Host inflammatory response and development of complications of Chlamydia trachomatis genital infection in CCR5-deficient mice and subfertile women with the CCR5delta32 gene deletion

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T cell immunity protects against diseases caused by the obligate intracellular bacterium Chlamydia trachomatis. Incidentally, host inflammatory response that includes T cells appears to also contribute to the pathogenesis of chlamydial diseases such as trachoma and tubal factor infertility (TFI). Therefore, designing effective prevention strategies requires a delineation of immune processes responsible for pathology and those mediating immunity, and identification of the immunogenetic factors predisposing to complication development. The chemokine receptor CCR5 is crucial for T cell activation and function since its deficiency causes suppression of T cell response. We investigated the hypothesis that the clearance of genital chlamydial infection in CCR5-deficient mice could be delayed in the short term; however, a beneficial effect could include protection against inflammation-related complications such as TFI. In a translational study in humans, we investigated the effect of a functional 32 bp deletion in the CCR5 gene on the risk of developing tubal pathology in Dutch Caucasian women with immunologic evidence [i.e., immunoglobulin G (IgG) responses] of chlamydial infection. When genetically-infected wild-type (WT) and CCR5 knockout (CCR5KO) mice were evaluated for microbiologic shedding of chlamydiae, there was a greater intensity of infection and delayed resolution in the knockout mice. However, compared to WT mice, the fertility of infected CCR5KO mice (measured by pregnancy rate) was only mildly affected in the short term and unaffected in the long term (70% vs 30% reduction in the short term, and 50 vs 0% in the long term, respectively). Immunobiologic analysis revealed that the diminished capacity of CCR5KO to control acute chlamydial infection correlated with the relatively low chemokine [interferon-inducible protein 10 (IP-10) and regulated upon activation normal cell expressed and secreted (RANTES)] and cytokine (mainly interferon-gamma and tumor necrosis factor-alpha) expression corresponding to a poor early T-helper I response. However, the reduced incidence of complications in the CCR5KO mice appears to correlate with the low activity of long term inflammatory mediators. Besides, the translational studies in humans revealed that among patients with positive anti-chlamydial IgG responses, tubal pathology correlated with a low incidence of CCR5delta32 deletion (7%), while women without tubal pathology had higher incidence of the CCR5delta32 deletion (31%) as compared to controls (19%). Thus, in mice and humans the inflammation associated with CCR5 function may predispose to development of complications of chlamydial infection, such as TFI.

Key words: Chemokine receptors, chlamydia, gene deletion, genetic predisposition to disease, immunogenetics

The genus Chlamydia comprises obligate intracellular, Gram-negative-like bacteria that cause numerous oculo-genital and respiratory infections in humans, animals and birds. Trachoma, caused by C. trachomatis serovars A, B, Ba and C, is the world’s most common preventable blinding disease. It is epidemic in several developing nations, including Africa, south east Asia, and the Middle East, with an estimated 150 million people affected, of whom six million are irreversibly blinded or severely visually impaired [1]. Genital infections by the different genovars of C. trachomatis constitute the most common bacterial sexually transmitted diseases (STDs) in the United States and several other
industrialized nations, including the United Kingdom, Germany, Japan, and France. Pelvic inflammatory disease (PID) and tubal factor infertility (TFI) are major complications of genital infection, occurring in approximately 40% and 10% of untreated infections, respectively, and constituting an enormous morbidity and socioeconomic burden of chlamydial infections [2-5]. The lymphogranuloma venereum (LGV) infections are invasive and often ulcerative with lymphatic tissue involvement (e.g., inguinal bubo) [6,7], which are endemic in certain developing nations including parts of Africa, Asia, South America, and the Caribbean [8]. The recent epidemic outbreak of LGV disease among male homosexuals in Europe is attracting considerable public health attention in many countries [9]. Ulcerative STDs in general are a major risk factor for HIV acquisition. Moreover, reports suggesting that genital chlamydial infection may be on the rise [10,11], and could predispose to HIV-related acquired immunodeficiency disease [12-18] and human papilloma virus-associated cervical dysplasia, have heightened these concerns [19].

There is an urgent need to develop intervention and prevention measures to control chlamydial infections in the human population. However, a better understanding of the pathobiology of the disease is crucial for efforts to design preventive measures including the use of targeted immunomodulators and selective anti-inflammatory agents to control the onset of pathologies, and the application of effective vaccines as prophylaxis. Although T cell immunity is crucial for chlamydial control [20-22], clinicopathologic and experimental studies have suggested that the pathogenesis of complications such as trachoma and TFI is due to the deleterious host inflammatory immune response against the infectious agent. Thus, studies defining the key elements of protective immunity against Chlamydia, and establishing the parameters for vaccine selection and evaluation, are often confounded by the complexities of chlamydia biology, serovariation, infection manifestations and induction of paradoxical immune effectors that can be both protective and pathologic [23-27]. This double-edge effect poses a dilemma to the effort to dissect the role of host immune response on pathogenesis and immunity. In this respect, various inflammatory chemokines and cytokines and strains of mice with differential susceptibilities to chlamydial infection have been evaluated to define some of the immunopathogenic factors responsible for chlamydial disease [28-34]. Among other findings, toll-like receptor 2 (TLR2) deficiency caused decreased secretion of specific inflammatory cytokines, and a significant reduction in oviduct and mesosalpinx pathology even without affecting the course of the infection in mice [30]. Also, cytokines and molecules indicative of T cell activation were upregulated in active trachoma [35]. These observations and others would suggest that there are key immunobiologic pathways of T cell activation with limited redundancies that often complicate such studies. The chemokine receptor CCR5 is a member of the 7-transmembrane, G protein-coupled receptor superfamily, functioning as an important chemokine receptor that is preferentially expressed on certain leukocytes (monocytes, cytotoxic T cells, and CD4, T-helper 1 (Th1) and dendritic cells), and binding specific chemokines [e.g., regulated upon activation normal cell expressed and secreted (RANTES), macrophage inflammatory protein 1 (MIP-1) alpha and MIP-1 beta] that activate and induce Th1-like cells [36-40]. As a crucial receptor involved in T cell activation and function, a deficiency of CCR5 is associated with a suppression of T cell induction and leukocyte migration under certain infectious and non-infectious conditions [41-43], suggesting that it plays a role in both infection-related immune and inflammatory processes. The effect of a targeted suppression of the critical specific T cell response on both immune-mediated microbial clearance and the development of complications of chlamydial infection is largely unknown.

We investigated the hypothesis that the suppression of T cell response against genital chlamydial infection in CCR5-deficient mice could delay the clearance of the pathogen in the short-term, but it could also confer a beneficial effect by protecting the animals from complications such as TFI. In addition, in a translational study in humans, we investigated if the functional 32 bp deletion in the CCR5 gene (CCR5delta32) had an effect on the risk of developing tubal pathology in women with serologic evidence [immunglobulin G (IgG) responses] of C. trachomatis infection. The results supported the working hypothesis and could pave the way for a more detailed analysis and definition of the specific host-related immune effectors and immunopathologic processes underlying the pathogenesis of chlamydial disease.

**Materials and Methods**

**Knockout and wild-type mice**

Female CCR5 knockout (CCR5KO or CCR5<sup>−/−</sup>) and the wild-type (WT) control (i.e., CCR5<sup>+/+</sup>) mice, on
(C57BL/6J) background, 5-8 weeks old, were obtained from The Jackson Laboratory, Bar Harbor, MA, USA. All animals were fed with food and water ad libitum, and maintained in laminar flow racks under pathogen-free conditions of 12-h light and 12-h darkness.

**Chlamydia stocks and antigens**

Stocks of *Chlamydia muridarum* (the *C. trachomatis* agent of mouse pneumonitis or MoPn) used for infections were prepared by propagating elementary bodies (EBs) in McCoy or HeLa cells, according to standard procedures [44]. Chlamydial stock titers were expressed as inclusion-forming units per milliliter (IFU/mL). Chlamydial antigens were prepared by growing the agent in HeLa cells and purifying EBs over renografin gradients, followed by inactivation under ultraviolet (UV) light for 3 h.

**Animal Infection**

Groups of CCR5KO and control mice were intra-vaginally infected with $10^6$ IFU of MoPn. The status of the infection was monitored by periodic cervico-vaginal swabbing of individual animals and isolation of chlamydiae in tissue culture [44]. Experiments were repeated 2 times to give 10-12 mice per group.

**Chlamydia-induced cell activation, cytokine and chemokine secretion by leukocytes**

The profile of cytokines and chemokines secreted by leukocytes from chlamydial-infected CCR5KO and WT mice was compared by measuring the levels of specific cytokines and chemokines released following in vitro restimulation of the total splenic cells with UV-inactivated chlamydiae. CCR5KO and control mice were genitally infected with MoPn as described above and at various times postinfection (7, 14, 21 and 28 days) splenic cells were isolated from infected mice and $2 \times 10^5$ cells were stimulated with $10 \mu$g/mL of chlamydia antigen and incubated at 37°C in 5% carbon dioxide incubators for 120 h. At the end of the incubation period, the supernatants were collected and assayed for the Th1 cytokine interferon-gamma (IFN-γ), and the Th1 chemokines interferon-inducible protein 10 (IP-10) and RANTES, using a quantitative enzyme-linked immunosorbent assay (Cytoscreen™ Immunoassay Kit; BioSource International, Camarillo, CA, USA) according to the supplier’s instructions. The concentration of the cytokine and chemokines in each sample was obtained by extrapolation from a standard calibration curve generated simultaneously.

Data were calculated as the mean values (± standard deviation) of triplicate cultures for each experiment. The results were derived from at least 3 independent experiments.

**Animal fertility studies**

Animals were infected with $10^6$ IFU/mouse with MoPn. Two weeks and 5 weeks postinfection, groups of animals were mated with male counterparts by placing 3 females to 1 male, and subsequently observed and weighed daily for 19 days to determine pregnancy, as previously described [45]. The numbers of pregnant mice in the different groups were enumerated after 19 days in each case.

**CCR5delta32 gene deletion in women with subfertility**

The study cohort included 256 Dutch Caucasian 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 cohort has been described elsewhere [46]. Tubal pathology was defined as extensive periadnexal adhesions and/or distal occlusions of one or both tubes, and 50 women had severe tubal pathology based on these criteria. Chlamydial antibodies were assessed by indirect microimmunofluorescence (MIF) test for anti-*C. trachomatis* IgG antibodies, as described previously [47,48]. A positive *C. trachomatis* IgG MIF test was defined as a titer ≥1:32. A healthy Dutch Caucasian control group (n = 145) was included to assess the general frequency of the CCR5delta32 genotypes in the Dutch Caucasian population. Genomic DNA was extracted from blood using the MagNaPure LC isolator according to the manufacturer’s instructions (Roche Molecular Biochemicals, Mannheim, Germany). The human CCR5 gene delta 32 deletion was determined by polymerase chain reaction (PCR), with the sense primer CCR5-d32S: 5’-CAAAAAGAAGGTCTTCTAACTACC-3’ and anti-sense primer CCR5-d32AS: 5’-CCTGTGCCTCTTCTTCTCATTTG-3’ under the following PCR conditions: 5 min at 94°C, followed by 35 cycles of 60 sec at 94°C, 60 sec at 55°C, and 60 sec at 72°C, and the cycling programme was followed by 7 min at 72°C with final storage at 4°C (Perkin Elmer PE9700). The PCR product was electrophoresed on 3% agarose and the following 3 fragment patterns were identified: WT CCR5 gene (1.1) 189 bp, homozygote CCR5 mutant gene (2.2) 157 bp, and the heterozygote CCR5 gene (1.2) 189 bp + 157 bp.
Statistical analysis
The levels of cytokines in samples from different experiments were analyzed and compared by performing a 1- or 2-tailed $t$ test, and the relationship between different experimental groupings was assessed by analysis of variance. Minimal statistical significance was judged at $p<0.05$. The chi-squared test or Fisher exact test was used for comparison of CCR5d32 genotype frequencies between patient (sub) groups and/or controls. Statistical Package for the Social Sciences (SPSS) 10.0 for Windows (SPSS Inc., Chicago, IL, USA) was used for statistical analysis.

Results

High intensity of infection and delayed clearance of genital chlamydial infection in CCR5KO mice
CCR5 is a crucial chemokine receptor that supports the activation and induction of specific T cells during infectious and non-infectious inflammatory processes. We investigated the effect of CCR5 deficiency on the ability of mice to control and clear genital chlamydial infection. Fig. 1 shows results from studies that compared the course of genital chlamydial infection in CCR5KO and control (WT) mice. The data revealed that within the first week of infection, there was no difference in the level of infectivity of CCR5KO and WT mice. However, by the second and fourth weeks, the ability of CCR5KO mice to control the infection was compromised, with a higher intensity of infection revealed by the isolation of higher chlamydiae from the mice. By the fifth week, all WT mice had cleared the infection but the CCR5KO mice remained infected ($0.0$ vs $\log_{10} 3.0$ IFU/mL, respectively). The results suggested that the deficiency of CCR5 could have adversely affected the ability of the mice to elicit the required T cell response that is known to clear chlamydiae in mice [20].

Protection of CCR5-deficient mice from certain complications of chlamydial infection
The effect of the diminished capacity of CCR5KO mice to clear genital chlamydial infection on the infertility that is commonly associated with a genital infection [45] was studied. Infected mice were mated at 2 and 5 weeks after the initial infection, and the fertility was assessed by the number of pregnancies recorded. The mating at different time periods was targeted at evaluating the short- and long-term effect of the infection on fertility, since the WT mice cleared their infection at this latter time. Interestingly, at 2 weeks post-genital infection, WT mice exhibited a significantly lower fertility (with $<40\%$ pregnancy rate) than CCR5KO mice ($>70\%$; $p<0.021$) [Fig. 2A]. Furthermore, at 5 weeks post-genital infection, all the CCR5KO mice exhibited $100\%$ fertility whereas the control mice scored approximately $50\%$ (Fig. 2B). These results suggested that the immune competence of the host is possibly a relevant factor in the development of the long-term complications of chlamydial infection such as infertility.

To test this proposition, we evaluated the likely immune correlates of this inverse relationship between the ability to clear chlamydial infection and the development of complications in the context of CCR5 integrity.

Immunologic correlates of clearance of infection and protection from disease
The direct immunobiologic impact of CCR5 deficiency includes the limitation of T cell activation and reduction or elimination of recruitment of leukocytes to inflammatory sites of infection [41-43]. Since these processes are mediated by chemokines and cytokines, their production by WT and CCR5KO leukocytes that are exposed to chlamydiae was evaluated. When splenic cells containing T cells and other leukocytes were exposed to chlamydiae, the antigen-specific IFN-\(\gamma\) and tumor necrosis factor-alpha (TNF-\(\alpha\)) response was expectedly elevated in the cells from the WT mice (Fig. 3A). The results presented in Fig. 3A also revealed that
In a translational study involving subfertile Dutch Caucasian women, we investigated by PCR method whether a functional 32 bp deletion in the CCR5 gene had an effect on the risk of developing tubal pathology in women with serologic evidence of *C. trachomatis* infection based on IgG responses. Results revealed that in the control women (n = 145) the CCR5delta32 WT genotype (1.1) was present in 81%, the heterozygous genotype 1.2 in 18%, and the 2.2 homozygous mutant genotype in 1% (with a total of 19% being carriers of the mutant allele). Also, the subfertility cohort (n = 256) had frequencies of the WT (1.1 = 80%), heterozygous (1.2 = 19.5%), and homozygous mutant genotypes (2.2 = 0.5%) essentially identical to the control women. Therefore, there was no difference in the frequency of the CCR5delta32 gene deletion among subfertile (20%) and control (19%) women.

However, in women with laparoscopically confirmed tubal pathology (n = 50), cariership of the delta32 deletion was lowered to 14%, while in women without tubal pathology (n = 206) cariership of the delta32 deletion remained at the normal level (21%). This suggested that the incidence of tubal pathology was lower in women carrying the CCR5 mutation. Since only a proportion of the women with proven *C. trachomatis* infection (MIF IgG responses) actually develop late complications such as tubal pathology, we wondered whether CCR5-mediated inflammatory response has a role in the development of chlamydial-associated complications. Therefore, to directly investigate the role of CCR5 status on the incidence of chlamydial-associated TFI, we hypothesized that as in mice, a functional deficiency in CCR5 gene will moderate tubal pathology in chlamydia-infected women. To test this hypothesis, we compared the frequency of delta32 deletion in women with *C. trachomatis* IgG responses who developed tubal pathology (TP+CT+, n = 28) and women with *C. trachomatis* IgG responses without tubal pathology (TP–CT+, n=13). The results revealed that there was a significant difference between the TP+CT+ women, with only 7% cariership of the 32 bp deletion.
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in the CCR5 gene, and the TP–CT+ women with 31% carriership (odds ratio 5.8). Fig. 5 presents a summary of these results.

**Discussion**

The ability of *Chlamydia* to induce both protective and immunopathogenic immune responses poses a phenomenal challenge to research efforts to define the conditions favoring either response, and vaccine design
research to skew the responses along the protective pathway. A detailed knowledge of the conditions engendering pathogenesis could lead to the design of targeted immunopharmacologic strategies to avert pathologies. In this respect, the recent findings from an interleukin 10-deficient dendritic cell-based cellular vaccine [20,49] suggested that a fast and vigorous Th1 response after an infection will rapidly arrest chlamydial replication, clear the infection, eliminate residual antigens and prevent the establishment of a latent infection. On the other hand, it was suggested that an inadequate or suboptimal Th1 response delays clearance of the pathogen, leading to the establishment of a latent or persistent infection, which fuels a low-grade chronic immune response that causes tissue damage. This proposition is supported by several experimental and clinical findings relating to the cytokine and leukocyte responses that are associated with the onset of chlamydial diseases [31,50-52].

The microbiologic and host factors governing the development of complications of chlamydial disease have not been defined adequately. The increasing experimental and clinical evidence that certain host genetic factors are crucial for the manifestation of specific disease phenotypes in certain individuals has led to the proposition that host genetic and immunologic or inflammatory processes are relevant for the development of complications of chlamydial disease [53-55]. In this respect, clinical evidence from a sibling cohort study in a trachoma-hyperendemic setting indicated that persistence and inadequate clearance of chlamydiae (possibly due to a delayed or inadequate Th1 response) were hallmarks of individuals who suffered severe trachoma [56]. Also, different isolates of C. trachomatis causing symptomatic and asymptomatic infections in women exhibited similar growth characteristics in vivo and in vitro [53], suggesting that the clinical outcome was mostly host dependent. In addition, history of prior infection, age, and the hormonal status relating to the phase of estrous cycle and HLA alleles were significant factors in the development of PID and infertility following genital chlamydial infection [57-62]. Furthermore, various inflammatory chemokines and cytokines and strains of mice with differential susceptibilities to chlamydial infection have been analyzed to define some of the immunopathogenic factors responsible for chlamydial disease [28-34,63]. Among other findings, TLR2 deficiency caused decreased secretion of specific inflammatory cytokines, and a significant reduction in oviduct and mesosalpinx pathology without affecting the course of the infection in mice [30]. The lack of manifestation of the effect of TLR2 deficiency on the clearance of chlamydial infection was probably due to the natural redundancy in the TLR signaling system, such that the absence of TLR2 was duly compensated by other pathways. However, the reduction in tissue pathology could be associated with the suppression of inflammatory response caused by the inhibition of TLR signaling. Also, cytokines and molecules indicative of T cell activation were upregulated in active trachoma [35], and animals that exhibit a high incidence of ascending genital chlamydial infection tend to secrete a higher TNF-α in response to an infection [28,29]. These observations strongly support the involvement of host inflammatory response in the pathogenesis of the complications of chlamydial infection.

We have experimentally demonstrated in this study that CCR5-related inflammatory response is crucial for the development of TFI following genital chlamydial infection. In translational immuno-genetic and pathobiologic clinical studies in humans, functional defect in CCR5 also appears to moderate...
the development of tubal pathologies associated with genital chlamydia infection in women. These data are corroborated by previous propositions that certain host factors are relevant for the development of the complications of chlamydial infection. Specifically, the 32 bp deletion in the CCR5 gene, which results in a truncated protein with impaired signal-transduction capacity, was associated with resistance to human immunodeficiency virus type 1 (HIV-1) infection [64, 65]. Recently, it has been suggested that heterozygosity for CCR5delta32 was also associated with spontaneous hepatitis C viral clearance and with significantly lower hepatic inflammatory scores [66]. Our experimental studies indicated that a deficiency of specific anti-chlamydial Th1 response led to a suppression of Th1 response and delayed clearance of genital chlamydial infection. However, CCR5KO mice were protected from the complication of the genital infection relating to infertility. In addition, C. trachomatis-exposed women with CCR5delta32 deletions appear to be protected from tubal pathology as well, suggesting a crucial role for CCR5-related specific Th1 and inflammatory responses in the pathogenesis of infectious tubal pathologies.

Perhaps this study represents the first concurrent demonstration of a strong causal relationship between CCR5-related specific Th1 and inflammatory response and development of complications of genital chlamydial infection in both animal models and humans. The implications include the fact that although Th1 response is crucial for chlamydial control, there are host conditions that could skew the response toward pathology. Such conditions may include the involvement of immunopathogenic chlamydial antigens [26] and vaccine design effort may focus on defining the existence of clonotypic T cells that recognize such antigens or develop additional strategies to eliminate them from promising vaccine candidates. T cell clones reactive against specific chlamydial antigens have been isolated from the synovial fluids of patients suffering chlamydia-induced reactive arthritis [67]. In addition, since an early and relatively robust T cell response is protective against subsequent development of complications of chlamydial infection [20,28], these results from the CCR5KO system may suggest that the lack of an early T cell activation caused the delay in resolution of the infection; however, the persisting suppression of T cell activation prevented the chronic host inflammatory response that induces pathologies. Therefore, an early treatment of chlamydial infection with antimicrobials followed by specifically targeted anti-inflammatory agents may hold promise as a strategy for preventing the complications of chlamydial infection. Finally, it is pertinent to mention that although previous studies along this proposition yielded conflicting results [68-72], the selective use of specifically targeted anti-inflammatory agents in combination with antibiotics could prove useful in the management of chlamydial infections to avert pathology. In fact, inflammatory processes induced by several species of Chlamydia could be suppressed by certain non-steroidal anti-inflammatory drugs, including aspirin and indomethacin [73].

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