CXCR5 regulates *Chlamydia* tubal pathology in mice and humans


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Abstract

Regulation of immune responses is critical for controlling inflammation and disruption of this process can lead to tissue damage. We report that lack of the CXCR5 chemokine receptor resulted in an increase of CD4 cells and fibrosis in the upper genital tract (UGT) of Cxcr5-/- weeks after Chlamydia muridarum genital infection. A similar phenotype was found using single nucleotide polymorphism analysis in C. trachomatis infected women attending a sub-fertility clinic. Women who developed tubal pathology after a C. trachomatis infection had a decrease in the frequency of CXCR5 SNP +10950 T>C. Further studies in Cxcr5-/- mice showed that oviduct tissue contained more Th1 cells without altering the resolution of infection. The number of activated NKT: CD69+NKT cells and production of Th1 associated cytokines and chemokines was increased in Cxcr5-/- mice. NKT cell depletion abrogated increased production of cytokines and chemokines. Further, chlamydial organisms directly activated type I and type II NKT cells which implies that C. muridarum activates NKT cells and cannot be regulated in the absence of CXCR5. We have identified a novel function of the CXCR5 chemokine receptor which appears to regulate NKT cell activation and UGT pathology in mice and possibly contributes to inter-individual differences in tubal pathology in humans.
Introduction

*Chlamydia trachomatis*, an obligate intracellular bacterium, causes the most cases of bacterial sexually transmitted infections (STIs) in the US resulting in about three million new cases annually.\(^1\) Inflammation and subsequent fibrosis from infection results in immune-mediated pelvic inflammatory disease (PID) and/or infertility, and can develop in as much as 40% of infected females. Alarmingly, this risk significantly increases following re-infection.\(^2\) Although female infection is easily detected and treated with antibiotics, treated individuals can acquire another infection in six months\(^3\) implicating repeated inflammatory insults as causing PID and infertility. The cause of fibrosis following *Chlamydial* genital infection is not known.

Eradication of *C. trachomatis* infection shortens inflammation and prevents development of inflammation and infertility. Many labs have shown that eradication of *Chlamydia* is dependent on CD4 T cells.\(^4\) IFNγ secreting T helper type 1 (Th1) cells have been shown to eliminate *Chlamydial* infection in vivo.\(^5\) In addition, natural killer T (NKT) cells have also been shown to be involved in the clearance of *Chlamydia* infection. Yang *et al.* showed a profound effect of NKT cell activation on *Chlamydia* lung infection that altered cytokine and subsequently affected outcomes of infection in a murine model. They found that activation and cytokine production of NKT cells enhanced *C. muridarum* lung infection.\(^6,7\)

The antigen receptors expressed by NKT cell differ from conventional T cells in that they recognize glycolipids expressed by bacteria and other sources, instead of protein antigens.\(^8\) CD1d-reactive NKT cells are classified into two subsets based on TCR usage: type I NKT cell with invariant TCR, iVα14 in the mouse and iVα24 in human; and type II with diverse TCRs.\(^9\) Both subsets play important roles in autoimmune diseases, tumor surveillance, and infectious diseases. Extensive studies have shown that upon activation, type I NKT cells cause a cytokine burst, down regulation of TCR and apoptosis.\(^10,11\) Type II NKT cells are less studied, but support protective roles in graft-versus-host disease (GVHD)\(^12\) and the response to hepatitis B virus in a mouse model.\(^13\) Depending on the stimulating antigens, type I NKT cells express and induce different cytokine secretion profiles; either a Th1-type profile, secreting IFN-γ and TNF-α, a Th2-type, secreting IL-4 and IL-13, or a combination of the two.\(^5\) Although some studies have provided evidence that NKT cells are activated by *Chlamydia* infection, the precise antigens, mechanisms and cytokine profiles remain unclear.

Formation of lymphoid aggregates within infected genital tract (GT) tissue is a hallmark of local immunity during *Chlamydial* genital infection.\(^14\) These structures are called tertiary lymphoid tissues (TLO) and resemble the organization seen in secondary lymphoid organs.\(^15\) Organized lymphoid tissue facilitates cell activation and cytokine secretion and is mediated
by the chemokines CCL19/CCR7, CXCL12/CXCR4, and CXCL13/CXCR5.\textsuperscript{16,17} We have previously found that in vitro infection with \textit{Chlamydia} organisms induces high levels of CXCL13, a ligand for the chemokine receptor CXCR5, by fallopian tube tissue.\textsuperscript{1} CXCR5 is important for formation of tissue lymphoid aggregates following infection with a range of pathogens and disruption of this chemokine pathway alters the immunity.\textsuperscript{18} CXCR5 is present on a number of immune cell types including NKT and CD4 cells.\textsuperscript{19} The level of CXCR5 expression distinguishes among NKT cells subsets.\textsuperscript{20} However, the role of CXCR5 in \textit{Chlamydia} genital infection is not known.

In the current study, we report that the lack of expression of CXCR5 on subsets of NKT cells activated in reproductive tissues by a \textit{Chlamydia} genital infection causes local secretion of enhanced amounts of pro-inflammatory cytokines and chemokines following \textit{C. muridarum} genital infection. Dysregulated cytokine and chemokine secretion facilitates the accumulation of Th1 cells and NKT cells in the oviduct and results in increased chronic inflammation and the formation of fibrosis in the upper genital tract (UGT) of \textit{Cxr5-/-} mice. We also report that humans with CXCR5 SNP at position +10950 are protected from the development of tubal infertility following \textit{C. trachomatis} genital infection. Further, we show evidence that \textit{C. muridarum} contains antigens that activate both type I and II NKT subtypes. Thus, the CXCR5 gene influences the outcome of \textit{Chlamydia} genital infection in mice and humans.

**Methods**

\textbf{Animals, Chlamydia and Challenge of mice.} A breeding colony was established with \textit{Cxr5-/-} mice (8 generations in C57BL/6) obtained from Martin Lipp, Delbrück-Center for Molecular Medicine, Berlin, Germany. Female C57BL/6 mice, 5-6 weeks old (Harlan Sprague-Dawley, Indianapolis, IN) were housed according to American Association of Accreditation of Laboratory Animal Care guidelines. Animal experimental procedures were approved by the UCLA Institutional Animal Care and Use Committee. \textit{Chlamydia muridarum} (MoPn) was grown on confluent McCoy cell monolayers, purified on Renograffin gradients and stored at -80°C in SPG buffer (sucrose-phosphate-glutamine) as previously described (47). Mice were hormonally synchronized by subcutaneous infection with 2.5 mg of medroxyprogesterone acetate (Depo Provera, Upjohn, Kalamazoo, MI) in 100 μl saline 7 days prior to a vaginal challenge with 1.5 x 10\textsuperscript{5} IFUs of \textit{C. muridarum} under anesthetization. Infection was monitored by measuring infection forming units (IFU) from cervical–vaginal swabs (Dacroswab Type 1, Spectrum Labs, Rancho Dominguez, CA) as previously described. Homogenates of oviduct tissues were harvested and various times and the number of \textit{C. muridarum} plaques were measured by plaque assay as described.\textsuperscript{40}
Histology, Hematoxin & Eosin and Trichrome stain. GT’s were removed at day 49 post-infection, fixed in 10% formalin overnight, and subsequently, 70% ethanol. Tissues were embedded en bloc in paraffin, sectioned (5 mm), and stained with either hematoxylin and eosin or Gormori trichrome. Two hematoxylin and eosin (H&E) sections per mouse were masked and scored by a veterinarian pathologist in comparison to aged-matched, hormone treated, uninfected controls using the following scoring scheme as described: 0 = normal, 1 = rare foci, 2 = scattered (1 to 4) aggregates, 3 = numerous aggregates (> 4), 4 = severe diffuse infiltration. The two scores from the uterine horn and oviducts of individual mice were averaged. Immunohistochemical analysis was performed on frozen sections prepared from frozen blocks 49 days following infection. Staining was carried out as previously described except using the following primary antibodies: CD4, CD8, CD19, CD11a, Ly6G/C, CD14, and NK1.1 (PharMingen, San Diego, CA). Positive staining cells (brown) were scored on two masked sections per mouse: 1+, 1-10 cells; 2+, 11-20 cells; 3+, 21-30 cells, or 4+, 31-40 cells per 20x field and aggregates were noted. Photographs were generated by scanning the microscope slides with an Olympus DP10 color digital video camera.

Immunohistochemistry. Sections from mouse GTs were snap-frozen into blocks and 5 µm frozen sections of the entire GT were prepared from the blocks by the UCLA Tissue Procurement Laboratory (TPCL) as previously described. The frozen sections were fixed in acetone, washed in PBS, and incubated in a mixture of methanol and 30% H2O2 for 30 minutes. The tissue was then blocked with avidin followed by biotin (Vector Laboratories, Burlingame, CA), as well as goat sera in order to reduce nonspecific binding. The tissue was then rinsed with PBS, at which point the primary antibody was added and incubated for 45 minutes at room temperature in the humidified box. After rinsing with PBS, the biotinylated secondary was added and incubated for an additional 45 minutes at room temperature in a humidified box. Streptavidin conjugated to horseradish peroxidase (Pierce) was added and the tissue was incubated as in the previous steps. The bound enzyme was visualized with metal-enhanced diaminobenzidine substrate (Thermo Scientific, Rockford, IL) followed by immunoperoxidase counterstain. The stained tissue was preserved with a thin layer of crystal mount (Fisher Scientific) and was comparatively ranked using a 20x objective with a 5 point scale determined from 0 to the greatest number of cells observed.

Detection of C. muridarum by PCR. Total Genomic DNA was prepared from the oviducts of d9 and d49 post infected mice using the Easy-DNA kit from Invitrogen (Carlsbad, Calif.) according to the manufacturer’s instructions. PCR reactions were performed using a 1X concentration of AmpliTaq Gold Master Mix (Applied Biosystems, CA). Briefly, the 50 ul reaction mix contained 300 ng total genomic mouse DNA, 250 U (0.05 U/ml) AmpliTaq Gold DNA polymerase, Gene Amp PCR Gold Buffer (30 mmol/L Tris-HCL, 100 mmol/L
KCl, pH 8.05), 400 mM dNTP, 5 mmol/L MgCl₂, and 0.5 uM each of forward and reverse oligonucleotide primers. AmpliTaq Gold DNA polymerase activation was for 5 min at 95 °C, followed by 35 cycles for 30 s at 95 °C, 45 s at 55 °C, 45 s at 72 °C followed by 1 cycle for 10 min at 72 °C. Two individual sets of primers for *C. muridarum* were utilized to determine the presence or absence of *C. muridarum* DNA.\(^4\) In addition, as a control for the integrity of the purified genomic mouse DNA, as well as, the absence of PCR reaction inhibitors in each purified sample, primers to the mouse nidogen-F gene were used. The primers used in the study are as follows: for *C. muridarum*, MOMP-forward, 5’-AA TCCCGCTTATGGCAAGCATATG-3’; MOMP-reverse, 5’-AGACCAACTAAGTTAAGGCGGC-3’; 16s rRNA-forward, 5’-CGTTAATACCCGGCCTGGATTTG-3’; 16s rRNA-reverse, 5’-GCCCCGATCTTTGACAATAC-ACT-AAC-3’; for mouse, nidogen-F-forward, 5’-CCAGCGACAGAATACCACCACC-3’; and nidogen-F-reverse, 5’-GGACATACTCTGCTGCCATC-3’. As a negative control, DNA was extracted from an uninfected normal mouse and subjected to amplification. As a positive control, DNA was extracted from renografin purified EBs and subjected to amplification. To determine the sensitivity of the PCR reaction, *C. muridarum* genomic copies ranging from 10⁵ down to 10¹ were spiked with 300 ng of uninfected mouse DNA and amplified using the MOMP primers as described above. The sensitiviy of the PCR was determined to be 10 copies per reaction using the ompA primer set.

**Lympholyte isolation and FACS identification.** Spleen (Spl), iliac lymph nodes (ILN), GTs or oviducts were harvested from individual mice. Single cell suspensions were attained by dissociating lymphocytes from Spl and ILN. Lymphocyte isolation from GTs or oviducts was carried out as described previously.\(^5\) Briefly, the entire GT or oviduct was removed and cut into 0.5cm pieces that were then rinsed with Ca-Mg-free Hanks’ balanced salt solution (HBSS). The tissue was incubated in a mixture of 5mM EDTA in HBSS at 37°C for two 15min periods with gentle stirring. The tissue was then incubated with RPMI1640 containing 2% bovine calf serum, antibiotics, 25mM HEPES and 1.5mg/ml collagenase (Sigma, USA) and incubated at 37°C with stirring for two periods of one hour. The isolated cells were pooled together and separated on a 40/75% discontinuous Percoll gradient (Pharmacia, Piscataway, N.J.) centrifuged at 2000rph at 22°C for 20min. Mononuclear cell pellets were resuspended in RPMI 1640 at 4°C until use. Lymphocytes isolated from different organs were incubated in RPMI 6140 in the presence of PMA and ionomycin. Brefeldin A (Sigma) was added 4h before the end of culture. The cells were then stained with fluorochrome-labeled antibodies against CD3 (clone 145-2C11), CD4 (clone GK1.5), α-GalCer-CD1d-tetramer, CD69 (cloneH1.2F3) and CXCR5 (clone 2G8) for 20 min on ice. After being washed, the cells were incubated with Cytofix/Cytoperm (Biolegend) for 1hr and the stained with fluorochrome-conjugated anti-IFN-γ antibody for 20 min on ice, washed again, resuspended in Cell Fix solution, and analyzed on LSR II.
**NKT cell depletion, CD1d blocking and in vitro cell culture.** Mice were intravaginally infected with *Chlamydia muridarum* as described earlier. On day 7 post-infection, lymphocytes were isolated from spleens. Cells were either treated with NKT depletion, CD1d blocking, NKT depletion + CD1d blocking, or with α-GalCer (250ng/ml), untreated cells served as control. For NKT depletion: Cells were stained with PE-α-GalCer-CD1d-tetramer on ice for 30min. NKT were depleted by using EasySep Mouse EP Positive Selection Kit (Stemcell Technologies, Canada) according to manufacture’s protocol. The depletion efficiency was greater than 90% tested by FACS. For CD1d blocking: cells were treated with anti-CD1d monoclonal antibody (clone 1B1) (10ug/ml) or isotype control for 30min prior to culture. Cells were then cultured in RPMI 1640 in presence of EB for 3 day, supernatants were collected until further use.

**Cytokine measurement and multi-analyte ELISArray.** Production of cytokines was measured in supernatants from cell cultures. Supernatants were stored at -80°C until analysis by enzyme-linked immunorobent assays specific for IFN-γ, performed according to the manufacturer’s specifications (R&D Systems, Minneapolis, Minn). Microbial-induced cytokines were analyzed by multi-analyte ELISAArray kit (SABiosciences, Frederick, MD). The assay was carried out according to manufacturer’s protocol. Calibrated standards were provided by the manufacturer.

**In vitro antigen detection assay.** In order to detect antigens from MoPn recognized by NKT in non-classic MHC I CD-1d receptor, *in vitro* antigen detection assay was carried out as described previously (Emmanuel Tupin). Briefly, 96-well flat bottom plate was coated with soluble mCD1d protein for 1hr at 37°C. After washing and blocking, sonicated MoPn was added to the well at various concentrations and incubated for 2-24 h at 37°C, α-GalCer and vehicle served as positive and negative controls, respectively. Following washings, 5×10⁴ invariant NKT hybridoma cells: 1.2, 1.4, or variant NKT hybridoma 19 were added and cultured overnight. IL-2 released in the supernatant was measured by ELISA as a readout of NKT cell activation.

**Immunogenetic studies**

**Patients**

**Dutch STD cohort**

Women of Dutch Caucasian (DC) origin (n = 543) the STD outpatient clinic in Amsterdam, the Netherlands in the period of July 2001 – December 2004. These women were consecutively included as the first part of a large prospective study. Among them, those who
had no Chlamydia infection, based on negative Ct-DNA and without Ct serology, served as controls. Participants were asked to sign an informed consent and fill out a questionnaire, regarding their complaints at that moment, varying from increased discharge, having bloody discharge during and/or after coitus, recent abdominal pain (not gastrointestinal or menses related) and/or dysuria. A cervical swab was taken for the detection of Ct-DNA by PCR(53). Peripheral venous blood was collected for the analysis of IgG antibodies against Ct (Medac Diagnostika mbH, Hamburg, Germany). A titre of $\geq 1:50$ was considered positive. Samples with grey zone values, e.g. cut off $\pm 10\%$, were repeated and considered positive when the result was positive or again within the grey zone. Infections with Candida albicans, Neisseria gonorrhoea, Trichomonas vaginalis and Herpes simplex virus 1/2 may result in symptoms similar to Ct infection. Therefore, an infection status was recorded for these microorganisms. Microorganism detection was done according to methods described by Ouburg et al.\textsuperscript{54}

**Dutch Subfertility cohort**

The study cohort included 259 DC women who presented with subfertility at the Research Institute Growth and Development (GROW) and the Department of Obstetrics and Gynaecology, Academisch Ziekenhuis Maastricht, The Netherlands. This subfertility group has been described elsewhere.\textsuperscript{41,58} For this study, women with or without clinically defined tubal pathology (n=56) were selected. Tubal pathology was defined as extensive periadnexal adhesions and/or distal occlusions of one or both tubes. Chlamydial antibodies were assessed by indirect microimmunofluorescence (MIF) test for anti- Ct IgG-antibodies. A positive CT IgG MIF test was defined as a titer $\geq 1:32$.

**Finland study population**

The study population considered of 114 infertile women who had attended the In Vitro Fertilization Unit, Department of Obstetrics and Gynaecology, Helsinki University Hospital, Helsinki, Finland in 1990-2005. Controls were selected from a group of 176 female blood donors. The selected cases consisted of 24 infertile women with laparoscopically verified tubal factor infertility (TFI). The control group consisted of 16 female blood donors whose buffy coat specimens were provided by the Finnish Red Cross Blood Transfusion Service (Oulu, Finland). The specimens were transported to the laboratory at room temperature within 24 h of donation. The specific immune responses of CT have been described elsewhere and CT status was determined by CT specific serology and cell stimulation as described previously.\textsuperscript{59}

**Cxcr5 SNP detection**

Genomic DNA was extracted from blood using the MagNaPure LC isolator as described.\textsuperscript{41} A healthy DC control group (n=130) was included to assess the general frequency of the three studied SNPs CXCR5 +3439 C>T (rs497916), +9086 T>C (rs12363277), +10950
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T>C (rs3922) in the general DC population. The CXCR5 SNPs were determined using the standard TaqMan analysis. The three CXCR5 SNPs tag 96.6% of the haplotypes, consisting of rs497916, rs566416, rs543524, rs12363277, rs613791, rs598207, rs3922, and rs676925. This haplotype spans 7KB of the CXCR5 gene.

**Statistics**

Student’s *t*-test and analysis of variance (ANOVA) followed by Bonferroni’s correction was performed using SigmaStat 2.03 (SPSS Inc. Chicago, IL). Groups were considered statistically different at *p < 0.05 and **p<0.01. Data are presented as mean ± SD or SEM differences as indicated. Differences in human *cxcr5* genotype and haplotype distributions were analyzed using χ² and Fisher Exact test, where appropriate and *p<0.05 was considered statistically significant. CXCR5 haplotypes were inferred using PHASE v2.1.1 and SNPHAP.

**Results**

**Lack of Cxcr5 gene causes increased tissue pathology and accumulation of Th1 cells in the oviduct.** Human genital infection with *C. trachomatis* can result in fibrosis and infertility in 40% of women following infection.2 The cause of fibrosis is yet undetermined and mouse models are used for investigation of the mechanism(s). C57BL/6 mice infected with *C. muridarum* (MoPn), the murine pathogen of *C. trachomatis*, variably exhibit slight signs of inflammation seven weeks (D49 post infection) after resolution of infection in upper GT (uterine horns and oviducts) tissue (UGT).21 However, a genital infection of *Cxcr5*-/− mice on a C57BL/6 background resulted in increased UGT inflammation as compared to WT C57BL/6 mice. Examination of H&E stained tissues by a veterinarian pathologist, found the infiltration in *Cxcr5*-/− mice to be dominated by lymphocytes compared to WT C57BL/6 mice (Figure 1, A and 1B).

In addition, trichrome stained sections from *Cxcr5*-/− mice revealed an increase of blue collagen fibers indicating fibrosis as seen in Figure 1C (arrows). We also noted great distension of oviducts not encased in fibrotic tissue (Figure 1C, arrowhead). In contrast, WT C57BL/6 mice only showed a small amount of blue staining collagen fibers (Figure 1D, asterisks). The immune infiltrate in WT and *Cxcr5*-/− mice was quantitated for lumen dilation, fibrosis, lymphocytic infiltration, and flattened mucosal folds. This finding is quite remarkable since intraluminal oviduct fibrosis after MoPn infection is marked in all C3H/HeJ and BALB/c mice but slight in C57BL/6, which is resistant to developing infertility.22 Although there was variability in the WT mice, we found a statistical increase in chronic but not acute immune cells (Figure 1E). Taken together, these data demonstrate that the lack of the chemokine
receptor, CXCR5, strikingly increases inflammation and fibrosis of UGT tissue in C57BL/6 background mouse following chlamydial genital infection.

Figure 1: The absence of CXCR5 results in UGT inflammation following *Chlamydia muridarum* infection. Mice were infected with MoPn, the GTs were harvested 48 days after infection and processed en bloc for paraffin sections. H&E sections of oviducts from (A) Cxcr5-/- or (B) C57BL/6 mice. Gormori trichrome stain of oviducts 7 wks after infection with MoPn from (C) Cxcr5-/- or (D) WT mice. Arrows = fibrosis, arrowhead = dilated oviduct and * = fibrosis. (E) H&E stained sections were scored in comparison to aged-matched, hormone treated, uninfected controls using the following scoring scheme: 0 = normal, 1 = rare foci, 2 = scattered (1 to 4) aggregates, 3 = numerous aggregates (> 4), 4 = severe diffuse infiltration. *p<0.025 by Student’s *t* test of WT vs. Cxcr5-/- mice, n=6 mice or 12 oviductsgrp.
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After chlamydial genital infection, WT mice form lymphoid aggregates in the UGT upon resolution of infection with *C. muridarum* which are comprised primarily of CD4 cells (Figure 2A and (23)). We also observed CD4+ lymphoid aggregates in Cxcr5-//- mice following chlamydial genital infection. However, the aggregates were larger in size compared to those in WT mice. The aggregates were primarily CD4 cells, as we only noted a slight increase of CD8+ cells (Figure 2A). Intriguingly, the aggregates in the UGT of Cxcr5-//- but not WT mice were surrounding both (arrow) and luminal (arrowhead) cells. We also noted a slight increase in neutrophils and B cells with this unusual pattern of surrounding epithelial cells in Cxcr5-//- mice (Figure 2B). We then performed flow cytometry on isolated oviducts from Cxcr5-//- and WT mice and noted a significant increase in the number of CD4 cells from Cxcr5-//- mice. Approximately 50% of the CD4 cells from CXCR5/-/- mice secreted IFNγ which is about twice as much as found in WT mice (data not shown). Further analysis revealed that CD4+IFN-γ+ Th1 cells were statistically elevated in the oviducts of Cxcr5-//- mice weeks after chlamydial genital infection (Figure 2C). Thus, mice lacking the CXCR5 chemokine receptor sustain an infiltrate of Th1 lymphocytes at the mucosa in the UGT.24,25

To further investigate the number of Th1 cell numbers during infection, we measured the number of Th1 cells in the GT of mice following genital inoculation with *C. muridarum*. WT and Cxcr5-//- mice were infected, lymphocytes collected, stimulated with PMA and ionomycin, and analyzed by FACS for CD4+IFNγ+ Th1 cells at various points during infection as indicated. Our data showed that Cxcr5-//- mice had fewer numbers of Th1 cells in the GT during the peak of genital infection (day 7) but had significantly more Th1 cells than the WT group as the mice began to resolve the genital infection (day 14) indicating that Th1 cells are retained as the infection resolves in Cxcr5-//- mice compared to WT mice (Figure 3A).
Figure 2: Th1 cells accumulate in the GTs of Cxcr5-/- mice following infection. (A) IHC staining of CD4 or CD8 cells within UGT from Cxcr5-/- or control C56BL/6 mice 7 wks after MoPn infection. Frozen sections were stained with anti-CD4 or anti-CD8 and appear as (brown) colored cells. Representative images from n=6 mice in each group where two sections from each mouse were stained with one of the antibodies. (B) Duplicate sections from mice in (A) were stained with antibodies that identify multiple cell types. The slides were digitally scanned and comparatively ranked on a 5 category scale. (C) Single cell suspensions of oviduct cells from Cxcr5-/- or control WT mice and were stained with CD4 and to identify Th1 cells and 96-904 CD4 cells were analyzed for expression by flow cytometry. The mean frequency ±SD per CD4 T cells were plotted. **p<0.01 (Bonferroni’s modified t test), each group consists of 3-4 sample points.
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Figure 3: Cxcr5−/− mice have Th1 cells in spleen and GT. Mice were with MoPn as indicated. At the various time points post-infection, lymphocytes were isolated from (A) GT or (B) spleen, stimulated with PMA and ionomycin and characterized by flow cytometry. Dotplots were gated on CD3 and CD4. Bars show the mean frequency of Th1 cells per group ± SD of 4/group. *p < 0.05 as determined by Bonferroni’s t test.
The chemokine receptor, CXCR5, is needed for development of Peyer’s patches and certain lymph nodes. Cxcr5-/- mice lack Peyer’s patches, axillary, inguinal, parathymic, mediastinal and iliac lymph nodes but facial, cervical, mesenteric lymph nodes and spleen are present. Therefore, we examined the number of Th1 cells in the spleens to assess the overall number of Th1 cells. Likewise, increased numbers of Th1 in the spleen were seen at recovery (d14) of genital infection in Cxcr5-/- mice compared to WT controls (Figure 3 B). Neither strain had any measurable Th2 cells in the GT or spleen (data not shown). These data indicate that Cxcr5-/- mice develop sufficient numbers of Th1 cells responsive to a C. muridarum infection. Although Th1 cells lacking CXCR5 require a longer period of time to reach the GT, they accumulate in the GT following infection with C. muridarum.

**Lack of the cxcr5 gene does not affect susceptibility or time of resolution to a C. muridarum genital infection.**

We next evaluated the chlamydial burden as measured in vaginal swabs to determine whether increased survival of organism in the reproductive tract contributed to the sustained lymphocyte infiltrate. We noted that WT and Cxcr5-/- mice were equally susceptible to chlamydial genital infection. We noted a slight increase in the number of organisms in the Cxcr5-/- GTs which is consistent with fewer numbers of Th1 cells in the GT during peak infection (Figure 4A). Cxcr5-/- mice do not have the GT draining ILNs which could explain the temporary increased number of organisms during the infection course. However, Cxcr5-/- mice had elevated numbers of Th1 cells in the spleen and also possess mesenteric lymph nodes which could supply Th1 cells needed for resolution of infection. Further analysis showed that WT and Cxcr5-/- mice resolved the infection at equivalent rates (not shown). To determine whether the sustained Th1 cell infiltrate observed in Cxcr5-/- late after infection was due to small numbers of chlamydiae remaining in oviduct tissue of previously infected mice, we performed PCR for MoPn during the time of infection (day 9 and day 49) on oviduct tissue with WT and Cxcr5-/- mice. Our results indicated that during the acute phase of infection, Cxcr5-/- and WT mice were PCR positive for the 16s rRNA and ompA genes. However none of the mice were PCR positive at the late time point during which Th1 cell infiltrates were observed (Figure 2). Although the bacterial load in Cxcr5-/- mice was temporarily increased, the lack of CXCR5 expression did not significantly alter bacteria clearance.
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![Graph](image)

**Figure 4**: *Chlamydia muridarum* does not exist in the UGT of WT or *Ccsr5*-/-- mice after resolution of infection in the lower GT. (A) Vaginal swabs were collected throughout the course of infection in *Ccsr5*-/-- and WT mice. *p*<0.05 by ANOVA, *n*=11. (B) Total genomic DNA was purified from the oviducts of mice on d9 and d49 post-infection and subjected to PCR to detect *ompA* and *16s rRNA* for *C. muridarum*.

**CXCR5 +10950 T>C correlates with lack of tubal infertility following C. trachomatis infection in humans.**

There is considerable variability in both susceptibility to acquiring a *C. trachomatis* infection and development of genital tract pathology following infection. To determine if the genetic variation in the *CXCR5* gene contributes to this variability in *C. trachomatis* (CT) infected humans we undertook a (SNP) analysis for genetic variation in the *CXCR5* gene using...
TaqMan analysis. There are three SNPs in the human CXCR5 gene +3439 C>T, +9086 T>C, +10950 T>C the distribution of these SNPs in our study population is shown in Supplemental Figure 1. However, the functional consequences, i.e., gain or loss of CXCR5 chemokine function is not known. We then evaluated these SNPs for contribution to the susceptibility of *C. trachomatis* genital infection by comparing women attending a STD clinic for potential uncomplicated *C. trachomatis* infection. As shown in Figure 5A, carriage of CXCR5 +10950 T>C did not differ significantly between women with and without *C. trachomatis* genital infection. However, other alleles did influence susceptibility since carriage of the CXCR5 +9086 C allele was significantly reduced in *C. trachomatis* infected women compared to controls (P: 0.049; OR: 0.5; 95%CI: 0.25–1.0) and *C. trachomatis* infected women had a significantly lower frequency of the CXCR5 haplotype IV (+3439 T / +9086 C / +10950 C) compared to CT negative women (P: 0.04; OR: 0.4; 95%CI: 0.2 – 1.0) (Supplemental Table 2). These results suggest that carrying the CXCR5 +10950 does not contribute to susceptibility of acquiring a genital infection with *C. trachomatis*.

We next evaluated whether different variants of the CXCR5 gene correlated with the development of tubal pathology following chlamydial genital infection. We identified that CXCR5 +1095 T>C was differentially distributed between women who developed tubal infertility following *C. trachomatis* infection versus those that did not develop tubal infertility following CT infection. Women developing tubal infertility had a statistically significant decrease in CXCR5 +10950 CC frequency, both in the cohort from the Netherlands (p=0.03; OR: 0.1; 95%CI: 0.02-0.85), and the cohort from Finland (p=0.04; OR: 0.14; 95%CI: 0.02-0.9) (Figure 5B). Combining both groups revealed an even more significant protection (p=0.002; OR = 0.1; 95%CI: 0.04-0.5). Therefore, SNP +10950 T>C of CXCR5 appears to protect against development of tubal pathology (TP) in women who had a previous CT infection. The CXCR5 gene influences chlamydial genital infection and tubal pathology in humans and mice, suggesting that the murine model could be used for understanding the role of CXCR5 gene in chlamydial genital infection.
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**Figure 5**: Distribution of the *CXCR5* SNP+10950 T>C (rs3922) in a STD cohort from Amsterdam and two tubal pathology cohorts. Genomic DNA was extracted from peripheral blood. Distribution of *CXCR5* SNP+10950 T>C (rs3922) was determined in samples (A) women with and without a positive *C. trachomatis* PCR on cervical swab (N.S.), n=543 or (B) women from NL (n=56) and FIN (n=114) that were positive for *C. trachomatis* and clinical evidence of subfertility (p=0.002; OR = 0.1; 95%CI: 0.04-0.5) by χ² and Fisher Exact test, (CT: *C. trachomatis*; TP: Tubal pathology; NL: Netherlands, FIN: Finland.
The absence of the cxcr5 gene increases the number of activated NKT cells at early stage following MoPn infection.

We noted an accumulation of CD4 cells dominated by IFNγ producing cells in oviducts following a chlamydial genital infection which indicated a continual activation of Th1 cells in the local tissue. However, chlamydial organisms or DNA was not found in oviduct tissue suggesting Th1 activation was cytokine driven. We therefore investigated NKT cells, which secrete a variety of cytokines, activate other cell types and have regulatory functions that control the magnitude of immune responses. There are two subtypes NKT cells: type I and type II, which can be defined phenotypically. Type I NKT cells are the best characterized and easily identified by staining with α-GalCer loaded CD1d tetramers. We examined the number of type I NKT cells (CD3+, NK1.1+, α-GalCer-CD1d-tetramer+) that were activated as indicated by CD69 expression. We found that activated type I NKT cells were found in the GT and spleen of infected mice, and is consistent with reports that this cell type is important for early host defense. During peak infection (day 7), we observed that the number of activated type I NKT cells in Cxcr5−/− mice were temporarily higher in comparison to WT mice at the local infection site (GT) (Figure 6). However, during the resolution phase (day 14), the number of activated NKT cells was equivalent between the strains. This finding suggests NKT cell activation is influenced by bacterial burden in the GT. Interestingly, we only found activated type I NKT cells in the spleens of Cxcr5−/− mice during the resolution phase (day 14) (Figure 6B). C. muridarum is capable of disseminating to the spleen and possibly suggests that type I NKT cells present in the spleen at this time are activated by a few disseminated chlamydiae bacteria. Further, these data imply that a lack of CXCR5 allows a greater degree of activation in type I NKT cells.

CXCR5 is expressed on NKT cells and the level of expression varies with NKT cell type and maturity. As can be seen in Figure 7A, CXCR5 is expressed by a subset of type I NKT cells. As expected, the lack of CXCR5 expression reduced the migration of NKT cells to the GT and spleen (Figure 7B). Although CXCR5 reduces the migration of some NKT cells to the GT, our data shows that NKT cells can enter GT tissue via other chemokine receptors. Furthermore, the increased number of activated NKT cells in the GT implies that CXCR5 is also important for regulation of NKT cell activation.
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**Figure 6:** Cxcr5-/- mice have a greater number of activated NKT cells in GT and spleen. Mice were infected with MoPn. At the various time points post-infection, lymphocytes were isolated from different tissues and characterized by flow cytometry. (A) Dotplots were gated on gated CD3+, NK1.1+ cells and show the percentage of α-GalCer-tetramer+ NKT cells that express CD69 in Cxcr5-/- and WT mice. (B) and (C): Bar graphs show the mean events of CD69+ cells out of 102 NKT cells per group + SD in GT and spleen, respectively. *p < 0.05 and **p<0.01 as determined by Bonferroni’s t-test, n= 4/group.
Figure 7: Cxcr5-/– mice show lower numbers of total NKT cells during infection due to lack of CXCR5 expression. Single cell suspensions of lymphocytes were collected from GTs and spleens at the indicated time points after MoPn infection, stimulated with PMA and ionomycin, and stained for NKT cells using α-GalCer tetramer, CD3, CD4, CD8 and CXCR5. Dotplots were gated on CD3+, NK1.1+, α-GalCer-tetramer+, cells (NKT cells) and show expression of CXCR5. (A) Representative dotplots showing NKT gating and expression of CXCR5, 7 days after infection. (B) Bars show the mean frequency of NKT+ cells per group + SD of the number of total NK cells (NK1.1+/CD3-) 4/grp. *p<0.05 or **p<0.01, n= 5 data points.

Lack of CXCR5 causes increased cytokine and chemokine production.

NKT cells are well recognized regulatory cells. They have been shown to regulate immune responses by bridging innate and adaptive immunity through communication with various cell types, including DC, NK cells, T cells and B cells directly or through production of cytokines and chemokines. We hypothesized that the increased activation of NKT cells seen in Cxcr5-/– mice 7 days after infection would also result in increased cytokine production when stimulated with chlamydiae elementary bodies (EB). We tested this hypothesis with IFNγ production. As can be seen in Figure 8A, splenic cultures from Cxcr5-/– mice produced approximately 10-fold greater amounts of IFNγ compared to cultures from WT mice. We then used various means to deplete cultures of NKT cells in order to determine if
NKT cells were the source of increased IFNγ. Depletion of type I NKT using the α-GalCer-CD1d tetramer effectively depleted greater than 90% of type I NKT cells and significantly diminished IFNγ production (Figure 8A). Stimulation with α-GalCer increased production of IFNγ above that of WT cells and demonstrated that type I NKT cells contribute in part, to the increased IFNγ production. Analysis of CXCR5 expression revealed that CXCR5 expression was not limited to type I NKT cells (data not shown). We examined IFNγ production from all NKT cells by blocking CD1d antigen presentation with anti-CD1d antibody. This also significantly reduced IFNγ levels. In addition, the combination of both treatments reduced IFNγ production in splenocytes from Cxcr5-/- mice to that of untreated WT control splenocyte cultures. This confirmed that the increased levels of IFNγ seen in Cxcr5-/- mice are largely produced by type I NKT cells as well as other non-classical NKT cells.

**Figure 8:** *In vitro* depletion of NKT cell function abrogates increased production of IFN-γ and other cytokines and chemokines in Cxcr5-/- mice. Mice were infected with MoPn on d7 and lymphocytes were isolated from spleens and treated with various conditions to deplete NKT cells with 95% efficacy. Cells were cultured with EB for 3 days. (A) The level of IFN-γ in cell culture supernatants with different NKT cell depleting/blocking treatments. *p<0.05; **p<0.01: compare each treated group to its own untreated counterpart using Bonferroni’s modified t test, n=4. (B) Multiple cytokines and chemokines levels were measured in supernatants from a-GalCer depleted plus anti-CD1d treated cell cultures (Depletion+blocking).
C. muridarum infection induces the production of cytokines and chemokines consistent with an inflammatory response and differentiation of Th1 and Th17 cells. As expected, splenic cultures of WT cells stimulated with EB produced a variety of inflammatory cytokines and chemokines associated with a Th1 and Th17 response and no production of IL-4 (data not shown). Cultures from Cxcr5-/- produced a 2 to 10-fold increase in the level of most of cytokines and chemokines analyzed (Figure 8B). We then examined supernatants depleted with α-GalCer-CD1d tetramer and blocked with anti-CD1d antibody (described above as “treated” groups) to reduce the activity of all NKT cells. We found that the combination of depletion+blocking of CD1d function reduced all of these cytokines/chemokine levels analyzed to at or below that of WT cultures (Figure 8B). In addition, combination treatment was able to reduce all chemokines and cytokines associated with a Th1/Th17 response from cultures of Cxcr5-/- mice to that of WT levels. Only secretion of IL-6 by Cxcr5-/- was partially reduced by depletion of all NKT cells. These data show that depletion of all NKT cells reduces the level of many cytokines/chemokines produced by DCs. Activated NKT cells are important for amplifying the Th1 differentiation capacity of dendritic cells and this data further suggests that activation of NKT cells by C. muridarum, induces enhanced cytokine/chemokine production by DC and amplifies Th1 cell production in Cxcr5-/- mice.

Chlamydia muridarum activate type I and type II NKT cells.

We next determined if chlamydial organisms contained antigens which directly activated type I and II NKT cells. Sonicated C. muridarum EBs at various concentrations was added to plates coated with recombinant, soluble mouse CD1d and incubated with NKT cell hybridomas. IL-2 release by the activated hybridoma cells provides a bioassay for TCR engagement. Our analysis found that a C. muridarum sonicate induced IL-2 secretion and activation of two type I and a type II NKT cell hybridoma (Figure 9A, B and data not shown). The findings from this assay, which contains only a TCR ligand and is free of any antigen presenting cells, indicates that C. muridarum organism contains lipid antigens which can bind and activate type I and II NKT cells. It is likely that diverse lipid antigens activate NKT cells since type II NKT cells express a diverse repertoire of TCRs and likely recognize multiple antigens (9). Chlamydia trachomatis does not have the enzymes for producing sphingolipids. However, the organism incorporates host lipids into the chlamydiae membranes.

Chlamydia muridarum activate type I and type II NKT cells.
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**Figure 9:** *C. muridarum* is recognized by type I and type II NKT cells. Various dilutions of MoPn sonicate or α-Gal were added to cultures of (A) a type I NKT cell hybridoma 1.2 and (B) a type II NKT cell hybridoma 1.9 and IL-2 was measured in triplicate by ELISA. Experiments were repeated 2-3 times.

**Discussion**

We have identified a novel form of immune regulation in the GT that depends on expression of the chemokine receptor, CXCR5. In the absence of this receptor, NKT cells become highly activated following *C. muridarum* infection and augment production of many pro-inflammatory cytokines and chemokines associated with DC, macrophages, epithelial, Th1, Th17 and NKT cells. The increased activation of NKT cells does not affect chlamydial clearance as both strains resolve infection at statistically similar times. However, NKT cell activation results in the expansion of CD4 cells in the oviducts of which the majority is Th1 cells. The end result is increased chronic inflammation and fibrosis. Although we used *C. trachomatis* infection in this study, the regulation of NKT cells by CXCR5 may cause chronic inflammation in other diseases.
Other studies support a role for NKT cells in chlamydial infection and either facilitate clearance or contribute to pathology in mice depending on the species and site of infection.\textsuperscript{6,7} These studies focused on the cytokine environment produced by NKT cells.\textsuperscript{28} Activation of NKT cells induces numerous functions including interaction with nearly every hematopoietic cell type and cytokine production is only one function. Our data shows that NKT cell activation as defined by expression of CD69, assists expansion of Th1 cells potentially by DC activation which helps to bridge innate and adaptive immunity.\textsuperscript{30} In addition, the time during which NKT cells were activated in Cxcr5\textsuperscript{-/-} mice correlated with elevated secretion of multiple cytokines \textit{in vitro} and implies that signaling CXCR5 is important for regulating the activation state and cytokine/chemokine secretion of NKT cells. There are precedents for a proposed role for a chemokine receptor in regulating type I NKT cell activity. For example, CXCR6 and its ligand have been shown to regulate the homeostasis of type I NKT cells in the liver, although trafficking to that site was not affected.\textsuperscript{40}

In our system, lack of CXCR5 does effect the overall trafficking of NKT cells. NKT cells have regulatory function and necessary for dampening Th1 cell responses. We have found fewer total NKT cells in the oviducts of Cxcr5\textsuperscript{-/-} mice (Supplemental Fig. 1A). However, we also found a similar increase of activated NKT cells (CD3\textsuperscript{+}, NK1.1\textsuperscript{+}, α-GalCer-CD1d-tetramer\textsuperscript{+}, CD69\textsuperscript{+}) in the oviducts of Cxcr5\textsuperscript{-/-} mice late after infection (Supplemental Fig 1B) which preserves the ratio of Th1:activated CD69\textsuperscript{+}NKT between WT and Cxcr5\textsuperscript{-/-} mice. This is consistent with numerous studies that show that NKT cells tend to have an activating role on the immune response.\textsuperscript{30} NKT cells appear to have multiple roles in chlamydial genital infection and could possibly be directly cytotoxic or activate other cell types with cytotoxic function, such as CD8\textsuperscript{+}TNFα\textsuperscript{+} cells, to mediate tissue damage.\textsuperscript{39}

This is the first study to provide direct evidence that antigens housed within chlamydial elementary bodies directly activate NKT cell subsets. Type I and type II NKT cells are activated by EB suggesting that there are multiple antigens responsible for activating this cell type since there is a diversity of TCR on these cell types.\textsuperscript{30} The antigens have not been identified yet, but type I and II NKT cells are both activated by presentation of a self or microbial lipid on CD1d molecules. We did not find evidence for persistent or residual \textit{C. muridarum} using a chlamydial plaque assay or PCR for the \textit{omp1} or \textit{16srRNA} for \textit{C. muridarum} in oviduct tissue late after infection (Fig 2).\textsuperscript{40} \textit{Chlamydia trachomatis} does not have the enzymes for producing sphingolipids.\textsuperscript{37} However, the organism incorporates host lipids into the chlamydiae membranes suggesting that host derived lipids from epithelial cells activate NKT cells.\textsuperscript{37,38}
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We have also shown a similar influence of CXCR5 on tubal pathology in humans. Women carrying the variant CXCR5 +10950 CC did not develop the level of tubal pathology seen in subfertile women after CT infection compared to those who did not have this CXCR5 allele, indicating a protective effect of the mutant allele. Haplotype analysis of the CXCR5 gene with the 3 identified CXCR5 SNPs (+3439 C>T, +9086 T>C and +10950 T>C) revealed that those carrying haplotype IV (TCC), present less frequently with a *C. trachomatis* infection compared to other CXCR5 haplotypes (Supplemental Table 1 and 2). The results indicate a protective effect of specific CXCR5 genotypes and haplotypes against *C. trachomatis* infections and the development of the late complication, tubal pathology. Interestingly, humans with the CXCR5 SNP+10950 T>C, were equally susceptible to *C. trachomatis* genital infection compared to carriers of other CXCR5 SNPs. Other studies have also found a SNP analysis of other chemokine genes correlates to tubal pathology. Barr et al., found that the CCR5-related inflammatory response was demonstrated to be crucial for the development of tubal factor infertility. They showed that in women with anti-chlamydial IgG responses, tubal pathology correlated with a low incidence of functional CCR5Δ32 deletion (7%), while women without tubal pathology had higher incidence of the CCR5Δ32 deletion (31%). Thus, in mice and humans the inflammation associated with CXCR5 and CCR5 function may predispose to development of complications of chlamydial infection, such as tubal factor infertility.

The host response is determined by interaction of *Chlamydiae* and their pathogen-associated molecular pattern (PAMP) with components of the innate host response. This interaction in turn is based on the individual genetic variation. Other studies have identified additional genes which also contribute to the variability in acquiring a chlamydial infection and immunopathology. Recently, Bailey et al. estimated the relative contribution of host genetics to the total variation in lymphoproliferative responses to chlamydial antigens by analyzing these responses in 64 Gambian pairs of twins from trachoma-endemic areas. Proliferative responses to serovar A EB antigens were estimated in monzygotic and dizygotic twin pairs. They found a stronger correlation and lower within-pair variability in these responses in monzygotic compared to dizygotic twin pairs. The heritability estimate was 0.39, suggesting that host genetic factors contributed almost 40% of the variation. This study reinforces the concept of compiling genetic traits that influence infection and genital tract pathology for the development of a sub-fertility risk profile to advance disease prevention profiles and identify individuals for immune-modulatory therapeutics. This approach is currently the focus of EpiGenChlamydia in Europe.
There is a strong association between repeated *C. trachomatis* genital infection and development of tubal inflammation and infertility.\textsuperscript{2,3} These differences are mainly based on a combination of bacterial factors, host factors, epidemiologic and demographic factors, such as co-infections, and the mutual interaction of these factors.\textsuperscript{14} Additionally, there are reported inter-individual differences in the clinical course of *C. trachomatis* genital infection such as transmission, symptoms, persistence or clearance of infection, and the development of late complications.\textsuperscript{2,43,44} It is difficult to correlate these to persistence of infection, especially when the detected serovar is not identical to the previous one.\textsuperscript{43-46} Possibly repeated of NKT cells with multiple serovars following chlamydial genital infection in those with altered CXCR5 expression and function could be responsible for inter-individual differences. This knowledge will be beneficial for the design of therapeutic strategies and vaccine development against *Chlamydia* infection.

Our study is the first describing the protective effect of CXCR5 gene polymorphisms in the development of *C. trachomatis* infection and late complications of this disease. As we have shown in the knockout mouse model, an increased NKT activity leads to an increased bacterial burden in the genital and late complications of this disease. In humans, carriage of the variant allele, +10950 T>C, might result in an altered function of the CXCR5 gene, and thereby an altered expression of NKT cells thus leading to less late complications of *C. trachomatis* infections such as tubal pathology. Although the exact pathways and mechanism(s) need to be elucidated, the knockout mice study apparently resembles a good model for understanding the pivotal role of CXCR5. We therefore hypothesize that in human, the exact mechanisms underlying the course of disease might be the same as in the knockout mice model. Therefore, more studies are needed of human CXCR5 genetic polymorphisms on the function of this chemokine receptor. One possibility would be to study the influence of CXCR5 gene on regulation of NKT cell activation between *C. trachomatis* and the host, and to find out whether CXCR5 gene function in humans is decreased or increased in the variant allele. As mentioned above, the three CXCR5 SNPs studied here tag 96.6% of the haplotypes and more studies are needed to study the effects of the other SNPs on *C. trachomatis* as well as other microbes that activate NKT cell function.

**Acknowledgements**

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Disease and Laboratory of the Municipal Health Service, Amsterdam, The Netherlands for the collection of the Dutch STD samples. The study was supported by the Helsinki University Hospital Research Funds and Oulu University Hospital. We also thank Ronika Sitapatrick and Su-Yin Kok for excellent technical assistance.
Supplemental figures and tables

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Supplemental table 1. Distribution of the CXCR5 SNPs in two tubal pathology cohorts and an STD cohort from Amsterdam. Genomic DNA was extracted from peripheral blood and PCR was performed for three CXCR5 SNPs; +3439 C>T, +9086 T>C and +10950 T>C. CT: Groups were compared using χ² and Fisher Exact test, where appropriate. p<0.05 was considered statistically significant. CT: C. trachomatis; TP: Tubal pathology.

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Supplemental table 2. Haplotypes of CXCR5 in in two tubal pathology cohorts and an STD cohort from Amsterdam. Genomic DNA was extracted from peripheral blood and PCR was performed for three CXCR5 SNPs; +3439 C>T, +9086 T>C and +10950 T>C. CXCR5 haplotypes were inferred using PHASE v2.1.1 (55, 56) and SNPHAP (57). Groups were compared using χ² and Fisher Exact test, where appropriate. p<0.05 was considered statistically significant. CT: C. trachomatis; TP: Tubal pathology.
Supplemental figure 1: Cxcr5-/− mice have a higher number of activated NKT cells in the oviduct. Single cell suspensions were prepared from the oviducts of WT and Cxcr5-/− mice at the indicated time points after infection. The cells were stained and the (A) mean ± SD of CD3+, NK1.1+, a-GalCer-CD1d tetramer+ cells among NK cells or (B) mean ± SD expression of CD69 was determined on gated on CD3+, NK1.1+, a-GalCer-CD1d tetramer+ cells. N= 4/grp. *p < 0.05 as determined by Bonferroni’s t test.
Supplemental figure 2: Oviduct tissue from WT or Cxcr5−/− mice do not contain chlamydiae by plaque assay. Homogenates of oviduct tissue from WT and Cxcr5−/− mice were performed at the indicated time points after infection. The number of chlamydial plaques were determined. Bars indicate the mean number of plaques + SEM n=6/grp. *p < 0.005 as determined by two-way repeated measures ANOVA.
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