TLR4 IN *CHLAMYDIA TRACHOMATIS* INFECTIONS: KNOCKOUT MICE, STD PATIENTS AND WOMEN WITH TUBAL FACTOR SUBFERTILITY

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SUMMARY

Chlamydia trachomatis *is the most prevalent sexually transmitted bacterium in the world with almost 100 million new cases each year, some of which will develop tubal pathology. Clear differences in its clinical course of infections have been observed, and recently it has been shown that 40% is based on host genetic factors. We used an integrated approach based on infection of Toll-like receptor 4 (TLR4) knockout mice and immunogenetic analysis of female sexually transmitted disease (STD) patients (susceptibility) and women with* C. trachomatis-*associated tubal factor subfertility (severity). The results in TLR4 knockout mice suggest that the protection against reinfection is more solid in normal as compared to the TLR4-deficient mice. In humans the functional* TLR4 *single nucleotide polymorphism studied was not involved in the susceptibility to infection. However,* C. trachomatis *immunoglobulin (Ig) G-positive subfertile women with tubal pathology were more than twice as likely to be carriers of the mutant* TLR4 +896 G allele as compared to *those without tubal pathology; however this observation did not reach statistical significance. In conclusion, both the murine model and the human immunogenetics studies show a slight effect upon* TLR4 *deficiency in the severity of infection but not in the susceptibility to infection.*

INTRODUCTION

Although *Chlamydia trachomatis* infection is a predominant cause of tubal pathology in subfertile women (1), not all women develop this complication following infection. The susceptibility, course and outcome of infectious diseases are determined by environmental factors (e.g., co-infection), bacterial factors (e.g., virulence) and host factors (e.g., immunogenetic differences between individuals).

C. trachomatis bacterial factors have been studied in relation to the clinical course of infection, such as symptomatic versus asymptomatic infection, lower versus upper genital tract infection, and clearance versus persistence. In previous studies, no strong associations were found between the different serovars of *C. trachomatis* and the clinical course (2-10). Host immune factors are considered more important determinants of the inter-patient variability in the susceptibility, course and outcome of infectious diseases in general (11-16) and *C. trachomatis* infections in particular (17). Recently, pattern recognition receptors (PRRs) of the Toll-like receptor (TLR) family have been shown to be essential components of the host innate immune system by playing a

role in the recognition of pathogens and initiation of the immune response. TLR4 recognizes *Chlamydial* lipopolysaccharide (LPS) via its coreceptor cluster of differentiation (CD) 14 (18, 19). CD14 exists in a membrane-bound type (mCD14) and a soluble type (sCD14). Both mCD14 and sCD14 are able to form a complex with LPS and the LPS-binding protein (LBP). Combined with TLR4, this complex induces the nuclear factor κ B (NFκB) signal transduction cascade. Its end product NFκB initiates the innate immune response by binding to specific DNA sequences in the nucleus, thereby enhancing the production of proinflammatory cytokines and acute phase proteins.

To investigate the role of specific genes and the proteins they encode, such as PRRs, in the susceptibility, course and outcome of diseases, different strategies can be employed. Two well-defined methods are the knockout (KO) mouse model and the human candidate gene approach.

The KO mouse model offers the opportunity to remove entire genes of interest from the genome, in order to compare the course and outcome (e.g., duration, amount of shedding, upper genital tract progression) of *C. trachomatis* infection and reinfection between KO mice and control mice, which possess the gene of interest. Previously, we developed a murine model using a human genital isolate of *C. trachomatis* in interferon (IFN)-γ and IFN-γ receptor KO mice, in which we were able to demonstrate a major role of IFN-γ in controlling *C. trachomatis* infection (20).

The human candidate gene approach, which is based on determining the frequencies of functional single nucleotide polymorphisms (SNPs) within phenotypically defined groups, can be used to investigate the relevance of genes in the susceptibility, course and outcome of diseases. SNPs in genes encoding for PRRs may influence receptor function, thereby leading to an aberrant immune response and an increased risk of adverse outcome of the disease. The *TLR4* +896 A>G SNP has been associated with hyporesponsiveness to LPS (21). In a previous study, we did not observe an association between the *TLR4* +896 A>G SNP and tubal factor subfertility, but the cohort was relatively small (22).

For the present study, the first objective was to assess the role of TLR4 in C3H/HeJ (*TLR4* deficient) and C3H/HeN (*TLR4* functional) mice. Secondly, we enlarged the cohort of subfertile women that was previously described (22) and added a cohort of STD patients with and

without *C. trachomatis* infections to investigate the role of *TLR4* +896 A>G SNP in susceptibility to *C. trachomatis* infections and in the development of *C. trachomatis*-associated tubal factor subfertility.

MATERIALS AND METHODS

Murine model

Mice

Seven-week-old female C3H/HeJ (*TLR4* deficient) and C3H/HeN (*TLR4* functional) mice were purchased from Jackson Laboratories (USA) and allowed to acclimate for 2 weeks prior to use. Mice were maintained in accordance with American Association of Accreditation of Laboratory Animal Care guidelines, and were provided food and water ad libitum in an environmentally controlled BL-2 containment room with a 12-hour light/dark cycle.

C. trachomatis

A type pure and *Mycoplasma* free strain of *C. trachomatis* serovar D was propagated, titrated and isolated in cycloheximide-treated McCoy cell monolayers using standard techniques. Density gradient concentrated stock cultures were suspended in transport media and frozen at −70 °C until used.

Genital tract infection

In order to induce prolonged diestrous and thus enhance the infection rate, progesterone in the form of medroxy-progesterone acetate (Depo-Provera®, Pharmacia & Upjohn Co., USA) was administered subcutaneously in 2.5 mg doses, 10 and 3 days prior to infection. The mice were inoculated intravaginally with 10 μL of a *C. trachomatis* elementary body suspension containing 1×10^5 inclusion forming units. Control mice for the reinfection arm received 10 μL of sterile transport media and were treated in every way similar to infected mice. They served as control mice for the reinfection arm of the experiment. All mice were progesterone-treated and infected with *C. trachomatis* in a similar manner 8 weeks later. All procedures were performed under protocols approved by the City of Hope National Medical Center and Beckman Research Institute Research Animal Care Committee.

Assessment of infection

The presence of *C. trachomatis* in the lower genital tract was determined by culturing material obtained by swab-

bing the vaginal vault and ectocervix every 2–7 days with a Dacron tipped urethral swab that was stored in transport media at −70 °C until tested. Specimens were plated onto McCoy cell monolayers in duplicate 96-well plates, centrifuged and incubated at 37 °C for 72 h. One plate was fixed, stained with iodine and enumerated for iodine straining inclusions, while the other plate was stored at −70 °C and used to verify the status of primary culture negative specimens. An animal was considered productively infected if culture was positive on at least 1 day postinfection, and a specimen was considered positive if inclusions were observed in either the primary or secondary cultures. At the end of the experiment, all mice were visually examined for hydrosalpinx, or other gross upper genital tract pathology.

Human study population

STD cohort

Women of Dutch Caucasian origin (N = 614), under the age of 33 (ranging 14-33 years), visiting the STD outpatient clinic in Amsterdam, the Netherlands, in the period of July 2001 to December 2004 participated in this cohort. 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 *C. trachomatis* DNA by polymerase chain reaction (PCR) (23). Peripheral venous blood was collected for the analysis of IgG antibodies against *C. trachomatis*. A titer of ≥ 1:50 was considered positive. Samples with gray-zone values, e.g., cutoff 10% or more, were repeated and considered positive when the result was positive, or again within the gray zone.

Subfertile women

The human study was performed in women who visited the Academic Hospital Maastricht between December 1990 and November 2000 because of subfertility. In all patients blood was drawn at their initial visit for a *Chlamydia* immunoglobulin (Ig) G antibody test (CAT). All spare sera were cryopreserved. Only patients who had undergone a laparoscopy and tubal testing as part of their fertility work-up were included in the present study. Since the prevalence of SNPs may depend on ethnic background, only Dutch Caucasian women were included. Patients who had undergone previous pelvic

surgery (except for an uneventful appendectomy or Caesarean section) were excluded.

Two independent investigators, who were unaware of the CAT results, scored 259 successive laparoscopy reports to assess the presence of tubal pathology. Tubal pathology was defined as extensive periadnexal adhesions and/or distal occlusion of at least one tube (24). In case of disagreement, consensus was reached by consultation. Of the 259 women who underwent a laparoscopy, only women who had tubal pathology (according to the above mentioned definition) or had no tubal pathology (no periadnexal adhesions and patent tubes) were included in the present study. Women who had minor or non-*C. trachomatis*-related abnormalities (any periadnexal adhesions and/or proximal occlusion of at least one tube) were excluded.

In the Netherlands, for retrospective analysis of anonymized patient data and stored sera, no ethics committee approval is required. In the fertility clinic of the Academic Hospital Maastricht, all couples are informed at intake about possible use of their anonymized data and stored sera for research purposes, and a "no objection procedure" is followed. Only patients who did not object participated in the present study.

C. trachomatis *IgG antibody testing*

IgG antibodies to *C. trachomatis* were detected using the species-specific *Chlamydia pneumoniae* IgG microimmunofluorescence (MIF) test (AniLabsystems, Finland), as described previously (25). This species-specific test is able to detect IgG antibodies to both *C. pneumoniae* and *C. trachomatis* (using an antigen derived from a *C. trachomatis* lymphogranuloma venereum [LGV] strain, serovar L2). We previously studied the test performances of five commercially available *C. trachomatis* IgG tests, including the *C. trachomatis* IgG spot in the *C. pneumoniae* MIF (AniLabsystems) (26). In our hands, the *C. trachomatis* IgG titer obtained by the *C. pneumoniae* MIF (AniLabsystems, Finland) had the best predictive value for tubal factor subfertility (26). Therefore, we used this test in the present study. The cut-off titer used for a positive test was 32.

Immunogenetic analysis

For the immunogenetic analyses, genomic DNA was extracted from the cryopreserved serum samples using either the MagNaPure LC isolator according to the manufacturers' instructions (Roche Molecular Biochemicals,

Germany) or the High Pure PCR Template Preparation (HPPTP) Kit according to the manufacturers' instructions (Roche Molecular Biochemicals, Germany). Both techniques provide enough DNA for reproducible genetic analyses.

TLR4 +896 A>G gene polymorphism

Genotyping of the *TLR4* +896 A>G SNP (rs4986790) was performed with forward primer 5'-TTT ACC CTT TCA ATA GTC ACA CTC A-3' and reverse primer 5'-AGC ATA CTT AGA CTA CCT CCA TG-3'. PCR for restriction fragment length polymorphism (RFLP) analyses was performed on a thermal cycler GeneAmp 9700 (Perkin-Elmer Cetus, USA). PCR conditions were as follows: initial denaturation at 94 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s and extension at 72 °C for 30 s. The cycling was followed by a final extension step at 72 °C for 5 min, followed by cooling to 4 °C. The 102 bp amplicons were digested overnight at 37 °C with NcoI (New England Biolabs, UK) resulting in amplicons that either were cut in two fragments of 80 bp and 22 bp (G allele) or were not restricted (A allele). These fragments were analyzed by electrophoresis on 4% low melting agarose gels (Tebu-Bio, the Netherlands) stained with ethidium bromide.

Control group for background genotyping

For ethnic-matched background genotyping, genomic DNA was extracted from whole blood of 166 healthy Dutch Caucasian employees of the VU University Medical Center. They gave written informed consent for use of their anonymized sera to serve as control sera for genetic research purposes.

Statistical analyses

The genotype distribution was tested for Hardy-Weinberg equilibrium to assess Mendelian inheritance. Fisher's exact or chi-square tests were used to compare the genotypes between *C. trachomatis* IgG-positive and IgG-negative subfertile women with and without tubal pathology and the healthy control group. P < 0.05 was considered statistically significant.

RESULTS

Murine model

The median duration of infection between the previously noninfected control groups of each strain were similar

Table I. *Duration of genital tract infection and culture results in C3H/HeJ (TLR4 deficient) and C3H/HeN (TLR4 functional) mice follow-ing* C. trachomatis *infection or mock-infection (at t = 0) and* C. trachomatis *infection (at t = 56).*

	Time		t = 0			t = 56		t = 58	t = 60	t = 63	t = 66	t = 70
	Mouse	Infection	Infection duration	Median duration	Infection	Infection duration	Median duration	Culture results on indicated day (inclusion forming units)				
C3H/HeJ	C1	CT	28	26	CT	7	7	6,300	1,850	360	0	0
(*TLR4*	C2	CT	24	26	CT	7		26,870	1,190	400	0	0
deficient)	C3	CT	35	26	CT	4		17,580	10	0	0	0
	C4	CT	24	26	CT	21		3,180	150	680	660	250
	J1	Mock	-	-	CT	28	31.5	33,900	5,370	12,900	1,680	1,390
	J2	Mock	-		CT	14		13,000	8,900	4,220	70	280
	J5	Mock	-		CT	35		28,810	14,040	21,150	8,200	2,950
	J6	Mock	-		CT	42		24,230	5,600	23,400	4,160	1,670
C3H/HeN	B1	CT	17	28	CT	4	4	68,120	2,770	0	0	0
(*TLR4*	B2	CT	35	28	CT	4		35,010	20	0	0	0
functional)	B3	CT	21	28	CT	4		6,240	+	0	0	0
	B25	CT	35	28	CT	4		33,450	100	0	0	0
	I1	Mock	-	-	CT	24	26	72,800	4,970	8,720	830	1,120
	I2	Mock	-		CT	24		23,400	13,520	10,460	120	110
	I4	Mock	-		CT	28		18,510	30,160	48,360	2,310	290
	I5	Mock	-		CT	35		22,360	67,600	10,920	3,350	530

CT, *C. trachomatis* serovar D; Mock, infection with sterile transport media; *TLR4,* Toll-like receptor 4.

(Table I). This was similar to what was observed during the initial infection arm of the experiment. In the first week the amount of chlamydiae shedding in the controls groups was slightly higher in the wild-type mice as com-pared to the *TLR4*-deficient mice. Upon reinfection, a significant level of protection was observed in both mouse strains when compared to the initial infection in the appropriate age- and conditions-matched control group: C3H/HeJ (*TLR4* deficient), 31.5 vs. 7 days; and C3H/HeN (wild-type), 26 days vs. 4 days. Remarkably, none of the wild-type mice was infected 4 days after rein-fection, while 75% of the *TLR4*-deficient mice were still *C. trachomatis* positive after 1 week, and one mouse during the complete follow-up period of 2 weeks.

However and although small, there is a suggestion that the protection was more solid in C3H/HeN compared to the *TLR4*-deficient C3H/HeJ mice. Finally, at the conclu-sion of the experiment there was no hydrosalpinx or other gross upper tract pathology observed in any mice of either strain.

Human candidate gene approach

Of the 259 women who underwent laparoscopy, 227 participated in the present study. Of these, 43 (19%) had tubal pathology and 184 (81%) did not. Thirty-two women had minor or non-*C. trachomatis*-related abnor-malities and were excluded. *C. trachomatis* IgG antibod-ies were present in 39 women, of whom 26 (67%) had tubal pathology and 13 (33%) did not have tubal pathol-ogy. *C. trachomatis* IgG antibodies were absent in 188 women, of whom 17 (9%) had tubal pathology and 171 (91%) did not have tubal pathology.

Susceptibility of infection

The genotype distribution for the STD cohort in shown in Table II and was in Hardy-Weinberg equilibrium showing Mendelian inheritance. The women who were positive versus those who were negative for *C. trachomatis* DNA had an equal genotype distribution as did the more extreme subgroup comparison *C. trachomatis* DNA and *C. trachomatis* serology-positive women versus the

C. trachomatis DNA and *C. trachomatis* serology-negative women (Table II).

Severity of infection

The genotype distribution was in Hardy-Weinberg equilibrium in the subfertile women and the ethnic-matched control group. The overall genotype distribution in the cohort of subfertile women was 88% AA, 12% AG and 0% GG. The genotype distribution in subfertile women with tubal pathology (86% AA, 14% AG, 0% GG) did not differ from the distribution in subfertile women without tubal pathology (89% AA, 11% AG, 0% GG). Introduction of *C. trachomatis* IgG serology, with special attention to *C. trachomatis* IgG-positive subfertile women with and without tubal pathology, did alter the observed genotype distribution, and a clear trend (OR: 2.9) was noticed towards a higher frequency of *TLR4* +896 A>G SNP carriage in *C. trachomatis* IgG-positive subfertile women with tubal pathology (19% *G) as compared to those without tubal pathology (8% *G). The results are summarized in Table III.

DISCUSSION

The current study used an integrated approach to study the role of *TLR*4 in the susceptibility to and severity of infection based on both the murine model and human candidate gene approaches in women. The results of the KO mouse model indicate an important level of protection against *C. trachomatis* reinfection, as reflected by a faster clearance, as compared to mice that were infected only once. Our data suggest that the protection is more solid in *TLR4* functional mice as compared to the *TLR4*-deficient mice. *C. trachomatis* IgG-positive subfertile women with tubal pathology were more than twice as likely to be carriers of the mutant *TLR4* +896 G allele compared with those without tubal pathology. This difference, however, was not statistically significant due to sample size limitations.

Although the *TLR4*-deficient mice had clear differences in the level of shedding and duration of infection after reinfection, the role was minimal and as anticipated. It has previously been shown that TLR2, but not TLR4, is more essential for recognition of a different *Chlamydia* species (*C. pneumoniae*) (27), LPS-signaling (28) and the development of murine tubal pathology (29) respectively. Our results are consistent with these findings, indicating that *TLR4* does play only a modest role in the recognition of *C. trachomatis* and that signaling through *TLR2* may be a more prominent element in the immune response. Indeed Karimi et al. recently showed that specific *TLR2*

Table II. TLR4 *genotype distribution in female STD patients.*

TLR4 +896 A>G	N	1.1 (AA)		1.2 (AG)		2.2 (GG)	
		n	%	n	%	n	%
Total STD patients	614	530	86.3	83	13.5	1	0.2
DNA+	193	169	87.6	24	12.4	0	0.0
DNA+, IgG+	140	121	86.4	19	13.6	0	0.0
DNA−	421	361	85.7	59	14.0	1	0.2
DNA−, IgG−	331	283	85.5	47	14.2	1	0.3

Table III. TLR4 *genotype distribution in women with subfertility.*

TLR4 +896 A>G	N	1.1 (AA)		1.2 (AG)		2.2 (GG)	
		n	%	n	%	n	%
All subfertile women	227	200	88.1	27	11.9	0	0.0
TP+	43	37	86.0	6	14.0	0	0.0
TP−	184	163	88.6	21	11.4	0	0.0
CT + TP+	26	21	80.8	5	19.2	0	0.0
CT + TP−	13	12	92.3	1	7.7	0	0.0
Control group	166	148	89.2	17	10.2	1	0.6

TP, tubal pathology; CT, *C. trachomatis* IgG; Control group: ethnically matched healthy employees of the VU University Medical Center, Amsterdam, the Netherlands

haplotypes were associated with the development of tubal pathology and the severity of infection (30).

The current lack of a strong association between the *TLR4* SNP studied and *C. trachomatis*-associated tubal factor subfertility may be explained by several other factors besides the suggested prominent role of TLR2, rather than TLR4, in the immune response to *C. trachomatis*. First, a recent study has shown that only the rare homozygous carriers of the *TLR4* +897 A>G SNP are less responsive to LPS, whereas heterozygous carriage does not affect LPS responsiveness (31). Our study cohort consisted of women with a normal *TLR4* genotype or heterozygous *TLR4* +897 A>G SNP carriers, whereas no patients were homozygous SNP carriers. However, LPS is not the only ligand for TLR4 and both human heat shock protein 60 (hHSP60) and *Chlamydial* HSP60 (cHSP80) are also ligands for TLR4. Secondly, the recognition of *C. trachomatis* involves a complex system of multiple PRRs. Partial, or complete loss of receptor function may be compensated by other receptors, preserving an adequate immune response via other pathways. Deregulation of the immune response, leading to a more

severe course of *C. trachomatis* infection, may only occur when different pathways are affected concomitantly, i.e., when multiple SNPs in multiple PRR genes (in a so-called carrier trait) are present. This hypothesis has already been tested for several diseases (12, 32-35), and a preliminary study has shown promising results for *Chlamydia*-associated tubal pathology (36).

In the current study, the most prominent effect was seen between *C. trachomatis*-positive women with and without serologically confirmed *C. trachomatis* infection. We added cHSP60 serology to the assessment (data not shown) since TLR4 also responds to cHSP60, and increased expression of cHSP60 has been reported in infertile women (37). Interestingly, all women positive for *C. trachomatis* IgG and cHSP60 IgG, and with the mutant G allele in *TLR4*, had tubal pathology. This potentially interesting phenomenon will be further explored in larger cohorts, and by adding SNPs in other TLRs and genes involved in the recognition and inflammatory processes upon infection. This is of importance since not all women with tubal pathology are positive for *C. trachomatis* IgG and cHSP60 IgG and yet have the G allele in TLR4.

CONCLUSIONS

TLR4 functional mice seem to be more protected against *C. trachomatis* reinfection as compared to the *TLR4*-deficient mice. In humans, the single *TLR4* +896 A>G SNP does not play a major role in susceptibility as shown in women with uncomplicated *C. trachomatis* infection. In women with serologic responses to *C. trachomatis* and carrying the *TLR4* SNP, the risk of developing tubal pathology was higher but did not reach statistical significance. Since other receptors may compensate for the loss of function in patients carrying the *TLR4* SNP, further studies are necessary to determine if carrying multiple SNPs in multiple PRR genes and having serologic responses to cHSP60 has a more profound impact on the development of tubal pathology following *C. trachomatis* infection as compared to the risk associated with carrying a single SNP in a single gene.

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DISCLOSURE

The authors have nothing to disclose.

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