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Gene Knockout Mice Establish a Primary Protective Role for Major Histocompatibility Complex Class II-Restricted Responses in *Chlamydia trachomatis* Genital Tract Infection

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Mice with disrupted β_2-microglobulin ($\beta_2m^{-/-}$), I-A (class $II^{-/-}$), or CD4 ($CD4^{-/-}$) genes were examined for their capacity to resolve *Chlamydia trachomatis* genital tract infection. C57BL/6 and $\beta_2m^{-/-}$ mice resolved infection similarly and were culture negative by 4 to 5 weeks following infection. Conversely, major histocompatibility complex (MHC) class $II^{-/-}$ mice failed to resolve infection, and $CD4^{-/-}$ mice showed a significant delay (2 weeks). Secondary challenge of C57BL/6, $\beta_2m^{-/-}$, and $CD4^{-/-}$ mice established that acquired protective immunity, which was characterized by an infection of shortened duration and reduced shedding of infectious organisms, developed. Serological analysis of C57BL/6 and $\beta_2m^{-/-}$ mice by enzyme-linked immunosorbent assays revealed no striking differences in the immunoglobulin subclass specificity of the anti-*Chlamydia* response, although some differences were observed in the magnitude of the immunoglobulin G2a (IgG2a) and IgG2b responses. Class $II^{-/-}$ mice produced lower-titered serum anti-*Chlamydia* antibodies of all isotypes. The serum antibody responses of $CD4^{-/-}$ mice were similar to those of C57BL/6 mice, except that the anti-*Chlamydia* IgA response was delayed by approximately 3 weeks. Analysis of vaginal washes for *Chlamydia*-reactive antibodies revealed the presence of IgG2a, IgG2b, and IgA in C57BL/6 and $\beta_2m^{-/-}$ mice and primarily of IgA in $CD4^{-/-}$ mice. Vaginal washes from class $II^{-/-}$ mice were consistently antibody negative. Interestingly, the *Chlamydia*-specific IgA response in the vaginal washes of $CD4^{-/-}$ mice was delayed, but its appearance coincided with decreased shedding of infectious organisms and resolution of infection. Our results demonstrate that MHC class II-restricted T-cell responses are necessary for the development of protective immunity to *Chlamydia* genital tract infection and that local (vaginal) anti-*Chlamydia* IgA antibody coincides with the resolution of infection. A substantive role for MHC class I-restricted T-cell responses in protective immunity to *Chlamydia* genital tract infection was not confirmed.

Despite the availability of effective antimicrobial therapy, infections caused by the obligate intracellular bacterial pathogen *Chlamydia trachomatis* continue to be a major cause of blindness and infertility worldwide. One approach to the control and prevention of *Chlamydia* infection and its sequelae is through immunoprophylaxis. However, past vaccination attempts in humans and experimental animals have been ineffective, since immunization confers only a minor degree of protection and might instead exacerbate the disease upon reinfection (10, 42, 45). Perhaps our inability to design an effective vaccine results from our incomplete understanding of the immune responses that contribute to the resolution of infection and protection against reinfection.

Studies of *C. trachomatis* infection in humans and experimental animals provide evidence that infection leads to the development of protective immunity; however, resistance wanes over time and reinfection is possible (2, 5, 14, 26, 31, 36). Both humoral and cell-mediated immune (CMI) responses are elicited following *Chlamydia* infection. Serum anti-*Chlamydia* antibodies are consistently detected following infection, but their in vivo role in protective immunity is not well understood. In certain animal models of *Chlamydia* infection, antibodies appear to provide some degree of protection (29, 30, 33); however, in the murine models of pneumonia and genital tract infections, antibody is not believed to play a primary protective role (26, 44). Protective immunity to murine *Chlamydia* genital tract infection is transferred to naive animals with immune lymphocytes (28), thus demonstrating that CMI is of preeminent importance.

T cells function as both immunologic regulatory cells, such as $CD4^+$ helper cells for antibody production, and as effector cells, such as $CD8^+$ cytotoxic T cells. However, a consensus has not been reached regarding the relative importance of these T-cell subpopulations in immunity to *Chlamydia* infection. Mice experimentally infected with *C. trachomatis* control their infections within several weeks without antibiotic therapy, providing a useful model for evaluating the host immune response to infection. Rank et al. showed that genital tract infection of T-cell-deficient nude mice with *Chlamydia* results in a chronic infection that persists for >9 months (32) and that the transfer of spleen cells enriched for either T or B cells brings about resolution of infection (23, 28). In contrast, studies by Tuffrey et al. found that nude mice resolve genital infection in a manner similar to that for infection in immunologically intact animals and that the adoptive transfer of immune cells does not confer additional protection (40, 41). These conflicting observations cannot readily be resolved but might be explained by differences in experimental design and the infecting strain of *C.*

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trachomatis. Studies to address the relative contributions of subpopulations of T cells in resolving *Chlamydia* infection by the in vivo depletion of $CD4^+$ or $CD8^+$ T cells with monoclonal antibodies (MAbs) have also been inconclusive (19, 20, 27), although the data tend to suggest that $CD4^+$ T cells play a more dominant role. T-cell lines and clones have been propagated and tested for their ability to resolve *Chlamydia* infection (12, 13, 28, 37). These studies demonstrate that both $CD4^+$ and $CD8^+$ T cells function in host immunity against *Chlamydia* infection, with the former being more efficient in resolving infection.

To delineate the possible role of $CD4^+$ and $CD8^+$ T cells in the development of protective immunity, we have taken an alternative approach by examining infection in specific gene knockout mice. Gene targeting is a method by which specific genes are altered in embryonic stem cells and subsequently passed through the germ line. This method has been used to generate mice that are devoid of cell surface expression of major histocompatibility complex (MHC) class I, MHC class II, or CD4 molecules. MHC class I-deficient animals ($\beta_2m^{-/-}$) have been generated by inactivation of the gene for β_2-microglobulin (17), which is required for the proper assembly and cell surface expression of the MHC class I molecule; as a result, these mice are deficient in $CD8^+$ T cells. Mice devoid of cell surface expression of MHC class II molecules (class $II^{-/-}$) are derived by inactivation of the $I\text{-}A_\beta$ gene and are deficient in $CD4^+$ T cells (11). Mice with targeted disruption of the CD4 gene ($CD4^{-/-}$) have impaired development of the CD4 T-cell lineage and decreased but not absent helper T-cell activity (16). In the present study, we examined the capacity of MHC class I ($\beta_2m^{-/-}$)-, MHC class II (class $II^{-/-}$)-, or CD4 ($CD4^{-/-}$)-deficient mice to resolve *C. trachomatis* genital tract infection.

MATERIALS AND METHODS

Organism. The *C. trachomatis* strain mouse pneumonitis (MoPn) was grown in HeLa 229 cells, and elementary bodies were purified by discontinuous density centrifugation (6).

Animals. C57BL/6J mice were purchased from the Jackson Laboratories (Bar Harbor, Maine). β_2-Microglobulin-deficient ($\beta_2m^{-/-}$) mice (17) were bred and maintained in the animal facilities of the Rocky Mountain Laboratories. MHC class II-deficient (class $II^{-/-}$) mice were purchased from GenPharm International (Mountain View, Calif.) (11). Breeding pairs of CD4-deficient ($CD4^{-/-}$) mice (16) were obtained from N. Killeen (University of California, San Francisco) and were bred and maintained in the animal facilities of the Rocky Mountain Laboratories. Female mice of 7 to 12 weeks of age were used throughout the study. The strains of knockout mice used in this study are not considered inbred but were backcrossed (≥5 times) with C57BL/6 ($H\text{-}2^b$) mice.

Infection. C57BL/6 (control), $\beta_2m^{-/-}$, class $II^{-/-}$, and $CD4^{-/-}$ mice received 2.5 mg of depoprovera (medroxy-progesterone acetate) subcutaneously at 10 and 3 days before vaginal infection. The mice were infected by placing 5 μl of 250 mM sucrose–10 mM sodium phosphate–5 mM L-glutamic acid (pH 7.2; SPG) containing 1,500 inclusion-forming units (IFU) (100 50% infective doses [ID_{50}]) of *C. trachomatis* MoPn into the vaginal vault. The course of infection was followed by swabbing the vaginal vault with a Calgiswab (Spectrum Medical Industries, Los Angeles, Calif.) at various times following infection and by enumerating IFUs by isolation onto HeLa cell monolayers. Inclusions were visualized by indirect immunofluorescence with the MoPn-specific anti-major outer membrane protein (MOMP) MAb MP-33b (a kind gift from X.-Y. Zhang, Boston University) and with fluorescein-tagged goat anti-mouse immunoglobulin G (IgG) (46). Animals that recovered from primary infection were rechallenged, following depoprovera treatment, as described above.

Evaluation of serum and secretory antibody responses. Antibody responses were assayed by enzyme-linked immunosorbent assays (ELISA) as described previously (38, 39). IgM, IgG1, IgG2a, IgG2b, IgG3, and IgA anti-*Chlamydia* antibodies were detected with monospecific, alkaline phosphatase-conjugated anti-mouse Ig sera (class and subclass specific; Southern Biotechnology Associates, Birmingham, Ala.) and then with substrate (5 mg of *p*-nitrophenyl phosphate [Sigma Chemical Corp., St. Louis, Mo.] into 10 ml of 0.1 M 2,2-amino-2-methyl-1,3-propanediol [pH 10.3; Zymed, South San Francisco, Calif.]). A_{405} was measured with an ELISA reader (Dynatech, Alexandria, Va.). Preimmune sera were used as negative controls. ELISA titers were expressed as the highest serum dilution giving an absorbance (optical density at 405 nm [OD_{405}]) of at least 0.3 and 3 times that observed for preimmune sera (OD_{405} consistently < 0.1).

Vaginal secretions were collected by rinsing the vaginal vaults of metafane-anesthetized mice twice with 40 μl of 10 mM phosphate-buffered saline (PBS) containing 0.5% bovine serum albumin. The washes were pooled and clarified by centrifugation, and the resulting supernatants were frozen at −20°C until they were assayed. Collection of secretions as described resulted in recovery of approximately 50 to 60 μl of wash. Vaginal washes were diluted further with ELISA buffer (0.05 M Tris buffer [pH 7.5] with 0.15 M NaCl) to bring the total volume to 800 μl. Fourfold serial dilutions were prepared and analyzed as described above. Because the true volume of vaginal fluid cannot be determined accurately, the data are presented as the numbers of animals with positive (class- or subclass-specific) responses over the total numbers tested. A positive response was defined as an absorbance (OD_{405}) of at least 0.3 and 3 times that observed for preimmune vaginal washes. However, it should be noted that when positive responses were detected, IgA responses were generally 4- to 64-fold higher than IgG responses.

Histology and immunodetection of *Chlamydia* inclusions. Mice were sacrificed at various times following infection; the entire genital tract was removed, fixed in 10% buffered formalin, and embedded in paraffin. Thin sections were stained with hematoxylin and eosin and were evaluated by a veterinary pathologist (Histo-Path of America, Temple Hills, Md.).

Chlamydia inclusions were detected in genital tract tissue by indirect immunoperoxidase staining. Thin sections were deparaffinized by incubation at 55°C for 40 min and then transferred to xylene (three changes, 3 min each, 22°C). All other reactions were done at 22°C. Endogenous peroxidase activity was blocked by incubation of the deparaffinized tissue for 30 min in methanol containing 0.5% H_2O_2. Tissue was rehydrated by sequential 3-min incubations in 100, 95, 70, and 50% ethanol and then PBS. Tissues were incubated in PBS containing 3% horse serum for 15 min and then washed with PBS to block nonspecific antibody binding. Nonspecific binding of avidin and biotin was blocked by using an avidin-biotin blocking kit (Vector Laboratories, Burlingame, Calif.) according to the manufacturer's procedure. Inclusions were detected by incubating tissues with anti-MoPn MOMP MAb MP-33b for 1 h in a humidified chamber. Tissues were washed with PBS, incubated for 1 h with biotinylated horse anti-mouse Ig (Vector Laboratories), washed with PBS, incubated for 30 min with streptavidin peroxidase (Biogenex, San Ramon, Calif.), and then washed with distilled water. Inclusions were visualized by adding 3,3′-diaminobenzidine (DAB; Vector Laboratories) substrate and counterstained with 3% methyl green.

DTH. Delayed-type hypersensitivity (DTH) responses were assessed by injecting hind footpads of naive or infected mice (74 days following primary infection) with 25 μl of either SPG or heat-inactivated (80°C, 30 min) MoPn elementary bodies (0.8 μg of protein per ml of SPG). Footpad swelling represents the difference in foot thickness before inoculation and at 24 h postinoculation. The animals were euthanized after measurement, and the footpads were excised, fixed in buffered formalin, and processed and stained with hematoxylin and eosin as described above. Sections were evaluated blindly and grouped according to the severity of the lesion and degree of mononuclear cell infiltration. Grades of 0 to 4 were assigned as follows: 0, normal; 1 to 2, focal dermal suppuration; and 3 to 4, focal dermal suppuration with moderate to marked dermal edema, dermal fibrin exudation, and mild to moderate dermal interstitial and perivascular mononuclear cell infiltration.

Statistical analysis. Student's *t* testing of log-transformed data was used to analyze differences between IFU counts of control and experimental groups.

RESULTS

Time course of *Chlamydia* genital tract infection. Female C57BL/6, $\beta_2m^{-/-}$, class $II^{-/-}$, and $CD4^{-/-}$ mice were infected vaginally with *C. trachomatis*. Infection was monitored by swabbing the vaginal vault at scheduled intervals following infection and by enumerating IFUs by isolation onto HeLa cell monolayers (Fig. 1). C57BL/6 and $\beta_2m^{-/-}$ mice resolved genital tract infections by 4 to 5 weeks postinfection, and shedding of infectious chlamydiae was not significantly different at any time point. Conversely, class $II^{-/-}$ mice remained culture positive throughout the study (>70 days) and continued to shed chlamydiae throughout the observation period ($P < 0.001$ at ≥14 days). Although $CD4^{-/-}$ mice recovered from primary infection, resolution was delayed compared with immunocompetent C57BL/6 mice ($P < 0.05$ at days 22, 28, and 35).

C57BL/6, $\beta_2m^{-/-}$, and $CD4^{-/-}$ mice, which resolved primary infections, were rechallenged to assess acquired immunity. Most animals in each group could not be reinfected (culture negative); however, those that became infected (4 of 12 C57BL/6, 4 of 10 $\beta_2m^{-/-}$, and 1 of 6 $CD4^{-/-}$ mice) had decreased shedding of infectious chlamydiae (4 to 6 $\log_{10}$ lower than that found following primary challenge). Therefore, three

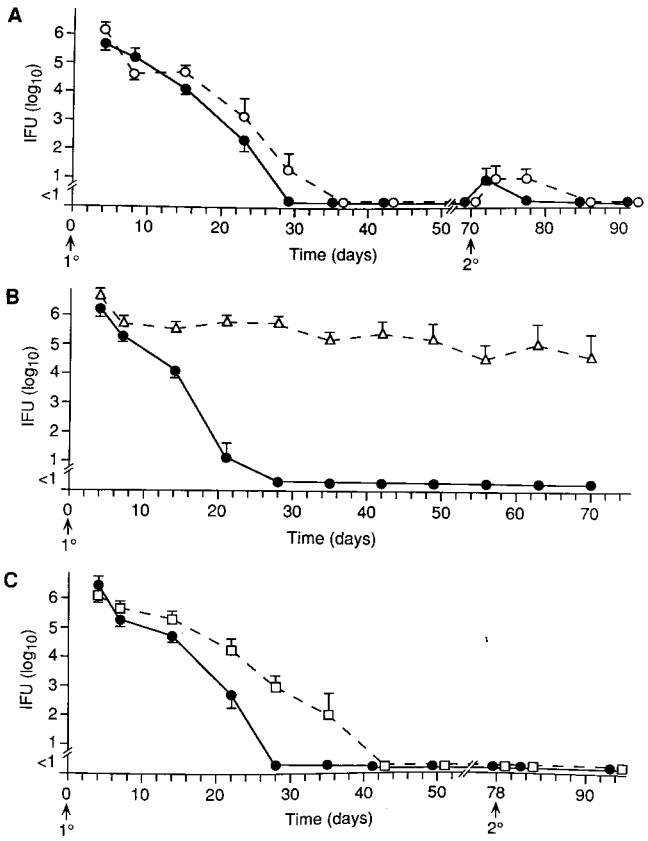

FIG. 1. Time course of *Chlamydia* genital tract infection. Infection was monitored by swabbing the vaginal vault at various times after infection and by enumerating IFUs by isolation on HeLa cell monolayers. Inclusions were visualized by indirect immunofluorescence with an MoPn MOMP-specific MAb (MP-33b) and fluorescein-tagged goat anti-mouse IgG. Data are mean IFUs ± standard errors of the means for 6 to 12 animals per group. (A) C57BL/6 (●) and $\beta_2m^{-/-}$ (○) mice; (B) C57BL/6 (●) and class $II^{-/-}$ mice (△); (C) C57BL/6 (●) and $CD4^{-/-}$ (□) mice. 1°, primary infection; 2°, secondary infectious challenge.

different outcomes to primary genital tract infection were observed: $\beta_2m^{-/-}$ mice resolved infection in a manner similar to that for immunocompetent control mice (C57BL/6); class $II^{-/-}$ mice were unable to resolve infection; and $CD4^{-/-}$ mice showed delayed resolution of infection. In addition, all animals that recovered from primary infection showed marked protection to reinfection, as revealed by decreased shedding of infectious chlamydiae.

Histopathological evaluation and immune detection of chlamydiae. Figure 2A to I shows representative examples of the inflammation observed in genital tract tissues (vagina, uterine horn, and oviduct) at various times following primary infection. A marked acute suppurative response, consisting primarily of neutrophils, was observed early during infection (4 to 7 days postinfection). By 14 days postinfection, the inflammatory response was primarily subacute and the cellular infiltrate of the submucosal epithelium was composed predominantly of mononuclear cells (histiocytes and lymphocytes with few neutrophils). The inflammatory response waned in C57BL/6, $\beta_2m^{-/-}$, and $CD4^{-/-}$ mice as the infection subsided (42 days postinfection), but inflammation persisted in class $II^{-/-}$ mice. Oviduct ectasia and hydrosalpinx were frequently observed in all strains of mice by 5 to 8 weeks postinfection. These results are of interest because they demonstrate that tubal ectasia and hydrosalpinx could occur in the absence of T-cell-dependent responses (class $II^{-/-}$ mice). Therefore, perhaps, in the murine model of *Chlamydia* genital tract infection, the acute inflammatory response and its products might be significant contributors to disease pathogenesis and infertility.

Immunoperoxidase staining was used to evaluate and localize *Chlamydia* infection in genital tract tissue (Fig. 2J to L). Chlamydiae initially colonized the vaginal and cervical epithelium, but by 7 to 14 days the infection had ascended from the vagina to colonize the uterine horns and oviducts in all four mouse strains. Inclusions were invariably found to be localized to the mucosal epithelium and were not detected in any strain of mice once vaginal cultures were negative. *Chlamydia* inclusions were visualized in the genital tract epithelia of class $II^{-/-}$ mice throughout the study (>70 days).

Serological analysis following primary and secondary infectious challenge. An ELISA was used to determine whether differences existed among the mouse strains in immunoglobulin class and subclass specificities of the anti-*Chlamydia* antibody responses. The isotype specificities of the serological responses in C57BL/6 and $\beta_2m^{-/-}$ mice were similar, although $\beta_2m^{-/-}$ mice produced somewhat lower titers of IgG2a and IgG2b antibodies (Fig. 3A). Titers of IgM and IgG3 were low in both strains, and the IgG1 response was seldom positive (<16). MHC class $II^{-/-}$ mice, which did not recover from infection, consistently produced very low titers of IgM, IgG2b, and IgG3 antibodies, and their IgG1, IgG2a, and IgA responses were consistently negative (Fig. 3B). $CD4^{-/-}$ mice, which resolved infection more slowly than control mice, produced titers of anti-*Chlamydia* IgM, IgG2a, and IgG2b similar to those of control mice; however, serum IgA titers were not detected in $CD4^{-/-}$ mice until after they had become culture negative (Fig. 3C). The antibody responses of C57BL/6, $\beta_2m^{-/-}$, and $CD4^{-/-}$ mice were unchanged following secondary challenge, which supports the culture results demonstrating that mice were immune to reinfection.

Immunoglobulin class and subclass specificity of anti-*Chlamydia* antibodies in vaginal washes. Vaginal washes were analyzed by ELISA to evaluate potential differences in the secretory antibody responses of the four mouse strains. Anti-*Chlamydia* IgA antibodies were detected in vaginal washes from C57BL/6, $\beta_2m^{-/-}$, and $CD4^{-/-}$ mice during the resolution of genital tract infections (18 to 32 days postinfection) (Table 1). The presence of IgG2a and IgG2b antibodies in vaginal washes was quite variable, and these subclasses were detected only in some infected animals. Anti-*Chlamydia* antibodies were never detected in the vaginal washes of class $II^{-/-}$ mice, regardless of immunoglobulin class. At the time of and 15 days following secondary infectious challenge, C57BL/6, $\beta_2m^{-/-}$, and $CD4^{-/-}$ mice had anti-*Chlamydia* IgA in vaginal washes and were protected, whereas IgG2a and IgG2b antibodies were present only in the vaginal washes of some mice. Noteworthy is the observation that unlike C57BL/6 and $\beta_2m^{-/-}$ mice, $CD4^{-/-}$ mice had undetectable serum anti-*Chlamydia* IgA (Fig. 3C) until after the infection had resolved (>32 days); however, *Chlamydia*-specific IgA was detected in vaginal washes much earlier (<32 days) (Table 1). Collectively, these data demonstrated that a correlation existed between the presence of local (vaginal) IgA antibodies and the resolution of genital tract infection. Furthermore, the presence of anti-*Chlamydia* IgA in vaginal washes of $CD4^{-/-}$ mice in the absence of serum IgA (Table 1 [day 32]; Fig. 3 [day 32]) suggests that IgA from vaginal washes was produced locally and was not plasma derived.

DTH responses in gene knockout mice. The helper T-cell component of the immune response is divided into two functionally distinct populations (21): Th1 cells are involved in

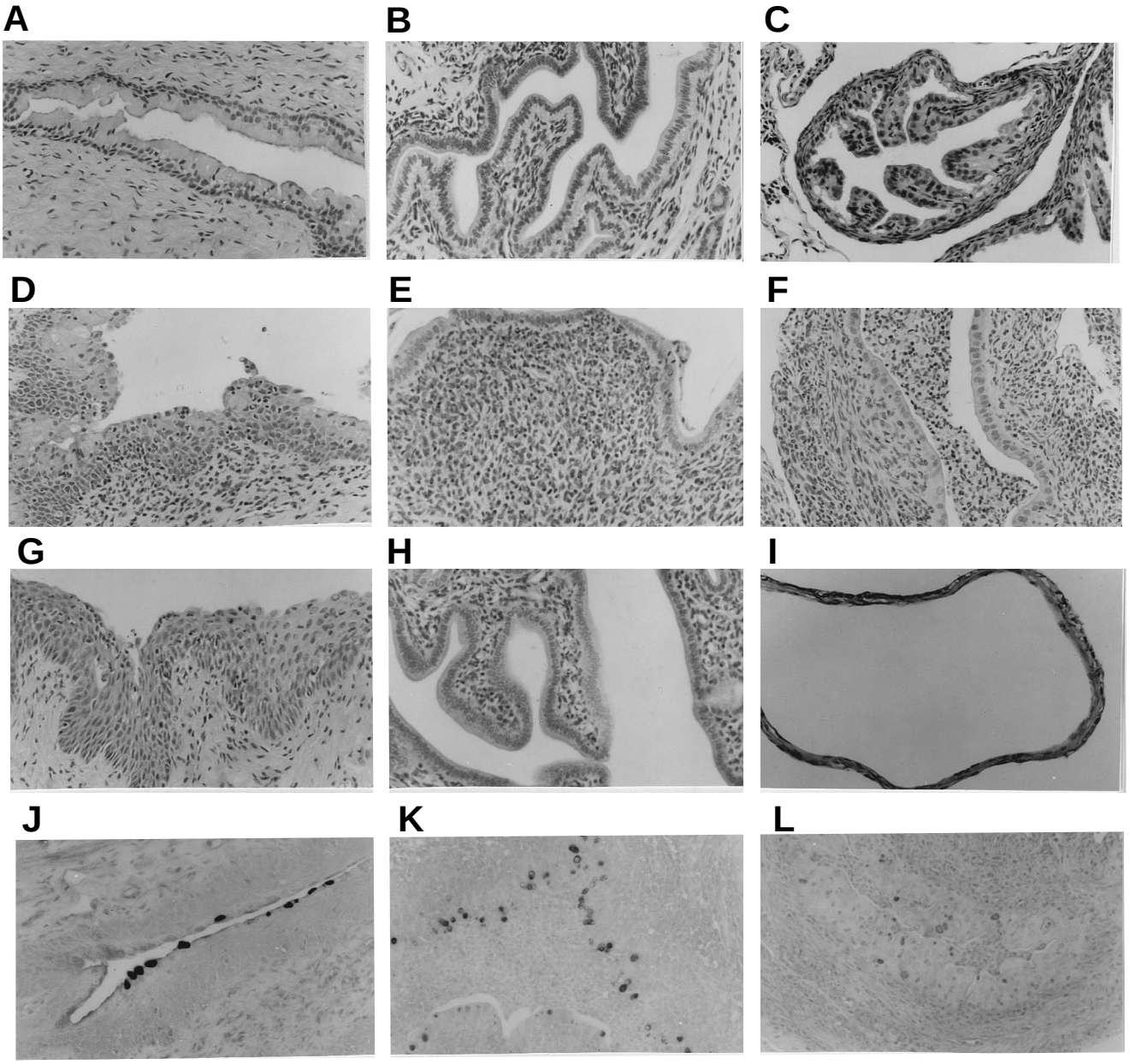

FIG. 2. Histopathological evaluation of *Chlamydia*-infected genital tract tissue. Representative examples of the inflammatory response observed in the vaginas (A, D, and G), uterine horns (B, E, and H), and oviducts (C, F, and I) of noninfected (A, B, and C), day 14 postinfection (D, E, and F), and day 42 postinfection (G, H, and I) C57BL/6 mice. Data are shown for C57BL/6 mice only. C57BL/6, $\beta_2m^{-/-}$, and $CD4^{-/-}$ were indistinguishable by histopathology; however, the genital tract tissues of class $II^{-/-}$ mice, which did not resolve infection, remained inflamed (acute to subacute) throughout the infection. *Chlamydia* inclusions were detected in epithelial cells of the vagina (J), uterine horns (K), and oviducts (L) by 7 days postinfection. Data are shown for C57BL/6 mice only; however, ascending infection was observed in all strains of mice.

DTH responses, and Th2 cells help in antibody class switching and synthesis. The data presented thus far demonstrated the importance of MHC class II-restricted T-cell responses in acquired immunity to *Chlamydia* infection and provided indirect evidence for a protective role of secretory IgA. To determine whether a DTH response (Th1 response) was elicited following infection of control or gene knockout mice, animals were challenged in the hind footpads with either heat-killed chlamydiae or buffer alone. Footpad swelling was not observed for any mice receiving buffer alone. However, C57BL/6 and $CD4^{-/-}$ mice, which recovered from primary infection, developed intense DTH responses when they were challenged with antigen ($P < 0.001$), whereas class $II^{-/-}$ mice, which did not recover from infection, failed to elicit positive responses (Fig. 4).

Histopathological studies of all mice that received buffer injections had normal dermal biopsies (data not shown). In mice receiving antigen, two distinct types of lesions were readily apparent (Table 2). Naive C57BL/6 (6 of 6) and infected class $II^{-/-}$ (6 of 6) mice developed only focal suppurative lesions associated with direct antigen deposition, which characteristically was composed of a dense focal infiltrate of

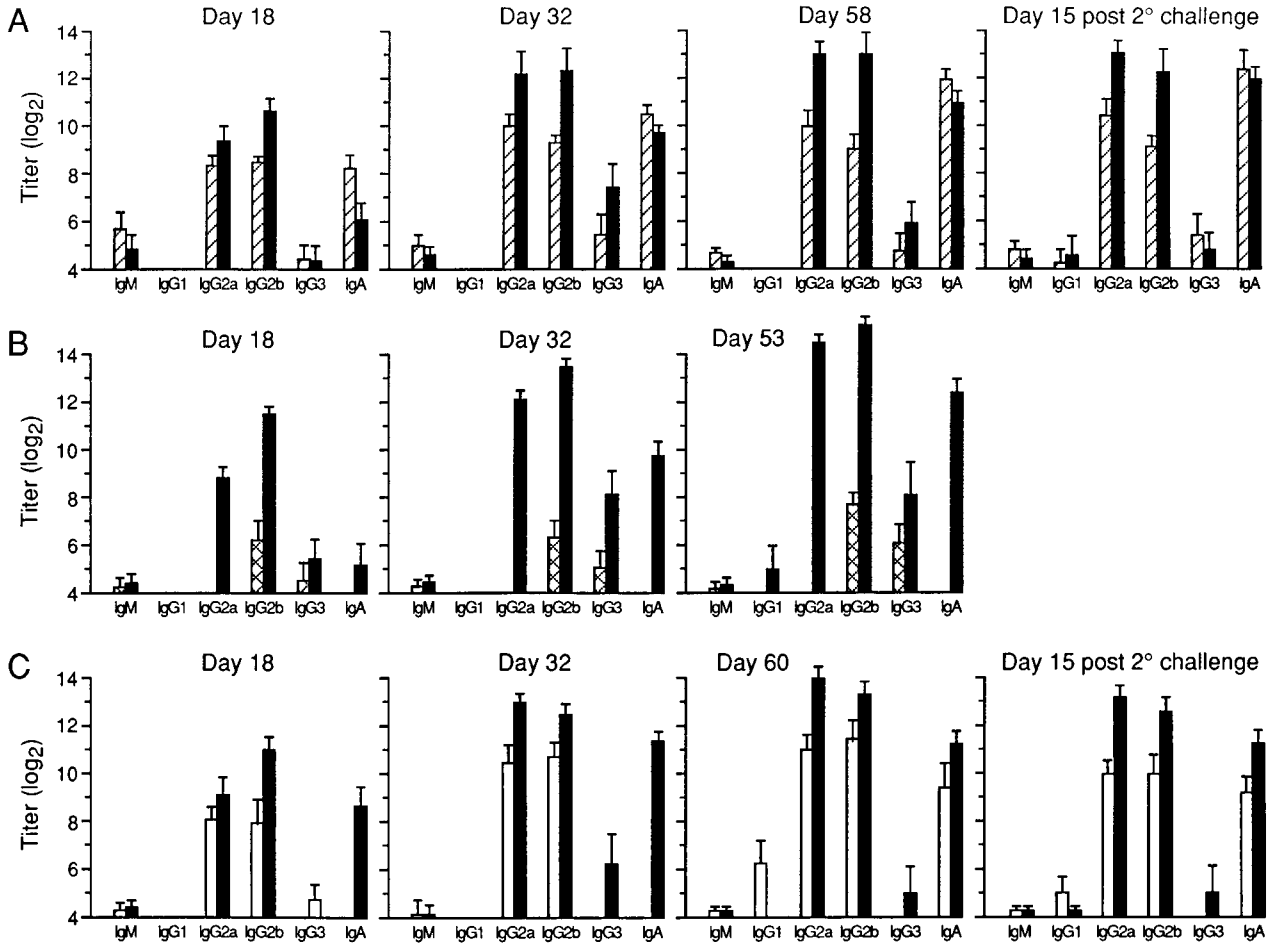

FIG. 3. Serum immunoglobulin class- and subclass-specific anti-*Chlamydia* responses of C57BL/6 (■), $\beta_2m^{-/-}$ (▨), class $II^{-/-}$ (☒), and $CD4^{-/-}$ (□) mice following primary (1°) and secondary (2°) infectious challenges. ELISA titers are expressed as the highest serum dilution giving an absorbance (OD_{405}) of at least 0.3 and 3 times that observed for sera of noninfected mice (OD_{405} consistently < 0.1). (A) C57BL/6 and $\beta_2m^{-/-}$ mice; (B) C57BL/6 and class $II^{-/-}$ mice; (C) C57BL/6 and $CD4^{-/-}$ mice. Days indicate times following primary or secondary challenge.

neutrophils with fewer eosinophils and histiocytes. Infected C57BL/6 (9 of 10) and $CD4^{-/-}$ (5 of 6) mice typically had lesions that contained a similar suppurative focus but that also contained superficial dermal interstitial and perivascular infiltrates of histiocytes, lymphocytes, and mast cells and moderate to marked focal dermal interstitial edema with variable fibrin exudation adjacent to the site of antigen deposition. Therefore, DTH responses developed in mice that recovered from *Chlamydia* genital tract infection. However, further investigations are needed to define more accurately the kinetics of the DTH response and whether Th1 or Th2 or both types of responses contribute to resolution of and immunity to *Chlamydia* infection.

DISCUSSION

Identification and characterization of immune responses that are necessary and sufficient to bring about the resolution of and protection against *C. trachomatis* infection are needed.

TABLE 1. Anti-*C. trachomatis* antibody responses in vaginal washes[a]

Mouse strain	Antibody response (no. of animals positive/total no. tested)											
	18 days post-primary challenge			32 days post-primary challenge			53 days post-primary challenge			15 days post-secondary challenge		
	IgG2a	IgG2b	IgA	IgG2a	IgG2b	IgA	IgG2a	IgG2b	IgA	IgG2a	IgG2b	IgA
C57BL/6	6/18	7/18	18/18	7/18	5/18	18/18	16/17	13/17	16/18	3/10	2/10	10/10
$\beta_2M^{-/-}$	0/6	1/6	5/6	1/6	0/6	6/6	2/6	0/6	6/6	1/6	0/6	6/6
MHC class $II^{-/-}$	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6	NT[b]	NT	NT
$CD4^{-/-}$	1/6	0/6	1/6	3/6	1/6	6/6	1/6	2/6	6/6	0/6	0/6	6/6

[a] Vaginal washes were collected and analyzed as described in Materials and Methods. Data are the numbers of mice with positive (class- or subclass-specific) anti-*Chlamydia* antibodies/total numbers of animals tested. Data are shown for IgG2a, IgG2b, and IgA only, since anti-chlamydial IgM, IgG1, and IgG3 were never detected in vaginal washes. Vaginal washes obtained prior to infection were negative for anti-*Chlamydia* antibodies.

[b] NT, not tested.

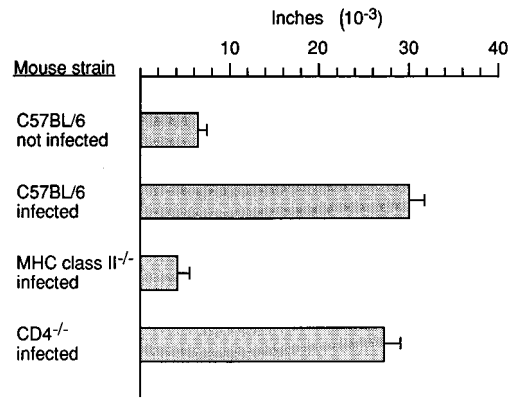

FIG. 4. DTH responses in normal and gene knockout mice. Seventy-four days post-primary infection, mice were tested for *Chlamydia*-specific DTH responses. Footpad swelling was measured at 24 h following antigen inoculation. Data are presented for antigen inoculation only. Footpad swelling was not observed in any infected or noninfected mice following buffer inoculation. Data are the means ± standard errors of the means for six to eight mice per group.

T cells, and in particular $CD4^+$ T cells, play a vital role in immunity to this obligate intracellular pathogen (19, 32). The cytokines produced by $CD4^+$ T cells influence immunoglobulin class switching and antibody production and are important in cell-mediated responses (21). Studies addressing the importance of effector immune responses in protective immunity to *Chlamydia* genital tract infection have been problematic. In vivo depletion of lymphocyte subpopulations suggests that $CD4^+$ T cells, $CD8^+$ T cells, or combinations of lymphocyte subpopulations are involved in the resolution of infection (19, 27, 28). In addition, attempts to transfer protective immunity by the passive administration of lymphocyte subpopulations have not provided definitive evidence for any single cell population (23, 28). In these studies, nonimmune and immune splenic lymphocytes, B cells, or T cells were equally capable of resolving *Chlamydia* genital tract infections in nude mice. Although no aspect of protective immunity to genital tract infection has been definitively identified, *Chlamydia*-specific T-cell clones have been isolated and found to resolve infections in nude mice, which are otherwise incapable of resolving infections (13, 28). The ability of these clones to provide protection from reinfection has not been explained, nor have the effector components of the immune response that contribute to the resolution of infection been identified. Because questions remain regarding the identification of elements of the protective immune response that confer immune protection, we chose to use mutant mice that fail to express MHC class I, MHC class II, or CD4 molecules on their cell surfaces to assess the importance of T-cell subpopulations on the development of protective immunity and acquired resistance to *Chlamydia* genital tract infections.

$CD8^+$ cytotoxic T lymphocytes (CTL) contribute importantly to the development of immunity to several intracellular bacterial pathogens (15). Because of the obligate intracellular lifestyle of *C. trachomatis*, it has been thought that CTL may also be involved in immunity to this pathogen. Past attempts to identify *Chlamydia*-specific CTL in vitro have been problematic (18, 24, 25). However, two laboratories have recently reported cytolysis of *Chlamydia*-infected cells by $CD8^+$ lymphocytes (3, 37). In one study, Beatty and Stephens found that the in vitro lysis of *Chlamydia*-infected cells by $CD8^+$ T cells could be detected only if the target cells were manipulated to express increased levels of ICAM-1 (3). Classical MHC class I-restricted cytolysis of *Chlamydia*-infected cells by a CTL line was demonstrated by Starnbach et al., and this CTL line had some protective activity when adoptively transferred into infected mice (37). Others have also reported some protective efficacy of $CD8^+$ T-cell clones or lines against *Chlamydia* genital tract infections of T-cell-deficient nude mice (12, 28).

Another approach to studying the involvement of MHC class I-restricted CTL responses in host immunity is to use $\beta_2m^{-/-}$ mice. These mutant mice have been used in other infectious disease models, and in many instances infection is exacerbated (9, 34). $\beta_2m^{-/-}$ mice have a profound deficiency in the development of mature $CD8^+$ T cells, although it does not appear to be absolute (17, 49). Following strong immunizations, such as allogeneic skin grafts or injection of live tumors, $\beta_2m^{-/-}$ mice develop $CD8^+$ T-cell responses (1, 48). Furthermore, $\gamma\delta$ T cells develop normally in $\beta_2m^{-/-}$ mice, and MHC class II-restricted cytotoxic T cells have been detected (7, 22). In a murine model of influenza virus infection, in which MHC class I-restricted $CD8^+$ CTL are primary mediators of virus clearance, $\beta_2m^{-/-}$ mice resolve infection, thus suggesting that alternative protective immune mechanisms exist (8). We found that $\beta_2m^{-/-}$ mice resolved *Chlamydia* genital tract infections with the kinetics of normal mice and were protected against rechallenge. These results do not necessarily argue against $CD8^+$ CTL activity in immunocompetent mice but instead suggest that MHC class I-restricted responses are not essential for either the resolution of genital tract infection or the development of acquired immunity. Indeed, previous experiments suggest that $CD8^+$ T cells provide some degree of protective immunity to *Chlamydia* infection (12, 28, 37). The mechanism by which $CD8^+$ T cells confer protection is unknown, and further studies are needed to determine if cellular cytotoxicity and/or the elaboration of the chlamydia-inhibitory cytokine gamma interferon (IFN-γ) contributes to the protective response.

To assess the role of helper T cells in the immune response to *Chlamydia* infection, we used mutant mice that lacked either MHC class II (class $II^{-/-}$) or CD4 ($CD4^{-/-}$) cell surface molecules. Class $II^{-/-}$ mice show a nearly complete absence of $CD4^{-/-}$ lymphocytes in the peripheral immune system and have a severely impaired ability to respond to T-cell-dependent antigens (11). However, normal to increased levels of peripheral $CD8^+$ T cells are found in class $II^{-/-}$ mice (11), and under certain conditions these mice clear virus infection and mount both primary and secondary virus-specific CTL responses (4). We found that class $II^{-/-}$ mice produced very low levels of *C. trachomatis*-specific antibody, had greatly diminished DTH responses, and were incapable of resolving genital tract infection. Therefore, in contrast to $\beta_2m^{-/-}$ mice, MHC class II-restricted responses are essential for the development of anti-*Chlamydia* protective immune responses. Conversely, $CD4^{-/-}$ mice have

TABLE 2. Histopathology of DTH responses[a]

Mouse strain	Histopathology grade		
	0 (normal)	1 to 2	3 to 4
Naive C57BL/6	0/4	4/4	0/4
Infected C57BL/6	0/10	1/10	9/10
Infected class $II^{-/-}$	0/6	6/6	0/6
Infected $CD4^{-/-}$	0/6	1/6	5/6

[a] Tissues were evaluated and graded as described in Materials and Methods. Data are shown for antigen challenge only, since all mice (infected and normal) that were challenged with buffer had normal biopsies. Data are the numbers of biopsies with a particular histopathology grade/total numbers examined. Infected mice were tested for DTH 74 days following primary infection.

impaired development of the CD4 T-cell lineage (~90% of the peripheral αβ T cells are $CD8^+$) and decreased but not absent helper T-cell activity (16). $CD4^{-/-}$ mice resolved infection (although resolution was somewhat delayed) and produced DTH responses to *Chlamydia* antigen that were indistinguishable, in both the intensity and the histopathology of the reaction, from responses elicited in infected control mice. *C. trachomatis*-specific antibodies were produced at nearly normal levels in $CD4^{-/-}$ mice; however, the anti-*Chlamydia* IgA responses in the vagina and serum were delayed. The resolution of genital tract infections in $CD4^{-/-}$ mice coincided with the production of vaginal IgA but not serum IgA. Our findings are consistent with previous studies showing that in women with uncomplicated *C. trachomatis* infections, an inverse correlation exists between *C. trachomatis*-specific secretory IgA and recovery of the organism from the cervix (5). Thus, we have demonstrated that MHC class II-restricted helper T-cell responses are a necessary component of host immunity to *Chlamydia* genital tract infection. In addition, a correlation exists between the presence of local (vaginal) but not serum anti-*C. trachomatis* IgA and the resolution of infection (Table 1).

Helper T cells are grouped into Th1- or Th2-type helper cells on the basis of the cytokines they secrete (21). Th1 cells are mediators of CMI responses, such as DTH, and secrete IFN-γ and interleukin-2 (IL-2). In contrast, Th2 cells secrete IL-4, IL-5, IL-6, and IL-10, which are involved in B-cell activation, growth, proliferation, and differentiation. The role of Th1 and Th2 helper T-cell responses in the resolution of murine *Chlamydia* genital tract infection and in acquired resistance to reinfection has not been firmly established. Recently, however, Igietseme et al. demonstrated that persistently infected T-cell-deficient nude mice resolved infection following the adoptive transfer of a $CD4^+$ Th1 T-cell clone (13). The protective Th1 clone secreted IL-2, tumor necrosis factor alpha, and IFN-γ, whereas a nonprotective Th1 T-cell clone secreted significantly lower levels of IFN-γ. Since IFN-γ inhibits *Chlamydia* growth (35, 43, 47), it was postulated that protection may have been mediated through this cytokine. A common feature of both $CD4^+$ and $CD8^+$ protective T-cell clones is the secretion of IFN-γ. Therefore, perhaps this cytokine is a necessary component of the immune-mediated resolution of *Chlamydia* infections. Although we found that MHC class II-restricted responses were necessary to resolve infection, additional studies are needed to determine the specific contributions of cytokines and Th1- and Th2-type responses.

Tubal scarring, ectopic pregnancy, and infertility are serious sequelae that frequently follow upper genital tract *Chlamydia* infections in women. Tubal scarring appears to result from the host's immune response to infection, but the precise immunological mechanism(s) is unknown. During the course of the present studies, we observed that mice in all groups (C57BL/6, $\beta_2m^{-/-}$, class $II^{-/-}$, and $CD4^{-/-}$) developed tubal ectasia and hydrosalpinx following infection. Therefore, ascending infection with the subsequent development of hydrosalpinx occurred regardless of the immune status of the animal. These data suggest either that there are multiple immune mechanisms that lead to tubal blockage or at least that in the murine model of infection, tubal blockage results from a T-cell-independent mechanism. A common feature of all mouse strains tested was the marked acute inflammatory response that accompanied ascending infection. Class $II^{-/-}$ mice do not resolve *Chlamydia* infection; however, a marked acute-subacute inflammatory response persists, and animals develop tubal blockage and hydrosalpinx. Therefore, the acute inflammatory response alone was not sufficient to bring about the resolution of infection but might have contributed to the development of hydrosalpinx. Further studies are needed to address the role of polymorphonuclear neutrophils and mediators of acute inflammation in the development of hydrosalpinx in this animal model.

The use of gene knockout mice has provided some useful insights into the host's immune response to *Chlamydia* infection. Although MHC class I-restricted $CD8^+$ CTL may be elicited and may function in normal mice, our results fail to substantiate an essential role for such responses in the resolution of *Chlamydia* genital tract infection. In contrast, MHC class II-restricted responses are absolutely required for immune protection. As with many infectious organisms, the immune response to chlamydiae is complex, and further characterization of T-cell and antibody responses and of antigens to which these responses are directed will be necessary for the design of effective immune intervention strategies.

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