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Identification of Homing Receptors That Mediate the Recruitment of CD4 T Cells to the Genital Tract following Intravaginal Infection with *Chlamydia trachomatis*

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Murine genital infection induced with the mouse pneumonitis biovar of *Chlamydia trachomatis* (MoPn) elicits a short-lived protective immunity mediated primarily by Th1 CD4 cells. To understand the development of local cell-mediated immunity against *C. trachomatis* infection, we investigated the mechanism(s) which mediates CD4 lymphocyte migration to the genital mucosa by identifying molecules that could support this process. We found that primarily CD4 cells were recruited to the genital tract (GT) during primary and challenge MoPn infection. Peak levels were found 21 days after primary inoculation (15.4% ± 2.7%) and 7 days (31.3% ± 8.5%) after challenge but diminished after resolution of infection. The CD4 cells appeared to be recruited to the GT in response to infection since these cells expressed the profile of activated, or memory, cells. We also observed up-regulation of homing receptors containing LFA-1 (CD11a) and α4 (CD49d) on GT CD4 cells over the course of infection. Furthermore, the mucosal homing receptor chain, β7, but not the peripheral homing receptor chain β1 (CD29), was detected on GT CD4 cells. MoPn-infected GT tissue expressed the endothelial cell ligands vascular cell adhesion molecule 1 (VCAM-1), intracellular adhesion molecule 1 (ICAM-1), and mucosal vascular addressin cell adhesion molecule 1 (MAdCAM-1), which correspond to the homing receptors on GT CD4 cells. Interestingly, VCAM-1 and MAdCAM-1 were not expressed in the GTs of uninfected mice but were temporarily induced following infection, indicating that expression of endothelial ligands in the GT are regulated by chlamydial infection. These data suggest that recruitment of CD4 cells to the GT is mediated through LFA-1:ICAM-1 and α4β7:MAdCAM-1-VCAM-1 interactions.

Chlamydia trachomatis is a major cause of sexually transmitted disease and infertility in humans (56). Reproductive disability, resulting from salpingitis, can be avoided by antimicrobial treatment early after infection. However, the majority of genital infections are subclinical and are not detected for early treatment. Therefore, eliciting protective immunity against *C. trachomatis* infection may be the most effective approach to reducing or preventing the sequelae of salpingitis (8). *C. trachomatis* produces a localized genital infection, and resolution in the murine model is mediated by chlamydia-specific CD4 cells (39, 46, 64). Natural infection in humans also elicits protective immunity although it is only short-lived (27, 58). This transient protective immunity has been demonstrated in both the murine and guinea pig models of chlamydial genital infection (45, 50). Furthermore, the loss of protective immunity is associated with a loss of chlamydia-specific T-cell proliferative responses in the genital tract (23). Therefore, sustaining protective immunity may depend on maintaining the presence of chlamydia-specific T cells in the genital tract.

Most mature lymphocytes recirculate between blood and secondary lymphoid tissues but not routinely through other tissues (16). The ability of lymphocytes to gain access to these sequestered sites is through the expression of vascular addressins on specialized high endothelial cells lining blood vessels in a particular tissue (4, 7). These molecules are comprised of mucin-like glycoproteins, such as the mucosal vascular addressin cell adhesion molecule 1 (MAdCAM-1) (62) and glycosylation-dependent cell adhesion molecule 1 (GlyCAM-1) (63), or belong to the immunoglobulin superfamily, most notably, intracellular adhesion molecule 1 (ICAM-1) (13) and vascular cell adhesion molecule 1 (VCAM-1) (54). These molecules can be differentially induced on endothelial cells in vitro by the cytokines tumor necrosis factor alpha (TNF-α), interleukin-1 (IL-1), IL-4, and gamma interferon (IFN-γ) as well as *Escherichia coli* lipopolysaccharide (LPS) (4, 7). In addition, the expression of these endothelial ligands is tissue specific. For instance, the expression of MAdCAM-1 is limited to mucosa-associated tissues (62) while the expression of E-selectin is confined to subcutaneous sites (44). Thus, initiation of an inflammatory response or infection at a localized site may regulate the expression of endothelial cell ligands and the types of cells that can gain access to that site.

Following the induction of endothelial cell ligands, lymphocytes expressing the complementary homing receptor(s) can then mediate lymphocyte binding to endothelial cell ligands and subsequent migration into tissues (4, 7, 60). This is a multistep process that requires the activation of integrin homing receptors, which can occur as a consequence of T-cell activation (15, 59). T-cell activation also induces changes in other cell surface molecules such as down-regulation of CD62L expression (20, 34). These changes appear to permanently alter the recirculation pattern of lymphocytes by allowing access to tissue sites of inflammation and decreasing recirculation through peripheral lymph nodes (35). Although all lymphocytes possess integrin homing receptors, the regulation of integrin activation is poorly understood and may be influenced by a number of factors present at the site of infection or inflammation such as chemokines (31) or the initial site of lymphocyte activation (26).

The genital tract lacks its own organized lymphoid tissue, so

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lymphocytes found in the genital tract during infection must first be recruited from the central circulation (41). Currently, nothing is known regarding the mechanisms of lymphocyte recruitment to the genital tract. To understand the production and maintenance of local cell-mediated immunity within the genital tract mucosa, we have characterized the types of lymphocytes recruited to the genital tract during both primary and challenge genital infection with the mouse pneumonitis biovar of *C. trachomatis* (MoPn). We have further identified homing receptors expressed on genital tract CD4 cells as well as the endothelial cell ligands that are induced by MoPn infection within the genital tract.

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MATERIALS AND METHODS

Animals. Female BALB/c mice, 4 to 6 weeks old, were purchased from Harlan Sprague-Dawley (Indianapolis, Ind.) and were given food and water ad libitum. Mice were allowed to acclimate to the AAALAC-accredited housing environment for approximately 1 week before experimentation was begun.

Antibodies. The following anti-mouse monoclonal antibodies were used for flow cytometry and immunohistochemistry. Hybridomas secreting rat anti-Thy1.2 (TIB107, immunoglobulin G2b [IgG2b]), anti-CD4 (TIB207, IgG2b), anti-B220 (TIB146, IgM), anti-kappa light chain (HB58, IgG1), and anti-ICAM-1 (CRL 1878, IgG2b) were purchased from the American Type Culture Collection (Rockville, Md.), and the undiluted supernatants were used for staining. Anti-CD11b (clone M1/70, IgG2b), anti-CD8α (53-6.7, IgG2a), anti-CD49d (9C10, IgG2a), anti-CD29 (9E67, IgG2a), anti-CD45RB (16A, IgG2a), anti-CD62L (Mel-14, IgG2a), anti-CD44 (IM7, IgG2b), anti-β7 (M293, IgG2a), anti-CD11a (M17/4, IgG2a), and anti-VCAM-1 (429, IgG2a) are rat monoclonal antibodies that were purchased from PharMingen (San Diego, Calif.) and used at concentrations ranging from 10 to 25 μg/ml. Rat anti-CD3 (29B, IgG2b) was purchased from Gibco BRL (Gaithersburg, Md.) and used at 10 μg/ml. Hamster anti-murine γδ T-cell receptor conjugated to fluorescein isothiocyanate (GL3, IgG) and a mouse anti-*C. trachomatis* LPS (CHL-888, IgG3) were also purchased from PharMingen and used at 20 and 33 μg/ml, respectively. A rat IgG2bκ myeloma protein (IR863) (17) and the rat monoclonal IgG2a (R35-95, PharMingen) were used as negative control antibodies. Rat monoclonal antibodies directed against MAdCAM-1 (MECA-367) and GlyCAM-1 (MECA-79) were kindly provided by Phil Streeter (Monsanto Corp., St. Louis, Mo.).

Infection. All groups were injected subcutaneously with 2.5 mg of DEPO-PROVERA (Upjohn, Kalamazoo, Mich.) in 100 μl of sterile phosphate-buffered saline (PBS). DEPO-PROVERA stimulates mice to an anestrus state and thus eliminates the variabilities in the rate and severity of infection due to the estrous cycle (52). Seven days later, while under sodium pentobarbital anesthesia, all mice were inoculated with 10^7 inclusion-forming units of MoPn grown in McCoy cells. Infection was monitored every 3 days after inoculation by obtaining cervicovaginal swabs (Dacroswab Type 1; Spectrum Labs, Houston, Tex.) from five mice from each group (29). The swabs were stored at −70°C in sucrose-phosphate buffer (57) until analysis.

Isolation of lymphoid cells. Single cell suspensions of iliac lymph nodes (ILN) mesenteric lymph nodes (MLN) and spleens were prepared from pooled tissues from 5 to 10 mice per group in Dulbecco's modified Eagle's medium (DMEM) (Gibco BRL), minced with scissors, and expressed through a 70-μm-pore-size nylon mesh. Whole genital tracts were harvested from 5 to 10 mice per group, minced with scissors, and subjected to collagenase digestion (type I, 5 mg/ml; Sigma, St. Louis, Mo.) for 45 min at 37°C. Single cell suspensions were prepared by expressing the digests through a 70-μm-pore-size nylon mesh.

Flow cytometry. Single cell suspensions (2×10^5 to 4×10^5 cells) were stained in DMEM containing 1% bovine serum albumin (BSA) (Sigma) and 0.1% sodium azide (staining buffer) by the microplate method as previously described (33). For single color analysis, cells were first incubated with rat anti-mouse cell surface markers, (see below) for 25 min on ice and then washed twice with DMEM containing 10% BSA. The cells were then resuspended in a goat anti-rat IgG-conjugated fluorescein isothiocyanate (BioSource International, Camarillo, Calif.) at a concentration of 20 μg/ml with 10% autologous mouse serum for 25 min on ice. Following the washing step described above, the cells were fixed in PBS containing 1% paraformaldehyde and kept at 4°C until analysis.

Identification of cell surface markers on the CD4 population was performed by using two-color staining. The cell suspensions were first stained for CD4 as described above. Biotinylated monoclonal antibodies were then added and incubated on ice for 25 min, washed, and then incubated with 20 μl of streptavidin conjugated to phycoerythrin (Becton Dickinson, Sunnyvale, Calif.). The cells were fixed in PBS containing 1% paraformaldehyde as described above.

Flow cytometry was performed on a fluorescence-activated cell-sorting analyzer equipped with a 488-nm argon laser and Lysys II software (FACScan; Becton Dickinson). The instrument was calibrated with beads (CaliBRITE; Becton Dickinson) by using AutoCOMP software, and the same settings were used throughout the study. Dead cells were excluded on the basis of forward angle and 90° light scatter, and 10,000 gated cells were analyzed for each sample. To obtain an accurate percentage of the different cell types in the genital tract, whole collagenase digests were stained directly without any further enrichment procedures. To strengthen the data analysis, we analyzed the percent fluorescence of genital tract cells collected within a leukocyte gate derived from forward angle and 90°-light-scatter dot plot of ILN and MLN. Approximately 30% of the genital tract cells in this gate were leukocytes, depending on the time point after infection. The percentage of CD4 cells expressing a certain cell surface marker was determined by gating on CD4-positive cells. For all markers analyzed, the percentage of positive cells was determined by subtracting the value obtained with the negative control antibody. This value ranged from 6.9 to 7.4% for all tissues and control antibodies.

Immunohistochemistry. The lower genital tract, including the vagina, cervix, and uterine fundus, was removed, and a longitudinal incision was made. The resulting tissue was placed cut side down in OCT freezing media (Fisher Scientific, Pittsburgh, Pa.) to prepare frozen blocks as previously described (17). The sagittal frozen sections were fixed in acetone, washed in PBS, and incubated in methanol-H_2O_2 for 30 min to quench endogenous peroxidase activity. Tissue biotin sites were blocked by the addition of avidin followed by biotin. After a tissue blocking step with goat serum, the primary antibodies were incubated on the tissue section for 45 min at room temperature in a humidified chamber and then washed. A goat anti-rat IgG $F(ab')_2$ antibody conjugated to biotin at 14 μg/ml (BioSource International) followed by streptavidin conjugated to horseradish peroxidase (Zymed, San Francisco, Calif.) was then added and incubated for 45 min. The bound enzyme was visualized with the ImmunoPure metal-enhanced DAB substrate kit (Pierce, Rockford, Ill.) and preserved with crystal mount (Fisher Scientific). To detect antichlamydial LPS, a goat anti-mouse IgG antibody conjugated to alkaline phosphatase (Southern Biotechnology Associates, Birmingham, Al.) at 2 μg/ml was incubated as described above and visualized with 5-bromo-4-chloro-3-indolyl phosphate–nitro blue tetrazolium chloride (Boehringer-Mannheim, Indianapolis, Ind.) (28). The numbers of T and B cells in the entire section were counted and expressed per measured square millimeter of area. Photographs were generated by scanning the microscope slides with a color video camera (Sony Electronics, Inc., San Jose, Calif.) and Pax-it! Software (Midwest Information Systems, Inc., Franklin Park, Ill.).

Statistics. Statistical differences in the percentages of cells expressing a certain cell surface marker over the course of MoPn infection between the MLN, ILN, and genital tract were detected by two-way analysis of variance and the Student-Newman-Keuls method for posthoc analysis with SigmaStat software (Jandel Scientific, San Rafael, Calif.). Chi-square analysis was used for statistical analysis of the number of cells per square millimeter.

RESULTS

Characteristics of leukocyte influx during MoPn genital infection. In the murine model of chlamydial genital infection, eradication of the organism is accomplished almost entirely by cell-mediated immunity and not by humoral immunity (51). Accordingly, we reasoned that since an MoPn infection is confined to epithelial cells in the genital mucosa, which lacks any organized lymphoid tissue (41), the immune cells responsible for resolution of infection would be actively recruited to the site of infection. We then examined the types of leukocytes present in the genital tract of infected mice following MoPn vaginal inoculation by flow cytometric techniques.

As shown in Fig. 1A and F and Table 1, only minimal numbers of leukocytes were present in the uninfected genital tracts of mice. By day 7 after infection, appreciable numbers of leukocytes could be found, and the majority of cells (21.5%) were MAC-1^+ (CD11b), which include granulocytes, monocytes, and macrophages (Fig. 1B, thin line, and Table 1). We also detected an increase in the number of T cells expressing the pan T-cell marker Thy1.2 from 6.5 to 13.0% on day 7 after infection (Fig. 1A and B, heavy line, and Table 1). This trend continued for T cells, and the number peaked at 23.9% at 21 days following infection and then dropped to 16.3% following resolution of MoPn infection (Fig. 1C and D, heavy line, and Table 1). This pattern of T-cell kinetics in the genital tract coincided with that seen when genital tract cells were monitored functionally by proliferation to MoPn in vitro (6). Likewise, the percentage of MAC-1^+ cells also returned to baseline following resolution of the infection.

Interestingly, we did not detect any increase in B lympho-

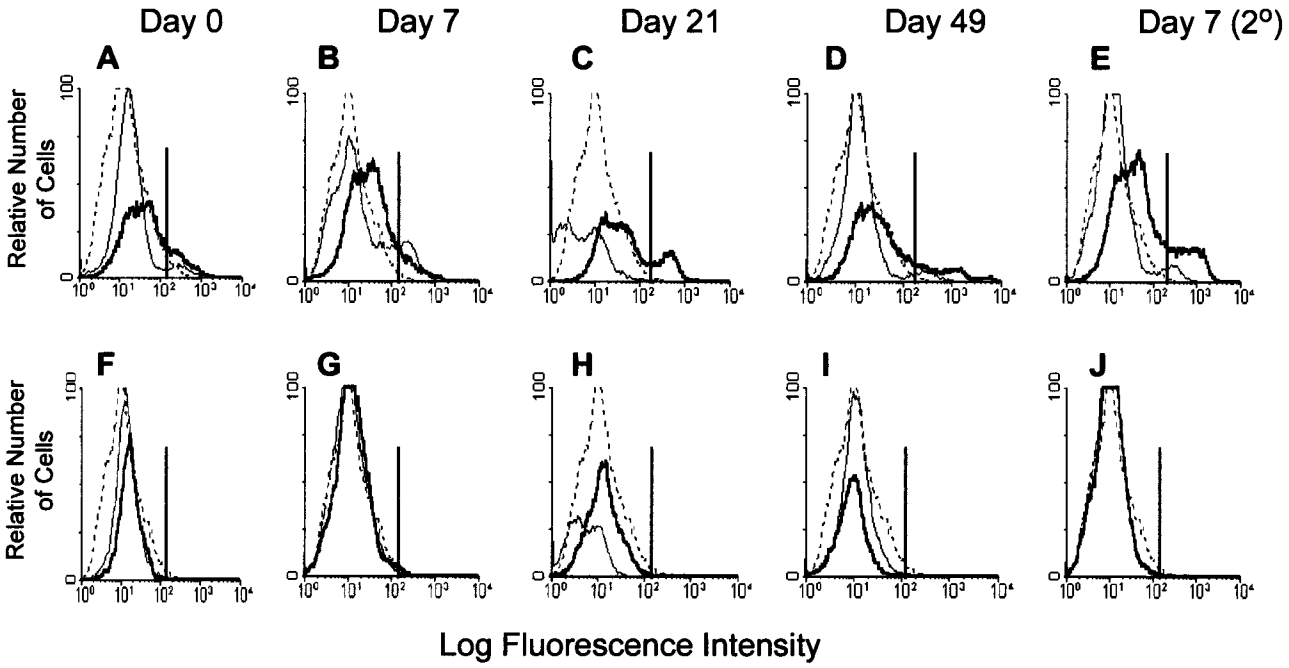

FIG. 1. Leukocyte influx within the genital tract during MoPn infection. Whole genital tracts from 4 to 10 mice per group were harvested and pooled on days 0, 7, 21, and 49 after a single vaginal inoculation of MoPn. A group of mice was given a second vaginal inoculation on day 49, and the genital tracts were harvested 7 days later (2°). Isolated genital tract cells were stained for flow cytometric analysis. (A to E) Representative histograms of Thy1.2 (heavy line)- and MAC-1 (CD11b [thin line])-stained cells; (F to J) representative histograms of B220 (heavy line)- and Ig-kappa-chain (thin line)-stained cells. The dotted line represents staining with an irrelevant isotype-matched control antibody.

cytes in the genital tract during infection by using two different B-cell markers that would detect mature B cells and plasma cells (Fig. 1F to J and Table 1). The pan B-cell marker, B220, is present on both mature B cells and plasma cells (12), and the kappa light chain is expressed on approximately 95% of mature murine B cells. We confirmed this finding by counting the number of T cells stained with anti-Thy1.2 and the number of B cells stained with anti-kappa Ig in frozen sections of genital tract tissues. The number of T cells significantly increased ($P < 0.001$) from 24.6 ± 22.1 cells/mm^2 of area (mean ± standard deviation, n = three to five mice) before infection to 62.82 ± 22.4 at 7 days after inoculation compared to only 0.13 ± 0.2 and 6.5 ± 6.8, for anti-kappa-positive B cells and plasma cells, respectively. Furthermore, no increase in B cells was found throughout the course of infection (data not shown).

Following recovery of a genital infection with MoPn, mice develop protective immunity against reinfection that wanes over time. Three weeks after resolution of infection, mice can be reinfected after challenge, but the infection is of lower intensity and shorter in duration (29). When the mice were reinfected at this time, the percentage of T cells in the genital tract 7 days later was more than twofold greater than that observed 7 days after a primary MoPn infection (Fig. 1E, heavy line, and Table 1). Thus, T cells appear to be recruited to MoPn-infected genital tracts of mice in a transient manner, with apparently greater numbers of T cells being recruited following reinfection.

Many groups have investigated which T-cell subset is primarily responsible for protective immunity against MoPn infection. Both CD4 (43, 64) and CD8 (61), but not γδ T cells (66), appear to be capable of providing immunity. However, there is some disagreement on the role of CD4 and CD8 cells

TABLE 1. Characterization of the leukocyte influx within the genital tract during MoPn infection

Day of sampling after infection	% of cell positive for[a]:					
	Thy1.2	CD4	CD8	B220	Ig kappa	MAC-1
Single inoculation						
0[b]	6.5 ± 3.4	0.2 ± 0.2	0.0 ± 0.0	0.0 ± 0.0	1.3 ± 0.7	2.9
7	13.0 ± 3.4	7.6 ± 2.5	2.7 ± 1.4	0.0 ± 0.0	2.0 ± 1.0	21.5
21	23.9 ± 0.9	15.4 ± 2.7	3.9 ± 2.0	2.0 ± 2.0	1.5	0.8
49	16.3 ± 4.7	6.3 ± 5.1	1.8 ± 1.5	1.8 ± 1.8	4.2 ± 2.1	0
Second inoculation						
7	31.3 ± 10.7	31.3 ± 8.5[c]	2.2 ± 1.2	3.6 ± 3.6	2.4 ± 1.2	3.5

[a] Datum points for days 0, 7, 49 and 7 represent the means ± standard errors of the means for three experiments. The means for day 21 and Ig kappa staining were obtained for two experiments. MAC-1(CD11b) staining was analyzed once for each time point, and Ig kappa staining on day 21 was performed only once.

[b] The percentages of Thy1.2- and CD4-positive cells were significantly greater than those of the other cell types by analysis of variance ($P < 0.05$).

[c] Posthoc analysis revealed that the percentage of CD4 cells was significantly increased ($P < 0.05$).

TABLE 2. The kinetics of genital tract CD4 cells expressing an activated, memory, phenotype during MoPn infection

Day of sampling after infection	% of CD45RB^{high} CD4 cells[a]			% of CD62L^{high} CD4 cells			% of CD44^{high} CD4 cells (MFI)[c]		
	GT[b]	ILN	MLN	GT	ILN	MLN	GT	ILN	MLN
Single inoculation									
0	4.1 ± 1.1	27.4 ± 1.7	23.1 ± 0.6	26.9 ± 5.3	39.3 ± 8.6	36.3 ± 8.6	39.9 (584)	23.5 (433)	23.0 (27)
7	9.2 ± 3.0	40.0 ± 4.0	21.7 ± 7.6	21.3 ± 6.8	38.0 ± 10.7	27.8 ± 12.8	76.8 (999)	2.4 (306)	3.6 (233)
49	7.4 ± 1.6	31.9 ± 2.8	30.0 ± 5.8	33.4 ± 7.6	37.3 ± 7.9	39.1 ± 11.7	31.3 (348)	9.9 (233)	6.9 (261)
Second inoculation									
7	7.9 ± 4.1	31.8 ± 1.9	28.8 ± 8.1	25.5 ± 5.2	38.1 ± 11.8	35.6 ± 12.6	55.4 (684)	18.6 (346)	6.9 (209)

[a] Each datum point represents the mean ± standard error of the mean for three experiments, with the exception of CD44, which was performed once for each time point.
[b] The percentage of CD4$^+$ CD45RB^{high} cells in the genital tract (GT) was statistically lower ($P < 0.001$) than those in ILN and MLN.
[c] MFI, mean fluorescence intensity of the positive cell population.

in protecting mice from MoPn infection. We examined the type of T cell(s) recruited to the genital tract following MoPn infection. As shown in Fig. 2A to E and Table 1, the percentage of only CD4 cells was found in statistically increased numbers ($P < 0.05$) following both a primary and a secondary inoculation. Although no significant increase in the percentage of CD8 or γδ T cells (Fig. 2F to J, heavy line) was detected, we did find a modest increase 7 days after infection. The percentage of CD4 cells was statistically increased on day 21 but not on day 49, suggesting a transient recruitment of these cells. Furthermore, the increase in CD4 cells observed 7 days following a challenge inoculation was statistically greater than that found 7 days after a primary infection ($P < 0.05$) (Table 1). This pattern of CD4 recruitment is similar to the kinetics of a recall response where a more rapid immune response ensues following a second exposure to antigen.

Phenotype of genital tract CD4 cells. The kinetics of CD4 cells appearing in the genital tract after MoPn infection suggested an active recruitment of these cells to infected tissues. The ability of cells to enter tissue where they do not commonly recirculate is dependent on the activation state of the lymphocytes (20). The activation state of cells can be determined phenotypically by the levels of expression of certain cell surface molecules. The cell surface molecules, CD45RB, CD62L, and CD44, have been used in murine systems to differentiate activated, or memory, CD4 cells from naive CD4 cells. Both CD45RB and CD62L are down-regulated following activation, while levels of CD44 are expressed at a greater level (34).

We examined the phenotype of CD4 cells isolated from the genital tract by double staining the isolated cells for CD4 and either CD45RB, CD62L, or CD44. Figure 3 shows the level of expression of these markers after gating on the CD4-positive

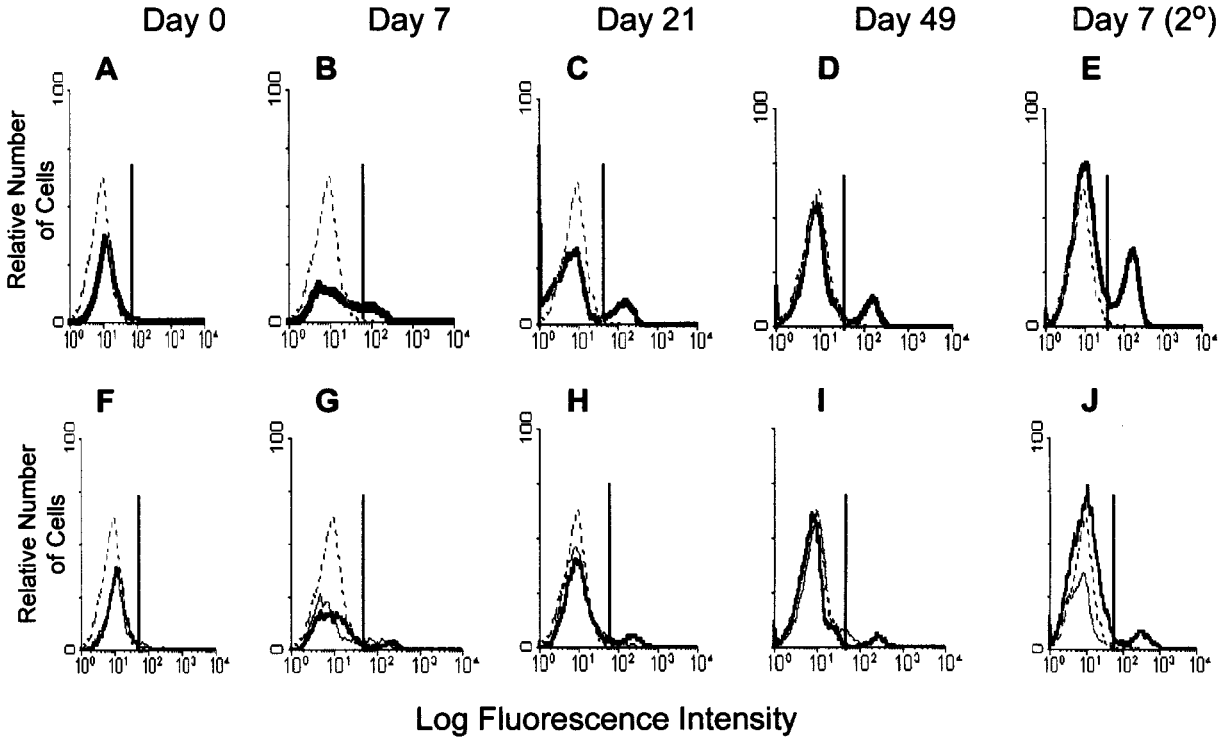

FIG. 2. Recruitment of T-cell subsets to MoPn infected genital tract. Whole genital tracts from 4 to 10 mice per group were harvested at the times described in the legend to Fig. 1 and stained for flow cytometric analysis. (A to E) Representative histograms of CD4 (heavy line)-stained cells; (F to J) representative histograms of CD8-stained cells (heavy line) and γδ T cells (thin line). The dotted line histogram represents staining with an irrelevant isotype-matched control antibody.

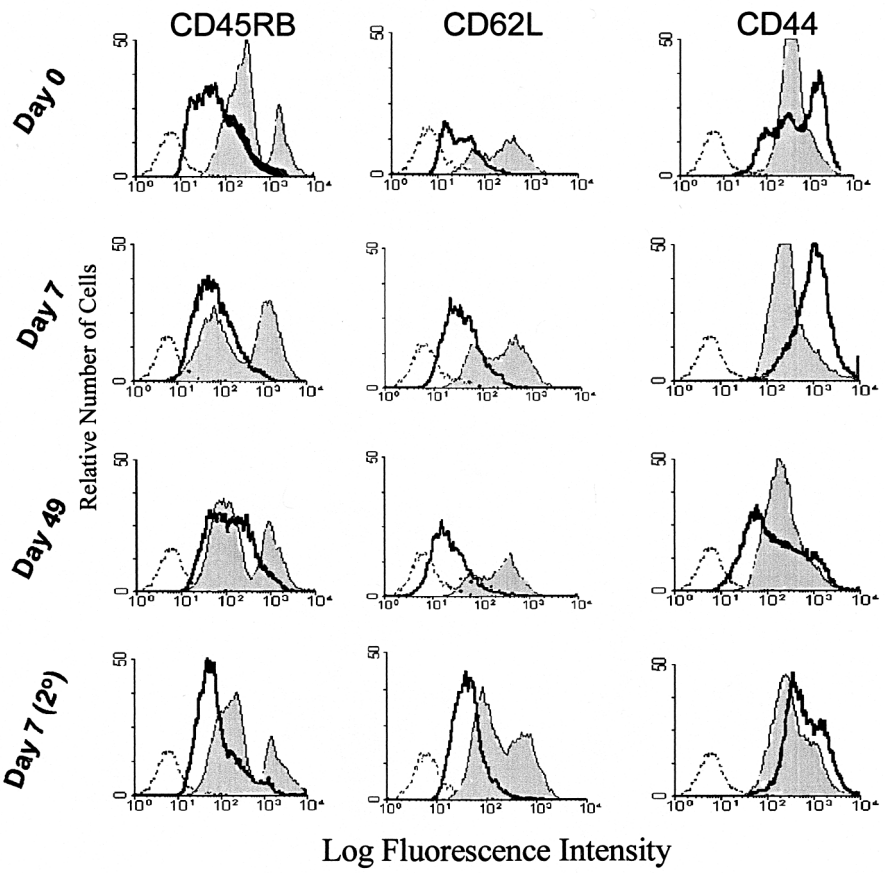

FIG. 3. Phenotype of CD4 cells recruited to MoPn infected genital tract. ILN and genital tracts from groups of 4 to 10 mice were harvested 0, 7, and 49 days after a single MoPn vaginal inoculation and 7 days following a challenge infection (2°). Like tissues were pooled, and the genital tracts were digested with collagenase. The cell populations were dual-stained for CD4 and CD45RB, CD62L, or CD44. Histograms represent the expression of various cell surface molecules on CD4 cells isolated from the ILN (shaded histograms) and genital tract (heavy lines). For each panel, the dotted line represents CD4 cells stained with the control antibody.

population in the ILN and the genital tract throughout the course of infection. As can be seen in Fig. 3 (leftmost panels), CD4 cells isolated from ILN contained populations expressing both high and low levels of CD45RB ($CD45RB^{high}$ and $CD45RB^{low}$, respectively) (shaded histograms) throughout the course of infection. In contrast, CD4 cells isolated from the genital tract expressed only low surface levels of CD45RB (Fig. 3 heavy lines). Furthermore, the percentage of $CD4^+$ $CD45RB^{high}$ cells in the genital tract was statistically lower throughout the course of a primary and secondary MoPn infections (Table 2) ($P < 0.001$). Likewise, the level of CD62L expression on CD4 cells was lower in the genital tract than in the ILN (Fig. 3, middle panels). Although, the percentage of $CD4^+$ $CD62L^{high}$ cells was not statistically lower than those in the ILN and MLN, it was consistently lower at all time points analyzed (Table 2).

The CD44 glycoprotein is expressed at high levels only on a minor subpopulation of CD4 and CD8 cells but is up-regulated following primary antigen stimulation (5). This molecule binds to hyaluronate in the extracellular matrix and is postulated to retain antigen-activated cells within tissues containing antigen by adherence to the extracellular matrix. It is clear in Fig. 3 (right panels) that CD4 cells isolated from the genital tract (heavy lines) on day 0 exhibited two populations, one with high surface levels of CD44 and one with lower surface levels of CD44. Also note in Fig. 3 that the percentage of genital tract CD4 cells expressing high levels of CD44 increased following exposure to MoPn and then returned to baseline levels following resolution of infection. The level of CD44 on genital tract CD4 cells during infection was two- to threefold higher than those seen on CD4 ILN (Fig. 3, shaded histograms) and MLN (Table 2). This pattern coincides with the appearance of CD4 cells in the genital tract during the course of infection (Fig. 2 and Table 1). Thus, CD4 cells isolated from the genital tract during MoPn infection exhibited the phenotype of activated, or memory, cells, and these data provide additional evidence that CD4 cells are actively recruited to the genital tract during MoPn genital infection.

Homing receptor expression on genital tract CD4 cells. Recruitment of cells to sites of inflammation is not a random process and is regulated by the expression of homing receptors on activated CD4 cells. Two molecules, CD11a/CD18 (LFA-1) and CD49d (α4 integrin) forming a receptor with the β1 or β7 chain, have been shown to be involved in the homing of CD4 cells to sites of inflammation (25, 68). Both of these molecules are up-regulated following antigen activation. As shown in Fig. 4A, both of these molecules are expressed at higher levels on CD4 cells isolated, after infection, from the genital tract (heavy lines) than from ILN (shaded histograms). The level of expression of CD11a on GT CD4 cells as determined by mean fluorescence intensity changed throughout the course of infection, with a peak at day 7 and gradual diminishing after resolution of infection (Fig. 5A). This increase was also seen on genital tract CD4 cells following a second MoPn vaginal challenge but oc-

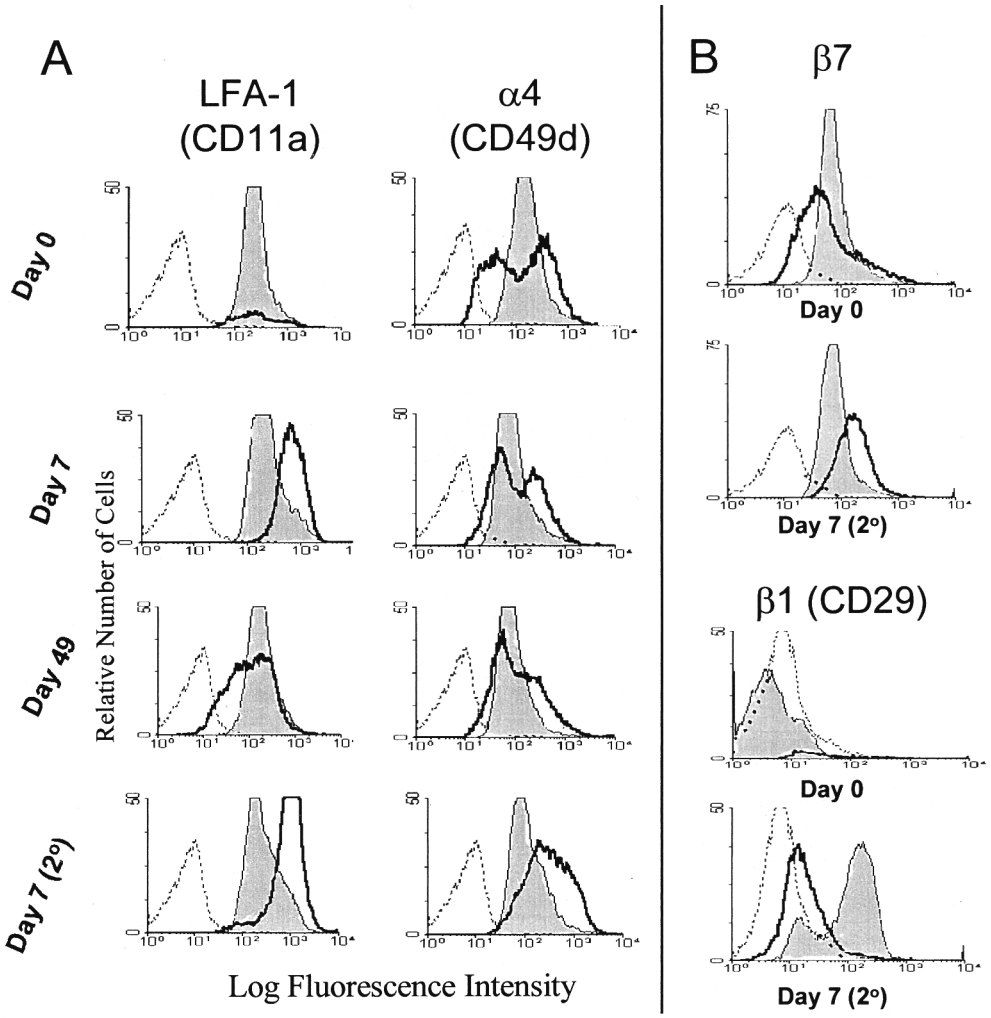

FIG. 4. Integrin expression on CD4 cells within the genital tract during infection. ILN and genital tracts from groups of 4 to 10 mice were harvested 0, 7, and 49 days after a single MoPn vaginal inoculation and 7 days following a challenge infection (2°). Like tissues were pooled, and the genital tracts were digested with collagenase. The cell populations were dual-stained for CD4 and CD11a, CD49d, β7, or β1 (CD29). Histograms represent the expression of various cell surface molecules on CD4 cells isolated from the ILN or spleen (shaded histograms) and genital tract (heavy lines). For each panel, the dotted line represents CD4 cells stained with the control antibody.

curred at an accelerated rate, with a peak as early as 5 days after infection. Analysis of CD49d on genital tract CD4 cells prior to infection revealed two distinct populations expressing either high or low levels (Fig. 4A). Likewise, the expression of CD49d on genital tract CD4 cells also followed a similar pattern of increased expression following infection with significantly greater levels on genital tract CD4 cells than on ILN or MLN CD4 cells ($P < 0.01$) (Fig. 5B) throughout the course of infection.

Expression of particular homing receptors on CD4 cells also regulates migration into different tissues. For instance, T cells that preferentially recirculate to Peyer's patches express the mucosal homing receptor comprised of CD49d (α4 integrin) and β7 integrin chains but not α4β1 (2). Since the vaginal mucosa is thought to be part of the common mucosal immune system (38), we examined genital tract CD4 cells for expression of the mucosal homing receptor. As shown in Fig. 4B (top panels), genital tract CD4 cells analyzed after challenge expressed higher levels of the mucosal homing receptor, β7, than did CD4 cells isolated from ILN. While this increase was not statistically different (mean fluorescence intensity ± standard deviation, 117 ± 20.4 for the genital tract and 110.2 ± 29.4 for ILN), the ILN drains the genital tract, and CD4 cells found in these tissues would also be expected to express the mucosal homing receptor. Furthermore, we observed a selection for the $\beta7^{high}$ population in the genital tract following infection (Fig. 4B). In contrast, expression of the peripheral homing receptor chain, β1, was seen on less than 2% of CD4 cells isolated from the genital tract, with a mean fluorescence intensity of only 33.1 ± 4.9 after infection (Fig. 4B, bottom panels, heavy lines) or at any other time point analyzed throughout the course of a primary or secondary infection (data not shown). This was not due to a detection problem since β1 expression was observed on the majority of splenocytes (Fig. 4B, bottom-most panel, shaded histogram). Thus, CD4 cells that are recruited to the genital tract during MoPn infection express the phenotype of a mucosal homing CD4 cell, $\alpha4\beta7^{high}\beta1^{low}$.

Expression of endothelial cells ligands within the genital tract during infection. The entry of lymphocytes into secondary lymphoid organs or tissues requires not only the expression of certain homing receptors but also the expression of the complementary ligands on specialized high endothelial cells

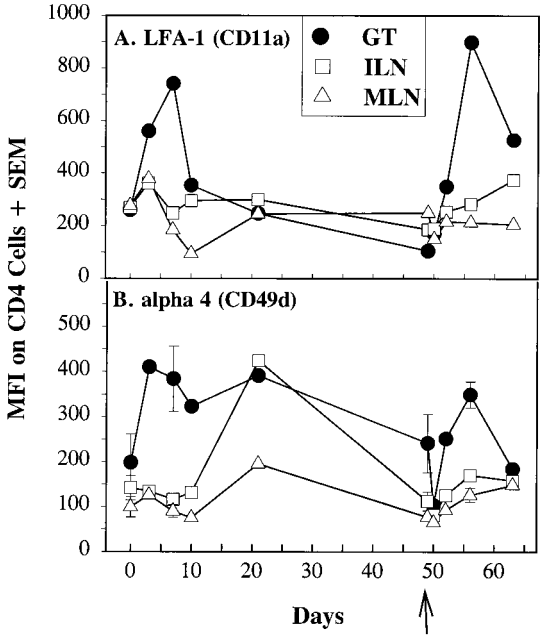

FIG. 5. Homing receptor expression on genital tract (GT) CD4 cells over the course of MoPn infection. ILN, MLN, and genital tracts were harvested on various days after a primary MoPn infection. Some groups of mice were rechallenged with a second MoPn infection on day 49 (arrow), and samples were harvested at various days after infection. Like tissues were pooled, and the genital tracts were digested with collagenase. The cell populations were dual-stained for CD4 and either LFA-1 (CD11a) (A) or α4 (CD49d) (B). In panel A, each datum point represents a single determination of the mean fluorescence intensity of CD11a expression on CD4 cells. In panel B, the datum points for days 0, 7, and 49 after primary infection and day 7 after a secondary infection represent the mean fluorescence intensities ± the standard errors of the means (SEM) of three experiments for α4 (CD49d) expression. Expression of CD49d was significantly greater ($P < 0.01$) in genital tract CD4 cells than in ILN or MLN CD4 cells.

that line blood vessels within tissues. VCAM-1 has been shown to mediate the homing of T cells to sites of inflammation through CD49d adhesion (32). We found that genital tracts from naive mice did not express VCAM-1 (Fig. 6A). However, this molecule was induced following MoPn vaginal inoculation. As seen in Fig. 6B, numerous venules in the lower genital tract stained intensely with a monoclonal antibody against VCAM-1. Interestingly, expression of VCAM-1 disappeared upon resolution of infection (Fig. 6C). This pattern was seen in four of four mice analyzed. ICAM-1 is also involved in T-cell homing to inflammatory sites through interactions with CD11a (40). In contrast to VCAM-1, ICAM-1 was found on venules of uninfected mice (Fig. 7C), on epithelial cells lining the lower genital tract 3 days after infection (Fig. 7D), and was greatly increased 7 days after MoPn infection to the extent that virtually the entire lower genital tract stained positive (data not shown).

We also examined the lower genital tract for expression of the mucosal endothelial cell ligand, MAdCAM-1, and peripheral ligand, GlyCAM-1. As seen for VCAM-1, no expression of MAdCAM-1 was observed in uninfected mice (Fig. 6D). Likewise genital tract infection with MoPn induced MAdCAM-1 expression on venules (Fig. 6E) in three of five mice but did not induce expression of GlyCAM-1 (data not shown). The expression of MadCAM-1 also subsided following resolution of infection (Fig. 6F). Thus, MoPn infection induces expression of genital tract endothelial ligands which correspond to those homing receptors found on CD4 cells recruited to the genital tract. Taken together, these data implicate a role for VCAM-1, MAdCAM-1, and ICAM-1 in the recruitment of protective CD4 cells to the genital tract.

Most tissues, with the exception of secondary lymphoid organs, do not constitutively express endothelial cell ligands and therefore do not routinely support the recirculation of lymphocytes. However, the inflammatory cytokines TNF-α and IL-1 can induce the differential expression of endothelial cell ligands derived from different vascular sites (7). Since *E. coli* LPS stimulates secretion of inflammatory cytokines and also directly induces the expression of ICAM-1 and VCAM-1 on human umbilical epithelial cells in vitro (7), we examined LPS expression in the lower genital tracts of infected mice. As seen in Fig. 7, we could detect chlamydial LPS on epithelial cells lining the lower genital tract 3 days after inoculation but not on epithelial cells in uninfected mice (Fig. 7A and B). Interestingly, this pattern coincided with the early expression of ICAM-1 on epithelial cells (Fig. 7D), suggesting that chlamydial LPS could also induce the expression of ICAM-1 and most likely other endothelial ligands in the genital tract. Taken together, the transient recruitment of CD4 cells to the genital tract suggests that the types of endothelial cell ligand and the local expression regulate the recruitment of protective CD4 cells, which in turn appears to be controlled by *C. trachomatis* infection.

DISCUSSION

In this study we have shown that CD4 lymphocytes are the predominant lymphocyte population recruited to the genital tract following MoPn infection. This report is the first to investigate mechanisms of CD4 cell recruitment to the vaginal mucosa during a natural infection and also the first report to identify adhesion molecules involved in this process. As others have shown, the genital mucosae of uninfected mice have relatively few resident lymphocytes (14, 42), but after infection we observed a significant increase in CD4 population but not in the CD8 and γδ populations, and the CD4 subset was further increased upon a second infection. The rapid influx of CD4 cells following a second infection is consistent with the pattern of a memory response. This is further substantiated by the phenotypes $CD45RB^{low}$, $CD62L^{low}$, and $CD44^{high}$ observed on CD4 cells isolated from the genital tract. Furthermore, we have identified receptor ligand pairs that may play a role in the regulation of T-cell recruitment to the genital mucosa. These data coupled with the kinetics of the proliferative response to MoPn within the genital tract indicate (6) that protective CD4 cells are recruited to the genital tract during MoPn infection and that this event is regulated by LFA-1:ICAM-1 and α4β7:MAdCAM-1-VCAM-1 interactions. Although ICAM-1 may play a role in CD4 trafficking to the genital mucosa, this molecule is constitutively expressed and may also support recirculation in noninflamed tissue.

These results support the findings of others that in the murine model of *C. trachomatis* genital infection, CD4 cells are sufficient to confer protection against a vaginal challenge (22, 64). While the transfer of CD8 lines and clones specific for MoPn can also confer protection (21, 47), the depletion of CD8 cells in vivo (37), as well as studies in class I knockout mice (39), attests to the importance of CD4 cells in a natural infection. In light of the fact that chlamydiae can elicit CD8 cells, the observation that very few CD8 cells are recruited to the MoPn-infected genital tract further supports a subordinate role for this cell type in chlamydial genital infection. Moreover, these data demonstrate that individual T-cell populations can be selectively recruited to sites of infection.

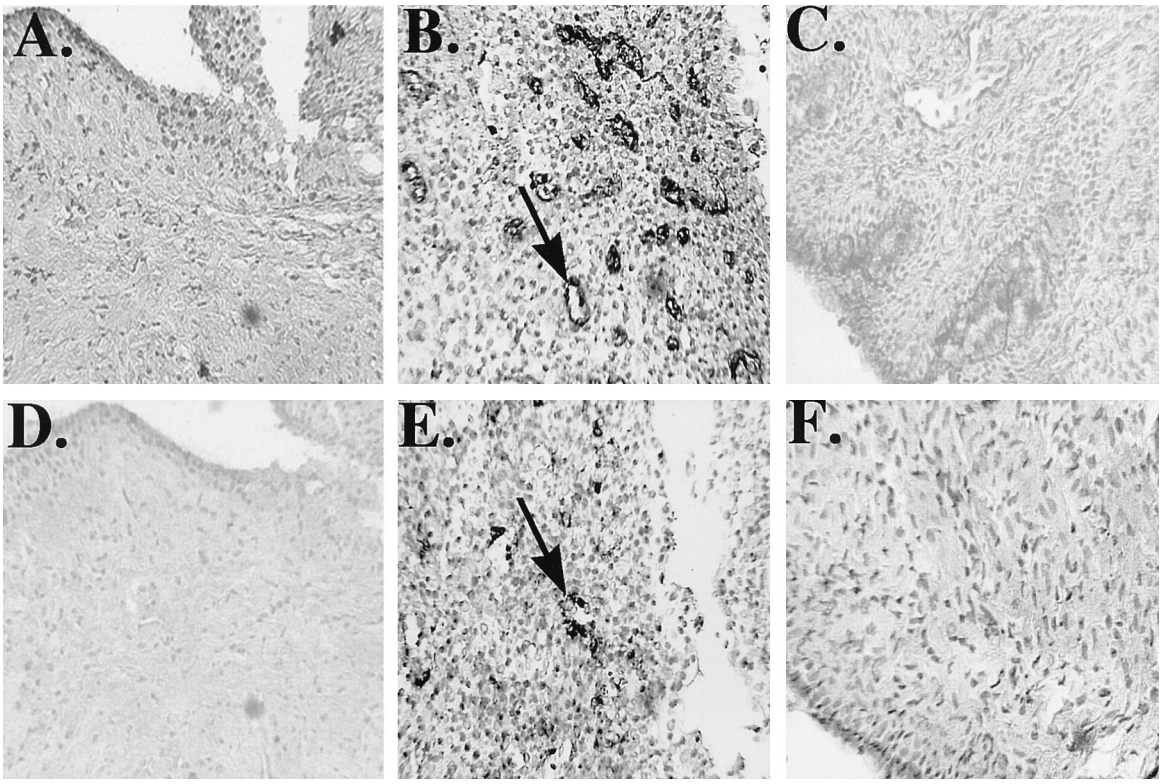

FIG. 6. VCAM-1 and MAdCAM-1 expression within the genital tract during MoPn infection. Frozen sections of lower genital tracts from mice harvested on days 0 (A and D), 7 (B and E), and 35 (C and F) were stained with monoclonal antibodies against VCAM-1 (A to C) or MAdCAM-1 (D to F). Arrows designate venules that stained positive for VCAM-1 (B) and MAdCAM-1 (E) 7 days after MoPn infection. Magnification, ×160 (A, C, D, and F) and ×250 (B and E).

The inability to transfer protection against a primary genital infection with anti-MoPn IgG- or IgA-secreting hybridomas (9) and the ability of B-cell-deficient mice to resolve infection and develop immunity to reinfection (48, 65) demonstrate that cell-mediated immunity, and not humoral immunity, is primarily responsible for the protective immunity in mice. However, humoral immunity may play a minor role in protection since Morrison et al. reported an association between the appearance of anti-MoPn IgA antibodies in vaginal washes and the resolution of MoPn genital infection in CD4-knockout mice (39). Our observation that B cells are not actively recruited to the genital tracts in MoPn-infected mice provides a possible explanation for the above findings. Although B-cell homing to genital tissues of mice has been reported, there were twofold-fewer labeled MLN B cells found in the cervix and vagina than in peripheral lymph nodes following adoptive transfer (38). Furthermore, this study was performed with naive mice, and the results may be influenced by lack of vascular addressins in the genital tract. The most likely role of B cells in MoPn genital infection may be in reducing the incidence of reinfection by production of specific antibody. In support of this, Cotter et al. observed a decrease in the number of oviducts containing MoPn inclusions in mice that were implanted with an anti-MoPn IgA-secreting hybridoma, indicating that high levels of specific antibody can confer protection in the genital tract (9). Also Su et al. recently reported that B-cell-deficient μMT/μMT-knockout mice had a higher incidence of reinfection than did control mice (65). Other models support this role for B cells since resolution and full protection from reinfection require both cell-mediated responses and specific antibody in the guinea pig model of genital infection (49, 53), and plasma cells have been observed in fallopian tubes during *C. trachomatis* salpingitis in humans (67). Although the B cells are not recruited to the site of local infection, mice develop a vigorous serum anti-MoPn response dominated by the IgG2a isotype (29) as well as anti-MoPn IgA in vaginal secretions (45).

The vaginal mucosa has been categorized as part of the common mucosal immune system based on the migratory patterns of lymph node cells (38). We have confirmed this association by demonstrating that genital tract CD4 cells express the mucosal homing receptor CD49d (α4)β7 and not the peripheral homing receptor α4β1. While all CD4 cells have been reported to express α4β1, the monoclonal antibody that we used to identify the β1 chain reacts with a conformational epitope on activated molecules (30). However, we were able to detect β1 expression on 5 to 50% of spleen cells (Fig. 4B) and cervical lymph node CD4 cells (data not shown). We also ruled out the possibility that collagenase digestion could be destroying cell surface markers on CD4 cells during the isolation procedure by comparing the mean fluorescence intensities of splenocytes isolated with and without collagenase. Of all the markers tested, only CD44 expression was decreased (data not shown). However, CD44 expression is extremely high on genital tract CD4 cells, even after collagenase digestion. Therefore, following a vaginal MoPn infection, memory CD4 cells expressing the phenotype $\alpha4\beta7^{high}\beta1^{low}$ are enriched in the genital tract. This phenotype correlates with the pattern of adhesion molecules expressed on venules within the genital tract during infection. Since only $\alpha4\beta7^{+}$ cells can bind to MAdCAM-1, we expected to find this molecule within infected genital tract tissue (Fig. 6E). We also detected venules staining intensely positive for VCAM-1 (Fig. 6B). Although α4β7 has

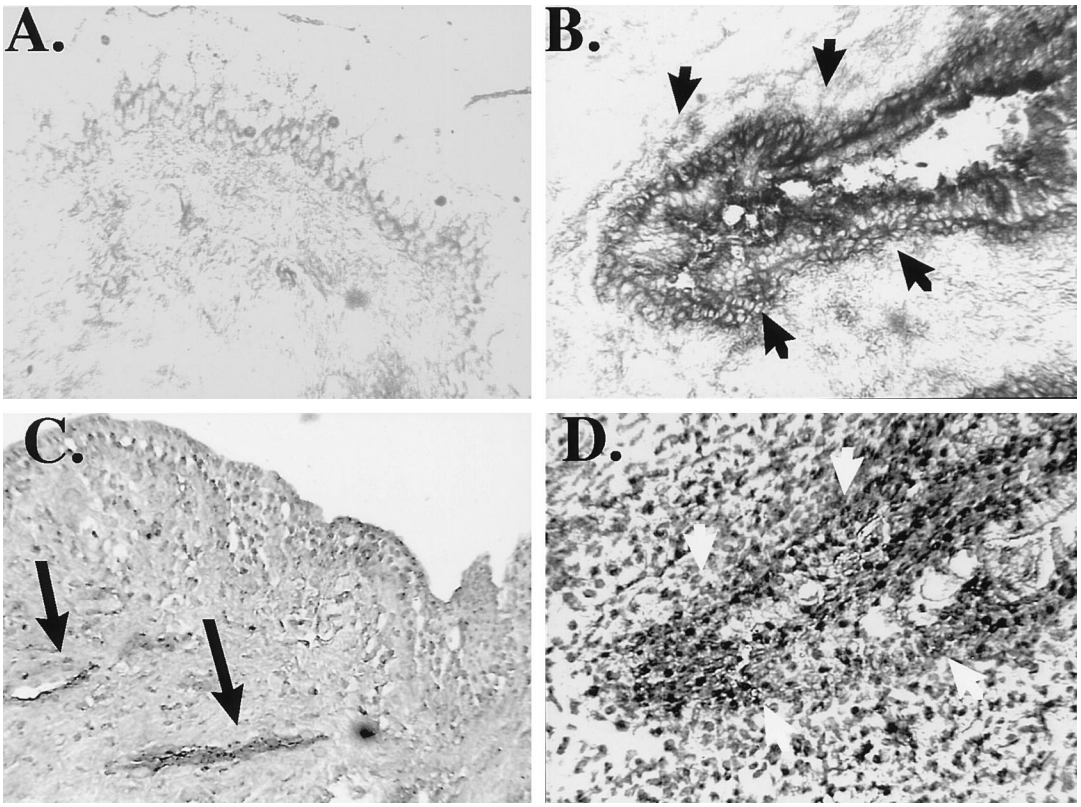

FIG. 7. Correlation of chlamydial LPS and induction of ICAM-1 in the genital tract following MoPn infection. Consecutive frozen sections of the lower genital tracts from mice harvested on day 0 (A and C) or day 3 (B and D) were stained with monoclonal antibodies against chlamydial LPS (A and B) or ICAM-1 (C and D). (B) Black arrowheads designate vaginal epithelial cells lining the endocervix that stained positive for LPS; (D) white arrowheads designate ICAM-1 staining. (C) Arrows show ICAM-1-positive endothelial cells in venules of naive mice. Magnification, ×160 (A and C) and ×250 (B and D).

been shown to bind VCAM-1 (1, 55), adherence to VCAM-1 tends to favor the $\alpha4\beta1^{high}$ subset of CD4 cells regardless of the levels of β7 expression (55). However, in the absence of β1, adherence to VCAM-1 is enriched for $\alpha4\beta7^{high}$ memory CD4 cells (55). Furthermore, at high densities of VCAM-1, $\alpha4\beta7^{high}$ memory cells have be shown to bind slightly better than $\alpha4\beta7^{-}$ memory CD4 cells (55). Based on these findings, it is likely that α4β7-VCAM-1 interactions also mediate entry of CD4 cells into the genital tract during MoPn infection. Certainly, other cell types could utilize α4β1-VCAM-1 interactions to gain entry to the genital tract during infection.

We were intrigued with the finding that the recruitment of CD4 cells to the genital tract was transient. Previously it has been shown in the mouse and guinea pig models of chlamydial genital infection that T-cell proliferative responses to chlamydial organisms in the genital tract diminished following the clearance of chlamydiae from the genital tract (23, 50). However, diminished responsiveness does not necessarily correspond to a decrease in the number of cells. This study is the first to demonstrate that CD4 cells are recruited to the genital tract and are retained for only a short period of time. Moreover, this finding may provide an explanation for the short-lived protective immunity observed with chlamydial genital infection. In support of this idea, Coulson and Wilson have demonstrated that the presence of *Schistosoma mansoni*-specific T cells within murine lungs at the time of intravenous challenge confers protection (10). Thus, the eradication of replicating MoPn from the genital tract may be responsible for the exodus of antigen-specific CD4 effector cells from that site and in turn may result in diminished protective immunity against future challenge. This finding has obvious implications for the design of immunization strategies aimed at producing protective cell-mediated immunity at peripheral sites. Nevertheless, it is encouraging that upon reinfection, CD4 cells are recruited more rapidly to the infection site. This finding may have both positive and negative aspects. Certainly, the rapid recruitment of cells is essential to limit a challenge infection. However, there is evidence that reproductive pathology occurs via delayed-type hypersensitivity-like mechanisms (51) and that the cells involved in this type of response may also be recruited to the genital tract by the same mechanisms.

Although, purified *C. trachomatis* LPS can induce NF-κB translocation and TNF-α secretion, it is 100-fold-less potent than *E. coli* LPS (24). In this study, we noted that on consecutive sections of the lower genital tract, epithelial cells which stained positive for *C. trachomatis* LPS also expressed ICAM-1, suggesting that chlamydial LPS could also induce the up-regulation of endothelial ligands, as has been shown for *E. coli* LPS. However, the induction of endothelial cell ligands within the genital tract following MoPn infection could also be due to inflammatory cytokines that are produced locally and not due to LPS directly. Infection with MoPn has been shown to elicit IL-1α, IL-1β, and IL-6 in vivo during a lung infection (36) and also TNF-α and IFN-γ within the genital tract following vaginal inoculation (11, 43). Recently, some bacterial heat shock proteins from *E. coli* have also been shown to up-regulate ICAM-1 expression and induce CD62E and VCAM-1 on cultured human umbilical epithelial cells (18). These findings emphasize the regulatory capacity of bacteria to control leukocyte migration at the site of infection.

Unlike that in other models for studying the induction of endothelial ligands in other tissues during inflammation (19), we observed not only the induction of VCAM-1 and MAdCAM-1 but also the down-regulation of these molecules following the clearance of chlamydiae from the genital tract. While this may be due to elimination of specific antigen from the genital tract, reports demonstrate the persistence of chlamydial organisms within tissues that are culture negative (3). Possibly, other factors could actively down-regulate the expression of endothelial cell ligands. Nevertheless, this observation provides a unique opportunity to study mechanisms regulating the expression and down-regulation of endothelial cell ligands. Taken together, these data indicate that chlamydial LPS and/or inflammatory cytokines induced through MoPn infection elicit VCAM-1 and MAdCAM-1 expression on venules within the genital tract and induce the expression of ICAM-1 on vaginal epithelial cells and other areas throughout the genital tract. Furthermore, the regulation of these molecules may then control the magnitude of protective immunity within the vaginal mucosa.

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