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Chlamydia suis and *Chlamydia trachomatis* induce multifunctional CD4 T cells in pigs

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ABSTRACT

Chlamydia trachomatis infections are the most prominent bacterial sexually-transmitted disease worldwide and a lot of effort is put into the development of an effective vaccine. Pigs have been shown to be a valuable animal model for *C. trachomatis* vaccine development. The aim of this study was to decipher the T-cell-mediated immune response to chlamydial infections including *C. trachomatis* and *C. suis*, the chlamydia species naturally infecting pigs with a demonstrated zoonotic potential. Vaginal infection of pigs with *C. suis* and *C. trachomatis* lasted from 3 to 21 days and intra-uterine infection was still present after 21 days in 3 out of 5 *C. suis*- and 4 out of 5 *C. trachomatis*-inoculated animals and caused severe pathological changes. Humoral immune responses including neutralizing antibodies were found predominantly in response to *C. suis* starting at 14 days post inoculation. The T-cell-mediated immune responses to *C. trachomatis* and *C. suis*-infections started at 7 days post inoculation and consisted mainly of $CD4^+$ T cells which were either IFN-γ single cytokine-producing or IFN-γ/TNF-α double cytokine-producing T-helper 1 cells. IL-17-producing $CD4^+$ T cells were rare or completely absent. The T-cell-mediated immune responses were triggered by both homologous or heterologous re-stimulation indicating that cross-protection between the two chlamydia species is possible. Thus, having access to a working genital *C. suis* and *C. trachomatis* infection model, efficient monitoring of the host-pathogen interactions, and being able to accurately assess the responses to infection makes the pig an excellent animal model for vaccine development which also could bridge the gap to the clinical phase for *C. trachomatis* vaccine research.

1. Introduction

Besides *in silico* and *in vitro* studies, vaccine development mostly relies on animal models and choosing the right animal model is crucial for a successful translation into humans. Mice are the most frequently used animal model but before going into clinical trials, vaccine candidates should be tested in at least one other species. Large animal models are particularly beneficial for predicting the vaccine outcome in humans [1]. Thus, many studies use non-human primate (NHP) models to test promising vaccine candidates but although NHPs deliver valuable information the use of NHPs is very controversial due to their high economical and ethical costs [1,2]. Pigs have several advantages as large animal models for vaccine design and testing including a similar physiology and immune system [1,3].

The porcine female genital tract shows several similarities to humans in regard to the reproductive cycle and immune system [4] making pigs a valuable animal model for studying sexually transmitted infections (STIs) like *Chlamydia trachomatis* (*Ct*) infections. *Ct* infections are the most prevalent bacterial STIs worldwide, and cause long-term complications such as infertility, chronic-pelvic pain and ectopic pregnancies [5]. So far, *Ct* infections are screened and treated by antibiotics with limited success and a lot of effort is put in the development of a protective vaccine. To date, this effort includes three studies on *Ct* vaccine candidates in pigs [6–8] and two more recent studies in mini-pigs [9,10] showing promising results by a decreased bacterial load and the induction of IFN-γ, mucosal IgA and serum neutralization

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antibodies. But due to previous limitations in the porcine immunological toolbox, the immune response analysis of antigen-specific $CD4^+$ T_H1 cells was limited to investigating IFN-γ production in ELISAs without determining its cell source or examining bulk $CD4^+$ T cells in blood of infected animals without identifying chlamydia-specific cells. Antigen-specific $CD4^+$ T_H1 cells, however, are crucial for the control and elimination of infection, and for the induction of a protective memory immune response in *Ct* infections [11,12]. Thus, the aim of this study was to analyse chlamydia infection and the resulting immune response in pigs with a focus on the $CD4^+$ T-cell immune response including the detection of multifunctional $CD4^+$ T cells. These cells possess a robust effector function, have a high long-term memory potential and have been shown to be good correlates of protection in *C. muridarum* infections [13]. We also included *C. suis* (*Cs*) in our studies, due to its high prevalence in pigs, similar disease outcomes as *Ct*, and the zoonotic potential demonstrated by its presence in pharyngeal and rectal samples of slaughterhouse employees [14].

2. Materials and methods

A detailed description of the materials and methods can be found in Supplementary Information 1.

2.1. Chlamydiaceae

The *Cs* strain S45, originally isolated in Austria [15], and *Ct* serovar E strain Bour, a *Ct* reference strain with high prevalence [16], were purchased from ATCC and grown on HeLa cells using standard techniques [17] and purified as previously described [18]. Titration was performed on HeLa cells and infection was analysed via flow cytometry (FCM) as previously described [19].

2.2. In vivo infection experiment

Fifteen synchronized 26-week-old female pigs were inoculated in standing estrous intra-vaginally and intra-uterine each with 10^8 inclusion forming units (IFU) *Cs* or *Ct* in sucrose-phosphate-glutamine (SPG) buffer, or MOCK-inoculated with SPG buffer alone. Clinical monitoring and rectal temperature recording was performed every other day. Blood, serum and vaginal swabs were collected at 3, 7, 10 and 14 dpi and at necropsy which was performed on three consecutive days 21, 22 and 23 dpi, collectively referred to as "21 dpi" (Fig. 1). Pigs were sacrificed using a captive bolt gun followed by exsanguination. At necropsy, the genital tracts and draining lymph nodes (iliac and uterine) were collected to monitor gross pathological changes and for sample collection. Experimental procedures are in accordance with the Animal Research Ethics Board (AREB) of the University of Saskatchewan (AREB # 20130095).

2.3. Swab, serum and blood sampling

Swab samples were collected into 1 ml of PBS, shaken for 1 h at 4 °C and spun for 1 h at 13,000*g* to pellet chlamydial elementary bodies (EBs). The pellet was lysed in 100 µl alkaline lysis buffer, neutralized and 2 µl of the supernatant was used for subsequent qPCR analysis. For serum collection, blood was collected into SST tubes (BD Biosciences, San Jose, CA), incubated for at least 30 min, spun for 20 min at 2000*g* and 20 °C and stored at −20 °C. Complete blood counts (CBCs) were determined from EDTA blood using a Hemavet 950 system (Drew Scientific Group, Miami Lakes, FL). PBMC isolation was performed via density gradient centrifugation.

2.4. Preparation of single cell suspensions from lymph nodes

Lymph nodes were cut into small pieces and pressed through cell strainers (BD Biosciences) into 50 ml tubes. Cell strainers were flushed with ice-cold PBS to elute the single cell suspensions.

2.5. Genital tract handling

Genital tracts were examined for gross pathological changes. Then, uterine horns were flushed with 40 ml PBS for immunotyping of infiltrated immune cells via FCM and for the detection of chlamydia in the uterine horns via qPCR. Horn flush cells were pelleted via centrifugation and 1 ml of the supernatant was used for chlamydia detection via qPCR upon treatment as described for the swab samples above. The pelleted cells were immunotyped via FCM as described below. Biopsy samples of altered tissue were homogenated using a Mini Bead Beater with zirconia beads (BioSpec Products, OK) and lysed in an alkaline lysis buffer. DNA content was determined using a NanoDrop spectrophotometer (ThermoFisher Scientific) and 200 ng DNA were subsequently used per qPCR reaction.

2.6. Detection of chlamydia via qPCR

Chlamydial DNA content was analysed via Taqman qPCR assay as described before [18]. A standard curve of *Cs* EBs was included on every plate in order to determine the concentration of chlamydial EBs in the test samples.

2.7. Immunotyping in blood, draining lymph nodes and uterine horn flushes

Single-cell suspensions were stained for T-cell subset discrimination markers as summarized in Table 1. Cells were recorded on a FACS Calibur using the CELLQuest Software (BD Biosciences) and data analysis was performed with FlowJo version 7.6.5 (FLOWJO LLC, Ashland, OR).

2.8. Serum IgG detection

Anti-chlamydial MOMP serum IgG levels were detected via ELISA. A mix of three partial MOMP proteins (ab67705-ab67707, Abcam, Cambridge, MA) were used for plate coating, before a 1:100 dilution of serum in PBS was added. Anti-pigIgG(H+L)-alkaline phosphatase (KPL, Gaithersburg, MD) in combination with p-nitrophenyl phosphate (PNPP, ThermoFisher Scientific) was used for the color substrate reaction and color change was recorded on an iMark microplate reader (BioRad, Hercules, CA). An internal control was included on every plate and data were normalized to this control in order to compensate for plate-to-plate variations.

2.9. Neutralizing antibody determination

Neutralizing antibodies in serum were detected by incubating heat-inactived serum with *Cs* elementary bodies (EBs) for 30 min at 37 °C in a final serum dilution of 1:10. This mixture was used to infect HeLa cells and infection was analysed via flow cytometry as described elsewhere [19]. Percent suppression was calculated for each animal using the following formula:

$$\%\ \text{suppression} = 100 - \left(\frac{\%\ \text{infection [x dpi]}}{\%\ \text{infection [}-2\ \text{dpi]}}\right) \times 100$$

where "x dpi" is the day of the calculated percent suppression.