Influence of chemical compounds and cell-autonomous immunity on the replication of sexually transmitted pathogens

Ph.D. Thesis

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INTRODUCTION

Sexually transmitted diseases (STDs) are a persistent and significant problem globally. Worldwide more than 1 million STDs are acquired every day and the incidence is increasing. *Chlamydia trachomatis (C. trachomatis)* infections are one of the most prevalent STDs. Many STDs have no symptoms or only mild symptoms that may not be sufficient to recognize and treat the disease. The lack of eradication leads to the uncontrolled spread of STDs. Persistent and latent-reactivating infections also make eradication hard or impossible. *Chlamydia* and different herpesvirus species prefer an intracellular lifestyle where the infection persist for months-years to lifetime.

My thesis focuses on the *Chlamydia* and HSV-2 growth-altering effects of various compounds, and also aims to identify murine defense genes involved in the elimination of *Chlamydia* strains.

Taxonomy and microbiology of *Chlamydia* genus

The order *Chlamydiales* contains several species, including *C. trachomatis* and *C. pneumoniae.* Based on serological characteristics *C. trachomatis* species can be grouped into 2 biovars which can be further divided into various serovars. Infections related to the urogenital serovars D-K include urethritis, cervicitis and pelvic inflammatory disease in adults. Perinatal transmission of the genital serovars leads to the conjunctivitis, pharyngitis and pneumonia in newborns. Prevalence of LGV biovars is lower, but can be high in certain areas of Africa, Southeast Asia, India, the Caribbean, and South America. In the last 10 years, LGV prevalence increased in North America and Europe causing outbreaks among men who have sex with men.

Chlamydiae are obligate intracellular bacteria that propagate prominently in the epithelial cells of the respiratory and urogenital tract. They exist in two morphological forms, the infectious elementary body (EB) and the non-infectious, but metabolically active reticulate body (RB). The EB attaches to the cell surface components of mucosal epithelial cells such as heparin sulfate proteoglycan and enters the host cell by various ways. Following the entry, the EBs are transported to the perinuclear area of the cell, where they are located in a large membrane bound compartment, the so-called inclusion. During primary differentiation the membrane bounded and lysis-protected EBs transform into RBs. Following the differentiation, RBs replicates by binary fission in the inclusion. After 24-72 hours the second differentiation takes place, where the replicated RBs differentiate again into EB. At the end of the cycle, infectious EBs are released by exocytosis or extrusion, ready to infect other cells.

Pathogenesis of *Chamydia trachomatis* infection and the host response

Epithelial cells are the main target of the *Chlamydia* infections, they are not professional immune cells, although they possess a so-called autonomous cell immunity. Toll-like receptors (TLR) expressed on the innate immune cells and the epithelial cells recognize pathogen-associated molecular patterns (PAMPs) of the *Chlamydia*. *Chlamydiae* have several components that may serve as PAMPs. TLR2 and TLR4 has been described as a receptor for chlamydial ligands, such as lipopolysaccharide (LPS) and heat shock proteins (HSPs). The ligation of the TLRs by PAMPs of infected epithelial cells, induces the production of pro-inflammatory metabolites. Pro-inflammatory metabolites attract monocytes and dendritic cells (DCs). Attracted monocytes differentiate to phagocytic cells, the activated macrophages. Activated macrophages after phagocytosing the pathogen produce more cytokines. These cytokines are chemokines, attract and activate further immune cells, neutrophil and natural killer (NK) cells. NKs are cytotoxic cells as well as neutrophil cells, they reduce direct chlamydial infection and limit spreading. Infected epithelial cells, DCs, macrophages and neutrophils present the antigen on their major histocompatibility complex (MHC) receptors. These antigen presenting cells by presentation the antigen, activate T lymphocytes, hence, initiate a cell-mediated and humoral immune response. T lymphocytes include CD4+ T helper cells and CD8+ T cytotoxic cells. Epithelial cells presenting the antigen by MHCI

receptors activate CD8+ cells, while professional antigen presenting immune cells presenting them on MHCII receptors activate the CD4+ cells. CD8+ T cells induce death of infected cells. CD4+ cells based on their profile of cytokine production are divided further to Th1 and Th2 cells. Th1 response is directed against intracellular pathogens. Th1 cells produce interferon-gamma (IFN-γ) cytokine, inducing cellular immunity and activating innate immune cells which participates in chlamydial infections related inflammation. Another part of the adaptive immunity is the B lymphocyte response. B lymphocytes differentiate to the plasma cells producing antibody and accomplish immunological memory against the pathogen. In genital mucosa, specific antibodies are secreted for the resolution of primary and secondary infections. These antibodies include monoclonal immunoglobulin A (IgA) and immunoglobulin G (IgG). Antibodies recognizes specific epitopes in MOPMs and HSP proteins of *Chlamydia*. MOMP specific monoclonal antibodies neutralize the extracellular form of *Chlamydia* and enhances the cellular immune responses during reinfection. The HSP specific antibodies in serum shows elevated level in case of serious infections. Innate immunity is the first line of defense against *Chlamydia* infections, while the adaptive immune response is involved in final elimination of the bacterium and provides protection against recurrent infections by creating immunological memory. At the tissue level, the adaptive and innate immunity are strongly linked, the communication and coordination of its elements are accomplished by cytokines. The pro-inflammatory cytokines produced first by the infected epithelial cells, include interleukin-1-alpha and interleukin-1-beta (IL-1α, IL-1β), tumor necrosis factor-alpha (TNFα), IL-8 and IL-6. IL-8 attracts of leukocytes to the site of the infection and enhances the expression of adhesion molecules ICAM-1 and VCAM-1. Several other cytokines such as IL-10, IL-12, IL-15 are implicated in the infections with different activating or regulatory roles. IFN-γ, a key cytokine in chlamydial infections is produced by NK and Th1 cells [48]. Among the diverse effects of IFN-γ, it induces the expression of the host enzyme indoleamine 2,3-dioxygenase (IDO) in infected cells influencing tryptophan availability in these cells. Tryptophan degradation occurs via the kynurenine pathway, where the IDO catalysis first step of the catabolism, the oxidation of L-tryptophan to N-formylkynurenine. Since *Chlamydia* is a tryptophan auxotroph, the limitation of tryptophan availability is an effective defense strategy of the host cell. IFN-γ also upregulates other host defense genes, such as the inducible nitric oxide synthase (iNOS) enzyme, which catalyzes the production of reactive nitrogen intermediates, most notably nitric oxide (NO). IFN-γ also downregulates host transferrin receptors on the infected cells, resulting in intracellular iron deficiency and limitation of the pathogen's replication. Besides orchestrating anti-chlamydial defense responses, IFN-γ and the other locally produced cytokines have a complex effect on tissue-level gene/protein expression and metabolism, including the production of reactive oxygen species, matrix metalloproteases, elastases, collagenases, cathepsins. The production of these proteins during acute and chronic inflammation eventually leads to tissue remodeling, fibroblast proliferation and extracellular matrix production.

Despite the significant *Chlamydia*-induced inflammation, the majority of the infections caused by *C. trachomatis* are asymptomatic and therefore remain untreated. In the male population the infections remain asymptomatic in 75% of cases, while in female populations it may reach up to 76.7%.

Impact of vaginal gels on *Chlamydia trachomatis* infection

The *Chlamydia* transmission is greatly influenced by components of the cervico-vaginal microenvironment including vaginal lactobacilli and indole-positive bacteria. Vaginal gels can be introduced into this microenvironment as lubricants or therapeutic gels. Vaginal gels are present during sexual intercourse and due to their spatial and temporal presence these gels may have significant impact on the acquisition of *Chlamydia* infection and other STDs. A major component of vaginal gels is the gelling agent itself. Hydroxyethyl cellulose (HEC) is a commonly used gelling agent that can be found in lubricants and therapeutic gels. Despite their potential importance in transmission, the impact of gelling agents is not well/described.

In vivo models of *Chlamydia* infections

The prevention of infections via vaccination would be a solution, although effective vaccines have not yet been developed. Mouse models are most frequently used for vaccine development, but the differences between the

human and murine immune systems, including the so-called cell-autonomous immunity makes the mouse models difficult to compare with humans. Cell-autonomous immunity is an intrinsic feature of host cells, which launches defense mechanisms that interfere with the growth of intracellular pathogens. Typically, these defense genes are inducible, IFN-γ is a prominent inducer cytokine. It has been described earlier that the major intracellular anti-chlamydial defense mechanism in human cells is the IFN-γ induced IDO expression, which leads to the degradation of the intracellular tryptophan pool and eventually the death of the tryptophan-auxotroph *C. trachomatis*. This elimination mechanism is effective *in vitro* for both the human *C. trachomatis* and the genetically closely related murine *Chlamydia* species *C. muridarum*. Nevertheless, *in vitro* data showed that IDO is not induced by *Chlamydia* infection and/or IFN-γ in mouse epithelial cells. Instead, microarray analysis of IFN-γ treated and *Chlamydia* infected murine epithelial cells revealed that the IFN-inducible GTPases are the suspected host genes that interfere with the developmental cycle of human *Chlamydia* strains. The murine *Chlamydia* strain developed mechanism(s) to deactivate the GTPase response and render this elimination mechanism ineffective. Despite this, the *C. muridarum* strain is rapidly eliminated from the murine cervicovaginal tract, hence yet unknown elimination mechanisms exist in mice that are effective against the murine *Chlamydia* strain *in vivo*.

Herpes simplex virus

The herpes simplex virus belongs to the family of Herpesviridae, comprising two species: HSV-1, which causes herpes labialis and HSV-2, the causative agent of herpes genitalis. HSV-1 or HSV-2 can both cause herpes genitalis, but the majority of cases is caused by HSV-2, while the prevalence of HSV-1 is increasing, mainly in young people, due to the practice of oral sex.

Microbiology and pathology of herpes simplex virus-2

The HSV virions are relatively large virus particles consisting of double stranded linear DNA encased within an icosahedral protein cage capsid. The primary transmission of HSV-2 occurs through direct contact and is usually asymptomatic. The virion attacks the epithelial cells and neurons. The virus could be recurrent and spread to the place of the first contact via the sensory nerve, at the side of the infection. The recurrence could be caused by numerous physiological and environmental factors such as fever, emotional influences, hormonal changes, trauma, stress, exhaustion, immunosuppression or other infection. People with symptomatic herpes genitalis and people who shed HSV asymptomatically can transmit the virus to their sexual partners. Both primary and recurrent HSV infection in pregnant women can result in intrauterine viral transmission causing congenital HSV infections in newborns.

Antiviral therapy of herpes simplex virus

HSV encodes a viral thymidine kinase, which could be utilized to increase selective toxicity only in the infected cells. Acyclovir (ACV), valacyclovir, penciclovir (PCV) and famciclovir have a selective mechanisms of action since they are about a hundred times more likely phosphorylated by the virus TKs than the host cells TKs. After the first phosphorylation, the subsequent phosphorylations are performed by host kinases resulting the triphosphate form that can incorporate to the newly synthesized DNA strand. Once these nucleoside analogs incorporated they terminate the DNA synthesis due to their modified sugar constituents. Currently these nucleoside analogs are standard first-line drugs against HSV infections.

Drug-resistance of herpes simplex virus

A large number of therapeutic agents can be used for the treatment of HSV infections however, infections represent a persisting treatment concern due to drug-resistant HSV, especially in the immune-compromised

population. Resistance can be expressed through four mechanisms. The resistant strain could (1) lack TK activity or decreased activity or (2) production of low amounts of viral TK. It is also possible that (3) the viral TK protein has altered substrate specificity, and the last and most unlikely case is when (4) viral DNA polymerase gene (DNA pol) has altered substrate specificity. The global prevalence of ACV-resistant HSV infections was reported between 2.5% and 10% for immune-compromised patients. Strains resistant to ACV are almost always cross-resistant to other TK-dependent drugs such as PCV and famciclovir.

Novel antiviral treatment

Despite of its disadvantages, ACV is still the first choice for treatment. A broad spectrum of antiviral compounds displays antiviral activity against different enveloped viruses including the HSV-2 acyclovir resistant strains. Still, there is an urgent need for discovery and development of effective and novel anti-viral agents with low toxicity which are able to ameliorate viral infections and avoid side effects. In this regard, medical plants and their natural substances could offer safe platforms. Essential oils from various aromatic medicinal plants are highly active against some viral infections, for example: balm oil, tea tree oil and peppermint oil demonstrate a significant antiherpetic activity *in vitro*. These essential oils are also highly active against ACV-resistant HSV strains. We should also remember the fact that these antiviral drugs do not cure these infections. Antiviral treatment decreases the severity and length of the outbreaks and helps the sores heal faster, keeps new sores from forming, and decreases pain and itching. The viruses that cause these infections accomplish latency in the body even between outbreaks. As long as the issue of total eradication of viral infections remains unsolved, a development of new drugs against HSV remain important.

AIMS OF THIS STUDY

Aim 1: *In vitro* and *in vivo* monitoring of the effect of hydroxyethyl-cellulose a major gelling agent of vaginal gels on *C. trachomatis* growth.

Aim 2: Identification of murine defense genes that could be involved in the elimination of the murine *Chlamydia* strain and murine genes that could be effective against the human *Chlamydia* strains.

Aim 3: To identify novel antiviral compounds extracted from the berry of sea buckthorn, *Elaeagnus rhamnoides (E. rhamnoides)*.

MATERIALS AND METHODS

Cell lines

In this study HeLa 229 (ATCC), McCoy (ECACC) and Vero cells (ATCC) were used.

Chlamydia strains

C. trachomatis (serovar D, UW3/CX reference strain, and serovar E strain DK20; ATCC) and *C. muridarum* strain Nigg [62] were propagated in McCoy cells. *C. pneumoniae* CWL029 strain ATCC was propagated in HEp-2 cells (ATCC).

Extracted compounds from *Elaeagnus rhamnoides* and *Rumex aquaticus*

The extracted compounds were provided by our cooperation partner, Judit Hohmann. Compound 1 (6,9-dihydroxy-1-ox-14-noreudesm-5,7,9 triene), compound 2 (2-hydroxy-7-isopropyl-1-methoxy-4-methyl-1,4-naphthoquinone), compound 3 (methoxy-substituted phenylpropane dimer) and compound 4 (caulexin C) were extracted from the berry of *E. rhamnoides*. Compound 5 (musizin), compound 6 (musizin-8-O-glucoside) compound 7 (torachrysone-8-O-glucoside) and compound 8 (2-methoxystipandron) are substituted naphtalenes, extracted from *R. aquaticus*.

Preparation of hydroxyethyl cellulose solution in vaginal simulant buffer

The aqueous solution of HEC were made by dissolving 30 mg of the HEC polymers in 1 ml of physiological salt solution (0.9% w/v NaCl), following, an 2-fold dilutions in the vaginal simulant. The applied HEC concentration range was between 1.5 – 0.023% w/v. The compound for 1 L vaginal simulant buffer were of NaCl 3.51 g/l; KOH 1.40 g/l; Ca(OH)2 0.222 g/l; bovine serum albumin 0.018 g/l; lactic acid 2.00 g/l; acetic acid 1.00 g/l; glycerol 0.16 g/l; urea 0.4 g/l; glucose 5.0 g/l which were dissolved in distilled water. The pH of the vaginal simulant was adjusted to a pH 4.2 or pH 7.0, with NaOH and HCl solutions.

Investigation of impact of hydroxyethyl cellulose on *Chlamydia trachomatis* serovars D and E growth in HeLa cells

HeLa 229 cells were placed into 96-well plates at a density of 4 x 10^4 cells/well in 100 µl of minimal essential medium (MEM) with Earle's salts supplemented with 10% heat-inactivated fetal bovine serum. The next day at 90% confluence of the cells were subjected to the infection after twice washing with 100 µl/ well with phosphate buffer saline (PBS) pH 7.4. Before the infection, the inoculum of EBs was pre-incubated in HEC 2-fold dilution (concentration range 1.5-0.023 % w/v) with *C. trachomatis* D and E strains at pH 4,2 and pH 7.0. In the control group vaginal simulant buffer at pH 4.2 and pH 7.0 was alone pre-incubated with EBs for 1h 37°C, 5% CO_2 as well. The pre-incubated inoculum of all groups were suspended in 0.5% (w/v) glucose medium and added to the cell layers. The infection was accomplished by incubating for 60 min at 37°C, 5% CO_2 without centrifugation. Following the infection, the cells were washed twice with PBS, and a culture medium containing 0.1 µg/ml cycloheximide was added. After the 48 hour incubation, the medium was removed from the cell monolayer, washed twice with PBS and 100 µl Milli-Q (MQ) water was added to the cells. The cell lysis was accomplished by two freeze-thaw cycles; cells with the supernatant were subjected to quick freezing (-80°C, 15 min) and a quick thawing on a plate shaker. The cell lysates with the supernatant were thoroughly mixed including the edges of the wells using a multichannel pipette. The mixed lysates were used as a template for the qPCR analysis.

Monitoring the growth of *Chlamydia trachomatis* serovars D and E by direct quantitative PCR

After the accomplished cell lysis, the mixed lysates were used as a template in the qPCR. The qPCR was performed using the Bio-Rad CFX96 real time system. The SsoFast EvaGreen qPCR Supermix master mix and *C. trachomatis pykF* gene specific primer pair were used. The primer sequences were the following: *pykF-F:* 5′-GTTGCCAACGCCATTTACGATGGA-3′, and *pykF-R:* 5′-TGCATGTACAGGATGGGCTCCTAA-3′. 5 µl SsoFast EvaGreen supermix, 1–1 µl forward and reverse primers (10 pmol each), 1 µl template and 2 µl MQ water was the consistent of the PCR mixture with a 10 µl final volume. 40 PCR cycles of 20 s at 95 °C and 1 min at 64 °C were performed with a 10 min at 95 °C polymerase activation for the first step. The fluorescence intensity was measured at the end of the annealing-extension step. The melting curve analysis was used to get the specificity of amplification. For each PCR, the cycle threshold (Ct) corresponding to the cycle where the amplification curve crossed the base line was

determined. To evaluate the statistical differences between the samples (3 biological replicates for each condition) Student's *t*-test was used.

Monitoring the growth of *Chlamydia trachomatis* strains D and E by chamber slide method

Chamber slides with 16 wells consisting of a removable, plastic chamber attached to a specially treated standard glass slide were used to culture HeLa cells for infection with *Chlamydia*. The cells were transferred into the wells of the chamber slides at a density of 4×10^4 cells/well in 100 µl of MEM culture medium. The slides were incubated for 1 h at room temperature in order to reduce the edge effect and then overnight at 37 °C under a 5% CO_2 atmosphere to obtain a 90% confluent cell layer. The infections were accomplished in way as was it described above (Investigation of impact of HEC on *Chlamydia* strains D and E growth in HeLa cells) except in this case were used only the highest concentrations for all groups. Briefly the pre-incubated EBs with HEC at pH 4.2 and 7.0 and with vaginal simulant at pH 4.2 and pH 7.0 were added to the cells. The infection was cultivated by incubating for 60 min at 37 °C, 5% CO_2 without centrifugation. Following the infection, the cells were washed twice with PBS, and a culture medium containing 0.1 µg/ml cycloheximide was added. After the 48 hour incubation cells were fixed for immunofluorescence staining. Anti-chlamydia LPS antibody was labeled with Alexa-647, and a 1:200 dilution was used for the detection of chlamydial inclusions. Fluorescence signals were analyzed with an Axon GenePix Personal 4100A DNA chip scanner and GenePix Pro (version 6.1) software as published earlier. In this investigation 4 biological replicates for each condition were evaluated.

Investigating the *in vivo* effect of hydroxyethyl cellulose on growth of *Chlamydia trachomatis* serovar D

To monitor the effect of HEC *in vivo*, 6-8 week old female BALB/c mice were treated s.c. with 2.5 mg medroxyprogesterone acetate 1 week before infection. Mice were inoculated intra-vaginally with $1x10^5$ inclusion forming units (IFU) of *C. trachomatis* serovar D mixed with HEC (1.5% w/v) and as a control group without HEC. After 3 day post infection cervicovaginal microenvironment was subjected for investigation by harvesting cervicovaginal washes from mice. The mice were intra-vaginally washed with 100 µl SPG. Afterward the samples were subjected to two freeze-thaw cycles, freezing at -80°C, 45 min and thawing on a plate shaker at the room temperature. Following the last thaw step the recoverable IFU (on McCoy cells, which were seeded on glass slips in 24-well plate) from samples was evaluated by using traditional immunofluorescence microscopy. day before the infection.

Infection of BALB/c and C57BL/6 mice with *Chlamydia pneumoniae* and *Chlamydia muridarum* and processing of the lung tissues

Pathogen-free 6-week-old female BALB/c mice were obtained from the Charles River Laboratories (Hungary), C57BL/6 mice obtained from BRC Animal House (Szeged, Hungary). The mice were maintained under standard husbandry conditions at the animal facility of the Department of Medical Microbiology and Immunobiology, University of Szeged, and were provided with food and water ad libitum. Before infection, the mice were mildly sedated with an intraperitoneal injection of 200 µl of sodium pentobarbital (7.5 mg/ml); they were then infected intra-nasally with 4×10^5 IFU *C. pneumoniae* (BALB/c) or 1×10^3 IFU of *C. muridarum* (BALB/c and C57BL/6) in 20 µl SPG buffer. Control mice were treated with 20 µl SPG buffer only. The mice were anaesthetized and sacrificed 7 days after infection. The lungs were removed and homogenized with acid-purified sea sand. Half of each homogenized lung was processed for total RNA extraction, and the other half was suspended in 1 ml of SPG for the detection of viable *Chlamydia* and to test the quantity of the tryptophan derivates. The lungs of three mice from both groups were fixed in 10% neutral buffered formalin solution (Sigma) for histopathological evaluation. All experiments were approved by the Animal Welfare Committee of the University of Szeged and conform to the Directive 2010/63/EU of the European Parliament.

Culturing of *Chlamydia pneumoniae* and *Chlamydia muridarum* from BALB/c mouse lungs

Homogenized lungs from individual mice were centrifuged (10 min, 400g), serial dilutions of the supernatants were inoculated onto McCoy cell monolayers and centrifuged (1 h, 800g). After 48-h incubation the cells were fixed with acetone and stained with monoclonal anti-*Chlamydia* LPS antibody and FITC-labeled anti-mouse IgG. The number of the recoverable *Chlamydia* inclusions was counted under a UV microscope and expressed as IFU/lung.

Inhibition of indoleamine 2,3-dioxygenase by 1-methyl-DL-tryptophan in BALB/c mice

Seven days before infection with *C. muridarum* the drinking water of 8-weeks-old female mice (n=4) was changed to that containing 2 mg/ml IDO inhibitor 1-methyl-DL-tryptophan (1-MT), dissolved in 10 mmol/l NaOH supplemented with Stevia sweetener. Control mice (n=4) received the Stevia-sweetened drinking water with 10 mmol/l NaOH without 1-MT. The solution was delivered in autoclaved water bottles, protected from light, and changed every other day. The infection of mice and the estimation of recoverable viable *C. muridarum* from the lungs at 7 day post infection were carried out as described previously.

Total RNA extraction and cDNA synthesis from *Chlamydia* infected and uninfected BALB/c and C57BL/6 mouse lungs

Total RNA was extracted from homogenized lung tissues of *C. muridarum* infected mice BALB/c (n=3) and C57BL/6 mice (n=5), *C. pneumoniae* infected BALB/c mice (n=3) and uninfected controls (n=3) with Tri Reagent according to the manufacturer's protocol (Sigma). Total RNA quantity (OD260) and purity (OD260/280) were measured by a NanoDrop spectrophotometer.

Quantitative PCR validation of the IDO1 and IDO2 RNA-Seq data

For qPCR 1 μg of total RNA was reverse transcribed using the Maxima Reverse Transcriptase according to the manufacturer's protocol with random hexamer priming. qPCR was performed in a Bio-Rad CFX96 real-time system. The qPCR was performed with the SsoFast EvaGreen qPCR Supermix master mix and the murine specific primer pairs *IDO1*: 5'-GCTTCTTCCTCGTCTCTCTATTG-3', 5'-TCTCCAGACTGGTAGCTATGT-3'; *IDO2*: 5'-CCTGGACTGCAGATTCCTAAAG-3'; 5'-CCAAGTTCCTGGATACCTCAAC-3'; *beta-actin*: 5'-TGGAATCCTGTGGCATCCATGAAAC-3', 5'-TAAAACGCAGCTCAGTAACAGTCCG-3'. To check the amplification specificity, the qPCR was followed by a melting curve analysis. Ct were calculated for IDO1, IDO2 and beta-actin genes, and the normalized gene expressions were calculated by the $_{\Delta}$Ct method (Ct_{IDO1}-Ct_{actin} or Ct_{IDO2}-Ct_{actin}). Statistical comparison of qPCR data was performed by comparing the $_{\Delta}$Ct values of uninfected and infected lung samples (n=3) by using the Student's t-test as described earlier.

Herpes simplex virus-2 strains

The HSV-2 strain (donated by Dr. Ilona Mucsi, University of Szeged, Szeged, Hungary) was grown in Vero cells and the titer was determined in the same cell line by using the plaque titration method.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

MTT assay was carried out to identify the highest non-toxic concentration of compounds 1-3 and 5-8 with potential anti-viral activity on Vero cells.

Assays for testing antiviral activity

The antiviral activity of sea buckthorn compounds was investigated in Vero cells. Cells were seeded in 96-well plates and were infected with HSV-2 at MOI of 0.01. After a 1 h adsorption period, the inoculum was removed, the cultures were washed twice, and culture medium containing the plant compounds in different concentrations was added. After a 24-hour incubation period, the cultures were washed with PBS, and finally 100 mL MQ was added to the cells. After, two quick freeze-thaw cycle, the cells were subjected to lysis.

Measuring antiviral activity by direct quantitative PCR

DNA release from the infected cells was achieved by two freeze-thaw cycles. 1 mL of the lysates were used directly in a qPCR assay. Each antiviral test was performed in 3 parallel wells. The qPCR assay was performed using a Bio-Rad CFX96 real time system, as described earlier. Briefly, a HSV-2 gD2 gene specific primer pair was applied during the qPCR process. The primer sequences were the following: gD2: 50-TCA GCG AGG ATA ACC TGG GA-30, 50-GGG AGA GCG TAC TTG CAG GA-3'. The qPCR mixture consisted of 5 mL SsoFast™ EvaGreen® Supermix, 1-1 µL of forward and reverse primers (10 pmol/mL each) and 1 µL template, and 2 µL MQ water was added to get a final volume of 10 µL. After a 10-min polymerase activation step at 95 °C, 40 PCR cycles of 20 s at 95 °C and 1 min at 64 °C were performed. Fluorescence intensity was detected at the end of the annealing-extension step. The specificity of amplification was confirmed by melting curve analysis. For each PCR, the Ct corresponding to the cycle where the amplification curve crossed the base-line was determined.

Determination of $TCID_{50}$ by the virus yield reduction technique

The virus yield in the supernatants of infected and plant compound-treated Vero cells was determined by the traditional dilution method. Vero cells (60,000 cells/well) were seeded onto 96-well flat-bottomed plates and cultivated for 24 h at 37 C at 5% CO_2 to produce a semi-confluent monolayer. Then the growth medium was removed and 10-fold dilutions of HSV-2 in the absence of the test compound (virus control), as well as compound-treated HSV-2-infected cell supernatants were added in quadruplicate, and plates were incubated at 37 C until typical cytopathic effect (CPE) was visible. After 48 h, the CPE of the virus was examined using an inverted microscope, and virus titers were estimated according to the Reed-Muench method, expressed as $TCID_{50}$/mL. The test compounds' antiviral activity was measured as the reduction of viral titer (log_{10}) in the presence of each compound, compared to the virus titer of the control sample.

RESULTS

Monitoring the impact of hydroxyethyl cellulose on the growth of *Chlamydia trachomatis* serovar D and E by quantitative PCR

After the pre-incubation of *C. trachomatis* EBs in pH4.2 vaginal fluid measured by qPCR 48 hours post infection shows a concentration-dependent enhancement effect of HEC on the chlamydial growth. The *C. trachomatis* serovar D maximum growth increase was 23.7 fold at the maximal 1.5% w/v HEC concentration, and a noticeable, but non-significant growth enhancement tendency could be detected up to a concentration of 0.188% w/v HEC. HEC at pH7 enhanced the chlamydial growth significantly with a 13.8 fold growth increase at a concentration of 1.5% w/v. Interestingly, in the case of *C. trachomatis* serovar E, the maximum growth increase (22.25 and 26.1 fold at pH 4.2 and pH7 respectively) was observed at the second highest HEC concentration (0.75% w/v) at both pH 4.2 and pH7 indicating a different HEC-EB interaction between the serovars.

Monitoring the impact of hydroxyethyl cellulose on the growth of *Chlamydia trachomatis* strains D and E by chamber slide method

To validate the qPCR results, we performed the automatic *Chlamydia* inclusion counting using the ChlamyCount measuring system, at pH4.2 or pH7 at 1.5% w/v and 0.75% w/v HEC concentrations for serovar D and serovar E, respectively. Inclusion counts showed similar, albeit lower growth enhancement than the chlamydial genome measurements by qPCR with a 5.9-6.5 fold increase for serovar D and 5.95-6.05 fold increase for serovar E. This difference is likely due to the fact, that ChlamyCount measures the chlamydial inclusion number, while qPCR measures the bacterial genome content of the inclusions.

Investigating the *in vivo* effect of hydroxyethyl cellulose on growth of *Chlamydia trachomatis* serovar D

The *in vivo* data also showed that HEC significantly increased the growth of *C. trachomatis* serovar D in the mouse genital tract, with a 2.57 fold enhancement 3 days post infection. It is important to note, that the chlamydial EBs were not pre-incubated with HEC before the infection, indicating an immediate growth enhancing effect of HEC *in vivo*.

***Chlamydia* infection and *Chlamydia*-induced histopathology in BALB/c mouse lung tissues**

In our animal model different doses of the two *Chlamydia* strains were used (4 x 10^5 IFU of *C. pneumoniae* and 1 × 10^3 IFU of *C. muridarum),* based on the results of our former experiments where the lower dose of the murine pathogen *C. muridarum* induced similar growth and histopathology than the human pathogen *C. pneumoniae*. Indeed, recoverable IFUs from the *C. muridarum* and *C. pneumoniae* infected BALB/c lungs were similar at 7 days post infection. Both infections induced a lymphoid hyperplasia, with the interstitial accumulation of lymphoid, plasmocytoid cells and macrophages in the widened bronchus walls. At this time of the infection, the presence of neutrophils were marginal, the major leukocyte populations were lymphocytes and macrophages. Control lung tissue showed thin alveolar septa without the clear presence of inflammatory cells, but a small number of pulmonary macrophages could be detected.

Impact of *Chlamydia muridarum* infection on the global gene expression of BALB7c mouse lung tissues

To explore the global gene expression changes induced at the tissue level by the murine *Chlamydia* strain we performed an Illumina next generation RNA sequencing of mouse lung tissues infected with *C. muridarum* 7 days post infection. RNA-seq analysis revealed that 755 murine genes had a higher expression and 251 genes had a lower expression than the uninfected control. The extent of up-regulation and the number of up-regulated genes was higher (1.48-345 fold), than in the case of the down-regulated genes (1.5-14.36 fold). The most highly up-regulated gene was the *CXCL11* (*I-TAC*), and several cytokines/ chemokines were among the highly upregulated genes including *CXCL9* (*MIG*), *CXCL10* (*IP-10*), *CCL8* (*MCP2*), *CCL2* (*MCP1*), *IFNG*, *IL21*, *IL10*, as well as already described defense genes *IRG1*, *IIGP* and *IDO1*. The most highly down-regulated gene was cDNA sequence *BC023719* with a 14.36 fold of down-regulation, and the functions of the most highly down-regulated genes were diverse.

Antimicrobial genes induced by *Chlamydia muridarum* infection in BALB/c mouse lung tissue

A prominent identified Gene Ontology molecular functional category was “GTP binding”. Most of the GTPases in this category are involved in defense responses against intracellular pathogens. Essentially all four classes of murine IFN-inducible GTPases were found to be up-regulated including various guanylate-binding proteins (*GBP2-9*, *GBP11*), the myxovirus resistance protein-1 (*MX1*), immunity-related GTPases *IRGM1* (*LRG47*),

IRGM2 (*GTPI*), *IRGA6* (*IIGP*), *IRGM3* (*IGTP*), *IRGB6* (*TGTP1-2*), *IRGB10* (*Gm12250*) and a very large IFN-inducible GTPase *GVIN1*.

qPCR validation of *IDO1* and *IDO2* RNA-sequencing data

The fact that the *IDO1* gene was highly upregulated (24.76 fold) in the infected lungs was an unexpected finding since the *IDO1* was found to be non-inducible by *C. trachomatis* or *C. muridarum* infection *in vitro* in murine epithelial and other cells. *IDO1* was detected as a significantly changed gene (*P=0.037*) between the *C. muridarum* infected and uninfected samples, while the *IDO2* was detected non-significant. However, both *IDO1* and *IDO2* genes had more sequence reads in the infected samples than uninfected ones.

Yet, in the case of *IDO2*, the read numbers were not high enough to be detected as a significantly changed gene. We used qPCR as an independent method to validate RNA-Seq data for *IDO1* and *IDO2*. Also, in order to test whether the *IDO1-2* induction is unique to the murine *Chlamydia* strain, we measured the *IDO1-2* gene expressions in *C. pneumoniae* infected lung samples. qPCR data supported the RNA-Seq data in the case of *C. muridarum* infection with a 20.38 +/- 11.3 fold and 38.2 +/- 25.2 fold of upregulation of *IDO1* and *IDO2*, respectively. qPCR also showed a similar extent of up-regulation 15.5 +/- 14.1 fold and 88.9 +/- 73.9 fold for *IDO1* and *IDO2,* respectively in the *C. pneumoniae* infected lung tissues.

IDO1-2 protein expression in *Chlamydia muridarum* and *Chlamydia pneumoniae* infected BALB/c mouse lung tissues

Moderate IDO1-2 positivity could be detected in the cytoplasms of bronchial and occasionally alveolar epithelial cells and moderate/strong positivity was detected frequently in macrophages in the *C. pneumoniae* and *C. muridarum* infected mouse lung tissues. *C. pneumoniae* and *C. muridarum* infections lead to similar IDO1-2 positivity in these cells. The control, uninfected lung tissues were also contained IDO1-2 positive bronchial epithelial cells, and a small number of IDO1-2 positive macrophages.

IDO 1-2 activity in *Chlamydia muridarum* and *Chlamydia pneumoniae* infected BALB/c mouse lung tissues

To determine whether the expressed IDO1 and IDO2 proteins were functional, we performed a HPLC analysis of the infected and control lung tissues of mice included in the gene expression and immunohistochemistry measurements. We measured IDO1-2 activity by measuring the total tryptophan level, and the level of the tryptophan degradation metabolite kynurenine. The applied HPLC method could not detect kynurenine in the uninfected lungs, while the *C. muridarum* and *C. pneumoniae* infected lungs contained 369.6 +/- 199.8 nM and 508.7 +/- 176.6 nM. The tryptophan levels were comparable in the infected samples, 2757.2 +/- 201.5 nM and 3054.1 +/- 418.1 nM in the *C. muridarum* and *C. pneumoniae* infected lungs, respectively. The tryptophan levels in the uninfected mice's lungs were slightly lower, 1569.1 +/- 246.9 nM. Since we could not control the cell numbers in the infected and control tissues, we normalized the samples by using the kynurenine/tryptophan ratios as described previously. The kynurenine/tryptophan ratios ranged from 0.12-0.22 in the *C. muridarum* infected samples, 0.13-0.20 in the *C. pneumoniae* infected samples and it was 0 in the control samples. To assess the impact of IDO activity on *C. muridarum* growth we inhibited IDO1-2 by 1-MT treatment starting from seven days before infection to seven days post infection. 1-MT treatment lead to a moderate but significant, 1.98 fold increase in *C. muridarum* recoverable IFU at 7 days post infection.

IDO 1-2 mRNA expression and activity in *Chlamydia muridarum* infected C57BL/6 mouse lung tissues

To explore whether the *Chlamydia*-induced IDO1-2 activity could be observed in another mouse strain, we performed qPCR and HPLC analyses of *C. muridarum* infected and control lung tissues of C57BL/6 mice. qPCR data showed a significant increase of IDO1 mRNA level in the *C. muridarum* infected lungs (fold of up-regulation range: 8.14-13.88), and while the IDO2 mRNA up-regulation was not significant, an up-regulation tendency could be observed (fold of up-regulation range: 1.71-21.49). HPLC analysis of tryptophan and kynurenine contents showed that uninfected C57BL/6 mice lungs contained a small amount of kynurenine (0.045-0.075 kynurenine/ tryptophan concentration ratios), and the *C. muridarum* infection significantly increased the IDO activity (0.185-0.773 kynurenine/ tryptophan concentration ratios).

Antiviral activity of compounds 1-3 and 5-8

The cytotoxicity of compounds 1-3 and 5-8 was tested at a concentration of 100-0.78 µM. All the compounds were dissolved in DMSO and diluted in culture medium. The maximum concentration of DMSO showed no cytotoxicity for Vero cells. After 24 h of incubation, cell viability was determined by the MTT test. The compounds showed a CC_{50} value higher than 100 µM, except for compound 8, which was rather toxic for Vero cells with a CC_{50} value of 6.25 µM. Applying traditional virus yield reduction assay, compounds 1 and 3 was found to cause a 2 $\log_{10}$ and 3.49 $\log_{10}$ reduction of HSV-2 yield, respectively, at a concentration of 12.5 µM, compared to the virus titer of untreated control samples. This finding is in line with literature data, which demonstrate that acyclovir, the gold standard of anti-HSV therapy, produces a 1-5.06 $\log_{10}$ reduction of HSV-2 yield at a concentration of 6.25 µM. Compound 2 exerted antiviral activity at concentrations of 50 µM or higher only. Musizin (5) has antiviral activity at a concentration of 12.5 µM, causing a 2.33 $\log_{10}$ reduction in virus yield. The anti-HSV-2 effect of musizin (5) demonstrated in our experiments is concordant with the result of Gescher et al. who reported the anti-HSV-1 activity of this compound. To validate our results, the direct qPCR method was used to determine the level of HSV-2 growth inhibition induced by serial dilutions of the compounds in the virus-infected cells. Similarly, to our findings from the yield reduction assay, inhibition curves based on the qPCR results showed that the most potent compounds against HSV-2 were compounds 1 and 3. The maximum HSV-2 growth corresponded to a DNA concentration of Ct ~15 in the direct qPCR assay. The compound concentration that decreased HSV-2 growth and corresponding DNA content by 50% (IC_{50}), increased the qPCR Ct value by approximately one cycle. Also, the compound concentration that inhibited HSV-2 growth by 90% (IC_{90}), raised the Ct value by ~3.32 cycles. In case of compounds 1 and 3 the IC_{50} and IC_{90} values were between 6.25 and 12.5 µM, while the IC_{50} value for compound 2 was ~25 µM and the IC_{90} value for compound 2 was between 25 and 50 µM. Musizin (5) also exerted a high antiviral activity with an IC_{50} of ~12.5 µM and IC_{90} of 25-50 µM. Torachrysone-8-O-glucoside (7) had no effect on HSV-2 growth in the concentration range applied, while the IC_{50} value for musizin-8-O-glucoside (6) was ~25 µM and the IC_{90} value was between 50 and 100 µM. 2-methoxystipandron (8) did not show any antiviral effect at the non-toxic concentration of 6.25 µM.

DISCUSSION

Aim1:

Interestingly, our results are different from those of Sater et al. who used the lymphogranuloma venereum strain *C. trachomatis* L2 and showed a concentration- and pH-dependent inhibitory effect of HEC on chlamydial growth *in vitro*. However, there are important differences between the two studies, including the fact that we used a complex buffer which may mimic better the physicochemical properties of the vaginal fluid than the phosphate and acetate buffers used by Sater et al.. Moreover, we observed the growth enhancing effect at 1.5-0.75% w/v (15000-7500 g/ml) HEC concentrations, the concentrations that are common in the vaginal gels, while Sater et al. used significantly lower HEC concentrations (2-200 g/ml). Instead of serovar L2, we also used the more prevalent urogenital serovars D and E. While *C. trachomatis* D and L2 have minor genetic differences, there are several phenotypic differences between the two serotypes. Previous studies showed that

their early interactions with epithelial cells are different, including the fact, that the centrifugation and dextrane pretreatment of host epithelial cells increased the infection efficacy of urogenital *C. trachomatis* serovars but had no impact on serovar L2. In addition, serovar E infection is heparin independent while serovar L2 infection exhibits a strong heparin dependency. Since probably HEC influences the early interactions between the EBs and the host cells, this effect may be different between the LGV and urogenital serovars. Altogether, our study shows that vaginal gel components, such as the gelling agent HEC have a significant growth enhancing effect on two prevalent *C. trachomatis* urogenital serovars. This enhancing effect was observed *in vitro* over a wide pH range, at lower concentrations, and also *in vivo*. Since the growth enhancement can theoretically lower the minimal number of bacteria required for infection transmission, these results suggest the need for testing current and future vaginal gels to determine their growth enhancing effects on *C. trachomatis* and on other sexually transmitted pathogens.

Aim 2:

RNA sequencing analysis of the *C. muridarum* infected lungs revealed that the expression of a wide variety of host genes were altered, and several up-regulated genes could contribute in the *Chlamydia*-induced inflammation and anti-chlamydial defense of the murine host. Genes related to both the innate and adaptive immunity were found to be induced in the *C. muridarum* infected lung tissue. The major functional groups were related to cytokine/chemokine expression, chemotaxis, signal transduction, antigen presentation, cell division and innate antimicrobial defense. According to the cellular theory of chlamydial pathogenesis, non-immune cells trigger the inflammation by secreting pro-inflammatory cytokines and chemokines. While the cellular source of the cytokines/chemokines cannot be identified by a tissue-level gene expression analysis, *in toto* the strong gene expression imprint of chemotaxis induction and cellular influx could be identified in the *C. muridarum* infected mouse lungs. The autocrine-paracrine effects of the secreted cytokines and the cell-to-cell interactions between resident and novel cells could result a milieu that induced a complex gene expression including the induction of certain anti-chlamydial genes.

Several members of the IFN-inducible GTPase family were found to be highly induced. Although we did not measure the gene expression changes induced by the *C. pneumoniae,* previous studies showed that both the murine and human *Chlamydia* strains were able to induce IFN-inducible GTPases. Novel GTPase genes were also found to be highly up-regulated after *C. muridarum* infection, and could be involved in the anti-chlamydial defense. Altogether, the differential sensitivity to the IFN-inducible GTPases could explain the fact that despite using 400 fold more *C. pneumoniae* IFU than *C. muridarum,* we recovered comparable IFUs from the infected lungs. The other known anti-chlamydial gene *iNOS* was also up-regulated (14 fold). iNOS induction has been shown to be an important mechanism in the later phase elimination of *C. muridarum* infection from the mouse genital tract and also in RAW 264.7 murine macrophages. *IRG1*, another IFN-inducible gene was found to be highly up-regulated (252.7 fold) after *C. muridarum* infection. A CXC chemokine, *MIG* was also highly up-regulated (127 fold) after *C. muridarum* infection. Interestingly, the size, the cationic charge and amphipathic nature of CXC chemokines are similar to certain antimicrobial peptides. We showed previously that MIG had a concentration-dependent direct toxicity to the elementary bodies of *C. muridarum*, *C. trachomatis* and *C. pneumoniae*.

RNA sequencing and qPCR revealed that the *IDO1* and *IDO2* genes were also highly induced in the infected lungs. To identify the source of IDO activity, we performed IDO1-2 IHC in infected and control lung tissues. We found that lung bronchial epithelial cells had a moderate level of IDO1-2 positivity both in the control and infected tissues, indicating a lower-level, steady-state expression. A higher level of IDO1-2 positivity was detected in leukocytes, prominently in macrophages, in both the uninfected and infected tissues, but the number of positive cells was higher in the *Chlamydia*-infected tissues. The higher number of IDO1-2 positive macrophages might be a result of *in situ* IDO1-2 induction and/or the influx of already IDO1-2 positive monocytes into the inflamed tissue. It is also possible that the IDO1-2 positive macrophages were activated locally resulting a higher IDO1-2 activity. According our gene expression data one of the prominent networks induced by *C. muridarum* infection was the IFN signaling pathway, therefore the IFN impact of *IDO1-2* gene induction was clearly present in the *Chlamydia*-infected tissues. HPLC detection of the tryptophan degradation product kynurenine demonstrated that in the uninfected murine lung tissues IDO activity was not detectable, hence the low level IDO1-2 protein positivity detected in uninfected epithelial cells and macrophages did not

yield significant tryptophan catabolism, IDO1-2 enzymes were induced and functionally active in both the murine and human *Chlamydia* infected lung tissues. A quantitative IHC was not performed, but the observation of similar level of IDO1-2 IHC positivity in epithelial cells before and after infection indicates that the IDO1-2 was not induced and IDO1-2 activity might not be involved in the elimination of *Chlamydia* from the murine lung epithelial cells. Further quantitative studies needed to clarify the exact role of murine epithelial IDO expression. The fact that C57BL/6 mouse lungs also showed *Chlamydia* infection induced IDO activity, supports the possibility that the observed IDO induction is not a mouse strain-specific response.

There are limitations of our study that need further investigations. We could not detect IDO 1-2 inducibility in epithelial cells, but the applied IHC was not a quantitative method. Since epithelial cells are the sites of chlamydial replication, the isolation of lung epithelial cells and the measurement of their steady state and infection-induced IDO activity are critical points and a goal we are currently pursuing. The other obvious targets to assess IDO activity are the isolated epithelial cells of the uninfected and infected murine urogenital tract. The role of IDO2 is not defined. The lung IDO2 mRNA was clearly induced by *Chlamydia* infection, but its RNA-seq read numbers were significantly lower than IDO1 reads. In order to assess the potential role of IDO2, the IDO2 protein concentrations in isolated lung epithelial cells and macrophages has to be measured and compared to IDO1. Also, chemical inhibition of IDO showed a significant, albeit limited phenotypic effect. This could be due to the limited defensive role of IDO1-2 or the incomplete inhibition of the enzymes. Further studies with optimized IDO inhibition protocol and more time points post infection are needed.

Chlamydia can avoid intracellular defense responses by using metabolic shunt, inactivating cellular effector proteins such as IFN-inducible GTPases, or avoiding the induction of the intracellular effectors. Our, *in vivo* study showed that -at the tissue level- various antibacterial mechanisms are switched on and IDO1-2 could be related to this effector repertoire.

Aim 3:
The dose dependent inhibitory effect of certain sesquiterpenes (1, 2), phenylpropane dimer (3), naphthalenes (5-7), and 14-naphthoquinone (8) derivatives isolated from *E. rhamnoides* (1-3) and *R. aquaticus* (5-8) were investigated against HSV-2 virus infected Vero cells, by applying the traditional virus yield reduction test and the qPCR method. Compounds 1 and 3, as well as musizin (5) were demonstrated to have a potent anti-HSV-2 activity. In case of glucosides, such as musizin-8-O-glucoside (6), only a moderate anti-HSV-2 activity was observed. Isolating two new 14-noreudesmane sesquiterpenes (1, 2) from sea buckthorn, and demonstrating the antiviral properties of several compounds present in the fruit peel of the plant are significant novelties of our study. Based on our results, sea buckthorn is worth being further studied, as it could be a potential source of agents with considerable anti-HSV-2 activity, and thus it might provide alternative drug candidates for the treatment of patient populations infected with acyclovir- and penciclovir-resistant strains of the virus.

THE FOLLOWING ARE CONSIDERED NOVEL

AIM 1:

The gelling agent, HEC have a significant growth-enhancing effect on the two prevalent *C. trachomatis* urogenital serovars. This enhancing effect was observed *in vitro* over a wide pH range, at lower concentrations, and also *in vivo*. Since the growth enhancement can theoretically lower the minimal number of bacteria required for infection transmission, these results suggest the need for testing current and future vaginal gels to determine their growth enhancing effects on *C. trachomatis* and on other sexually transmitted pathogens.

AIM 2:

Infection with two different *Chlamydia* strains in BALB/c murine lungs, showed highly expressed IDO 1-2 amongst other host defense genes. It is a novel finding, since IDO was considered as a unique human anti-chlamydial defense gene.

IDO 1-2 activity were also increased in *C. muridarum* infected C57BL/6 lung tissues, indicating that this phenomenon is not mouse strain specific.

AIM 3:

Out of three extracted compounds from *E. rahmnoides* two had a potent anti-HSV-2 activity, compound 1 (a sesquiterpen) and compound 3 (a phenylpropane heterodimer). Among the derivatives isolated from *R. aquaticus* compound 5 (musizin) showed a very potent anti-HSV-2 activity while compound 6 (musizin-8-O-glicoside) has a moderate anti-HSV-2 activity.

SUMMARY

STDs are a persistent problem globally, worldwide more than 1 million STDs are acquired every day, and these numbers tend to increase. Amongst bacterial STDs *C. trachomatis* infections are the most prevalent.

The transmission of the urogenital serovars of *C. trachomatis* can be significantly influenced by vaginal gels. HEC is a commonly used gelling agent that can be found in various vaginal gels. We investigated the effect of HEC on the *C. trachomatis* serovar D growth and found that it had a growth-enhancing effect, both *in vitro* and *in vivo*. In addition, *in vitro* investigation of the impact of HEC on *C. trachomatis* serovar E growth also showed enhancing effect.

The prevention of the *Chlamydia* infections with vaccination, would be a potent option to decrease the prevalence of chlamydial infections. Mouse models are generally used for vaccine development, to study the immune response and histopathology associated with *Chlamydia* infection. An important question, regarding murine models is the *in vivo* identification of murine host genes responsible for the elimination of the murine and human *Chlamydia* strains. *C. muridarum* infected BALB/c lung transcriptome revealed that several genes with direct anti-chlamydial functions were induced at the tissue level, various members of the IFN-inducible GTPase family, the CXCL chemokines *CXCL9*, *CXCL11*, immunoresponsive gene 1, iNOS and lipocalin-2. IDO1-2, the previously described potent anti-chlamydial host enzymes were also highly expressed in the infected murine lungs. This finding was novel, since IDO was considered as a unique human anti-chlamydial defense gene. Besides a lower level of epithelial cell positivity, immunohistochemistry showed that IDO1-2 proteins were expressed prominently in macrophages. Detection of the tryptophan degradation product kynurenine and the impact of IDO inhibition on *C. muridarum* growth proved that the IDO1-2 proteins were functionally active. IDO1-2 activity also increased in *C. muridarum* infected C57BL/6 lung tissues, indicating that this phenomenon was not mouse strain specific.

Another prevalent, sexually transmitted intracellular pathogen is HSV-2. Various therapeutic agents can be used for the treatment of HSV-2 infections, however, infections by drug resistant HSV represent a persisting treatment concern, especially in the immune-compromised population. To identify a possibly novel antiviral compound extracted from the berry of sea buckthorn, we investigated the *in vitro* antiviral effects of these compounds against HSV-2. Applying the traditional virus yield reduction test and qPCR methods, we found, that two extracted compounds from *E. rahmnoides* have a potent anti-HSV-2 activity, compound 1 (sesquiterpen) and 3 (phenylpropane heterodimer). Derivatives isolated from *R. aquaticus* compound 5 (musizin) showed a very potent anti-HSV-2 activity as well, while compound 6 (musizin-8-O-glicoside) had a moderate anti-HSV-2 activity.

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PUBLICATIONS RELATED TO THIS THESIS

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