Changes in Cytokine Levels during Reactivation of 
Toxoplasma gondii Infection in Lungs

GREGORY A. FILICE,¹,²* CONNIE R. CLABOTS,¹ PAUL E. RICIPUTI,¹
OSCAR GONI-LAGUARDIA,¹ AND CLAIRE POMEROY¹,²†

Infectious Disease Section, Veterans Affairs Medical Center,¹ and Infectious Disease Division, University of Minnesota,² Minneapolis, Minnesota

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We studied cytokine proteins and mRNAs in mice with two forms of Toxoplasma gondii pneumonia resulting from reactivation of infection. In the first form, mice were infected with T. gondii, developed and recovered from systemic disease, and then developed pneumonia 3 weeks later. As pulmonary inflammation developed, levels of cytokine mRNAs for gamma interferon (IFN-γ), interleukin-2 (IL-2), IL-4, and IL-10 increased in bronchoalveolar lavage (BAL) cells or lung tissue, and the level of IFN-γ protein increased in BAL fluid. The second form of pneumonia occurred as a complication of primary cytomegalovirus (CMV) disease in mice with dormant T. gondii infection. During CMV disease, IL-2 mRNA levels decreased in lung tissue, IL-10 protein levels increased in lung tissue, and IL-10 protein levels increased in BAL fluid. As the mice recovered from CMV disease, T. gondii infection was reactivated in the lungs and was manifested as T. gondii pneumonia. During CMV-induced T. gondii pneumonia, IFN-γ, IL-2, IL-4, and IL-10 mRNA levels increased in BAL cells or lung tissue, and both IFN-γ and IL-2 protein levels increased in BAL fluid. We concluded that both forms of T. gondii pneumonia are accompanied by increases in both type 1 T-helper and type 2 T-helper cytokine levels in lungs. The mechanism of CMV-induced reactivation of T. gondii infection in lungs may involve local decreases in IL-2 levels and/or increases in IL-10 levels.

Severe postnatal toxoplasmosis is often the consequence of reactivation of infection in people with compromised immunity (4). Its frequency has increased dramatically with the spread of AIDS. Although much has been learned about host defenses against primary Toxoplasma gondii disease, less is known about the pathogenesis of reactivation. Pneumonia is the second most common manifestation of toxoplasmosis in persons with AIDS (27), and pneumonia is present in some congenitally infected infants (2, 33). During local inflammatory events, lung cytokine and cellular responses are often different from systemic processes (7, 24). We studied lung cytokine responses in mice with T. gondii pneumonia due to reactivation of T. gondii infection. In order to compare local cytokine responses to systemic ones, we studied cytokine concentrations in serum and cytokine mRNA levels in spleens.

Two forms of pneumonia from the reactivation of dormant T. gondii infection have been extensively characterized (3, 13, 28–30). The first occurs approximately 3 weeks after mice recover from primary infection (29). The second occurs after mice with dormant T. gondii infection are infected with murine cytomegalovirus (CMV). CMV disease occurs during the week after CMV infection, followed by days severe T. gondii pneumonia (29). T. gondii disease and CMV disease are each immunosuppressive in mice and humans, and these pathogens frequently coexist in lesions of human disease (29). Immunosuppression by either T. gondii or CMV disease may sufficiently depress host defenses to allow reactivation of latent infection with the other pathogen, especially in a person in whom host defenses are already suppressed.

We have previously reported that both forms of T. gondii reactivation pneumonia are preceded by a decrease in lung CD4⁺ lymphocyte levels and that pneumonia is characterized by large increases in lung CD8⁺ and CD4⁺ lymphocyte levels (28, 30). When the severity of CMV disease is diminished with antiviral therapy, T-lymphocyte changes are attenuated and T. gondii reactivation is prevented (3, 13).

In both mice and humans, the type 1 T-helper (Th1) subset of CD4⁺ lymphocytes is associated with strong cell-mediated immunity and control of infectious diseases, while the Th2 subset is associated with strong antibody responses, especially immunoglobulin A and immunoglobulin E, allergic reactions, and diminished cell-mediated immunity (1). Th1 lymphocytes secrete primarily gamma interferon (IFN-γ), interleukin-2 (IL-2), and tumor necrosis factor; Th2 lymphocytes secrete primarily IL-4, IL-5, IL-6, and IL-13 (1). Analogous CD8⁺ lymphocyte subsets, termed Tc1 (for type 1 cytotoxic) and Tc2, have similar cytokine patterns (26, 34). Based on these characteristics and our previous work, we hypothesized that reactivation of T. gondii infection in lungs results from a decrease in Th1 cytokine levels or a transient increase in Th2 cytokine levels and that control of T. gondii reactivation is due to cells secreting Th1 cytokines. We were especially interested in the role of IFN-γ, which is central to the control of this pathogen in both acute and chronic infections (10, 11, 38, 39, 41).

MATERIALS AND METHODS

Mice, T. gondii, and CMV infection. Specific-pathogen-free BALB/cAnN mice (Harlan, Indianapolis, Ind.) were maintained in American Association for Accreditation of Laboratory Animal Care-accredited facilities as previously described (29). At 6 weeks of age, they were inoculated intraperitoneally with 3 × 10⁵ T. gondii C56 tachyzoites (29). From days 2 to 21 after infection, sulfadiazine (400 mg/liter) was added to their drinking water to keep the mice from dying during primary disease. As previously described (29), mice developed systemic T. gondii disease and recovered during the first 10 days.

After recovery, mice appear well until 5 weeks after infection, when they develop toxoplasma pneumonia (termed primary reactivation pneumonia). These mice...
do not have detectable disease elsewhere in their bodies. Most mice recover without treatment, and survivors remain well for months.

The Smith strain of murine CMV was maintained by salivary gland passage in BALB/c mice (29). *T. gondii*-infected mice and controls were infected intraperitoneally with a sublethal quantity of CMV ($\leq 2 \times 10^4$ PFU). This inoculum of CMV is slightly smaller than the amount that would kill BALB/c mice with dormant *T. gondii* infection and two- to threefold larger than lethal doses for uninfected BALB/c mice (29). We have demonstrated that mice that have dormant *T. gondii* infection and are then inoculated with CMV have a systemic illness lasting 7 to 10 days with no evidence of pneumonia (29). However, 14 days after CMV inoculation, mice reproducibly develop tachypnea and cyanosis and are found to have *T. gondii* pneumonia without evidence of *T. gondii* disease elsewhere (termed CMV-induced pneumonia [29]).

**Cell, fluid, and tissue collection.** Serum was prepared from blood samples obtained by orbital plexus puncture. Bronchoalveolar lavage (BAL) was performed as previously described (29). For each mouse, the inferior vena cava was severed to drain the lungs of blood. Ten 1-ml volumes of phosphate-buffered saline (PBS) with 0.6 mM EDTA (pH 7.2) (PBS-EDTA) was used for lavage of the bronchi. Cell counts were obtained as previously described (30) and used to estimate the amount of RNA in samples. The supernatant from the first milliliter of fluid was collected for measurements of cytokine concentrations in order to minimize dilution with PBS-EDTA. Lung tissue was obtained after BAL to represent cells and tissues not easily removed by lavage. BAL pellets and spleen and lung tissues were snap frozen in liquid nitrogen in RNase-free microcentrifuge tubes.

**RNA extraction, cDNA, and PCR.** RNA was extracted with phenol and chloroform after inactivation and dissolution of proteins with guanidinium thiocyanate (6). Briefly, frozen tissue was homogenized in lysis solution (3.2 M guanidinium thiocyanate, 20 mM sodium citrate, 100 mM sodium acetate, 0.05 M 2-mercaptoethanol, 0.4% Sarkosyl). RNA was extracted in a solution containing phenol-chloroform-isooamyl alcohol (25:25:1), precipitated in isopropanol, washed with 75% ethanol, and quantified by optical density measurements.

RNA was converted to cDNA with Moloney murine leukemia virus reverse transcriptase (32). Briefly, 0.05 mg of random hexamer primers (Promega, Madison, Wis.) per ml, 1,000 U of RNasin (Promega) per ml, and 4 μg of RNA were heated for 5 min at 65°C (32). Then, 10^6 U of Moloney murine leukemia virus reverse transcriptase (Gibco/BRL, Grand Island, N.Y.) per ml was added, and the solution was incubated at 37°C for 1.5 h. The reaction was stopped with heat (95°C, 5 min), and the samples were diluted fivefold.

PCR was performed as previously described (18). Briefly, the basic reaction mixture contained 1 to 5% cDNA transcribed from 4 μg of total RNA, 10 mM Tris (pH 8.3), 1.5 mM MgCl$_2$, 50 mM KCl, 0.1% Triton X-100 (Fisher Biotech, Itasca, Ill.), 0.2 M deoxynucleoside triphosphates, 0.4 μM each sense and antisense primers (18), and 0.625 U of *Bacillus thuringiensis* DNA polymerase (Fisher Biotech). The reaction was carried through 32 cycles, each consisting of 93°C for 40 s, 55°C for 20 s, and 72°C for 40 s; the samples then were incubated at 72°C for 10 min for complete extension.

PCR products were separated by polyacrylamide gel electrophoresis, stained with ethidium bromide, and quantitated by densitometry (UV Max Technologies, Inc., Fremont, Calif.) by comparison to standards run in parallel. In each experiment, serial dilutions of a plasmid containing cytokine DNA sequences of interest, each containing a spacer, were run in parallel for quantitation (32). Quantitation was confirmed for selected samples by amplification in a compet-
Systemic

T. gondii disease

Primary reactivation pneumonia

CMV disease

CMV-induced pneumonia

![Graph showing IFN-γ concentrations in BAL and serum](Image)

**FIG. 2.** IFN-γ concentrations in BAL and serum expressed logarithmically. Mice were inoculated with *T. gondii* on day 0 and with CMV on day 99. Error bars represent standard deviations. Data are representative of three separate experiments.

Both the amounts of IFN-γ mRNA or protein and the relative increases in the amounts of IFN-γ mRNA or protein were greater than those for other cytokines. There was a gradual, small increase in serum IFN-γ levels (Fig. 2).

There was a severalfold increase in the amount of IL-2 mRNA in BAL cell samples during primary reactivation pneumonia, an earlier, more prolonged increase in lung samples, and an increase in spleen samples (Fig. 3). The amount of IL-2 mRNA was the lowest of the four cytokines that were studied, but the results were reproducible. IL-2 protein concentrations in BAL fluid or serum samples obtained during primary reactivation pneumonia were inconsistent (data not shown).

There were substantial increases in the amounts of IL-4 mRNA in BAL cell and lung samples during primary reactivation pneumonia (Fig. 4). The concentrations of IL-4 mRNA in spleen samples were mostly below concentrations that could be reliably measured, and these data are not presented. IL-4 protein concentrations in BAL fluid or serum samples were inconsistent during primary reactivation pneumonia (data not shown).

There was a striking increase in the amounts of IL-10 mRNA in BAL cell and lung samples during primary reactivation pneumonia (Fig. 5). The amount of IL-10 mRNA increased slightly in spleen samples in the experiment shown in Fig. 5, and there were greater increases in the other two experiments. Concentrations of IL-10 protein were between 20 and 100 pg/ml and did not change consistently in BAL fluid or serum samples obtained during primary reactivation pneumonia (data not shown).

**CMV disease.** For IFN-γ, IL-2, and IL-10, there were increases in cytokine proteins in serum and/or in cytokine mRNA in spleens during CMV disease. IFN-γ protein concentrations increased in serum (Fig. 2), and concentrations of IFN-γ mRNA increased substantially in spleens (Fig. 1). There were no substantial changes for IFN-γ in lungs.

**RESULTS**

**Primary reactivation pneumonia.** Expressed as a proportion of total RNA, the amount of IFN-γ mRNA increased substantially in BAL cell, lung, and spleen samples during primary reactivation pneumonia (Fig. 1). The amount of IFN-γ protein increased substantially in BAL fluid during the peak of pulmonary infiltration, 40 days after *T. gondii* inoculation (Fig. 2).
By 5 days after CMV inoculation, IL-2 protein concentrations in serum increased an average of sixfold (from 12 to 84 pg/ml; \(P, 0.03\)). IL-2 mRNA concentrations in spleen samples increased substantially but briefly during CMV disease in all experiments (Fig. 3). There was little change in BAL cell IL-2 mRNA concentrations, but IL-2 mRNA concentrations decreased substantially in lung samples in two of three experiments (Fig. 3).

IL-4 protein concentrations in serum and BAL fluid did not change during CMV disease. IL-4 mRNA concentrations did not change substantially during CMV disease in BAL cell or lung samples (Fig. 4).

IL-10 protein concentrations increased in BAL fluid during the first 5 days of CMV disease from 58 to 210 pg/ml, but the difference was not significant (\(P, 0.07\)). IL-10 protein concentrations increased in serum during the first 5 days of CMV disease from 50 to 70 pg/ml, but the difference was not significant (\(P, 0.10\)). During this 5-day interval, there were increases in IL-10 mRNA concentrations in lungs in two of three experiments and in spleens in three of three experiments (Fig. 5).

**CMV-induced pneumonia.** There was evidence from measurements of mRNA, protein, or both that the amounts of all four cytokines increased in lungs during CMV-induced pneumonia, similar to the increases seen during primary reactivation pneumonia. IFN-\(\gamma\) protein levels increased substantially in BAL fluid during the early phase of the pulmonary inflammatory response, 14 days after CMV inoculation (Fig. 2). There was a corresponding increase in serum IFN-\(\gamma\) protein levels (Fig. 2). IFN-\(\gamma\) mRNA levels also increased substantially in BAL cell and lung but not spleen samples (Fig. 1).

IL-2 protein levels increased in BAL fluid during the first 14 days of CMV-induced pneumonia, from 11 to 52 pg/ml (\(P, 0.02\)). IL-2 protein levels also increased in serum during this time period, from 12 to 113 pg/ml (\(P, 0.02\)). IL-2 mRNA levels increased in BAL cell and lung samples during CMV-induced pneumonia but remained near the baseline in spleen samples (Fig. 3).

IL-4 protein levels did not change substantially in BAL fluid or serum samples during CMV-induced pneumonia. IL-4 mRNA concentrations increased in serum (Fig. 2). There was a corresponding increase in serum IL-4 protein levels (Fig. 2). IL-4 mRNA concentrations increased in spleens, but not BAL cell or lung samples, during CMV-induced pneumonia (Fig. 5).

**FIG. 3. IL-2 mRNA concentrations in BAL cells, lung tissue, and spleen tissue.** Mice were inoculated with *T. gondii* on day 0 and with CMV on day 93. The data are representative of three separate experiments.
levels increased in BAL cell and lung samples during CMV-induced pneumonia (Fig. 4).

IL-10 protein levels did not change much in BAL fluid or serum samples during CMV-induced pneumonia. IL-10 mRNA levels increased in BAL cell samples during CMV-induced pneumonia but not consistently in other samples (Fig. 5).

**DISCUSSION**

There was evidence for substantial increases in the levels of all four cytokines, IFN-γ, IL-2, IL-4, and IL-10, in lungs during both forms of *T. gondii* pneumonia. The most striking increases were observed for IFN-γ. IFN-γ protein levels in BAL fluid and IFN-γ mRNA levels in BAL cell and lung samples increased more than 10-fold during pneumonia. Increases in IFN-γ mRNA levels in lungs during primary reactivation pneumonia were accompanied by increases in spleen IFN-γ mRNA levels (Fig. 1). Later, during CMV disease, increases in spleen IFN-γ mRNA levels were substantially greater than the increases observed during either primary or CMV-induced pneumonia. This maximal spleen IFN-γ mRNA activity probably reflected the systemic nature of CMV disease. IFN-γ protein levels increased substantially in serum during CMV disease as well (Fig. 2). From the magnitude of increases of IFN-γ protein and mRNA levels, it appeared that inflammation occurring during the two forms of pneumonia was driven predominantly by Th1 cytokines. IFN-γ is known to be a key cytokine in models of primary *T. gondii* infection (35, 36, 40–42).

IL-2 mRNA levels also increased substantially in lung tissue and BAL cells during pneumonia. The amounts of spleen IL-2 mRNA did not parallel the changes in lungs, emphasizing the localized nature of the inflammatory response. A sharp increase in the amounts of spleen IFN-γ mRNA (Fig. 1). The main physiological activities of IL-2 are to stimulate clonal T-cell proliferation, natural killer cell differentiation into lymphocyte-activated killer cells, and activation of B lymphocytes, cytotoxic T lymphocytes, and macrophages (21). Previous observations suggest that IL-2 is important for effective control of primary toxoplasma infection (8, 15, 22, 37).

Interestingly, during CMV disease, IL-2 mRNA levels decreased in lung samples. A decrease in IL-2 and perhaps Th1 or Tc1 (26, 34) lymphocyte activity might have been responsible for the subsequent reactivation of *T. gondii* infection in lungs. The decreased IL-2 mRNA levels in lungs during CMV disease are consistent with our earlier observations that there is a consistent decrease in the levels of CD4+ cells in lung lavage populations just before either primary reactivation pneumonia or CMV-induced pneumonia (28, 30). Others have reported that mouse splenic lymphocytes are unresponsive to *T. gondii* antigens during the first week of primary *T. gondii* infection and that this finding is associated with diminished IL-2 production by these lymphocytes (17).

Although the increases in the levels of the Th1 cytokines IFN-γ and IL-2 were most impressive, increases in the levels of IL-10 and IL-4 suggested that these cytokines may have modulated the inflammation driven primarily by IFN-γ and IL-2. IL-10 mRNA levels increased in BAL cell and lung samples during primary reactivation pneumonia and in BAL cell samples during CMV-induced pneumonia. IL-10 is closely associated with Th2 activity, but IL-10 is also secreted by other T cells, B cells, macrophages, keratinocytes, and mast cells (9, 25). The source of IL-10 in our experiments was not known.

The main physiological activities of IL-10 are to inhibit cytokine secretion by Th1 lymphocytes, Th2 lymphocytes, and mononuclear phagocytes. IL-10 suppresses the synthesis
of IFN-γ and inhibits macrophage effector and antigen-presenting activities (9, 25). IL-10 has been associated with immunodepression during acute *T. gondii* infection (12, 16, 19, 20, 23) and appears to protect mice from excessive, potentially lethal inflammation during *T. gondii* disease (12). It is likely that one effect of increased IL-10 levels in mouse lungs during primary reactivation pneumonia and CMV-induced pneumonia was to limit the inflammation that occurred in response to local *T. gondii* replication. In support of this notion, recent unpublished data from our laboratory indicate that depletion of IL-10 allows inflammation to worsen.

During CMV disease, IL-10 mRNA levels increased in lung samples in two of three experiments, and there was a nearly fourfold increase in BAL fluid IL-10 protein levels in all experiments. The increases in IL-10 protein levels in BAL fluid were not quite statistically significant; there was substantial variability from mouse to mouse. In view of the effects of IL-10 on Th1 cell function, it is of interest that IL-2 mRNA levels decreased in lungs during CMV disease. Our results suggest the intriguing possibility that IL-10 produced during CMV disease may have led to reactivation of *T. gondii* infection in lungs. Experiments to answer this question are ongoing. Spleen IL-10 mRNA levels also increased and may have been involved in modulating the systemic inflammatory response during CMV disease.

IL-4 is secreted by Th2 cells and shifts immune responses toward Th2 domination (5). Specifically, IL-4 stimulates B cells and increases their ability to process antigens and to produce immunoglobulins, especially IgE (5). In our experiments, the amounts of IL-4 mRNA increased in BAL cells and lung tissue during primary reactivation pneumonia and CMV-induced pneumonia, but the amounts of IL-4 mRNA and the degree of the increase were smaller than those for IFN-γ or IL-10. It appeared that IL-4-producing cells were present in the lungs during times of intense lung inflammation but that the amounts produced were more limited than for IFN-γ. Since only small amounts of IL-4 were produced and since IL-10 has so many other potential sources, it seems likely that Th2 cells were
present in the lungs but that they did not predominate in the inflammatory response, as has been observed for some other infections (31). It did not appear that a predominance of Th2 cells was responsible for the reactivation of T. gondii infection, as we had initially hypothesized.

In mice with reactivation of T. gondii infection, disease was limited to the lungs, and pulmonary inflammatory responses often differed from those elsewhere in the body (7, 24). We used two complementary approaches to describe cytokines present in mice before, during, and after toxoplasma pneumonia. One was to measure the levels of cytokines directly in BAL fluid and serum. BAL fluid reflected lung cytokines that diffused into the bronchi. Serum reflected cytokine secretion throughout the body. Since neither fluid was taken directly from lung parenchyma, we also measured the levels of cytokine mRNA in BAL cells and lung tissue. The advantage of measuring mRNA levels directly was that we could be confident that the mRNA was produced in vivo. On the other hand, post-translational events may have altered the relationship between mRNA production and secretion. Another approach sometimes used is to remove cells and measure cytokine production in vitro in response to mitogens or antigens. This approach measures the types of cells that can be cultured and what their capabilities are but not what the cells are doing in the complex milieu of inflammatory sites. The strength of our experimental approach was that it provided information about pathophysiological events occurring in vivo during the disease process in the relevant organ and in the whole mouse.

Increased cytokine mRNA concentrations were not always reflected in BAL fluid cytokine protein concentrations for IL-2, IL-4, and IL-10. Cytokine protein concentrations were variable between experiments and more difficult to interpret. It was likely that cytokines secreted in focal areas of inflammation in the lungs were degraded locally or removed in the circulation before they diffused into BAL fluid. There is no feasible way to measure cytokine protein concentrations bathing individual cells within inflammatory foci.

Our results indicated that both Th1 and Th2 cytokines were present in mouse lungs during T. gondii pneumonia and that decreases in IL-2 and/or increases in IL-10 levels during CMV disease may have induced the reactivation of T. gondii infection. If true for humans, these observations may be critical for understanding the reactivation of T. gondii infection. They would also be important for the development of rational approaches to prevent or control this or other opportunistic infections in humans by modulation of immune responses.

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REFERENCES


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