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

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ABSTRACT

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 using the guinea pig model of *C. caviae* inclusion conjunctivitis. We hypothesize 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 with rabbit anti-neutrophil antiserum, and immune responses and immunopathology were evaluated during the first 7 days of infection. Results show 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 IL-5 and anti-inflammatory TGF-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.
INTRODUCTION

*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 pathologic 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 non-human primates, the latter because of issues of expense. However, an outstanding model for chlamydial inclusion conjunctivitis is available using the guinea pig infected with *C. 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). Monnickendam 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 co-workers 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 pathologic 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 a producer of chemokines that attract immune cells to infectious sites and as a source 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 on 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 shows 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).

Our goal was to investigate the role of neutrophils in the pathologic response and early adaptive immunity in the guinea pig model of chlamydial inclusion conjunctivitis. To achieve this objective, we developed a rabbit antiserum 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 down-regulating 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 lbs, 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 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 antiserum. Guinea pig neutrophil antiserum was produced in two New Zealand female rabbits, according to procedures described previously (11,37,41). The antiserum was treated to remove antibodies to guinea pig lymphocytes, monocytes and platelets. Briefly, neutrophils were isolated from guinea pig blood using 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 cytospins and were 97% viable, as measured by Trypan blue exclusion. Two rabbits were immunized with guinea pig neutrophils eight times at two to three 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 on the backs of the rabbits at 5 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 $2 \times 10^6$ 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 minutes to destroy complement. To eliminate antibodies specific for guinea pig MHC I molecules, antisera were adsorbed at least 5 times with a 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 (PVDF) 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 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 anti-monocyte antibodies, indirect immunofluorescent staining of guinea pig blood mononuclear cells was employed using antisera as the primary antibody. Pre-immune sera served as negative isotype controls, and the secondary antibody was a FITC-conjugated goat anti-rabbit antibody IgM & IgG (H + L chain specific) (Southern Biotech, Birmingham, AL).

Elimination of platelet antibodies was confirmed during in vivo testing of antisera for the ability to deplete neutrophils. Briefly, normal, non-infected guinea pigs were administered either 1.0 or 1.5 mL antisera from rabbit 1 (n=2) or rabbit 2 (n=2) intraperitoneally every 24 hr 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. Chlamydiae were passaged, prepared for infection, and quantified by standard methodology (39). Guinea pigs were anesthetized with sodium pentobarbital (Nembutal; 32 mg/kg body weight) and infected in the conjunctivae of both eyes by instilling 20 µl of SPG containing $10^4$ 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 antiserum was administered intraperitoneally every 24 hrs beginning the day before infection (Day -1) and continued until termination of experiments on Day 7 post infection. Control animals (NRS-controls) were administered 1 mL of sterile filtered, heat inactivated normal rabbit serum (Pel-Freez Biologicals, Rogers, AR). No animals exhibited signs of serum sickness prior to euthanasia on day 7 post infection. To confirm that the neutrophil antisera was effective in depleting neutrophils *in vivo*, absolute neutrophil counts of the peripheral blood and myeloperoxidase immunohistochemistry staining of conjunctival sections were performed. Peripheral blood was collected from the lateral saphenous vein in the back feet of guinea pigs while under ketamine anesthesia (22). To confirm the ability of the antisera to diminish neutrophil recruitment into chlamydial infected tissue; immunohistochemistry staining for myeloperoxidase, a neutrophil-specific enzyme, was performed on 5 µm paraffin-embedded
conjunctivae sections. A rabbit polyclonal antibody to myeloperoxidase (Cat#RB-373; Thermo Scientific, Fremont, CA) diluted 1:100 and the Ultra Vision Detection System, Anti-Polyvalent, HRP/AEC Kit (Thermo Scientific) was used according to manufacturer's instructions.

**Infection course.** The course of infection was followed by measuring conjunctival IFU.

Conjunctival material for the isolation and quantification of chlamydiae was collected from guinea pigs as previously described while under ketamine anesthesia (20 mg/kg) (6). Briefly, conjunctivae were swabbed using a Dacron swab which was placed in 2-sucrose-phosphate transport medium and frozen at -70°C until needed. Swabs were processed for isolation and determination of number of IFU by standard techniques (36). To assure conjunctivae swabbing did not affect gross ocular pathology scoring, conjunctival swabs were collected from the left eyes 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 erythema, edema, and exudation as previously described (39). In order to maintain consistency and subjectivity, one investigator scored the pathology, blinded to the experimental group. 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. A Veterinary Pathologist, blinded to the experimental design, evaluated sections for mucosal epithelial erosion based on a 0-to-4+ scale.

**C. caviae-specific antibody titers in serum and tears.** To obtain tears, guinea pigs were anesthetized with sodium pentobarbital and a small (1 x 0.2 cm), absorbent, sterile sponge (Ear wick, DeRoyal, Powell, TN) was placed under the left eye conjunctiva (the eye NOT used to evaluate and score gross pathology). After 15 minutes the sponge was removed and frozen at –
20°C until further processing. To obtain serum, peripheral blood was obtained from the lateral
saphenous veins while the guinea pigs were under anesthesia. Serum samples were collected on
days 0, 3, and 6 post infection, and tears were collected on days 0, 4, and 6 post infection.

An ELISA using Renografin-purified elementary bodies as the antigen, as previously
described (38), was utilized to measure C. caviae-specific IgA and IgG antibodies in serum and
C. caviae-specific IgA in tears. The antibodies for IgA detection by indirect ELISA were rabbit
anti-guinea pig alpha chain (primary antibody) at 1:500 dilution, followed by horse radish
peroxidase-labeled goat anti-rabbit IgG (H+L chain specific) (secondary antibody) at 1:1500
dilution. The antibody for IgG detection in a direct ELISA was a horse radish peroxidase-labeled
rabbit anti-guinea pig IgG (H+L chain specific) at a 1:6000 dilution. All antibodies were
purchased from MP Biomedicals, Irvine CA.

**Flow cytometry analysis of lymphocytes in C. caviae infected conjunctivae.** For analysis
of numbers and types of lymphocytes present, infected conjunctivae were harvested and
processed individually to produce a single-cell suspension as described (47). Approximately, 1-2
x 10^5 cells were stained for individual cell surface markers or isotype controls (5 µg/mL) for 20
minutes on ice. 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), then fixed with 1% paraformaldehyde/phosphate buffered saline. For indirect
immunostaining, cells were washed, incubated with a fluorescent-tagged secondary antibody
before treatment with blue fixable dye and fixation with paraformaldehyde. The antibodies used
in direct immunostaining were RPE-labeled mouse anti-guinea pig CD4 (clone CT7), FITC-
labeled mouse anti-guinea pig CD8 (clone CT6), and purified mouse anti-guinea pig CD45
(clone IH-1). Allopycocyanin (APC) was conjugated to purified anti-guinea pig CD45 using an
APC conjugation kit (AbD Serotec, Oxford, United Kingdom), according to manufacturer instructions. The antibody used in the indirect immunostaining techniques was a purified anti-B-cell subset (MsGp10). Secondary antibody was a 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 FACS Aria cell sorter (BD Biosciences, San Jose, CA), and data were analyzed using FCS Express 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 RNA later (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 conjunctivae sample, 0.5 µg total RNA was treated with RNase-free DNase (Promega, Madison, WI) for 25 minutes at 37°C, followed by incubation at 70°C for 10 minutes to inactivate DNase. The RNA samples were then converted into cDNA using oligo dT and random hexamer primers and the Superscript III Reverse Transcriptase kit from Invitrogen Life Technologies according to 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 (47). No-template controls and DNA 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. Fold 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 IFN-α, TGF-β1,
CXCL8 (IL-8), TNF-α and 18S rRNA were published by Allen et al. (3); sequences for IL-5 and GM-CSF were published by Kubo et al. (19); and sequence for CCL2 (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. (Coralville, IA).

Statistical analysis. For experiments recording 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 posthoc 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 \( p < 0.05 \).

Experimental Design. A guinea pig, neutrophil-depleting antiserum (produced in rabbits) was used to deplete neutrophils in guinea pigs during the first seven days of a primary *C. caviae* ocular infection. Briefly, female guinea pigs (450-500g) 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 hours beginning the day before infection (day-1); and ii) a control group, injected with normal rabbit serum (NRS) in the same manner. Twenty hours after first treatment of antiserum or NRS (day 0 of infection), all guinea pigs were infected in the conjunctival sac with \( 10^4 \) *C. caviae* inclusion forming units (IFU). The infection course was followed and confirmed by i) enumeration of IFU collected from the conjunctivae on days 3 and 6 post infection; and ii) 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 chlamydia-specific IgA and IgG levels. On day 7 post infection, 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 post infection to prepare conjunctival histological sections for histopathology and myeloperoxidase immunohistochemistry staining. Finally, 5 uninfected guinea pigs were euthanized to collect normal conjunctivae for histological sections and quantitative PCR.

RESULTS

Anti-neutrophil antiserum effectively depletes neutrophils in guinea pigs. To confirm that anti-neutrophil 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 $10^4$ IFU C. caviae. As shown in Figure 1A, by 24 hours after the first antiserum treatment (day 0) and at every point thereafter, guinea pigs had significantly reduced neutrophils ($p<0.001$) compared to 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 post infection 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 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 activated
neutrophils. In contrast, very few myeloperoxidase positive neutrophils and no diffuse, red stain
were present in the infected conjunctivae of the neutrophil-depleted group (Fig. 1B). These
results correlate with the absolute neutrophil counts shown in Figure 1A and demonstrate that
anti-neutrophil antiserum treatment was effective in depleting neutrophils from peripheral blood
and chlamydia-infected conjunctivae.

**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 post infection (Fig. 2). Neutrophil depletion did not
significantly affect numbers of IFU on days 3 or 6 post infection ($p=0.131$ and $p=0.089$,
respectively, as determined by an unpaired $t$ test). In normal conjunctival infections with $10^4$ IFU
of *C. caviae*, one normally finds a peak response between day 4 to day 6 following infection and
the infection resolves by days 15-18 (48).

**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 post
infection and continuing until day 7 (day of euthanasia), guinea pigs in the neutrophil depleted
group had significantly less ocular pathology ($p<0.001$) than NRS-control group. In both groups
pathological changes first became evident on day 3, peaked on days 4-5 and were in decline by
day 6 post infection. The ocular pathology in the neutrophil depleted group showed the same
pattern of change as the NRS-control, but was consistently and significantly less.
To determine if microscopic histopathology of the conjunctivae correlated with the gross ocular pathology, H&E stained conjunctival sections from both groups taken on days 4 and 7 post infection were scored by a veterinary pathologist for conjunctival mucosal epithelium damage (Fig. 3B). There was significantly less mucosal epithelium erosion in the neutrophil depleted group ($p<0.05$). Interestingly, the ocular pathology scoring (Fig. 3A) and histopathology scores (Fig. 3B), both based on independent 0-4 scale, were very comparable on days 4 and 7 post infection, lending support to their validity. Visual representation of the difference in histopathology between the two groups is shown in Figure 3C. H&E sections from NRS-control animals showed severely damaged mucosal epithelium with original structure barely discernible. In contrast, sections from neutrophil depleted animals show a slightly damaged mucosal epithelium with the barrier structure basically intact (note yellow bracket). Taken together, these data support a role for neutrophils in promoting conjunctival tissue damage during primary *C. caviae* ocular infections.

**CD4$^+$ and CD8$^+$ T cells are decreased in chlamydial infected conjunctivae following neutrophil depletion.** A study investigating the role of neutrophils in the pregnant mouse model of *C. abortus* indicates 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 effect of neutrophil depletion on T and B lymphocyte recruitment into *C. caviae* infected conjunctivae on day 7 post infection. Results are presented as absolute numbers (Fig. 4A) and percentage (Fig. 4B) of B and T cells in total live cells per infected conjunctiva. Neutrophil depletion had no significant effect on absolute numbers or percentage 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 with 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 were measured in tears and serum. IgG was measured only in serum due to insufficient volume of tear samples. In tears (Fig 5A), there was a low baseline titer of IgA in uninfected animals (day 0) capable of binding *C. caviae* EBs. On days 3 and 6 post infection, *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 on (*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 post infection, 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 down-regulating chlamydial-specific IgA production during ocular *C. caviae* infection.

Conjunctival cytokine/chemokine expression profile is 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 post
infection to evaluate cytokine/chemokine profiles, because this time point represents an intersection between innate and adaptive immune responses. As shown in Figure 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 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 post infection. Additionally, no significant differences were observed in proinflammatory cytokines TNF-α and IL-1β; activation marker MHC II, and 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 affect 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 post infection. Further, neutrophils may function beyond microbial killing and clearance to help modulate the adaptive response by down-regulating 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 12-15 days with clearing of chlamydiae in 3-4 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 post infection), suggesting that neutrophil function as a phagocytic cell is not essential in clearing chlamydiae. Although no similar studies of chlamydial ocular infections are available for comparison with our results, there are neutrophil-function studies in chlamydial genital infections that show diverse IFU results (4,5,20). In mice with *C. 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 post infection. 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. where 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 hours post infection, 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 hours.

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 show 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 does not point to neutrophils as the only agents of pathology. Neutrophil depletion significantly decreased pathology, but it 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 (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 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 affect on adaptive immunity. With neutrophil depletion, CD8⁺ and CD4⁺ T cell recruitment to infected conjunctivae 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 conjunctivae contain abundant areas of mucosal 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 substantiates 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<sup>+</sup> and CD8<sup>+</sup> T cells at infectious foci in the liver (28). In summary, our data suggests that neutrophils help modulate adaptive immunity by promoting T cell recruitment and down-regulating chlamydia-specific IgA antibody production. Mechanisms by which neutrophils may modulate the adaptive response most likely involve cytokine and chemokines released by neutrophils during infections. Neutrophils produce multiple cytokines and chemokines, some of which directly recruit T cells, such as 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 show evidence that neutrophils may down-regulate IgA humoral responses in ocular chlamydial infections; most likely by down-regulating 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 down-regulate these two cytokines is obscure. However, TGF-β is likely to play an important role since it has multiple functions during an immune response; which include down-regulation of inflammation and promotion of IgA production (21).

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

The authors are indebted to Dr. Steven A. Schichman for his valued editorial assistance with manuscript preparation.

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REFERENCES


# TABLE 1. Primers for quantitative RT-PCR

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*Allen et al (22), a designed with Beason Designer software (Bio-Rad); bKubo et al.(23); d Lyons et al (24)*
Figure Legends.

Figure 1. Anti-neutrophil antiserum treatment effectively depleted neutrophils from peripheral blood and *C. caviae* infected conjunctivae of guinea pigs. Guinea pigs (n=5/group) were administered 1.0 ml of rabbit neutrophil-depleting antiserum or normal rabbit serum (NRS-control) intraperitoneally every 24 hrs beginning the day before infection (day-1). On day 0, all animals were infected in the conjunctiva with $10^4$ IFUs *C. caviae*. A) Peripheral blood neutrophil counts were performed on guinea pigs on days -1, 0, 3 and 6 post infection. The experiment was repeated and values for all animals (n=10 per treatment group) are shown as mean ± SEM. *p<0.001* by two-way analysis of variance (ANOVA) with repeated measures (Day & Group) with the posthoc Holm Sidak method for multiple comparison procedures. B) Histological sections of *C. caviae* infected conjunctivae harvested on day 4 post infection were stained for myeloperoxidase. Red = myeloperoxidase/neutrophils (arrows); Blue = nuclei of cells. Diffuse, red background staining in NRS-control represents free myeloperoxidase molecules released from neutrophils into microenvironment. Magnification 200X. Photomicrographs are representative of responses in each group (n=5/group).

Figure 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* (10^4 IFU) on days 3 and 6 post infection (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 mean ± S.D. 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.
Figure 3. Neutrophil depletion significantly decreased ocular pathology during a *C. caviae* ocular infection.  

A) Gross ocular pathology in NRS-control and neutrophil-depleted guinea pigs was visually assessed and scored on a daily basis as described in Materials and Methods. The experiment was repeated and values for all animals (10 per group) are shown as mean ± S.E.M. Data was analyzed using a Mann-Whitney Rank Sum Test (*p*<0.001).  

B) H&E stained conjunctival sections (5 µm) from NRS-control and neutrophil-depleted guinea pigs on day 4 (n=4 animals per group) and day 7 (n=5 animals per group) were evaluated by a veterinary pathologist for mucosal epithelium erosion using a 0 to 4+ scaling system. Data is shown as mean ± S.E.M. and for each day the two groups were statistically compared using a Mann-Whitney Rank Sum Test (*p*<0.05). C) H&E stained sections of guinea pig, infected conjunctivae on day 4 post infection revealed severely damaged mucosal epithelium in NRS-control guinea pigs, but much less damage in neutrophil-depleted animals. A yellow bracket spans conjunctival mucosal epithelium showing close to normal mucosal structure in neutrophil-depleted conjunctiva. Magnification 400x. Photomicrographs are representative of responses in each group.

Figure 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 post infection 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. In flow cytometric analysis, a gate was set on live, intact cells using 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 is presented as the mean ± S.D. of 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).

Figure 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 post infection 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 mean ± S.D. Data for IgA in tears was analyzed using a two-way analysis of variance (ANOVA) with repeated measures (Day & Group) with the posthoc 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).

Figure 6. Cytokine/chemokine mRNA expression in *C. caviae*-infected conjunctivae was significantly altered in neutrophil depleted guinea pigs. On day 7 post infection guinea pigs were euthanized and conjunctivae harvested for quantitative PCR analysis as described in Materials and Methods. The experiment was repeated and values for all animals (n=10 per treatment group) are shown as mean ± S.D. Fold increase of mRNA was determined from the threshold cycle (Ct) values normalized for 18S rRNA expression and then normalized to the values from conjunctivae of healthy, uninfected guinea pigs. Data from the two groups was statistically compared with an unpaired t test (*p<0.05; **p<0.02).
Fig 1.

A

![Graph showing neutrophil levels post infection.](image)

B

![Images comparing NRS-control and Neutrophil-depleting antiserum.](image)
Fig. 2

Post Infection
Day 3 Day 6
Inclusion forming Units (IFU)

Neutrophil-depleting antiserum
NRS-control

0
1x10^6
2x10^6
3x10^6
4x10^6
5x10^6

Fig. 2
Fig 3.

A

![Graph showing Pathology Score over Day Post Infection for Neutrophil-depleting antiserum and NRS-control](image)

B

![Bar graph showing Mucosal Erosion Score for Neutrophil-depleting antiserum and NRS-control](image)

C

![Images of tissue sections labeled NRS-control and Neutrophil-depleting antiserum](image)
Fig. 4

A

B cells CD4 CD8

Absolute Number / Conjunctiva

0
1e+5
2e+5
3e+5
4e+5
5e+5
6e+5

Neutrophil-depleting antiserum
NRS-control

% of Conjunctival Cells

0
10
20
30
40
50

Neutrophil-depleting antiserum
NRS-control

A

B
Fig. 5

A  IgA Tears

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B  IgA & IgG Serum

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Fig. 6

A) Cytokines

B) Chemokines

Fold Increase mRNA