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Influence of *Chlamydia trachomatis* infection on the barrier function of porcine oviduct epithelial cells

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submitted by

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Abstract

Chlamydia trachomatis (*Ct*) infections cause the most prevalent bacterial sexually transmitted disease worldwide causing infertility and ectopic pregnancies. An additional detrimental side effect of *Ct* infections is that patients are at higher risk of experiencing co-infections with sexually transmitted viruses such as human immunodeficiency virus (HIV). It has been previously demonstrated that *Ct* infection of porcine oviduct epithelial cells (pOECs) redirects claudin-4, a tight-junction protein, from the cell surface to the intracellular *Ct* inclusions. This redirection of claudin-4 indicates that *Ct*-infected cells have a lower tight-junction barrier function. Thus, this study aims to show the effect of *Ct* infection on pOECs regarding their barrier integrity. pOECs were isolated and infected with *Ct* to measure the trans-epithelial electrical resistance (TEER). By comparing the TEER between *Ct*-infected and non-infected pOECs, we could demonstrate that *Ct* infection leads to a significant decrease in the TEER of pOECs as early as eight hours post-infection (hpi) with *Ct* titers ranging from 1:8 through 1:128,000. This effect was dose-dependent. The reproductive cycle of *Ct* in pOEC takes ~ 48 hours before *Ct* are released via cell lysis. Since *Ct* inhibits host cell apoptosis and a drop in TEER was observed as early as 8 hpi, it is unlikely that this effect is caused by *Ct*-induced cell death. Taken together this data suggests a *Ct*-induced disruption of the epithelial barrier integrity which could explain the higher risk of *Ct* patients for co-infections with HIV. Thereby, this project justifies further investigation into how *Ct* infection might affect the permeability of pOECs barriers for viruses like HIV.

Zusammenfassung

Chlamydia trachomatis (*Ct*) Infektionen sind Ursache für die weltweit häufigste bakterielle Geschlechtskrankheit, und können zu Unfruchtbarkeit und ektopischen Schwangerschaften führen. Ein weiterer Nebeneffekt liegt darin, dass PatientInnen eine höhere Chance für Ko-Infektionen mit sexuell übertragbaren Viren, wie dem Human Immunodeficiency Virus (HIV), haben. Es wurde bereits gezeigt, dass *Ct*-Infektion in Eileiter-Epithelzellen des Schweins (pOECs) zu einer Umleitung von Claudin-4, einem Tight-Junction-Protein, von der Zelloberfläche zu intrazellulären *Ct*-Einschlusskörpern führt. Diese Umleitung von Claudin-4 deutet darauf hin, dass *Ct*-infizierte Zellen eine geringere Barrierefunktion aufweisen. Ziel dieser Studie ist daher, die Auswirkungen von *Ct*-Infektionen auf pOECs hinsichtlich ihrer Barrierefunktion zu untersuchen. pOECs wurden isoliert und mit *Ct* infiziert, um den trans-epithelialen elektrischen Widerstand (TEER) zu messen. Durch den Vergleich des TEER zwischen *Ct*-infizierten und nicht-infizierten pOECs konnten wir zeigen, dass eine *Ct*-Infektion bereits acht Stunden nach der Infektion (hpi) zu einer signifikanten Abnahme des TEER von pOECs führt, wobei die *Ct*-Titer von 1:8 bis 1:128.000 reichten. Diese Wirkung war dosisabhängig. Der Replikationszyklus von *Ct* in pOECs dauert ~ 48 Stunden, bevor die Chlamydien durch Zelllyse freigesetzt werden. Da *Ct* die Apoptose der Wirtszelle inhibiert und ein Rückgang des TEER bereits nach 8 hpi beobachtet wurde, ist es unwahrscheinlich, dass dieser Effekt durch *Ct*-induzierten Zelltod verursacht wurde. Zusammengefasst deutet dies auf eine gestörte Epithelbarriere hin, welche das höhere Risiko für Ko-Infektionen mit HIV erklären könnte. Dieses Projekt rechtfertigt somit weitere Untersuchungen über den Einfluss von *Ct*-Infektionen auf die Barrierefunktion von pOECs hinsichtlich ihrer Durchlässigkeit gegenüber Viren, wie HIV.

Table of contents

1 Introduction

1.1 Overview of *Chlamydia trachomatis*

Chlamydia trachomatis (*Ct*) is an obligate intracellular bacterium which can be divided into several serovars, causing different diseases. Serovars A-C cause ocular trachoma, leading to blindness and visual impairments. Serovars D-K are mainly sexually transmitted and cause inflammation in the genital tract. This leads to urethritis and epididymitis in men and pelvic inflammatory disease and ectopic pregnancies in women (Brunham and Rey-Ladino 2005, Murray and McKay 2021).

1.1.1 Replicative cycle

The replicative cycle of *Ct* in host epithelial cells is biphasic. *Ct* exists in one of two distinct forms, the elementary body (EB) or the reticulate body (RB). The EBs are mainly extracellular and infectious but metabolically inactive, whereas RBs are the metabolically active intracellular form (Brunham and Rey-Ladino 2005, Murray and McKay 2021).

Upon transmission, EBs enter epithelial cells through use of the Type III Secretion System (T3SS) complex. The T3SS resides in the outer membrane of EBs and is responsible for initiating internalisation of the EBs after binding to the host epithelial cells. For successful binding of the EB to the host cell two steps are necessary. First membrane proteins of the EB interact electrostatically with heparan sulphates in the membrane of epithelial cells. The second step is the interaction and binding of pathogen antigens to host receptors such as the mannose receptor or the cystic fibrosis transmembrane conductance regulator. Once these two binding steps are accomplished, effector proteins are released by the T3SS into the host epithelial cells. One of these effectors is the translocated actin-recruiting phosphoprotein (Tarp) needed for internalisation of the EB into the host cell (Murray and McKay 2021). Tarp can directly bind actin through C-terminal actin-binding domains and enhance actin oligomerisation. Secondly, Tarp can indirectly cause actin remodelling through activating a Rac1-dependent pathway. This activates several regulatory proteins necessary for actin reorganisation. Both of these mechanisms lead to cytoskeletal rearrangements in the host cell which facilitate *Ct* internalisation (Gitsels et al. 2019).

Once internalised, EBs are contained in inclusions. These inclusions are non-lysosomal vacuoles with the ability to dissociate from the endocytic pathway. After the inclusions are established EBs differentiate into metabolically active RBs. The inclusion membrane is

modified through chlamydial proteins to facilitate the intracellular replication (Fields and Hackstadt 2002, Gitsels et al. 2019, Murray and McKay 2021). These modifications are necessary to interact with the exocytic pathway of the host cell for nutrient acquisition and fusion with exocytic vesicles. Through this *Ct* obtain membrane lipids like cholesterol and sphingomyelin, ATP, glucose, amino acids and other necessary molecules for replication. Moreover, RBs are also able to modify the host immune pathways through these modifications (Gitsels et al. 2019, Murray and McKay 2021).

In the established inclusions RBs multiply through binary division. After multiple division rounds RBs differentiate back into EBs. This process is asynchronous however it is largely unknown what exactly happens during this process (Murray and McKay 2021). These EBs are then released from the host cell for a new infection round. There are two possible ways *Ct* can exit the host cell. One is through induction of host cell lysis. First lysis of the inclusion membrane is induced by a cysteine protease. Then the host cell plasma membrane is ruptured. This process is dependent on calcium signalling (Gitsels et al. 2019, Murray and McKay 2021). The second way of *Ct* release is by extrusion, which keeps both the host cell and inclusion intact. This way functions similar to exocytosis, with budding from the host cell. It is not destructive and may also play a role in chlamydial persistence, as some *Ct* is left in the host cell after release (Hybiske and Stephens 2007, Murray and McKay 2021).

1.1.2 Host immune response to *Chlamydia trachomatis* infection

1.1.2.1 Innate immunity

Antigen-presenting cells are necessary to activate the adaptive immune response of the host. *Ct* pathogen-associated molecular patterns (PAMPs) are recognised by several different pattern recognition receptors. These receptors are involved in the activation of the immune response through dendritic cells (DCs). DCs then produce numerous pro-inflammatory cytokines, especially Interleukine-12 (IL-12) which activates a T-helper 1 type immune response. Another important cytokine is Interferon gamma (IFNγ) which helps in clearance of *Ct* infection through tryptophan starvation (Brunham and Rey-Ladino 2005, Murray and McKay 2021).

1.1.2.2 Adaptive immunity

After chemokine release, lymphocytes and other immune cells are recruited to the epithelial cells in the genital tract and form immune inductive sites (Brunham and Rey-Ladino 2005). CD4+ and CD8+ T-lymphocytes are present during infection. The role of CD8+ cells is still

unclear. However, they are suspected to partake in both protection and driving pathology (Murray and McKay 2021). The role of CD4+ cells, especially CD4+ T-helper 1 cells (Th1 cells), is determined to be crucial for eliminating *Ct* infection (Brunham and Rey-Ladino 2005, Murray and McKay 2021).

Although an antibody response alone is not sufficient to prevent and clear a primary *Ct*-infection, it is beneficial for the prevention and clearance of *Ct*-reinfections (Brunham and Rappuoli 2013). B-cells are thought to produce neutralising antibodies, which limit infection. B-cells might also enhance the T-cell response in specific cases, through enhanced antigen presentation to T-cells (Brunham and Rey-Ladino 2005, Murray and McKay 2021).

Overall, the immune response against *Ct* infections is driven by CD4+ Th1 cells, with CD8+ T-cells and B-cells playing a smaller role (Brunham and Rey-Ladino 2005).

1.1.3 Evasion of host cell defences

Chlamydia have developed several strategies to escape the defence mechanisms of the host cell. First *Ct* can adapt its morphological structure. *Ct* has diverse surface antigens to avoid detection by antibodies. Furthermore, lipopolysaccharides (LPS) are present at a lower level and also less potent in activating inflammatory responses. Replication inside the inclusion brings additional protection. Targeting through antibodies is less likely and inclusion proteins in the membrane can interrupt the immune response through interfering in signalling processes or blocking production of inflammatory cytokines (Brunham and Rey-Ladino 2005, Murray and McKay 2021).

If conditions in the host cell are not viable for replication, RBs move into a persistent state in which they cannot replicate. This is caused through a lack of nutrients, which can be intentionally induced by the host cell. One example would be tryptophan starvation as mentioned above. This tryptophan starvation can either kill *Ct* or in low moderation induces the persistent state of RBs. This persistent state can also be induced through numerous other events such as lipid or micronutrient starvation (Brunham and Rey-Ladino 2005, Gitsels et al. 2019, Murray and McKay 2021).

Another important mechanism of host defence evasion is the inhibition of cell apoptosis. As *Ct* replicate in intracellular inclusions, it is necessary to keep the host cell intact. Infection with *Ct* inhibits mitochondrial induced apoptosis through blocking the release of cytochrome c. Bax- and Bak-activation, both effectors of mitochondrial apoptosis, are inhibited during *Ct* infection.

This results in the inhibition of host cell death, ensuring *Ct* can replicate inside the host cell (Waguia Kontchou et al. 2022).

1.1.4 Pathology

Ct infections cause infertility, ectopic pregnancies, and chronic pelvic pain in women (Brunham and Rey-Ladino 2005). In men, infections cause epididymitis and proctitis (Hocking et al. 2023). An additional detrimental side effect of *Ct* infections is that patients are at higher risk of experiencing co-infections with sexually transmitted viruses such as human immunodeficiency virus (HIV) (Brunham and Rey-Ladino 2005).

1.1.5 Epidemiology

Chlamydia trachomatis is the cause for one of the most common sexually transmitted infections that are curable. In a study of Rowley et al. funded partly by the World Health Organisation (WHO), the global prevalence and incidence of urogenital chlamydia infections in women and men between the ages of 15 to 49 for the year 2016 were estimated. For women the global prevalence was estimated at 3.8 % and for men at 2.7 %. The highest prevalence of *Ct* infection in men was determined in the African Region (4 %), the highest prevalence in women however was estimated for the Region of the Americas (7 %). Based on the categorisation in high- and low-income countries, territories and areas, *Ct* prevalence was highest in upper-middle income countries. For 2016 the estimated global incidence rates were 34 cases per 1000 women and 33 cases per 1000 men. The estimated incidence rate was highest in the Region of the Americas. Globally 127,2 million new *Ct* infections were estimated for 2016 (Rowley et al. 2019).

According to data for the year 2020, published on the official website of the WHO, the global prevalence of *Ct* infection was 4 % in women and 2.5 % in men. Worldwide a total of 128.5 million new *Ct* infection cases were estimated. These estimates are similar to the study mentioned above, which estimated prevalence for the year 2016 (World Health Organisation 2023).

1.2 Protective role of epithelial cells against pathogens

Epithelial cells play a big role in protection against pathogens, providing both a physical and chemical barrier. Part of the physical barrier are tight junctions, which hinder pathogens in entering between the cells of the epithelium. As a part of the chemical barrier epithelial cells produce mucus and secrete anti-microbial molecules. If pathogens are able to enter the cells regardless, an immune response is activated (Alberts et al. 2015).

As mentioned above, *Ct* infect epithelial cells in the genital tract where they reproduce intracellularly. However, epithelial cells are able to recognise the pathogen through innate immune receptors. Then epithelial cells secrete numerous cytokines and chemokines which stimulate inflammation and recruit immune cells to the infected sites (Brunham and Rey-Ladino 2005, Darville and Hiltke 2010).

Tight junctions are located between epithelial cells and are responsible for keeping the integrity of the epithelial layer, an important part of the physical barrier against pathogen invasion. Tight junctions can change, depending on different stimuli, which is needed for the passage of selected molecules. However, a change in the tight junction integrity can also be caused by pathogens (Blaskewicz et al. 2011).

The integrity and permeability of the tight junction epithelial barrier can be measured through several methods. One of them being the trans-epithelial electrical resistance (TEER) measurement, a non-invasive way to measure changes of the tight junctions in live cells. It is accomplished via measuring the ionic conductance of the epithelial layer (Srinivasan et al. 2015).

1.3 Pig models and *Chlamydia trachomatis* infection

Pigs are the natural host to *Chlamydia suis* (*Cs*) but are also receptive to *Ct*, a closely related chlamydia species (Amaral et al. 2020). Furthermore, pigs share many biological features with humans. Though there are some differences in the anatomy of the female and porcine genital tract, the overall structure is very similar. Especially the Fallopian tubes, the site for *Ct* infection, show little differences in construction, both having a singular epithelial cell layer and ciliated and non-ciliated secretory cells. Additionally, the hormonal cycle of women and sows is very similar, with only a slight difference in length (Amaral et al. 2020, Lorenzen et al. 2015).

The immune systems of humans and pigs share many similarities as well. Immune cells and mediators have similar functions in both and antibody levels are about the same as well (Lorenzen et al. 2015). Furthermore, it has been shown that pigs infected with *Ct* develop a CD4+ Th1 immune response, with CD4+ cells that mainly produce IFNγ or IFNγ and Tumour Necrosis Factor alpha (TFNα) (Käser et al. 2017). These similarities to humans make the pig a good animal model for *Ct* research (Amaral et al. 2020, Käser et al. 2017, Lorenzen et al. 2015).

1.4 Aim of this study

Porcine oviduct epithelial cells (pOECs) are the counterpart to human Fallopian tube epithelial cells. It has been previously demonstrated that *Ct* infection of pOECs redirects claudin-4, a tight-junction protein, from the cell surface to the *Ct* inclusions in which *Ct* replicate intracellularly (Amaral et al. 2021). Based on this redirection of claudin-4 we hypothesize that *Ct*-infected cells have a lower tight-junction barrier function. To test this hypothesis, this study aims to show the effect of *Ct* infection on pOECs regarding their barrier integrity.

To accomplish this, pOECs were isolated based on an established protocol. Next, a *Ct* stock was generated, and its titer determined via flow cytometry. With these *Ct*, pOECs were infected to not only monitor *Ct* growth but also to use the xCelligence instrument to measure the TEER. The TEER is a non-invasive way to quantify the integrity of tight junctions in epithelial cell cultures (Srinivasan et al. 2015). By comparing the TEER between *Ct*-infected and non-infected pOECs, the effect of *Ct* on the pOEC barrier integrity could be evaluated.

2 Materials and Methods

2.1 *Chlamydia trachomatis* propagation

To produce *Ct* stocks, large-scale propagation in 6-well plates was performed. Initially, two plates were infected following the standard protocol for infection using centrifugation. Two days post-infection McCoy cells were seeded in two new 6-well plates. These were then infected on the next day using *Ct* from the first plates. This cycle was repeated again, this time infecting eight new 6-well plates with *Ct* from the previous two plates. After another three days of infection, the *Ct* were harvested from the infected cells.

The *Ct* stocks used to infect the first plates were of the strain *Ct E Bour* originally purchased from ATCC and then propagated in the Kaeser lab at North Carolina State University, Raleigh, USA.

2.1.1 Cultivation of McCoy cells in 6-well plates

First McCoy cells were seeded in culture medium (DMEM (PAN-Biotech GmbH, Germany) + 10% fetal calf serum (FCS) (Sigma-Aldrich, USA) + Na-Pyruvate) into T175 cell culture flasks (Greiner Bio-One International GmbH, Austria). For propagation of *Ct*, McCoy cells were seeded into 6-well plates (Greiner Bio-One International GmbH, Austria) at a density of $1*10^6$ cells/well. At the time of infection, 24 hours after seeding, cells were about 90 % confluent.

2.1.2 Infection of McCoy cells with *Chlamydia trachomatis*

The infection was initially done, using an already-established infection protocol. First, the culture medium was removed, and the infection medium was directly put onto the cells (2 ml/well). The infection medium was produced by diluting four different *Ct* stock to a concentration of 1:60. In the next step, the plates were centrifuged for one hour at 37 °C at 900 g. Afterwards, the plates were left in the incubator at 37 °C for an additional hour. Lastly, the infection medium was taken off and new culture medium with Cycloheximide (1:1000 dilution, Thermo Fisher Scientific Inc., USA) was added.

After the initial round of infection using thawed *Ct* stocks the new plates were infected with the *Ct* harvested from the first plates. This process is described below.

2.1.3 Harvesting of *Chlamydia trachomatis* using sonication

For harvesting the *chlamydia* supernatants from the plates were transferred into 50 ml tubes and kept on ice. 1 ml PBS (DPBS, PAN-Biotech GmbH, Germany) was added to each well to

prevent the cells from drying out. Cells were scraped and the PBS containing the McCoy cells was transferred into different 50 ml tubes. The plates were washed with 1 ml PBS per plate, which was also added to the tubes. The cell suspension collected in these tubes was sonicated using a water bath to break open the cells. The tubes were sonicated for one minute, rested on ice for one minute, and were then sonicated for another minute.

Afterwards, all tubes were centrifuged (400 g, 4 °C, 10 min). The supernatant was taken off and the cell pellet was discarded. The supernatant of those tubes which contained the scraped cells was sonicated once more and centrifuged again, to break any remaining McCoy cells open.

Then all supernatants were combined for another centrifugation step (400 g, 4 °C, 10 min) to pellet any remaining cells away. The supernatant containing *Ct* was transferred to highspeed centrifugation tubes and spun for 30 min at 24 000 g at 4 °C. This final pellet now contained the desired *chlamydia* which could either be used for another round of infection or frozen for further use.

To infect new plates with the isolated *Ct*, the complete pellet was resuspended in the amount of culture medium needed to infect all new 6-well plates. After the first round of infection one of the *Ct* stocks was not propagated further. As it was determined via visual inspection of the cells, that none of the McCoy cells were infected properly. Thus, further propagation was only done for three out of four initial *Ct* stocks. This propagation procedure was repeated until there were eight plates infected with *chlamydia* of each initial *Ct* stock. Then the *Ct* were harvested and frozen.

To freeze the *Ct*, the final pellets after high-speed centrifugation were resuspended in SPG-Buffer and 1 ml aliquots were made. The *Ct* stocks (*Ct* A, *Ct* B, *Ct* C) were stored at -80 °C. These aliquots were thawed for infection of cells and diluted in the right amount of culture medium to reach the desired concentration.

2.1.3.1 SPG Buffer

This buffer was prepared by dissolving 75 g sucrose (ICN Biomedicals, USA), 87 ml Na_2HPO_4 (0.2 M), 13 ml NaH_2PO_4 (0.2 M) and 0.72 g L-glutamic acid (L(+)-Glutamic acid, 99 %, Thermo Fisher Scientific Inc., USA) in 1 litre of water. The pH was adjusted to 7.4 with NaOH (2 M). After, the buffer was filtrated for sterilisation and stored in 100 ml aliquots at 4 °C.

2.1.4 Harvesting of *Chlamydia trachomatis* by foaming the cells

For the first round of *Ct* isolation two different methods of breaking up the McCoy cells were tested. The first one was described above using sonication in a water bath. The second method was to foam the supernatant and cells using a 1000 µl pipette.

Contrary to described above, it is not necessary to separate the cell culture supernatant from the cells for this method. Using a cell scraper, the cells are directly scraped in the supernatant. Then using the pipette, the whole supernatant is foamed by pipetting the medium with the cells up and down while deliberately sucking in air and producing small bubbles. This foam is transferred to 50 ml tubes and centrifuged the same as the other tubes (400 g, 4 °C, 10 min).

After this, all steps are the same for both sonication of the cells as well as the foamed cells.

2.2 Isolation of porcine oviduct epithelial cells (pOECs)

2.2.1 Harvesting of oviducts from porcine genital tracts

pOECs were isolated out of oviducts from two pigs. These pOECs were then cultivated and infected as needed to monitor the trans-epithelial electrical resistance.

For isolation, the oviducts were placed in big petri dishes and PBS was added. Any connective tissue was removed using forceps and scissors and the oviducts were placed into clean petri dishes. The oviducts were opened using a small scissor and the tissue was cut into pieces about 1 cm each. The tissue pieces of each oviduct were placed into 20 ml of the prepared enzyme solution in a 50 ml tube. The tubes were left on a 360° rocker at 4°C overnight.

The tubes were labelled with 2401 and 2402 for each animal. Additionally, both oviducts of one animal were kept separate as well. Therefore, four tubes labelled 2401-01, 2401-02, 2402-01 and 2402-02 were used to isolate pOECs.

2.2.1.1 Enzyme solution

200 ml enzyme solution were prepared the following: 2 ml Anti-Anti (Gibco™ Antibiotic-Antimycotic, Life Technologies Corporation, USA), 1 ml dispase (Gibco™ Dispase II, Life Technologies Corporation, USA) and 2.4 g pancreatin (Thermo Fisher Scientific Inc., USA) were added to 200 ml of DMEM/F12 (PAN-Biotech GmbH, Germany).

2.2.2 Isolation of pOECs without scraping (pOEC-)

The next day the enzyme solution was transferred into new tubes and the tissue pieces were placed into petri dishes, keeping the animals as well as oviducts separate. Any remaining liquid

on the tissue pieces was removed and added to the tubes. 10 ml cold PBS was put into the petri dishes to ensure the tissue pieces wouldn't dry. The cells from the tissue pieces were harvested separately.

To stop the enzymatic activity 5 ml FCS was added to 20 ml enzyme solution. The solution was filtered through a 50 µm filter and cells were counted. The tubes were centrifuged for 5 min at 600 g at 4 °C. The resulting cell pellets were resuspended in 20 ml pOEC-culture medium and the pOECs were cultivated in two T175 flasks for each animal in a total volume of 35 ml pOEC-culture medium.

pOECs from tube 2401-01 were seeded at a density of $24.5*10^6$ cells per flask. pOECs from tube 2401-02 were seeded at a density of $17.7*10^6$ cells per flask. pOECs from tube 2402-01 were seeded at a density of $22.5*10^6$ cells per flask. pOECs from tube 2402-012 were seeded at a density of $16.8*10^6$ cells per flask.

2.2.2.1 pOEC culture medium

The culture medium used for pOEC cultivation was prepared by using 500 ml DMEM/F12 and adding the following components: 25 ml 5 % FBS, 5 ml Glucose (200 mg/ml, Gibco™ Glucose solution, Life Technologies Corporation, USA), 100 µl Gentamicin (10 µg/ml, Gentamicin sulfate, PAN-Biotech GmbH, Germany), 500 µl Insulin-Transferrin-Selenium (ITS) (0.1 %, Gibco™ Insulin-Transferrin-Selenium, Life Technologies Corporation, USA), 12.5 µl epidermal growth factor (EGF) (0.0025 %, Gibco™ EGF Recombinant Protein, Life Technologies Corporation, USA) and 5 ml Anti-Anti (1 %).

2.2.3 Isolation of pOECs with scraping (pOEC+)

Cells from the tissue pieces, which were put in PBS, were cultivated separately from pOECs isolated through overnight incubation in the enzyme solution alone.

The top side of the oviduct lumen of each tissue piece was scraped using a scalpel. The PBS now containing the scraped epithelial cells was transferred to 50 ml tubes and the tissue pieces were discarded. The petri dishes were washed with 5 ml of PBS which was also added to the tubes. 4 ml FCS was added to each PBS cell solution.

The tubes were also centrifuged at 600 g at 4 °C for 5 min. The resulting cell pellet was resuspended in 10 ml cold accutase (Gibco™ StemPro™ Accutase™ Cell Dissociation Reagent, Life Technologies Corporation, USA) per tube to break down the collagen. The cells were incubated at 37°C for a total of 30 minutes in a water bath. After the first 15 minutes of

incubation, the cell solution was resuspended shortly and put back for the second 15 minutes of incubation.

After this incubation step the cell solution was spun again at 600 g at 4 °C for 5 min. The resulting cell pellets were resuspended in 20 ml pOEC-culture medium for each tube and filtered through a 50 µm filter. Then cells were counted and cultivated in T175 flasks. As this method resulted in a larger number of isolated cells, only pOECs from animal 2401 were cultivated. pOECs were cultivated in 35 ml pOEC-culture medium per flask. Three T175 flasks of pOECs isolated from tube 2401-01 were cultivated at a seeding density of about $24.5*10^6$ cells. pOECs isolated from tube 2401-02 were cultivated in four flasks with a seeding density of $25.7*10^6$ cells.

pOECs from animal 2402 were frozen immediately in freezing medium (50 % RPMI 1640 (PAN-Biotech GmbH, Germany), 40 % FCS, 10 % DMSO (Sigma-Aldrich, USA) at a concentration of $4*10^7$ cells/cryo vial. This resulted in seven vials for 2402-01 and five vials for 2402-02.

2.2.4 Cultivation of pOECs

The cells isolated through incubation in the enzyme solution alone and those isolated through additional scraping were cultivated separately to monitor possible differences. After two days of cultivation, the pOECs from tubes with scraping were washed once with 10 ml PBS and the pOEC medium was changed. This step ensured the removal of any non-adherent cells, dead cells and other unwanted cell debris.

After another two days, pOECs reached 100 % confluency in all flasks and were harvested and frozen. The pOECs were pooled for each animal 2401 and 2402 but the distinction between cells isolated through scraping and those isolated without scraping was kept.

First, the medium was taken off and cells were washed once with 10 ml PBS. Then the cells were incubated at 37 °C in 3 ml Trypsin (1x, PAN-Biotech GmbH, Germany) for around 6 minutes. Additionally, a cell scraper had to be used to detach the cells completely. To stop the trypsinization, 7.5 ml of pOEC medium was added to each flask. The cells were transferred into 50 ml tubes and centrifuged for 6 minutes at 400 g at 4 °C.

The cell pellets were resuspended in new medium and pooled for each animal. Scraped and unscraped cells were kept separately. The cells were centrifuged again and resuspended in the necessary amount of freezing medium, using 1 ml freezing medium per tube and freezing

$3*10^6$ cells/cryovial. 25 cryovials could be frozen with scraped pOECs from animal 2401. With unscraped cells from animal 2401, eight cryovials were produced. Unscraped cells from animal 2402 produced five cryovials. The vials were stored at -80 °C and transferred to -150 °C after a few days.

For use in experiments, these frozen pOECs were thawed and cultivated in flasks for a few days before transferring them into the experimental plates.

2.3 Flow cytometry staining of infected cells

Flow cytometry (FCM) staining of infected cells was performed to titrate the necessary antibodies and to determine the titer of the produced *Ct* stocks.

2.3.1 Cultivation of McCoy cells in 24-well plates

First McCoy cells were seeded in culture medium into T175 cell culture flasks (Greiner). To titrate the primary and secondary *chlamydia* antibody using FCM, McCoy cells were cultivated in 24-well plates (Greiner) at a density of $0.2*10^6$ cells/well. At the time of infection, 24 hours after seeding, cells were about 90% confluent.

2.3.2 Titration of antibodies for FCM

2.3.2.1 Infection of 24-well plates

The infection of McCoy cells in 24-well plates was performed the same way as for 6-well plates described above. However, 500 µl of infection medium were used per well.

2.3.2.2 FCM staining

Infected McCoy cells were harvested 24 hpi. The supernatant was taken off using a vacuum pump to contain the infectious medium. The cells were washed once with PBS and then incubated in 200 µl Trypsin (1x) for 10 min at 37 °C. Trypsinization of the cells was stopped by directly adding 20 µl FCS into each well.

Afterwards, the cells were transferred into a 96-well plate and centrifuged for 4 min at 500 g at room temperature. The supernatant was removed using a multichannel pipette, and cells were washed once in 200 µl PBS. Each washing step consisted of resuspending the cells in the washing solution and then centrifuging the plate once (4 min, 500 g, RT).

After the first washing step, cells were incubated in 200 µl eBio Fix/Perm buffer (eBioscience™ Intracellular Fixation & Permeabilization Buffer Set, Thermo Fisher Scientific Inc., USA) for 30 min at 4 °C. After fixation, the supernatant was flipped off for removal, as the cells weren't

infectious anymore. Then the cells were washed twice with 200 µl eBio Perm/Wash (eBioscience™ Intracellular Fixation & Permeabilization Buffer Set, Thermo Fisher Scientific Inc., USA).

To titrate the primary anti-Chlamydia antibody (anti-Chlamydia & Chlamydophila mouse monoclonal, PROGEN Biotechnik GmbH, Germany) the cells were stained with five different concentrations of the antibody (4 µl, 2 µl, 1 µl, 0.5 µl, 0.25 µl). The cells were incubated with the antibody for 10 min at room temperature. Then the cells were washed twice in eBio Perm/Wash. The secondary anti-mIgG3-AF488 antibody (Goat Anti-Mouse IgG3-AF488, SouthernBiotech, USA) was tested in four different dilutions (1:50, 1:100, 1:200, 1:400). The cells were incubated again for 10 min at room temperature in the dark and then washed another two times.

To measure the staining on the flow cytometer (CytoFLEX LX, Beckman Coulter, Inc., USA) the cells were resuspended in 250 µl Wash/Perm buffer.

2.3.2.3 Analysis

To determine the best combination of the primary anti-Chlamydia antibody and the secondary anti-mIgG3-AF488 antibody *Ct*-infected cells were analysed visually (Supplementary Figure 1) and through calculating the staining index (Table 1). For visual inspection of the results, a clear distinction between *Ct*-positive and *Ct*-negative cells was examined. To calculate the staining index (SI) the median fluorescence intensity (MFI) of *Ct*-inclusion positive McCoy cells was analysed. The staining index (SI) was calculated with the following formula:

$$\mathrm{SI} = \mathrm{MFI}\ Ct\text{-inclusion positive cells} / \mathrm{MFI}\ Ct^{-}\ \mathrm{cells}$$

2.3.3 Titer determination of *Chlamydia trachomatis* stocks

To determine the titer of the produced *Ct* stocks, McCoy cells were infected with different concentrations of *Ct* and the infected cells were analysed using flow cytometry.

2.3.3.1 Infection of 24-well plates

The infection of McCoy cells in 24-well plates was performed the same way as for 6-well plates described above. However, 500 µl of infection medium were used per well. The plates were infected with Ct from the three produced stocks in several dilutions: 1:8, 1:32, 1:128, 1:512, 1:2,000, 1:8,000, 1:32,000, 1:128,000, 1:512,000, 1:2,000,000, 1:8,000,000.

2.3.3.2 FCM staining

Infected McCoy cells were harvested 24 hpi. The supernatant was taken off using a vacuum pump to contain the infectious medium. The cells were washed once with PBS and then incubated in 200 µl Trypsin (1x) for 10 min at 37 °C. Trypsinization of the cells was stopped by directly adding 20 µl FCS into each well.

Afterwards, the cells were transferred into a 96-well plate and centrifuged for 4 min at 500 g at room temperature. The supernatant was removed using a multichannel pipette, and cells were washed once in 200 µl PBS. Each washing step consisted of resuspending the cells in the washing solution and then centrifuging the plate once (4 min, 500 g, RT).

After the first washing step, cells were incubated in 200 µl eBio Fix/Perm buffer for 30 min at 4 °C. In later experiments Live/Dead staining of the cells was performed before fixation. This was accomplished by incubating the cells in 2.5 µl viability dye per well (eBioscience™, Fixable Viability Dye eFluor™ 780, Thermo Fisher Scientific Inc., USA) for 10 min at room temperature. After fixation, the supernatant was flipped off for removal, as the cells weren't infectious anymore. Then the cells were washed twice with 200 µl eBio Perm/Wash.

Staining of *Ct* inside the cells was done using the primary anti-Chlamydia antibody (4 µl/well) and the secondary anti-mIgG3-AF488 antibody (1:100 dilution). Incubation time for both antibodies was 10 min at room temperature each. After both staining steps, the cells were washed twice in eBio Perm/Wash.

To measure the staining on the flow cytometer the cells were resuspended in 250 µl Wash/Perm buffer.

2.3.3.3 Infection Analysis via flow cytometry

To calculate the amount of inclusion forming units (IFU) per millilitre of the *Ct* stocks the percentage of *Ct*-inclusion positive McCoy cells was determined. The IFU/ml were then calculated with the following formula:

$$\mathrm{IFU/ml} = \text{total cell number} * \text{dilution factor} * (\%\ Ct\text{-inclusion positive cells}/100)$$

2.4 Modification of infection protocol:

During the FCM staining of *chlamydia*-infected cells for titer determination, problems emerged while propagating *Ct*-infected McCoy cells in 24-well plates. The cells kept dying after undergoing the infection protocol as described above. This cell death was observed across all

Ct concentrations as well as in uninfected controls. Different possible sources of errors were tested, which led to modifications of the infection protocol.

During all experiments the plates were infected using the following *Ct* dilutions: 1:400, 1:1,600, 1:6,400, 1:25,600, 1:102,400.

2.4.1 Problem source: Cycloheximide

An excess concentration of Cycloheximide in the culture medium was initially thought to be the problem therefore, a new flask of culture medium was prepared. Infected cells were treated normally, and the new culture medium was used for cultivation. Only one well per plate was cultivated in the old culture medium. This comparison showed no difference and cell death was still observed across all wells, regardless of the culture medium, *Ct* concentration and in the negative controls.

2.4.2 Problem source: centrifugation

Next, the centrifugation step was investigated as the possible source of error. To ensure no part of the cell layer was without medium during the process, the volume of infection medium was increased to 1 ml in 24-well plates and to 3 ml in 6-well plates. Additionally, three different centrifugation speeds were evaluated: 900 g, 500 g, and no centrifugation at all. This also showed no particular effect on cell viability and cell death was still visible across all plates.

2.4.3 Problem source: cell culture plates and McCoy cells

The next possible problem could have been with the used cell culture plates or with the McCoy cells we propagated. Therefore, 24-well plates from a different manufacturer (TPP Techno Plastic Products AG, Switzerland) were compared to the regularly used ones (Greiner Bio-One International GmbH, Austria) and two different McCoy cell batches were used. One was propagated at the Unit of Immunology and the other was propagated at the Clinical unit for Swine.

The best possible combination with the least number of dead cells noted within this experiment were McCoy cells from the Clinical unit for Swine in 24-well plates from Greiner. However, the results still didn’t explain the cell death happening across all plates and no real conclusion could be drawn.

2.4.4 Problem source: infection medium change

Lastly, it was investigated whether the medium change during infection was causing the problem. Therefore, three 24-well plates were infected with *Ct.*

However, one half of the plate was infected using the initial protocol, taking off the culture medium completely and adding 1 ml of infection medium. For the second half of the plate, 500 µl of culture medium was left on the cells and 500 µl of infection medium was added to each well. This infection medium contained double the *Ct* concentration as it would be diluted through the left-over culture medium in each well. In addition, the centrifugation speed test was performed once more, testing 900 g, 500 g and no centrifugation.

This experiment showed that the complete medium change was the cause of cell death in all previous experiments. Cells died within 10 minutes after the change. In comparison, the cells where only half of the culture medium was changed to infection medium didn't show any signs of cell death.

2.5 *Chlamydia*-staining of infected cells for fluorescence microscopy

For fluorescence microscopy McCoy cells were cultivated on Poly-D-Lysin-treated cover slips in 24-well plates. The plates were infected using the infection protocol described above. 72 hpi the coverslips were inspected under the microscope and those with the most viable looking cells were chosen for fluorescence microscopy.

The coverslips (Microscope Cover Glasses, Paul Marienfeld GmbH & Co. KG, Germany) were removed from the plate and transferred into a fresh plate to wash them once in 1 ml PBS. The cells were incubated in 500 µl ice-cold 100 % methanol for 10 min in the freezer for fixation and permeabilization. Then the cells were incubated for 5 minutes in 1 ml PBS + 2 % FCS to block any unspecific binding sites. The coverslips were dried off using a Kimwipe (Kimtech Science, Kimwipe, KIMBERLY-CLARK PROFESSIONAL™, KIMBERLY-CLARK GmbH, USA). For staining with the primary antibodies, 20 µl of the antibody suspension was put on a fresh microscopy slide (Süsse Labortechnik GmbH & Co. KG, Germany) and each coverslip was placed on one drop with the cells facing down. The coverslips were incubated for 10 minutes in a wet chamber at 37 °C to prevent drying. The primary antibodies used were an anti-Chlamydia antibody (4 µl/well) and an anti-claudin-4 (CLDN-4) antibody (1:100 dilution, Invitrogen™ Claudin 4 Polyclonal Antibody, Life Technologies Corporation, USA).

Afterwards, the coverslips were washed once in 1 ml PBS and then incubated for 10 minutes with the secondary antibodies in the wet chamber. The secondary antibodies used were the anti-mIgG3-AF488 antibody (1:100 dilution) and the anti-rabbit-Alexa555 antibody (1:200 dilution, Goat Anti-Mouse IgG3, Human ads-AF555, SouthernBiotech, USA). Next, the

coverslips were washed once in 1 ml PBS and then once in 1 ml methanol to remove any salt residue from PBS. Methanol was used as it dries faster than ddH_20.

Once the coverslips were completely dried, they were mounted onto microscopy slides using 4 µl of a mounting medium, containing 4',6-Diamidino-2-phenylindol (DAPI) (Invitrogen™ ProLong™ Diamond Antifade Mountant with DAPI, Life Technologies Corporation, USA), per slide. The coverslips were left to dry overnight and then pictures were taken on a fluorescence microscope (AxioImager Z2, Zeiss, Carl Zeiss GmbH, Austria).

2.5.1 Poly-D-Lysin coating of coverslips

The coverslips were first sterilised by submerging them in 70 % Ethanol for 30 seconds and then left to dry on Kimwipes. Then one side of the coverslips was covered with 70 µl of Poly-D-Lysin (Gibco™ Poly-D-Lysin, Life Technologies Corporation, USA) and left to incubate for two hours. After this, the Poly-D-Lysin was removed from the coverslips, collected in a new tube and stored at 4 °C as this could be used again. The coverslips were dried by putting them at a 90° angle on a Kimwipe. The now coated coverslips were stored in the dark at room temperature until used for microscopy experiments.

2.6 Measurement of trans-epithelial electrical resistance (TEER)

To measure the trans-epithelial electrical resistance (TEER) of the cells during an infection cycle, pOECs and McCoy cells were cultivated in 96-well ePlates (E-Plate 96 PET, Agilent Technologies, Inc., USA). First, a background measurement was taken using 100 µl of pOEC-medium in each well. Then pOECs or McCoy cells were seeded at the desired density in 200 µl medium/well. Before putting the ePlate into the plate reader (xCELLigence RTCA SP, Agilent Technologies, Inc., USA), it was left at room temperature under the laminar hood for 30 minutes. This is a recommendation of the manufacturer to reduce possible edge effects which might occur. After this waiting period the plate was placed in the plate reader in the incubator at 37 °C and the measurement was started.

The setup for the data acquisition was as follows:

1) background measurement: one sweep of each well
2) cell kinetic: 1 sweep every 30 minutes, 145 times
3) infection kinetic: 1 sweep every 30 minutes, 145 times

When the second step of measurement was completed, the plate was infected with *Ct in* dilutions from 1:8 up to 1:8,000,000. For this 100 µl of the culture medium was removed and

100 µl of the infection medium with double the desired *Ct* concentration was added to each well. As the ePlates cannot be centrifuged the infectious medium was left on the cells for the whole duration of the experiment and data acquisition was started right away.

2.6.1 Impedance and cell index

On the bottom, the ePlates are covered with microelectrodes, which are used to measure the cellular impedance. If no cells are present, the electron impedance mainly depends on the ion environment in the medium and at the electrodes. Once cells attach at the bottom of each well the ion environment changes and the impedance rises. The impedance continues to increase as long as the cells divide and grow. Once they reach confluency the impedance reaches a plateau phase.

The xCelligence instrument measures the cellular impedance as a parameter called cell index (CI). CI is a quantitative measurement of the cells in each well according to $CI(t) = \frac{Z_t - Z_0}{Z_{nominal}}$. Z_t is the impedance at the measure time point, Z_0 the impedance without cells (Agilent Technologies, Inc.).

2.6.2 Statistical analysis

TEER experimental data was analysed in GraphPad Prism (GraphPad 10.0 software, Inc., USA). A two-way ANOVA was performed for the dataset and multiple comparisons were done. Differences were defined as significant for $p<0.05$.

3 Results

3.1 Harvesting of *Chlamydia trachomatis* via sonication or by foaming the cells

McCoy cells which were infected with *Ct* harvested through sonication or foaming showed similar amounts of inclusions in each cell. Therefore, both methods worked similarly well in harvesting *Ct* from infected cells. Based on the better options for standardisation, we continued with the sonication method.

3.2 Titration of antibodies for FCM

To establish and optimize the analysis of *Ct* infection in McCoy cells using FCM, the required antibodies were first titrated. The primary antibody was an anti-Chlamydia antibody and the secondary antibody an anti-mIgG3-AF488 antibody. To determine the best antibody combination, we cross-titrated both antibodies. As mentioned in the methods section, the concentration of the primary antibody ranged between 0.25 and 4 µl and the secondary antibody was diluted to concentrations ranging from 1:50 to 1:400. The results of this antibody titration were inspected visually as well as through calculating the SI. As explained in the methods section, the SI was calculated by dividing the MFI of *Ct*-infected cells by the MFI of non-infected cells.

After visual inspection, a combination of 1-4 µl of primary antibody with a 1:100 dilution of the secondary antibody was concluded as the optimal combination, as this led to the best distinction between *Ct*-positive cells and *Ct*-negative cells as seen in Supplementary Figure 1. The SI ranged from 5.23 (0.25 µl primary antibody with 1:100 dilution of secondary antibody) to 52.13. The highest SI (52.13) was achieved with the combination of 4 µl primary antibody and a 1:100 dilution of the secondary antibody (Table 1). Thus, this combination of primary and secondary antibodies was used for all FCM experiments.

Table 1. Staining Index of antibody combinations.

Sample ID	*Ct* infection	Primary Ab [µl]	Secondary Ab	*Ct* Incl+ [Median]	*Ct*- [Median]	Staining Index (SI)
Ct-_4_50.fcs	Negative	4	1:50	n/a	21207	n/a
Ct-_4_100.fcs	Negative	4	1:100	n/a	13904	n/a
Ct-_4_200.fcs	Negative	4	1:200	n/a	14495	n/a
Ct-_4_400.fcs	Negative	4	1:400	n/a	11818	n/a
Ct_1_50.fcs	Positive	1	1:50	735000	24027	30,59
Ct_1_100.fcs	Positive	1	1:100	553000	16884	32,75
Ct_1_200.fcs	Positive	1	1:200	401000	15201	26,38
Ct_1_400.fcs	Positive	1	1:400	157299	13849	11,36
Ct_2_50.fcs	Positive	2	1:50	437000	23094	18,92
Ct_2_100.fcs	Positive	2	1:100	283947	16068	17,67
Ct_2_200.fcs	Positive	2	1:200	240875	14238	16,92
Ct_2_400.fcs	Positive	2	1:400	198352	14014	14,15
Ct_4_50.fcs	Positive	4	1:50	665000	22730	29,26
Ct_4_100.fcs	**Positive**	**4**	**1:100**	**882000**	**16918**	**52,13**
Ct_4_200.fcs	Positive	4	1:200	717000	15629	45,88
Ct_4_400.fcs	Positive	4	1:400	563000	14726	38,23
Ct_05_50.fcs	Positive	0,5	1:50	203529	21376	9,52
Ct_05_100.fcs	Positive	0,5	1:100	141613	15051	9,41
Ct_05_200.fcs	Positive	0,5	1:200	152691	14070	10,85
Ct_05_400.fcs	Positive	0,5	1:400	115692	12692	9,12
Ct_025_50.fcs	Positive	0,25	1:50	102721	19629	5,23
Ct_025_100.fcs	Positive	0,25	1:100	85768	13523	6,34
Ct_025_200.fcs	Positive	0,25	1:200	71330	11889	6,00
Ct_025_400.fcs	Positive	0,25	1:400	75401	11472	6,57

3.3 Titer determination of *Ct* stocks

After producing the *Ct* stocks, we tried to determine the titer of each stock. Due to the unexpected issues with McCoy cell death, we were only able to determine the titer of one of the three stocks (*Ct* A). We infected McCoy cells with this stock in several dilutions ranging from 1:400 to 1:102,400. The wider range of *Ct* dilutions, as described in the methods section, was deemed unnecessary after initial experiments, as the infection rates were either too high or too low for titer calculation. The IFU/ml was then calculated by using the percentage of *Ct*-inclusion positive cells and the corresponding dilution factor in the formula as described in the methods section.

The percentage of *Ct*-infected cells ranged from 74.8 % (1:400 dilution) to 0.63 % (non-infected cells) and the percentage of *Ct*-inclusion positive cells ranged from 4.4 % (1:6400 dilution) to 0 % (non-infected cells) (Figure 1). To calculate the IFU/ml titer of the produced *Ct* stock the percentage of *Ct*-inclusion positive cells with the 1:102,400 dilution was used. This

dilution was chosen, based on experience from previous experiments, where an infection rate of 1 % was optimal to calculate the titer. Based on this the calculated titer resulted in $2.47*10^8$ IFU/ml for our *Ct* stock.

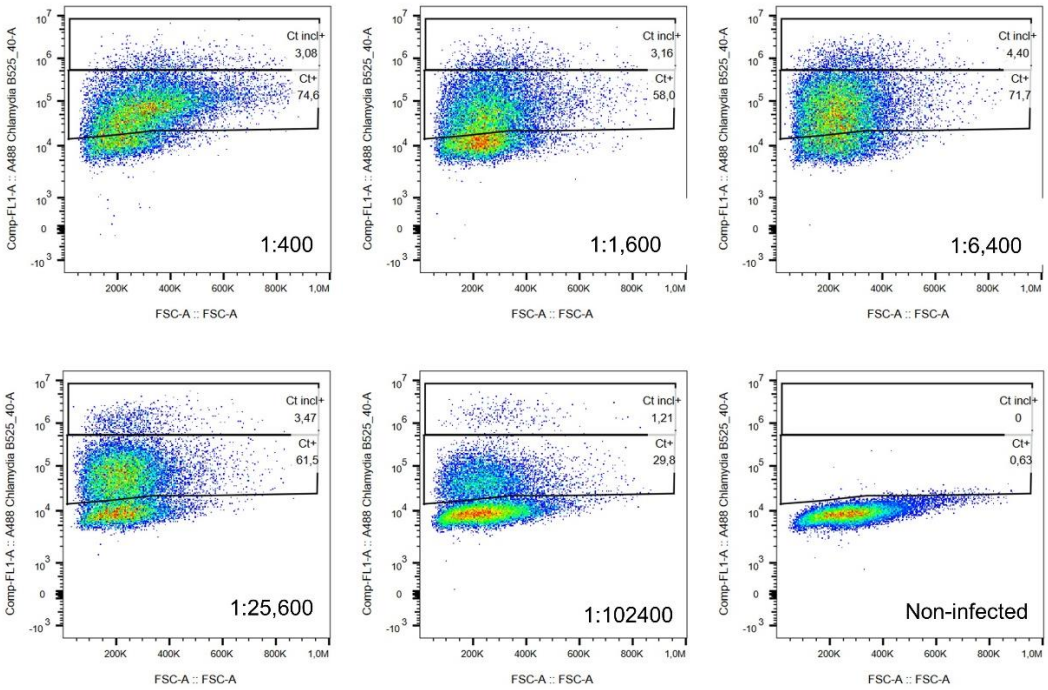

Figure 1. Titration of *Chlamydia trachomatis* on McCoy cells via flow cytometry. McCoy cells were infected with a 4-fold serial dilution of *Ct*. The cells were harvested 24 hpi, stained for *Ct* and the infection was analysed via FCM. This figure shows the density plots (FSC/*C. trachomatis*) of *Ct*-infected McCoy cells. The dilution factor is in the right bottom corner of each picture and the numbers inside the gates show the percentage of *Ct*-positive (*Ct*+) and *Ct*-inclusion positive (*Ct* incl+) cells.

3.4 Fluorescence microscopy of *Chlamydia trachomatis* infected McCoy cells

To visualise the *Ct* infection in McCoy cells fluorescence microscopy was performed on cells at 72 hpi. This experiment was performed on McCoy cells showing signs of cell death. Therefore, typical *Ct*-inclusions are not visible in the cells. In the 1:6,400 dilution some *Ct* are diffusely distributed around the cells. In the 1:256,000 dilution it seems some very small inclusions inside the cells are visible. In comparison, the non-infected control shows no *Ct* infection at all (Figure 2).

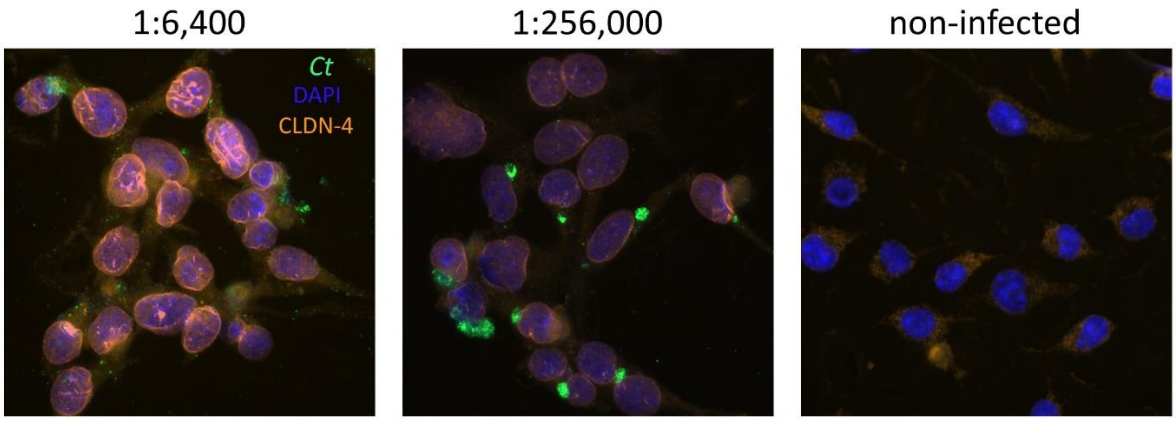

Figure 2. Fluorescence microscopy of *Ct*-infected McCoy cells. Cells were fixed and permeabilized with ice-cold methanol and stained for fluorescence microscopy 72 hpi: claudin-4 (CLDN-4, orange) visualizes the host cell membrane, 4',6-Diamidino-2-phenylindol (DAPI, blue) to stain the host cell nucleus, and an anti-Chlamydia antibody (green) to show *Ct* infection. The numbers above each picture show the dilution factor for the *Ct* infection.

3.5 Effect of *Chlamydia trachomatis* infection on pOEC TEER

3.5.1 Seeding density experiment

First, we performed a titration of pOECs to determine the best seeding density and cell type for the *Ct*-infection in the ePlates. Since we infected our cells at the time of confluency, another goal was to determine the time needed for the cells to reach confluence in the ePlates. pOECs harvested with scraping (pOEC+) and pOECs harvested without scraping (pOEC-) as well as McCoy cells were tested in seeding densities ranging from 10,000 to 40,000 cells per well. The measurement was conducted over 72 hours initially and then prolonged for another 71 hours. However, these measurements were not conducted as one large experiment in the software but as two separate, which affected the quality of the results during the second time span (Figure 3B).

In Figure 3A a rise of the cell index can be seen for all seeding densities and all cell types (pOEC+, pOEC-, McCoy). McCoy cells generally showed the lowest cell indices, while pOEC+ showed the highest cell indices for all seeding densities. pOEC+ showed a seeding density-dependent increase of the cell index, with higher seeding densities causing a higher increase of the cell index. At the end of the first time span the cell index seemed to have reached its highest point for pOEC+, whereas for pOEC- and McCoy cells it seemed the cell index might still go higher (Figure 3A). This is the reason why another measurement for 71 hours was

started, as we wanted to observe the full time kinetic of the cell index for each cell type until the plateau phase.

Since a new experiment had to be started in the software, the background measurement and the last measured cell index for each well were not transferred, leading to negative cell indices. The cell indices of pOEC- rose for approximately five hours before they reached the plateau phase and did not change much for the rest of the experiment. The cell indices of pOEC+ rose for ten hours before they reached the plateau phase. The cell index of pOEC+ with a seeding density of 10,000 cells/well stayed up the longest before it went down later during the experiment. The cell indices of the McCoy cells dropped initially but then rose again slightly until the 22.5-hour mark. After this, the cell indices began to drop for the higher seeding densities (20,000-40,000 cells/well) while the cell index of the lowest seeding density stayed in the plateau phase the longest (Figure 3B).

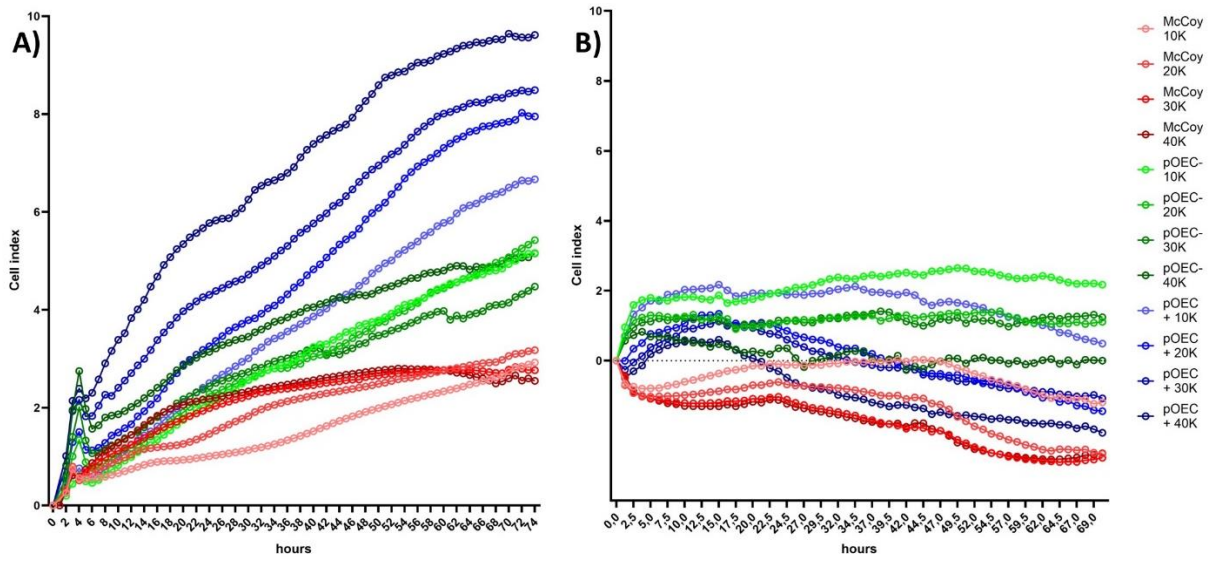

Figure 3. Cell seeding density of pOECs and McCoy cells. Cells were seeded on the ePlates in seeding densities from 10,000-40,000 cells/well. On the right of both figures, the cell type and seeding density is shown. Colour coding is the same for both figures. A) shows the TEER over the duration of the first 72 hours. B) shows the TEER over the duration of the second 71 hours.

3.5.2 Effect of *Ct* infection on the TEER of pOECs

To measure the effect of *Ct* infection on pOECs, pOEC+ were seeded at a density of 10,000 cells/well and cultivated for 72 hours. Then the cells were infected with different *Ct* dilutions. The TEER was measured for a total of 148 hours. In the first 72 hours the TEER rose steadily for all pOECs until it reached a plateau phase at about 56 hours of cultivation. The

cells were infected with *Ct* at the 72-hour mark. For the *Ct* dilutions 1:8-1:128 the TEER showed a significant drop as early as 8 hpi. About 20 hpi a significant difference to the non-infected cells could be seen in all *Ct* dilutions except in the three highest (1: 512,000, 1:2,000,000 and 1:8,000,000) (Figure 4A). The drop in the TEER was dependent on the amount of *Ct* with which the pOECs were infected. Higher *Ct* concentrations correlated with a bigger drop of the cell index. At 24 hpi the cell index of non-infected pOECs was at 9.3 while the cell index of *Ct*-infected cells dropped significantly between 5.2 (1:8 dilution) and 8.4 (1:128,000 dilution). At 30 hpi the drop in TEER was even greater, ranging between 4.4 (1:8 dilution) and 8.2 (1:128,000 dilution). 36 hpi the TEER dropped significantly with measurements ranging between 3.7 (1:8 dilution) and 7.8 (1:512,000 dilution) (Figure 4B).

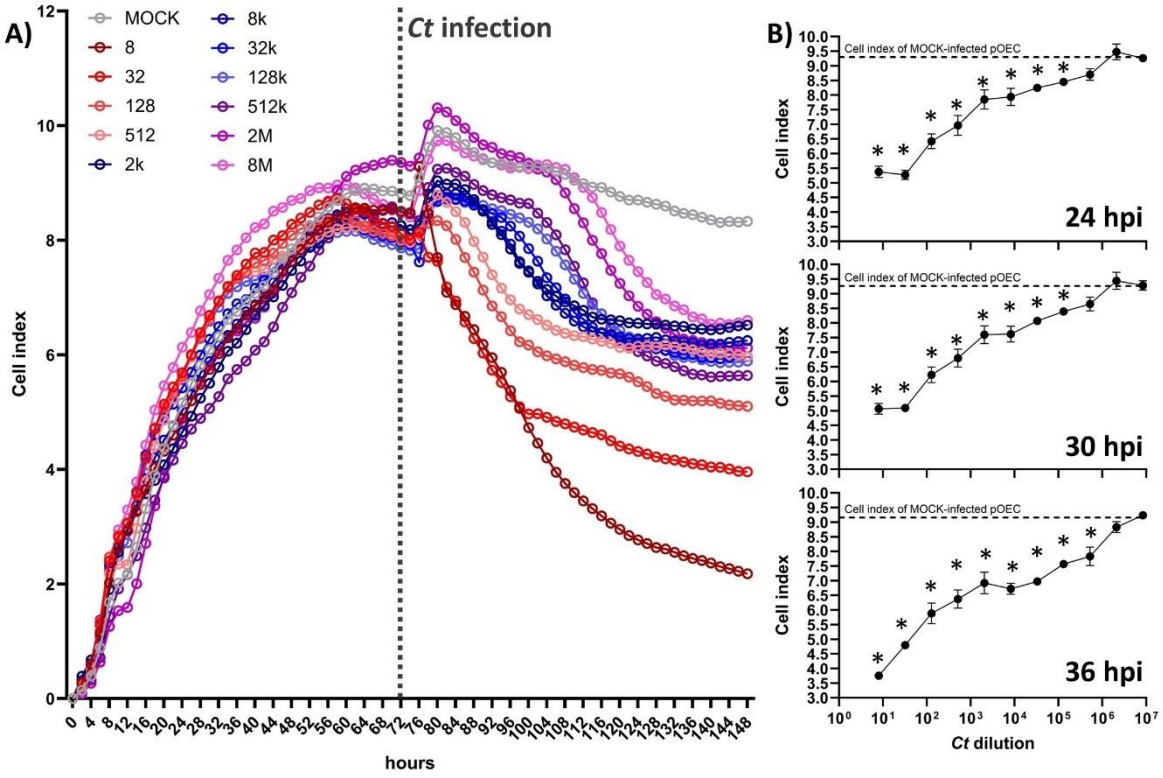

Figure 4. Effect of *Ct* infection on pOEC TEER. pOECs were seeded on the ePlates and infected with a 4-fold serial dilution of *Ct*. A) shows the TEER over the duration of 148 hours. The cells were infected after 72 hours of cultivation. In the top left corner, the *Ct* dilution factors are shown. B) shows the difference in cell index between *Ct*-infected and non-infected cells for all *Ct*-dilutions at the time points 24, 30 and 36 hpi. The cell index of the non-infected pOECs is depicted as a dotted line. A two-way ANOVA was used for comparison. * = $p<0.05$.

4 Discussion

4.1 Cell death during *Chlamydia trachomatis* infection of McCoy cells

As mentioned above, during cultivation of *Ct*-infected McCoy cells for FCM staining, we experienced cell death after changing the culture medium for infection. This effect was irrespective of cell centrifugation or *Ct* infection. However, it was only visible in cells where the entirety of the culture medium was taken off and not in cells which retained half of the initial culture medium during the infection process. A literature search on possible factors causing cell death in culture revealed many different reasons: first, fluctuating temperature and lack of CO_2 are mentioned as a cause of cell death; secondly, accumulation of toxins or incorrect osmotic pressure could lead to apoptosis of the cells (Segeritz and Vallier 2017). However, temperature change or lack of CO_2 can be ruled out as pre-warmed culture medium was used and cells were out of the incubator for only a very short time span, as the whole infection process took no longer than ten minutes at the most. Accumulation of toxins or incorrect osmotic pressure could also be ruled out as possible reasons for cell death, seeing as cell death was observed regardless of cycloheximide concentration and no contamination of the culture medium was observed. Moreover, the same medium was used for the infection process which was normally used for the propagation of McCoy cells.

A more plausible explanation seems to be that during proliferation the cells secrete specific molecules into the culture medium, which are needed for cell growth. McCoy cells are mouse-derived fibroblast cells. It is widely known that fibroblast growth factors play amongst other things a role in cell proliferation (Thisse and Thisse 2005), meaning removal of those could lead to cell death. During the initial infection process, the complete medium was changed meaning possibly secreted growth factors were completely removed from the cells. This correlates also with our observation that cell death can be prevented if half of the initial culture medium is retained on the cells, leaving some of the secreted growth factors available for the cells to continue proliferation.

4.2 Fluorescence microscopy of *Chlamydia trachomatis* infected cells

This part of the thesis was a start in re-establishing a fluorescence microscopy protocol for *Ct*-infected cells. The goal was to test the fluorescent antibodies as well as the mounting medium with integrated DAPI and determine the best concentrations. To accurately determine what concentrations would lead to the best result, an experiment with a small antibody titration

might be necessary. However, due to time constraints only one initial experiment was conducted.

This experiment was conducted with *Ct*-infected McCoy cells that showed signs of limited viability during propagation. This influenced the *Ct*-infection. Amaral et. al showed that in fully healthy cells *Ct* should have formed large *Ct*-inclusions at 72 hpi, which would take up most of the space inside the cells (Amaral et al. 2021). In comparison, our results showed no *Ct*-inclusions at the higher *Ct* concentration and only very small *Ct*-inclusions in the lower concentration (Figure 2). These results suggest that a successful *Ct*-infection is to some extent dependent on the health of the cells, since unhealthy cells seem to cause reduced growth of the *Ct*-inclusions. *Ct* depend on the host cell during the replicative cycle for nutrient acquisition (Gitsels et al. 2019). Limited viability of the cells might cause a limited access to nutrients, which could be an explanation for the reduced size of inclusions in unhealthy cells. Thus, improving the health of the *Ct*-infected cells should be an important factor in future experiments to accurately visualize *Ct*-infections using fluorescence microscopy.

4.3 Seeding density effect on cell index

The experiment was conducted to test the seeding density combined with the amount of time it took the cells to reach confluence in the ePlates to later use in the *Ct*-infected pOEC TEER measurement. For this pOEC+ were used, as they showed a seeding-density-dependent rise of the cell index and reached the beginning of the plateau phase the earliest at 72 hours (Figure 3A). Since we wanted to infect the cells once they reached confluency, this time frame was chosen for the first kinetic of the TEER experiment (Figure 4A). The seeding density of 10,000 pOEC+/well was picked as the cell index dropped the latest during the second half of the experiment (Figure 3B). This ensures that non-infected cells are still in the plateau phase during most of the TEER measurement of the *Ct*-infected cells, making it easier to visualise a drop of the TEER in *Ct*-infected cells compared to non-infected controls.

Although this experiment showed some limitations regarding the quality of the results (negative cell indices during the second phase), the overall kinetic of the cell indices could still be evaluated. For experiments where a direct assessment of the cell index is necessary, it would be crucial to plan the set-up accordingly for the complete measurement to be conducted as one with a proper background reading.

4.4 *Chlamydia trachomatis* infection affects the barrier integrity of pOECs

It has been previously demonstrated that claudin-4 is re-localized to the *Ct* inclusion in *Ct*-infected pOECs (Amaral et al. 2021). Based on this we tried to determine the effect of *Ct* infection on pOECs through analysing the TEER, a measurement used for the integrity of the epithelial cell layer, over a period of 148 hours. We saw a decrease in the TEER as early as 8 hpi for high *Ct* concentrations and a later decrease for lower *Ct* concentrations (Figure 4A). Amaral et al. showed that claudin-4 is re-localised to the *Ct* inclusion in the time span of 12-36 hpi (Amaral et al. 2021). This correlates with the decrease in TEER in almost all tested *Ct* concentrations at 24, 30 and 36 hpi (Figure 4B). This drop in TEER was also observed to be dose-dependent, seeing as pOECs infected with lower *Ct* concentrations showed a later and more moderate decrease compared to higher *Ct* concentrations.The reproductive cycle of *Ct* in pOECs takes about 48 hours (Amaral et al. 2021), before *Ct* are released via cell lysis or extrusion (Murray and McKay 2021). During this cycle, *Ct* inhibit apoptosis of the host cells to facilitate their growth inside intracellular inclusions (Waguia Kontchou et al. 2022). As we witnessed a drop in the TEER as early as 8 hpi, for high *Ct* concentrations, or 20 hpi, for almost all *Ct* dilutions, it is highly unlikely that this effect is caused by *Ct*-induced cell death or host cell defence mechanisms.

Therefore, the combination of this decrease of the TEER in *Ct*-infected pOECs as early as 20 hpi with the decrease in membrane-bound claudin-4 (Amaral et al. 2021) strongly suggests that the drop of the TEER is caused by a disruption of the epithelial barrier integrity due to *Ct* infection of the pOECs.

Certainly, this study shows limitations. Most importantly, based on time restraints caused by the unexpected issues with McCoy cell death, only one experiment was conducted on the development of the pOEC TEER. Although our results correlate with the findings of Amaral et al. 2021 it would be beneficial to conduct this experiment again, to show the reproducibility of these findings. Additionally, it might be interesting to pair the TEER experiment with fluorescence microscopy at specific timepoints to directly correlate the decrease in TEER with a re-localization of claudin-4. Furthermore, methods to verify the survival (e.g. FCM-based live/dead stains) and adhesion (crystal violet stainings) of *Ct*-infected McCoy cells could further corroborate that the *Ct*-induced TEER reduction is in fact caused by a reduction of pOEC barrier function.

In conclusion, this study demonstrates a clear drop in the pOEC TEER during infection with *Ct*. As *Ct* inhibits host cell apoptosis, the TEER reduction is most likely caused by a *Ct* infection-induced reduction of barrier integrity in pOECs. A reduced epithelial barrier integrity can lead to an increased permeability for viruses. Thereby, this study provides a potential insight into the higher risk of *Ct* patients for co-infections with HIV. In turn, this project justifies further investigation into how *Ct* infection might affect the permeability of pOECs to viruses like HIV.

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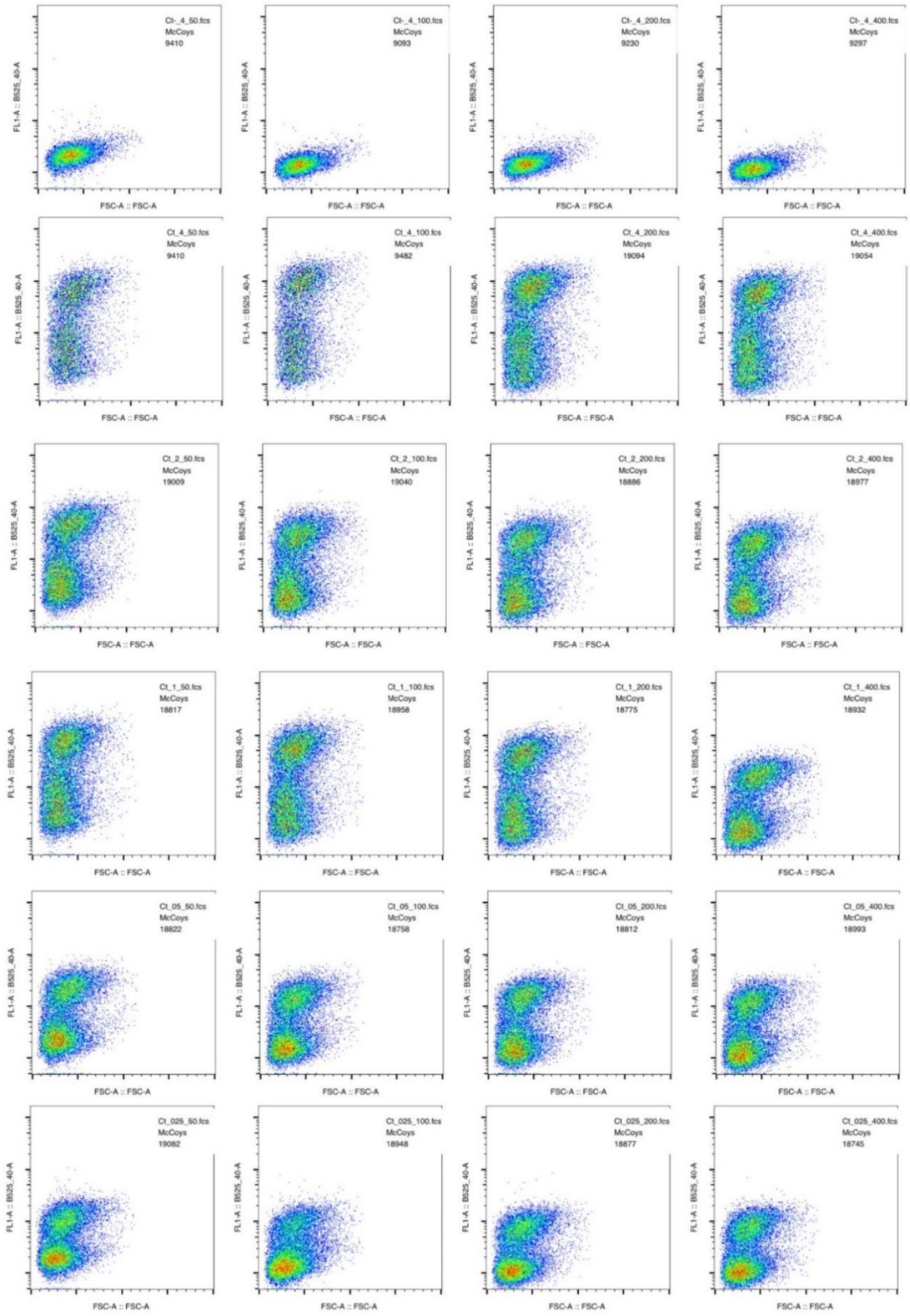
6 List of abbreviations

Anti-Anti	Antibiotic-Antimycotic
Th1 cells	CD4+ T-helper 1 cells
CI	Cell index
CLDN-4	Claudin-4
Cs	*Chlamydia suis*
Ct	*Chlamydia trachomatis*
DAPI	4',6-Diamidino-2-phenylindol
DC	Dendritic cell
EB	Elementary body
EGF	Epidermal growth factor
FCM	Flow cytometry
FCS	Fetal calf serum
HIV	Human immunodeficiency virus
hpi	Hours post-infection
IFNγ	Interferon gamma
ITS	Insulin transferrin selenium
IFU	Inclusion forming units
LPS	Lipopolysaccharides
PAMPS	Pathogen-associated molecular patterns
pOECs	Porcine oviduct epithelial cells
pOEC+	pOECs harvested with scraping
pOEC-	pOECs harvested without scraping
RB	Reticulate body

Tarp	Translocated actin-recruiting phosphoprotein
TEER	Trans-epithelial electrical resistance
TFNα	Tumour Necrosis Factor alpha
T3SS	Type III Secretion System
WHO	World Health Organisation

7 List of tables and figures

8 Supplement



Supplementary Figure 1

9 Declaration of independence

I hereby declare that I have written this thesis independently and that no other sources or aids were used other than those stated. All text passages taken from external sources have been labelled.

I have carried out the decisive work myself and all contributors to this work have been listed with their contribution to the work.

This thesis has not been submitted or published elsewhere.

Vienna, on the 13.06.2024 Katharina Maria Krenmayr