity of SHP-1 is defective in asthmatic airway epithelial cells, because our data demonstrate significantly decreased SHP-1 tyrosine phosphorylation and phosphatase activity in asthmatic airway epithelial cells after challenge with *M. pneumoniae* infection.

Reduced SHP-1 phosphorylation levels and phosphatase activity in asthmatic airway epithelial cells might arise from the genetic mutation of the SHP-1 gene itself or from the lower expression or phosphorylation of SHP-1 upstream partners in asthma. Defective SHP-1 activation in asthmatic airway epithelial cells induced by *M. pneumoniae* may account for the observed enhanced IL-8 production that was not observed in nonasthmatic airway epithelial cells. This observation is further supported by the inhibitory effect of SHP-1 overexpression, which reduced *M. pneumoniae*-induced IL-8 production in asthmatic cells. To our knowledge, defective SHP-1 activity in asthmatic airway epithelial cells after an infectious challenge has not been previously described.

Activation of the TLR2-signaling pathway is critical in *M. pneumoniae*-induced mucin and proinflammatory cytokine production (12, 13). After binding to TLR2, *M. pneumoniae* induces IL-8 production in human epithelial cells through activating MAPKs, ERK1/2, JNK, as well as NF-κB (19). Our study shows that *M. pneumoniae* induced significant time-dependent nuclear translocation of NF-κB and phosphorylation of PI3K/Akt in asthmatic airway epithelial cells (Figs. 5B, 6A) compared with nonasthmatic cells, resulting in significantly higher IL-8 production in asthmatic cells. Furthermore, inhibition of PI3K, Akt, and NF-κB significantly attenuated *M. pneumoniae*-induced IL-8 production in asthmatic airway epithelial cells (Fig. 5A, 5C).

To our knowledge, we demonstrate for the first time that PI3K and Akt...
play important roles in the signaling pathway of *M. pneumoniae*-induced IL-8 production. NF-κB activation could be PI3K/Akt independent or dependent; the latter is supported by a number of studies (55, 56). In addition, SHP-1 knockdown significantly enhanced mycoplasma-induced Akt phosphorylation (Fig. 5B), NF-κB nuclear translocation (Fig. 6B), and IL-8 production in nonasthmatic airway epithelial cells; the latter was dramatically inhibited by PI3K/Akt or NF-κB inhibitor (Fig. 5C). SHP-1 overexpression obviously inhibited mycoplasma-induced Akt phosphorylation and NF-κB nuclear translocation in nonasthmatic and asthmatic cells (Figs. 5B, 6B). Taken together, these results show that SHP-1 plays a critical role in attenuating mycoplasma-induced IL-8 production through the inhibition of Akt phosphorylation and NF-κB nuclear translocation in airway epithelial cells.

The cross-talk between SHP-1 and TLR-mediated proinflammatory signaling pathway has been well demonstrated in hematopoietic cells (57–59) but not in epithelial cells. SHP-1 acts at various receptors through recruitment to phosphorylated ITAM on inhibitory receptors, which deactivates certain critical proinflammatory mediators and terminates the downstream signaling pathways. Similar to hematopoietic cells, we demonstrated in airway epithelial cells that SHP-1 negatively regulates TLR-mediated production of proinflammatory cytokines by inhibiting activation of NF-κB and deactivating PI3K/Akt signaling through dephosphorylation of PI3K, leading to subsequent abrogation of the phosphorylation of Akt. SHP-1 can also inhibit ubiquitin ligase TNFR-associated factor 6-dependent signal transduction activated by the ligand for the cytokine receptor RANK (52), which is also important in TLR-mediated production of proinflammatory cytokines. Furthermore, our present study demonstrates an *M. pneumoniae*-induced dynamic association between SHP-1 and TLR2 in both nonasthmatic and asthmatic airway epithelial cells. However, the dynamic recruitment of SHP-1 to TLR2 is defective in asthmatic airway epithelial cells in the setting of *M. pneumoniae* infection (Fig. 4), which may contribute to the defective inhibitory effect of SHP-1—signaling pathway. It is possible that defective structure and function of TLR2 and/or SHP-1 lead to defective recruitment of SHP-1 to TLR2. Alternatively, the IL-4- and IL-13—signaling pathways, which are critical for the asthma phenotype, might sequester SHP-1 from TLR2 and induce overactivation of TLR2-mediated proinflammatory signal transduction. To test this possibility, we performed Western blot of phosphorylated Stat3/Stat6 and coimmunoprecipitation of SHP-1/Stat3 and SHP-1/Stat6. Western blot analysis showed that mycoplasma infection did not induce significant Stat3 and Stat6 phosphorylation (data not shown) in either nonasthmatic or asthmatic airway epithelial cells. There were no significant differences in the amounts of SHP-1 associated with Stat3 or Stat6 between nonasthmatic and asthmatic airway epithelial cells prior to mycoplasma infection (Supplemental Fig. 4). In addition, time-course treatment with mycoplasma did not induce dynamic association between SHP-1 and Stat3/Stat6 in asthmatic and nonasthmatic airway epithelial cells (Supplemental Fig. 4), which indicates that IL-4/IL-13-related signaling pathways may not sequester more SHP-1 and are unlikely to contribute to the defective SHP-1 activity in asthmatic cells under the challenge of mycoplasma infection. Elucidation of the mechanisms that drive reduced SHP-1 phosphatase activity in asthmatic airway epithelial cells requires further investigation. The defect in SHP-1 in asthmatic subjects may increase the susceptibility to other TLR ligands that are known to induce asthma exacerbations, such as rhinovirus, influenza virus, and respiratory syncytial virus (60). It is not known whether *M. pneumoniae* infection induces SHP-1 redistribution and nuclear translocation, and the importance of cytoplasmic versus nuclear SHP-1 in regulating *M. pneumoniae*-induced inflammation also requires further investigation.

In conclusion, we identified SHP-1 as a critical regulator of *M. pneumoniae*-induced inflammation in primary human airway epithelial cells cultured directly from well phenotyped subjects with asthma and nonasthma controls. Defective SHP-1 activation in asthmatic airway epithelial cells contributes to the increased activation of PI3K/Akt and NF-κB after *M. pneumoniae* infection, which further increases TLR2-mediated IL-8 production (Fig. 7). Deficiency of SHP-1 function in human asthmatic airway epithelial cells in the setting of exogenous insults, such as *M. pneumoniae* infection, may enhance the inflammatory response in human asthma following insults, such as infection, and lead to exacerbation of the disease. Our findings establish a link between SHP-1 and asthmatic airway epithelial cell inflammation. Elucidating the role of SHP-1—modulated signaling pathways in *M. pneumoniae*-induced exacerbation of airway inflammation in asthma will shed light on the innate immune regulation and dysfunction in asthma and offer new therapeutic avenues through which to decrease asthma exacerbations.


Supplemental Figure 1:

Asthma
Non-asthma

IL-8 Fold Change

* *
Supplemental Figure 2:

M. pneumoniae

P-Akt (60 kDa)

Asthma Non-asthma

M. pneumoniae

Akt (60 kDa)
Supplemental Figure 3:

A

**M. pneumoniae**

- - + +

**P65 of Nuclear Extract (65 kDa)**

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**P65 of Cytoplasmic Extract (65 kDa)**

Asthma

Non-asthma

B

**With M. Pneumoniae**

- - + +

**Asthma**

**Non-asthma**

**Without M. Pneumoniae**

- - + +

**Asthma**

**Non-asthma**

With virus

No virus

Ctr virus

With virus

No virus

Ctr virus

*
Supplemental Figure 4:

**Stat3 IP**

- **SHP-1** (68kDa)
- **Stat3** (86kDa)

**Stat6 IP**

- **SHP-1** (68kDa)
- **Stat6** (110kDa)
Supplemental Figure Legends

Figure S1. *M. pneumoniae*-induced IL-8 production in non-asthmatic and asthmatic airway epithelial cells with or without transduction of control adeno-associated virus. To test whether rAAV alone caused non-specific effects on IL-8 levels in epithelial cells, apical supernatant collected 48 hr after *M. pneumoniae* infection from cells with or without control rAAV treatment was used to perform IL-8 Elisa. “No virus”: airway epithelial cells without control rAAV transduction were treated with *M. pneumoniae* for 48 hours; “Ctr virus”: control rAAV transduced airway epithelial cells were treated with *M. pneumoniae* for 48 hours. *p* < 0.01 compared with non-asthmatic cells. Data representing the fold change of IL-8 from cells without mycoplasma infection are presented as means ± SEM (n=10 for non-asthma; n=12 for asthma). rAAV alone did not cause significant effect on IL-8 levels in both non-asthmatic and asthmatic airway epithelial cells.

Figure S2: *M. pneumoniae*-induced Akt phosphorylation in total cell lysates of non-asthmatic and asthmatic cells with or without control adeno-associated virus transduction. Immunoblot analysis of phosphorylated Akt (P-Akt) levels in airway epithelial cells after 2 hr treatment with *M. pneumoniae*. Lane 1, 3: airway epithelial cells without control rAAV transduction; Lane 2, 4: airway epithelial cells transduced with control rAAV. rAAV alone did not cause non-specific effects on the levels of Akt phosphorylation in cells with or without *M. pneumoniae* infection.
Figure S3: *M. pneumoniae*-induced NF-κB activation in non-asthmatic and asthmatic airway epithelial cells with or without control adeno-associated virus transduction. (A) Immunoblot of cytoplasmic and nuclear protein lysates against p65 in non-asthmatic and asthmatic airway epithelial cells without mycoplasma infection or challenged with mycoplasma for 2 hr. Lane 1, 3: airway epithelial cells without control rAAV infection; Lane 2, 4: airway epithelial cells infected with control rAAV. (B) NF-κB activation was quantified in non-asthmatic and asthmatic airway epithelial cells without mycoplasma infection or 2 hours after mycoplasma infection. The NF-κB DNA binding activity is reported as ng of bound p65 protein per 20 μg of nuclear extracts. “No virus”: airway epithelial cells without control rAAV transduction; “Ctr virus”: control rAAV transduced airway epithelial cells. * p<0.01 compared with cells without mycoplasma infection in the same group. Data are presented as means ± SEM values (n=10 for non-asthma; n= 12 for asthma). rAAV alone did not cause non-specific effects on the activity of NF-κB.

Figure S4: *Mycoplasma pneumoniae* infection did not induce dynamic association of Stat3/SHP-1 and Stat6/SHP-1 in airway epithelial cells from non-asthmatic and asthmatic subjects. Following a time-course treatment employing *M. pneumoniae* infection, epithelial cells lysates (500 μg) were immunoprecipitated with 2μg of rabbit anti-human Stat3 or Stat6 antibody (Cell Signaling Technology, Danvers, MA). The immunoprecipitates were analyzed by SDS-PAGE and immunoblotting using mouse anti-human SHP-1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) to determine the total amount of SHP-1 co-immunoprecipitated with Stat3 or Stat6 in the samples. The
PVDF membrane was then stripped and re-probed with mouse anti-human Stat3 or Stat6 antibody to determine the amounts of Stat3 or Stat6 in the immunoprecipitates and quantified by densitometry. Shown are representative data from five asthmatic and five non-asthmatic subjects. There was no significant difference of the amounts of SHP-1 associated with Stat3 or Stat6 between non-asthmatic and asthmatic airway epithelial cells, before and after the treatment with mycoplasma.