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Transcervical Inoculation with *Chlamydia trachomatis* Induces Infertility in HLA-DR4 Transgenic and Wild-Type Mice

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ABSTRACT *Chlamydia trachomatis* is the leading cause of infection-induced infertility in women. Attempts to control this epidemic with screening programs and antibiotic therapy have failed. Currently, a vaccine to prevent *C. trachomatis* infections is not available. In order to develop an animal model for evaluating vaccine antigens that can be applied to humans, we used *C. trachomatis* serovar D (strain UW-3/Cx) to induce infertility in mice whose major histocompatibility complex class II antigen was replaced with the human leukocyte antigen DR4 (HLA-DR4). Transcervical inoculation of medroxyprogesterone-treated HLA-DR4 transgenic mice with 5×10^5 *C. trachomatis* D inclusion forming units (IFU) induced a significant reduction in fertility, with a mean number of embryos/mouse of 4.4 ± 1.3 compared to 7.8 ± 0.5 for the uninfected control mice ($P < 0.05$). A similar fertility reduction was elicited in the wild-type (WT) C57BL/6 mice (4.3 ± 1.4 embryos/mouse) compared to the levels of the WT controls (9.1 ± 0.4 embryos/mouse) ($P < 0.05$). Following infection, WT mice mounted more robust humoral and cellular immune responses than HLA-DR4 mice. As determined by vaginal shedding, HLA-DR4 mice were more susceptible to a transcervical *C. trachomatis* D infection than WT mice. To assess if HLA-DR4 transgenic and WT mice could be protected by vaccination, 10^4 IFU of *C. trachomatis* D was delivered intranasally, and mice were challenged transcervically 6 weeks later with 5×10^5 IFU of *C. trachomatis* D. As determined by severity and length of vaginal shedding, WT C57BL/6 and HLA-DR4 mice were significantly protected by vaccination. The advantages and limitations of the HLA-DR4 transgenic mouse model for evaluating human *C. trachomatis* vaccine antigens are discussed.

KEYWORDS *Chlamydia trachomatis*, *Chlamydia* vaccine, HLA-DR4 transgenic, transcervical inoculation, tubal infertility

Chlamydia trachomatis is the most common sexually transmitted bacterial infection (1, 2). If left untreated, *C. trachomatis* can ascend to the endometrium, reach the fallopian tubes, and cause upper genital tract pathology, including tubal factor infertility (3–7). Since *C. trachomatis* infections can be asymptomatic, it may be difficult to determine to whom and when antibiotic treatment should be given (1, 8). Thus, an effective means of reducing or eliminating *C. trachomatis*-induced infertility is vaccination (9–13). Unfortunately, there is still no licensed *C. trachomatis* vaccine for humans.

Efforts to develop a human vaccine against C. *trachomatis* ocular infection were initiated more than 100 years ago (14–19). However, whole-organism-based vaccines induced only short-lived immunity and, in some cases, even exacerbated ocular pathology upon reexposure to *C. trachomatis* (20, 21). The failure of the human vaccine trials stimulated the search for a subunit vaccine to prevent genital tract infection using the *Chlamydia muridarum* mouse model (11, 12). *C. muridarum* infection of the mouse genital tract can induce infertility similar to that observed in women following a

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C. trachomatis infection (22–24). As a result, several protective antigens from *C. muridarum* have been identified (11, 25–28). However, the homologous *C. trachomatis* antigens have not been evaluated due to lack of a convenient model for induction of infertility. It is known that intravaginal inoculation with *C. trachomatis*, a procedure closest to natural infection, fails to cause tubal pathology, partly due to the ability of the innate immune response in the lower genital tract of the mouse to rapidly eliminate human chlamydial organisms (29). To address this limitation, Carmichael et al. (30) used an intrabursal inoculation model to compare different *C. trachomatis* serovars for their ability to induce infertility in C3H/HeN mice pretreated with medroxyprogesterone. These authors found that *C. trachomatis* serovar D caused a significant reduction in fertility. The intrabursal inoculation model, however, involves survival surgery and bypasses both the cervical barrier and the endometrial lumen where vaccine-induced immunity may localize. Interestingly, it has been shown that transcervical inoculation with *C. trachomatis*, a procedure that does not involve surgery but still bypasses the cervical barrier, can cause both infection and inflammation in the mouse upper genital tract (31). This inoculation route has recently been used for evaluating the efficacy of an inactivated *C. trachomatis* vaccine to protect the genital tract of mice against infection and inflammatory pathology (32). However, it remains unclear whether transcervical inoculation with *C. trachomatis* serovar D can induce infertility. Addressing this question may allow us to develop a model for evaluating whether a human *C. trachomatis* vaccine can preserve the reproductive function of the mouse genital tract.

In the current study, we tested whether transcervical inoculation with *C. trachomatis* D could induce infertility in mice whose major histocompatibility complex class II (MHC-II) antigen is replaced with the human leukocyte antigen DR4 (HLA-DR4). These mice have already been utilized to identify $CD4^+$ T-cell epitopes in several microorganisms and human tumor-associated antigens and in vaccination studies (28, 33–36). Since both the *C. trachomatis* vaccine antigens and the MHC-II antigen are of human origin, the vaccine antigens identified in this model should be applicable to humans. We found that transcervical inoculation of medroxyprogesterone-treated HLA-DR4 transgenic mice with 5×10^5 inclusion forming units (IFU) of *C. trachomatis* D (strain UW-3/Cx) induced a significant reduction in fertility, and a similar reduction was also elicited in wild-type (WT) C57BL/6 mice. Moreover, the transcervical inoculation-induced infertility correlated with enhanced recovery of *C. trachomatis* D from the mouse genital tract and robust systemic lymphocyte responsiveness but limited levels of serum neutralization antibodies. In addition, intranasal immunization of HLA-DR4 and WT mice with live *C. trachomatis* D elementary bodies (EBs) elicited similar levels of protection against a transcervical challenge in both strains of mice. In conclusion, transcervical inoculation with *C. trachomatis* D in HLA-DR4 mice can be a useful model for evaluating human *C. trachomatis* vaccines.

RESULTS

Transcervical inoculation with *C. trachomatis* D significantly reduces fertility in HLA-DR4 and WT mice. As shown in Table 1, the mean number of embryos in both uterine horns of HLA-DR4 mice was significantly different between the transcervically *C. trachomatis* D-infected (4.4 ± 1.3 embryos/mouse) and the fertility control (7.8 ± 0.5 embryos/mouse) group ($P < 0.05$). The number of embryos in the right uterine horns was also significantly different between the two groups ($P < 0.05$). The transcervical inoculation-induced infertility was also observed in WT C57BL/6 mice. In infected mice the mean number of embryos/mouse was 4.3 ± 1.4 while the fertility control mice had 9.1 ± 0.4 embryos/mouse ($P < 0.05$). The numbers of embryos in the right and left uterine horns were also significantly different between the two groups ($P < 0.05$).

HLA-DR4 mice are more susceptible to *C. trachomatis* D transcervical infection than WT animals. Vaginal cultures for *C. trachomatis* D were collected twice a week for the first 4 weeks and once a week for two additional weeks for a total of 10 cultures/mouse (Fig. 1A and B). All mice infected transcervically had positive cultures as early as day 4 postinoculation. Some HLA-DR4 mice continued to shed for the 43 days of

TABLE 1 Fertility results of HLA-DR4 and WT mice infected transcervically with *C. trachomatis* serovar D

Mouse strain and infection status	No. of embryos/mouse (mean ± 1 SE)		
	Total	Right horn	Left horn
HLA-DR4			
Transcervically infected	4.4 ± 1.3[a]	2.3 ± 0.8[a]	2.1 ± 0.6
Noninfected (fertility control)	7.8 ± 0.5	4.8 ± 0.3	3.0 ± 0.3
WT			
Transcervically infected	4.3 ± 1.4[a]	2.6 ± 0.8[a]	1.6 ± 0.6[a]
Noninfected (fertility control)	9.1 ± 0.4	5.0 ± 0.5	4.1 ± 0.4

[a]$P < 0.05$ by Student's *t* test for values compared to those of the respective noninfected strain.

observation while WT mice stopped by day 35. Of the 100 vaginal cultures collected, 75% (75/100) from the HLA-DR4 mice were positive for *C. trachomatis* D while only 38% (38/100) from the WT C57BL/6 mice were positive ($P < 0.05$) (Fig. 2A). The total numbers of *C. trachomatis* D IFU recovered were also significantly different between the HLA-DR4 (289,316 IFU) and WT (8,925 IFU) mice ($P < 0.05$) (Fig. 2B). In addition, the mean time (days) to negative cultures was significantly different between the HLA-DR4 (37 ± 3 days) and WT (23 ± 3 days) mice ($P < 0.05$) (Fig. 2C). Using Wilcoxon's rank-sum test to compare the areas under the curve (AUC), the vaginal shedding was significantly different between the two groups (59,324 versus 1,478 arbitrary units; $P < 0.05$) (Fig. 1A and B).

Characterization of the immune responses following *C. trachomatis* D transcervical infection. To monitor antibody responses, blood was collected from all mice at 42 days post-transcervical infection. As shown in Fig. 3A, WT C56BL/6 mice had higher IgG antibody titers than the HLA-DR4 animals. At day 42, the transcervically infected WT mice had a *C. trachomatis* D-specific IgG geometric mean titer (GMT) of 67,559 (range, 25,600 to 204,800) by enzyme-linked immunosorbent assay (ELISA) while the HLA-DR4 mice had a GMT of 9,701 (range, 6,400 to 12,800) ($P < 0.05$).

The IgG2c/IgG1 ratio was used to monitor the bias toward a Th1 phenotype (Fig. 3A) (37). Based on this ratio, the WT C57BL/6 mice (IgG2c/IgG1 = 58,813/696, or 84) had more Th1-biased responses than HLA-DR4 mice (2,786/919, or 3). The HLA-DR4 mice also displayed reduced titers of serum IgM (115; range <100 to 400) and IgA (303; range, <100 to 1,600) than WT mice (for IgM, 303 [range, <100 to 400]; for IgA, 1,393 [range, 400 to 6,400]) although these differences were not statistically significant. The IgG antibody titers in the vaginal washes were also not significantly higher in WT (127; range, 80 to 160) than in HLA-DR4 (20; range, 10 to 80) mice ($P > 0.05$).

The serum antibodies were further evaluated for their ability to neutralize *C. trachomatis* D infectivity *in vitro* (Fig. 3A). HLA-DR4 mice had a lower neutralizing GMT (44; range, <10 to 160) than WT (452; range, 200 to 1,600) mice, which correlates with both the reduced fertility and prolonged shedding ($P < 0.05$).

Western blotting was performed with serum samples collected before infection and at day 42 post-transcervical infection. As shown in Fig. 4 (lanes 6 and 7), HLA-DR4 mice had weaker antibody responses than WT mice following transcervical infection. HLA-DR4 serum reacted with a band in the molecular mass range of 60 kDa, likely corresponding to the 60-kDa cysteine-rich protein and/or the 60-kDa heat shock protein, and a band corresponding to the major outer membrane protein (MOMP; 42 kDa). The WT mice, on the other hand, had antibodies to other proteins, including proteins of ~150-kDa (probably polymorphic membrane proteins), an ~26-kDa protein, and lipopolysaccharide (LPS), suggesting that the humanization of the C57BL/6 mice with MHC-II quantitatively and qualitatively affected antibody responses.

Lymphoproliferative T-cell responses were determined at day 42 following transcervical infection (Fig. 3B). As in the case of the humoral immune responses, more robust T-cell responses were measured in T cells from WT mice stimulated with *C. trachomatis* D EBs than in those of HLA-DR4 animals. The value in WT mice was 10,259 ± 952 cpm

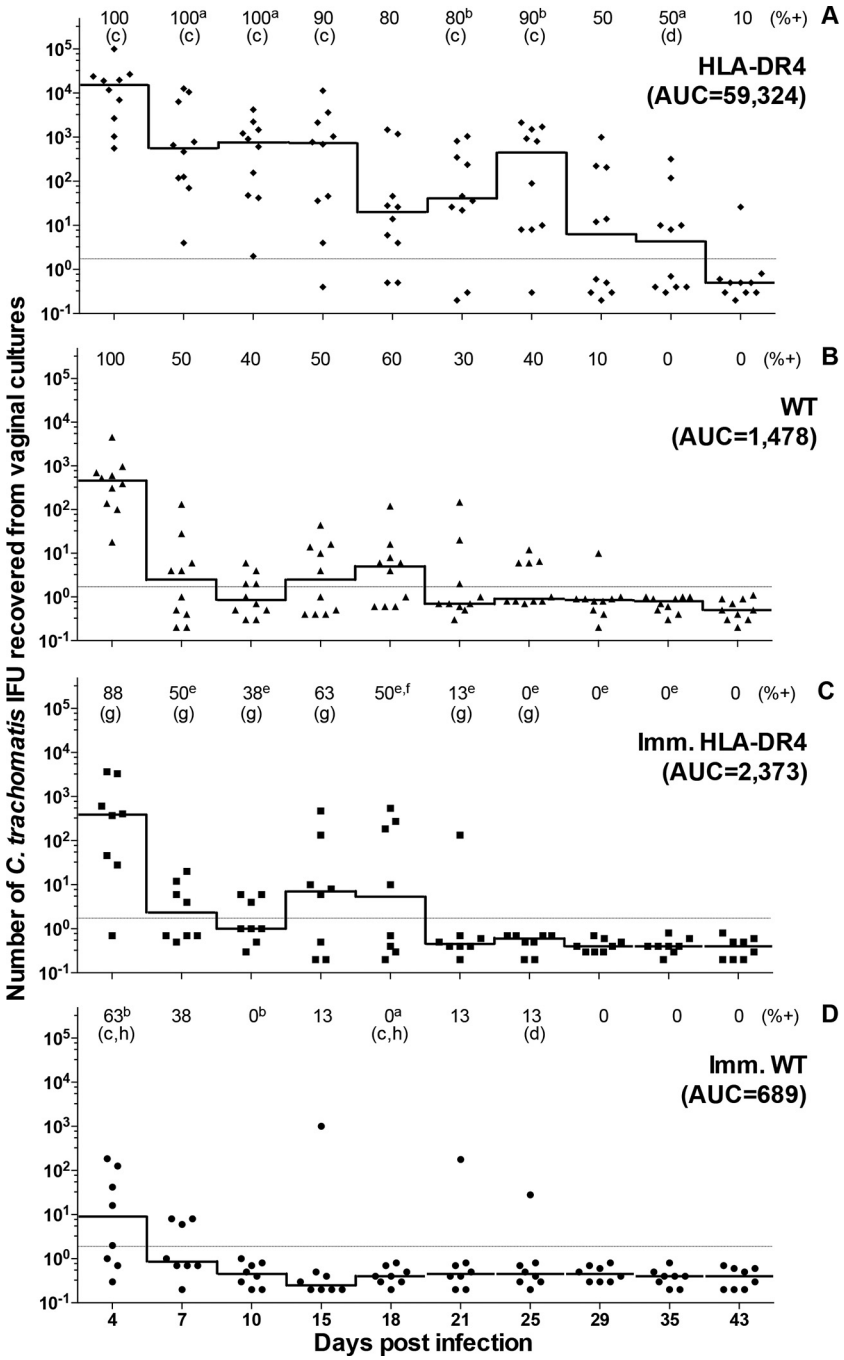

FIG 1 Number of *C. trachomatis* D IFU recovered per mouse from vaginal cultures and length of the infection. (A and B) Mice were infected with *C. trachomatis* D transcervically. (C and D) Mice were immunized (Imm) intranasally with live *C. trachomatis* D and 4 weeks later challenged transcervically. Vaginal cultures were collected for a period of 6 weeks following the transcervical infection. Each mouse is represented by a dot, and the median number of IFU per group is shown by a continuous line. The limit of detection was 2 IFU/culture. The area under the curve (AUC) was calculated in arbitrary units (analyzed by a Wilcoxon matched-pairs signed-rank test). The top row of numbers indicates the percentages of mice with positive vaginal cultures. Significance is indicated by lowercase letters as follows: a, $P < 0.05$ by Fisher exact test for values compared to those of WT mice; b, $P < 0.1$ by Fisher exact test for values compared to those of WT mice; c, $P < 0.05$ by Mann-Whitney U test for values compared to those of WT mice; d, $P < 0.1$ by Mann-Whitney U test for values compared to those of WT mice; e, $P < 0.05$ by Fisher exact test for values compared to those of HLA-DR4 mice; f, $P < 0.1$ by Fisher exact test for values compared to those of immunized WT mice; g, $P < 0.05$ by Mann-Whitney U test for values compared to those of HLA-DR4 mice; h, $P < 0.05$ by Mann-Whitney U test for values compared to those of immunized HLA-DR4 mice.

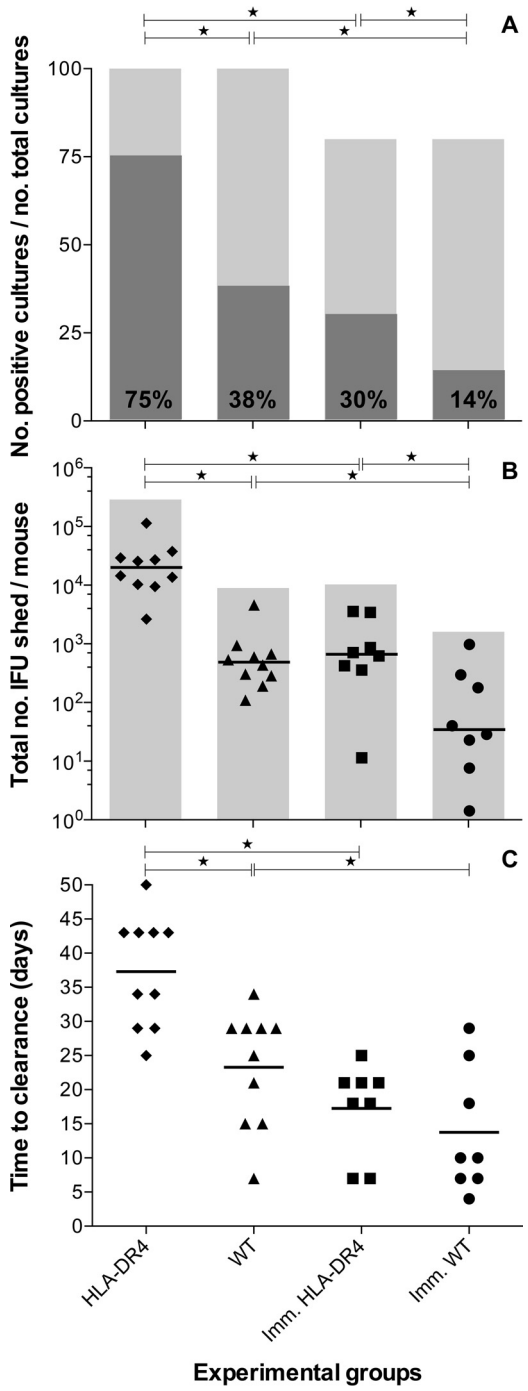

FIG 2 *C. trachomatis* D IFU yields and length of vaginal shedding. (A) Number of positive vaginal cultures over the total number of cultures collected during the 6 weeks of observation. (B) Total number of *C. trachomatis* D IFU recovered per group (shaded area) and per mouse during the 6 weeks of vaginal culture. Each mouse is represented by a symbol, and the median number of IFU for each group is indicated by a continuous horizontal line. (C) Number of days the vaginal cultures remained positive. Each mouse is represented by a symbol, and the mean number of days to clearance is represented by a continuous horizontal line. Statistically significant differences ($P < 0.05$) are marked with an asterisk and were determined by a Fisher's exact (A), Mann-Whitney U (B), or Student's *t* (C) test.

while that in HLA-DR4 mice was 3,246 ± 260 cpm ($P < 0.05$). Interestingly, opposite results were obtained when the T cells were stimulated with concanavalin A (ConA) (20,648 ± 1,034 cpm versus 89,952 ± 1,191 cpm, for WT versus HLA-DR4 mice, respectively). Levels of gamma interferon (IFN-γ) in supernatants from EB-stimulated

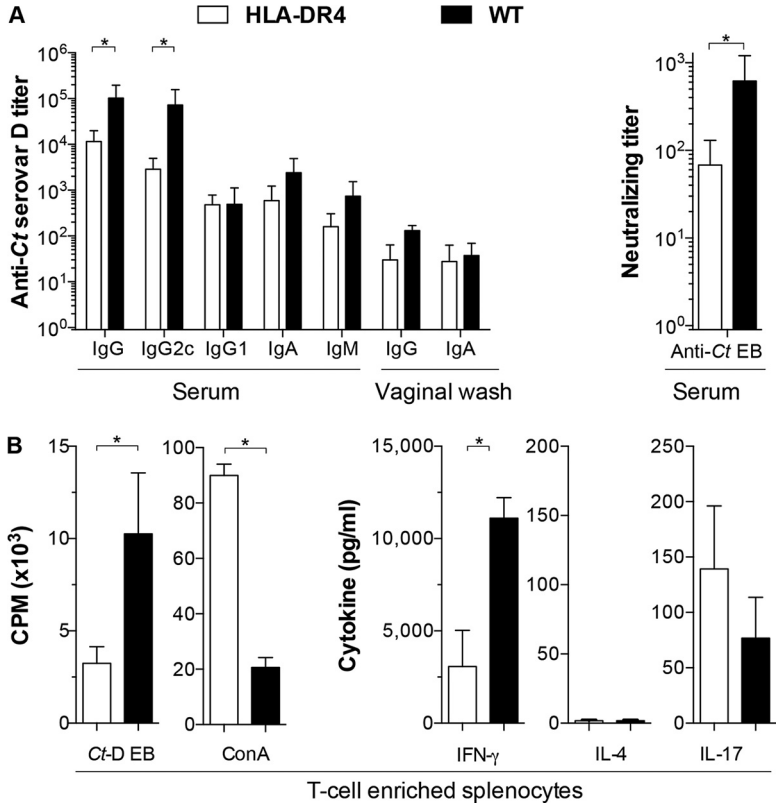

FIG 3 Characterization of the humoral and cell-mediated immune responses in HLA-DR4 and WT mice following transcervical infection with *C. trachomatis* D. Serum, vaginal washes, and T cells from the spleen were collected at day 42 following transcervical *C. trachomatis* D infection. (A) ELISA antibody titers in serum and vaginal washes and serum neutralizing antibody titers. (B) Lymphoproliferative responses from T cells stimulated with *C. trachomatis* D EBs and levels of cytokines in supernatants from *C. trachomatis* D EB-stimulated T cells. Statistically significant differences ($P < 0.05$) are marked with an asterisk (Student *t* test). *Ct*, *C. trachomatis*.

T cells were also higher in WT (11,111 ± 552 pg/ml) than in HLA-DR4 (3,075 ± 980 pg/ml) mice ($P < 0.05$). Interleukin-17 (IL-17) levels were higher in HLA-DR4 than in WT mice, but there was no significant difference between the two groups. Levels of IL-4 were below the limits of detection in both groups of mice.

Characterization of the immune responses following *C. trachomatis* D intranasal vaccination. To determine if protective immune responses could be elicited by vaccination, naive mice were inoculated intranasally with 10^4 IFU of *C. trachomatis* D and challenged transcervically 4 weeks later. Antibody responses following intranasal immunization were evaluated in blood and vaginal washes. Similar to results following transcervical inoculation, WT mice developed more robust antibody responses than HLA-DR4 animals (Fig. 5A). For example, at day 28 postimmunization, the IgG GMT in WT mice was 204,800 (range, 204,800 to 204,800) while in HLA-DR4 mice it was 36,204 (range, 25,600 to 51,200) ($P < 0.05$). The IgG2c/IgG1 ratio was also higher in WT mice (IgG2c/IgG1 = 144,815/2,263, or 64) than in HLA-DR4 animals (12,800/800, or 16). The IgA GMT in serum was also higher in WT than in HLA-DR4 mice. No IgM was detected in serum. Serum neutralizing titers were higher in WT mice (566; range, 100 to 3,200) than in HLA-DR4 mice (25; range, 25 to 25) ($P < 0.05$). Levels of IgG and IgA in vaginal washes were also lower in HLA-DR4 mice than those of the WT animals but were not statistically different ($P > 0.05$).

Western blotting performed with serum samples from WT mice vaccinated intranasally with *C. trachomatis* D showed broader immune responses than samples from HLA-DR4 mice (Fig. 4, lanes 4 and 5). Serum from WT mice recognized bands with molecular masses corresponding to ~150 to 170 kDa (likely polymorphic membrane

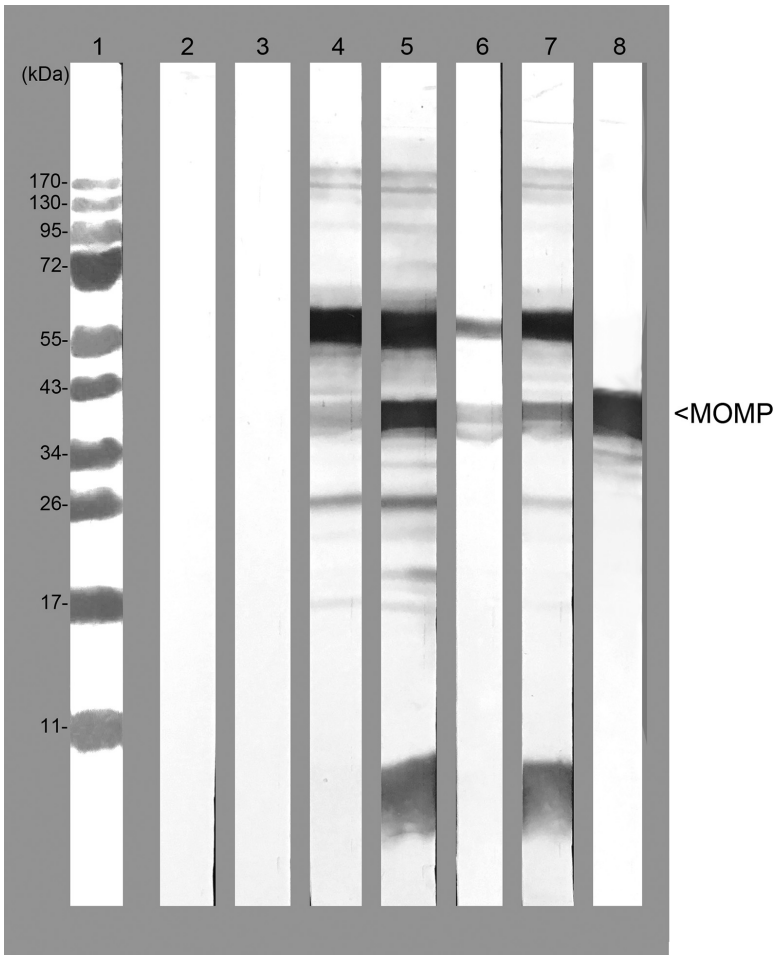

FIG 4 Western blot of serum samples from intranasally immunized mice and nonimmunized mice infected transcervically with *C. trachomatis* D. *C. trachomatis* D EBs were run on a 10% SDS-PAGE gel and blotted to nitrocellulose paper. EBs were probed with sera collected from HLA-DR4 (lane 2) and WT (lane 3) mice before infection. Sera from mice immunized intranasally were tested the day before the transcervical challenge (lane 4, HLA-DR4; lane 5, WT mice). Sera from HLA-DR4 (lane 6) and WT (lane 7) mice at day 42 were probed following the transcervical infection. *C. trachomatis* D EBs were probed with monoclonal antibody (E4) to MOMP (lane 8). Lane 1, molecular mass standards.

proteins), ~60 kDa (heat shock protein 60 and/or a 60-kDa cysteine-rich protein), 40 kDa (MOMP), ~26- and ~20-kDa proteins, and LPS while serum from HLA-DR4 recognized bands only at ~150 to 170, 60, 40, and 26 kDa.

T-cell proliferative responses to *C. trachomatis* D EBs were also more robust in WT than in HLA-DR4 intranasally immunized animals (Fig. 5B). The level in WT mice reached 11,243 ± 1,358 cpm while that in HLA-DR4 was 5,885 ± 313 cpm ($P < 0.05$). In contrast, T cells from HLA-DR4 mice stimulated with ConA responded more strongly than those from WT mice (108,046 ± 3,116 cpm for HLA-DR4 versus 35,706 ± 2,182 cpm for WT mice) ($P < 0.05$). Levels of IFN-γ in EB-stimulated T-cell supernatants were 11,063 ± 1,385 pg/ml for WT mice and 7,790 ± 619 pg/ml for HLA-DR4 mice ($P < 0.1$). Levels of IL-17 in supernatants were higher in the HLA-DR4 (455 ± 51 pg/ml) than in the WT (132 ± 26 pg/ml) mice ($P < 0.05$). IL-4 was below the limit of detection for both strains.

Intranasal immunization with live *C. trachomatis* D elicits protection in HLA-DR4 and WT mice against a transcervical challenge. Four weeks after the intranasal immunization with live *C. trachomatis* D, mice were challenged transcervically, and vaginal cultures were collected as described previously. Both strains of vaccinated mice were protected compared to their respective nonimmunized strains. This difference is

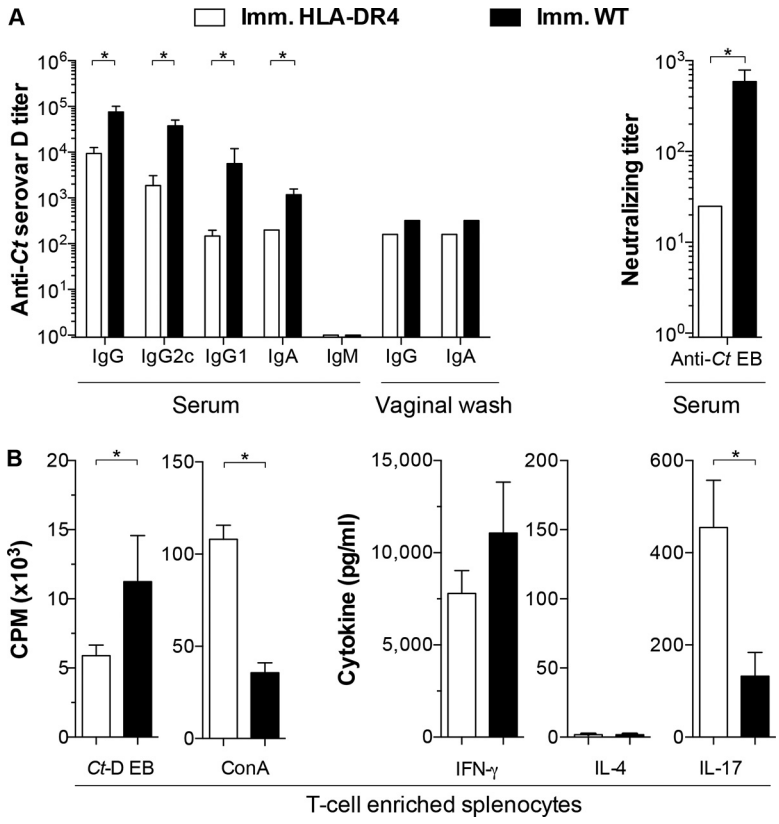

FIG 5 Characterization of the humoral and cell-mediated immune responses in HLA-DR4 and WT mice following intranasal immunization with *C. trachomatis* D. Serum, vaginal washes, and spleen T cells were collected at day 28 following intranasal *C. trachomatis* D immunization. (A) ELISA antibody titers in serum and vaginal washes and serum neutralizing antibody titers. (B) Lymphoproliferative responses from T cells stimulated with *C. trachomatis* D EBs and levels of cytokines in supernatants from the *C. trachomatis* D EB-stimulated T cells. Statistically significant differences ($P < 0.05$) are marked with an asterisk (Student *t* test).

more striking for the HLA-DR4 than for the WT mice probably, at least in part, because the HLA-DR4 mice were more susceptible to transcervical infection. For example, 75% (75/100) of the vaginal cultures of the nonvaccinated HLA-DR4 mice were positive, but only 30% (25/80) of cultures from the immunized group were positive (Fig. 2A) ($P < 0.05$). Similarly, the total number of *C. trachomatis* D IFU recovered and the time to clearance were also significantly different (Fig. 2B and C) ($P < 0.05$). This difference between immunized and nonimmunized HLA-DR4 mice is very apparent when areas under the curve are compared (2,373 versus 59,324 arbitrary units; $P < 0.05$) (Fig. 1A and C).

A direct comparison of protection achieved between the two vaccinated strains of mice shows that similar protection levels were elicited in WT and HLA-DR4 mice. As shown in Fig. 1C and D, all but one HLA-DR4 mouse had positive cultures on day 4 postchallenge in contrast to 5 of 8 WT mice with negative cultures, with HLA-DR4 mice having a median (range) number of IFU shed of 389 (<2 to 3,654) versus 9 (<2 to 185) for WT mice ($P < 0.05$). Between the HLA-DR4 and the WT immunized mice significant differences were observed in the percentages of mice with positive cultures (30% [24/80] of HLA-DR4 and 14% [11/80] of WT mice), total number of IFU shed (10,268 and 1,605, respectively), and median number of *C. trachomatis* D IFU shed/mouse (689 versus 36, respectively) ($P < 0.05$) (Fig. 2A and B). However, the areas under the curve (2,373 versus 689 arbitrary units) and the mean number of days to clearance (17 $\pm$ 2 versus 14 $\pm$ 13 days) were not significantly different between the two groups of mice ($P > 0.05$) (Fig. 1C and D and 2C).

DISCUSSION

In the current study, we demonstrated that transcervical inoculation with *C. trachomatis* D reduces fertility in HLA-DR4 transgenic mice. Importantly, the transcervical inoculation-induced infertility was reproduced in the WT C57BL/6 mice, which both validates the reproducibility of the model and suggests that the infertility is not associated with either a mouse strain or a human MHC class II haplotype. The latter finding is consistent with previous observations made in the *C. muridarum* mouse model revealing that it is the $CD8^+$, but not $CD4^+$, T-cell response that plays a critical role in promoting infertility (38). Indeed, both human and mouse data suggest that protective immunity is more likely MHC class II restricted and $CD4^+$ T cell dependent (28, 39–41). Thus, an HLA-DR4-restricted *C. trachomatis* antigen that shows protection in the HLA-DR4 mouse model is unlikely to exacerbate pathology in humans. In addition we showed that intranasal immunization with live *C. trachomatis* D protects both strains of mice against a transcervical challenge.

The *C. trachomatis*-induced infertility caused by transcervical inoculation of HLA-DR4 mice paralleled extensive organism recovery from vaginal cultures and also correlated with dampened cellular and humoral immune responses, including neutralizing antibodies. The question is whether vaccine antigen-primed neutralization antibody responses can be recalled to control the extensive number of live organisms in genital tissues, a premise testable in this model. WT C57BL/6 mice are known to possess a strong bias toward the Th1 phenotype (42–44). Interestingly, our current study revealed that replacing the mouse MHC class II antigens with the human HLA-DR4 attenuated the extreme Th1 bias, which may render the HLA-DR4 mice more suitable for screening vaccine antigens for susceptible humans. The previous success in inducing protective cellular immunity against *C. muridarum* infection in the genital tract of HLA-DR4 mice with proteins that are highly conserved in both *C. trachomatis* and *C. muridarum* suggests that the HLA-DR4 mice are suitable for evaluating T-cell antigens for vaccine development (28).

Transcervical infection bypasses the cervical barrier that may represent a critical site where the vaccine antigen-induced immunity develops. Therefore, this model may miss vaccine formulations that induce immunity against ascending infection. This concern can be addressed by monitoring the descending infection. Vaccine-induced cervical immunity should be able to prevent both ascending and descending infection. By monitoring live organisms in vaginal swabs after transcervical inoculation, we can still determine whether a given vaccine antigen is able to induce anti-ascending/descending immunity in this model. Indeed, here we also showed that intranasal immunization with live *C. trachomatis* D induces immunity that significantly reduces descent of transcervically inoculated *C. trachomatis* organisms into the lower genital tract, confirming previous results (32).

Although the numbers of IFU, testing times, and Depo-Provera treatments differed for the intranasal and transcervical inoculations with *C. trachomatis* D, similar humoral and cellular immune responses were observed in both strains of mice. However, more robust immune responses were obtained in WT than in HLA-DR4 mice independently of the route of inoculation. Antibody titers in serum and vaginal washes were quite high in both strains of mice. Based on the IgG2c/IgG1 ratio in serum and IFN-γ levels in supernatants from T cells stimulated with *C. trachomatis* D EBs, both routes elicited Th1-biased immune responses consistent with the Th1 bias of C57BL/6 mice (42–44). Interestingly, T-cell proliferative responses, using ConA for stimulation in contrast to using *C. trachomatis* D EBs, were higher in HLA-DR4 mice than in WT mice, suggesting that these mice do not have generalized weaker cell-mediated responses. Also, levels of IL-17 in supernatants from EB-stimulated T cells were higher in HLA-DR4 mice than in WT animals. IL-17 is thought to play a critical role in the generation of Th1 immunity and the induction of memory immune responses, and, therefore, it is important in *Chlamydia*-elicited vaccine immunity (45–47). These findings also suggest that HLA-DR4 mice may help elucidate immune mechanisms involved in vaccine-induced protection.

More work is necessary to reach definitive conclusions about the use of HLA-DR4 mice in vaccination studies. Interestingly, however, although the HLA-DR4 mice

mounted weaker immune responses to live *C. trachomatis* D, the protection levels elicited by intranasal vaccination against transcervical challenge were equivalent in the two strains of mice. For example, using the AUC as a parameter to determine protection against vaginal shedding, the HLA-DR4 mice had a decrease of 25-fold (59,324/2,373 arbitrary units) while the WT mice had a decrease of 2-fold (1,478/689 arbitrary units). It could be argued that the AUC is still 3.4 (2,373/689 arbitrary units) higher in HLA-DR4 than in WT mice, but an apparent higher susceptibility to infection, and not weaker protective immune responses, may account for that difference.

It is recognized that although DR4 (beta chain allele, DRB*0401) is a common haplotype of MHC class II (MHC-II) in humans, the DR4-selected antigens may not work in individuals who lack DR4. This concern is alleviated by the fact that immunodominant antigens selected by DR4 likely contain multiple epitopes recognized by other MHC-II haplotypes. In addition, the incorporation of multiple antigens into a vaccine will increase human MHC-II recognition frequency (11). Finally, the DR4-selected antigens could also be analyzed in mice expressing other human MHC-II alleles associated with normal fertility after chlamydial infection, such as DQA*0501 or DQB*0402 (48). The antigens presentable by multiple MHC-II haplotypes should be prioritized for incorporation into *C. trachomatis* vaccines.

In summary, transcervical inoculation with *C. trachomatis* D significantly reduced fertility in HLA-DR4 transgenic and WT C57BL/6 mice. Based on the number of positive vaginal cultures, the number of IFU recovered, and time to clearance, HLA-DR4 mice were more susceptible than WT mice to a *C. trachomatis* D transcervical infection. Furthermore, as determined by vaginal shedding, intranasal immunization of HLA-DR4 and WT C57BL/6 mice with live *C. trachomatis* D protected against a transcervical challenge with this organism. This novel animal model opens new opportunities to test subunit vaccine antigens that can potentially be used in humans.

MATERIALS AND METHODS

***C. trachomatis* D stocks.** *C. trachomatis* D (strain UW-3/Cx) was obtained from the American Type Culture Collection (ATCC; Manassas, VA) and was grown in HeLa-229 cells using Eagle's minimal essential medium supplemented with 5% fetal bovine serum (FBS) (49). Density gradient-purified EBs were stored at −80°C in 0.2 M sucrose, 20 mM sodium phosphate (pH 7.4), and 5 mM glutamic acid (SPG medium) (50). Bacterial stocks were titrated in HeLa-229 cells.

Transcervical infection and intranasal vaccination of mice. Six-week-old female WT and transgenic C57BL/6 mice [B6.129S2-H2Ab^{tm1Gru} Tg (HLA-DRA/H2-Ea, HLA-DRB1*0401/H2-Eb)1Kito; model 4149F] were purchased from Taconic Biosciences, Inc., and housed at the University of California, Irvine (UCI), vivarium (28). For transcervical infection, mice were anesthetized and inoculated with 5×10^5 IFU of *C. trachomatis* D via the cervical os by using a nonsurgical embryo transfer device (ParaTechs Corp., Lexington, KY) (28). Four days prior to transcervical infection, mice were treated subcutaneously with 2 mg of Depo-Provera (Pfizer).

To determine if HLA-DR4 and WT mice can be protected by vaccination, 10^4 IFU of *C. trachomatis* D was delivered intranasally, and Depo-Provera-treated mice were challenged transcervically 4 weeks later with *C. trachomatis* D (5×10^5 IFU).

Immunoassays. Blood was drawn from the periorbital region, and genital secretions were collected by washing the vagina twice with 20 μl of phosphate-buffered saline (PBS). *C. trachomatis* D-specific antibody titers were determined by an enzyme-linked immunosorbent assay (ELISA) as previously described (51). Briefly, multiwell plates (Corning Glass Works, Corning, NY) were coated overnight with 1 μg of purified *C. trachomatis* D EBs per well, and serial dilutions of samples were added. The antigen-antibody reactions were detected with horseradish peroxidase-conjugated goat anti-mouse antibodies. The following antibodies were utilized: IgG, IgM, and IgA (Kirkegaard and Perry Laboratories, Inc., Gaithersburg, MD), IgG1 (BD Pharmingen, San Diego, CA), and IgG2c (Jackson Immuno Research, West Grove, PA). The substrate, 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic) acid, was used for color development. The plates were scanned at 405 nm in an ELISA reader (Labsystem Multiscan, Helsinki, Finland). Titers were calculated after subtracting the optical density (OD) ±2 standard deviations (SD) of preinfected mouse samples.

Western blotting was performed with *C. trachomatis* D EBs as previously described (52, 53). Following transfer of the proteins to a nitrocellulose membrane, nonspecific binding was blocked, and the serum samples were added and incubated overnight. The membrane was washed and incubated with horseradish peroxidase-conjugated goat anti-mouse Ig antibody followed by visualization of the bands by the addition of hydrogen peroxide and 4-chloro-1-naphthol.

In vitro neutralization assays were performed as published by Peterson et al. (49). Briefly, 1×10^4 IFU of *C. trachomatis* D was added to serial dilutions of mouse serum made with Ca^{2+}- and Mg^{2+}-free PBS, pH 7.2, and supplemented with 5% guinea pig serum. After incubation for 45 min at 37°C, the mixture

was inoculated by centrifugation into HeLa-229 cells grown on shell vials. Following 40 h of incubation at 37°C, the monolayers were fixed and stained with a monoclonal antibody (MAb) to *C. trachomatis* MOMP (MAb E4) (54). The titer of a sample was the dilution that yielded 50% neutralization relative to neutralization of the control serum from naive mice.

A T-cell lymphoproliferative assay (LPA) was performed at week 7 postinfection using splenocytes, as previously described (52). In short, splenic T cells were collected, and UV-inactivated *C. trachomatis* D EBs were added at a concentration of 1 EB to 1 T cell plus antigen-presenting cell (APC). APCs were prepared by irradiating splenocytes with 3,300 rads of ^{137}Cs. Negative-control wells received medium alone, and positive controls received concanavalin A (5 μg/ml). At 96 h of incubation, cell proliferation was measured by addition of 1 μCi of [*methyl*-^{3}H]thymidine (47 Ci/mmol; Amersham, Arlington Heights, IL) per well. The mean count was obtained from triplicate cultures.

Levels of IFN-γ, IL-4, and IL-17 were determined, using a commercial kit (BD Pharmingen, San Diego, CA), in supernatants from splenic T cells stimulated as described above (55).

Genital cultures. Following the genital challenge, vaginal swabs were cultured twice weekly for the first 4 weeks and then at 7-day intervals for an additional 2 weeks (52). HeLa-229 cells grown in 48-well tissue culture plates were inoculated with 10-fold dilutions of the vaginal swab cultures and incubated for 40 h at 37°C. The monolayers were fixed with methanol, and chlamydial inclusions were stained using MAb E4 as described above. The limit of detection was 2 IFU/mouse/culture.

Fertility studies. At 7 weeks following *C. trachomatis* D transcervical inoculation, mice were caged with male WT C57BL/6 mice, and pregnancy was determined by weighing the animals. Mice that gained weight were euthanized, and the number of embryos in each uterine horn was counted. Mice that did not become pregnant were caged again with a different male mouse that had fathered a litter during the first mating (52). Two groups of noninfected mice, of the same age as those infected, were used as fertility controls. The numbers of embryos in both uterine horns were counted.

Statistics. A two-tailed unpaired Student's *t* test, a Mann-Whitney rank U test, a Wilcoxon matched-pairs signed-rank test, and Fisher's exact test were used for statistical analyses with the program SigmaStat, version 3.5. Differences were considered significant for P values of <0.05 (a P value of <0.1 was noted in a few analyses to indicate a trend to significance).

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