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The Combination of the Gastrointestinal Integrin ($\alpha 4\beta 7$) and Selectin Ligand Enhances T Cell Migration to the Reproductive Tract during Infection with *C. trachomatis*

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Abstract

Problem—*Chlamydia trachomatis* cause sexually transmitted infection and reproductive dysfunction worldwide. Identifying a population of endocervical T cells to target in vaccine development is likely to enhance efficacy of a vaccine and reduce reproductive tract dysfunction.

Method of Study—Endocervical samples were obtained from young women and flow cytometric analysis was used to identify lymphocytes that appeared in the genital tract in response to bacterial sexually transmitted infections caused by *C. trachomatis*.

Results—Increased numbers of $\alpha 4\beta 7$ +CLA+ memory T cells, a unique T cell phenotype, were found in the endocervix of human females infected with *C. trachomatis*.

Conclusion—A unique population of memory T lymphocytes expressing both $\alpha 4\beta 7$ and CLA gain access to reproductive tract tissues during a sexually transmitted infection with *C. trachomatis* and should be considered in development of vaccines against sexually transmitted infections.

Keywords

cell migration; chemokines; *Chlamydia*; human; reproductive tract

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³Abbreviations used in this paper: GT, genital tract; MLN, mesenteric lymph node; ILN, iliac lymph node; $\alpha 4\beta 7$, mucosal integrin; CLA, cutaneous leukocyte antigen; STI, sexually transmitted infection; WT, wild type; DC, dendritic cell.

INTRODUCTION

Sexually transmitted infections caused by *C. trachomatis* induce infection in the upper reproductive tract¹. Animal models of chlamydial genital tract (GT) infection have demonstrated that CD4 Th1 cells are necessary for eradication of chlamydiae from the murine GT^{2, 3}. The anatomical location of initial antigen exposure can define the homing properties of a memory T cell population irrespective of the antigenic stimulus. Investigation of murine and human systems 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, 5}. The human female reproductive tract is a member of the common immune system and expresses the ligand for $\alpha 4\beta 7$ integrin, MadCAM-16. Thus, mucosal homing mechanisms are likely to control leukocyte entry into the GT.

An ideal vaccine would promote expansion of a memory T cell population which can rapidly home to the primary site of tissue invasion and effectively eradicate the pathogen. Memory T cells acquire a unique set of homing properties upon entry into tissue sites and this process is called tissue imprinting^{7, 8}. Tissue imprinting imparts the ability of memory cells to rapidly migrate back to the original tissue site. Lymphocyte migration to tissues in humans is mediated by a finite set of adhesion molecules. Two examples are the mucosal homing receptor, $\alpha 4\beta 7$ integrin and the peripheral homing receptor, cutaneous lymphocyte antigen (CLA). Expression of $\alpha 4\beta 7$ integrin or CLA enables exclusive migration of lymphocytes to mucosal tissues and peripheral tissue sites such as the skin, respectively.

Induction of homing receptors important for rapid lymphocyte migration to the female GT would enhance efficacy of a vaccine for chlamydial genital infection. 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 tissues⁶. 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). To confirm that lymphocyte migration to the human GT employs both mucosal and non-mucosal T cell migration pathways, we examined expression of the mucosal homing receptor ($\alpha 4\beta 7$ integrin) and non-mucosal homing receptor (CLA) on endocervical T cells isolated from humans during acute infection with a bacterial STI that causes infection in the upper region of the reproductive tract; *C. trachomatis*.

MATERIALS AND METHODS

Study Subjects

Females who presented to the UCLA Student Health Center for a gynecological examination and cultures for sexually transmitted infections (STI) were eligible for enrollment in the study. All study subjects were defined as negative by wet prep for *Candida*, *T. vaginalis*, and evidence of bacterial vaginosis. They showed a normal cytological appearance of the Papanicolaou (PAP) smear did not have ulcerative genital lesions and had a CBC within the normal range. They were not pregnant or menstruating, had not used vaginal creams or suppositories, had not douched within the past 2 days, and had not taken any antibiotics during the previous 14 days prior to sample collection. The use of contraceptive devices was not included as an exclusion criterion for the study. If endocervical bleeding was induced during sample collection it was not found to be excessive. Eligible study subjects donated a

peripheral blood sample and two endocervical brush samples from the pelvic exam. The study was reviewed and approved by the UCLA Institutional Review Board for human studies.

Antibodies

Murine IgG anti-human antibodies against CD3-PerCP (clone SK7), CD45RO-APC (clone UCHL1), CD103 (clone BerACT8), CLA-FITC (clone HECA452), mouse IgG (clone X40), and mouse-IgG-FITC (clone X40) were purchased from BD Biosciences, San Jose, CA. Murine IgG anti-human CD11c-FITC (clone MCA 1848F) was purchased from Serotec, Raleigh, NC. Murine anti- $\alpha 4\beta 7$ integrin (clone ACT-1) was kindly provided by Dr. Michael Briskin at Merrimack Pharmaceuticals.

Flow cytometry

Two endocervical brush samples (Medscand Cytobrush Plus Cell Collector, Owens & Minor, Mechanicsville, VA) and peripheral blood were collected by a nurse practitioner from 40 study subjects described above and kept at room temperature in RPMI media (Gibco, Carlsbad, CA). Cells were released from the cytobrush by gentle rotation. 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 described⁹. Isolated cells were first incubated with $10 \mu\text{g/ml}$ 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 $20 \mu\text{g/ml}$ 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 (FACSscan; 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.

Cervical chemokines

A nasal sponge with attached string (4.5 cm $\times$ 1.5 cm $\times$ 2.0 cm; Ivalon Surgical Products, M-PACT, Eudora, KS) was inserted by a nurse practitioner into the vagina of each study subject to the level of the ectocervix. After 30 minutes, the sponges were removed and frozen at -70°C. Reproductive tract secretions were eluted from the sponges by incubation in a Spin-X microcentrifuge tube (Fisher Scientific, Rockville, MD) in 300 μL of sterile PBS with 0.5% BSA and 0.05% Tween 20 and a protease cocktail inhibitor (Roche Applied Science, Indianapolis, IN) for 1 h on ice followed by centrifugation through a 0.2 μM cellulose acetate filter. Spin-X filters were pre-blocked with sterile PBS with 2% BSA and 0.05% Tween-20 for 30 min at 25°C, centrifuged and washed twice with sterile PBS. Samples were kept on ice and promptly loaded into an ELISA plate prepared for a specific cytokine assay. Chemokine levels for CXCL10, CXCL11, CCL17, CCL25, CCL27 and CCL28 were performed using DuoSet ELISA assays according to the manufacturer's specifications (R&D Systems, Minneapolis, MI).

Statistics

Statistical differences in numbers of leukocytes recovered in endocervical brush samples and chemokine levels in reproductive tract secretions were compared in STI positive and negative study subjects by Student's t-test. 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

Infections in the reproductive mucosa induce an accumulation of various leukocyte subsets in the endocervix of females with non-ulcerative STIs (HIV-negative subjects with *C. trachomatis*, *N. gonorrhoeae* or *T. vaginalis* infections)¹⁰. We developed a method to identify leukocyte subsets in endocervical brush samples and peripheral blood mononuclear cells (PBMC) using flow cytometry. Forty study subjects were enrolled in the project as described in the Materials and Methods section. The subjects were females between the ages of 18–24 and included diverse racial categories (1, Alaska Native; 1, Pacific Islander; 1 African American; one mixed race, 14 whites and 24 of unreported racial category). Eleven subjects were excluded from the study because the yield of endocervical cells was too few to analyze. The subjects were categorized as STI positive by polymerase chain reaction (PCR) assay of endocervical swabs for *Neisseria gonorrhoeae* and *Chlamydia trachomatis* (Hoffmann-La Roche Ltd Roche, Basel, Switzerland). Twelve study subjects were positive for *C. trachomatis* and negative for *N. gonorrhoeae*. Controls (16) were negative for *C. trachomatis* and *N. gonorrhoeae*. Peripheral blood samples from each individual were used to create gates for leukocyte subsets and were applied to the paired endocervical sample. Three cell types could easily be distinguished in these samples (supplemental Fig. 1); granulocytes, monocytes and lymphocytes (top to bottom).

For each endocervical sample, we calculated the percent of individual leukocyte subsets among total endocervical cells on a flow cytometer and compared these values between humans that tested positive for *Chlamydia trachomatis* and negative for *Neisseria gonorrhoeae* on endocervical samples (Hoffmann-La Roche Ltd Roche, Basel, Switzerland) with those that were negative for both pathogens at the time of examination. Granulocytes were the predominant cell type present in the endocervix of both STI (+) and STI (–) individuals ranging from 49–55% (Fig. 1A). However, the number of granulocytes was higher in peripheral blood samples and ranged from 61–63% (see supplemental figure). There was no statistical difference in any of the cell types between those testing positive for STI compared to the negatives. We further characterized the lymphocytes in the endocervix by staining with CD3, CD45RO and CD103. As shown in Fig. 1B, we found a significant increase in memory T cells (CD3+CD45RO+) in endocervical samples from individuals with an STI compared to uninfected controls. CD103 is expressed on T cells within epithelial surfaces and facilitates interaction with epithelial cells via E-cadherin¹¹. Although not significant, we also found a greater percentage of endocervical memory cells with increased expression of CD103 from individuals with an active STI compared to STI negative controls indicating that memory T cells are retained within the endocervix of infected individuals at a higher rate compared to uninfected controls (Fig. 1B).

To examine the dependence of mucosal ($\alpha 4\beta 7$) or non-mucosal (CLA) migration pathways on trafficking of cells to endocervical tissue during an STI, we performed flow cytometric staining. We stained the processed endocervical and peripheral blood samples for CD3, CD45RO, $\alpha 4\beta 7$ and CLA from STI positive and STI negative individuals and a representative dotplot of endocervical cells is shown in figure 2A. We found that the STI positive group had a significantly increased number of memory T cells that expressed both the mucosal homing receptor ($\alpha 4\beta 7$) and the peripheral homing receptor (CLA). This is a unique population in the endocervix because generally, $\alpha 4\beta 7$ & CLA are not co-expressed on the same memory T cell¹² and is only rarely seen ($< 2\%$) in peripheral blood¹³. This

double-positive population of memory T cells identified a unique subtype of memory T cells that were recruited to the reproductive mucosa during infection (Fig. 2B).

Trafficking patterns not only depend on expression of homing receptors on lymphocytes and corresponding adhesion molecules on the endothelium but also require chemokines and chemokine receptors. The combination of chemokines secreted by certain tissues in conjunction with homing receptors identifies specific migration patterns to specific tissues¹². Accordingly, we further defined the unique lymphocyte trafficking pattern of the GT in humans by measuring a wide array of chemokines in cervical secretions. We analyzed chemokines that are associated with lymphocyte homing to various tissues; CCL17 (trachea, bronchi and synovial joints), CCL25 (small intestine and colon), CCL27 (skin), and CCL28 (salivary and mammary glands). We also measured chemokines found in most tissues under inflammatory conditions; CXCL10 and CXCL11. We did not find a significant increase in any of the tissue-specific chemokines as shown in Figure 3 but we did see a trend of increased levels of the inflammatory chemokines, CXCL10 and CXCL11 in cervical secretions from individuals with an STI compared to STI negative controls (Fig. 3). In addition, we found that the receptor for CXCL10 and CXCL11 (CXCR3) was expressed on 75% of endocervical T lymphocytes with high expression levels of $\alpha 4\beta 7$ from three STI (+) samples which contained sufficient cells for analysis (Fig. 4) confirming the utility of inflammatory cytokines in this setting. Unfortunately, there were no STI (-) samples with sufficient cells for CXCR3 expression analysis.

DISCUSSION

In this study, we show that humans infected with the bacterial STI; *C. trachomatis* had a significantly increased concentration of this unique population of memory T cells among endocervical lymphocytes. These data are the first to identify a unique population of memory T lymphocytes expressing both $\alpha 4\beta 7$ and the selectin-ligand, CLA, which access female reproductive tract tissues during an active STI infection in humans. Previous studies have shown that $\alpha 4\beta 7$ +CLA+ T cells exist, but their presence in peripheral blood is low¹³. Further, tissue compartments differ from peripheral blood by representation of various leukocyte populations and it is not unexpected to find an increase of $\alpha 4\beta 7$ +CLA+ T cells in reproductive tract tissues.

Regulation of lymphocyte homing 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 activation¹⁵. 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^{7, 16}. 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.

Chemokine receptor expression also changes on human T cell clones during activation¹⁷. We found that approximately 70% of $\alpha 4\beta 7$ +CLA+ endocervical cells from three individuals also expressed CXCR3. These data suggest that human DCs may also regulate the migration of human T cells to the reproductive mucosa by inducing selective expression of homing receptors and chemokine receptors on memory T cells. Vaccines against STI should be designed to target certain DC subsets to induce T cell homing receptors and chemokine receptors for efficient migration to human reproductive mucosa. Possibly, the accumulation of $\alpha 4\beta 7$ memory T cells via the CXCR3 chemokine receptor functions as a positive feedback mechanism to increase the number of anti-chlamydial effector T cells since $\alpha 4\beta 7$

expressing cells have an increased propensity to secrete IFN γ compared to non- $\alpha 4\beta 7$ bearing T cells¹⁴.

This study indicates that vaccine design for STI of the human genital mucosa also include molecules induced by the inflammatory response. However, it is important that a specific migratory pathway is targeted for selection of Th1 cells and not any inflammatory leukocytes to avoid tissue hypersensitivity¹⁸. It is possible to enhance the trafficking of certain lymphocyte populations with molecules associated with inflammation. Recently, trafficking to the lamina propria of the intestinal tract, an extra-lymphoid tissue, was shown to occur by a population of T lymphocytes that co-expressed $\alpha 4\beta 7$ and a ligand for P-selectin and accumulation of this population was enhanced by IL-12¹⁹. The authors further showed that the regulation of selectin expression is mediated through the post-translational activity of enzymes Fuc-T VII and C2GlcNAcT-1 and rely on IL-12 for expression. Dendritic cells that produce Th1 mediated responses secrete IL-12. Thus, dendritic cell secretion is central to resolution of chlamydial genital infection not only for induction of Th1 cells but of homing receptors and chemokine receptors associated with rapid lymphocyte migration to the GT.

These findings have important implications for vaccine design. It is accepted that activated effector/memory T cells preferentially migrate to tissues which are linked to the secondary lymphoid organs where antigen was first encountered. As an example, oral antigens induce effector/memory cells that preferentially accumulate in intestinal tissues. Mechanistically, this occurs is by the activation of T cells with dendritic cells that induce specific patterns of homing and chemokine receptors⁷. In this example, dendritic cells from Peyer's patches and not spleen or peripheral lymph nodes induced expression of $\alpha 4\beta 7$ and CCL25. Our report suggests that lymphocyte homing receptor expression should be considered in vaccine design against pathogens of the female reproductive tract. Possibly, a vaccine which targets immune inductive sites that stimulate $\alpha 4\beta 7$ and CLA, i.e. mucosal sites, would likely result in a more efficacious vaccine.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Leukocyte subset expression in the endocervix

Endocervical cells were collected using a cytobrush, from individuals following pelvic examination. The cells were processed and stained for flow cytometric analysis as described in materials and methods. Cells were analyzed in a flow cytometer and expressed as those with an STI (solid bars) and STI negative controls (striped bars). A) Granulocytes, lymphocytes and monocytes were quantitated by FSC vs SSC. B) Expression of CD3, CD45RO and CD103 by endocervical cells. * $p < 0.02$, Student's t-test. The data were compiled from 6–15 endocervical samples.

Figure 2. Expression of $\alpha 4\beta 7$ and CLA on memory lymphocytes

Endocervical and peripheral blood cells were collected from individuals following a pelvic examination. The cells were processed and stained for four-color flow cytometric analysis as described in materials and methods. A) Representative dotplots $\alpha 4\beta 7$ and CLA expression on CD3+CD45RO+ memory T cells. B) The samples were gated on CD3+CD45RO+ cells and percent of cells expressing $\alpha 4\beta 7$ and/or CLA was determined. *p=0.047, Student's t-test. The data were compiled from 3–9 endocervical samples.

Figure 3.

Chemokine levels in cervical secretions of *Chlamydia* infected and uninfected individuals. A sterile nasal sponge was placed in the vagina of subjects following annual pelvic examination. After 30 minutes the sponge was removed. Vaginal secretions were eluted as described in the materials and methods and various chemokines were measured by ELISA. Data were compiled of vaginal secretions from 12–16 subjects.

Figure 4.

The majority of $\alpha 4\beta 7^{\text{hi}} \text{CLA}^{\text{hi}}$ cells express CXCR3. Representative flow histogram of endocervical cells from a *Chlamydia* positive individual were stained as described in materials and methods for CD3, $\alpha 4\beta 7$, CLA and CXCR3. The expression of CXCR3 on CD3+ T cells with various expression levels of $\alpha 4\beta 7$ and CLA is shown.