Changes in cell recruitment in the female reproductive tract during chlamydial infection

Thierra Christopher[1,2], Flutur Latifi[2], Vjollca Konjufca[2]

[1]SI Bridges to the Baccalaureate, School of Biological Sciences, Southern Illinois University Carbondale

[2]Department of Microbiology, Southern Illinois University Carbondale

Introduction

Chlamydia spp. are gram-negative, anaerobic bacteria that can cause sexually transmitted infections (STI), pneumonia, and trachoma. *Chlamydia trachomatis* (CT) causes the most reported STIs worldwide and in the US. In 2020, WHO reported around 128.5 million new chlamydial cases worldwide, with the highest prevalence among sexually active adolescents and young adults, especially women between 15 to 24 years old. About 70% of chlamydial genital infections are asymptomatic in women and 50% are asymptomatic in men at the time of diagnosis. Therefore chlamydial STIs are of undiagnosed and untreated. When untreated, *Chlamydia* may ascend to the upper female reproductive tract (FRT) and can cause serious health complications like pelvic inflammatory disease (PID), ectopic pregnancy, endometriosis, and ultimately infertility in women.

Chlamydia initially infects the epithelial cells of the lower FRT, and if the infection is not cleared, *Chlamydia* ascends to the uteri, and oviducts causing tubal inflammation (hydrosalpinx) causing tubal infertility. In the mouse model the mouse-specific *Chlamydia muridarum* (Cm) ascends to the oviducts by 7-14 days post infection (dpi), depending on the dose of infection.

Upon infection of epithelial cells with *Chlamydia*, the cell autonomous immunity is initially activated and leads to early production of IFN- γ. The importance of IFN- γ in *Chlamydia* clearance is well established. As *Chlamydia* proceeds to infiltrate the epithelium of the genital tract, cell receptors recognize antigens present in the cell and activate the innate immunity. This leads to recruitment of neutrophils, macrophages and tissue resident lymphoid cells at the site of infection. Neutrophils are dispensable for *Chlamydia* clearance, while the role of macrophages is not well understood. Innate lymphoid cells are responsible for the IFN- γ production at this early stage of infection. As infection continues, humoral and cellular adaptive immunity is activated. The activation of adaptive immunity induces an inflammatory response that clears the bacteria but also causes scarring of the infected tissue which results in infertility. Therefore, it is essential to understand the components of immune response responsible for *Chlamydia* clearance and mechanisms involved in pathogenesis. Activated B cells have shown to be protective against reinfection, and *Chlamydia* specific IgA in genital secretions have shown to neutralize *Chlamydia* and decrease ascension and pathology. $CD4^+$ T cells are the main producers of IFN-γ and are thus essential in clearing *Chlamydia* infection. Identifying the immune mechanisms responsible for the tissue scarring and for *Chlamydia* clearance is important for identification of new therapeutical targets and vaccines.

Here we examined the recruitment of immune cells in different FRT regions at 7 and 14 dpi using in situ analysis of frozen FRT tissue sections. The complete characterization of and the role of specific immune cells in clearing chlamydial infections is yet to be defined.

Methodology

Ethics Statement. Animal experiments were conducted using recommendations by the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. Southern Illinois University's Institutional Animal Care and Use Committee approved all procedures involving animals. Prior to infection, animals were anesthetized using 1-3% isoflurane delivered by a precision vaporizer in a stream of oxygen. Mice were humanely euthanized using CO2 and cervical dislocation was performed to ensure proper euthanization of mice.

Per-vaginal (PV) infection with Cm. C57BL6/J mice (6-8 weeks-old) were sub-cutaneously (SC) injected with 2.5 mg medroxyprogesterone and 5 days later were PV infected with a $2x10^5$ Cm IFUs in 10 µl of SPG. At 7 or 14 dpi mice were euthanized and uteri, combined ovaries and oviducts (O+O) were collected for Cm enumeration with *Chlamydia* Pathfinder (Bio-Rad) or for in-situ analysis by immunofluorescence microscopy (IFM) as detailed below.

Staining and Analysis of Tissue Sections by IFM: At 7 or 14 dpi, tissues of the FRT were surgically excised following the animal euthanization. Tissues were frozen in Tissue-Tek OCT compound on dry ice and then stored in a -80℃ freezer. Tissues were then sectioned to make 5-7µm thick cryosections, which were then fixed in 4% paraphormaldehyde (PFA). After fixation, tissues were stained with fluorescence-conjugated monoclonal antibodies and phalloidin. All antibodies were used in a 1:100 dilution. Antibodies that were used include Cm-FITC, Alexa Fluor 350 Phalloidin, F4/80-PE, Ly6C-FITC and CD11b-APC. Stained tissues were then imaged on a Leica DM4000 B fluorescent microscope equipped with a Q imaging QIClick camera. All analysis of images was conducted using Volocity software.

Results

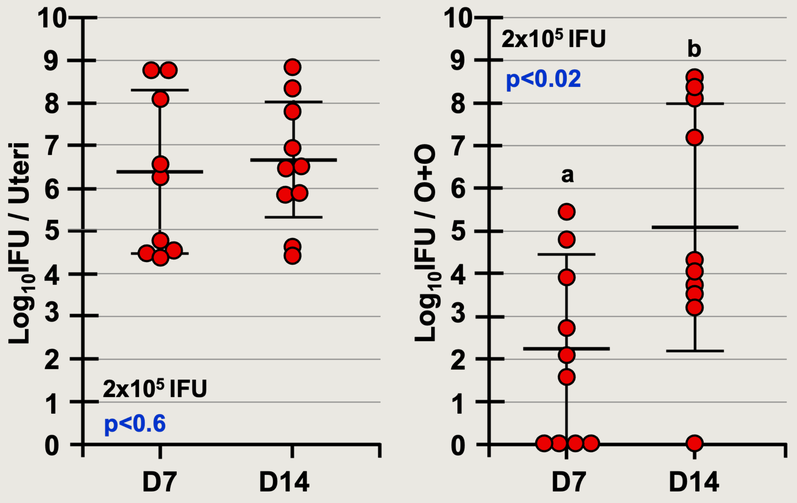

Figure 1. *Chlamydia* ascends and infects ovaries and oviducts (O+O) at day 14 pi. Chlamydia titers in (A) uteri and (B) O+O at 7 and 14 days pi with $2x10^5$IFUs. Data are representative of one experiment and are expressed as the mean ± SD. Group means that do not share a superscript are significantly different from each other ($p<0.02$).

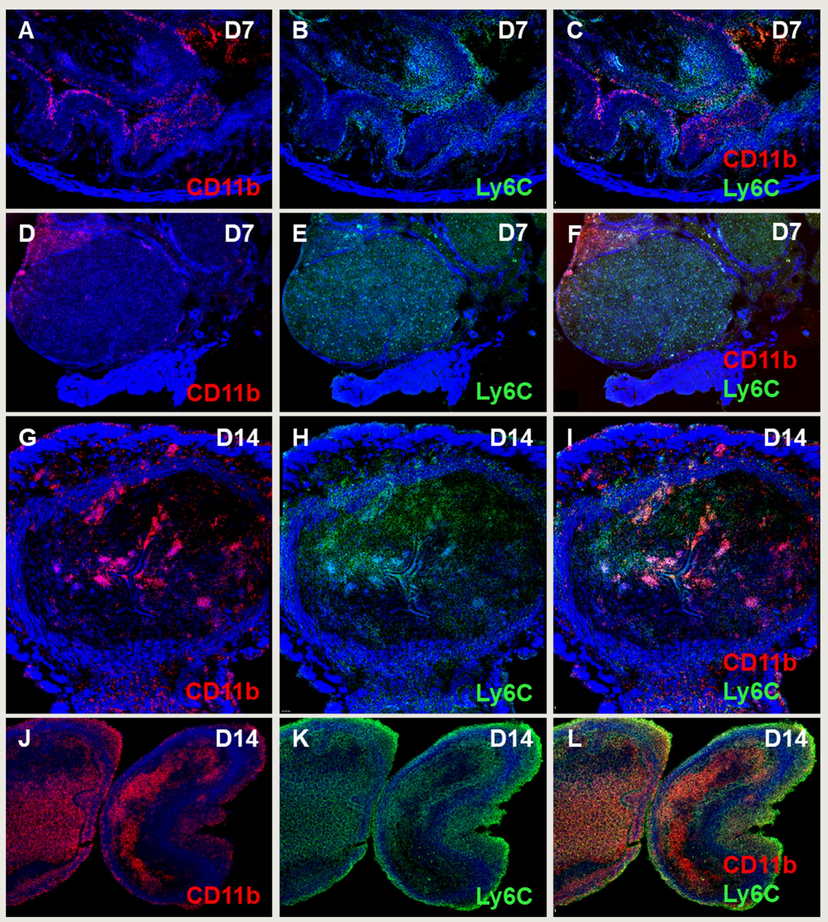

Figure 2. O+O of *Chlamydia* infected mice show higher recruitment of $CD11b^+Ly6c^+$ cells at day 14 pi. Recruitment of $CD11b^+$, $Ly6C^+$, and $CD11b^+Ly6C^+$ cells in the uteri (A-C) at 7 days pi and (G-I) at 14 days pi. (D-F) Recruitment of $CD11b^+$, $Ly6C^+$, and $CD11b^+Ly6C^+$ cells in the O+O at 7 days pi and (J-L) at 14 days pi. Sectioned tissues were labelled with monoclonal antibodies and Alexa Fluor 350 Phalloidin to visualize the *in-situ* cell recruitment and tissue architecture.

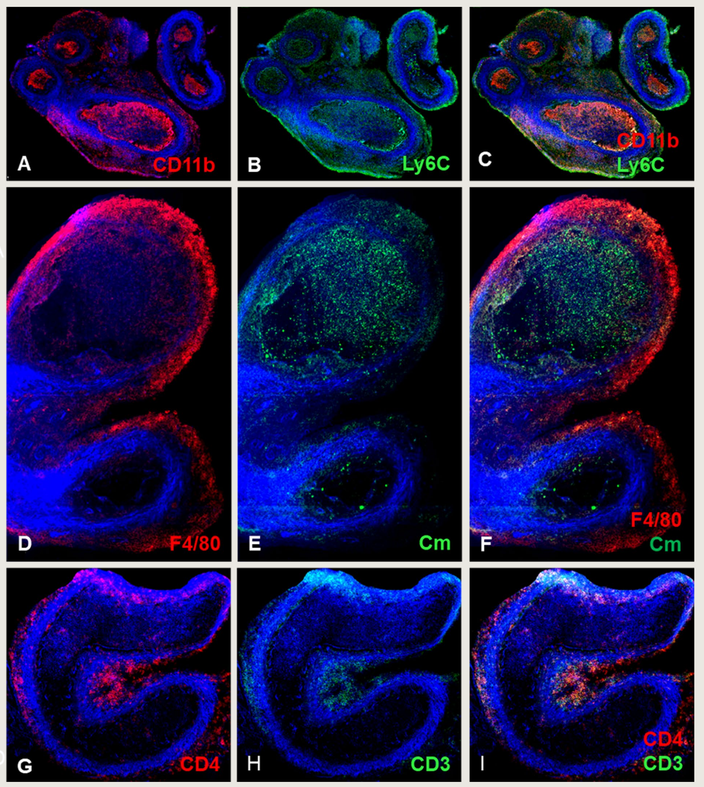

Figure 3. T cells and $F4/80^+$ cells are also recruited at the level of O+O at day 14 pi. Recruitment of (A-C) $CD11b^+Ly6C^+$ cells (D-F) $F4/80^+$ cells and (G-I) of $CD4^+$ and cells at the level of O+O 14 days pi. Sectioned tissues were labelled with monoclonal antibodies and Alexa Fluor 350 Phalloidin to visualize the *in-situ* cell recruitment and tissue architecture.

Conclusions

Chlamydia infects epithelial cells of the lower FRT and ascends to the upper FRT, resulting in a high *Chlamydia* burden at day 7pi in the uteri and day 14pi in the ovaries and oviducts. Chlamydial infection induces an immune response that triggers immune cell recruitment at the site of infection. Utilizing IFM, we have visualized recruitment of certain immune cells at the site of infection and have shown the in-situ distribution of these cells at different levels of the FRT at different timepoints. Our images show the following:

- There is recruitment of CD11b+Ly6C+ cells in the lumen of the uteri at day 7 pi. Parallel with the increase of the Chlamydia burden in uteri at day 14 pi, there is an increase of recruitment of CD11b+Ly6C+ cells as well.
- At day 14 pi, there is also a lateral invasion of the peripheral wall of the uteri with CD11b+Ly6C+ cells that reaches the muscular layer of the uterine wall.
- As infection propagates to the ovaries and oviducts at day 14pi, there is a higher recruitment of double positive cells $CD11b^+Ly6c^+$ in the lumen of the O+O, compared to day 7pi.
- The double positivity for CD11b and Ly6C marker suggests the presence of inflammatory monocytes, but further analyses should be conducted to characterize these cells, as other cell types might share the same markers (e.g. Myeloid-Derived Suppressor Cells-MDSC).
- Presence of Ly6C+CD11b- cells is noted in the ovaries and oviducts at day 7 pi. This phenotype might suggest for plasmacytoid dendritic cells, but further analyses should be conducted to characterize the cells.
- At day 14 pi, besides CD11b+Ly6C+ cells, there is recruitment of CD4+ T cells, as well as F4/80+cells in the ovaries of infected mice. In contrary to CD11b+Ly6C+ cells, T cells and F4/80+cells have a distinct distribution with higher concentration in the lateral wall of the ovaries.

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