

***Chlamydia pneumoniae* infection enhances cellular proliferation and reduces steroid responsiveness of human peripheral blood mononuclear cells via a tumor necrosis factor- α -dependent pathway**

Y. S. Cho*, T.-B. Kim*, T.-H. Lee*, K.-A. Moon*, J. Lee*, Y.-K. Kim†, K.-Y. Lee* and H.-B. Moon*

*Department of Allergy and Clinical Immunology, Asan Medical Center, University of Ulsan College of Medicine, Seoul, Korea and †Department of Internal Medicine, Seoul National University College of Medicine, Seoul, Korea

Summary

Background Although epidemiological studies have found an association between *Chlamydia pneumoniae* infection and severe asthma, the causality and underlying mechanism are largely unknown. We hypothesized that *C. pneumoniae* infection increases the proliferation and enhances the survival of immune and inflammatory cells, resulting in reduced responsiveness to corticosteroids and suggesting that the underlying mechanism is related to a TNF- α -dependent pathway.

Methods Human peripheral blood mononuclear cells (PBMCs) were cultured *in vitro* in the presence or absence of *C. pneumoniae* infection. Responsiveness to corticosteroids was assayed by adding dexamethasone, and the underlying mechanism was investigated by treating cells with infliximab that is a chimeric anti-TNF- α monoclonal antibody. Cellular proliferation and apoptosis was assessed by thymidine uptake and counting apoptotic cells using flow cytometry.

Results Cellular proliferation was significantly higher in *C. pneumoniae*-infected PBMCs than in uninfected PBMCs, which is more prominent in Th2-dominant microenvironment. The anti-proliferative and pro-apoptotic effect of corticosteroid were significantly reduced in *C. pneumoniae*-infected PBMCs compared with uninfected PBMCs. The proliferative effect of *C. pneumoniae* infection and the reduced response to corticosteroid were effectively reversed by blocking the TNF- α pathway at least partially.

Conclusion *C. pneumoniae* infection enhanced the proliferation and survival of immune and inflammatory cells, resulting in steroid resistance. The reversal of these phenomena by the TNF- α inhibitor suggests that TNF- α may play an important role in the induction of steroid dependence or resistance. A TNF- α inhibitor may therefore be a candidate agent for managing steroid-dependent or -resistant severe asthma.

Keywords *Chlamydia pneumoniae*, corticosteroid, peripheral blood mononuclear cell, TNF- α

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Introduction

The clinical manifestations of bronchial asthma are phenotypically heterogeneous, with some asthmatics experiencing a severe clinical course in symptoms and lung function. *Chlamydia pneumoniae*, an obligate intracellular pathogen, is a common cause of human respiratory tract infections [1]. Recent seroepidemiological data suggests that infection with this organism is associated with severe steroid-dependent or -resistant asthma [2–4], persistent airflow limitation in adult onset asthma [5], and acute exacerbation of asthma [6], as well as with the development of asthma in children [7, 8]. However, the association between *C. pneumoniae* infection and severe asthma is not sufficient to prove causality.

Recently, some possible mechanism explaining this correlation could be inferred from the results of other studies. For example, *C. pneumoniae* has been shown to infect and replicate in monocytes and macrophages, as well as in epithelial, endothelial, and smooth muscle cells, leading to unlimited proliferation without killing infected host cells. Furthermore, infection with *C. pneumoniae* has been found to increase cellular secretion of various pro-inflammatory cytokines, including TNF- α , IL-1 β , IL-4, IL-6, and IL-8, and to decrease MHC I expression [9–11]. These characteristics may be associated with the development of inflammation or the aggravation of pre-existed inflammation of infected tissue, as observed in asthmatic airways.

In addition, *C. pneumoniae*-infected epithelial cells and peripheral blood mononuclear cells (PBMC) have been shown to be resistant to apoptosis induced by potent apoptotic stimuli such as staurosporine and photo-activated chemotherapeutic agents [12–16]. Although the effect of corticosteroids on *C. pneumoniae*-infected PBMCs has not

Correspondence: Hee-Bom Moon, Department of Allergy and Clinical Immunology, Asan Medical Center, University of Ulsan College of Medicine, 388-1, Pungnap-2dong, Songpa-gu, Seoul, Korea.
E-mail: hbmooon@amc.seoul.kr

been evaluated, it is likely that *C. pneumoniae* infection alters the effects of corticosteroids on cell survival and on the death of immune cells. Corticosteroids are the most effective drugs used to treat asthmatics, and corticosteroid-induced apoptosis of inflammatory cells is one of the mechanisms by which these drugs manifest their anti-inflammatory activities in asthma [17]. Thus, *C. pneumoniae* infection in asthmatics might induce a more severe clinical phenotype and a perpetuation of airway inflammation through microbial development of at least partial resistance to corticosteroids.

To date, however, the exact mechanism by which *C. pneumoniae* infection affects the survival and proliferation of immune system cells is largely unknown. This mechanism may be closely associated with the aggravation of allergic inflammation of asthmatic airways. In many immune-mediated diseases, various pro-inflammatory cytokines are linked in a network with TNF- α at its apex [18]. Furthermore, TNF- α secretion by monocytes and macrophages upon infection by pathogenic microbes such as *C. pneumoniae* may enhance lymphocyte survival [11]. Thus, *C. pneumoniae* infection may alter allergic inflammation in asthmatics through a pathway involving TNF- α .

We hypothesized that *C. pneumoniae* infection of immune cells may induce increased cellular proliferation and decreased responsiveness to corticosteroid treatment, and that the mechanism underlying these phenomena is related, at least in part, to a TNF- α pathway. We therefore used an *in vitro* model of *C. pneumoniae* infection of PBMCs to evaluate the interaction of *C. pneumoniae* and immune system cells.

Materials and methods

Culture and inoculum preparation of *Chlamydia pneumoniae*

C. pneumoniae strain TW 183 was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA) and propagated in HEp-2 cells (ATCC) grown in Eagle's minimum essential medium (Sigma, St Louis, MO, USA) supplemented with 10% fetal calf serum, L-glutamine (2 mM), non-essential amino acids, gentamycin (10 mg/L), vancomycin (50 mg/L), and amphotericin B (2 mg/L). Confluent monolayers were infected with *C. pneumoniae* by centrifuging (2000 g, 35 °C, 45 min) infectious inocula onto the host cells. Supernatants were replaced by serum-free growth medium containing cyclohexamide (1 mg/L, Sigma). Infected cells were cultured in a 35 °C, 5% CO₂ saturated humidified incubator, harvested on day 3 or 4, and disrupted by two cycles of freezing and thawing and ultrasonification, and similar harvests were pooled. After centrifugation at 1000 r.p.m. for 5 min to remove cell debris, the bacteria were concentrated by high-speed centrifugation at 25 000 g for 25 min. Pellets were resuspended in PBS, pH 7.4, mixed with an equal volume of sucrose-phosphate-glutamic acid buffer, aliquoted, and frozen at -70 °C until use.

C. pneumoniae titers were assessed by an immunofluorescence (IF) assay. HEp-2 cells were infected with serial dilutions of bacterial stock, cultured for 65 h, fixed with methanol-acetone (1:1), and incubated with antibody to major outer membrane protein (mouse IgG3 anti-*C. pneumoniae* monoclonal antibody (mAb); Dako, Cambridgeshire, UK), followed by fluorescein

isothiocyanate (FITC)-conjugated secondary antibody (goat F(ab')₂ anti-mouse IgG; Sigma). After counting *C. pneumoniae* inclusions under a fluorescence microscope and correcting for dilution factors, bacterial titres were expressed as inclusion-forming units (IFU/cell).

Human peripheral blood mononuclear cells preparation and infection with *Chlamydia pneumoniae*

PBMCs were isolated from the blood of study subjects through centrifugation on a standard Ficoll gradient. The interface containing the mononuclear cell fraction was washed with Dulbecco's PBS by centrifugation and resuspended in RPMI 1640 medium (BioWhittaker, Walkersville, MD, USA), and cultured in RPMI 1640 medium supplemented with 10% heat-inactivated (30 min at 56 °C) fetal calf serum, 40 μ mol/L L-glutamine, 100 U/mL penicillin G sodium, 100 U/mL streptomycin, and 20 mmol/L HEPES buffer solution (GIBCO BRL Life Technologies, Grand Island, NY, USA). Immediately after *Chlamydia* inoculation (0.6 IFU/cell), the samples were centrifuged at 35 °C for 1 h at 550 g to facilitate contact between cells and bacteria. *C. pneumoniae* infection in PBMCs was confirmed by an IF assay, described above.

The study protocol was approved by an independent ethical review board of the institute, and written informed consent was obtained from each study subject.

Cellular proliferation and responsiveness to corticosteroid of *Chlamydia pneumoniae*-infected peripheral blood mononuclear cells

First, we evaluated whether cellular proliferation after *in vitro* *C. pneumoniae* infection is enhanced and whether the effect is different in Th2-dominant microenvironment. Cellular proliferation in *C. pneumoniae*-infected or uninfected PBMCs exposed to a Th2-dominant microenvironment was assessed using PBMCs isolated from *Dermatophagoides farinae*-sensitized atopic subjects and cultured with 10 μ g/mL of Der f 2 (kindly donated from Professor Sang-Heon Cho, Seoul National University, Seoul, Korea) and 20 ng/mL of human IL-4. As controls, we used PBMCs isolated from non-atopic subjects and cultured with or without *C. pneumoniae* (0.6 IFU/cell) in the absence or presence of Der f 2 or IL-4 for 72 h. Cellular proliferation was determined using ³H-thymidine uptake previously described. In brief, 0.5 μ Ci ³H-thymidine was added to each well, and cells from the ³H-thymidine-pulsed wells were collected into polystyrene test tubes after 16 h. Excess radioactive material was washed out by sequential centrifugations in PBS, pH 7.4, and the radioactivity in each cell sample was measured using a scintillation counter, with the results reported as counts per minute (c.p.m.).

To evaluate responsiveness to corticosteroid treatment, the PBMCs from each experimental group described above were cultured in the presence or absence of dexamethasone (10⁻⁹, 10⁻⁷, or 10⁻⁵ M). Cellular proliferation was measured after 72 h. Assuming that individual variations in basal c.p.m. values may render the mean value of actual figures biased, the percent decrease in cellular proliferation was calculated based on the c.p.m. value obtained from dexamethasone-free cultures (basal proliferation) of each enrolled sample under each experimental condition.

Effect of Chlamydia pneumoniae infection in peripheral blood mononuclear cells on corticosteroid-induced apoptosis of lymphocytes

Although the results of the experiments described above showed that the cellular proliferation in *C. pneumoniae*-infected PBMCs was significantly enhanced in Th2-dominant microenvironment (Fig. 1), similar degree of decreased responsiveness to corticosteroid was observed in both Th2 dominant and control microenvironment (Fig. 2). We, therefore, decided to perform further experiments for responsiveness to corticosteroid using only the PBMCs obtained from control subjects.

PBMCs were plated at 2×10^5 cells per well in 96-well round bottom culture plates and were grown in 100 μ L of culture medium containing dexamethasone (10^{-9} – 10^{-5} M) or *C. pneumoniae* (0.6 IFU/cell). To evaluate the pro-apoptotic effect of corticosteroid on lymphocytes, we measured cellular apoptosis by flow cytometry after 12, 36, and 72 h in culture. Apoptosis was measured by staining PBMCs with propidium iodide (PI) and an FITC-conjugated mAb to Annexin V (Annexin V-kit; MedSystems Diagnostics GmbH, Vienna, Austria). To determine apoptosis within specific cell populations, samples were labelled with PE-conjugated mAb to CD4 or CD8 (DK-2600; Dako A/S, Glostrup, Denmark) and FITC-conjugated mAb to Annexin V. Flow cytometric analysis was performed using an FACS operating system with Cellquest software (Becton Dickinson, Mountain View, CA, USA). The total population of viable cells was gated according to their typical forward and right angle light scattering. The percentage of cells positive for Annexin V or PI/Annexin V was determined relative to living cells.

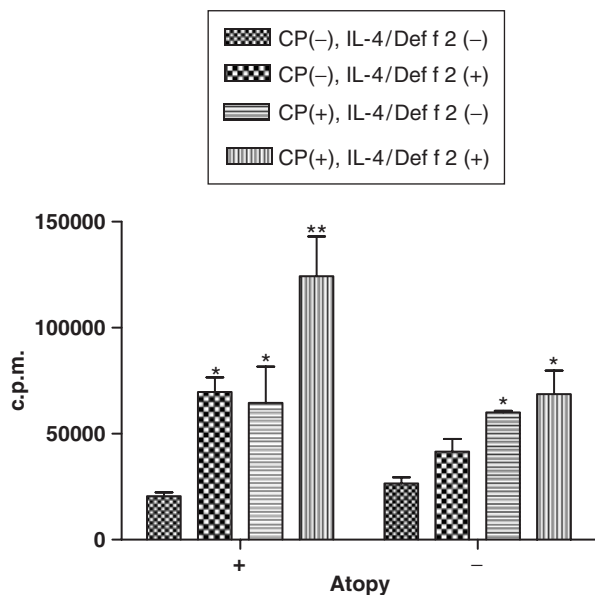


Fig. 1. Peripheral blood mononuclear cells (PBMCs) were stimulated in the presence or absence of a combination of IL-4 (20 ng/mL) and Der f 2 (10 μ g/mL) for 72 h following infection with CP (0.6 inclusion-forming unit (IFU)/cell). And then, the cellular proliferation was determined by ³H-thymidine incorporation following incubation for 16 h. The results were presented as mean $\pm$ SEM. * $P < 0.05$, compared with uninfected PBMCs not exposed to IL-4 and Der f 2. ** $P < 0.05$, compared with infected PBMCs exposed to IL-4 and Der f 2 in the atopic group. CP, *Chlamydia pneumoniae*; c.p.m., means counts per minute; $n = 5$ in each experiment.

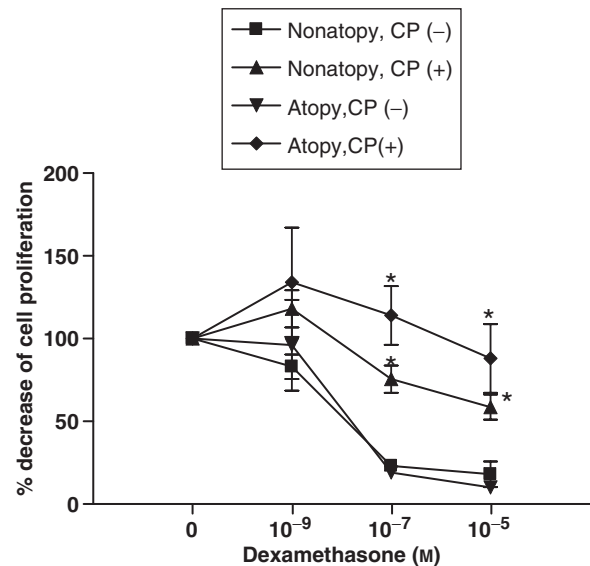


Fig. 2. CP-infected (0.6 inclusion-forming unit (IFU)/cell) or uninfected peripheral blood mononuclear cells (PBMCs) were cultured in the various concentrations of dexamethasone (10^{-9} , 10^{-7} , and 10^{-5} M) for 72 h. Then, the cellular proliferation was measured using ³H-thymidine uptake. The percent decrease in cellular proliferation was calculated based on the c.p.m. value obtained from dexamethasone-free cultures of each enrolled sample under each experimental condition. The results were presented as mean $\pm$ SEM. * $P < 0.05$, compared with uninfected PBMCs. c.p.m., counts per minute; CP, *Chlamydia pneumoniae*; $n = 7$ in each experiment.

Effect of tumor necrosis factor- α blocker on cellular proliferation and responsiveness to corticosteroid of Chlamydia pneumoniae-infected peripheral blood mononuclear cells

At first, TNF- α concentrations in the culture supernatants of each experimental condition were measured using a commercial ELISA kit (R&D Systems, Minneapolis, MN, USA). To evaluate the effect of blocking TNF- α , we added various concentrations of infliximab, an antagonistic mAb to TNF- α (0.1, 1, 10 ng/mL) to each culture, as described above, and we measured cellular proliferation and apoptosis after 72 h at the concentration of 10^{-7} M of dexamethasone in culture. The degree of cellular proliferation or apoptosis in *C. pneumoniae* infected PBMCs was compared with that in uninfected PBMCs in each concentration of infliximab.

Statistical analysis

All data are presented as mean $\pm$ SEM. Statistical comparisons were made using the Mann–Whitney *U*-test. *P*-values of < 0.05 were considered statistically significant.

Results

Chlamydia pneumoniae infection enhances peripheral blood mononuclear cell proliferation in T-helper type 2-dominant microenvironment and reduces responsiveness to corticosteroids

In the atopic group, we found that cellular proliferation was significantly enhanced by IL-4 and Der f 2 treatment, and further enhanced after *in vitro* *C. pneumoniae* infection. In

contrast, the non-atopic group showed no response to IL-4 or Der f 2, but did show a significant increase in cellular proliferation following infection with *C. pneumoniae* (Fig. 1).

In the absence of *C. pneumoniae* infection, proliferation of PBMCs was inhibited by dexamethasone in a dose-dependent manner, in both a Th2-dominant microenvironment of atopic group and under control conditions (Fig. 2). The anti-proliferative effect of corticosteroid was significantly lower in *C. pneumoniae*-infected PBMCs than in uninfected PBMCs in both the control and Th2-dominant microenvironments (Fig. 2).

Chlamydia pneumoniae infection inhibits steroid-induced apoptosis of lymphocytes

We evaluated our hypothesis that *C. pneumoniae* infection enhances cell survival by reducing the pro-apoptotic effect of corticosteroids on T lymphocytes. We found that the pro-apoptotic effect of corticosteroid on CD4⁺ (Table 1, Fig. 3) and CD8⁺ (data not shown) T lymphocytes was reduced in *C. pneumoniae*-infected PBMCs compared with uninfected PBMCs.

Tumor necrosis factor- α inhibitor reverses the effect of *Chlamydia pneumoniae* infection

In response to *C. pneumoniae* infection, we found that TNF- α secretion was up-regulated for at least 72 h, whereas dexamethasone significantly reduced production of TNF- α (Fig. 4). In addition, infliximab treatment effectively reduced TNF- α levels in the culture supernatants under various experimental conditions (Fig. 4).

We have observed the effect of infliximab on cellular proliferation in the absence of dexamethasone. In the atopic group, we found a two-component proliferation increase. IL-4/Der f 2 treatment was not effectively suppressed by blockade of TNF- α , but that the increased cellular proliferation induced by *C. pneumoniae* infection was almost completely reversed by infliximab treatment (Fig. 5). In the non-atopic group, infliximab effectively suppressed the enhanced cell proliferation induced by *C. pneumoniae* infection.

In dexamethasone-treated cultures, infliximab also partially inhibited the cellular proliferation enhanced by *C. pneumoniae* infection in a dose-dependent manner when compared with the degree of proliferation in uninfected PBMCs (Fig. 6). The reduced pro-apoptotic effects of corticosteroid on T lymphocytes in *C. pneumoniae*-infected PBMCs was also

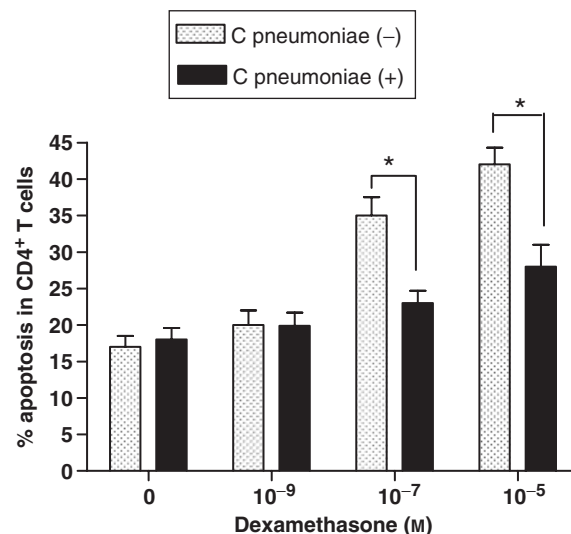


Fig. 3. *Chlamydia pneumoniae*-infected (0.6 inclusion-forming units (IFU)/cell) or uninfected peripheral blood mononuclear cells (PBMCs) were cultured in the medium containing dexamethasone (10^{-9} – 10^{-5} M) for 72 h. And then, the apoptosis of CD4⁺ T cells was measured by flow cytometry after staining PBMCs with propidium iodide (PI), an fluorescein isothiocyanate-conjugated monoclonal antibody (mAb) to Annexin V, and PE-conjugated mAb to CD4. The percentage of cells positive for Annexin V or PI/Annexin V was determined relative to living cells. The results were presented as mean $\pm$ SEM. * P < 0.05, compared with uninfected PBMCs. n = 7 in each experiment.

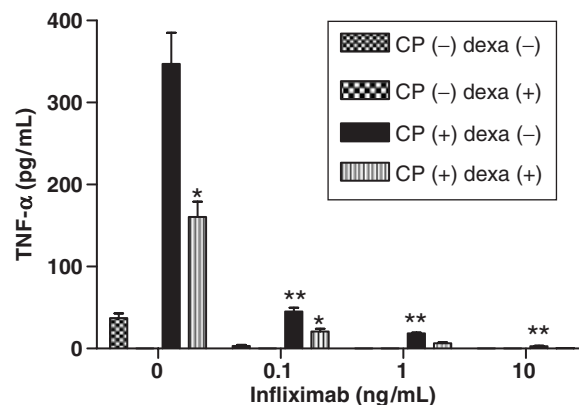


Fig. 4. *Chlamydia pneumoniae*-infected (0.6 inclusion-forming units (IFU)/cell) or uninfected peripheral blood mononuclear cells (PBMCs) were cultured with or without dexamethasone (10^{-7} M) and the various concentrations of infliximab (0.1, 1, 10 ng/mL) were added. After 72 h culture, the levels of TNF- α were measured in the culture supernatants by using ELISA method. * P < 0.05, compared with the values of culture supernatants of *C. pneumoniae*-infected PBMCs without dexamethasone treatment. ** P < 0.05, compared with the values of culture supernatants without infliximab treatment. n = 5 in each experiment.

Table 1. Percentage of apoptotic CD4⁺ T cells in each experimental condition

	Dexta (-), %	Dexta (10^{-7} M), %
12 h, CP infection -	6.1 $\pm$ 2.7	12.3 $\pm$ 2.8
CP infection +	4.8 $\pm$ 2.6	7.9 $\pm$ 2.7
36 h, CP infection -	11.9 $\pm$ 4.3	29.8 $\pm$ 4.9
CP infection +	8.2 $\pm$ 2.4	19.7 $\pm$ 2.8*
72 h, CP infection -	18.3 $\pm$ 4.2	32.9 $\pm$ 3.9
CP infection +	16.2 $\pm$ 3.5	26.2 $\pm$ 2.5*

* P < 0.05, compared with uninfected peripheral blood mononuclear cells. CP, *Chlamydia pneumoniae*; Dexta, dexamethasone; n = 5 in each experiment.

reversed by treatment with infliximab in a dose-dependent manner (Fig. 7).

Discussion

We have shown here that *in vitro* *C. pneumoniae* infection of PBMCs enhanced cellular proliferation, especially in Th2 microenvironments, and reduced the effects of corticosteroid

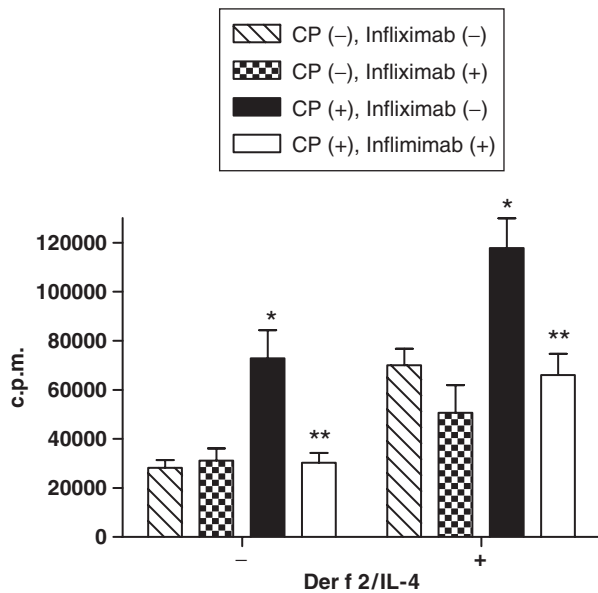


Fig. 5. *Chlamydia pneumoniae* infected (0.6 inclusion-forming units (IFU)/cell) or uninfected peripheral blood mononuclear cells (PBMCs) were cultured with or without a combination of IL-4 (20 ng/mL) and Der f 2 (10 µg/mL) in the medium containing infliximab (10 ng/mL) or not. After 72 h culture, the cellular proliferation was determined by using ³H-thymidine uptake method. The results were presented as mean ± SEM. **P* < 0.05, compared with the proliferation of *C. pneumoniae* uninfected PBMCs. ***P* < 0.05, compared with PBMC proliferation in the absence of infliximab. PBMCs obtained from *Dermatophagoides farinae*-sensitized atopic subjects. *n* = 5 in each experiment.

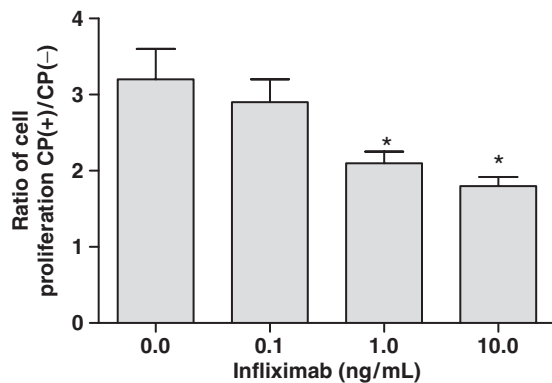


Fig. 6. Cellular proliferation was measured after 72 h cultures of *Chlamydia pneumoniae*-infected (0.6 inclusion-forming units (IFU)/cell) or uninfected peripheral blood mononuclear cells (PBMCs) at the concentration of 10^{-7} M of dexamethasone and various concentrations of infliximab (0.1, 1, 10 ng/mL). The degree of cellular proliferation in *C. pneumoniae*-infected PBMCs was compared with that in uninfected PBMCs in each concentration of infliximab. The results were presented as mean ± SEM. **P* < 0.05, compared with the ratio of cell proliferation in the absence of infliximab. *n* = 7 in each experiment.

on immune and inflammatory cells. Furthermore, blocking the TNF-α pathway effectively reversed these phenomena at least partially.

The clinical manifestations of asthma patients are heterogeneous, with some showing severe phenotypes that lead to considerable difficulties in their management. Although the mechanisms underlying severe asthma are not completely understood, patients with severe asthma may have higher airway inflammation than patients with mild and moderate

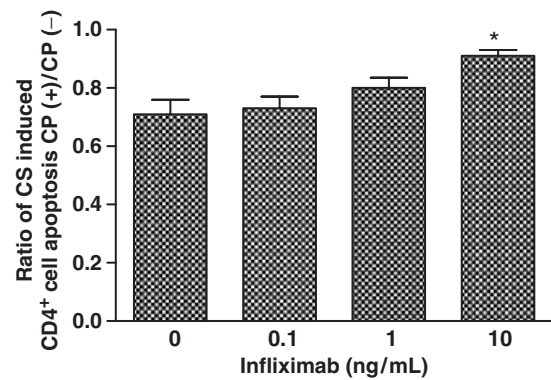


Fig. 7. Corticosteroid-induced apoptosis was measured after 72 h cultures of *Chlamydia pneumoniae*-infected (0.6 inclusion-forming units (IFU)/cell) or uninfected peripheral blood mononuclear cells (PBMCs) at the concentration of 10^{-7} M of dexamethasone and various concentrations of infliximab (0.1, 1, 10 ng/mL). The degree of cellular apoptosis in *C. pneumoniae*-infected PBMCs was compared with that in uninfected PBMCs in each concentration of infliximab. The results were presented as mean ± SEM. **P* < 0.05, compared with the ratio of apoptosis in the absence of infliximab. *n* = 7 in each experiment.

asthma. Although no conclusions can be drawn about the causal relationship between *C. pneumoniae* infection and asthma, several arguments favour the hypothesis that *C. pneumoniae* is involved in the pathogenesis of asthma that presents with a severe clinical course and irreversible airway obstruction [2–4, 19].

We have attempted to evaluate the hypothesis that intracellular infection with bacteria, such as *C. pneumoniae*, is associated with enhanced proliferation and survival of immune and inflammatory cells. We have shown here that *C. pneumoniae* infection augmented PBMC proliferation, suggesting that *C. pneumoniae* infection may be associated with augmentation of the underlying inflammation in asthma patients.

In our study, it seems that there are two components to the cellular proliferation – one induced by Der f 2/IL-4 and the other induced by *Chlamydia* infection. That is, the proliferation of *C. pneumoniae*-infected PBMCs of atopics was further significantly enhanced after exposure to allergen and IL-4, suggesting that PBMCs exposed to this microenvironment may be more susceptible to increased intracellular replication and may induce more enhanced immune responses. These results might be caused by the immune stimulating effect of allergen or the suppressive effect of IL-4 on the production of INF-γ, the most important cytokine for protecting host cells from chlamydial infection [20]. However, IL-4 alone may not play a major role in the enhancement of cellular proliferation in view of unremarkable effect of IL-4 in non-atopic group. In the mean time, the cellular proliferation induced by IL-4/Der f 2 treatment was not effectively suppressed by blockade of TNF-α, while that caused by *C. pneumoniae* infection was almost completely reversed by infliximab treatment. Collectively, these results suggest that the functional mechanism by which cellular proliferation is induced may differ between allergens and infectious agents such as *Chlamydia*. Further studies would be needed to investigate the exact mechanisms by which this occurs.

Corticosteroids are the most important class of drug used for asthma management; however, clinical responsiveness to corticosteroid treatment is quite heterogeneous in asthma

patients. Patients with severe asthma are relatively resistant to the therapeutic effects of corticosteroids and require higher amounts of these drugs, even systemic medication. Although the exact anti-asthmatic mechanism of corticosteroids has not been clarified, these agents have shown suppressive effects on cellular and molecular pathways, including the inhibition of pro-inflammatory transcription factors such as nuclear factor- κ B (NF- κ B) and anti-proliferative and pro-apoptotic effects on immune and inflammatory cells. In evaluating our second hypothesis, that *C. pneumoniae* infection enhances resistance to the therapeutic effects of corticosteroids, we found that resistance to corticosteroid treatment was induced following *C. pneumoniae* infection of immune cells, as shown by assays of cell proliferation and corticosteroid-induced apoptosis. These results suggest that steroid resistance is induced by *C. pneumoniae*, and, to our knowledge, this finding is the first to show a causal relationship between *C. pneumoniae* infection and corticosteroid resistance in immune cells.

We also investigated the role of a TNF- α blocker in the increase cellular proliferation and steroid resistance in *C. pneumoniae*-infected immune cells. TNF- α , a cytokine produced by macrophages and monocytes upon infection with *C. pneumoniae*, has been shown to play a central role in many immunologic and inflammatory diseases. TNF- α has various immunoregulatory activities, including enhancing the growth of T and B lymphocytes, activating macrophages, and inducing the expression of pro-inflammatory mediators such as IL-6, IL-1, vascular adhesion molecules, intercellular adhesion molecule, and IL-8, all of which may be associated with the aggravated clinical course observed in asthma. In addition, TNF- α is one of the prime activators of the transcription factor NF- κ B, which may be associated with the development of steroid resistance. We found that secretion of TNF- α was dramatically increased when PBMCs were infected with *C. pneumoniae* and that a TNF- α inhibitor effectively reversed the effects of *C. pneumoniae* infection on immune cells at least partially.

In the absence of dexamethasone, the increased cellular proliferation induced by *Chlamydia* infection was suppressed to baseline levels by treatment with infliximab. However, the *Chlamydia*-associated corticosteroid resistance as analyzed by cellular proliferation was partially reversed by infliximab in the presence of dexamethasone, suggesting that processes other than TNF- α signalling may be involved in the development of altered corticosteroid sensitivity. The cellular proliferation may occur through complicated mechanisms, as corticosteroids directly affect cell survival and cellular proliferation independent of TNF. This suggests that the mechanism governing the TNF-based response to *Chlamydia* infection and corticosteroids may be quite complex. Further work will be required to clarify the exact mechanism of these differences.

With respect to the effect of infliximab on steroid-induced T cell apoptosis, a further reverse effect was observed compared with the effect on the cellular proliferation. Considering that infliximab has been reported to directly induce apoptosis in T cells in a manner independent of TNF blockade [21, 22], the effect of infliximab on corticosteroid-induced apoptosis observed in our study suggests that either infliximab has additional direct pro-apoptotic effects, or that there are complex interactions between corticosteroid, TNF, cellular proliferation, and apoptosis.

Taken together, these findings in our study suggest that some degree of steroid resistance observed in severe asthma may be dependent, at least in part, on the TNF- α pathway. In addition, our results also suggest that the drugs, which could block TNF- α pathway may be a candidate agent for the management of severe steroid-dependent or -resistant asthmatics. However, confirmation of a causal relationship between severe asthma and chronic *Chlamydia* infection and the investigation of the exact role and the risks of spreading the underlying chronic indolent infection when using infliximab, must be clarified. Furthermore, it should be considered that the *in vitro* conditions examined here may be quite different from the conditions *in vivo*.

In conclusion, the results of this study suggest that *C. pneumoniae* infection, may induce an increase in inflammatory cells and may aggravate the underlying inflammation through a TNF- α pathway locally in asthmatic airways. However, further studies should be needed to clarify the exact functioning mechanism of TNF- α on immune cells exposed to *C. pneumoniae* and corticosteroid.

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References

- Grayston JT, Campbell LA, Kuo CC et al. A new respiratory tract pathogen: *Chlamydia pneumoniae* strain TWAR. *J Infect Dis* 1990; 161:618–25.
- Von HL, Vasankari T, Liippo K, Wahlstrom E, Puolakkainen M. *Chlamydia pneumoniae* and severity of asthma. *Scand J Infect Dis* 2002; 34:22–7.
- Hahn DL, Bukstein D, Luskin A, Zeitz H. Evidence for *Chlamydia pneumoniae* infection in steroid-dependent asthma. *Ann Allergy Asthma Immunol* 1998; 80:45–9.
- Black PN, Scicchitano R, Jenkins CR et al. Serological evidence of infection with *Chlamydia pneumoniae* is related to the severity of asthma. *Eur Respir J* 2000; 15:254–9.
- ten Brinke A, van Dissel JT, Sterk PJ, Zwinderman AH, Rabe KF, Bel EH. Persistent airflow limitation in adult-onset nonatopic asthma is associated with serologic evidence of *Chlamydia pneumoniae* infection. *J Allergy Clin Immunol* 2001; 107:449–54.
- Miyashita N, Kubota Y, Nakajima M, Niki Y, Kawane H, Matsushima T. *Chlamydia pneumoniae* and exacerbations of asthma in adults. *Ann Allergy Asthma Immunol* 1998; 80: 405–9.
- Hahn DL, McDonald R. Can acute *Chlamydia pneumoniae* respiratory tract infection initiate chronic asthma? *Ann Allergy Asthma Immunol* 1998; 81:339–44.
- Espósito S, Blasi F, Arosio C et al. Importance of acute *Mycoplasma pneumoniae* and *Chlamydia pneumoniae* infections in children with wheezing. *Eur Respir J* 2000; 16:1142–6.

- 9 Kutlin A, Roblin PM, Hammerschlag MR. Effect of prolonged treatment with azithromycin, clarithromycin, or levofloxacin on *Chlamydia pneumoniae* in a continuous-infection model. *Antimicrob Agents Chemotherapy* 2002; 46:409–12.
- 10 Rodel J, Woytas M, Groh A et al. Production of basic fibroblast growth factor and interleukin 6 by human smooth muscle cells following infection with *Chlamydia pneumoniae*. *Infection and Immunity* 2000; 68:3635–41.
- 11 Kaukoranta-Tolvanen SS, Teppo AM, Laitinen K, Saikku P, Linnavuori K, Leinonen M. Growth of *Chlamydia pneumoniae* in cultured human peripheral blood mononuclear cells and induction of a cytokine response. *Microb Pathogen* 1996; 21:215–1.
- 12 Carratelli CR, Rizzo A, Catania MR et al. *Chlamydia pneumoniae* infections prevent the programmed cell death on THP-1 cell. *FEMS Microbiol Lett* 2002; 215:69–74.
- 13 Airene S, Surcel HM, Tuukkanen J, Leinonen M, Saikku P. *Chlamydia pneumoniae* inhibits apoptosis in human epithelial and monocyte cell lines. *Scand J Immunol* 2002; 55:390–8.
- 14 Rajalingam K, Al-Younes H, Muller A, Meyer TF, Szczepek AJ, Rudel T. Epithelial cells infected with *Chlamydia pneumoniae* are resistant to apoptosis. *Infect Immun* 2001; 69:7880–8.
- 15 Redecke V, Dalhoff K, Bohnet S, Braun J, Maass M. Interaction of *Chlamydia pneumoniae* and human alveolar macrophages: infection and inflammatory response. *Am J Respir Cell Mol Biol* 1998; 19:721–7.
- 16 Caspar-Bauguil S, Puissant B, Nazzari D et al. *Chlamydia pneumoniae* induces interleukin-10 production that down-regulates major histocompatibility complex class I expression. *J Infect Dis* 2000; 182:1394–401.
- 17 Ohta K, Yamashita N. Apoptosis of eosinophils and lymphocytes in allergic inflammation. *J Allergy Clin Immunol* 1999; 104: 14–21.
- 18 Feldmann M, Brennan FM, Maini RN. Role of cytokines in rheumatoid arthritis. *Ann Rev Immunol* 1996; 14:397–440.
- 19 von HL. Role of persistent infection in the control and severity of asthma: focus on *Chlamydia pneumoniae*. *Eur Respir J* 2002; 19:546–6.
- 20 Airene S, Surcel HM, Bloigu A, Laitinen K, Saikku P, Laurila A. The resistance of human monocyte-derived macrophages to *Chlamydia pneumoniae* infection is enhanced by interferon-gamma. *APMIS* 2000; 108:139–44.
- 21 Mitoma H, Horiuchi T, Hatta N et al. Infliximab induces potent anti-inflammatory responses by outside-to-inside signals through transmembrane TNF-alpha. *Gastroenterology* 2005; 128:376–92.
- 22 van den Branda J, Hommes DW, Peppelenbosch MP. Infliximab induced T lymphocyte apoptosis in Crohn's disease. *J Rheumatol* 2005; 74 (Suppl.):26–30.