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Lymphokine-Mediated Inhibition of *Chlamydia* Replication in Mouse Fibroblasts Is Neutralized by Anti-Gamma Interferon Immunoglobulin

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Experiments were carried out to characterize immunologically mediated chlamydial persistence in cell culture. Mouse fibroblasts were activated to restrict *Chlamydia psittaci* 6BC replication by including mitogen (concanavalin A)-induced spleen cell supernatant fluids from immunized animals in the growth medium. When mouse fibroblasts were incubated with lymphokine for 24 h before infection and then with growth medium after infection (preinfection treatment), chlamydial replication was delayed but eventually detected. No substantial chlamydial growth occurred, even with extended incubation times when mouse fibroblasts were continuously exposed to lymphokine before and after infection. Low levels of infectious chlamydiae were produced in preinfection-treated mouse fibroblasts but not in mouse fibroblasts subjected to continuous lymphokine exposure. Incubation of lymphokine with anti-murine gamma interferon immunoglobulin neutralized the observed lymphokine-mediated activity, but incubation in the presence of anti-murine alpha plus beta interferon serum did not alter lymphokine activity. We conclude that the lymphokine components responsible for activating fibroblasts to restrict *C. psittaci* replication exhibits properties similar to gamma interferon.

The genus *Chlamydia* comprises two species of obligate intracellular procaryotic pathogens that replicate exclusively within membrane-bound cytoplasmic vesicles of susceptible eucaryotic host cells (18). *Chlamydia* strains are widely distributed in nature, and although overt clinical infections are common in both humans (principally, *Chlamydia trachomatis* and less frequently *Chlamydia psittaci*) and animals (*C. psittaci*), subclinical, occasionally lifelong infections are even more frequent (5, 15, 18). Persistent chlamydial infections contribute to the transmission of these pathogens, and the potential for subclinical infection to progress to symptomatic disease places the infected individual at continual risk.

In areas of the world where trachoma remains prevalent, chronic infections maintain *C. trachomatis* reservoirs in endemic communities (19). Sexual transmission of *C. trachomatis* is prevalent worldwide. Females with apparent or inapparent cervical infections may be predisposed to canalicular ascending complications, leading to pelvic inflammatory disease. In addition, neonatal infection (conjunctivitis, pneumonitis, and possibly enteritis) acquired at parturition can result from overtly or subclinically infected women (15). Persistent *C. psittaci* infections of exotic birds and occasionally domestic flocks continues to represent a reservoir for human pulmonary and systemic disease (20), and persistent *C. psittaci* infection of bovine and ovine species maintains a high prevalence in herds and has resulted in significant morbidity and mortality for newborn calves and sheep (5).

Epidemiological investigations (7, 10, 11) have provided information concerning the prevalence of persistent, inapparent chlamydial infections and their tendency to recrudesce under a variety of stressful and traumatic situations and in the immunocompromised, yet little is known concerning factors that contribute to chlamydial persistence or the equilibria that exist between chlamydiae and their hosts that may influence whether infections will manifest as clinical disease or inapparent persistence.

Several cell culture systems have been reported to result in chlamydial persistence. Morgan and Bader (12) and more recently Hatch (8) and Pearce and Allen (16) found that competition between host cells and chlamydiae for essential nutrients, especially amino acids, resulted in intracellular chlamydial persistence that was reversed when the depleted amino acids were restored. Lee and Moulder (9) and Moulder and co-workers (14) provided data to support the

hypothesis that *C. psittaci* and *C. trachomatis* latency resulted from an acquired structural or metabolic change or both in the chlamydial cell. The reported reorganization of chlamydiae to a so-called cryptic form may occur in response to changes in the host cell, but the alteration of form is most certainly a chlamydial-specified process. Nutritional considerations were also reported (13) to play a role in the maintenance of latently (cryptic chlamydial forms) infected cells and in the reactivation of these cells to exhibit overt chlamydial replication. It has been shown previously (1–3) that chlamydial latency may also occur as a result of the host response to infection. Lymphokines (LKs) produced by spleen cells taken from *C. psittaci*-inoculated mice activated macrophages in cell culture to inhibit the intracellular replication of *C. psittaci*. In this report, we extend those findings to show that LK-mediated chlamydial latency induced in fibroblasts apparently requires stimulation of spleen cells from previously sensitized animals, and the material secreted by these cells is similar in several respects to gamma interferon (IFN-γ).

MATERIALS AND METHODS

Fibroblast and *C. psittaci* growth. Mouse fibroblasts (L cells, clone 929) were propagated as monolayers in medium 199 (K.D. Biologicals, Kansas City, Mo.) supplemented with 1.2% sodium bicarbonate, 10% (vol/vol) heat-inactivated fetal bovine serum (K.C. Biologicals), and 100 μg of streptomycin sulfate and 10 μg of gentamicin sulfate (standard medium) per ml. Confluent monolayers were infected with 10 times the 50% tissue culture infective dose ($TCID_{50}$) of *C. psittaci* 6BC. The inoculum was adsorbed on a rotary shaker for 60 min and aspirated, and the infected cells were incubated for 2 days at 37°C in standard medium containing 2 μg of cycloheximide per ml in a 5% CO_2 in air atmosphere. After incubation, infected cells were trypsinized, sonicated, pooled with the collected growth medium, and centrifuged at 500 × *g* for 10 min to collect cell debris and then for 30 min at 10,000 × *g* to collect chlamydiae. The pellet was suspended in phosphate-buffered saline containing 0.2 M sucrose and 2% heat-inactivated fetal bovine serum (chlamydial buffer). The number of viable elementary bodies was quantitated by the $TCID_{50}$ method (8).

Preparation of LKs. LKs were induced in concanavalin A (ConA)-incubated spleen cell suspensions from A/J or LAF mice after the animals had been inoculated subcutaneously with 2×10^2 to 5×10^2 viable *C. psittaci*. Mock LKs were produced by incubating spleen cells from normal (uninoculated) mice in the presence of ConA. Spleens were removed 10 days after subcutaneous inoculation. Typically, 6 ml of splenocytes per 100-mm-diameter culture dish at a density of 5×10^6 cells per ml was incubated for 24 h in standard medium containing 5 μg of ConA per ml.

Fibroblast activation and measurement of chlamydial replication. L cells were plated onto Lab-Tek slides (5×10^4 to 1×10^5 cells per well), allowed to adhere and spread at 37°C, and then treated with standard medium containing 10% (vol/vol) LK or mock LK supernatant fluids. Treated cells were incubated for 18 h to 24 h. The medium was aspirated, and the cells were infected with a *C. psittaci* inoculum adjusted to maximally infect ≤50% of the host cells. The inoculum was adsorbed for 60 min on a rotary shaker (37°C) and then aspirated. Standard medium with (continuous exposure) or without (preinfection treatment) 10% LK or mock LK was added, and the cells were returned to the incubator. Evidence for chlamydial growth was determined by staining fixed cells with Giemsa and examining them by light microscopy for inclusion development.

Alternatively, evidence for chlamydial growth was obtained by measuring the amount of acid-insoluble radioactivity incorporated into cycloheximide-treated cells incubated in the presence of ^3H-amino acids. For these experiments, 2×10^6 L cells were plated onto 60-mm-diameter cell culture dishes. The cells were LK treated or mock treated as before and then infected with two to five times the $TCID_{50}$ of *C. psittaci*. Infected cells were incubated for 16 to 18 h and then treated with 2 μg of cycloheximide per ml and 30 min later with 2 μCi of ^3H-amino acids (specific activity, 51.6 Ci/mM; New England Nuclear Corp., Boston, Mass.). Cells were incubated for an additional 4 to 6 h, washed extensively, trypsinized, pelleted, suspended in cold 10% trichloracetic acid, and boiled for 10 min, and acid-insoluble counts were collected onto filters. Dried filters were counted in a toluene-based scintillant with a Searle liquid scintillation spectrometer. Addition of cycloheximide inhibited greater than 95% of host cell incorporation without affecting chlamydial development or LK-mediated activity when added just before the addition of ^3H-amino acids. Appropriate uninfected controls were included in each experiment.

Neutralization of LK activity. LK preparations were incubated for 60 min at 37°C in the presence of excess (225 U/ml) rabbit anti-murine gamma interferon (anti-Mu IFN-γ) immunoglobulin or rabbit anti-murine alpha plus beta interferon (anti-Mu IFN-α+β) serum (126 U/ml); each preparation was purchased from Enzo Biochem Inc., New York, N.Y. One neutralizing unit was defined as the reciprocal of the highest antiserum dilution that neutralized 10 U of Mu IFN. After treatment, preparations were added to L cell monolayers, incubated overnight, and then infected and treated as described above to evaluate LK-mediated activity. Appropriate antiserum and LK controls were included in each experiment.

Photomicroscopy. Photomicrographs were obtained for fixed and stained monolayers with a Nikon FX 35 camera, an AFX exposure meter, and an Optiphot phase-contrast bright-field microscope.

RESULTS

***C. psittaci* inhibition in LK-treated mouse fibroblasts.** Data presented in Table 1 show that when L cells were incubated for 24 h in medium containing 10% (vol/vol) LK and then infected with less than 1 $TCID_{50}$ of *C. psittaci*, the fraction of inclusion-bearing cells observed 18 to 22 h after infection was significantly ($P < 0.001$) reduced when compared to either cells incubated in standard medium or cells incubated in

TABLE 1. Effect of LK treatment on *C. psittaci* inclusion development in L cells

Samples	% Inclusion-containing cells[a] ± SD	% Of control	Significance[b]
Control (standard media)	27.3 ± 1.66	100	
10% Mock (preinfection treatment)	29.0 ± 1.50	106	$P > 0.20$
10% Mock (continuous exposure)	27.1 ± 2.76	99	$P > 0.20$
10% LK (preinfection treatment)	7.5 ± 1.04	27	$P < 0.001$
10% LK (continuous exposure)	9.4 ± 0.85	34	$P < 0.001$

[a] Mean of 17 to 53 individual determinations.
[b] Significant differences ($P < 0.05$) were calculated by the two-tailed Student *t* test.

medium containing mock LK supernatant fluid. A similar extent of inclusion reduction was observed for L cells exposed to LK for only a 24-h period before infection (preinfection treatment) and for L cells exposed to LK before and subsequent to infection (continuous exposure).

We consistently observed that those inclusions present in LK-treated L cells were much smaller than inclusions present in untreated or mock-treated cells, and therefore the degree of inhibition as measured by inclusion development actually represented a minimal approximation of the real reduction in chlamydial growth. This hypothesis was tested by measuring the amount of chlamydial protein synthesis in LK-treated L cells during a 4 to 6-h pulse of ^{3}H-amino acids. The incorporation of labeled precursor specifically into chlamydial protein was facilitated by adding cycloheximide to the infected cells 30 min before the addition of radiolabeled amino acids. Addition of cycloheximide at this point reduced host cell protein synthesis by greater than 95% without affecting chlamydial growth or LK-induced activity. The increase in counts per minute shown in Fig. 1 for infected versus uninfected L cells represents chlamydiae-specific protein synthesis as reflected by labeled amino acid incorporation into the acid-insoluble fraction. The amount of radioactivity was reduced essentially to background levels when L cells were treated with LK for 24 h before infection. Therefore, the small inclusions observed in a low percentage of LK-activated cells did not synthesize protein to an extent that was measurable in this assay, and the greater than 90% reduction in biosynthesis probably more accurately reflects the profound inhibition

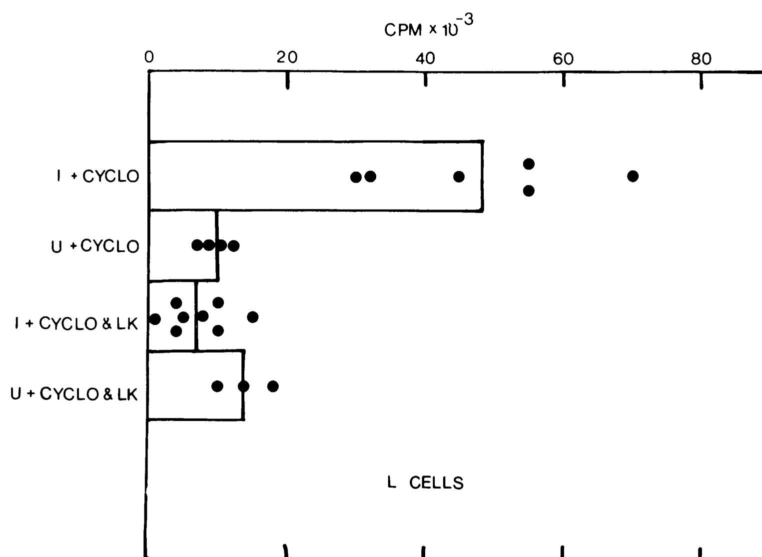

FIG. 1. Effect of LK preinfection treatment of *C. psittaci* protein synthesis. L cells were incubated in standard medium or in medium containing 10% (vol/vol) LK for 24 h before infection with 5 $TCID_{50}$ of *C. psittaci*. Sixteen hours after infection, 1 μg of cycloheximide was added per ml, and 30 min later 2 μCi of ^{3}H-amino acids was added per ml. Cells were incubated for 4 h, and then acid-insoluble radioactivity (cpm) was measured. Cycloheximide (CYCLO) reduced L cell protein synthesis by greater than 95% (not shown). Results with infected (I) and uninfected (U) cells treated with or without LK are presented. Counts per minute of infected cells in the presence of cycloheximide (I + CYCLO) represent *C. psittaci*-specific protein synthesis. Each point represents an individual determination. Bars represent mean values.

of chlamydial development that occurs in LK-activated fibroblasts.

Maintenance of persistence in LK-activated L cells. When preinfection-treated or continuously exposed cells were harvested and tested for the presence of infectious chlamydiae, a low but measurable number of infectious elementary bodies (EDs) were recovered from preinfection-treated cells that were harvested 44 h after infection (Table 2). Negligible numbers of infectious EBs were recovered from continuously exposed cells. Thus, despite the appearance of equivalent percentages of inclusion-containing cells in preinfection-treated samples, maturation of the intracellular chlamydiae to the infectious form of the organism was dramatically reduced when compared to controls. The low number of EBs recovered from preinfection-treated cells was sufficient to cause progression of the infection, as evidenced by the data shown in Table 3. For these experiments, L cells were initially infected with a very low multiplicity of infection of *C. psittaci*. The experiment was designed so that more than one cycle of intracellular development occurred, and at least two cycles were required to cause a 100% infection. A nearly 100% infection was observed by 3 days in mock-treated samples, and preinfection-treated cells were nearly completely infected by 5 days after inocula addition. The enhanced chlamydial growth observed after mock LK treatment was a consistent finding. Similar results occurred when cycloheximide, and inhibitor of eucaryotic protein synthesis, was added to *C. psittaci*-infected L cells. Thus, although mock LK preparations were devoid of activity that resulted in the restriction of chlamydial replication, a decrease in host cell macromolecular synthesis as a result of mock LK treatment might have promoted chlamydial growth. Alternatively, since mitogen (ConA) was present in the mock LK preparations, enhanced growth could have resulted from some undefined mitogenic effect. In contrast, continuously exposed cells had a very low percentage of inclusion-containing cells throughout the course of the experiment. Thus, once preinfection-treated L cells began supporting chlamydial growth, complete maturation to EB, occurred, and the entire host cell population was susceptible to infection. These data also corroborate a previous observation (1–3) that uptake of *C. psittaci* is unaffected in LK-activated cells. Continuously exposed cells, however, never released infectious EBs, and the cell population remained protected from spread of the infection even under conditions of extended incubation times. The photomicrographs shown in Fig. 2 illustrate the progression of infection in mock-treated, preinfection-treated, and continuously exposed cells at different time points after addition of the *C. psittaci* inocula. Note the lag in inclusion development in all LK-treated samples when compared to controls and the maintenance of persistence in continuously exposed L cells.

Neutralization of LK-mediated host cell activation. It has been shown previously (1–3) that anti-murine immunoglobulin G (IgG) or anti-murine IgM sera did not neutralize LK activity. LK-containing supernatant fluids were incubated for 60 min in the presence of excess rabbit anti-Mu IFN-γ immunoglobulin and then added to L cells. When these cells were infected 24 h later, there was nearly a complete ablation of LK-mediated activity (Table 4). No neutralization of LK activity was observed when supernatant fluids were incubated in the presence of excess rabbit anti-Mu IFN-α+β serum. Some L cell toxicity was seen in preparations treated with anti-Mu IFN-α+β serum. This probably was a major contributing factor to the 39% inhibition ($P < 0.05$) of inclusion-bearing cells observed for the anti-Mu IFN-α+β serum control and the partial neutralization observed when anti-Mu IFN-γ immunoglobulin and anti-Mu IFN-α+β serum were mixed and added to LK. A dosage effect (only half the amount of anti-Mu IFN-γ immunoglobulin was present) no doubt also contributed to the partial neutralization of LK-mediated activity. The anti-Mu IFN-α+β

TABLE 2. Effect of LK treatment on recovery of infectious chlamydiae in L cells

Sample (growth media)	No. of infectious chlamydiae recovered:[a]		% Of control	Significance[b]
	Per ml ± SD	Per host cell		
Standard media (control)	$2.63 \pm 0.57 \times 10^7$	25.2	100	
Mock treated (10%, vol/vol)				
Preinfection	$3.14 \pm 0.33 \times 10^7$	30.1	119	$P > 0.1$
Continuous exposure	$3.60 \pm 1.19 \times 10^7$	34.6	137	$P > 0.2$
LK treated (10%, vol/vol)				
Preinfection	$7.8 \pm 1.6 \times 10^5$	0.75	3	$P < 0.001$
Continuous exposure	$5.2 \pm 2.3 \times 10^4$	0.05	0.2	$P < 0.001$

[a] Chlamydia were harvested 44 h after infection of 1.04×10^6 L cells in replicate cultures. Calculations were made after infecting 10^5 L cells with serial dilutions of individual harvests.

[b] *P* values were calculated by the two-tailed Student *t* test.

TABLE 3. Effect of preinfection treatment and continuous LK exposure on the replication of *C. psittaci* in L cells initially infected with a low multiplicity of infection

Time after infection (h)	% Inclusion-containing cells ± SD			
	Standard medium (control)	10% Mock LK-treated (continuous exposure)	10% LK treated	
			Preinfection	Continuous exposure
6[a]	0	0	0	0
23	1.4 ± 1.2	1.2 ± 0.8	0.3 ± 0.3	0.2 ± 0.4
46	4.3 ± 2.5	5.5 ± 3.1	1.6 ± 0.2	0.5 ± 0.1
76	17.6 ± 10.4	67.4 ± 11.1	22.0 ± 3.7	2.4 ± 1.2
100	62.9 ± 23.2	96.6 ± 3.1	62.1 ± 10.7	1.9 ± 2.7

[a] Inclusions do not become microscopically observable microcolonies until approximately 14 to 18 h after infection.

serum toxicity limited the amount of antiserum used in these experiments. The results reported represent the maximum antiserum concentrations possible under the given conditions. In addition to the observed neutralization of LK-mediated activity in the presence of anti-Mu IFN-γ immunoglobulin, when LK-containing preparations were tested for the presence of antiviral IFN activity, 1,800 U/ml was found. Less than 10 U/ml was found in mock LK preparations (data not shown). Thus, the LK-mediated induction of chlamydial microbistatic activity appears to be related to the presence of IFN-γ in spleen cell supernatants from animals inoculated with *C. psittaci*.

DISCUSSION

It has been shown that factors produced by spleen cells from *C. psittaci*-inoculated mice

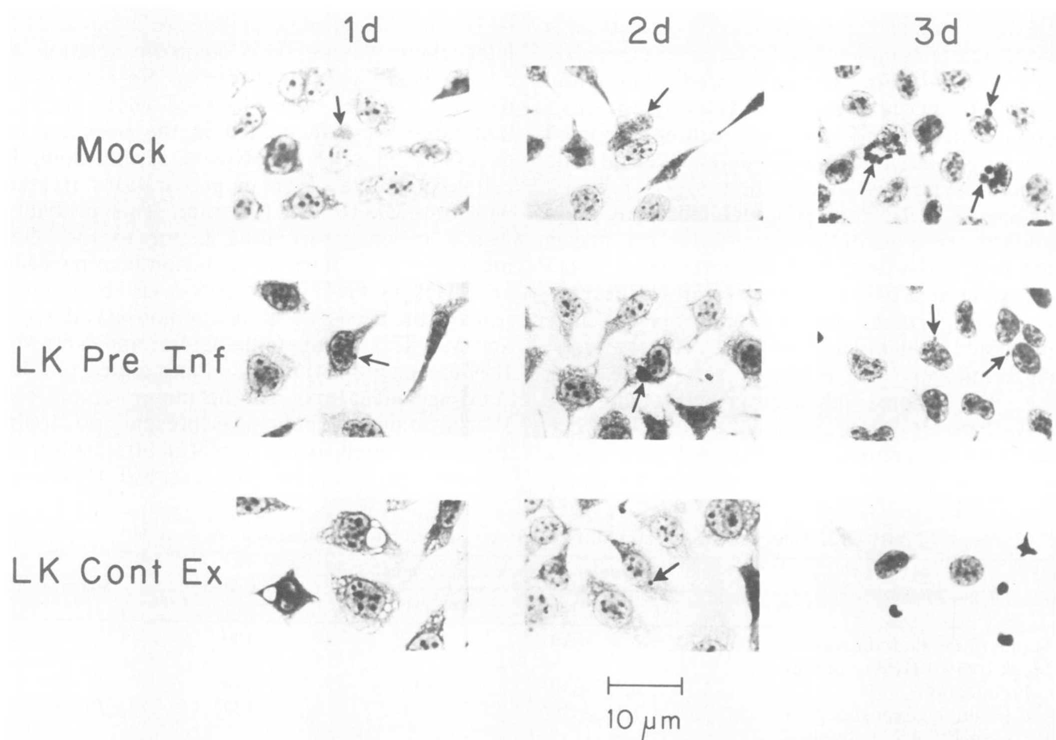

FIG. 2. Photomicrographs of *C. psittaci* development in L cells after mock LK treatment (Mock), preinfection LK treatment (LK Pre Inf), or continuous LK exposure (LK Cont Ex) after 1, 2, or 3 days (d) of infection. All micrographs were taken at a magnification of ×1,000. The bar represents 10 μm, and arrows point to chlamydial inclusions (intracellular microcolonies). Note paucity of inclusions in cells continuously exposed to LK at all time points and the size differences for early time points in all LK-treated samples.

TABLE 4. Effect of rabbit anti-Mu IFN-γ immunoglobulin and rabbit anti-Mu IFN-α+β serum on LK activity

Sample	% Inclusion-containing cells (mean ± SD)[a]	% Inhibition	Significance[b]
Control (standard medium)	30.1 ± 7.4	0	
Control (standard medium + anti-IFN-γ immunoglobulin)	29.4 ± 4.4	1	$P > 0.50$
Control (standard medium + anti-IFN-α+β serum)	18.0 ± 2.9	39	$P < 0.05$
LK + anti-IFN-γ immunoglobulin	24.2 ± 2.9	18	$P > 0.20$
LK + anti-IFN-α+β serum	6.8 ± 2.4	77	$P < 0.01$
LK + anti-IFN-γ immunoglobulin + anti-IFN-α+β serum	12.6 ± 2.4	57	$P < 0.02$
LK + standard medium	9.8 ± 1.9	67	$P < 0.02$

[a] L cells were incubated for 24 h as described in the text, washed, and then infected with *C. psittaci* adjusted to maximally infect 50% of the cells (30.1% were maximally infected in this experiment). All values are means of three to four separate determinations.

[b] P values were calculated by the two-tailed Student t test. Values of $P < 0.05$ are considered significantly different from the standard medium-incubated control.

activate a variety of host cells (1) to restrict intracellular chlamydial replication. The observed inhibition leads to chlamydial persistence, and therefore factors related to the immune response against chlamydial infection contribute to latency, and perhaps to inapparent chlamydial infections.

Of interest is our observation that the LK component responsible for fibroblast activation is neutralized in the presence of immunoglobulin directed against Mu IFN-γ. Other circumstantial data (3) corroborate this finding, in that the active component is stable at 56°C and labile at pH 2. In addition, these preparations have been found to contain antiviral IFN activity. It has been previously reported (4) that although *C. trachomatis* development is restricted in fibroblasts treated with exogenous IFN-α+β, *C. psittaci* 6BC replication is unaffected in these cells. Thus, if IFN-γ is responsible for activating fibroblasts to inhibit *C. psittaci* 6BC replication, then comparative studies with this chlamydial strain may lead to the discovery of distinctions between activation schemes for the various IFN classes. Rothermel et al. (17) provided data that implicate the participation of human IFN-γ in restricting *C. psittaci* replication in human monocytes. Therefore, IFN-γ may contribute to chlamydial persistence in a broader sense than described in the murine model presented here.

If the potential for including chlamydial persistence during the immune response against chlamydiae is also generally applicable, then these findings would help explain why chlamydial vaccine programs (6) have not been successful. Results of these experiments therefore draw attention to the importance of testing for persistence as well as protection and selecting antigen preparations that potentiate the latter and not the former in any future attempts at development of a vaccine against chlamydial infections.

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