# Investigation of drugs influencing the outcome of *Chlamydia pneumoniae* infection and the immune response induced by the pathogen

**Ph.D. thesis**

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# 1. Introduction

- **General characteristics of *Chlamydia* infections**

Chlamydiae are Gram negative, obligate intracellular bacteria with biphasic developmental cycle and can infect wide range of host species from amoebae to humans. *C. pneumoniae* is responsible for community-acquired atypical pneumonia, bronchitis, pharyngitis, and sinusitis and is implicated in the development of severe asthma and acute exacerbations.

Chlamydia infections elicit predominantly cellular responses and trigger IFN-γ production of $CD4^{+}$ and $CD8^{+}$ T-cells. IFN-γ activates the indolamine 2,3-dioxygenase (IDO) that catabolises L-tryptophan to N-formylkynurenine, resulting in the deprivation of tryptophan. As *Chlamydiae* are tryptophan auxotroph bacteria, this mechanism could lead to the elimination of the infectious chlamydia or cause persistent state. A transcriptome analysis of IFN-γ exposed, *Chlamydia*-infected murine epithelial cells revealed that IFN-γ significantly enhanced the expression of T-cell chemokines, MIG/CXCL-9, IP-10/CXCL-10, I-TAC/CXCL-11 and increased the expression levels of genes involved in neutrophil and monocyte attracting. IDO activity can be triggered by various factors such as IL-10, TNF-α, IFN-γ or oxidative stress and plays a pivotal role in eliminating viral or bacterial infections. A previous study demonstrated that neutralization of IL-17A in *C. pneumoniae* infected mice resulted in a higher burden of bacterial load, decreased neutrophil influx and diminished cytokine levels, suggesting that IL-17A could provoke anti-chlamydial activity indirectly through initiating neutrophilic inflammation.

- **The mechanism of infection-mediated asthma with focus on *C. pneumoniae***

Evidence of the association of *C. pneumoniae* infection with asthma disease severity have accumulated since several studies reported that persistent *C. pneumoniae* infection contributes to severe, chronic asthma and is a risk factor in developing asthmatic symptoms and exacerbations. Higher levels of *C. pneumoniae* antibodies provided evidence of the role of infection in the outcome of asthma. Of note, serological markers also determined a decline in lung function, reflecting an enhanced airflow limitation in *C. pneumoniae*-infected patients with asthma. *C. pneumoniae* can also affect cytokine responses in patients with asthma and amend therapeutic outcomes. *C. pneumoniae* can induce the secretion of IL-8, TNF-α in peripheral blood mononuclear cells (PBMCs), enhance the NF-κB activity, and trigger IFN-γ responses in airway epithelial cells.

- **Corticosteroid treatment in patients with asthma and its associations with respiratory infections**

Inhaled corticosteroids (ICSs) are regarded as the most effective treatment for asthma and chronic obstructive pulmonary disease (COPD) to reduce the risk of exacerbation and improve lung function, however, ICSs have been associated with increased risk of pneumonia. Earlier studies have provided controversial data about the potential risk of pneumonia in patients using ICSs and emphasise on their difference in the mechanism of action. It is well established that budesonide (BUD) and fluticasone propionate (FP) show differences in their pharmacokinetic, physicochemical and even in immunosuppressive properties, which can explain their distinct effects on respiratory infections and exacerbations.

- **Steroid-resistant asthma and its associations with *C. pneumoniae* infection**

Approximately 5% to 10% of asthmatic patients fail to fully respond to steroid therapy, and these patients also show higher mortality and morbidity rates. A diagnosis of steroid resistance is established when patients exhibit <15% improvement in the forced expiratory volume in one second (FEV1) during post-bronchodilator spirometry after 14 days of oral prednisolone therapy. Asthmatics with earlier *C. pneumoniae* infection are more likely to develop steroid-resistant asthma, and their positive serostatus is associated with an increased severity of asthma and airway neutrophilia.

IL-10 plays a crucial role in maintaining lung immune responses and participates in asthma pathogenesis by regulating and inhibiting Th2 responses. Asthmatic patients exhibit diminished IL-10 production in bronchoalveolar lavage (BAL) fluids and, to the best of our knowledge, there are no congruent data about the IL-10 production by the peripheral blood cells of these patients.

TNF-α responses play a significant role in AHR via eosinophil and neutrophil attraction, and *C. pneumoniae* is able to induce TNF-α production and trigger cellular proliferation, leading to decreased steroid responsiveness of peripheral blood mononuclear cells (PBMCs). A previous *C. pneumoniae* infection could have a long-term effect on TNF-α response, hence, we investigated TNF-α secretion by PBMCs of *Chlamydia*-specific IgG negative and positive patients.

Matrix metalloproteinases (MMPs) and their inhibitors are involved in the changes of the extracellular matrix and determine airway epithelium thickness. MMP-9 has a pivotal role in remodelling and was the first type of MMP to be investigated in asthma. *C. pneumoniae* affects MMP-9 and tissue inhibitor of metalloproteinase-1 (TIMP-1) production by PBMCs and weakens the impact of glucocorticoids on the secretion of MMPs.

## 2. Aims and objectives

- **Aim 1. Effects of ICS in *in vitro* and *in vivo C. pneumoniae* infection**

We investigated the effects of FP and BUD treatment in A549 cells and infected mouse model. We hypothesised that *C. pneumoniae* replication and infection-induced immune responses, especially anti-chlamydial IFN-γ, and IFN-related chemokine production (MIG/CXCL-9), IFN-γ triggered gene expressions (IDO1, IDO2, TDO) and cytokine production (IL-4, IL-10, IL-17A) could be influenced by the administration of ICSs. We assessed the effects of FP and BUD on the *in vitro* and *in vivo* growth of *C. pneumonia*e.

- **Aim 2. Differences in cytokine production between steroid-resistant and -sensitive asthmatics *in vitro***

We hypothesised that determining IL-10 responses in steroid-resistant and -sensitive asthmatics proves that a former infection induces alterations in a different manner in asthma phenotypes. MMP-9 is implicated in the remodelling process of the lung and is believed to be influenced by *C. pneumoniae* infection. As there are no data available regarding MMP-9 levels in steroid-sensitive and -resistant asthmatics, we intended to define differences in the patients' sera according to the *C. pneumoniae* serostatus and steroid responsiveness.

## 3. Materials and Methods

- ***In vitro* study design**

A549 human airway epithelial cells were pre-treated and incubated for 24 h at 37 °C, 5% $CO_2$ with FP or BUD or left untreated. The highest non-toxic drug concentrations (FP: 3.5 x $10^{-4}$ mM, BUD: 7 x $10^{-4}$ mM) determined by the 3- (4,5-dimethylthiazol -2yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) cytotoxicity test. After 24 h treatment, the wells were washed twice with phosphate buffered saline (PBS) and the cells were infected with *C. pneumoniae* at a multiplicity of infection (MOI) of 0.01. The cells were inoculated in 0.5% (w/v) glucose medium, and centrifuged (800*g*, 60 min), followed by the addition of FP or BUD to the wells. Control infected cells were left untreated. After infection, the plates were incubated at 37 °C, under 5% $CO_2$ for 48 h. Subsequently, the wells were washed twice with PBS and 100 μL sucrose-phosphate-glutamic acid (SPG) solution was added to each well. The plates were subjected to two freeze–thaw cycles with a quick freezing (-80 °C, 15 min) to obtain cell lysates, which were used directly as templates for quantitative polymerase chain reaction (qPCR). To evaluate *C. pneumoniae* propagation, direct qPCR was performed.

- **Inoculum preparation and immunostaining**

*C. pneumoniae* strain CWL-029, kindly gifted by Agathe Subtil (Pasteur Institute, Paris, France), was propagated on HEp-2 cells. The EBs were partially purified with concentration, and subsequently aliquoted in SPG, followed by storage at -80 °C until further use. Indirect immunofluorescence was performed to determine the concentration of infectious *C. pneumoniae* EBs. Serial dilutions of purified EBs were inoculated onto McCoy cell monolayers. After incubation for 48 h, the infected cells were fixed with acetone at -20°C and stained with monoclonal anti-Chlamydia LPS antibody and FITC-labelled anti-mouse IgG. The number of *C. pneumoniae* inclusions was counted under a UV microscope and the titre was expressed as inclusion forming units (IFU)/mL.

- **Corticosteroid treatment in mice**

FP and BUD powder were obtained, and dimethyl sulfoxide (DMSO) was used as a vehicle for the drugs. Mice were exposed to nebulized BUD (40 µg, 1000 µg/kg) and FP (25 µg, 625 µg/kg) in an inhalation chamber for 15 min once a day, as described previously. We used BUD and FP at equivalent concentrations with the ratio FP:BUD = 1:1.6, based on former clinical studies and the higher potency of FP.

- **Animals and experimental design**

Female BALB/c mice (6-8-weeks-old) were obtained from Charles River Laboratories (Hungary). The mice were kept under standard husbandry conditions at the animal facility of the Department of Medical Microbiology and Immunobiology, University of Szeged. Animals were fed regular mouse chow and provided with water *ad libitum*. The mice were randomly divided into three groups: the control, the BUD-treated and FP-treated (n=16 in each group). Mice received either BUD, FP, or vehicle alone, for three days prior to infection, and then for seven days after infection. On day 3, the mice were sedated with intraperitoneal injection of sodium pentobarbital (200 µL, 7.5 mg/mL) and were infected with $5x10^5$ IFU *C. pneumoniae* in 20 µL SPG. On day 10, i.e. seven days after infection, the mice were anaesthetised and sacrificed. The lungs were removed and homogenised with acid-purified sea sand using a mortar with a pestle. One half of the homogenised lungs was prepared for total RNA extraction, and the other half was suspended in 1 mL SPG for the detection of recoverable *C. pneumoniae* and for cytokine measurements. The experiments were implemented with the approval of the Animal Welfare Committee of the University of Szeged, Hungary and conformed to the Directive 2010/63/EU.

- **Culturing of *C. pneumoniae* from the lungs**

One half of the homogenised lungs was centrifuged (10 min, 400*g*) and serial dilutions of the supernatants were inoculated onto McCoy cell monolayers and centrifuged (60 min, 800*g*). The number of recoverable *C. pneumoniae* inclusions

was determined by indirect immunofluorescence as described earlier, and expressed in terms of IFU/mL.

- **mRNA extraction and cDNA synthesis**

Total RNA was extracted from the other half homogenised lung tissues of the control (n=12), as well as BUD- and FP-treated mice (n=12 for each group) using TRI reagent according to the manufacturer's protocol. Total RNA concentrations and purity were measured using a NanoDrop spectrophotometer. First-strand cDNA was synthesised from 2 µg of total RNA using Maxima First Strand cDNA Synthesis Kit, and 20 pM random hexamer primer in 20 µL reaction buffer according to the manufacturer's.

- **qPCR validation**

qPCR was performed in a Bio-Rad CFX96 real-time system with SsoFast™ EvaGreen® qPCR Supermix master mix and murine specific primer pairs. Cycle threshold (Ct) values were calculated for β-actin, IDO1, IDO2, TDO, IFN-γ, MIG/CXCL9, IP10/CXCL10, ITAC/CXCL11, VDR, and GR, and the relative gene expression levels were determined by the $2^{-(\Delta\Delta Ct)}$ method. The relative expression level was indicated as 2−(ΔΔCt), where ΔΔCt = ΔCt for the experimental sample – ΔCt for the control sample.

- **Lung histology**

Microscopic examination of the lungs of infected control, as well as BUD- and FP-treated mice (n=4 from each group) was performed. After the removal of the lungs, tissues of individual mice were immediately placed into plastic tubes, pre-filled with 10% formalin, resulting in 1:10 of tissue:formalin ratio. During dissection, tissue samples in the tube were cut into 1 mm slices and embedded into paraffin blocks. Four-micrometre sections were cut, and regular haematoxylin-eosin (HE) staining was performed. All tissue sections were examined by light microscopy.

- **Preparation of the *C. pneumoniae* antigen**

*C. pneumoniae* CWL29 were purified from infected Hep2 cells by density gradient centrifugation and inactivated with formaldehyde treatment. The protein content of the antigen was measured by spectrophotometry, and the antigen was stored at -80 °C until use.

- **Separation and stimulation of PBMCs**

PBMCs from 10 mL heparinized blood were separated using Ficoll gradient, $5 \times 10^5$ cells in three parallel wells were incubated in the presence of 2 µg/mL *C. pneumoniae* antigen or 10 µg/mL polyclonal mitogen (phytohemagglutinin, PHA) or left untreated in 200 mL RPMI medium. Supernatants of the stimulated wells were

harvested 48 h after treatment, aliquoted, and stored at -80 °C until performing the cytokine ELISA.

- ***C. pneumoniae*-specific enzyme-linked immunosorbent assay**

*C. pneumoniae*-specific antibodies from the patients and the controls were detected using the "NovaLisa TM *Chlamydia pneumoniae*" enzyme-linked immunosorbent assay [ELISA] kit. Fifty-fold diluted sera were tested in duplicate in accordance with the manufacturer's instructions for the presence of *C. pneumoniae*-specific IgG.

- **Cytokine and chemokine measurements from the lungs, supernatants of PBMCs and patients' sera**

The supernatants of homogenised lung tissues were centrifuged (12000*g*, 5 min) and ELISA for IFN-γ, IL-4, IL-10, IL17-A, MIG/CXCL-9 was performed according to the manufacturers' instructions. MIG/CXCL-9 concentration was determined using a mouse MIG/CXCL-9 ELISA Kit, and IL-17A was measured using Quantikine mouse IL-17 immunoassay. IFN-γ, IL-4 and IL-10 concentrations were detected with Invitrogen mouse ELISA kits. Sensitivity for IFN-γ, IL-4, IL-10, IL-17A and MIG/CXCL-9 measurements ranged between 15-2000 pg/mL, 4-500 pg/mL, 32-4000 pg/mL, 10.9-700 pg/mL and 2.741-2000 pg/mL, respectively.

The supernatants of the stimulated or untreated PBMCs obtained from patients, were centrifuged (5 min, 1200 rpm) and assayed for the concentrations of IL-10 and TNF-α using Human Mini ELISA Development cytokine kits , while the quantity of MMP-9 in the sera was determined using the human MMP-9 ELISA kit. The sensitivities of the IL-10, TNF-α and MMP-9 measurements were in the range of 23 to 3000, 16 to 2000, and 8.23 to 6000 pg/mL, respectively. The clarified supernatants and sera were tested in duplicate in accordance with the manufacturer's instructions.

- **Study population and participants**

Eighty adult patients with asthma were recruited from the outpatient departments and inpatient wards at the Department of Pulmonology (University of Szeged, Hospital of Chest Diseases, Deszk). The inclusion criteria included clinically stable asthma, persistent asthma symptoms, inhaled steroid use, absence of current exacerbation, and complete follow-up periods. The exclusion criteria included a history of HIV infection, current viral or bacterial infections, chronic immunosuppression or autoimmune disease, cancer, systemic intravenous corticosteroid use (in the past 30 days), and antibiotic treatment (in the past 30 days). As a control group, 40 non-asthmatic, healthy blood donors without obstructive lung diseases, nasal polyposis, allergic rhinoconjunctivitis, cancer, chronic heart disease, autoimmune diseases, and immunosuppression were selected. Patients'

demographic and clinical characteristics were recorded. To investigate cytokine production, 5 mL native and 5 mL unfractionated heparin anticoagulated blood samples were collected from each patient. Before collecting blood samples from the patients with asthma, post-bronchodilator tests were performed. After administering 400 μg inhaled salbutamol, dynamic lung volumes (the FEV1, forced vital capacity [FVC], FEV1/FVC, and forced expiratory flow at 25% to 75% of the pulmonary volume [FEF25/75]) were measured.

## 4. Results

- **FP suppressed *C. pneumoniae* replication in A549 cells**

We assessed *C. pneumoniae* growth in FP- and BUD-treated epithelial cells based on the cycle threshold (Ct) values. FP treatment resulted in significantly higher Ct values, indicating suppressed *C. pneumoniae* growth, compared to that measured in BUD-treated (32.35±0.51 vs. 30.81±0.55, $p<0.01$) and untreated control (32.35±0.51 vs. 31.41±0.39, $p<0.01$) cells.

- **FP inhibited *C. pneumoniae* growth in the lungs of mice**

We found that the viable number of *C. pneumoniae* was significantly lower in FP-treated mice compared to the control group and BUD-treated mice ($p<0.001$).

- **Effects of FP and BUD on *Chlamydia*-infected lung tissue histopathology**

In the haematoxylin-eosin (HE)-stained mouse lung tissues, a distinctive difference in the general blueish appearance of the background in the control and BUD-treated specimen was observed, which was caused by extensive lymphoid infiltration. Similarly, in the FP-treated mouse lung tissues, lymphocytic and plasmacytic infiltration was observed; centriacinar emphysema was also visible, with thin alveolar septa.

- **Effects of FP and BUD treatment on gene expressions related to IFN-γ and corticosteroid responses in *C. pneumoniae*-infected mice**

We revealed that the relative expression of IFN-γ was significantly enhanced in FP-treated mice ($p<0.001$) compared to BUD-treated and control mice. Our results indicated a significantly increased IDO2 expression in the FP-treated mice, compared to the control and BUD-treated group ($p<0.05$). Unexpectedly, we found that BUD significantly decreased the expression of MIG/CXCL9 ($p<0.05$) and IP-10/CXCL10 ($p<0.01$), compared to untreated *C. pneumoniae* infected control mice. Interestingly, our results demonstrated that FP treatment increased VDR expression significantly, compared to control and BUD-treated mice ($p<0.01$), whereas BUD treatment did not affect VDR expression.

- **Anti-chlamydial IFN-γ and MIG/CXCL9 protein production are enhanced by FP treatment**

According to our results, IFN-γ production was significantly increased after FP treatment, compared to untreated infected control ($p<0.05$). We found that FP-treated mice showed a higher protein level of MIG/CXCL9, compared to that in untreated controls and to the BUD-treated mouse lungs ($p<0.01$)

- **Effects of BUD and FP treatment on the secretion of Th17 and Th2 cytokines in *C. pneumoniae*-infected lung tissues**

We found a significantly elevated IL-17A level in FP-treated mouse lungs, compared to the control group ($p<0.01$). Our results indicated unaltered IL-4 production, whereas IL-10 levels changed in a different manner. We detected a significantly higher amount of IL-10 in FP-treated lung tissues, but not in the BUD-treated lungs, in comparison with the untreated infected mice ($p<0.05$).

- **Patient characteristics and demographics**

40 steroid-sensitive asthmatic patients (65% female, 35% male, with a mean age of 59 years) and 40 steroid-resistant asthmatic patients (68% female, 32% male, with a mean age of 63 years) were enrolled. The steroid-resistant group had a mean FEV1 value of 56% ± 0.2%, with a significant difference as compared with the sensitive group with a mean FEV1 value of 72% ± 0.22% ($p = 0.01$).

- ***C. pneumoniae*-specific serological status of asthmatic patients**

The control group representing the average Hungarian population exhibited a 67% seropositivity rate. Surprisingly, we observed a lower *C. pneumoniae* seropositivity rate in asthmatic patients than among the controls. In asthmatic patients, 42% of steroid-sensitive and 47% of steroid-resistant participants were *C. pneumoniae* IgG-positive.

- **IL-10 cytokine production in asthmatics in response to specific (*C. pneumoniae*) and nonspecific phytohemagglutinin (PHA) stimulation**

PBMCs obtained from steroid-resistant and -sensitive asthmatics were cultured with the *C. pneumoniae* antigen or phytohemagglutinin (PHA); or were untreated. Untreated PBMCs from *C. pneumoniae* seropositive, steroid-sensitive patients secreted a significantly higher amount of IL-10 than did those from the *C. pneumoniae* positive non-asthmatic blood donors ($p = 0.04$). The same tendency was observed in the seropositive steroid-resistant group, as their PBMCs spontaneously produced a higher amount of IL-10 than did those of seropositive non-asthmatic blood donors ($p = 0.0002$) Concerning steroid resistance, we compared IL-10 production in *C. pneumoniae* seropositive and seronegative asthmatics. We found that in cases of seropositivity, steroid-resistant patients exhibited significantly higher spontaneous IL-10 cytokine release than did seronegative individuals ($p = 0.02$).

- **TNF-α production in asthmatics in response to specific (*C. pneumoniae*) and nonspecific PHA stimulation**

PBMCs obtained from asthmatics and cultured without stimulation produced a higher amount of TNF-α than did those from non-asthmatics, however these differences did not reach significance. PBMCs from *C. pneumoniae* seropositive steroid-resistant participants spontaneously secreted a higher level of TNF-α than did seropositive steroid-sensitive patients (0.23 ± 0.16 ng/ml vs. 0.08 ± 0.06 ng/ml, $p = 0.05$).

- **MMP-9 production in steroid-sensitive and steroid-resistant asthmatic patients**

The serum level of MMP-9 was measured in *C. pneumoniae* seropositive and seronegative steroid-sensitive and steroid-resistant asthmatics. A significant difference in the serum MMP-9 level was observed among the steroid-resistant participants. *C. pneumoniae* seronegative patients exhibited significantly increased serum levels of MMP-9 as compared with those found in *C. pneumoniae* seropositive asthmatics ($p$=0.01). In association with *C. pneumoniae* seronegativity, a statistically significantly higher MMP-9 level was found in the sera of steroid-resistant patients than in steroid-sensitive patients ($p = 0.04$).

## 5. Discussion

- **Aim 1: The effects of ICSs on *C. pneumoniae* infection *in vitro* and in mice**

Collectively, our reported data inspire several exciting concepts that could have practical outcomes for respiratory physicians. Our *in vitro* results suggested that *C. pneumoniae* growth is suppressed in infected epithelial cells by FP, but not by BUD. Thus, we tested our hypothesis whether FP could inhibit the growth of *C. pneumoniae in vivo*. We found that the number of recoverable *C. pneumoniae* decreased in *C. pneumoniae*-infected mouse lungs after FP treatment, as observed in A549 epithelial cells. It is well established that the IFN-γ is the main regulator of *Chlamydia* elimination, and can trigger various mechanism leading to *Chlamydia* inhibition. IFN-γ exposure induced IDO activity which is responsible for tryptophan degradation. As *Chlamydiae* are tryptophan auxotrophs, increased IDO activity results in a restricted *Chlamydia* growth *in vitro* and *in vivo*. Moreover, IFN-γ enhances the expression of genes involved in innate immunity, thus, contributing further to the mechanisms inhibiting *Chlamydia* infections. Our findings revealed that FP can significantly induce IFN-γ not only at the transcription level, but also at the protein level, in contrast to BUD. Our results demonstrated that MIG/CXCL9 levels increased at the protein level in parallel with IFN-γ after FP administration, compared to that in untreated control and BUD-treated mouse lungs. Interestingly, FP did not alter the expression

of other IFN-γ-inducible chemokines, suggesting that other mechanisms may be playing a role in chemokine expression. Notably, BUD treatment significantly reduced the gene expression levels of the CXCL-10 and CXCL-11. Consistently, ICS treatments have a unique impact on IFN-γ response and chemokine production.

IDO activity is a hallmark of tryptophan depletion and suppression of *Chlamydia* growth in cell cultures and mice. Our results also indicate a significant increase in IL-10 production after FP administration in infected mice; conversely, BUD did not have an enhancing effect. Since it is well-known that FP can increase local secretion of IL-10 *in vivo*, we concluded that the observed IDO activity could have been derived from the additive effects of IFN-γ and IL-10.

Our current findings revealed that FP-treated mice produced significantly higher amounts of IL-17A in response to *C. pneumoniae* infection, suggesting that this increase in IL-17A production might also result in the development of *C. pneumoniae* inhibition, as observed in a previous study, IL-17A can synergize with IFN-γ, playing a protective role in *Chlamydia* infection.

Lastly, we analysed the relative expression of GR and VDR, and found that FP treatment significantly increased VDR expression compared to control and BUD-treated mice. VDR is one of the highly downregulated transcription factors in *Chlamydia*-infected murine cells, suggesting that the elevated VDR expression in our study was due to the influence of FP in mice.

To our knowledge, this is the first study that investigates the effects of FP and BUD on *C. pneumoniae* infection *in vivo* and *in vitro*.

- **Aim 2: The influence of *C. pneumoniae* in steroid- resistant and steroid-sensitive patients with asthma**

We hypothesized that a prior *C. pneumoniae* infection has an impact on IL-10 production in asthmatic patients. We examined cytokine production under different stimuli in steroid-resistant and steroid-sensitive patients. Untreated PBMCs from *C. pneumoniae* seropositive patients secreted a higher level of IL-10 than did those from controls. Our results indicated that increased IL-10 responses are derived from PBMCs, indicating that in asthma pathogenesis, systemic immune responses can alter disease severity and clinical features. In the *C. pneumoniae* seropositive groups, we detected greater spontaneous IL-10 secretion in steroid-resistant individuals than in steroid-sensitive individuals. Additionally, PBMCs from steroid-resistant *C. pneumoniae* seropositive asthmatics produced significantly higher IL-10 responses when untreated and under *C. pneumoniae* stimulation, supporting the notion that earlier *C. pneumoniae* infection contributes to an altered IL-10 response in steroid-

resistant asthmatics. In asthmatics, there is a positive correlation between disease severity and the IL-10 level.

PBMCs from steroid-resistant patients spontaneously secreted significantly higher levels of TNF-α than did those of steroid-sensitive patients, however, under *C. pneumoniae* stimulation no significant differences were detected between healthy volunteers, steroid-sensitive, and steroid resistant asthmatics.

In this study, we measured the serum MMP-9 levels to define differences related to the *C. pneumoniae*-specific IgG serostatus and steroid responsiveness. Steroid-resistant *C. pneumoniae* seropositive patients had a lower MMP-9 level than did seronegative patients.

Taken together, our findings revealed unknown features of asthmatic patients and strengthened the line of evidence that former infection could affect asthma mechanisms.

## 6. Summary

*C. pneumoniae* is a ubiquitous, intracellular bacterium and is implicated in the pathogenesis of obstructive lung diseases, especially playing a decisive and detrimental role in asthma. The associations between ICSs use and pneumonia was intensively examined, but data remain still controversial, as the ICSs showed distinct immunomodulatory effects with regard to cell functions, cytokine and chemokine production and to the expression of host defence genes with antimicrobial activity. We investigated whether FP or BUD can inhibit *C. pneumoniae* growth *in vitro* and found that FP suppressed the *C. pneumoniae*, in contrast to BUD. Concerning our observations, we aimed to test this effect *in vivo*, in a mouse model and investigate further underlying mechanism at gene expression and protein level. We detected significantly higher gene expression of IFN-γ, IDO2 and VDR that could contribute to a potential antimicrobial defence. Besides, we found that in *C. pneumoniae*-infected and FP treated mice IFN-γ response and an IFN-γ inducible chemokine, MIG/CXCL-9, IL-10 and IL-17 were significantly pronounced at protein level. All factors were previously described to reflect anti-chlamydial effects. Indeed, the number of viable *C. pneumoniae* cultured from FP-treated mice lungs was significantly lower compared to BUD-treated or control mice, suggesting the validity of our hypothesis.

Next, we intended to define distinct immunological features of steroid-resistant and -sensitive asthmatic patients based on cytokine production and *C. pneumoniae* serostatus. We observed that steroid-resistant and -sensitive patients show different pattern of IL-10 and TNF-α cytokine production after *C. pneumoniae* antigen stimuli,

based on whether PBMCs obtained from *C. pneumoniae* seropositive or seronegative patients. The differences were also manifested in baseline cytokine or MMP-9 levels. This study highlights that steroid-resistant and steroid sensitive patients are different regarding cytokine production of PBMCs, reflecting distinct immunophenotypes of patients with asthma.

Collectively, our reported data in both study inspire several exciting concepts in terms of *C. pneumoniae* infection, asthma treatment and their associations with patients' individual responsiveness to infections or conventional therapies. Thus, we believe our results could contribute to further translational asthma research and therapeutic choices as well.

## Considered novel findings

The following results are regarded to be state-of-the-art in *Chlamydia* and asthma research:

- FP suppressed *C. pneumoniae* growth *in vitro* and *in vivo;*
- FP promoted IFN-γ expression and production, along with IDO gene expression and IFN-γ -inducible MIG/CXCL-9 secretion at the protein level in *C. pneumoniae*-infected mouse lungs;
- FP triggered the production of IL-17A, thus contributing to the indirect inhibition of *C. pneumoniae* replication in mice;
- Interestingly, FP induced the expression of VDR *in vivo* that could exert additional immunomodulatory effects;
- FP was demonstrated to affect immune responses in a distinct manner, compared to BUD;
- PBMCs of the patients with steroid-resistant or -sensitive asthma act differently in response to stimulation with *C. pneumoniae* or a polyclonal mitogen stimulation, demonstrating altered IL-10 and TNF-α responses, based on the host's *C. pneumoniae* serological status.

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## Publications related to the subject of the Thesis:

1. **Dóra Paróczai**, Anita Sejben, Dávid Kókai, Dezső P.Virok, Valéria Endrész, Katalin Burián:
   Beneficial Immunomodulatory Effects of Fluticasone Propionate in *Chlamydia pneumoniae*-infected Mice
   *Pathogens* 2021, 10(3), 338
   Impact factor: 3.018, Q1

2. **Dóra Paróczai**, Tímea Mosolygó, Dávid Kókai, Valéria Endrész, Dezső P. Virok, Attila Somfay, Katalin Burián:
   *Chlamydia pneumoniae* Influence on Cytokine Production in Steroid-Resistant and Steroid-Sensitive Asthmatics
   *Pathogens* 2020 Feb 11;9(2):112
   Impact factor: 3.018, Q1

## Publications not related to the subject of the Thesis:

1. József Furák, **Dóra Paróczai**, Katalin Burián, Zsolt Szabó, Tamás Zombori (shared first author):
   Oncological advantage of nonintubated thoracic surgery: Better compliance of adjuvant treatment after lung lobectomy
   *Thorac Cancer* 2020 Nov;11(11):3309-3316
   Impact factor: 2.610, Q2

2. Dezso P. Virok , Tímea Raffai, Dávid Kókai, **Dóra Paróczai**, Anita Bogdanov, Gábor Veres, László Vécsei, Szilárd Poliska, László Tiszlavicz, Ferenc Somogyvári, Valéria Endrész and Katalin Burián:
   Indoleamine 2,3-Dioxygenase Activity in *Chlamydia muridarum* and *Chlamydia pneumoniae*-Infected Mouse Lung Tissues
   *Front Cell Infect Microbiol* 2019 Jun 12;9:192
   Impact factor: 4.123, Q1

## Abstracts related to the Thesis:

1. **Dóra Paróczai**, Dávid Kókai, Katalin Burián:
   The effects of inhaled corticosteroids and a beta-2 agonist on *C. pneumoniae*-infected airway epithelial cells
   European Respiratory Society (ERS) International Virtual Congress September 7-9, 2020

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