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Two different homing pathways involving integrin $\beta 7$ and E-selectin significantly influence trafficking of CD4 cells to the genital tract following *C. muridarum* infection

Kathleen A. Kelly, PhD^{1,2}, Ann M. Chan³, Anthony Butch, PhD¹, and Toni Darville, MD⁴

¹ Dept of Pathology & Laboratory Medicine, University of Pittsburgh Medical Center, Pittsburgh, PA 15260

² California NanoSystems Institute, University of Pittsburgh Medical Center, Pittsburgh, PA 15260

³ Department of Physiological Science, University of Pittsburgh Medical Center, Pittsburgh, PA 15260

⁴ Dept of Pediatrics, Children's Hospital of Pittsburgh, University of Pittsburgh Medical Center, Pittsburgh, PA 15260

Abstract

Problem—*Chlamydia trachomatis* causes STI and reproductive dysfunction worldwide which is not preventable with antibiotics. Identifying a population of endocervical T cells to target in vaccine development would enhance efficacy.

Method of Study—Trafficking of murine CD4+ lymphocytes to *Chlamydia muridarum* infected genital tract (GT) tissue *in vivo* was measured using adoptive transfer studies of fluorescent CD4+ T cells from integrin $\beta 7^{-/-}$ mice or mice which lack E-selectin on endothelial cells.

Results—Murine *in vivo* migration studies showed that lack of $\alpha 4\beta 7$ or E-selectin significantly reduced trafficking of CD4 T cells to the GT of mice infected with *C. muridarum*.

Conclusions—CD4+ T cells use at least two different adhesive mechanisms involving an integrin of the mucosal homing pathway and selectin pathway to accumulate in the GT during *C. muridarum* infection.

Keywords

cell migration; chemokines; *Chlamydia*; murine; lymphocytes

INTRODUCTION

Animal models of chlamydial genital tract (GT) infection have demonstrated that CD4 Th1 cells are necessary for eradication of chlamydiae from the murine GT. 1· 2 Using the mouse model, we have shown in direct comparison that primary exposure of chlamydial antigens via mucosal routes induces an immune response that more rapidly eradicates a subsequent genital infection compared to primary exposure of chlamydial antigens via a subcutaneous route. 3 This result is consistent with the tenet that the anatomical location of initial antigen exposure can define the homing properties of a memory T cell population.

Address correspondence and reprint requests to Dr. Kathleen A. Kelly, Department of Pathology & Laboratory Medicine, Geffen School of Medicine, University of California, Los Angeles, 10833 Le Conte Ave., mailroom A7-149 CHS, Los Angeles, CA 90095-1732. E-mail address: kkelly@mednet.ucla.edu.

Studies have shown that CD4 T cells primed in lymphoid organs which drain mucosal sites increase surface expression of $\alpha 4\beta 7$ integrin, also called the mucosal integrin; whereas those primed in lymph nodes draining peripheral or non-mucosal sites lose $\alpha 4\beta 7$ integrin expression following antigen exposure and instead express P-selectin ligand and accumulate in peripheral tissues. 4 Further, a murine T cell clone that mediates protection upon adoptive transfer expresses high surface levels of $\alpha 4\beta 7$ and these clones appear in the GT significantly earlier than non- $\alpha 4\beta 7$ -expressing lymphocytes. 5 In addition we found that the expression of ligands for adhesion of $\alpha 4\beta 7^+$ (MAdCAM) and non- $\alpha 4\beta 7^+$ cells (E-selectin, P-selectin, ICAM-1 and VCAM-1) were transiently induced in infected genital tissues indicating that expression of endothelial ligands in the GT is regulated by infection. 6 Taken together, these data indicate that both mucosal and non-mucosal homing interactions contribute to recruitment of murine CD4 T cells to the GT.

Identifying the set of homing receptors that are important for rapid lymphocyte migration to the female GT would enhance the accumulation of an anti-chlamydial specific cell mediated response and reduce infection levels. Previous *in vitro* studies revealing expression of adhesion molecules on human Fallopian tube tissue explants infected *in vitro* with *Chlamydia trachomatis* suggest that lymphocytes may use multiple adhesion pathways to migrate to human tissues. 7 We reported a significant upregulation of the mucosal adhesion molecule, MAdCAM-1, and also a non-mucosal adhesion molecule associated with inflammation: vascular adhesion molecule-1, VCAM-1. We also noted the marked increase of additional non-mucosal adhesion molecules; P-selectin and intracellular adhesion molecule-1 (ICAM-1). We sought to determine whether both a mucosal homing pathway and a peripheral homing pathway influence trafficking of CD4 T cells to the GT by using adoptive transfer and knockout mice depleted of $\beta 7$ -integrin on T cells (mucosal) and selectin molecules (inflammatory/peripheral) on endothelial cells.

MATERIALS AND METHODS

Animals

Female BALB/c (Harlan Sprague-Dawley, Indianapolis, ID), C57BL/6 mice (Jackson Laboratories, Bar Harbor, ME), beta 7 integrin knockout mice on the C57BL/6 background developed by Dr. Norbert L. Wagner 8 at the Institute for Genetics, at the University of Cologne, Germany and kindly provided by Dr. L Lefrancois at the University of Connecticut Health Sciences. 9 P-selectin and E-selectin knockout mice on the BALB/c background were kindly provided by Dr. Daniel C. Bullard, University of Alabama at Birmingham. 10 All mice were housed according to the American Association of Accreditation of Laboratory Animal Care guidelines. Experimental procedures and breeding colonies were approved by the UCLA Institutional Animal Care. All mice, 5 to 7 weeks of age, were first injected subcutaneously with 2.5 mg of Medroxyprogesterone acetate (Upjohn, Kalamazoo, MI) in 100 μ l of sterile phosphate-buffered saline. Medroxyprogesterone acetate drives mice into a state of anestrus thus eliminating the variability in the rate and severity of infection due to the estrus cycle. Seven days later, anesthetized mice were vaginally inoculated with 1.5×10^5 inclusion-forming units (IFU) of *C. muridarum* grown in McCoy cells (50% infective dose = 2.5×10^3 IFU). Infection was monitored by obtaining vaginal swabs (Dacroswab Type 1, Spectrum Labs, Houston, TX) every three days. Swabs were stored in sucrose-phosphate buffer at -70°C until analyzed.

Isolation of chlamydiae from cervical-vaginal swabs of mice

Swabs were prepared and chlamydial load was determined by quantifying the number of chlamydial inclusions as previously described. 11 12 The inclusion bodies within 20 fields

($\times 40$) were counted under fluorescence microscope and numbers of IFU per milliliter were calculated.

Antibodies

The following rat anti-mouse Abs were purchased from PharMingen, San Jose, CA; CD8-biotin (clone 53-6.7), kappa-biotin (clone 187.1) and CD45R-biotin (clone RA3-6B2) and were used for negative selection over magnetic columns with streptavidin conjugated to microbeads from Miltenyi Biotec, Auburn, CA.

Flow cytometry

Single cell suspensions (3×10^5 to 5×10^5 cells) were stained in DMEM containing 1% bovine serum albumin (Sigma, St Louis, MO) and 0.1% sodium azide using the microplate method as previously described. 6 Isolated cells were first incubated with $10 \mu\text{g}/\text{ml}$ of fluorescently tagged or unconjugated mouse anti-human cell surface markers (see “Antibodies”) for 25 min on ice and then washed twice with DMEM containing 10% bovine serum albumin. The cells were then resuspended in $20 \mu\text{g}/\text{ml}$ of PE-conjugated goat anti-mouse F(ab')₂ (Biosource International, Camarillo, CA) for 25 min on ice.

Following the washing step described above, the cells were fixed in phosphate-buffered saline containing 1% paraformaldehyde and kept at 4°C until analyzed. Flow cytometry was performed on a fluorescence activated cell sorting analyzer equipped with a 488-nm argon laser and CellQuest software (FACScan; Becton Dickinson, San Jose, Calif.). The instrument was calibrated with beads (CaliBRITE; Becton Dickinson), using AutoCOMP software. Dead cells were excluded on the basis of forward-angle and 90° light scatter, and 10,000 gated cells were analyzed for each sample.

Fluorescent Labeling

CD4 cells were purified from the spleens of beta 7 integrin knockout and wild type (WT) C57BL/6 mice on day 7 following a GT infection. To purify CD4 cells, CD8 cells and B cells were removed by negative selection over magnetic columns using anti-CD8, anti-kappa and anti-CD45R. The resulting lymphocytes were $>95\%$ pure for CD4 in select experiments. Purified CD4 cells or a *Chlamydia*-specific CD4 Th1 cell clone (Th1-MoPn)13 were labeled with the red fluorescent dye, PKH-26 (Sigma, St. Louis, MO) or the green fluorescent dye, BODIPY-green (Molecular probes, Carlsbad, CA) as previously described¹⁴

Adoptive Transfer of beta 7 integrin positive or negative CD4 cells

CD4⁺ cells were purified from the spleens of beta 7 integrin $-/-$ (knockout, $\beta 7^{-/-}$) mice 7 days after GT infection in order to enrich for *Chlamydia muridarum* responsive T cells and labeled with the red fluorescent dye, PKH-26 (Sigma, St. Louis, MO) as previously described.¹⁴ A second group of CD4⁺ cells which expressed beta 7 integrin ($\beta 7^{+/+}$) were purified from the spleens of C57BL/6 mice 7 days after infection and also labeled with the red fluorescent dye, PKH-26. With each adoptive transfer, an equal percent (50:50) of CD4⁺ cells also isolated from infected $\beta 7^{+/+}$ WT mice but labeled with a green fluorescent dye called BODIPY-green (Molecular probes, Eugene, OR) were added separately to both the red labeled ($\beta 7^{-/-}$) and ($\beta 7^{+/+}$) CD4 cells to normalize *in vivo* migration assays using donor cells from different strains of mice ($\beta 7$ knockout and wildtype cells). Thus, a 50:50 mixture of either red PKH-26 ($\beta 7^{-/-}$) (1×10^7) or red PKH-26 ($\beta 7^{+/+}$) (1×10^7) and green BODIPY ($\beta 7^{+/+}$) (1×10^7) cells from infected mice were adoptively transferred to recipient C57BL/6 mice infected 7 days previously with *C. muridarum*. GT tissues were harvested 18 hours after adoptive transfer and single cell suspensions were prepared as previously described.¹⁴ The percentage of red labeled cells is a measure of the dependence of cells expressing beta 7

integrin to enter the GT. Percentages that are greater than 50 indicate enhanced migration. As a positive control for $\alpha 4\beta 7$ migration, we also measured the ability of beta 7 integrin cells to accumulate in mesenteric lymph nodes as was previously described¹⁵.

Adoptive Transfer of Th1-MoPn clone to P and E-selectin^{-/-} mice

To examine the necessity of selectin molecules on the endothelium, we adoptively transferred 1×10^7 red fluorescent clone cells (Th1-MoPn) labeled with PKH-26 14 and P-selectin^{-/-}, E-selectin^{-/-} or BALB/c mice on day 7 following *C. muridarum* infection. Eighteen hrs after transfer, GT, iliac (ILN) and MLN tissues were harvested and single cell suspensions were prepared and measured on a flow cytometer. The percentage of clone Th1-MoPn (red fluorescent cells) was determined among total GT cells present in various tissues as previously described. 5

Genital tract (GT) homogenates

GT tissues were divided into the cervical-vaginal region (lower GT), uterine horns (middle GT) and oviducts (upper GT) with the ovaries removed as previously described¹⁶. Tissue sections from individual mice were placed in 1 ml of RNazol B (Tel-Test, Inc., Friendswood, Tex.), and homogenized as previously described¹⁷ using a hand-held homogenizer (Omni International, Warrenton, Va.). Aliquots of each homogenate were removed for isolation of chlamydiae. The remaining homogenate volumes were sonicated at 20 kHz for 1 min and then centrifuged at $900 \times g$ for 15 min at 10°C to remove cellular debris. Supernatants were filtered through 0.2- μ m-pore-size Acrodiscs (Gelman Sciences, Ann Arbor, Mich.) to remove chlamydiae, and samples were stored at -70°C until analyzed.

mRNA isolation and PCR analysis

Total RNA was isolated from murine GTs homogenized in RNazol B (Tel-Test, Inc., Friendswood, Tex.), quantitated on a spectrophotometer analysis at 260 nm wavelength and stored at -80°C until used. As a positive control, mice were injected i.p. with 30 μ g TNF α (R&D Systems, Minneapolis, MN) and the GT tissue harvested 4 hours later. Five μ g mRNA was reverse transcribed into cDNA using a reverse transcription kit (Pharmacia Biotech, Piscataway, NJ). The primers used were 5' - gAC AgC AgA AAA CTT TCg TgC - 3' (sense) and 5' - TCC AgC CAC TCA gTC TTg g - 3' (antisense) for cyclophilin and 5' - CgT CCT CAT TgC TCT ACT TgT - 3' (sense) and 5' - Cgg TgT TTC TgT TCC CAA AT - 3' (antisense) for E-selectin. The amplification buffer contained 50 mM KCl, 10 mM Tris-HCl (pH 8.3), and 2.5 mM MgCl. Specific oligonucleotide primer was added (200 ng per sample) to the buffer, along with 1 μ g of the reverse transcribed cDNA samples and a HotstartTaq DNA polymerase (Qiagen, Valencia, CA). The cDNA was amplified after determining the optimal number of cycles. The mixture was first incubated for 15 min at 95°C then was cycled 35 times at 95°C for 1 min 30 s and 58°C for 1 min 20 s, and elongated at 72°C for 2 min. These conditions allowed optimal amplification with little or no nonspecific amplification of contaminating DNA. After amplification, the sample was separated on a 3% agarose gel containing 0.3 μ g/ml (0.003%) ethidium bromide. The bands were visualized and photographed using UV transillumination.

Statistics

The percent of red fluorescent (PKH-26) labeled from wildtype (C57BL/6), $\beta 7$ -ntegrin knockout mice were compared by Student's t-test. In addition, the percent of red fluorescent (PKH-26) labeled Th1-MoPn clone cells within isolated GT cells were compared among Wildtype (BALB/c), P-selection^{-/-}, and E-selectin^{-/-} mice using ANOVA. The course of infection compared among mouse strains using repeated measures ANOVA. The above mentioned statistical tests were suggested by and performed using SigmaStat software based

on the distribution of the data and sample size (Jandel Scientific, San Rafael, Calif.). Groups were considered statistically different at p values of < 0.05 .

RESULTS

Most adhesion interactions are not species dependent so we utilized the mouse model of chlamydial genital infection to confirm the role of the mucosal homing receptor ($\alpha 4\beta 7$:MAdCAM-1) in lymphocyte migration to the chlamydial-infected GT *in vivo* during a sexually transmitted infection (STI). We have previously shown that $\alpha 4\beta 7$ was used by cloned CD4 cells specific for *Chlamydia muridarum* to gain entry to infected murine GT tissues 14. To examine a polyclonal T cell population, we focused on the beta 7 integrin chain to examine mucosal homing by adoptive transfer since T cell homing integrins are heterodimers of the alpha 4 integrin chain with other integrins to form mucosal as well as non-mucosal homing receptors. As expected, the adoptive transfer of an enriched population of CD4⁺ anti-chlamydial responsive cells required beta 7 integrin for migration to the MLN 15-18 verifying that our assay measures beta 7 integrin dependence. Analysis of infected GT tissues during a *C. muridarum* STI revealed that the absence of beta 7 integrin significantly reduced cell recruitment to the GT (Fig. 1A) and indicates that $\alpha 4\beta 7$ does impart an increased ability of lymphocytes to migrate to the infected genital mucosa. A set of mice were also monitored for the course of infection. We did not observe a difference between the groups in time of resolution (Fig. 1B) but noted a marked but not significant increase in the bacterial burden of mice lacking $\beta 7$ -integrin on their CD4 cells. This finding corroborated our transfer studies and suggests that the absence of a single lymphocyte adhesion pathway is not sufficient to significantly alter bacterial shedding. Thus, access of T cells to the GT during *C. muridarum* infection was enhanced by expression of beta 7 integrin.

To dissect the influence of non-mucosal homing integrins which bind to CLA (CLA:selectin) on GT migration during *C. muridarum* infection, we employed mice deficient for P-selectin and E-selectin which previously have been shown to bind to CLA expressed on human cells 19 and compared adoptive transfer of a protective *Chlamydia*-specific Th1 clone. 14 As can be seen in Figure 2A, the absence of E-selectin but not P-selectin on GT tissue endothelial cells significantly prevented the ability of the protective T cell clone (P-MoPn) to rapidly gain access to the GT during *C. muridarum* infection ($p < 0.05$). This effect was limited to infected tertiary tissue sites since no differences were found in the lymphoid organs of the iliac (ILN) and MLN (data not shown). We also monitored the course of infection in mice lacking selectin molecules on the endothelium. As seen with $\beta 7$ -integrin knockout mice, we did not observe a difference between mice lacking selectin adhesion molecules on the endothelium (Fig. 2B). This finding supports the idea that deletion of a single lymphocyte adhesion pathway is not sufficient to significantly alter bacterial shedding. Taken together, these data identify E-selectin as an important ligand expressed in chlamydial-infected GT tissues for mediating the binding and accumulation of T cells within GT tissues.

The inflammatory cytokine, TNF α , induces the expression of E-selectin on endothelial cells 20 and TNF α and other inflammatory cytokines are produced during chlamydial genital infection 21 The involvement of E-selectin in lymphocyte trafficking to the GT strongly suggests that inflammation enhances lymphocyte accumulation in the GT. To confirm this finding we attempted to demonstrate the appearance of E-selectin in the GT. We previously reported that non-infected murine GT tissues do not express P-selectin but rapid expression is seen by immunohistochemical analysis following *C. muridarum* infection 14. However, immunohistochemical analysis of E-selectin is technically difficult in murine tissues and does not conclusively show expression 22. Thus, we investigated the appearance of E-

selectin mRNA and found that E-selectin mRNA was present in the oviducts or upper GT region but not in the uterine horns or middle GT and only variably expressed in the lower GT of infected mice suggesting that inflammation is more apparent in the upper GT region. These results indicate that inflammation induced by chlamydial infection influences the trafficking of CD4 cells to the GT. *Chlamydia* infection is also associated with tissue damage 23 and it will be important to determine if upregulation of P and E-selectin molecules are associated with GT tissue damage.

DISCUSSION

Resolution of acute *Chlamydia* infection of the female reproductive tract requires Th1 CD4 T cells as shown using animal models. 24 We have previously shown in the murine model that CD4 cells expressing $\alpha 4\beta 7$ migrate more rapidly to the *C. muridarum*-infected murine GT. 5 These studies were performed using a Th1 clone specific for *C. muridarum* which provides protection from vaginal infection with *C. muridarum* when adoptively transferred to naive mice. In this study we confirmed that the mucosal pathway, dependent on the $\alpha 4\beta 7$ homing receptor, is involved in CD4 cell accumulation in the *C. muridarum* infected GT. Further, we show that E-selectin expressed on the endothelium also controls the accumulation of CD4 cells into the infected GT. These data are the first to show that multiple pathways regulate CD4 cell trafficking to the GT during chlamydial infection. These data further demonstrate that both a mucosal and non-mucosal pathway can mediate CD4 T cell trafficking to the GT.

Although we did not observe a significant difference in the course of infection in mice deficient in only one lymphocyte trafficking pathway, we noted the $\alpha 4\beta 7$ and selectin adhesion appear to influence different segments of the infection; early and late, respectively. For instance, mice lacking the mucosal homing pathway showed more variation in the early phase of infection consistent with our study showing that the mucosal trafficking pathway influences ability of cells to rapidly accumulate in the genital tract 5. Recently a mouse was made where the MAdCAM-1 adhesion molecule on endothelial cells can be conditionally knocked out. Experiments using this mouse lacking MAdCAM-1 at the initiation or resolution phase of the infection would substantiate this possibility. Further, crossing mice that lack E-selectin with those that lack MAdCAM-1 would be predicted to statistically increase chlamydial burden in the GT.

The regulation of lymphocyte homing has important implications for vaccine design and dictates that an efficacious vaccine stimulate the expression of $\alpha 4\beta 7$ on T cells. There is little evidence that induction of systemic T cell-mediated immunity alone can protect from infection in mucosal surfaces 25. Unfortunately, we don't understand how to induce T-cell mediated mucosal immunity but selective induction of the mucosal homing receptor would enhance the entry of T-cell mediated immune cells in mucosal tissues 26. Our current knowledge has demonstrated that induction of T cell lymphocyte homing receptors occurs during T cell activation. Mora, et. al., reported that murine dendritic cells (DC) program the expression of homing receptors on murine T lymphocytes during activation. 27 A subset of murine DCs found in the intestine preferentially induced expression of $\beta 7$ integrin compared to peripheral DCs, regardless of the source of T cells. In contrast, DCs isolated from peripheral tissues regulated the expression of homing receptors which bound selectin molecules on endothelial cells. 19, 28 Thus, murine DCs dictate the homing properties of T cells during activation and specific subsets of DCs appear to differentially program the homing properties of T cells.

Parallels can be drawn between the results of this migration study and *Chlamydia trachomatis* STI in humans. We have previously reported that the mucosal adhesion

molecule MadCAM and E & P-selectin are increased in the fallopian tube upon infection using the *in vitro* fallopian tube infection model 7. Although the murine analog which mediates the binding of lymphocytes to E-selectin has not yet been identified, in humans CLA binds to E-selectin. Also, human T cells expressing CLA bind to murine P and E-selectin molecules. 19 Thus, humans infected with *C. trachomatis* would be expected to use CLA and the mucosal homing receptor on lymphocytes to gain entry to the reproductive tract.

There is an association between Th1 cells and selectin molecules. For example, Th1 cell migration to other murine tissues requires selectin ligands. 29 Recently, Haddad et. al., 30 showed that the migration of $\alpha 4\beta 7$ Th1 lymphocytes to the lamina propria of the murine intestine depended on expression of P-selectin. Here, we report that a selectin molecule, E-selectin, is important in the migration of Th1 cells to the infected murine GT. This finding further delineates differences in T cell migration to the GT and intestine 31 and establishes a unique set of molecules that regulate CD4 T cell migration to the GT. Our prior studies using human Fallopian tube tissue also noted slight increases of selectin molecules in human Fallopian tube tissue upon infection with *Chlamydia* 7. Taken together, this study shows that adhesive pathways involving $\alpha 4\beta 7$ and E-selectin contribute to lymphocyte homing to the GT. Future studies using double knockout mice will help clarify the necessity of $\alpha 4\beta 7$ and E-selectin in vaccine models.

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Figure 1.

Beta 7 integrin, contributes to lymphocyte migration to the murine genital tract. A) CD4 cells purified from beta 7 integrin KO and WT C57BL/6 mice 7 days after infection with *C. muridarum*. Cells were label with the red fluorescent dye, PKH-26. To control for individual variability in the migration assay, CD4 cells were also purified from WT mice and labeled with the green fluorescent dye, BODIPY-green. The labeled red (beta 7+ or beta 7-/-) and green cells were mixed in equal proportions and adoptively transferred into 7 day post-*C. muridarum* infected WT C57BL/6 mice. Cells were harvested 18 hours later. n = 3 mice per group, *p<0.001 by t-test. B) Shedding of *C. muridarum* was determined every three days after vaginal inoculation with 1×10^5 IFU. Data points denote means from 5 mice. There

were no significant differences between groups. Representative of two experiments with similar results.

Figure 2.

The absence of E-selectin on endothelial cells influences the trafficking of CD4 cells to the GT. A) CD4 Th1 clone specific for *C. muridarum* was labeled with PKH-26 and adoptively transferred into BALB/c mice infected 7 days previously with *C. muridarum*. The cells were harvested 18 hours later. $n = 4$ mice per group, $*p=0.014$ by ANOVA. B) Shedding of *C. muridarum* was determined every three days after vaginal inoculation with 1×10^7 IFU. Data points represent the mean from 5 mice. There were no significant differences between groups. Representative of two experiments with similar results.

Figure 3.

E-selectin is present in the upper GT. GT tissues were harvested 7 days following vaginal infection as described and homogenates were prepared of the upper GT (oviducts, UGT), middle GT (uterine horns, MGT) and lower GT (cervico-vaginal region, LGT) from two representative mice (1 & 2). Homogenates of the spleen from a TNF α injected mouse served as positive control. PCR analysis was also performed on GT tissue using primers against the housekeeping gene, cyclophilin.