Essential Role for Neutrophils in Pathogenesis and Adaptive Immunity in *Chlamydia caviae* Ocular Infections

H. Marie Lacy,¹* Anne K. Bowlin,¹ Leah Hennings,² Amy M. Scurlock,³ Uma M. Nagarajan,¹,³ and Roger G. Rank¹

Departments of Microbiology and Immunology,¹ Pathology,² and Pediatrics,³ University of Arkansas for Medical Sciences and Arkansas Children’s Hospital Research Institute, Little Rock, Arkansas 72202

Received 29 November 2010/Returned for modification 20 January 2011/Accepted 1 March 2011

Trachoma, the world’s leading cause of preventable blindness, is produced by chronic ocular infection with *Chlamydia trachomatis*, an obligate intracellular bacterium. While many studies have focused on immune mechanisms for trachoma during chronic stages of infection, less research has targeted immune mechanisms in primary ocular infections, events that could impact chronic responses. The goal of this study was to investigate the function of neutrophils during primary chlamydial ocular infection by using the guinea pig model of *Chlamydia caviae* inclusion conjunctivitis. We hypothesized that neutrophils help modulate the adaptive response and promote host tissue damage. To test these hypotheses, guinea pigs with primary *C. caviae* ocular infections were depleted of neutrophils by using rabbit antineutrophil antiserum, and immune responses and immunopathology were evaluated during the first 7 days of infection. Results showed that neutrophil depletion dramatically decreased ocular pathology, both clinically and histologically. The adaptive response was also altered, with increased *C. caviae*-specific IgA titers in tears and serum and decreased numbers of CD4⁺ and CD8⁺ T cells in infected conjunctivae. Additionally, there were changes in conjunctival chemokines and cytokines, such as increased expression of IgA-promoting interleukin-5 and anti-inflammatory transforming growth factor β, along with decreased expression of T cell-recruiting CCL5 (RANTES). This study, the first to investigate the role of neutrophils in primary chlamydial ocular infection, indicates a previously unappreciated role for neutrophils in modulating the adaptive response and suggests a prominent role for neutrophils in chlamydia-associated ocular pathology.

*Chlamydia trachomatis*, an obligate intracellular bacterium, is the etiologic agent of trachoma, the world’s leading cause of preventable blindness. While most trachoma studies have addressed the mechanisms of disease during chronic scarring stages, there has been less research on inclusion conjunctivitis, the initial disease associated with a primary ocular infection. The study of inclusion conjunctivitis is important because events in the initial stages of infection may have an impact on the chronic pathological response and the development of protective immunity. The first response to a primary chlamydial infection, acute inflammation, has been studied extensively in genital and respiratory chlamydial infections, but there is little information on this response in chlamydial conjunctival infections.

It is difficult to study inclusion conjunctivitis in humans and in nonhuman primates, the latter because of issues of expense. However, an outstanding model for chlamydial inclusion conjunctivitis is available from guinea pigs infected with *Chlamydia caviae*, also known as the agent of guinea pig inclusion conjunctivitis. *C. caviae* was first isolated from the conjunctiva of laboratory guinea pigs by Murray (29) and was initially used as a model for chlamydial ocular infection (30–32). Nonnickerendam later characterized the model both immunologically and pathologically and demonstrated that a trachoma-like disease could be elicited by repeated infection (26, 27). In addition, Schachter and coworkers extensively documented the local and serum antibody responses to infection and reinfection (2, 24). A major advantage of the model is that one can quantify the gross pathological response in the conjunctiva of individual animals over the course of an infection and correlate the response to the number of organisms isolated from ocular swabs. Moreover, there is abundant conjunctival tissue for use in histopathologic, flow cytometric, and gene expression studies, such that all of these parameters can be assessed in a single animal.

The major cellular component of acute inflammation is the neutrophil. Historically, the neutrophil has been viewed as a professional phagocyte whose sole function in immunity is to engulf, kill, and clear bacteria. However, recent studies suggest that neutrophils play other roles in an immune response, both as producers of chemokines that attract immune cells to infectious sites and as sources of cytokines that affect innate and adaptive responses (34). Moreover, there is evidence that some cytokines produced by neutrophils may have a direct regulatory role in the subsequent adaptive immune response (46). Although neutrophils are part of the host’s response to eradicate pathogens, they have also been associated with host tissue damage in chlamydial infections, including guinea pig ocular chlamydial infection. A transmission electron microscopy study of *C. caviae* ocular infection in guinea pigs showed neutrophils in close association with infected mucosal epithelial cells and disrupted epithelial/basal laminal focal adhesions, suggesting that neutrophils may be actively involved in releasing epithelial cells from the conjunctival mucosal epithelium (40).

* Corresponding author. Mailing address: Chlamydia Research Group, Arkansas Children’s Hospital Research Institute, Slot 512-44, 13 Children’s Way, Little Rock, AR 72202. Phone: (501) 960-7565. Fax: (501) 364-2403. E-mail: HMLacy@uams.edu.

† Published ahead of print on 14 March 2011.
Our goal was to investigate the role of neutrophils in the pathological response and early adaptive immunity in the guinea pig model of chlamydial inclusion conjunctivitis. To achieve this objective, we developed a rabbit antisera to guinea pig neutrophils and used it to deplete neutrophils in guinea pigs during the initial stage of ocular infection with *C. caviae*. We determined that neutrophils not only played a role in ocular pathology during infection but were also involved in promoting T cell recruitment and downregulating chlamydia-specific IgA titers at the infected conjunctivae. To our knowledge, this is the first study to examine the role of neutrophils in primary chlamydial ocular infection.

**MATERIALS AND METHODS**

**Experimental animals.** Two female New Zealand rabbits, weighing 9 lb, were purchased from Myrtle’s Rabbitry, Inc. (Thompson Station, TN) and were housed separately. Female, Hartley strain guinea pigs (450 to 500 g) were obtained from Charles River Laboratories (Wilmington, MA) and were housed separately. Female, Hartley strain guinea pigs (450 to 500 g) were obtained from Myrtle’s Rabbitry, Inc. (Thompson Station, TN) and were housed separately in cages covered with fiberglass filter tops. All animals were housed in environmentally controlled rooms, maintained on a 12:12 light-dark cycle, and provided with food and water ad libitum. Animal experiments and protocols were approved by the Institutional Animal Care and Use Committee of the University of Arkansas for Medical Sciences and the Arkansas Children’s Hospital Research Institute.

**Production of guinea pig neutrophil-depleting antisera.** Guinea pig neutrophil antisera was produced in two New Zealand female rabbits, according to procedures described previously (11, 37, 41). The antisera was treated to remove antibodies to guinea pig lymphocytes, monocytes, and platelets. Briefly, neutrophils were isolated from guinea pig blood by using the standard techniques of dextran sedimentation, Ficoll-Hypaque gradient separation, and hypotonic lysis of erythrocytes (11). Preparations consisted of >95% neutrophils as determined by Wright-stained cytocentrifuge smears and were >97% viable, as measured by trypan blue exclusion. Two rabbits were immunized with guinea pig neutrophils eight times at 2- to 3-week intervals. For the first immunization, equal parts of neutrophil suspension and Freund complete adjuvant were emulsified, and 1 ml of the mixture was injected intradermally into the backs of the rabbits at five sites (0.2 ml per site). Booster inoculations of neutrophils were suspended in sterile saline and given intravenously (37). The number of neutrophils per immunization varied from 2 × 10^6 to 10^7. At various times after the first two booster inoculations, blood was collected from rabbits to obtain serum. Because immune responses can vary between animals, the antisera from the two rabbits were treated as separate reagents without pooling.

Before processing and characterization, antisera were heat inactivated at 56°C for 45 min to destroy complement. To eliminate antibodies specific for guinea pig major histocompatibility complex class II molecules, antisera were absorbed at least 5 times with 20% solution of fresh, saline-washed guinea pig red blood cells (RBC) isolated from peripheral blood by standard techniques (11, 37). After each adsorption procedure, antisera were filtered with sterile, low protein-binding Durapore (polycrylilide difluoride) 0.45-mm filters (Millipore, Billerica, MA). Elimination of MHC-I antibodies was monitored with a guinea pig RBC agglutination assay as previously described (37). To eliminate antibodies to guinea pig lymphocytes, monocytes, and platelets, the antisera were adsorbed at least 3 times with fresh, saline-washed lymphocytes, monocytes, and platelets isolated from guinea pig blood by standard techniques (11). To monitor removal of anti-lymphocyte and antimonocyte antibodies, indirect immunofluorescent staining of guinea pig blood mononuclear cells was employed using antisera as the primary antibody. Preimmune sera served as negative isotype controls, and the secondary antibody was a fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit antibody IgM and IgG (H+L chain specific; Southern Biotech, Birmingham, AL).

Elmination of platelet antibodies was confirmed during in vivo testing of antisera for the ability to deplete neutrophils. Briefly, normal, noninfected guinea pigs were administered either 1.0 or 1.5 ml antisera from rabbit 1 (n = 2 guinea pigs) or rabbit 2 (n = 2 guinea pigs) intraperitoneally every 24 h for 3 days. Blood was collected daily from each animal, as described below, to perform manual total white blood cell counts and peripheral blood white cell differentials on Wright-stained blood smears. Absolute values of peripheral blood neutrophils, lymphocytes, and monocytes were calculated from the results of these two tests (35). Successful removal of platelet antibodies by adsorption procedures was confirmed by performing platelet estimates on the blood smears according to standard procedures (35).

**C. caviae infection of guinea pigs.** *C. caviae* has been continually passaged in this laboratory for 36 years, first in yolk sac and then in tissue culture. McCoy cell-grown *C. caviae* was utilized. Chlamydial were passaged, prepared for infection, and quantified by standard methodology (39). Guinea pigs were anesthetized with sodium pentobarbital (Nembutal; 32 mg/kg of body weight) and infected in the conjunctivae of both eyes by instilling 20 μl of sucrose-phosphate-glutamic acid containing 10^8 inclusion-forming units (IFU) of *C. caviae* directly into the conjunctival sac. This dose ensures 100% infection and produces a strong pathological response that is easily quantified by gross observation.

**Neutrophil depletion in guinea pigs during *C. caviae* ocular infection.** To deplete neutrophils in guinea pigs during *C. caviae* ocular infection, 1 ml of sterile filtered neutrophil antisera was administered intraperitoneally every 24 h beginning the day before infection (day -1) and continued until termination of experiments on day 7 postinfection. Control animals (normal rabbit serum [NRS] controls) were administered 1 ml of sterile filtered heat-inactivated normal rabbit serum (Pel-Freeze Biologicals, Rogers, AR). No animals exhibited signs of serum sickness prior to euthanasia on day 7 postinfection. To confirm that the neutrophil antisera was effective in depleting neutrophils in vivo, absolute neutrophil counts of the peripheral blood and myeloperoxidase immunohistochemical staining on histologic sections of guinea pigs infected with *C. caviae* were measured. Peripheral blood samples were collected on days 0, 3, and 6 postinfection. To ensure conjunctivae swabbing did not affect gross ocular pathology scoring, conjunctival swabs were collected from the left eye only, while pathology scoring was performed only on the right eye.

**Pathology scoring.** Scoring of clinical ocular pathology was assessed visually on a daily basis and scored on a 0 to 4+ scale for evaluation of edema, chemosis, and exudation as previously described (39). In order to maintain consistency and subjectivity, one investigator, blinded to the experimental group, scored the pathology. To score for conjunctival histopathology, conjunctivae were excised immediately after euthanasia and fixed in 10% neutral buffered formalin. Tissues were embedded in paraffin, sectioned at 5 μm, and stained with hematoxylin and eosin (H&E). A veterinary pathologist, blinded to the experimental design, examined histologic sections for mucosal and submucosal inflammation, epithelial erosion, and keratinization.

**Conjunctival lymphocyte analysis.** To determine the absolute number of lymphocytes present, infected conjunctivae were harvested and processed individually to produce a single-cell suspension as described previously (47). Approximately, 1 × 10^7 to 2 × 10^7 cells were stained for individual cell surface markers or isotype controls (5 μg/ml) for 20 min on ice.
TABLE 1. Primers for quantitative reverse transcriptase PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sense primer</th>
<th>Antisense primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCL2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5'-TGGGACACAGATATGCTTGAG-3</td>
<td>5'-CGGATATTGTTAATCCTGATG-3</td>
</tr>
<tr>
<td>CCL5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5'-CGGATATTGTTAATCCTGATG-3</td>
<td>5'-CGGATATTGTTAATCCTGATG-3</td>
</tr>
<tr>
<td>CCL7&lt;sup&gt;c&lt;/sup&gt;</td>
<td>5'-TCACCTGCTTGAGCTGCACACG-3</td>
<td>5'-TCACCTGCTTGAGCTGCACACG-3</td>
</tr>
<tr>
<td>CXCL5&lt;sup&gt;d&lt;/sup&gt;</td>
<td>5'-GACGACCTGTCTCTTCTCTCTC-3</td>
<td>5'-GACGACCTGTCTCTTCTCTCTC-3</td>
</tr>
<tr>
<td>GM-CSF&lt;sup&gt;e&lt;/sup&gt;</td>
<td>5'-GCGAGCTCGGTTCACCATGTAC-3</td>
<td>5'-GCGAGCTCGGTTCACCATGTAC-3</td>
</tr>
<tr>
<td>IFN-γ&lt;sup&gt;f&lt;/sup&gt;</td>
<td>5'-ATGGCTGGAAGCTGAGCTGCACACG-3</td>
<td>5'-ATGGCTGGAAGCTGAGCTGCACACG-3</td>
</tr>
<tr>
<td>IL-1β&lt;sup&gt;g&lt;/sup&gt;</td>
<td>5'-GCCCAAAGATGCTGCACACGCTTCTCT-3</td>
<td>5'-GCCCAAAGATGCTGCACACGCTTCTCT-3</td>
</tr>
<tr>
<td>IL-5&lt;sup&gt;h&lt;/sup&gt;</td>
<td>5'-GTTCCTGGATTACCTGCAAGAA-3</td>
<td>5'-GTTCCTGGATTACCTGCAAGAA-3</td>
</tr>
<tr>
<td>MHC-II&lt;sup&gt;i&lt;/sup&gt;</td>
<td>5'-GCTCCATTACCTGACCTTCCTTC-3</td>
<td>5'-GCTCCATTACCTGACCTTCCTTC-3</td>
</tr>
<tr>
<td>TGF-β1&lt;sup&gt;j&lt;/sup&gt;</td>
<td>5'-GCATCGATGTTGCTGTCTCAGACAGGTGAG-3</td>
<td>5'-GCATCGATGTTGCTGTCTCAGACAGGTGAG-3</td>
</tr>
<tr>
<td>TNF-α&lt;sup&gt;k&lt;/sup&gt;</td>
<td>5'-CCTACTGCTCTTCTACCATACC-3</td>
<td>5'-CCTACTGCTCTTCTACCATACC-3</td>
</tr>
<tr>
<td>18S rRNA&lt;sup&gt;l&lt;/sup&gt;</td>
<td>5'-TGATCGGCGCTTTCCTTGTT-3</td>
<td>5'-TGATCGGCGCTTTCCTTGTT-3</td>
</tr>
</tbody>
</table>

<sup>a</sup> The primers for this gene were obtained from Allen and McMurray (3).

<sup>b</sup> The primers for this gene were designed using the Beacon Designer software (Bio-Rad).

<sup>c</sup> The primers for this gene were obtained from Kubo et al. (19).

<sup>d</sup> The primers for this gene were obtained from Lyons et al. (23).

For direct immunostaining, the cells were washed once, treated with a blue fluorescent reactive fixable dye (Live/Dead fixable dead cell stain kit; Invitrogen, Carlsbad, CA), and then fixed with 1% paraformaldehyde-phosphate-buffered saline. For indirect immunostaining, cells were washed and incubated with a fluorescent-tagged secondary antibody before treatment with blue fixable dye and fixation with paraformaldehyde. The antibodies used in direct immunostaining were R-phycocyanin (RPE)-labeled mouse anti-guinea pig CD4 (clone CTTF-1), FITC-labeled mouse anti-guinea pig CD8 (clone MHC-II), and purified mouse anti-guinea pig CD45 (clone IL-5). Allophycocyanin (APC) was conjugated to purified anti-guinea pig CD45 by using an APC conjugation kit (AbD Serotec, Oxford, United Kingdom) according to the manufacturer's instructions. The antibody used in the indirect immunostaining techniques was a purified anti-B cell subset (McGiP10). Secondary antibody was an RPE-conjugated goat anti-mouse IgM-IgG-IgA (H + L, Southern Biotech, Birmingham, AL). All primary antibodies were purchased from AbD Serotec. Flow cytometric analysis was performed using a FACSAria cell sorter (BD Biosciences, San Jose, CA), and data were analyzed using FCS Express software (De Novo Software). Dead cells were excluded from analysis.

Quantitative PCR analysis of cytokines and chemokines in *C. caviae*-infected conjunctivae. Conjunctivae were excised immediately after euthanasia of guinea pigs and stored in RNAlater (Ambion, Austin, TX) at −20°C until further use. Total RNA was extracted from homogenized conjunctivae with TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA). For each conjunctiva sample, 0.5 μg total RNA was treated with RNase-free DNase (Promega, Madison, WI) for 25 min at 37°C, followed by incubation at 70°C for 10 min to inactivate DNase. The RNA samples were then converted into cDNA using oligo(dT), random hexamers, and SuperScript III reverse transcriptase kit from Invitrogen Life Technologies according to the manufacturer's recommendations. Real-time PCR was performed using iQ SYBR green supermix (Bio-Rad Laboratories, Inc., Hercules, CA) in a Bio-Rad iCycler, as described previously (47). No-template controls and melting curve analysis were used as controls to ensure the lack of contaminating DNA in the RNA preparations and to rule out primer-dimer formation, respectively. Induction of mRNA was determined from the threshold cycle (*C*<sub>T</sub>) values normalized for 18S rRNA expression and then normalized to the value derived from conjunctivae of healthy, uninfected guinea pigs. Real-time primers for guinea pig genes evaluated in this project are listed in Table 1. Primer sequences for alpha interferon (IFN-α), transforming growth factor β1 (TGF-β1), CXCL5 (interleukin-8 [IL-8]), tumor necrosis factor alpha (TNF-α), and 18S rRNA were published by Allen et al. (3); sequences for IL-5 and granulocyte-macrophage colony-stimulating factor (GM-CSF) were published by Kubo et al. (19); the sequence for CCL2 (monocyte chemotactant protein 1 [MCP-1]) was published by Lyons et al. (23). All others were designed with Beacon Designer software (Bio-Rad). All primers were synthesized by IDT, Inc. (Corvallis, IA).

Statistical analysis. For experiments reporting repeated measures from individual animals, significant differences between the two animal groups (guinea pigs receiving neutrophil-depleting antiserum or guinea pigs receiving only normal rabbit serum) were determined by a two-way analysis of variance (ANOVA) with repeated measures (day and group) with Holm-Sidak post hoc analysis as a multiple comparison procedure. For experiments comparing the two groups at only one time point, an unpaired *t* test was used. Ocular pathology and conjunctival mucosal erosion scores were analyzed using the Mann-Whitney rank sum test. Differences were considered significant when *p* was <0.05.

Experimental design. A guinea pig, neutrophil-depleting antiserum (produced in rabbits) was used to deplete neutrophils in guinea pigs during the first 7 days of a primary *C. caviae* ocular infection. Briefly, female guinea pigs (450 to 500 g) were divided into two groups (*n* = 5 per group): (i) a neutrophil-depleted group, injected intraperitoneally with 1.0 ml of neutrophil antiserum every 24 h beginning the day before infection (day −1), and (ii) a control group, injected with NRS in the same manner. Twenty hours after the first treatment with antiserum or NRS (day 0 of infection), all guinea pigs were infected in the conjunctival sac with 10<sup>6</sup> IFU of *C. caviae*. The infection course was followed and confirmed by enumeration of IFU collected from the conjunctivae on days 3 and 6 postinfection and by daily visual scoring of gross ocular pathology of each animal. At various times during the infection, blood was collected to measure peripheral blood neutrophils, and blood and tears were collected to quantify chlamydial-specific IgA and IgG levels. On day 7 postinfection, the guinea pigs (*n* = 5 per group) were euthanized to harvest infected conjunctivae for histological sections, flow cytometry, and quantitative PCR. The entire experiment was repeated for a total of 10 animals per group. An additional 8 guinea pigs (*n* = 4 per treatment group) were infected and euthanized on day 4 postinfection to prepare conjunctival histological sections for histopathology and myeloperoxidase immunohistochemistry staining. Finally, five uninfected guinea pigs were euthanized to collect normal conjunctivae for histological sections and quantitative PCR.

RESULTS

Antineutrophil antiserum effectively depletes neutrophils in guinea pigs. To confirm that antineutrophil antiserum was effective in depleting peripheral blood neutrophils during the *C. caviae* ocular infection, blood neutrophil counts were performed on guinea pigs treated with antiserum and on control guinea pigs treated with NRS (Fig. 1A). Baseline neutrophil counts were performed the day before infection (day −1), immediately before the first injections of antiserum or NRS. On day 0, all guinea pigs were infected in the conjunctival sac of both eyes with 10<sup>6</sup> IFU of *C. caviae*. As shown in Fig. 1A, by 24 h after the first antiserum treatment (day 0) and at every point thereafter, antiserum-treated guinea pigs had significantly reduced neutrophils (*P* < 0.001) than control guinea pigs treated with NRS.

To verify that depletion of peripheral blood neutrophils reflected neutrophil depletion at the infection site, histological sections of conjunctivae from day 4 postinfection were immunohistochemically stained for myeloperoxidase, a specific marker for neutrophils (Fig. 1B). In NRS control animals, a heavy infiltration of neutrophils (red-stained cells) into the
infected conjunctival mucosal epithelium was evident (Fig. 1B). Also present in NRS control tissues was a diffuse, red background stain due to the presence of myeloperoxidase released from neutrophils. In contrast, very few myeloperoxidase-positive neutrophils and no diffuse, red stain were present in the infected conjunctiva of the neutrophil-depleted group (Fig. 1B). These results correlate with the absolute neutrophil counts shown in Fig. 1A and demonstrate that antineutrophil antiserum treatment was effective in depleting neutrophils from peripheral blood and Chlamydia-infected conjunctiva.

The early *C. caviae* infection course was not affected by neutrophil depletion. Neutrophils are well-characterized phagocytic cells that engulf, kill, and clear bacteria (1). To investigate the role of neutrophils in chlamydial clearance during ocular infections, IFU from conjunctival tissues were quantified on days 3 and 6 postinfection (Fig. 2). Neutrophil depletion did not affect the infection course (number of IFU) during the first 7 days of a primary *C. caviae* ocular infection. Conjunctival swabs were obtained from NRS control and neutrophil-depleted guinea pigs infected with *C. caviae* (10⁴ IFU) on days 3 and 6 postinfection (n = 5 per group). IFU were isolated from swabs and measured as described in Materials and Methods. The experiment was repeated, and values for all animals (n = 10 per group) are shown as means ± standard deviations. An unpaired *t* test was used to statistically compare data from both experimental groups on each day. No significant differences were noted on day 3 or day 6.

Neutrophil depletion significantly reduced ocular pathology during *C. caviae* infection. In order to follow the ocular infection clinically, pathological changes in the right eye of each guinea pig were visually assessed and scored once a day (Fig. 3A). Beginning on day 4 postinfection and continuing until day 7 (day of euthanasia), guinea pigs in the neutrophil-depleted group had significantly less ocular pathology (P < 0.001) than the NRS control group. In both groups pathological changes first became evident on day 3, peaked on days 4 to 5, and were in decline by day 6 postinfection. The ocular pathology in the neutrophil-depleted group showed the same pattern of change as the NRS control but was consistently and significantly less severe.

To determine if microscopic histopathology of the conjunctiva correlated with the gross ocular pathology, H&E-stained conjunctival sections from both groups taken on days 4 and 7 postinfection were scored by a veterinary pathologist for conjunctival mucosal epithelium damage (Fig. 3B). There was significantly less mucosal epithelial erosion in the neutrophil-depleted group (P < 0.05). Interestingly, the ocular pathology scores (Fig. 3A) and histopathology scores (Fig. 3B), both based on an independent scale from 0 to 4, were very comparable on days 4 and 7 postinfection, lending support to the validity of these scores. A visual representation of the differences in histopathology between the two groups is shown in Fig. 3C. H&E sections from NRS control animals showed severely damaged mucosal epithelium with the original structure barely discernible. In contrast, sections from neutrophil-depleted animals showed a slightly damaged mucosal epithelium with the barrier structure basically intact (note the yellow
neutrophils in promoting conjunctival tissue damage during primary *C. caviae* ocular infections. CD4⁺ and CD8⁺ T cells are decreased in chlamydia-infected conjunctivae following neutrophil depletion. A study investigating the role of neutrophils in a pregnant mouse model of *Chlamydia abortus* indicated that neutrophils may have a role in regulating T lymphocyte recruitment to infectious sites (28). To determine if this may also occur in *C. caviae* ocular infections, we used flow cytometry to examine the effects of neutrophil depletion on T and B lymphocyte recruitment into *C. caviae*-infected conjunctivae on day 7 postinfection. Results are presented in absolute numbers (Fig. 4A) and as percentages (Fig. 4B) of B and T cells per total live cells per infected conjunctiva. Neutrophil depletion had no significant effect on absolute numbers or percentages of B cells. However, neutrophil depletion resulted in significantly decreased numbers (Fig. 4A) and percentages (Fig. 4B) of conjunctival CD4⁺ and CD8⁺ T cells. The reduction of CD4⁺ and CD8⁺ T cells associated with neutrophil depletion suggests a link between neutrophils and adaptive immunity.

Chlamydia-specific IgA titers are significantly increased upon neutrophil depletion. Considering the reduced T cell recruitment with neutrophil depletion, we wanted to determine if neutrophils also affect humoral immunity. Therefore, *C. caviae*-specific IgA and IgG levels were measured in tears and serum. IgG was measured only in serum, due to insufficient volumes of the tear samples. In tears (Fig. 5A), there was a low baseline titer of IgA in uninfected animals (day 0) capable of binding *C. caviae* elementary bodies. On days 3 and 6 postinfection, *C. caviae*-specific IgA titers in both groups were significantly higher than baseline (*P* < 0.05), but neutrophil-depleted guinea pigs had significantly higher IgA titers than control animals (*P* < 0.01) on both days. The same pattern for IgA titers was found in serum (Fig. 5B). *C. caviae*-specific IgA titers in serum on day 6 were higher than baseline levels in both groups, but titers in neutrophil-depleted animals were significantly higher than controls (*P* < 0.05). In contrast, *C. caviae*-specific IgG titers in serum were not affected by neutrophil depletion (Fig. 5B). On day 6 postinfection, IgG titers in both groups rose above baseline (*P* < 0.05), but there was no difference in titers between control and
neutrophil-depleted animals. These data indicate that neutrophils may have a role in downregulating chlamydia-specific IgA production during ocular C. caviae infection.

Conjunctival cytokine/chemokine expression profiles are altered with neutrophil depletion. The findings of reduced CD4+ and CD8+ T cells and increased chlamydia-specific IgA with neutrophil depletion suggest that neutrophils have a role in regulating adaptive responses to chlamydial infections. To explore mechanisms by which neutrophils may modulate the adaptive response, we used quantitative PCR to assess changes in expression of several cytokines and chemokines associated with acute primary infections. We chose day 7 postinfection to evaluate cytokine/chemokine profiles, because this time point represents an intersection between innate and adaptive immune responses. As shown in Fig. 6A, transcripts of IL-5 and TGF-β1 were significantly higher in neutrophil-depleted animals (P < 0.05 and P < 0.02, respectively). Since both of these cytokines are essential for IgA production (TGF-β for IgA isotype switching [45] and IL-5 for IgA production [44]), their increased expression corroborated the increased chlamydia-specific IgA titers in neutrophil-depleted animals.

Two chemokines associated with T cell recruitment were altered in neutrophil-depleted animals (Fig. 6B). Expression of the T cell-recruiting chemokine CCL5 (RANTES) was significantly lower (P < 0.05), while CCL7 (MCP-3), a CCL5 antagonist (7), was significantly higher (P < 0.02) in neutrophil-depleted animals. Neither MCP-1 (a macrophage-recruiting chemokine) nor IL-8 (a neutrophil-recruiting chemokine) was affected by neutrophil depletion at this time point, day 7 postinfection. Additionally, no significant differences were observed in the proinflammatory cytokines TNF-α and IL-1β, the activation marker MHC-II, or neutrophil growth factor GM-CSF between neutrophil-depleted and NRS control animals (data not shown). The reduction of T cell-recruiting chemokine CCL5 expression in neutrophil-depleted animals corroborated the reduction in T cell recruitment observed in infected tissues. Taken together, these data suggest that neutrophils

FIG. 4. CD4+ and CD8+ T cells, but not B cells, were significantly decreased in C. caviae-infected conjunctivae in neutrophil-depleted guinea pigs. On day 7 postinfection guinea pigs were euthanized and conjunctivae harvested and processed into single-cell suspensions for immunofluorescent staining and flow cytometric analysis, as described in Materials and Methods. For the flow cytometric analysis, a gate was set on live, intact cells, using the Live/Dead cell stain kit (Invitrogen) to exclude debris and dead cells. The gated cells were then analyzed for the presence of CD4+ T cells, CD8+ T cells, and B cells. Data are presented as means ± standard deviations of the absolute number (A) and percentage (B) of lymphocyte subsets in total live cells analyzed per infected conjunctiva. Cell types in the two groups were statistically compared using an unpaired t test (*, P < 0.05; **, P < 0.005).

FIG. 5. C. caviae-specific IgA titers were significantly increased in tears and serum with neutrophil depletion during a C. caviae ocular infection, but C. caviae-specific IgG titers in the serum were unchanged. Tears were collected on days 0, 3, and 6, and serum was obtained on days 0 and 6 postinfection from guinea pigs with C. caviae ocular infections. C. caviae-specific IgA titers were measured by an indirect ELISA and C. caviae-specific IgG titers were measured by direct ELISA as described in Materials and Methods. The experiment was repeated, and values for all animals (n = 10 per group) are shown as means ± standard deviations. Data for IgA in tears were analyzed using a two-way ANOVA with repeated measures (day and group) with the post hoc Holm-Sidak method for multiple comparison procedures. For serum, levels of IgA or IgG of both treatment groups on day 6 were compared to each other with an unpaired t test (*, P < 0.05; **, P < 0.01).
infection produces an acute inflammatory conjunctivitis that affects adaptive immunity directly or indirectly by regulating local cytokine/chemokine levels.

**DISCUSSION**

The purpose of this study was to investigate the role of neutrophils in promoting immunopathology and regulating adaptive immune responses during chlamydial ocular infection. The results suggest that neutrophils are key contributors to host conjunctival tissue damage during ocular *C. caviae* infection but may not be essential for chlamydial clearance, at least on days 3 and 6 postinfection. Further, neutrophils may function beyond microbial killing and clearance to help modulate the adaptive response by downregulating humoral immunity and promoting T cell recruitment.

The guinea pig model of *C. caviae* ocular infection/trachoma has been characterized in terms of primary and chronic infection (2, 24, 26, 27, 30) and is an ideal model for our experiments. An experimentally induced primary ocular chlamydial infection produces an acute inflammatory conjunctivitis that clinically resolves in 12 to 15 days with clearing of chlamydiae in 3 to 4 weeks, depending on the infectious dose (24). There are few neutrophils present in normal, uninfected guinea pig conjunctivae, but neutrophil infiltration begins rapidly after *C. caviae* infection—within hours (24). Resolution of conjunctival infection requires both a cell-mediated Th1 response (R. G. Rank and H. M. Lacy, unpublished data) and humoral immunity (25).

It is accepted dogma that neutrophils play a critical role in bacterial clearance via their phagocytic capabilities. In our study, however, neutrophil depletion did not alter bacterial burden (at days 3 and 6 postinfection), suggesting that neutrophil function as a phagocytic cell is not essential in clearing chlamydiae. Although no similar studies of chlamydial ocular infection are available for comparison with our results, there are neutrophil function studies of chlamydial genital infections that show diverse IFU results (4, 5, 20). In mice with *Chlamydia muridarum* genital tract infections followed until total clearance of chlamydiae, neutrophil depletion did not alter the number of IFU isolated from swabs or tissues, prompting the authors to conclude that neutrophils play little or no role in clearing infection (20). Similar conclusions were drawn in a study using genetically different strains of mice (C3H and C57BL/6) with *C. muridarum* pulmonary infections (4). Neutrophils recruited to the lungs were significantly higher in C3H mice, yet these mice had significantly more IFU isolated from lungs on days 7 and 14 postinfection. The authors concluded that neutrophils are not efficient in clearance of chlamydial infection (4). Different results were shown in an earlier report by Barteneva et al., in which neutrophil depletion in mice with *C. muridarum* genital infections resulted in a longer and more severe infectious course (5). Finally, a recent preliminary study in our laboratory showed that neutrophil-depleted mice with *C. muridarum* genital infections had significantly increased IFU between 42 and 48 h postinfection, while control animals had no increase (R. G. Rank and P. B. Wyrick, unpublished data). Taken together, these data suggest that neutrophil impact on bacterial clearance may occur in the very initial stages of infection, possibly within the first 48 h.

Regarding the role of neutrophils in ocular immunopathology, our results suggest that neutrophils have a prominent role in producing conjunctival damage during primary chlamydial ocular infections. In neutrophil-depleted guinea pigs, ocular gross pathology and conjunctival histopathology were dramatically decreased. Interestingly, the ocular pathology scores of neutrophil-depleted and control animals showed the same pattern of pathology presentation, zenith, and decline. However, pathology in neutropenic guinea pigs was significantly less than in control animals at every time point. It is important to note that our data do not point to neutrophils as the only agents of pathology. Neutrophil depletion significantly decreased pathology but did not eliminate it. Studies using a different chlamydial model have shown similar results. Several groups using the mouse model of *C. muridarum* genital infection reported observational and descriptive accounts of a relationship between neutrophils and host tissue damage (12, 13, 16). Overall, results from our study strongly support the concept that neutrophils are key agents of immunopathology during primary chlamydial infections.

One possible mechanism by which neutrophils may cause...
host cell damage involves potent proteinases released by neutrophils during infection. Neutrophil elastase and cathepsin G are serine proteinases that degrade multiple bacterial proteins as well as multiple host proteins. Additionally, neutrophil elastase promotes activation of matrix metalloproteinase 9 (MMP-9) (14), another proteinase released by neutrophils and capable of degrading host proteins. Increased MMP-9 activity and increased numbers of neutrophils and macrophages containing MMP-9 have been found in conjunctival biopsy specimens from trachoma patients (15). Although there are other pathways by which neutrophils may promote host damage, neutrophil proteinases may be one of the more prominent, because they have been linked to pathology of acute inflammatory diseases at other mucosal sites, such as the respiratory and gastrointestinal tracts (17, 18, 33, 49).

Regarding the relationship between neutrophils and adaptive responses, our results indicate that neutrophils may have a modulating effect on adaptive immunity. With neutrophil depletion, CD8+ and CD4+ T cell recruitment to infected conjunctiva was significantly decreased. In addition, chlamydia-specific IgA in tears and serum was significantly increased. An increase in IgA as early as day 3 was somewhat surprising, but it must be taken into account that conjunctiva contain abundant areas of mucosa-associated lymphoid tissue (generically called MALT, but specifically for conjunctiva called CALT). Any immunogenic stimulus deposited in the conjunctiva would be immediately accessible to an immune-inductive site to elicit a rapid local antibody response. Moreover, conjunctival chemokine and cytokine data substantiate both of these adaptive immune changes. Expression of T cell-recruiting CCL5 (RANTES) was significantly decreased and CCL7, an antagonist to T cell recruitment (7), was increased, while cytokines IL-5 and TGF-β, both required for IgA production, were significantly increased. A study of neutrophil depletion in the pregnant mouse model of C. abortus infection showed similar results, wherein neutrophil depletion resulted in significantly reduced numbers of CD4+ and CD8+ T cells at infectious foci in the liver (28). In summary, our data suggest that neutrophils help modulate adaptive immunity by promoting T cell recruitment and downregulating chlamydia-specific IgA antibody production. Mechanisms by which neutrophils may modulate the adaptive response most likely involve cytokines and chemokines released by neutrophils during infection. Neutrophils produce multiple cytokines and chemokines, some of which directly recruit T cells, such as macrophage inflammatory protein 1α (MIP-1α), MIP-1β (42), MIP-3α, and MIP-3β (43). Neutrophils are also an important source of IL-12, the cytokine that instructs dendritic cells to orchestrate cell-mediated immunity (8, 9, 46).

We have provided evidence that neutrophils may downregulate IgA humoral responses in ocular chlamydial infections, most likely by downregulating TGF-β and IL-5, since both are increased when neutrophils are depleted and both are required for IgA production (10, 44, 45). The mechanism(s) by which neutrophils downregulate these two cytokines is obscure. However, TGF-β likely plays an important role, since it has multiple functions during an immune response which include downregulation of inflammation and promotion of IgA production (21).

In conclusion, evidence presented in this study suggests greater complexity in the neutrophil response to chlamydial ocular infection than previously understood. Neutrophils may shape antichlamydial adaptive responses at the same time they are promoting host tissue damage and yet not be essential in directly killing chlamydiae.

ACKNOWLEDGMENTS

We are indebted to Steven A. Schichman for his valued editorial assistance with manuscript preparation.

This work was supported by Children’s University Medical Group (CUMG) grant 030602 from the Arkansas Children’s Hospital Research Institute, NIAID grant AI059650 from the NIH, and the Arkansas Bioceiences Institute.

REFERENCES


