Drugs of Today 2009, 45(Suppl. B): 83-93

CCC: 1699-3993/2009

TLR9 KO MICE, HAPLOTYPES AND CPG INDICES IN *CHLAMYDIA TRACHOMATIS* INFECTION

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SUMMARY

Previous studies have investigated the role of Toll-like receptor (TLR)2 and TLR4 in susceptibility to and severity of Chlamydia trachomatis *infections. In this study we employ a unique integrated approach to study the role of the intracellular CpG DNA receptor: we use a murine knockout (KO) model to assess TLR9 relevance, study human TLR9 genotypes and haplotypes in sexually transmitted disease (STD) patients and subfertile women with or without tubal pathology and use in silico TLR9 CpG index calculations to assess potential immunostimulatory properties of the* Chlamydia *bacterium. Although no significant differences in the course of initial infections were observed between KO mice and wild-type mice the* TLR9 *KO mice showed a significant level of protection upon reinfection (P = 0.02). We did not observe significant differences in genotype frequencies between* C. trachomatis*-positive and* C. trachomatis*-negative women (STD patients). However, haplotype analyses revealed a trend between* C. trachomatis*-positive and* C. trachomatis*-negative women in the carriage of haplotype IV (P = 0.061; OR: 2.6; 95% CI: 1.0–6.8). In women with subfertility, odds ratios between 2 and 3 were found for tubal pathology risk, but they did not reach significance due to cohort size limitations. Finally, CpG sequence analysis showed mildly immunostimulatory properties for the genomic sequences of* Chlamydia *serovars B and D. Based on the murine model, human immunogenetic studies and in silico CpG index analyses, TLR9 seems to play a modest role in* C. trachomatis *infections. Extension of the human cohorts is necessary to significantly prove the effect in humans.*

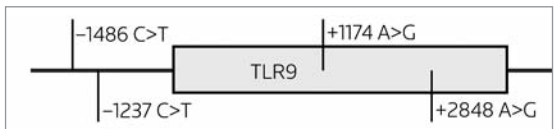
INTRODUCTION

Chlamydia trachomatis is the most common sexually transmitted bacterium around the world. Most infections will run an asymptomatic and uneventful course, although some may develop into chronic infections with the risk of late complications. These late complications include pelvic inflammatory disease, ectopic pregnancy and tubal pathology. The reasons why some women do develop late complications, while others clear the infection asymptomatically, remain largely unknown. Studies into bacterial factors have not been able to explain all observed differences in the clinical course of infection. It is known that the host immune system plays an important role in the course of infections and it has been shown that host genetic variation within the immune system can influence the susceptibility to and severity of diseases (1-3). It is therefore conceivable that host genetic variation may influence the susceptibility to and severity of *Chlamydia* infections.

The Toll-like receptor (TLR) family is a family of pathogen recognition receptors, able to recognize a variety of microbial compounds and, upon recognition, to initiate an inflammatory response against the invading microorganism. TLR9 is an intracellular receptor of CpG motifs, which are short sequences of unmethylated DNA, predominantly present in bacterial DNA. These motifs have immunostimulatory activity by inducing dendritic cell maturation, B-cell proliferation and production of cytokines (4-9).

TLR9 and its polymorphisms have been associated with a variety of diseases which include systemic lupus erythematosus (10), pneumococcal infection (11), pouchitis (12), malaria (13, 14), HIV (15) and asthma (16). The *TLR9* –1237 T>C polymorphism (rs5743836) has been associated with asthma (16) and pouchitis (12). Together with the *TLR9* +2848 G>A polymorphism (rs352140), this polymorphism is part of a four single nucleotide polymorphism (SNP) haplotype. Lazarus et al. found seven out of 16 theoretically possible haplotype combinations, and the –1237 and +2848 polymorphisms allow distinction between the four most common haplotypes (Fig. 1)(16).

We have previously studied these polymorphisms in relation to the risk of developing tubal pathology in sub-



Haplotype	–1486 C>T	–1237 C>T	+1174 A>G	+2848 A>G
I	T	T	A	G
II	C	T	G	A
III	T	C	G	A
IV	Other combinations			

Figure 1. *TLR9* SNP positions and haplotype as described by Lazarus et al. (16). The –1237 and +2848 SNP allow distinction between the four most common haplotypes. Haplotype IV consists of: TTGG, CTGG, CCGG, and TTGA (together 8% of the total cohort of Lazarus et al. [16]).

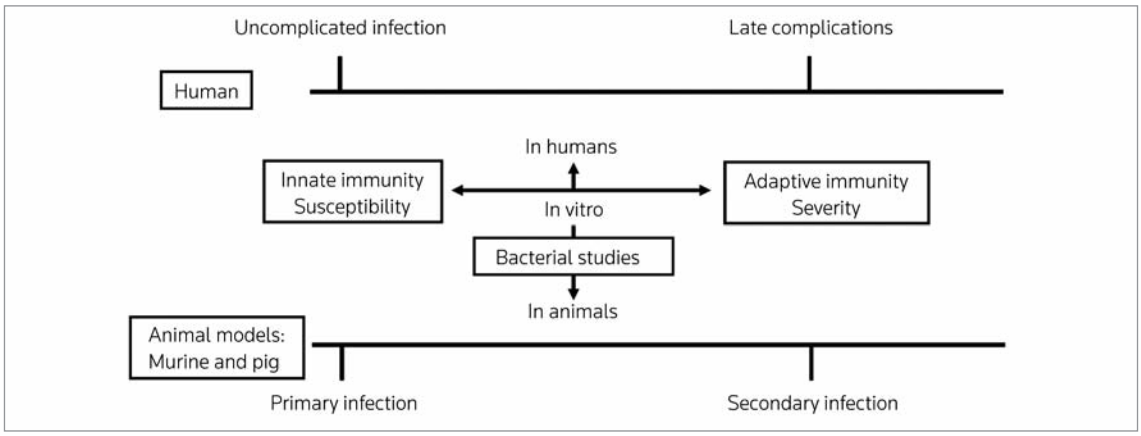


Figure 2. Integrated approach to study *C. trachomatis* infections, which promotes a synergism between epidemiology, immunogenetics, functional biology and clinical studies. Primary infection in the mouse may yield information about the role of innate immunity and the susceptibility to infection, which may translate to uncomplicated infections in humans (STD cohort study). The secondary infection (or reinfection) in mice helps identify relevant genes in the adaptive immunity or severity of infection, which may provide clues about chronic infection of late complications in humans (subfertility/tubal pathology cohort study).

fertile women and identified a trend towards increased risk to tubal pathology (17). Therefore, we are extending that study with a murine model and a cohort of women visiting a sexually transmitted disease (STD) outpatient clinic, to assess to effect of *TLR9* polymorphisms on the susceptibility to and severity of *C. trachomatis* infections. To assess this effect, we employed a translational model (Fig. 2) (18). Studies of *Chlamydia* infections in mice, which show similarities to human *Chlamydia* infections, may indicate potential genes of interest. Primary infections in knockout (KO) mice may help identify relevant genes for uncomplicated *Chlamydia* infection in humans, while the secondary infection in the mouse model may be translated to chronic infections and/or late complications observed in humans. Combining data generated from the mouse model and from studies in humans may thus provide extra information on *Chlamydia* pathogenesis.

This study explores the contribution of TLR9 to *Chlamydia* pathogenesis using three approaches: i) we use a murine KO model to assess TLR9 relevance; ii) we use immunogenetic approaches to study TLR9 genotypes and haplotypes in STD patients and women with and without tubal pathology; and iii) we use in silico *TLR9* CpG index calculations to assess potential immunostimulatory properties for the genomic sequences of *C. trachomatis*.

MATERIALS AND METHODS

Murine model

Mice

Permission to use C57BL/6 TLR9$^{-/-}$ was granted by Shizuo Akira, MD, PhD (Osaka University, Osaka, Japan) and mice were generously provided by Edouard Cantin, PhD (City of Hope National Medical Center and the Beckman Research Institute, Duarte, California, USA), from a colony maintained at the Animal Resource Center of the City of Hope National Medical Center and Beckman Research Institute. Aged-matched control C57BL/6 mice were purchased from Jackson Laboratories (Bar Harbor, Maine, 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.

Chlamydia trachomatis

A type-pure and mycoplasma-free strain of *C. trachomatis* serovar D (19, 20) was propagated, titrated and isolated in cycloheximide-treated McCoy cell monolayers using standard techniques (21). Density gradient concentrated stock cultures were suspended in transport media and frozen at –70 °C until used.

Genital tract infection

In order to induce a prolonged diestrous and thus enhance the infection rate, progesterone in the form of medroxyprogesterone acetate (Depo-Provera®, Pharmacia & Upjohn Co.) was administered subcutaneously in 2.5 mg doses, 10 and 3 days prior to infection (19, 22). Mice were inoculated intravaginally with 10 µL of an elementary body suspension containing 1 x 10^5 inclusion forming units (IFU). Control mice for the reinfection arm of the experiment received 10 µL of sterile transport media and were treated in every way similar to infected mice during the initial infection phase. All groups of mice were progesterone treated and infected 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 *Chlamydia* in the lower genital tract was determined by culturing material obtained by swabbing 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-positive on at least 1 day post-infection, and a specimen was considered positive if inclusions were observed in either the primary or secondary cultures.

STD cohort

Women of Dutch Caucasian (DC) origin, under the age of 33 years (range 14–33 years; median 22 years) and visiting the STD outpatient clinic in Amsterdam, the Netherlands, were included in this study (collection period: July 2001–December 2004), as described previously (23). The women were asked to sign an informed consent and to 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 (CT-DNA) by polymerase chain reaction (PCR; Cobas Amplicor; Hoffman–La Roche, Basel, Switzerland)(24). Peripheral venous blood was collected for the analysis of immunoglobulin (Ig)G antibodies against *C. trachomatis* (Medac Diagnostika GmbH, Hamburg, Germany). A titer of ≥ 1:50 was considered positive. Samples with gray zone values (e.g., cut-off 10% or more) were repeated and considered positive when the result was positive or again within the gray zone. A total of 696 women were included in this study.

Subfertility/tubal pathology cohort

The study was performed in women who visited the Academic Hospital Maastricht between December 1990 and November 2000 because of subfertility, as described previously (17). In all patients, blood was drawn at their initial visit for a *Chlamydia* IgG antibody test (CAT). Only patients who had undergone a laparoscopy and tubal testing as part of their fertility work-up were included in the present study. Patients who had undergone previous pelvic surgery (except for an uneventful appendectomy or Caesarean section) were excluded. The grade of tubal pathology was assessed in 259 successive laparoscopy reports by two blinded investigators (i.e., they were not made aware of the CAT analysis results). Tubal pathology was defined as extensive periadnexal adhesions and/or distal occlusion of at least one tube (25). In case of disagreement, consensus was reached by consultation. Of the 259 women who underwent a laparoscopy, 43 (17%) had tubal pathology (according to the above mentioned definition) and 184 (71%) had no tubal pathology (no periadnexal adhesions and patent tubes), and these 227 women participated in the present study. Thirty-two women (12%) had minor or non-*C. trachomatis*-related abnormalities (any periadnexal adhesions and/or proximal occlusion of at least one tube) and were excluded. IgG antibodies to *C. trachomatis* were detected using the species-specific *Chlamydia pneumoniae* IgG micro-immunofluorescence (MIF) test (AniLabsystems, Finland), as described previously (26).

Healthy controls

A healthy Dutch Caucasian control group (n = 150) was included to assess the general frequency of the *TLR9* genotypes in the Dutch Caucasian population.

Immunogenetic analyses

Since the prevalence of SNPs may depend on ethnic background, only Dutch Caucasian women were included in the present study. The *TLR9* –1237 T>C

(rs5743836) and *TLR9* +2848 G>A (rs352140) SNPs were analyzed using TaqMan analyses, as described previously (12).

CpG sequence analysis

To determine the relative prevalence of canonical stimulatory and inhibitory CpG motifs in the genomic sequences of the various bacteria, we employed a two-step approach. Genbank-retrieved FASTA formatted text files were uploaded to http://insilico.ehu.es and the frequency of all 4,096 possible hexamers were determined as described by Bikandi et al. (27). This information, and the general composition of each genome, were then entered into Microsoft Excel, and common-place formulas were used to count the occurrence of specific CpG motifs. As a measure of each genome's potential for TLR9 stimulation, the CpG index was calculated by comparing the frequency of stimulatory and inhibitory CpG motifs, as previously described (9).

Statistical analyses

Genotype distributions were tested for Hardy Weinberg Equilibrium to test for Mendelian inheritance. Fisher's Exact and chi-square tests were used, where appropriate, to assess differences in genotype distributions between different (sub)groups. $P < 0.05$ was considered statistically significant. *TLR9* haplotypes were inferred using PHASE v2.1.1 (28, 29) and SNPHAP (30).

RESULTS

Murine model

No differences were observed in either the level of shedding during the acute phase of infection or the median duration of infection between the control groups of each strain. This was similar to what was observed during the initial infection arm of the experiment. Upon reinfection, a significant level of protection was observed in the *TLR9*-deficient mice when compared with the initial infection in the age- and conditions-matched control group ($P = 0.02$), while only two mice in the genetically intact C57BL/6 group appeared protected, with one of the remaining two animals having an initial infection of only 2 days. The results are shown in Table I. Finally, at the conclusion of the experiment there was no hydrosalpinx or other gross upper genital tract pathology observed in any mice of either strain.

Immunogenetic analyses (single gene analyses)

STD cohort

Genotype distributions were in Hardy Weinberg equilibrium. No significant differences were observed in genotype frequencies, either in the total group or in the subgroup analyses. A more stringent definition of *C. trachomatis* positivity (presence of *C. trachomatis* DNA combined with a positive *C. trachomatis* IgG serology) did not alter the findings (Table II).

Subfertility/tubal pathology cohort

Overall, genotype distributions were comparable between cases and controls, and did not differ significantly. No statistical differences were observed between either the *TLR9* –1237 T>C or the *TLR9* +2848 G>A SNPs, and the risk of tubal pathology (Table III).

Immunogenetic analyses (haplotype analyses)

STD cohort

No statistically significant differences were observed between cases and controls, and/or between the subgroups (Table IV). Trend analyses, however, did reveal a trend between *C. trachomatis* DNA- and IgG-positive, and *C. trachomatis* DNA- and IgG-negative women in the carriage of haplotype IV (2.8% vs. 1.1%; $P = 0.061$; OR: 2.6; 95%CI: 1.0–6.8; Table IV).

Subfertility/tubal pathology cohort

The *TLR9* haplotype frequency distribution in the women who developed tubal pathology was I: 37.2%, II: 46.5%, III: 16.3% and IV: 0.0% (Table V). This distribution was comparable to the haplotype frequency distribution that was observed in the subfertile women who did not develop tubal pathology.

The *TLR9* haplotype frequency distribution in the women who developed tubal pathology after a *C. trachomatis* infection, compared with those who did not, was remarkable (21.2 vs. 11.5%, respectively; OR: 2.1); however this did not reach statistical significance (Table V).

Trend analyses

Trend analyses show a decreased carriage of haplotype IV in *C. trachomatis*-positive women without symptoms, with symptoms, and those who developed tubal pathology (*C. trachomatis*-positive asymptomatic [3.7%] > *C.*

Table I. *Primary culture and duration of infection results from TLR9 knockout and C57BL/6 female mice following genital tract infection with* C. trachomatis *serovar D.*

			Primary culture results on indicated day post-infection[2]					Infection duration (initial)[3]	Median duration of infection
Strain	Group[1]	Animal	2	4	7	10	14		
TLR9	Reinfected	E2	5,370	70	40	0	0	7 (10)	4.5
		E5	15,910	5,490	220	0	0	7 (14)	
		E16	14,460	0	0	0	0	2 (21)	
		E0	6,880	0	0	0	0	2 (10)	
	Control	K1	3,060	2,600	2,390	2,600	220	14	15.5
		K3	4,280	2,540	11,210	1,490	0	10	
		K15	6,240	4,450	5,780	0	10	17	
		K25	20,070	2,140	13,690	40	0	28	
C57BL/6	Reinfected	A1	20,180	520	180	130	220	21 (14)	12.5
		A2	3,580	+	0	0	0	4 (35)	
		A3	2,370	0	0	0	0	2 (17)	
		A24	2,310	1,910	6,930	2,830	110	28 (2)	
	Control	G2	6,070	2,020	1,020	0	0	7	12.0
		G3	1,960	2,890	490	0	60	14	
		G4	7,800	910	4,800	10	460	28	
		G5	8,260	4,330	11,440	30	0	10	

[1]Reinfected mice were initially infected 8 weeks prior to reinfection and control mice were age- and conditions-matched mice that were maintained and handled in every way similar to reinfected mice during the initial infection arm of the experiment; [2]Mice were sampled every 2–7 days postinfection with numerical values representing the number of inclusion forming units enumerated in primary culture and + representing a positive secondary culture of primary culture-positive negative specimens; [3]Duration of infection was assigned as the day on which the last culture-positive specimen was collected following infection, with the number in parenthesis being the initial infection duration of reinfected mice.

Table II. *TLR9 single nucleotide polymorphism (SNP) distribution in the STD and control cohorts.*

		–1237 T>C			+2848 G>A		
	n	TT (%)	TC (%)	CC (%)	GG (%)	GA (%)	AA (%)
Total	696	73.0	24.3	2.7	23.0	48.6	28.4
CT+	232	73.3	23.3	3.4	23.7	47.8	28.4
CT–	464	72.8	24.8	2.4	22.6	48.9	28.4
CT+ IgG+	160	76.3	21.3	2.5	25.0	48.1	26.9
CT– IgG–	363	70.8	27.0	2.2	22.0	49.3	28.7
Controls	150	71.3	28.7	0.0	16.0	48.0	36.0

Genotype frequencies of the *TLR9* –1237 T>C and +2848 G>A SNPs in the STD cohort, subdivided into *C. trachomatis* DNA positivity (CT+/–) and *C. trachomatis* serology (IgG+/–).

Table III. *TLR9 single nucleotide polymorphism (SNP) distribution in the subfertility cohort.*

			−1237 T>C			+2848 G>A	
	n	TT (%)	TC (%)	CC (%)	GG (%)	GA (%)	AA (%)
Total	227	68.3	27.8	4.0	19.8	45.8	34.4
TP+	43	72.1	23.3	4.7	18.6	37.2	44.2
TP−	184	67.4	28.8	3.8	20.1	47.8	32.1
CT+ TP+	26	61.5	34.6	3.9	11.5	38.5	50.0
CT+ TP−	13	76.9	23.1	0.0	23.1	46.2	30.8
CT− TP+	17	88.2	5.9	5.9	29.4	35.3	35.3
CT− TP−	171	66.7	29.2	4.1	19.9	48.0	32.2

Genotype frequencies of the *TLR9* −1237 T>C and +2848 G>A SNPs in the STD cohort, subdivided into *C. trachomatis* DNA positivity (CT+/−) and *C. trachomatis* serology (IgG+/−).

trachomatis-positive symptomatic [1.8%] > *C. trachomatis*-positive, tubal pathology-positive [0.0%]; P = 0.076, χ^2_{trend}: 3.14).

CpG sequence analyses

CpG stimulatory and inhibitory sequences and the CpG index were calculated for *C. trachomatis* serovars B (ocular infections) and D (urogenital infections) (Table VI). The CpG index of *C. muridarum* was also calculated, and represented in Table VI together with previously published herpes simplex virus (*HSV)* 1/2 CpG indices (9) for comparison.

All *Chlamydia* genomes showed mildly stimulatory indices, although not as strong as the previously published *HSV* CpG indices (7 [serovar B], 6.8 [serovar D] and 6.1 [muridarum] vs. 24.0 [HSV-1] and 25.8 [HSV-2]; Table VI).

DISCUSSION

Data collected from the murine model and the human cohorts indicate that *TLR9* does not strongly influence the susceptibility to acute *Chlamydia* infection. No differences were observed in the course of the primary infection in the mouse model, nor was the frequency of either *TLR9* SNP or any *TLR9* haplotype significantly

Table IV. *TLR9 haplotype distribution in the STD and control cohorts.*

		TLR9 haplotypes[1]			
	2n	I (%) TTAG	II (%) CTGA	III (%) TCGA	IV (%) Other
Total	1,392	45.3	39.8	12.9	1.9
CT+	464	44.8	40.1	12.3	2.8
CT−	928	45.6	39.7	13.3	1.5
CT+ IgG+	320	46.3	40.6	10.3	2.8
CT− IgG−	726	45.6	38.7	14.6	1.1
Controls	300	39.0	46.7	13.3	1.0

[1]Haplotypes as described by Lazarus et al. (16). Haplotypes inferred from the typing of the −1237 and +2848 single nucleotide polymorphisms.

Table V. *TLR9 haplotype distribution in the subfertility cohort.*

		TLR9 haplotypes[1]			
	2n	I (%) TTAG	II (%) CTGA	III (%) TCGA	IV (%) Other
Total	454	42.1	40.1	17.2	0.7
TP+	86	37.2	46.5	16.3	0.0
TP–	368	43.2	38.6	17.4	0.8
CT+ TP+	52	30.8	48.1	21.2	0.0
CT+ TP–	26	46.2	42.3	11.5	0.0
CT– TP+	34	47.1	44.1	8.8	0.0
CT– TP–	342	43.0	38.3	17.8	0.9

[1]Haplotypes as described by Lazarus et al. (16). Haplotypes inferred from the typing of the –1237 and +2848 single nucleotide polymorphisms.

different between groups, although a trend was observed towards increased carriage of haplotype IV in *C. trachomatis* DNA and IgG-positive women compared with *C. trachomatis* DNA and IgG-negative women (P = 0.06; OR: 2.6).

However, the murine model does indicate a role for the adaptive immune system in *Chlamydia* infection, as can be deduced from the protection observed in the *TLR9*-deficient mice upon reinfection (P = 0.02). We were unable to statistically significantly confirm the role of

Table VI. *CpG sequence analyses of different* Chlamydia *species, and another microorganism causing urogenital infections, herpes simplex virus.*

					CpG motif deviation[a]			
		Genome			Total	Consensus motifs		CpG
Bacterium	Associated pathology	Size (Mb)	G+C (%)	CpG/kb[b]	CpG[c]	Stimulatory[d]	Inhibitory[e]	index[f]
C. muridarum	Murine specific	1.07	40.3	81.4	75.5	85.9	76.0	6.1
C. trachomatis serovar B	Ocular infections	1.04	41.3	85.3	79.2	88.7	78.3	7.0
C. trachomatis serovar D		1.04	41.3	85.3	79.2	88.3	78.3	6.8
Herpes simplex virus 1 (9)	Urogenital infections	1.52	68.3	235.4	100.9	104.9	94.8	24.0
Herpes simplex virus 2 (9)		1.55	70.4	262.1	105.8	106.2	96.9	25.8

[a]Consensus stimulatory and inhibitory CpG hexamer motifs are based on published analyses (6, 31). They are used in this table as indicators of the general frequencies of stimulatory and inhibitory CpG hexamer motifs in each genome; [b]Number of CpG hexamer motifs (NNCGNN) occurring in each genome normalized to 100 kb; [c]Total frequency of CpG hexamer motifs (NNCGNN), expected number based on nucleotide composition of the genome; [d]Frequency of consensus stimulatory hexamer motifs (RRCGYY); [e]Frequency of consensus inhibitory hexamer motifs (NCCGNN and NNCGRN); [f]Calculated from frequencies of stimulatory less inhibitory consensus hexamer motifs as an indicator of stimulation versus inhibition multiplied by total CpG number (normalized to 1 kb) and the overall frequency of CpG (NNCGNN) (9).

TLR9 in the tubal pathology study, although a clear trend was observed, especially in *C. trachomatis*-positive women with versus without tubal pathology. Power analyses show that extension of this cohort may result in significant associations.

Trend analyses showed that carriage of haplotype IV decreases in increasing severity, although this did not reach statistical significance (P = 0.076). The combined haplotype results suggest that carriers of haplotype IV are at increased risk of acquiring a *Chlamydia* infection, although they may not progress to late complications. One might hypothesize that carriage of haplotype IV results in a decreased immune response against *Chlamydia* and may result in increased susceptibility, but the reduced immune response may at the same time result in less collateral damage in the fallopian tubes (decreased severity). The decreased immune response theory may be in line with recently published results showing reduced CD4 cell counts in carriers of *TLR9* mutations, suggesting a potential functional impairment of the immune system (32). Carriers of haplotype III might be at increased risk of developing late complication, but further studies are required to significantly prove the difference.

It should be noted that, although the murine model is a good representation of *Chlamydia* infection in humans, it has its limitations. Two major differences between mice and humans may influence the translational value of *TLR9* data obtained in the murine model of human *Chlamydia* infections. First, humans and mice recognize slightly different CpG motifs, although stimulatory indexes for any given motif are around 60–80% between the two species, indicating that oligodeoxynucleotides (ODNs) containing unmethylated CpG motifs may induce different responses in the two species. Second, in mice plasmacytoid dendritic cells (PDCs), myeloid dendritic cells, macrophages and B cells respond to CpG DNA, while in humans only PDCs and B cells respond to CpG DNA (33). Our finding could mean that while the genomes of *Chlamydia* are capable of CpG-induced TLR9 stimulation, differential responses in both the mouse model and our clinical studies depend more on variations in host genomes rather than the pathogen.

It has been shown that the amount of CpG encountered by TLR9 is of influence (34), and that different CpG motifs may elicit different responses (35-37), suggesting that the influence of *TLR9* polymorphisms may be dependent on the type of CpG-ODN encountered by *TLR9* (Fig. 3). Furthermore, Lundberg et al. have shown that the ratio between immunostimulatory and immunoinhibitory sequences influences the effect of CpG DNA stimulation (9). They defined a formula where the positive outcome represents a stimulatory effect, while a negative outcome represents an inhibitory effect, with the greater the difference between the sequences types determining the strength of the effect (9).

We have studied the CpG indices in the genomes of two *Chlamydia* serovars. Both the ocular *C. trachomatis* serovar B and urogenital *C. trachomatis* serovar D show mildly immunostimulatory indices, demonstrating that these specific serovars have the potential to stimulate the immune system. Although the effect is not as strong as that for *HSV1/2*, it does show that taking the specific serovars and their CpG indices into consideration in analyses of *Chlamydia* pathogenesis may be worthwhile. Interestingly, the CpG index of another urogenital pathogen, *Neisseria gonorrhoeae*, shows a very strong inhibitory index, making this an interesting target for study in relation to CpG indices, *TLR9* SNPs/haplotypes, and pathogenesis (unpublished findings; manuscript in preparation). Functional analyses and in vitro stimulation assays may reveal more on the interplay between CpG indices and *TLR9* polymorphisms, and the combined effect on pathogenesis.

Interactions have been described where the presence of *CARD15/NOD2* and *IL23R* polymorphisms may alter NOD2–TLR9 synergism (38, 39), and may thus influence the study of TLR9 in relation to *Chlamydia* pathogenesis. These interaction models involving multiple genetic loci are consistent with the findings in our *TLR9*$^{-/-}$ mice and our clinical analysis, in that the genetic background of the host supersedes the importance of the pathogen genome. Indeed, similar dependence on host genetic composition, as well as sex, has been demonstrated in HSV infection both in the animal model (40-42) and in human clinical trials (43, 44). Finally, a recent publication by Campino et al. reveals an effect of *cis*-variants

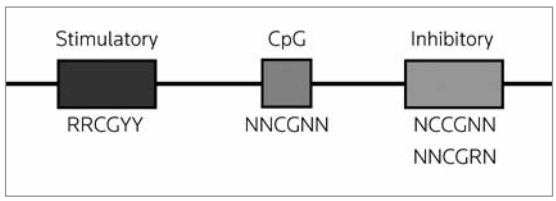


Figure 3. Immunostimulatory and immunoinhibitory sequences, based on Lundberg et al. (9).

acting on *TLR9* gene expression, although the authors were unable to identify the exact polymorphisms that caused the distortion in gene expression (45).

CONCLUSIONS

The data generated in this study show a potential, although limited, role for TLR9 in the pathogenesis of *Chlamydia* infections and especially in the late complications of infection. However, the study needs to be extended and other factors that may influence the role of TLR9, including specific serovars and their respective CpG indices, the presence of *NOD2* and *IL23R* polymorphisms, and *cis*/*trans* regulation of *TLR9*, need to be included to fully understand the role of TLR9 in *Chlamydia* infections.

ACKNOWLEDGMENTS

The mice were kindly provided by the Division of Virology, City of Hope National Medical Center and Beckman Research Institute, Duarte, California, USA (Head: Prof. Edouard M. Cantin). The aim of this work is in line with the European EpiGenChlamydia Consortium which is supported by the European Commission within the Sixth Framework Programme through contract no. LSHG-CT-2007-037637. See www.EpiGenChlamydia.eu for more details about this Consortium.

DISCLOSURE

The authors have nothing to disclose.

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