

Chlamydia pneumoniae Induces Interferon Gamma Responses in Peripheral Blood Mononuclear Cells in Children with Allergic Asthma

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Received 27 February 2017; Accepted in revised form 28 April 2017

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Abstract

Respiratory infections caused by *Chlamydia pneumoniae* have been associated with exacerbations of asthma. Cell-mediated immunity (CMI) is critical for maintaining immunity. We compared interferon (IFN)- γ responses in *C. pneumoniae*-infected peripheral blood mononuclear cells (PBMC) in paediatric patients \pm asthma. Presence of *C. pneumoniae* was tested from asthma patients ($N = 17$) and non-asthmatic controls ($N = 16$) (PCR). PBMC were infected for $1 \text{ h} \pm C. pneumoniae$ AR-39 (MOI = 0.1) and cultured for 48 h. IFN- γ levels were measured in supernatants (ELISA). *C. pneumoniae*-IgG antibodies in serum were determined (MIF). All subjects tested negative for *C. pneumoniae* (PCR). *C. pneumoniae*-induced IFN- γ production *in vitro* was more prevalent in asthma compared with non-asthma; levels of IFN- γ were higher in asthma compared with non-asthma ($P = 0.003$). There was no association between recent respiratory infection and positive IFN- γ responses. These data show that *C. pneumoniae* modulates IFN- γ responses in patients with asthma, even in absence of active infection.

Introduction

Asthma is a major health concern in the United States (U.S.) and worldwide [1]. The prevalence of allergic asthma has been increasing in the U.S.A, especially in children and minority populations [1]. Persistent respiratory infections caused by viruses and atypical bacteria account for a high number of asthma exacerbations and hospital admissions in patients with asthma and/or chronic obstructive pulmonary disease (COPD) [2].

Chlamydia pneumoniae, an obligate intracellular bacterium [3], is a common cause of community acquired respiratory infections in adults and children and has also been associated with exacerbations of asthma [4, 5]. Prior literature has shown that *C. pneumoniae* can cause prolonged respiratory infections in asthmatic and non-asthmatic individuals, with the potential for ongoing stimulation of the immune system [5–8].

Recent studies in our laboratory demonstrated that *C. pneumoniae* infection has the ability to induce allergic responses in PBMC of adult asthmatics, as evidenced by production of Th2 responses (e.g. interleukin (IL)-4) and immunoglobulin (Ig) E responses [9]. However, to our knowledge, few studies have investigated the possible role

of *in vitro* *C. pneumoniae*-mediated IFN- γ responses in paediatric asthmatic patients in the absence of an acute exacerbation.

Cell-mediated immunity (CMI) is important for host defence against primary *C. pneumoniae* infection [10]. Chlamydia infection elicits cytokine responses by direct infection of host epithelial cells, and by interaction with cells of the innate immune system [10]. In patients at risk for persistent infection, CMI responses (e.g. IFN- γ production) may be useful in epidemiologic studies as a biomarker for the detection of persistent *C. pneumoniae* infection. In this study, we examined the effect of *C. pneumoniae* infection on PBMC obtained from asthmatic children without recent exacerbation and non-asthmatic controls by comparing levels of IFN- γ .

Methods

Study design and population. Paediatric patients with allergic asthma ($N = 17$) and non-asthmatic controls ($N = 16$) were recruited between September 2009 and January 2010 from the outpatient department at Kings County Hospital Center, Brooklyn, NY, paediatric asthma clinic. Written informed consent and assent was obtained from patients

and their legal guardians. Inclusion criteria included a physician's diagnosis of asthma or a clinical diagnosis of allergic asthma [11] without recent symptoms of asthma exacerbation, but had history of recent upper respiratory tract infection. Exclusion criteria included history of chronic immunosuppressive or autoimmune disease, human immunodeficiency virus infection, cancer or systemic steroid use. Non-asthmatic control subjects were defined by absence of asthma, based on clinical criteria [12]. Peripheral blood (10 ml) and nasopharyngeal specimens were collected at the time of diagnosis, follow-up or referral to our clinic. This study was approved by the Institutional Review Board SUNY Downstate Medical Center, Brooklyn, NY, and the procedures followed were in accordance with institutional guidelines involving human subjects.

Detection of specific IgG *C. pneumoniae* antibodies. IgG *C. pneumoniae* Abs were measured using the microfluorescence (MIF) assay (AniLabsystems; Vantaa, Finland), as previously described [13, 14]; a titre of ≥ 16 was considered indicative of prior infection [9].

Real-time polymerase chain reaction (qPCR) for quantification of *C. pneumoniae*. qPCR was performed as previously described [15]. DNA from nasopharyngeal swabs and PBMC was extracted using the QIAamp DNA mini kit (Qiagen Inc., Valencia, CA, USA). *C. pneumoniae* DNA was detected by means of TAQMan (Roche Molecular Diagnostics, Basel, Switzerland) technology-based quantitative real-time PCR on the Light Cycler 2.0 platform (software version 4.0, Roche Molecular Diagnostics). All samples were analysed in triplicate, and samples with positive or indistinct results were reanalysed at least once. An additional replicate was checked for PCR inhibitors, and inhibited specimens were retested using a 1:10 dilution. A sample was considered positive if three of three assay results were positive in the triplicate test and if the average value for the PCR run was greater than or equal to 1.0. A sample was considered negative if the crossing point (CP) PCR value exceeded 45 cycles.

Chlamydial isolates. *C. pneumoniae* AR-39 (ATCC 53592) was obtained from the ATCC (American Type Culture Collection, Manassas, VA, USA) and purified as described previously [14]. Briefly, *C. pneumoniae* AR-39 was passaged several times in HEp-2 cell (ATCC LCL-23) monolayers to achieve high titres. *C. pneumoniae* elementary bodies (EBs) were purified by Urografin (Schering, Berlin, Germany) density gradient centrifugation and were resuspended in sucrose phosphate glutamate buffer and stored in aliquots (titre 1×10^8 IFU/ml) at -80°C . Titres were determined by infecting HEp-2 cells with serial dilutions of EB suspension aliquots, fixing cells at 72 h post-infection (p.i.), staining with fluorescein-conjugated murine monoclonal genus-specific antilipopolsaccharide Abs (pathfinder, Bio-Rad, Hercules, CA, USA) and counting the inclusions per well. Aliquots were frozen at -80°C until use.

Cell cultures. PBMC were separated from blood on a Ficoll-Paque (GE Healthcare, Chicago, IL) gradient (density 1.077). The PBMC were carefully removed using a transfer pipette (VWR Scientific, San Francisco, CA, USA). Cells were washed twice in RPMI-1640 medium (Life Technologies/GIBCO, Grand Island, NY, USA) with 10% foetal bovine serum (FBS) (Atlanta Biologicals, Norcross, GA, USA) and resuspended in complete RPMI 1640 (c-RPMI). c-RPMI contained RPMI-1640 Medium HEPES Modification (Sigma, St Louis, MO, USA) supplemented with 5 mM L-glutamine (Sigma) and 10% FBS (Atlanta Biologicals). Cells were counted on a hemocytometer (Fisher Scientific, Springfield, NJ, USA), and cell viability was evaluated, as judged by Trypan Blue (Fisher Scientific) exclusion; cell viability was $>95\%$. For each experimental condition, PBMC ($1.5 \times 10^6/\text{ml}$) were cultured in duplicate in a 24-well flat-bottom plate (1 ml/well) (CORNING; Corning, NY, USA) at 37°C in cRPMI medium in a humidified 5% CO_2 atmosphere for up to 48 h. Cell viability was determined at 0, 24 and 48 h ($>98\%$, 95% and 90% , respectively). Following a 2-h incubation to allow adherence, PBMC cultures were infected with *C. pneumoniae* or mock-infected. In some experiments, PBMC were allowed to adhere to the cell culture plates for 2 h, and then the non-adherent cells were removed and cultured separately from adherent cells. IFN- γ cytokine assays were run using supernatants from these cell populations.

In vitro infection with *C. pneumoniae*. PBMC were infected with *C. pneumoniae* by adding purified EB for 1 h and then incubating for 48 h p.i. at 37°C in cRPMI in a humidified 5% CO_2 atmosphere. The multiplicity of infection (MOI; 0.1) and time points (48 h p.i. for cytokines) used for analysis were selected by kinetic and dose-response studies (using MOI of 0.01–10) for optimization of the assay, which revealed peak concentrations and clear distinctive profiles for the respective outcome variables at these time points. (An MOI of 0.1 results in a clinically relevant bacterial load.) Adherent cells were stained with a fluorescein-conjugated murine monoclonal genus-specific antilipopolsaccharide antibody (Becton-Dickinson (BD) Biosciences, San Jose, CA) to confirm and quantify infection with *C. pneumoniae* at 72 h p.i. Two types of controls were used in infection experiments: identical volumes of heat-inactivated purified *C. pneumoniae* [16] and identical volumes of HEp-2 cell cultures not containing any bacteria processed the same way as the purified *C. pneumoniae* [14] based on dose-response experiments.

Cytokine assay: IFN- γ determination. For the *in vitro* quantitative determination of human IFN- γ content in cell culture supernatants, solid-phase sandwich ELISA assays were performed using the cytokine IFN- γ ELISA kit (BioLegend, San Diego, CA, USA), which was used according to the manufacturer's recommended procedure. A positive *in vitro* IFN- γ response to *C. pneumoniae* was

defined as >16 pg/ml (greater than average non-stimulated PBMC IFN- γ response \pm 2SD).

Statistical analysis. Statistical significance was determined by Fisher's exact test for categorical variables and Mann-Whitney U-test for continuous variables which were performed using the SPSS for Windows, version 12.0 software (Chicago, IL, USA). A two-sided *P*-value of <0.05 was taken to indicate statistical significance for all comparisons.

Results

Participant characteristics and demographics

We enrolled 17 patients with allergic asthma (47% female, 53% male) (mean age: 13 years) and 16 healthy non-asthmatic controls (48% female, 52% male) (mean age: 14 years). None of the healthy subjects had a history of asthma or allergic rhinoconjunctivitis. All asthma patients were classified as having moderate persistent asthma and were treated with inhaled corticosteroids (ICS). The ICS used were either fluticasone HFA (176, 220, or 440 mcg) fluticasone/salmeterol HFA (180 mcg), fluticasone/salmeterol DPI (500 or 1000 mcg), beclomethasone HFA (160 mcg) or Budesonide suspension (0.5 mg). In asthmatics, the mean forced vital capacity (FVC) (% predicted) was 85, the mean forced expiratory volume in 1 s (FEV₁) (% predicted) was 89, the FEV₁/FVC ratio was 81 and the mid-flow rate or forced expiratory flow occurring in the middle 50% of the patient's exhaled volume (FEF_{25%-75%}). The clinical characteristics of the enrolled subjects with asthma are shown in Table 1.

Serology and PCR testing for C. pneumoniae

All subjects (asthma and non-asthma) tested negative for *C. pneumoniae* DNA by PCR (nasopharyngeal swabs). The

Table 1 Participant characteristics.

Asthma (N = 17)	
Age, mean, years	13
Female (%)	47
Male (%)	53
FVC (% predicted)	85
FEV ₁ (% predicted)	89
FEV ₁ /FVC	87
FEF ₂₅₋₇₅ (% predicted)	72
URTI (%)	18
LRTI (%)	0

FVC, mean forced vital capacity; FEV₁, mean forced expiratory volume in 1 s; FEF₂₅₋₇₅, mid-flow rate or forced expiratory flow occurring in the middle 50% of the patient's exhaled volume; URTI, upper respiratory tract infection; LRTI, lower respiratory tract infection.

prevalence of IgG *C. pneumoniae* Abs and the median IgG *C. pneumoniae* titres were similar in both groups (*P* > 0.05).

Effect of C. pneumoniae infection on IFN- γ responses induced in vitro

C. pneumoniae-induced IFN- γ production *in vitro* was more prevalent in asthmatic patients compared with non-asthmatic controls (76% versus 13%; *P* = 0.007); levels of IFN- γ were higher in PBMC from patients with asthma compared with non-asthma (319 \pm 623 pg/ml, 113 \pm 271; *P* = 0.003) (Fig. 1). In contrast, uninfected PBMC produced similar levels of IFN- γ in asthma compared with non-asthma (12 \pm 4, 7 \pm 18; *P* = NS) (Fig. 1).

Relationship of IFN- γ responses to recent respiratory events

While 18% of patients with asthma had upper respiratory tract infections (URTIs) within the past 6 months, none of the patients with asthma had lower respiratory tract infections (LRTIs) within the past 6 months (Table 1). There was no association between recent respiratory infections or recent courses of oral corticosteroids (within 6 months prior to study enrolment) and either the presence of positive IFN- γ -responses or the amount of IFN- γ secreted (*P* = NS).

Relationship of IFN- γ responses and MIF titres

To evaluate the relationship between generation of IFN- γ responses by PBMC and MIF titres in subjects with

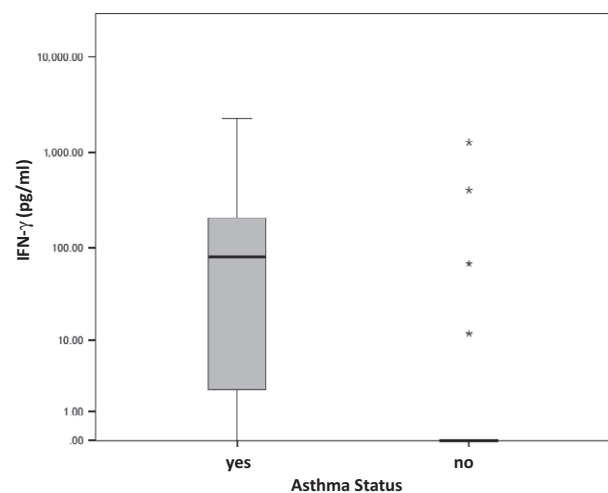


Figure 1 Effect of *C. pneumoniae* infection on IFN- γ responses induced *in vitro*. Human PBMC (1.5×10^6 /ml) were obtained from asthmatic subjects (*N* = 17) and non-asthmatic controls (*N* = 16) and cultured with *C. pneumoniae* (MOI = 0.1) for 48 h, and levels of IFN- γ were measured in supernatants (ELISA). Data are expressed as pg/ml [mean \pm standard deviation (SD)]. Cytokine response was defined as cytokine of interest in infected PBMC minus cytokine of interest in uninfected PBMC. Left bar: asthma; Right bar: no asthma.

asthma, we analysed the data set for association between the two biomarkers for infection. We found an equal distribution of IFN- γ values across categories of MIF titre ($P = 0.633$), indicating no association (Fig. 2).

Discussion

In the current study, the main preliminary observation was that *C. pneumoniae*-induced IFN- γ production was more prevalent in asthmatic patients compared with non-asthmatic controls and that *C. pneumoniae* infection led to a significant increase of IFN- γ levels in supernatants of PBMC from asthmatic patients, compared with non-asthmatics. These results may indicate recent or persistent *C. pneumoniae* infection in these patients.

We have previously shown that *C. pneumoniae*-infected PBMC from asthmatic adults had significantly increased levels of IL-4 and IFN- γ , while levels of IL-10 and IL-12 were low [9]. However, when uninfected PBMC from asthmatic adults were stimulated with *Lactobacillus rhamnosus* GG (LGG), levels of IL-4 were undetectable; levels of IL-10 and IFN-gamma increased significantly and there was a trend to increased levels of IL-12 [9]. The increased Th1 responses elicited by LGG *in vitro* in PBMC obtained from asthmatic patients may result in part, from morphologic properties, including the presence of a bacterial cell wall peptidoglycan [9, 16]. In previous studies, the Th2 bias observed in *C. pneumoniae*-infected PBMC from asthmatics may be due to differential stimulation and/or activation of receptors for pathogen-associated molecular patterns (PAMP) [9]. Thus, current and previous [9] work

demonstrates that *C. pneumoniae* is required for induction of *in vitro* IFN- γ responses in PBMC from subjects with asthma.

In contrast, other studies in our laboratory reported that in *C. pneumoniae*-infected PBMC from adult asthmatics compared with non-asthmatic controls, there was a significant increase in the levels of IL-4, while levels of IFN- γ were low [16]. This result suggests that *C. pneumoniae* infection may promote allergic inflammation and ongoing production of IgE [16]. However, the observed difference might be due to other elements of inflammation in this group of patients, and further immunological studies are required to evaluate this issue.

Another finding in the present study was that asthmatics and non-asthmatics had similar seroprevalence rates and titres of *C. pneumoniae* IgG, indicating that both groups probably had similar rates of past infection with *C. pneumoniae*. The findings of the present study are consistent and in agreement with the aforementioned study that also reported similar seroprevalence rates and titres of *C. pneumoniae* IgG in both asthmatics and non-asthmatics [16]. It could be that the differences in overall cytokine responses between asthmatics and controls cannot be attributed to differences in exposure alone [16]; other factors including the presence of memory lymphocytes following prior infection and length of time since infection may also play important roles in these response [16].

Recent evidence suggests that suppression of IFN- γ production may inhibit clearance of *C. pneumoniae* infection through CMI, in particular Th1 responses, which are crucial for infection clearance caused by *C. pneumoniae* [10]. Prior literature has described persistent infections in asthmatics, which may be associated with lack of strong Th1 responses [5, 17]. Wang, *et al.* observed that *C. muridarum*-infected IFN- γ gene knockout mice mounted strong delayed-type hypersensitivity (DTH) responses following footpad challenge with inactivated organisms [18]. (DTH is an *in vivo* manifestation of CMI [18].) The DTH responses in these mice were associated with Th2 cytokine production, and the inflammatory cells in these mice failed to target the cellular sites of chlamydial inclusions in infected tissues and failed to clear infection [18]. Thus, the authors concluded that both Th1- and Th2-associated DTH responses may account for the dual role that DTH is speculated to play in chlamydia protective immunity and immunopathology [18].

As of date, there exists no biomarker to detect chronic infection with *C. pneumoniae*, in the absence of documented persistent infection by culture or PCR. In the present study, all of our subjects were tested and found to be negative for *C. pneumoniae* by qPCR (nasopharyngeal swabs). It should be noted that persistent infections with *C. pneumoniae* may not be detected by PCR for several reasons: it may be low-level and latent and therefore harder to detect. In addition, the site of infection may not be the

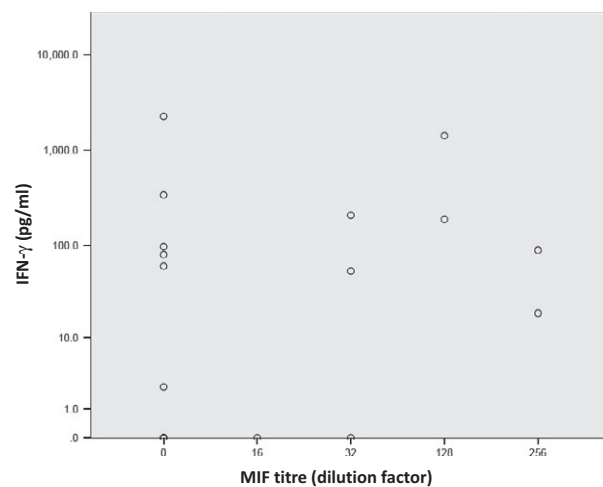


Figure 2 MIF IgG antibody response to *C. pneumoniae* and IFN- γ in subjects with asthma. IgG Ab responses to *C. pneumoniae* in serum were measured using MIF assay (see Methods); a titre of ≥ 16 was considered indicative of prior infection. IFN- γ levels were measured in cell culture supernatants from PBMC obtained from subjects with asthma ($N = 17$) (ELISA). MIF titre was expressed as dilution factor, and IFN- γ levels were expressed as pg/ml. Nonparametric P value (Mann–Whitney U-test; SPSS Statistics).

upper respiratory tract, which was sampled for this study. However, previous studies in our laboratory have demonstrated that nasopharyngeal specimens were suitable for direct detection of *C. pneumoniae* in patients with acute lower respiratory infections and asthma exacerbations [5]. There is no known biological reason that the incidence of infection with *C. pneumoniae* in patients with asthma should be higher than in control subjects from the same community [5]. Therefore, the presence of persistent infections with low bacterial loads may be more likely to occur.

Our last finding in the current study revealed that there was no association between a history of recent infections, asthma exacerbations or systemic corticosteroid use and CMI. This result may suggest that the observed differences in *C. pneumoniae*-induced CMI are not due to higher incidence of infections, but may be due to persistence of infections in children with asthma. Halme, *et al.* reported that in patients with *C. pneumoniae* infections, CMI to *C. pneumoniae* is specific and there is no cross-reactivity with other chlamydial species [10]. However, another possible explanation may be non-specific responses. Non-specific reactivity to other chlamydial species should occur at a similar frequency in control subjects. CMI in children with asthma may differ from healthy subjects without asthma and could lead to non-specific responses. This may be reflected in the fact that in the current studies, no differences were observed in IFN- γ responses of non-stimulated PBMC between asthma and control subjects.

Other major potentially infectious bacterial agents associated with asthma include *Mycoplasma pneumoniae* [19]; cytokine production observed in the current study was elicited by and specific to *in vitro* infection with *C. pneumoniae*, and thus, *M. pneumoniae* infection should not have affected our results.

This study has potential limitations including small sample size and serologic methods for confirmation of timing of infection may be difficult; thus, incidence of primary infection in patients is unknown. Future prospective studies that measure CMI responses to *C. pneumoniae* and its relationship to asthma inflammation could characterize patients with persistent infection that may benefit from treatment with antibiotics.

Acknowledgment

We would like to thank Kevin B. Norowitz, M.D., Department of Pediatrics, SUNY Downstate Medical Center for fruitful discussions.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial or not-for-profit sectors.

Disclosure

The authors declare no competing financial interest to disclose.

Conflict of Interest

The authors declare no conflict of interest to disclose.

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