Chemokine and Chemokine Receptor Dynamics during Genital Chlamydial Infection

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Current design strategies for vaccines against certain microbial pathogens, including Chlamydia trachomatis, require the induction and targeting of specific immune effectors to the local sites of infection known as the mucosal effector sites. Chemokines and their receptors are important mediators of leukocyte trafficking and of the controlled recruitment of specific leukocyte clonotypes during host defense against infections and during inflammation. We analyzed the dynamics of chemokine and chemokine receptor expression in genital mucosae during genital chlamydial infection in a murine model to determine how these molecular entities influence the development of immunity and the clearance of infection. A time course study revealed an increase of up to threefold in the levels of expression of RANTES, monocyte chemotactic protein 1 (MCP-1), gamma-interferon-inducible protein 10 (IP-10), macrophage inflammatory protein 1α (MIP-1α), and intercellular adhesion molecule type 1 (ICAM-1) after genital infection with the Chlamydia agent of mouse pneumonitis. Peaks levels of expression of RANTES, MCP-1, and MIP-1α occurred by day 7 after primary infection, while those of IP-10 and ICAM-1 peaked by day 21. Expression levels of these molecules decreased by day 42 after primary infection, by which time all animals had resolved the infection, suggesting an infection-driven regulation of expression. A rapid upregulation of expression of these molecules was observed after secondary infection. The presence of cells bearing the chemokine receptors CCR5 and CXCR3, known to be preferentially expressed on Th1 and dendritic cells, was also synchronous with the kinetics of immune induction in the genital tract and clearance of infection. Results demonstrated that genital chlamydial infection is associated with a significant induction of chemokines and chemokine receptors that are involved in the recruitment of Th1 cells into the site of infection. Future studies will focus on how selective modulation of chemokines and their receptors can be used to optimize long-term immunity against Chlamydia.

The pathological consequences of genital infection by Chlamydia trachomatis include major sequelae, such as pelvic inflammatory disease and infertility. The urgent need to develop an efficacious vaccine poses the major clinical and basic research challenge of defining the requirements for inducing and maintaining protective genital mucosal immunity. Recent studies of animal models indicated that rapid and early elicitation and recruitment of certain immune effectors (specifically dendritic and Th1 cells) into local genital mucosae are crucial for antichlamydial immunity (15, 20, 28). Thus, strategies for designing protective vaccines require the definition of novel approaches for optimizing the induction, recruitment, and retention of chlamydia-specific Th1 cells in genital mucosae.

In general, the mucosal immune response to a vaccine is influenced by many factors, such as the antigen, vector, adjuvant, route of delivery, and hormones associated with the estrous cycle (for genital mucosal response) (22). It is conceivable that these factors affect antigen uptake, mucosal immune induction, and homing, recruitment, and retention of immune effectors in infected sites. Chemokines are important mediators of leukocyte trafficking and of the controlled recruitment of specific lymphocyte clonotypes during immune induction and inflammation. Epithelial cells and certain other cell types comprising the innate immune effectors elaborate a number of cytokines and chemokines upon encountering mucosally routed antigens (24, 31). The CD4+ Th1 and CD8+ T cells and their cytokines, especially gamma interferon (IFN-γ), are crucial for antichlamydial immunity in mice (6, 12, 15, 16, 29, 30). The recruitment of Th1 cells into genital mucosae after chlamydial infection has been demonstrated (4, 15, 18). This recruitment would require obligatory interactions among chemokines, chemokine receptors, and cell surface molecules such as the major histocompatibility complex, addressins, coreceptors, and costimulatory and adhesion molecules (8, 9). Factors that regulate the induction, recruitment, and persistence of immune effectors (especially Th1 cells) in the genital mucosal site control long-term genital mucosal immunity and are particularly important for antichlamydial immunity. Although presently unknown, such factors may regulate the expression and functions of certain chemokines, chemokine receptors, and adhesion molecules. Interestingly, recent findings that chemokine receptors act as coreceptors (with the CD4 molecule) for entry of the human immunodeficiency virus into host cells have resulted in the identification of chemokines responsible for recruiting different leukocyte subpopulations or subsets (including Th1, Th2, and dendritic cells) that express the receptors. Among the members of the two major classes of chemo-

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kines (CC and CXC) and their receptors (CCR or CXCR), CCR3 and CXCXR3 have been clearly demonstrated to be preferentially expressed on Th1 cells, while CCR3 and CCR4 have been shown to be selectively expressed on Th2 cells (3, 25). Moreover, RANTES (regulated on activation, normal T cell expressed and secreted), macrophage inflammatory protein 1α (MIP-1α), MIP-1β, and IFN-γ-inducible protein 10 (IP-10) recruit Th1 cells (1, 3, 19, 25). Thus, certain chemokines and their receptors regulating the induction of protective immunity against C. trachomatis could serve as new markers for following the course and possibly predicting the outcome of genital chlamydial infection. In addition, an understanding of the biology of the factors that regulate the expression of Th1-associated chemokines and their receptors will provide new insights that may lead to the design of novel strategies to optimize local mucosal antichlamydial immunity by a potential vaccine.

In the present study, we investigated the dynamics of chemokine and chemokine receptor expression with respect to the recruitment of T cells and the resolution of chlamydial infection. The results revealed that the pattern of expression of chemokines and their receptors in the genital tract is crucial for the induction of protective immunity and the clearance of infection.

MATERIALS AND METHODS

Chlamydia stocks. Stocks of the C. trachomatis agent of mouse pneumonitis (MoPn) used to infect mice in vivo were prepared by propagating elementary bodies (EBs) in McCoy cells, as previously described (23). The titers of the stocks were determined by infecting McCoy cells with various dilutions of EBs, and the infectious titer was expressed in inclusion-forming units (IFU) per milliliter.

Animals, infection, and analysis of the course of disease. Female BALB/c mice (5 to 6 weeks old) were obtained from Harlan-Sprague Dawley (Indianapolis, Ind.). All animals were housed in the animal facility of the Morehouse School of Medicine, fed with food and water ad libitum, and maintained in laminar-flow racks under pathogen-free conditions of 12-h light and 12-h darkness. Mice (five per group) were infected intravaginally with 10^5 IFU of MoPn per mouse in a volume of 20 μl of phosphate-buffered saline (PBS) while under phenobarbital-induced anesthesia. Uninfected control mice received 30 μl of PBS. The course of the infection was monitored by periodic (every 3 days) cervicovaginal swabbing, as determined by infecting McCoy cells with various dilutions of EBs, and the infectious titer was expressed in inclusion-forming units (IFU) per milliliter.

The response levels of chlamydia-specific Th1 response and recruitment of Th1 cells into the genital tract. The levels of Th1 response and recruitment of Th1 cells into genital mucosa after infection were determined by measuring the responses of chlamydia-specific, IFN-γ-secreting T cells in the genital tract tissues of infected mice, as previously described (15). Briefly, immune T cells were prepared from the genital tract tissues of infected mice by the collagenase digestion method (10, 13) as follows. At the indicated time after infection, the animals in each group were sacrificed, and the genital tract between the vagina and ovaries (i.e., the cervix, uterus, and fallopian tubes) was excised from each mouse and placed in sterile HEPES (N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid)-buffered RPMI 1640 medium (Atlanta Biologicals, Norcross, Ga.). Explants were transferred to 7 ml of a 0.6-ml/gel concentration of filter-sterilized type I collagenase (Atlanta Biologicals). The tissues were minced, incubated at 37°C for 45 to 60 min, teased with forceps, and washed. The cells were enriched for T cells by the nylon wool adherence method (12, 13). Purified genital tract cells contained at least 97% CD3^+ cells, as determined by fluorescence-activated cell sorter analysis.

The response levels of chlamydia-specific Th1 cells induced in genital tissues were assessed by seeding purified T cells (10^5 cells per well) into 96-well tissue culture plates (Costar, Cambridge, Mass.) with syngeneic antigen-presenting cells (APCs) (2 × 10^5 cells per well), in the absence of UV-irradiated MoPn EBs (10 μg/ml of IFN-γ; Sigma) (i.e., the cervix, uterus, and fallopian tubes) was excised from each mouse and placed in sterile HEPES (N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid)-buffered RPMI 1640 medium (Atlanta Biologicals, Norcross, Ga.). Explants were transferred to 7 ml of a 0.6-ml/ml concentration of filter-sterilized type I collagenase (Atlanta Biologicals). The tissues were minced, incubated at 37°C for 45 to 60 min, teased with forceps, and washed. The cells were enriched for T cells by the nylon wool adherence method (12, 13). Purified genital tract cells contained at least 97% CD3^+ cells, as determined by fluorescence-activated cell sorter analysis. The response levels of chlamydia-specific Th1 cells induced in genital tissues were assessed by seeding purified T cells (10^5 cells per well) into 96-well tissue culture plates (Costar, Cambridge, Mass.) with syngeneic antigen-presenting cells (APCs) (2 × 10^5 cells per well), in the presence of UV-irradiated MoPn EBs (10 μg/ml of IFN-γ; Sigma) (i.e., the cervix, uterus, and fallopian tubes) was excised from each mouse and placed in sterile HEPES (N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid)-buffered RPMI 1640 medium (Atlanta Biologicals, Norcross, Ga.). Explants were transferred to 7 ml of a 0.6-ml/ml concentration of filter-sterilized type I collagenase (Atlanta Biologicals). The tissues were minced, incubated at 37°C for 45 to 60 min, teased with forceps, and washed. The cells were enriched for T cells by the nylon wool adherence method (12, 13). Purified genital tract cells contained at least 97% CD3^+ cells, as determined by fluorescence-activated cell sorter analysis.
cDNA from each sample as the housekeeping genes as well as primers specific to β-actin and a negative control containing no cDNA.

(iv) Semiquantitation of PCR product. A volume of 10 μl of each PCR product was combined with 2 μl of loading buffer and 2 μl of distilled water and electrophoresed on 2% agarose gels containing ethidium bromide in Tris-borate-EDTA buffer. We included aliquots containing the same amounts of the PCR products derived from β-actin primers as housekeeping genes. To quantitate and compare mRNA levels, the gels were photographed and the intensities of the bands were analyzed with Gelexpert software (NucleoTech, San Mateo, Calif.). The relative band intensities in each experiment were normalized to the mean intensity of the β-actin band. The ratio of sample intensities to β-actin intensity was plotted against the time points of the analysis.

Statistical analysis. The levels of IFN-γ in samples from different experiments were analyzed and compared by performing a one- or two-tailed t test, and the relationship between different experimental groupings was assessed by analysis of variance. Minimal statistical significance was based on a P of < 0.05.

### RESULTS

**Differential kinetics of chemokine induction during genital chlamydial infection.** We investigated the hypothesis that the pattern of local immune induction and recruitment of specific effector cells into the genital tract after genital chlamydial infection is due to the production of chemokines induced during the infection. Results presented in Fig. 1 show the pattern of expression of RANTES, MCP-1, MIP-1α, IP-10, and intercellular adhesion molecule type 1 (ICAM-1) in the genital tract of noninfected control mice and infected mice, as analyzed by a semiquantitative RT-PCR. Figure 2 shows the plots of the densitometric scans of the data shown in Fig. 1, normalized to β-actin expression analyzed simultaneously at each time point.

The results revealed that the expression of RANTES and MCP-1, which are functional in recruiting both Th1 and Th2 cells, was induced early and remained relatively upregulated during the primary and secondary infections. The expression levels of the Th1-inclined chemokines, MIP-1α and IP-10, were also induced early, but the profiles during the infection were irregular, especially for MIP-1α. Thus, the expression levels of MIP-1α and IP-10 were rapidly upregulated following genital infection, but while the expression of MIP-α was decreasing by day 21, IP-10 did not show a significant decrease in expression until day 42 after primary infection (P > 0.02). It is interesting that the expression of ICAM-1 followed the expression patterns of Th1-related chemokines (Fig. 1 and 2), peak-

![Days Post-infection

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FIG. 1. Chemokine induction during genital chlamydial infection. Female BALB/c mice were infected intravaginally with 10^5 IFU of MoPn. Total RNAs isolated from genital tract tissues at the indicated time points (days) after the primary (1°) and secondary (2°) infections were analyzed by a semiquantitative RT-PCR to assess the expression levels of RANTES, MCP-1, MIP-1α, IP-10, ICAM-1, and β-actin. NI, noninfected genital tissues; Inf, infection.
ing by day 21 and appearing to be reduced by day 42 (after the animals had cleared the primary infection) but becoming rapidly upregulated with the secondary infection. The results suggest that some of the chemokines responsible for recruiting protective effector cells into the genital tract are made during an infection but are downregulated after the resolution of the infection.

Patterns of local chemokine receptor expression during genital chlamydial infection, development of genital mucosal immunity, and clearance of chlamydia. We next determined the development of the protective Th1 response in genital mucosae and its relationship to the course of genital chlamydial infection. Figure 3 shows the kinetics of the Th1 response in genital mucosae following genital chlamydial infection, which appear to correlate inversely with the course of the infection (Fig. 4). The data indicated that Th1 cells are induced relatively early following an infection and emigrate after the resolution of the infection, as previously reported (13, 15). All of the animals analyzed resolved the infection by 4 to 6 weeks after the initiation of infection, as previously reported (15).

Dendritic and Th1 cells are important immune effectors in antichlamydial immunity. Since these cells preferentially express the chemokine receptors CCR5 and CXCR3 (3, 7, 19) and have been shown to produce Th1-related chemokines (26), we analyzed the kinetics of expression of these receptors in the genital tract after genital chlamydial infection and correlated the expression pattern with the functional Th1 response. Results presented in Fig. 5 show the patterns of expression of CCR5 and CXCR3 as analyzed by a semiquantitative RT-PCR from a representative experiment. Figure 6 shows the plots of the densitometric scans of the pooled mRNA levels from two separate experiments, normalized to β-actin expression at each time point. The results revealed that the expression of CCR5 and CXCR3 in the genital tract follows the same pattern as the Th1 response shown in Fig. 3 during the period analyzed for...
for a total of 10 animals per experimental group. Experiments were repeated twice, making a total of 10 animals per experimental group.

**DISCUSSION**

A major challenge in designing antichlamydial vaccines is to develop an immunization regimen capable of inducing and retaining a mucosal Th1 response in order to foster long-term protective benefits. Susceptibility to reinfestation after a primary genital chlamydial infection has previously been shown to be associated with the emigration of antigen-specific T cells from genital mucosae after the resolution of the infection (13). Chemokines are important immunoregulatory factors that play an important role in the induction and recruitment of leukocytes. The hypothesis investigated in the present study was that the patterns of chemokine and chemokine receptor expression would reflect the induction of a protective mucosal immune response against chlamydial infection. The results revealed that the expression of certain chemokines responsible for recruiting the relevant Th1 cells and dendritic cells into genital mucosae follows the course of the infection, such that MIP-1α and IP-10 were detected at high levels during the productive infection but were suppressed or attenuated after the resolution of the infection. This suppression of the induction of the immune effectors required for local resistance to reinfection is a major cause of susceptibility to reinfection (13). The challenge for vaccine strategy is to design a regimen that would retain CCR5- and CXCR3-positive cells in the genital tract in order to maintain long-term antichlamydial immunity. Our studies and those of others have revealed that the temporary protective immunity associated with intravaginal infection with live chlamydiae was associated with the induction of the α4β1-vascular cell adhesion molecule (VCAM) and α4β7-mucosal addressin cell adhesion molecule (MAdCAM) pathways for T-cell recruitment into genital mucosae (11, 17, 21). Furthermore, direct interaction between epithelial and T cells via the ICAM-1-leukocyte functional antigen 1 (LFA-1) adhesion pathway is required for the efficient killing of intracellular chlamydiae in infected cells (14). Future studies should define and molecularly characterize these adhesion, homing, trafficking, and interacting molecules in the mucosal inductive and effector sites involved in genital, respiratory, and ocular mucosal immunity so that they can be better targeted to optimize immunity against *Chlamydia* and other agents of sexually transmitted diseases. Moreover, since the recruitment and retention of immune effectors in the genital, respiratory, or ocular mucosae are important for maintaining long-term immunity against chlamydial infections (13), the molecular pathways and regulatory elements should be established.

Aside from the ability of certain chemokines and their receptors to regulate the induction of protective immunity against *C. trachomatis*, the results revealed that these factors can be used as markers for following the course and possibly predicting the outcome of genital chlamydial infection. For instance, if recruited T cells bear CCR5 or CXCR3 and the chemokines are RANTES, IP-10, and MIP-1α, a predominantly Th1 response leading to resolution of infection can be predicted (3, 19). However, if the recruited T cells bear CXCR3 and the predominant chemokines are eotaxin, MCP-3, and MCP-4, a Th2 response with delayed resolution of chlamydial infection is likely (25).

An understanding of the cellular and molecular immunologic basis of Th1 augmentation against *Chlamydia* by chemokines and their receptors may furnish information on how biochemical modulation of chemokines and their receptors can be exploited in the design of a vaccine against *C. trachomatis*.

**FIG. 4.** Course of genital chlamydial infection. Female BALB/c mice were infected intravaginally with 10⁵ IFU of MoPn. The course of the infection was monitored by periodic (every 3 days) cervicovaginal swabbing of individual animals. Chlamydiae isolated from cervicovaginal swabs in tissue culture were assayed by staining infected monolayers of McCoy cells with fluorescein isothiocyanate-labeled, genus-specific antibody as described in Materials and Methods. The animals were monitored for 6 weeks. Experiments were repeated twice, making for a total of 10 animals per experimental group.

**FIG. 5.** Chemokine receptor expression in genital mucosae during genital chlamydial infection. Female BALB/c mice were infected intravaginally with 10⁵ IFU of MoPn. Total RNAs isolated from genital tract tissues at the indicated time points (days) after the primary and secondary infections were analyzed by a semiquantitative RT-PCR to assess the expression of CCR5 and CXCR3, as described in Materials and Methods and the legend to Fig. 1. Lane M, molecular size markers. NI, noninfected genital tissues.

and other pathogens controlled by Th1 immunity. A plausible strategy would involve the coadministration of chemokines and vaccines, which may attract APCs to boost the immune response, or the local deposition of chemokines at the site of infection to foster the recruitment of effectors to clear the infection. Since the half-lives of chemokines are relatively short, it is unclear how the latter approach would be beneficial, but the former strategy is amenable to a gene transfer approach in which the chemokine is expressed simultaneously with antigen delivery.

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REFERENCES


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