

## Protection against Ascending Infection of the Genital Tract by *Chlamydia trachomatis* Is Associated with Recruitment of Major Histocompatibility Complex Class II Antigen-Presenting Cells into Uterine Tissue

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**A mouse model of ascending infection following intravaginal inoculation with a strain of *Chlamydia trachomatis* isolated from humans has been used to identify immune mechanisms associated with protection against genital infection. BALB/c and C3H mice differed in their susceptibilities to infection and inflammatory disease. In both mouse strains, ascension of the organism and recruitment of bone marrow-derived mononuclear leukocytes were evident in uterine tissue 1 week postinfection. By 3 weeks the organism had been cleared and inflammation had been resolved in the BALB/c mice, but both persisted in the C3H animals. In athymic nude BALB/c mice both the organism and inflammation persisted, indicating the influence of the hosts' immune response on the outcome of infection. Both BALB/c and C3H mice had a Th1 response in draining lymph nodes, with predominant production of gamma interferon and tumor necrosis factor alpha, low levels of interleukin-10, and no detectable levels of interleukin-4. However, the composition of the early uterine infiltrate differed in these two mouse strains. Cell surface labeling and analysis of light scatter properties by flow cytometry identified a population of large, CD45<sup>+</sup> major histocompatibility complex class II mononuclear cells, which were a prominent feature of the infiltrates in BALB/c mice but were present in significantly lower numbers in C3H mice. These cells expressed the costimulatory molecules CD86 and CD40 and stimulated allogeneic T cells, suggesting that these mononuclear cells are a population of antigen-presenting cells and that they may play a role in clearing antigen and protecting against inflammatory disease in BALB/c mice. An additional level of immunological control may thus exist in genital chlamydial infection.**

*Chlamydia trachomatis* is an obligate intracellular gram-negative bacterium which selectively colonizes epithelial cells in the human host. Infection of the genital tract with *C. trachomatis* serovars D through K is a major cause of sexually transmitted disease worldwide. Infection is insidious and, though often asymptomatic, can have serious consequences particularly for women. In some cases of cervical infection the organism ascends into the upper genital tract; this is a major cause of pelvic inflammatory disease with sequelae that include infertility and ectopic pregnancy (21).

Left untreated, genital chlamydial infections are chronic, and repeated infections are common, indicating that the natural immune response is poorly protective. However, the incidence of genital chlamydial infection falls with increasing age; this might be due to cumulative serovar-specific immunity mediated by a local antibody (3). Antibodies can neutralize infectivity in vitro and in vivo (40) but have not been identified as the dominant protective mechanism in animal studies; antibodies play little part in protection against primary infections (12, 26, 31), although they can protect against severe pathology (7) and can play a subsidiary role in defense against reinfection (31).

A predominant role for Th1 CD4<sup>+</sup> T cells and the produc-

tion of gamma interferon (IFN- $\gamma$ ) in controlling primary genital infection and preventing spread to other tissues has been implicated in cell transfer studies, antibody-mediated depletion experiments, and infections in gene knockout mice (8, 12, 15, 19, 30). However, in the absence of a functioning IFN- $\gamma$  system a poorly defined compensatory mechanism can operate (12, 37) and a mechanism dependent on interleukin-12 (IL-12) but independent of IFN- $\gamma$  may thus be important in the early stages of infection (25). IFN- $\gamma$  is less important in protection against reinfection than against a primary challenge (8, 37). Thus, a successful immune response against chlamydial infection is flexible and complex, with different mechanisms involved as the infection progresses.

Studies of infection in mouse strains with different susceptibilities to disease have proved useful in identifying protective immune mechanisms. We have identified mouse strain differences in disease susceptibility following intrauterine (i.u.) injection with a serovar-F strain of *C. trachomatis* from humans; C3H mice developed severe disease with prolonged salpingitis resulting in infertility, whereas BALB/c suffered less-severe inflammatory changes and remained fertile (32). Because direct injection of the organism into the upper genital tract did not allow us to distinguish between increased susceptibility to infection and sensitivity to pathological reactions, we developed a model of ascending infection following intravaginal (i.vag.) inoculation of this strain of *C. trachomatis*. Ascension of the organism into the uterus and oviducts of C3H mice was detected in association with inflammatory changes in genital tract tissue (29).

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In the present study we compare ascending infections in C3H and BALB/c mice and report increased susceptibility in C3H mice despite the development of a predominantly Th1 response and the production of IFN- $\gamma$  in the lymph nodes of both mouse strains. Instead, clearance of the organism and protection against inflammatory disease appeared to be associated with recruitment of major histocompatibility complex (MHC) class II antigen-presenting cells (APC) into uterine tissue early in infection. These cells may possibly play a role in the defense against genital chlamydial infection.

#### MATERIALS AND METHODS

**Mice.** Female BALB/c (*H-2<sup>d</sup>*), C3H (*H-2<sup>k</sup>*), BALB/c *nu/nu* (athymic, *H-2<sup>d</sup>*), and C57BL/10 (*H-2<sup>b</sup>*) mice aged 6 to 10 weeks and bred and housed at Northwick Park Institute for Medical Research were used for all experiments.

**Chlamydia strains.** For infection experiments, *C. trachomatis* N11 (serovar F [34]) was grown by centrifuge-assisted inoculation of McCoy cell monolayers in Dulbecco's minimal essential medium supplemented with 10% fetal calf serum (FCS), L-glutamine (100  $\mu$ g/ml), gentamicin (25  $\mu$ g/ml), and cycloheximide (1  $\mu$ g/ml). Elementary bodies (EB) were harvested after 48 h of culture and stored in sucrose-phosphate buffer (2SP) under liquid nitrogen. The infectious titer, expressed as inclusion forming units (IFU) per milliliter, was determined by titration on McCoy cell monolayers and enumeration of inclusions by dark ground microscopy following Giemsa staining. For *in vitro* assays, *C. trachomatis* L1/440/LN was grown in buffalo green monkey kidney cells, purified by centrifugation (80,000  $\times$  g, 4°C, 60 min) through 25% (vol/vol) Isopaque (Nycomed, Oslo, Norway), and inactivated by UV irradiation. The protein concentration was determined by the Lowry method.

**Infection.** Mice were pretreated with progesterone (Depo-Provera; Upjohn, Kalamazoo, Mich.) in order to enhance infection by maintaining target epithelium and to stabilize the reproductive tract in diestrus for immunological studies (35). Mice were given two 2.5-mg injections 7 days apart and infected on the same day as the second injection. For *i.v.* inoculation, mice were first anesthetized with a mixture of 1 part Hypnorm (fentanyl-fluanisone; Janssen Pharmaceuticals, Ltd., Olen, Belgium), 2 parts water, and 1 part Hypnovel (midazolam; Roche Products, Ltd., Hertfordshire, United Kingdom) in a dose of 0.1 ml/30 g administered intraperitoneally. Either N11 in 2SP or 2SP alone (25  $\mu$ l) was introduced into the vagina by using a pipette tip. Spermicide-free lubricating jelly was smeared over the vaginal opening to maximize retention, and the mice were maintained in an inverted position until recovery from anesthesia.

**Detection of infection.** Colonization of the lower genital tract was assessed by swabbing the vagina with nasopharyngeal swabs (Medical Wire and Equipment Co.) at 6 to 7 days postinfection. The swab was expressed in 1 ml of cold 2SP medium and then stored in liquid nitrogen until being cultured on McCoy cells as described above. Results are presented as the number of inclusions per monolayer (0.5 ml of the expressed swab). The statistical significance of the differences in the recovery of organisms was assessed by using an unpaired *t* test.

**Assessment of ascending infection.** Viable organisms were detected in uterine homogenates, which had been prepared in 2SP with a ground-glass homogenizer, by titration on McCoy cells as described above. The results are presented as IFU/monolayer. Each monolayer comprised approximately  $1.5 \times 10^6$  McCoy cells, and 0.5 ml (50%) of the homogenate was added.

**Detection of chlamydial DNA.** Nested PCR was used to detect chlamydial plasmid DNA as previously described (29), except that the sample preparation was modified as follows: 75  $\mu$ l of each homogenate was washed in Tris-buffered saline and resuspended in 50  $\mu$ l of a lysis buffer comprising 0.5% Nonidet P-40, 0.5% Tween-20, and 100  $\mu$ g of proteinase K per ml. Then, 5  $\mu$ l was used for the PCR. The primers for the first round of amplification were 5'-CCG ATG CAA GAT ATC GAG TAT GCG TTG TTA GG-3' and 5'-GAC CGG CCT CTA GCG CTG CG-3', amplifying a 473-base product (20). For the second round the nested set of primers were 5'-GAC TCT ACT GAG TAT ATT CTG AGG C-3' and 5'-CTA TCA AGC CTT CCC TTT ATA CGC-3', yielding a product of 377 bases. All PCRs were performed in 50 mM KCl-10 mM Tris-HCl-1.5 mM MgCl<sub>2</sub>-1% Triton X-100 (pH 8.8) with the addition of 1  $\mu$ M (each) primer, 0.4 mM deoxynucleoside triphosphate, and 1 U of *Taq* polymerase. Each round comprised 35 cycles of 94°C for 1 min, 57°C for 2 min, and 72°C for 4 min. PCR products were resolved by 1% agarose gel electrophoresis and DNA stained with ethidium bromide. Negative (water) and positive controls (DNA from *C. trachomatis*-infected cells) were included in each experiment.

**Flow cytometry to detect bone marrow-derived MC in uterine tissue.** The accumulation of CD45<sup>+</sup> mononuclear cells (MC) in genital tract tissue following infection with *C. trachomatis* was measured by flow cytometry. Uteri were placed in phosphate-buffered saline (PBS) on ice, cut into small pieces, and incubated in Ca- and Mg-free PBS containing 10 mM EDTA (pH 7.2) for 90 min at 37°C with gentle shaking. A single-cell suspension was obtained by pressing the tissue pieces through a 40- $\mu$ m (pore size) cell strainer (Falcon, Franklin Lakes, N.J.), and MC were obtained by centrifugation (1,250  $\times$  g, 20 min, room temperature) over Lympholyte-M (Cedarlane Laboratories, Toronto, Ontario, Canada). Cells in suspension were counted, resuspended in PBS supplemented with 1 mM

EDTA, 2% heat-inactivated FCS, and 0.02% sodium azide (PFAE). The cells were then incubated on ice with 20  $\mu$ l of heat-inactivated normal mouse serum and 1.5  $\mu$ l of Fc-Block (PharMingen, San Diego, Calif.) for 15 min to reduce the nonspecific binding of monoclonal antibodies. Cells were labeled with Cy-Chrome-conjugated rat anti-mouse CD45 (leukocyte common antigen; PharMingen) or the appropriate isotype control (Cy-Chrome-conjugated rat immunoglobulin G2b from PharMingen) at 4°C before being washed in ice-cold PFAE and fixed in 1% paraformaldehyde in saline (pH 7.4). Cells were analyzed on a FACScan flow cytometer (Becton Dickinson) with CellQuest software. The number of CD45<sup>+</sup> cells was calculated as the number of MC isolated times the proportion CD45<sup>+</sup> cells (as counted by a fluorescence-activated cell sorter), and the increase above the number in uninfected tissue was calculated by subtracting the mean ( $n \geq 2$ ) value for sham-infected controls.

**Histology.** Samples of oviduct and distal uterus were fixed in 10% buffered formalin and embedded in paraffin. Sections (4  $\mu$ m) were cut through various levels of the block, stained (hematoxylin and eosin), and examined in a "blinded" fashion. Inflammatory changes in oviducts were graded as follows:  $\pm$ , small patches of inflammatory cells in the mesovarium; +, patchy infiltrate in the ovarium, increased infiltration of the mesovarium, and the occasional presence of polymorphonuclear leukocytes (PMN) in the lumen; and ++, large numbers of inflammatory cells in the lumen and heavy infiltration of the mesovarium and mucosa, with mucosal folds still present. Severe salpingitis and hydrosalpinx formation, observed following *i.u.* infection, were not observed in these experiments. Uterine lesions were graded as follows:  $\pm$ , occasional inflammatory cells; +, patchy infiltrate; ++, moderate infiltrate with PMN in the lumen; or +++, severe infiltrate with exudate of PMN and lymphocytes in the lumen and involvement of the mucosa and ducts.

**Secondary responses of LNC.** Pooled draining (iliac) lymph nodes from groups of five infected or sham-infected mice 7 to 21 days posttreatment were pressed through cell filters (Falcon, Franklin Lakes, N.J.) into complete medium containing RPMI 1640 (Dutch modification; Sigma) supplemented with 10% (vol/vol) heat-inactivated FCS, L-glutamine (100  $\mu$ g/ml), penicillin (100 U/ml), streptomycin (100  $\mu$ g/ml), and  $5 \times 10^{-5}$  M 2-mercaptoethanol. Triplicate 20- $\mu$ l hanging drops containing 25,000 lymph node cells (LNC) per well were established in Terasaki plates (13), and UV-inactivated EB (serovar L1) were added at between 0.005 and 50  $\mu$ g/ml. Control cultures contained no antigen. Plates, inverted over saline, were incubated for 3 days at 37°C in a humidified atmosphere of 5% CO<sub>2</sub> in air, pulsed for 2 h with 1  $\mu$ l of [<sup>3</sup>H]thymidine (equivalent to 1  $\mu$ g of thymidine per ml at a specific activity of 2 Ci/mmol), and harvested by blotting onto filter papers. This technique, *i.e.*, the use of low-specific-activity thymidine in flooding conditions for a short pulse time, resulted in low counts that reflected the level of DNA synthesis without the complication of limiting availability of thymidine or excessive radiation damage (13). Radioisotope incorporation was determined by liquid scintillation counting.

**Detection of secreted cytokines.** Supernatants from 3-day hanging-drop cultures with or without 0.5  $\mu$ g of L1 EB per ml were analyzed by enzyme-linked immunosorbent assay (ELISA) for IFN- $\gamma$ , IL-4 (Genzyme, Cambridge, Mass.), and IL-10 (Ultra-Sensitive kit; Biosource International, Camarillo, Calif.) according to the manufacturers' protocols.

**Intracellular cytokine staining.** Cells from pooled iliac nodes of *i.v.*-infected mice were cultured with 0.5  $\mu$ g of L1 EB per ml at 10<sup>6</sup>/ml in 1-ml cultures in 24-well plates (Falcon, Franklin Lakes, N.J.). Monensin (3  $\mu$ M) was added for the final 2 h of culture before the cells were recovered after a total incubation of 96 h at 37°C. The cells were stained with fluorescein isothiocyanate (FITC)-conjugated antibodies to cell surface antigens before fixation and permeabilization with PermeaFix (Ortho, Raritan, N.J.) according to the manufacturer's instructions. The cells were stained with phycoerythrin-conjugated monoclonal antibodies to IFN- $\gamma$ , IL-4, IL-10, TNF- $\alpha$ , or isotype-matched control monoclonal antibodies (PharMingen). In some experiments excess exogenous cytokine was added or the permeabilization step was omitted to demonstrate the specificity of the labeling. Analysis was performed with the FACScan, and the cytokine labeling of the blast cells, which were identified on the basis of their light scatter properties, was determined.

**Phenotypic analysis.** Antibodies to the following markers were used: H-2A, CD11a, CD11b, CD11c, CD4 (clone RM4-4), CD8 $\alpha$ , CD19, CD45R/B220, CD40, CD80, CD86,  $\beta$ 7 integrin chain, CD103 (integrin  $\alpha_{IEL}$  chain),  $\alpha\beta$ TCR, and  $\gamma\delta$ TCR (all from PharMingen) and F4/80 (Serotec, Oxford, United Kingdom). The NLDC-145 hybridoma (14) was kindly provided by George Kraal (Free University, Amsterdam, The Netherlands). Isotype-matched control labelings were performed for all antibodies. Pools of cells from five or more animals were used for phenotypic analysis of uterine CD45<sup>+</sup> mononuclear cells. Flow cytometry was performed as described above, and backgating on CD45<sup>+</sup> cells was used to identify subpopulations with different light scatter properties.

**Isolation of CD11b<sup>+</sup> cells.** CD11b<sup>+</sup> cells were positively selected from uterine mononuclear cell suspensions by using the MiniMacs system (Miltenyi Biotec, Bisleigh, United Kingdom). Cells were labeled with FITC-conjugated anti-CD11b (PharMingen) in ice-cold PBS supplemented with 5 mM EDTA, and 0.5% bovine serum albumin. After the cells were washed twice in the same buffer, they were labeled with immunomagnetic anti-FITC beads (Miltenyi), and positive cells were selected by using the MiniMacs column according to the manufacturer's methodology. Following separation, more than 90% of the CD45<sup>+</sup> cells were found to be CD11b<sup>+</sup>.

TABLE 1. Ascending infection in control and *C. trachomatis* N1-infected mice<sup>a</sup>

Strain	Vaginal swab at day 6, % positive (mean IFU) <sup>b</sup>	PCR (% positive) at days:		Uterine culture, % positive (mean IFU) <sup>b</sup> at days:		Histology, % positive (severity)	
		7-10	21-24	7-10	21-24	Uterus	Oviduct
<b>N1 infected</b>							
BALB/c <i>nu/nu</i>	100 (25)	90	100	50 (14.9)	60 (7.3)	40 (± to +++)	30 (± to ++)
BALB/c +/+	60 (6.9)	80	20	0	0	0	0
C3H +/+	85 (6.2)	80	90	10 (11)	33 (9.7)	50 (+ to ++)	0
<b>Control</b>							
BALB/c <i>nu/nu</i>	0	0	0	0	0	ND	ND
BALB/c +/+	0	0	0	0	0	ND	ND
C3H +/+	0	0	0	0	0	ND	ND

<sup>a</sup> Mice were infected by i.vag. inoculation of  $4.5 \times 10^5$  IFU of *C. trachomatis* N1. Control animals were sham infected with vehicle alone. ND, not determined.

<sup>b</sup> Mean IFU = IFU per monolayer (0.5 ml per homogenate per swab).

**Allogeneic mixed leukocyte reaction (MLR).** CD11b<sup>+</sup> uterine mononuclear cells were irradiated (20 Gy) and used to stimulate allogeneic (C57BL/6 *H-2<sup>b</sup>*) LNC in hanging-drop cultures. Between 100 and 3,000 irradiated stimulator cells were added per well to 50,000 LNC in 20  $\mu$ l of complete medium. Control wells contained LNC alone. Proliferation after 3 days in culture was assayed by measuring [<sup>3</sup>H]thymidine incorporation as described above. Stimulation by mature spleen dendritic cells (DC), prepared by overnight culture of BALB/c spleen cell suspensions and isolation of low-density cells on metrizamide gradients, was assayed in parallel.

## RESULTS

**Mouse strain variation in ascending infection.** Disease is more severe in C3H mice than in BALB/c mice following i.u. infection with *C. trachomatis* N1, so we compared ascending infection after i.vag. inoculation in these two strains. Athymic BALB/c *nu/nu* mice were also included to determine the requirement for T cells in the control of ascending infection (Table 1). Groups of 20 animals were inoculated i.vag. with  $4.5 \times 10^5$  IFU, and the proportion of animals infected was determined by culture of vaginal swabs obtained 6 days postinfection. Approximately 60 to 100% of the animals were infected depending on the mouse strain. Significantly greater numbers ( $P < 0.05$ ) of viable organisms were recovered from BALB/c *nu/nu* mice than from immunologically intact BALB/c animals, suggesting that a T-cell-dependent immune response is involved in protection during the early stages of infection. A higher proportion of C3H mice than of immunologically intact BALB/c animals was colonized, but the recovery of viable organisms did not differ significantly between these two strains.

Ascending infection was detectable by PCR in almost all animals at one week postinfection, but chlamydial DNA was no longer detectable in the uteri of the majority of BALB/c mice by 3 weeks (Table 1). In contrast, DNA persisted in the uterine tissue of both BALB/c *nu/nu* and C3H mice, and ascending viable organisms were also recovered from these two mouse strains.

There was no evidence of histological disease in BALB/c mice, but mild inflammatory changes were observed in the uteri of half of the C3H mice. More severe uterine inflammation and infiltrate in the oviducts was observed in the BALB/c *nu/nu* mice (Table 1).

In summary, the results of this experiment demonstrated that there was a hierarchy of susceptibility to ascending infection: BALB/c *nu/nu* > C3H > BALB/c. This confirms the pattern of disease susceptibility observed following i.u. inoculation.

**Accumulation of CD45<sup>+</sup> MC in uterine tissue following i.vag. inoculation.** At 3 weeks postinfection, the number of

CD45<sup>+</sup> MC found in uterine tissue corresponded to the picture observed histologically; marked infiltrates were observed in BALB/c *nu/nu* and C3H mice but were significantly less ( $P < 0.001$ ) in BALB/c animals (Fig. 1). The numbers of CD45<sup>+</sup> MC obtained from BALB/c *nu/nu* and C3H mice did not differ significantly at this time point.

In contrast, a CD45<sup>+</sup> MC infiltrate was evident in the uterine tissues of all three strains earlier in the infection (Fig. 1). There was some variation in the mean sizes of infiltrate, but only the difference between the BALB/c *nu/nu* and the C3H mice reached statistical significance ( $P < 0.05$ ). There was a 7- to 14-fold increase in the number of these cells in infected animals compared with control animals assessed in parallel. By between 7 to 10 days and 21 to 24 days the BALB/c infiltrate resolved ( $P < 0.005$ ), but infiltrates for the other strains were not significantly changed.

**Both BALB/c and C3H mice produced Th1 responses in draining lymph nodes.** LNC from the draining (iliac) nodes of N1-infected but not sham-infected BALB/c and C3H mice mounted secondary responses in vitro when restimulated with inactivated serovar L1 EB (Fig. 2A). L1 EB, which are easier to grow and purify than N1 EB, were used for these in vitro studies because T-cell responses against *Chlamydia* sp. are predominantly against common, rather than serovar-specific, epitopes. Moreover, these two strains were grown on different cell lines and the potential problem of sensitization to cell antigens was reduced. Figure 2 illustrates the responses at 17 days postinfection. In additional experiments, responses were also observed at days 7, 14, and 21. These LNC responses were maximal at days 14 to 17, but no response was observed in spleen cells or inguinal lymph nodes at any of these time points (data not shown). Overall, the responses were not consistently greater in one strain than in the other.

Analysis of cytokines in supernatants from these hanging-drop cultures by ELISA indicated predominant production of IFN- $\gamma$ , with a small amount of IL-10; IL-4 was not detectable (<6.6 pg/ml) (Fig. 2B). Similar results were obtained with cells from both mouse strains, and the amount of IFN- $\gamma$  produced reflected the amount of LNC proliferation (Fig. 2A). A similar dominance of the Th1 cytokine was also observed in larger-scale 1-ml cultures, suggesting that the results obtained were not confined to the hanging-drop culture system (data not shown).

Production of cytokines was also evaluated by intracellular cytokine staining. LNC from infected mice were restimulated with EB, and FACSscan analysis was performed on activated blast cells gated on the basis of size and granularity. Similar

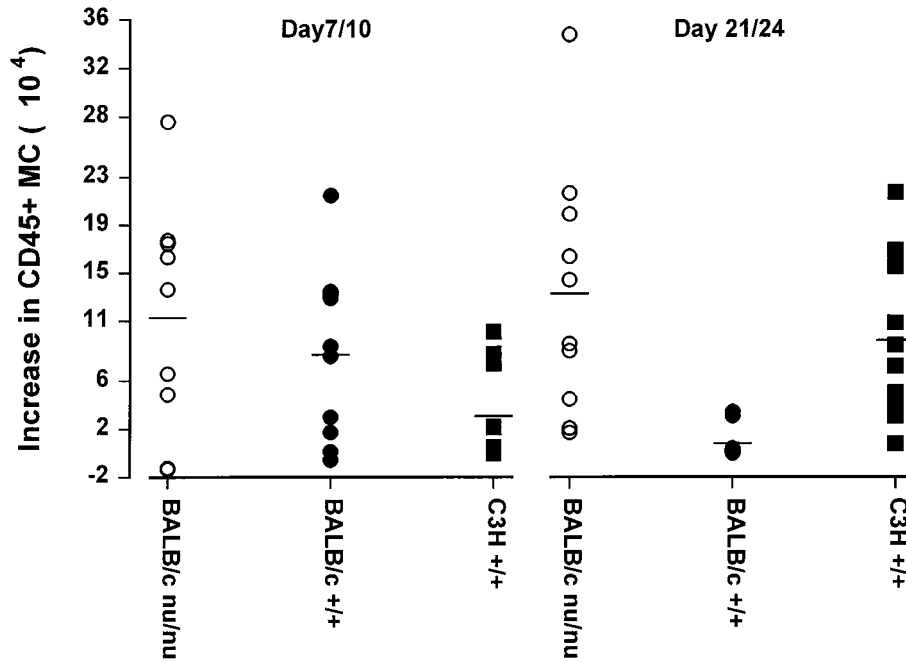


FIG. 1. Mouse strain variation in uterine CD45<sup>+</sup> MC infiltrate. Each symbol represents the results of an individual mouse. Uteri were obtained 7, 10, 21, and 24 days postinfection with  $4.5 \times 10^5$  IFU of *C. trachomatis* NI1 or with administration of vehicle alone. MC were obtained, and the percentages of CD45 staining were determined by flow cytometry. CD45<sup>+</sup> MC numbers were determined as follows: number of MC recovered times the proportion of CD45<sup>+</sup> cells. The increase in CD45<sup>+</sup> MC numbers in infected animals was obtained by subtracting a mean value ( $n = 2$ ) for uninfected mice of the appropriate strain. The results were indistinguishable at days 7 and 10 and also at days 21 and 24, and so the results were pooled for an early and late time point. The lines represent the mean values for each group.

staining was observed with cells from BALB/c and C3H mice; over 80% of the cells stained positive for TNF- $\alpha$  and 34 to 36% of the cells were  $\alpha\beta$ TCR, TNF- $\alpha$  positive (Fig. 3A). Staining could be completely blocked by the addition of excess recombinant TNF- $\alpha$ , and no labeling of nonpermeabilized cells was

observed (Fig. 3C). In addition, 80 to 85% of the  $\alpha\beta$ TCR<sup>+</sup> cells within this gate were positive for TNF- $\alpha$  (Fig. 3A), but only 5 to 7% of  $\alpha\beta$ TCR<sup>+</sup> small lymphocytes were positive for TNF- $\alpha$  (data not shown). These data suggest that the labeling observed with anti-TNF- $\alpha$  represents specific staining of intra-

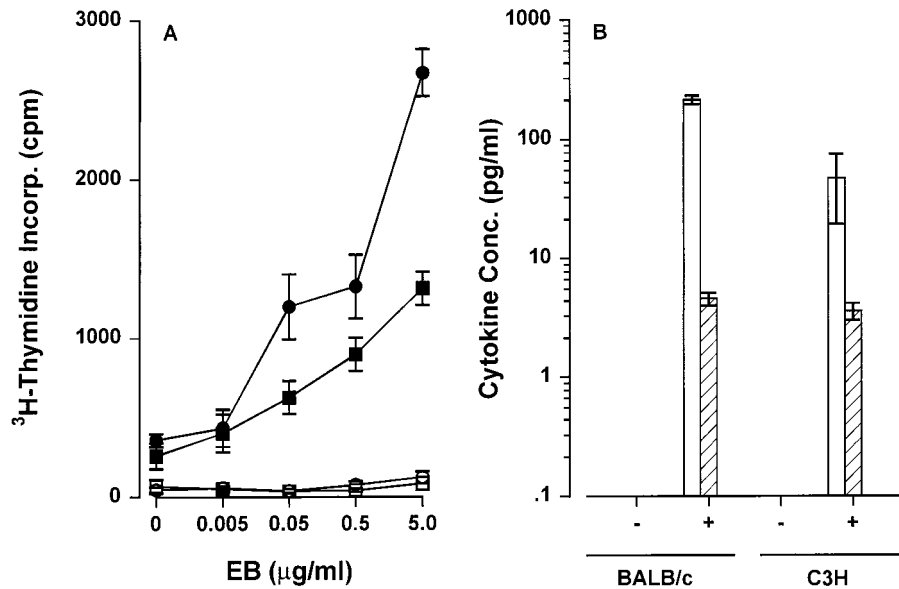


FIG. 2. Proliferative responses (A) and cytokine production (B) by pooled draining lymph node cells taken from groups of 5 mice 17 days post-i.vag. infection with  $4.5 \times 10^5$  IFU. Symbols (panel A): ●, BALB/c EB infected; ■, C3H EB infected; ○, BALB/c sham infected; □, C3H sham infected. Error bars encompass the standard deviation of triplicate cultures. In panel B, the LNC from EB-infected mice cultured with EB (+) or medium alone (-) are as indicated. The cytokines detected were IFN- $\gamma$  (□) and IL-10 (▨). No IL-4 was detected. Error bars encompass the standard deviation of duplicate wells. No cytokines were detected in the cultures of cells from uninfected mice. Results representative of two experiments are shown.

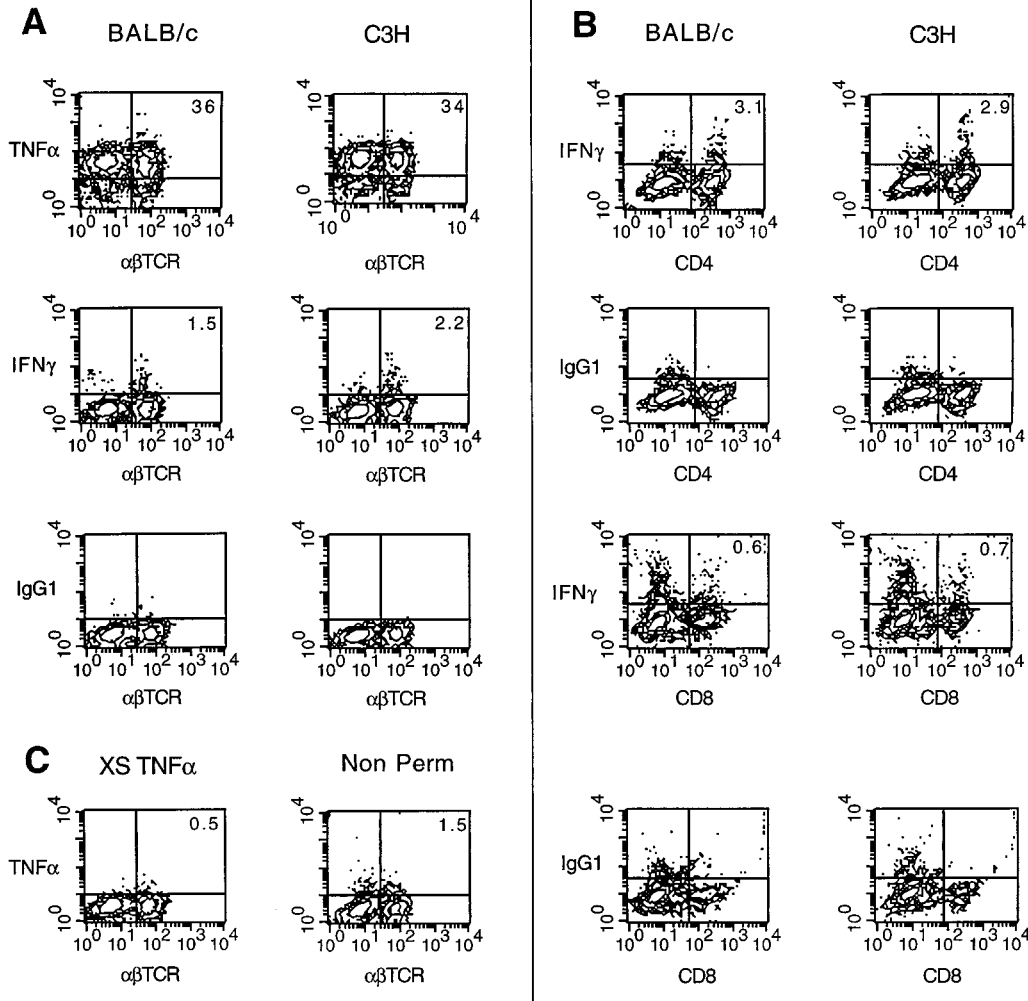


FIG. 3. Intracellular cytokine staining of LNC from mice infected with  $3.5 \times 10^5$  to  $4.5 \times 10^5$  IFU of *C. trachomatis* N11 and restimulated in vitro with 0.5  $\mu$ g of inactivated EB per ml. LNC were obtained 13 to 17 days postinfection. Gates for analysis were set on large cells on the basis of light scatter. (A) Double labeling with anti- $\alpha\beta$ TCR antibody. (B) Double labeling with anti-CD4 and anti-CD8. (C) Specificity controls for labeling of intracellular TNF- $\alpha$  (blocking with excess TNF- $\alpha$  and the need for permeabilization). Cells were from BALB/c mice and the control TNF- $\alpha$  labeling is shown in panel A. Each graph shows the percent positive cells in the upper right quadrant following subtraction of the isotype control staining results.

cellular cytokine produced following stimulation with *C. trachomatis*.

Small numbers of LNC blasts producing IFN- $\gamma$  were consistently observed in suspensions from both strains of mice (Fig. 3A and B), with the majority of these cells staining positively for CD4 (Fig. 3B). In contrast, IL-10 and IL-4 were not detected (data not shown). Together, these findings suggest that genital tract infection of both BALB/c and C3H mice stimulates a response in the draining lymph nodes with the production of TNF- $\alpha$  and predominantly Th1-associated cytokines.

**Mouse strain variation in uterine infiltrates.** Uterine MC suspensions indicated two major CD45<sup>+</sup> cell populations as determined by their light scatter properties: population 1 (R1) comprised small agranular cells, and population 2 (R2) comprised larger, more granular cells (Fig. 4). Comparison of light scatter plots for the individual mice used in the experiment illustrated in Fig. 1 revealed strain differences in the proportions of these two populations (Fig. 4). Whereas early uterine CD45<sup>+</sup> MC infiltrates from infected BALB/c mice comprised approximately equal proportions of R1 and R2 cells (ratio of

R2 to R1 =  $1.2 \pm 0.6$ ), the larger R2 cells were significantly underrepresented in C3H infiltrates; the ratio of R2 to R1 cells was significantly reduced in C3H mice compared to BALB/c mice ( $0.5 \pm 0.1$ ;  $P < 0.01$ ; Fig. 4), as was the absolute number of CD45<sup>+</sup> R2 MC ( $P < 0.05$ ; data not shown), suggesting a lack of accumulation of cells in R2 rather than increased numbers of R1 cells. This idea is supported by the observed trend for larger numbers of infiltrating CD45<sup>+</sup> MC in BALB/c rather than C3H uteri at days 7 and 10, although this difference did not reach statistical significance (Fig. 1) and by the cell surface phenotyping of the CD45<sup>+</sup> MC infiltrate (Fig. 5). BALB/c *nu/nu* infiltrates contained a significantly higher proportion ( $P < 0.001$ ) and number ( $P < 0.01$ ) of R2 cells than did the BALB/c animals.

To obtain sufficient cells for phenotyping of the uterine infiltrates, cell suspensions were pooled from five or more animals. The major difference between BALB/c and C3H mice was that MC suspensions prepared from the latter group contained markedly fewer CD45<sup>+</sup> cells expressing MHC class II antigens (Fig. 5), a finding consistent with the differences in

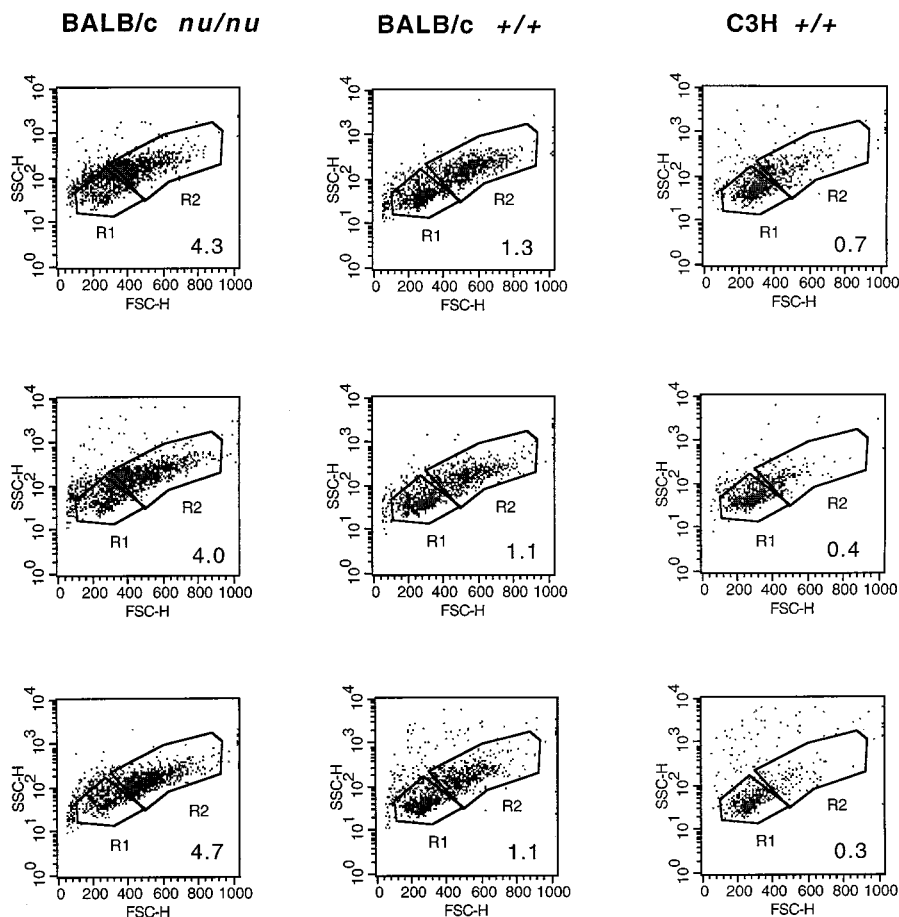


FIG. 4. Mouse strain differences in uterine  $CD45^+$  MC infiltrates at 7 to 10 days post-i.vag. infection with  $4.5 \times 10^5$  IFU of *C. trachomatis* NI1. Plots represent light scatter properties of infiltrates isolated from three individual mice from each strain. Samples were gated on  $CD45^+$  cells. All tissues were PCR positive for *C. trachomatis* and contained at least fivefold more  $CD45^+$  MC than did the control tissues. The numbers represent the ratio of large (R2) to small (R1) cells, and these values differed significantly between strains: BALB/c versus C3H,  $P < 0.01$ ; and BALB/c *nu/nu* versus BALB/c,  $P < 0.001$ .

light scatter properties observed in individual mice. There were similar proportions of both  $\alpha\beta$  and  $\gamma\delta$  T cells, with the predominant population being  $CD4^+ \alpha\beta$  T cells in both BALB/c and C3H infiltrates; small numbers of  $CD8^+ \alpha\beta$  T cells were also detectable. The majority of  $\gamma\delta$  T cells were  $CD4^- CD8^-$ . Small numbers of T cells were detectable in genital tract tissue from our nude mice. Staining with two commonly used B-cell markers, CD19 and B220 (CD45R), produced discordant results: a significant proportion of  $CD45^+$  MC from all three mouse strains stained positively with B220 but there was little labeling with CD19 (Fig. 5).

These data indicate a marked recruitment of large  $CD45^+$  MC into uterine tissue of BALB/c mice following i.vag. infection with *C. trachomatis*, a level of recruitment which was markedly smaller in C3H mice. Since BALB/c mice control infection more effectively, these cells may play a role in bacterial clearance and we therefore sought to characterize them further.

**Phenotype of large  $CD45^+$  MC in uteri of BALB/c mice.** MC preparations were pooled from the uteri of 10 BALB/c mice 7 to 8 days postinfection, and gates for flow cytometry analysis were set on large (R2)  $CD45^+$  cells. Most of these cells expressed high levels of MHC class II antigens, and subpopulations expressed the costimulatory molecules CD86 and CD40 (Fig. 6). CD80 was absent or very weakly expressed. This phe-

notype suggests a population of APC, but the lineage is not clear; the cells expressed CD11c, which is preferentially expressed on DC, but another DC marker, the DEC205 antigen identified by the antibody NLDC-145, was detected at low levels in only one of three experiments. Expression of the mature macrophage marker F4/80 was weak (Fig. 6). The cells expressed a number of surface molecules involved in intercellular adhesion, including CD11a, CD11b, and CD54, and a small subpopulation expressed the  $\beta 7$  integrin, which may be involved in migration to epithelia. Some cells also expressed CD4. Few cells expressing T-cell receptors or B-cell markers were detectable in this region (data not shown).

Small  $CD45^+$  MC in these preparations did not express MHC class II antigens, costimulatory molecules, or CD11c (data not shown). They were also  $CD11b^-$ , and the combination of high levels of expression of this marker on the large (R2) cells but its absence from the small cells allowed us to select positively the population of large cells for functional studies.

**$CD11b^+$  MC from BALB/c stimulate naive T cells.** Large MC from BALB/c uteri were enriched on the basis of CD11b expression, irradiated, and used to stimulate allogeneic T cells in an MLR. Small numbers of irradiated  $CD11b^+$  MC stimulated an allogeneic MLR (Fig. 7A), but they were approximately 10-fold less potent than mature spleen DC (Fig. 7B).

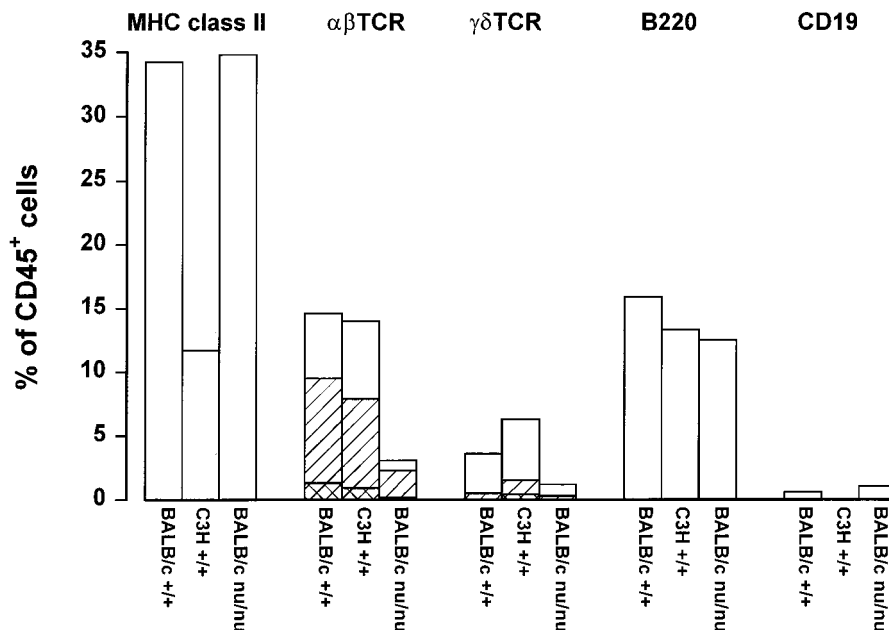


FIG. 5. Phenotypic variation in the composition of uterine CD45<sup>+</sup> MC infiltrate at 7 days postinfection with  $3.5 \times 10^5$  IFU of *C. trachomatis* NI1. Uterine tissue was pooled from five individual mice from each strain, and the MC were prepared. Analysis was performed on gated CD45<sup>+</sup> MC and, because of different levels of autofluorescence and isotype binding, large (R2) and small (R1) cells were analyzed separately and the results then combined. The percentage of cells binding isotype-matched control antibodies has been subtracted in all cases. Columns: ▨, CD4<sup>+</sup>; ▩, CD8<sup>+</sup>; □, CD4<sup>-</sup> CD8<sup>-</sup>.

## DISCUSSION

This study demonstrates that BALB/c mice are more able to limit ascending genital tract infection with *C. trachomatis* NI1 than are C3H mice and suggests that this control is associated with the recruitment of MHC class II cells into uterine tissue early in infection. Previously, we demonstrated that introduction of the organism directly into the uterus produces severe salpingitis and infertility in C3H mice but milder inflammation and no impact on fertility in BALB/c mice (32–34). In the current study we compared ascending infection following i.vag. inoculation to mirror natural infection and found evidence for differences in susceptibility. Spread of the organism into the uterus occurred early in infection in both strains, but in the BALB/c mice the organism was cleared from the tissues with concomitant resolution of inflammation. In contrast, in C3H mice the organism or components of it persisted for at least 3 weeks after infection and the inflammation did not resolve.

A comparison of ascending infection in wild-type BALB/c and athymic BALB/c *nu/nu* mice illustrated the importance of the host immune response and demonstrated the ability of *C. trachomatis* NI1 to persist in BALB/c tissue and provide an inflammatory stimulus in the absence of such a response. Shedding of bacteria, isolation from uterine homogenates, and histological changes were all greater in nude mice. This is consistent with the earlier finding (34) that, after unilateral i.u. injection, spread to the contralateral uterine horn is more common in nude mice. These data suggest that the control of ascending infection involves T-cell-dependent mechanisms, which is consistent with findings in other models of genital chlamydial infection. We are unable to conclude that inflammatory disease is T-cell independent in our model since low numbers of both  $\alpha\beta$  and  $\gamma\delta$  T cells were detected in the uterine tissue of our infected nude mice. Oviduct inflammation was only noted in the immunodeficient BALB/c *nu/nu* mice during the 3-week period in this study. We chose a limited time course

to enable the estrus cycle to be halted throughout without the need for additional hormone injections, which can induce genital tract pathology (unpublished observation). With a longer time course oviduct disease may also have developed in the C3H mice.

The findings that the host immune response is essential to limiting ascending infection and that BALB/c and C3H mice differ in their susceptibilities suggested that there may be a difference in the immune response of these two strains. We have attempted to identify this difference. In lung infection experiments with the mouse pneumonitis (MoPn) biovar of *C. trachomatis*, mouse strain differences in susceptibility have been identified (39), but in this case BALB/c display high susceptibility. Differences correlate with differences in cytokine production: susceptibility is associated with a shift away from the production of IFN- $\gamma$  and towards the production of IL-10 (39). Treatment with anti-IL-10 increases clearance of MoPn. In our model of ascending genital tract infection there was no evidence for differences in cytokine production. Draining lymph node cells from both BALB/c and C3H mice yielded predominantly IFN- $\gamma$  and TNF- $\alpha$  with a little IL-10. Although IFN- $\gamma$  and TNF- $\alpha$  have protective effects (8, 12, 27, 38) and have also been implicated in pathology (1, 6, 36), production by BALB/c and C3H lymph node cells was similar. Darville et al. have recently reported more severe genital tract disease in C3H mice than in C57 mice despite a dominant Th1 response in both strains (9). It remains possible that local differences in genital tract tissue, which are not reflected in the draining nodes, could contribute to this variation in response. The differences between findings in the genital tract and those in the MoPn lung model are probably related to the route of infection since intravaginal inoculation of BALB/c mice with MoPn induces a Th1 response (4).

Within 1 week of i.vag. inoculation there was evidence of spread of the organism into the uterus of both BALB/c and

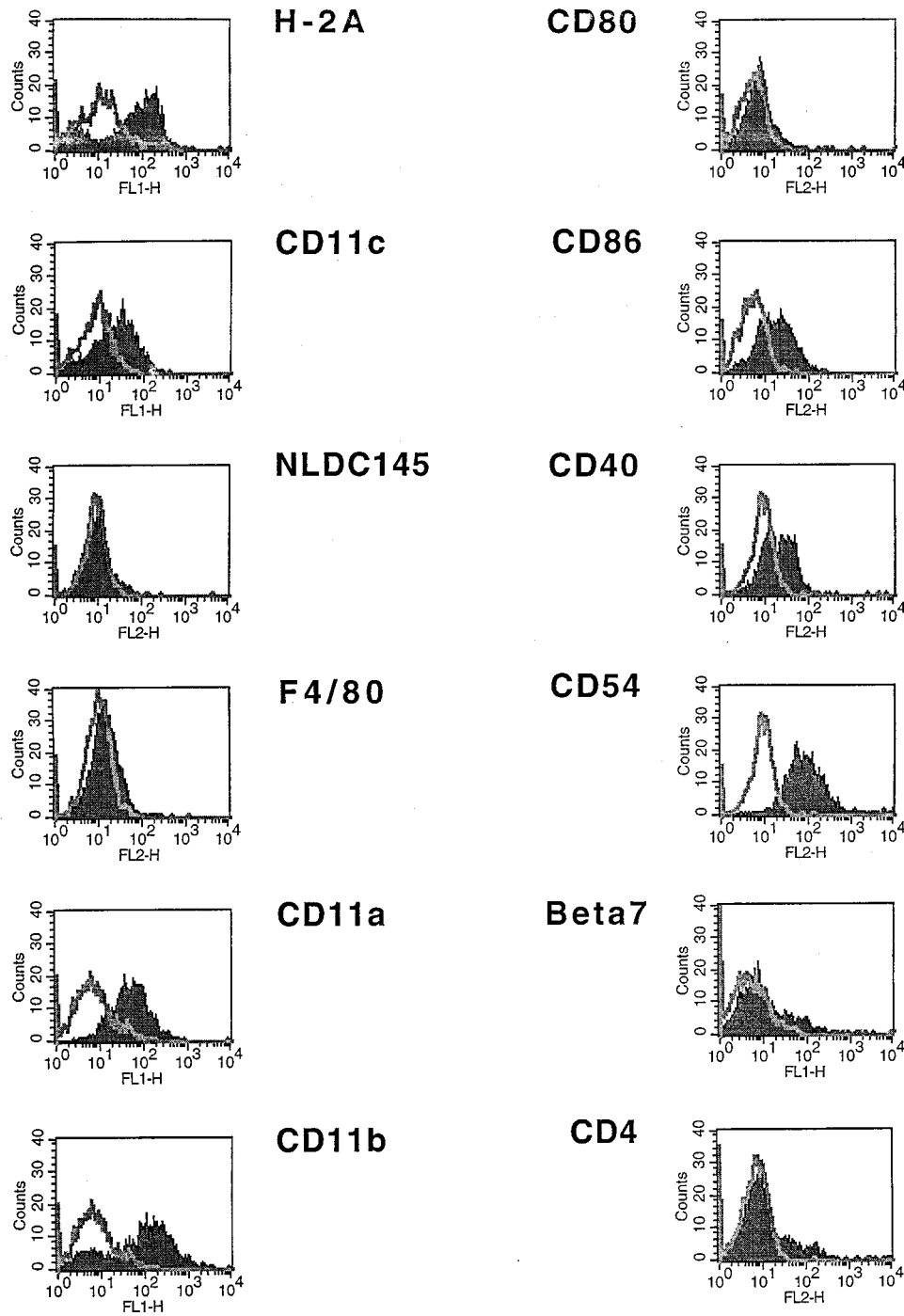


FIG. 6. Phenotypic characterization of large (R2) MC from BALB/c uteri. Tissues were pooled from 10 animals at 8 days postinfection with  $3.5 \times 10^5$  IFU of *C. trachomatis* N11, the MC were isolated, and the cells were gated for flow cytometry analysis on the basis of light scatter properties and expression of CD45. Histograms display the results for R2 CD45<sup>+</sup> MC. Solid histograms show labeling with the antibody noted in the figure; open histograms show binding of an isotype-matched control. The figure summarizes the results of three separate experiments.

C3H mice and the accumulation of CD45<sup>+</sup> MC in the tissue. When individual mice were examined there was a strong association between ascending infection and inflammatory changes in uterine tissue, suggesting that the accumulation of CD45<sup>+</sup> MC represents a reaction to the presence of the organism in the upper genital tract (29). The amounts of this early CD45<sup>+</sup> MC infiltrate were similar in BALB/c and C3H mice, but there

was a difference in composition; in BALB/c mice the infiltrate comprised a population of small agranular cells and a population of larger cells present in approximately equal numbers. In contrast, the infiltrate in C3H uteri lacked these larger cells. Phenotypic analysis revealed similar T-cell and B-cell infiltrates in the two mouse strains but only one-third the number of MHC class II-positive cells in C3H mice. This population

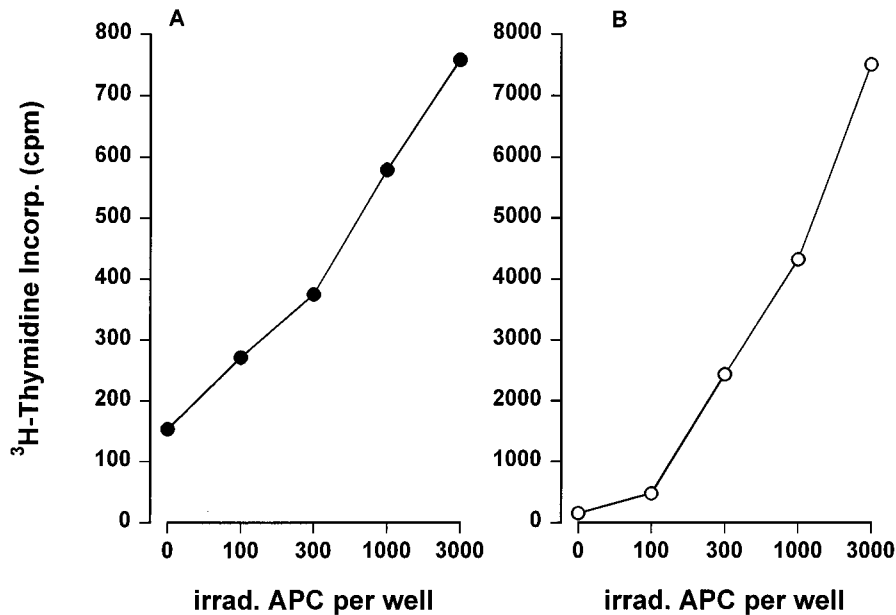


FIG. 7. Functional characterization of large (R2) MC from BALB/c uteri. Allogeneic (B10) T cells were stimulated by uterine MC obtained from a pool of 10 mice at 9 days postinfection with  $3 \times 10^5$  IFU of *C. trachomatis* N1 (A) or mature spleen DC isolated from uninfected BALB/c mice (B). Stimulator cells were irradiated (20 Gy) before addition to hanging-drop cultures. Each point represents the mean proliferation of triplicate cultures.

corresponded to the large cells identified by light scatter. A comparison of infected and control BALB/c mice indicated a selective increase in large cells following i.vag. infection (a five-fold increase as opposed to a three-fold increase in small CD45<sup>+</sup> MC), and these cells were not removed by perfusion of the genital tract tissue before isolation of MC (data not shown). Large CD45<sup>+</sup> cells were only a minor population of BALB/c peripheral blood MC (data not shown). Collectively, these data suggest that there is a selective accumulation of large MHC class II-expressing cells in the uterine tissue of BALB/c mice which is absent or significantly reduced in the more susceptible C3H mouse strain. Blander and Amortegui (2) reported that immunization of BALB/c mice with a detergent extract of MoPn induced a splenic T-cell response and enhanced infiltration of the genital tract upon infection, notably with eosinophils. However, this response was not protective, again suggesting that the composition of the infiltrate is important in determining the degree of protection. There may be additional differences in the composition of the infiltrates that are not revealed by this study, since the panel of markers we used only identified a maximum of approximately 70% of the CD45<sup>+</sup> cells (Fig. 5).

Expression of the costimulatory molecules CD86 and CD40, in addition to MHC class II antigens, suggests the large CD45<sup>+</sup> MC in BALB/c uteri are a population of APC. They were able to activate naive allogeneic T cells in an MLR, albeit less potently than mature splenic DC. These cells may be equivalent to the MHC class II<sup>+</sup> NLDC-145<sup>-</sup> DC population identified histologically in the murine cervix and vagina (23). Antigens introduced into the genital tract are believed to elicit systemic immune responses after acquisition by DC and transportation to the draining lymph nodes (22, 24). Studies with airway epithelia suggest that additional DC are recruited early in inflammatory responses (18) in response to chemokines and chemoattractants (17, 28). They form part of a specialized surveillance system designed to alert the immune system to the presence of pathogens at mucosal surfaces. Infection in C3H

mice may fail to induce these signals, or their DC (or DC precursors) may be poorly responsive to the signals induced.

We are currently unable to conclude with certainty that a difference in recruitment of uterine APC is the single critical factor which underlies the difference in disease susceptibility of BALB/c and C3H mice; there may be a number of factors involved. However, the failure to find differences in other aspects of the host response already demonstrated to be important in the control of chlamydial infection supports the notion that this recruitment is important. We are currently attempting to identify specific signals involved in the recruitment of DC into genital tissue during chlamydial infection in order to target these experimentally. The uterine APC population identified in BALB/c mice could play a number of important roles in the control of genital infection. These include the initiation of a systemic immune response, local presentation of chlamydial antigen in the tissue, production of cytokines or other compounds with antichlamydial effects, or an influence on the growth or differentiation of B cells. IL-12, which has a role early in chlamydial infection (25) and drives the development of a Th1 response (reviewed in reference 25), is produced by macrophages and DC (16). Stimulation via CD40 is potent at inducing IL-12 production by DC (5), and the cells of the uterine population we identified were CD40<sup>+</sup>. In addition to their influence on T-cell responses, DC generated from progenitors in vitro can interact with activated B cells to potentiate isotype switching towards immunoglobulin A production (10, 11).

The model we have described here closely mirrors the insidious nature of human infection with a low inoculum dose, ascending infection into the upper genital tract, and a low bacterial burden. Our findings are consistent with the importance of Th1 T-cell responses described in other models but suggest that the immunological control of genital infection is more complex: the ability to clear antigen from the tissue and therefore protect against immunopathology requires recruitment of APC as well as the appropriate T-cell response.

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