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Production of Colony-Stimulating Factors during Pneumonia Caused by *Chlamydia trachomatis*

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The colony-stimulating factors (CSFs) are cytokines involved in the production, differentiation, and activation of host phagocytes. During murine infection with *Chlamydia trachomatis* (MoPn), plasma CSF levels increased in euthymic (*nu/+*) and athymic (*nu/nu*) BALB/c mice. Levels declined later in infection, with the *nu/+* mice resolving the infection but the *nu/nu* mice succumbing by day 16. Either live or heat-killed *Chlamydia* organisms could induce CSF increases on day 7 postchallenge in *nu/+* mice; however, by day 14, only mice challenged with live organisms maintained high plasma levels. CSFs were also produced by spleen cells of *nu/+* and *nu/nu* mice in response to *Chlamydia* antigen. Spleen cell CSF production was detectable by days 3 to 5 postinfection. In *nu/+* mice, spleen cell CSF production was elevated throughout the rest of the time course but in *nu/nu* mice fell significantly at day 14. Like the plasma CSF activity (CSA) production, spleen cell CSA production on day 7 was seen in mice challenged with either live or heat-killed *Chlamydia* organisms, but on day 14 only *nu/+* mice challenged with live organisms maintained significant CSA production. To further characterize the T-cell dependence of CSA production, spleen cells of *nu/+* mice were depleted of T cells or T-cell subsets before producing supernatants. On day 14 postinfection, the $CD4^+$ lymphocyte was the major producer of CSFs. Additionally, there were different types of CSFs secreted by *nu/+* and *nu/nu* mice as determined by the ability of spleen cell supernatants to support the granulocyte-macrophage CSF/interleukin 3-dependent cell line FDCP-1. Supernatants from *nu/+* mice had 4 to 8 times the level of FDCP-1 CSF activity of the supernatants from *nu/nu* mice. These results support the evidence that *nu/+* mice were producing some CSFs by T-cell-dependent mechanisms. This is the first report of CSF production in vivo during *Chlamydia* infection. Furthermore, we show that CSFs are produced by both T-cell-dependent and T-cell-independent mechanisms. The capacity of the CSFs to increase the production and effector function of phagocytes may be important to host defenses.

The colony-stimulating factors (CSFs) are glycoproteins integrally involved in producing, differentiating, and activating phagocytic cells. The names of the CSFs were originally assigned according to the major type of phagocyte produced in the CSF bioassay (9, 10). Four major CSFs have been classified in this system: interleukin 3 (IL-3, or multi-CSF), which induces the formation of granulocytes, macrophages, and eosinophils; granulocyte-macrophage CSF (GM-CSF), which induces the formation of granulocytes and macrophages; macrophage CSF, which induces the formation of macrophages; and granulocyte CSF, which induces the formation of granulocytes.

The CSFs may be important mediators of host defenses against infectious pathogens (2, 8, 11, 13, 21). The capacity of CSFs to increase the number of available phagocytes is one host defense mechanism aimed at killing invading microorganisms. In addition to increasing phagocyte numbers, CSFs also modulate secretory capacity, chemotaxis, and killing mechanisms of phagocytes. Thus, the CSFs are important regulatory glycoproteins that enhance both the production and function of phagocytes.

Our model of *Chlamydia trachomatis* pneumonia, caused by the mouse pneumonitis agent (MoPn), has been developed to investigate host defenses against infectious challenge (14–19). In this model, we have used both athymic (*nu/nu*) and euthymic (*nu/+*) BALB/c mice to investigate the role of T cells in immunity. It has been previously shown that the *nu/nu* mouse is much more susceptible to infection than the *nu/+* mouse (18). In our current model, the *nu/nu* mouse can be protected by passive transfer of T lymphocytes (17). Studies on lymphocyte subsets indicate that early in the infection, the $CD4^+$ lymphocyte is the most important for transfer of protection (15).

The goal of this study was to measure the production of CSFs during *Chlamydia* pneumonia and describe the cell types responsible for CSF production.

MATERIALS AND METHODS

Mice. Male and female specific-pathogen-free *nu/+* and *nu/nu* mice from a BALB/c background were bred and maintained under barrier conditions at the Audie L. Murphy Veteran's Administration Hospital, San Antonio, Texas. These mice were free of pathogenic bacteria and virus as assessed by culture or serology. Mice were housed in rooms on a 12-h dark-light cycle and used between 6 and 12 weeks of age. Infected mice were housed in isolator units in separate rooms. Female C57BL/6J mice were purchased from Jackson Laboratories (Bar Harbor, Maine) and used

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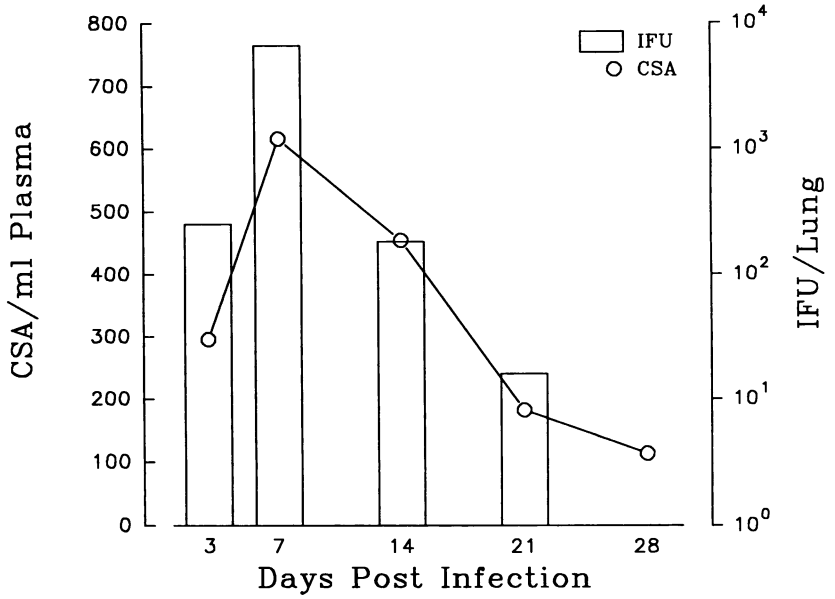

FIG. 1. Comparison of inclusion-forming units per lung and levels of plasma CSA during infection. Samples were harvested on the designated days after intranasal infection with MoPn.

between 8 and 16 weeks of age. Mice were fed rodent chow and water ad libitum.

MoPn. The biovar MoPn of *C. trachomatis* was maintained in embryonated hen's eggs (17). Mice were infected intranasally, after being anesthetized with sodium pentobarbital, with 10^2 to 10^5 inclusion forming units diluted in McCoy's modified 5A medium in a volume of 0.05 ml. MoPn grown in eggs was heat-killed by incubation at 60°C for 60 min. Purified elementary bodies were prepared by Renografin density gradient centrifugation of cell culture-derived *C. trachomatis* (17). The purified elementary bodies were killed under UV light before use as an antigenic stimulus. Quantitative culture of infected tissue was performed in McCoy cell monolayers and reported as inclusion forming units per organ (14).

Supernatant production. At various times postinfection, mice were sacrificed and the spleens were removed aseptically. Single cell suspensions were prepared by grinding the spleens between the frosted ends of glass microscope slides. The spleen cells were washed and resuspended in RPMI 1640 supplemented with 5% fetal bovine serum (HyClone Laboratories, Logan, Utah), 100 μg of vancomycin per ml, and 50 μg of gentamicin sulfate per ml. For the time course studies, spleens from individual mice were plated in 24-well plates at 2.5×10^6 cells per ml with or without 5 μg of elementary body antigen per ml. There were 3 to 5 mice per group at each time period.

For some experiments, spleen cells were separated into lymphocyte subsets by antibody-complement-mediated lysis before supernatant production. We used the monoclonal antibodies from hybridoma TIB 107 (anti-Thy 1.2), TIB 207 (anti-CD4), or TIB 210 (anti-CD8). The hybridomas were grown in tissue culture, and the antibody-containing supernatants were collected between 10 and 14 days of culture. To select for lymphocyte subsets, spleens from 3 to 5 mice were pooled and run through two cycles of antibody-containing supernatant and Low Tox-M rabbit complement (Accurate Chemicals, Westbury, N.Y.). Each lot of antibody and complement was tested to ensure depletion of the appropriate lymphocyte subset as assessed by flow-cytometric analysis. Postdepletion, the cells were plated with elementary body antigen as described for the time course studies.

CSF assays. Plasma was collected at various times postinfection by cardiac puncture from Metofane-anesthetized mice. Plasma or supernatants were placed into 35-mm dishes with 10^5 bone marrow cells from C57BL/6J mice in McCoy 5A medium supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml), amphotericin B (0.25 μg/ml), 15% fetal bovine serum, essential and nonessential amino acids, and 0.3% agar (6). Cultures were incubated for 7 days at 37°C in 7.5% CO_2. Colonies consisting of more than 50 cells were scored with a dissecting microscope. The amount of CSF activity (CSA) per ml was calculated by multiplying the number of colonies per plate by the inverse of the serum or supernatant dilution along the linear portion of the dose-response curve (20).

The growth-factor-dependent cell line FDCP-1 was also used to estimate CSA (8). The FDCP-1 cell line proliferates in response to both IL-3 and GM-CSF. Samples were serially diluted in triplicate in 96-well microtiter plates. Afterward, 10^4 FDCP-1 cells were added to each well and the cultures were incubated for a total of 48 h. Tritiated thymidine (0.5 μCi per well) was added during the last 6 h of culture. The cultures were harvested and counted by liquid scintillation spectroscopy.

Statistical analysis. Comparison of groups was performed by Student's *t* test with correction for unequal variance.

RESULTS

Time course of CSA production during infection. Our first experiment shows a time course of both quantitative *Chlamydia* counts and total serum CSA during infection of *nu*/+ mice (Fig. 1). *Chlamydia* counts were detectable on day 3 and maximized on day 7. Thereafter, the organism counts declined, until they were undetectable on day 28. Plasma CSA followed a similar time course. The normal values of CSA in uninfected *nu*/+ mice were generally between 50 and

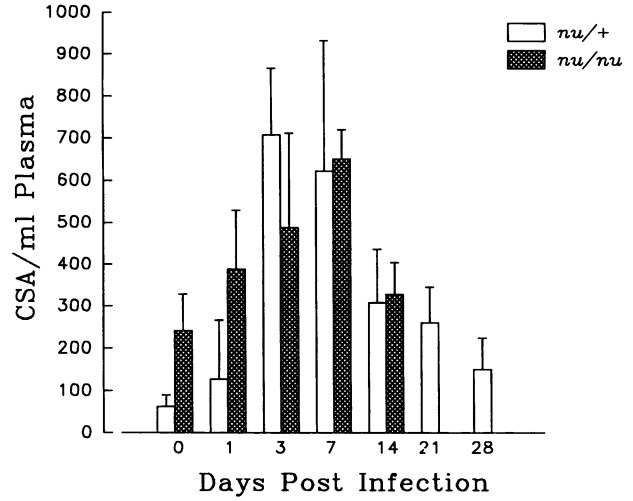

FIG. 2. Plasma CSA levels during infection in *nu*/+ and *nu/nu* mice. The only significant difference between *nu*/+ and *nu/nu* during the time course was at the baseline levels (day 0; $P = 0.011$).

150 U/ml. By day 3, plasma CSA levels were significantly elevated and maximized at day 7. Thereafter, CSA declined, until basal levels were reached by day 28. To determine whether the induction of CSA required live organisms, *nu*/+ mice were challenged with either live or heat-killed *Chlamydia* organisms, and plasma was harvested on days 7 and 14 postchallenge. Challenge with either live or heat-killed *Chlamydia* organisms induced increased CSA production on day 7 (2,800 ± 654 U of CSA per ml in *nu*/+ mice challenged with live *Chlamydia* organisms versus 1,840 ± 706 U of CSA per ml in *nu*/+ mice challenged with heat-killed organisms). However, by day 14, plasma CSA values in mice challenged with live organisms were significantly higher (1,680 ± 761 U of CSA per ml in mice challenged with live organisms versus 500 ± 120 U of CSA per ml in mice challenged with heat-killed organisms, $P < 0.025$). The maximum values of the latter experiment were higher than those of the former experiment, indicating some experiment-to-experiment variation in CSA values over the time course.

Comparison of CSA production in *nu*/+ and *nu/nu* mice during infection. Plasma CSA levels in *nu*/+ and *nu/nu* mice were measured over the time course of infection (Fig. 2). Plasma CSA values from uninfected (day 0) mice were 62 ± 36 U/ml. Maximum CSA was observed on day 3, followed by a slight decrease in activity on day 7. Thereafter, the serum CSA declined towards baseline levels, which were reached on day 28. In *nu/nu* mice, the baseline CSA level was higher than in the *nu*/+ mouse. The CSA levels were maximal on day 7 postinfection and declined on day 14. In *nu/nu* mice, this inoculum was lethal, and most mice were dead by day 16, so later CSA values were not obtainable in vivo for *nu/nu* mice. In this experiment, there were no statistically significant differences between results with *nu*/+ and *nu/nu* mice over the time course except for the day 0 values (baseline values) ($P = 0.011$).

Production of CSA by spleen cells following antigenic stimulation. The results of spleen cell production of CSA are reported in Table 1. Incubation of spleen cells from noninfected mice with MoPn antigen led to only low-level background CSA production (<10 U/ml) (data not shown). Furthermore, spleen cells from infected mice incubated without MoPn antigen did not produce CSFs above background

TABLE 1. Production of CSA by spleen cells[a]

Expt 1		Expt 2			Expt 3		
Day	*nu*/+	Day	*nu*/+	*nu/nu*	Day	*nu*/+	*nu/nu*
1	<10	1	<10	<10			
5	209	3	257	207			
10	180	7	134	172	7	262	447[b]
30	125	14	26[c]	<10	14	380[d]	83
		21	73	ND[e]			
		28	146	ND			

[a] Supernatants from individual mice (3 to 5 per group) were tested at each time period. *nu*/+ and *nu/nu* refer to the types of mice used (euthymic and athymic, respectively).
[b] $P = 0.010$ compared to *nu*/+ on day 7.
[c] $P = 0.016$ compared to *nu/nu* on day 14.
[d] $P = 0.001$ compared to *nu/nu* on day 14.
[e] ND, not done.

levels on any day during the time course (data not shown). In experiment 1, spleen cells from *nu*/+ mice did not produce significant CSA through day 1 postinfection. However, by day 3 postinfection, significant CSA levels were being secreted. Spleen cells maintained their ability to produce increased levels of CSA throughout the 30-day time course. In experiment 2, total CSA values from *nu/nu* and *nu*/+ mice were not significantly different during days 1 to 7 postinfection. Background levels were observed on day 1, with significant production occurring on day 3 for both *nu*/+ and *nu/nu* mice. The values from *nu/nu* spleen cells declined and by day 14 were significantly lower than those from *nu*/+ mice ($P = 0.016$). No other values were obtained from the *nu/nu* mice since by day 16 all the athymic mice had succumbed to the infection, but CSA values from *nu*/+ spleen cells remained elevated throughout the time course. In experiment 3, again both *nu*/+ and *nu/nu* mice produced high levels of CSA, with *nu/nu* mice having significantly higher values on day 7 ($P < 0.01$). However, on day 14, values in *nu*/+ mice were significantly higher than in *nu/nu* mice ($P < 0.001$). The differences between maximum CSA values in experiments 2 and 3 indicate that there is some experiment-to-experiment variation in production. To determine the requirement for live organisms for spleen cell production, mice were challenged with live or heat-killed *Chlamydia* organisms, and spleen supernatants were harvested at days 7 and 14 postinfection. Like the serum, spleen cells from *nu*/+ mice challenged with either live or heat-killed organisms produced CSA on day 7 (262 ± 73 U of CSA per ml in mice challenged with live organisms versus 270 ± 147 U of CSA per ml in mice challenged with heat-killed organisms). Yet by day 14, *nu*/+ mice challenged with live organisms maintained the capacity to produce CSA (380 ± 74 U of CSA per ml in *nu*/+ mice challenged with live organisms versus 43 ± 17 U of CSA per ml in *nu*/+ mice challenged with heat-killed organisms, $P < 0.001$). Additionally, *nu*/+ mice produced significantly more CSA than *nu/nu* mice challenged with either live or heat-killed *Chlamydia* organisms (380 ± 74 U of CSA per ml in *nu*/+ mice challenged with live organisms versus 83 ± 20 U of CSA per ml in *nu/nu* mice challenged with live organisms [$P < 0.001$] or versus 110 ± 67 U of CSA per ml in *nu/nu* mice challenged with heat-killed organisms [$P < 0.005$]).

Effect of T-cell depletion of CSA production by *nu*/+ spleen cells. The capacity of *nu*/+ mice to maintain CSA production on day 14 indicated that an intact T-cell compartment may be required for some of the CSA production at that time.

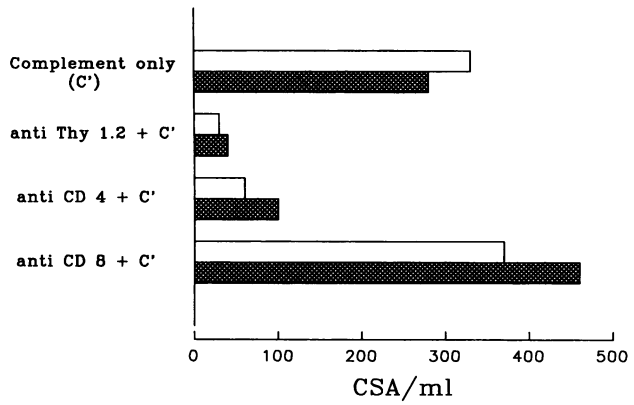

FIG. 3. The effect of T-cell or T-cell-subset depletion on supernatant production of CSA by *nu*/+ spleen cells 14 days postinfection. The results of two separate experiments with 5 mice per group are shown.

Therefore, we performed experiments in which spleen cells from infected *nu*/+ mice were placed on day 14 into culture with MoPn antigen after depletion of T cells or T-cell subsets (Fig. 3). In two separate experiments, total CSF secretion by spleen cells was 280 to 330 U/ml on this day. Treatment of spleen cells with anti-Thy 1.2 dramatically reduced the CSF secretion by immune spleen cells to 30 to 40 U/ml. Removal of $CD4^+$, or helper T cells, also reduced the production of CSFs to 60 to 100 U/ml. In contrast, anti-CD8 treatment did not reduce the secretion of CSFs with concentrations of 370 to 460 U/ml. These studies show that in vitro CSA production by *nu*/+ mice is strongly $CD4^+$ on day 14.

Production of IL-3 and GM-CSF by *nu*/+ and *nu/nu* spleen cells. We next performed experiments using the growth-factor-dependent cell line FDCP-1 to confirm the presence of IL-3 and GM-CSF. Spleen cell supernatants were produced from *nu*/+ and *nu/nu* mice 7 and 14 days postinfection and assayed (Fig. 4). Supernatants from *nu*/+ mice supported significant growth of cells at 1:160 and 1:80 dilutions on days 7 and 14, respectively. Supernatants from *nu/nu* mice supported significant growth only at a 1:20 dilution on both days. Therefore the *nu*/+ mice produce four- to eightfold more GM-CSF and/or IL-3. Thus, *nu*/+ mice are producing different types of CSFs from the *nu/nu* mice. Since GM-CSF and IL-3 are produced by T cells, these results suggest that there may be a T-cell dependence of CSA production in *nu*/+ mice.

DISCUSSION

In this article, we have shown for the first time that CSFs are produced in both *nu*/+ and *nu/nu* mice in vivo and in vitro in response to MoPn challenge. Early (day 7) responses, both in vivo and in vitro, could be induced with either live or heat-killed *Chlamydia* organisms. This is not surprising, since the capacity of killed organisms or subcellular bacterial fractions to induce changes in CSF production or other hematological responses has been documented with other models (4, 5, 22). These early host responses, which we have shown in vitro with both *nu*/+ and *nu/nu* mice, are most likely independent of T-cell control and part of the natural host defense mechanisms. Yet, high late (day 14) responses were sustained only by live organisms in *nu*/+ mice, indicating that induction of specific immunity may play a role in this phase of the response. This was confirmed by demonstrating that the in vitro CSF production on day 14 is strongly T-cell dependent, with the $CD4^+$ cells being the most important subset.

The facts that significant amounts of CSFs are produced in vivo and in vitro by T-cell-deficient *nu/nu* mice and that $CD4^+$ dependent CSFs are produced in vitro on day 14 in *nu*/+ mice suggest that more than a single type of CSF is produced in response to MoPn. This was confirmed by using the factor-dependent cell line FDCP-1. Supernatants from *nu*/+ mice support the growth of FDCP-1 cells four- to eightfold better than the *nu/nu* supernatants on both day 7 and day 14. The FDCP-1 cell line responds to both GM-CSF

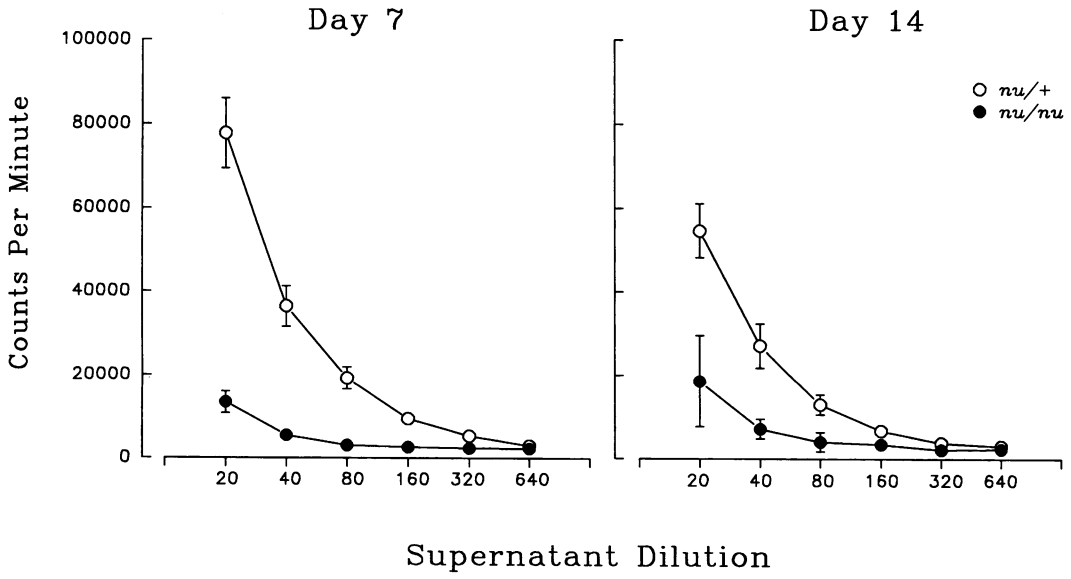

FIG. 4. Comparison of the capacity of supernatants from *nu*/+ and *nu/nu* mice on days 7 and 14 postinfection to support the growth of FDCP-1 cells. There were 3 to 5 mice per group.

and IL-3. IL-3 is produced exclusively by T cells, whereas GM-CSF is produced by T cells, macrophages, fibroblasts, and endothelial cells (9, 10). These other cellular sources of GM-CSF may contribute to the early production of CSF levels in both *nu/+* and *nu/nu* mice. Thus, the small amount of FDCP-1 activity in the *nu/nu* supernatants may result from the production of GM-CSF by these alternative sources. Although increased total supernatant CSA in *nu/+* versus *nu/nu* mice is apparent only on day 14 (Table 1), the lack of obvious T-cell dependence in total CSA on day 7 may suggest a compensatory production of T-cell-independent CSA by *nu/nu* mouse spleens at that time. This concept may be supported by the significantly higher CSA levels in *nu/nu* mice on day 7 in experiment 3 (Table 1), which clearly represent high production of a non-T-cell-dependent factor in these mice. It is tempting to speculate that granulocyte CSF (a non-T-cell-dependent cytokine) is produced during infection by both *nu/+* and *nu/nu* mice and may be the dominant CSF in the serum early in infection. Subsequently, GM-CSF and/or IL-3 are produced by primarily T-cell-dependent mechanisms in *nu/+* mice by spleen cells. Further studies with our model will be needed to characterize the types of CSFs produced at different times in the various host compartments (plasma, spleen, and lung).

Our earlier published data on the histology of the inflammatory response in *nu/+* and *nu/nu* mouse lungs suggests that cytokine production in the lung may mimic that observed in the spleen in these studies (3). The early pulmonary cellular response to infection in both types of mice is primarily the granulocyte. Although the *Chlamydia* organisms were found primarily in the type 1 epithelial cells, granulocytes containing intracellular organisms were seen by 10 h postinfection in both *nu/nu* and *nu/+* mice. By day 14, the infiltrate in the *nu/+* mouse lung changed to predominantly mononuclear cells, yet in the *nu/nu* mouse, the infiltrate was still primarily granulocytes. Challenge of *Chlamydia*-immune *nu/+* mice showed a different pattern of cellular reaction. The primary responding cell was the macrophage, and by 24 h, no organisms could be detected by electron microscopy. Thus, the in vivo difference in the phagocyte response between *nu/+* and *nu/nu* mice suggest that different CSFs are being produced during infection in the lung and in the spleen.

T cells may have multiple roles in the anti-*Chlamydia* response. It has been found that the *nu/nu* mouse is more susceptible than the *nu/+* mouse (17, 18). Passive transfer studies have shown that T cells were the primary cells responsible for the transfer of protection. Furthermore, the $CD4^+$ subset was the major contributor to protection (15). One proposed mechanism for the role of $CD4^+$ T cells could be the production of gamma interferon (IFN-γ). Previous studies from our and other laboratories indicate that removing IFN-γ in vivo by injecting a monoclonal anti-IFN-γ antibody renders mice more susceptible to chlamydial infection (14, 24). Furthermore, by using another *Chlamydia* serovar, others have shown that treatment of mice with recombinant murine IFN-γ reduces the number of organisms in the lungs, livers, and spleens (23). Additionally, we and others have shown that IFN-γ can inhibit the intracellular replication of chlamydiae in vitro (1, 12). Thus, IFN-γ is clearly one important cytokine in the immune response to MoPn.

Other cytokines have been shown to participate in host defenses. Recently, we have shown that T-cell clones against *Listeria monocytogenes* produce GM-CSF in addition to IFN-γ (7, 8). Supernatants from these clones were able to protect mice against lethal *Listeria* infection. By removing GM-CSF from these supernatants, protection was significantly diminished. Furthermore, immunotherapy with recombinant murine GM-CSF provided protection against *Listeria monocytogenes*.

Thus, host defenses against infectious challenge are clearly multifactorial. We believe that ultimate protective immunity against *Chlamydia* organisms results from the stimulation of T cells. The production of IFN-γ is probably one important mediator of cellular immunity. The studies reported here show that the largely T-cell-derived CSFs (IL-3 and/or GM-CSF) are also produced in response to *Chlamydia* infection. The role of these factors in host resistance remains to be defined.

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