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Chemokine Response in Mice Infected with *Mycobacterium tuberculosis*

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We show here that infection of murine macrophages with various strains of *Mycobacterium tuberculosis* induces the rapid in vitro expression of genes encoding chemokines macrophage inflammatory protein 1α and macrophage inflammatory protein 2, which recruit neutrophils to sites of infection, and macrophage-recruiting chemokines 10-kDa, interferon-inducible protein (IP-10) and macrophage chemotactic protein 1. Three strains of *M. tuberculosis*, Erdman and the clinical isolates CSU 22 and CSU 46, induced similar levels of secretion of macrophage chemotactic protein 1 from infected macrophage monolayers; however, the Erdman strain failed to induce levels of secretion of tumor necrosis factor alpha similar to those induced by either CSU 22 or CSU 46. Using a low-dose aerosol infection model, we also found that while the Erdman strain induced negligible increases in chemokine mRNA levels in the lungs, infection with either CSU 22 or CSU 46 resulted in greater levels of mRNA production for all four chemokines tested. The growth of these strains in the lungs was, however, equally well contained by acquired host immunity. These data allow us to hypothesize that the chemokine response in the lungs probably does not control the protective granulomatous response and that perhaps other T-cell- or macrophage-associated cytokines such as tumor necrosis factor alpha or interleukin 12 may be involved in this process.

Cell-mediated immunity to *Mycobacterium tuberculosis* infection is characterized by the sensitization of T cells that subsequently release cytokines which activate parasitized macrophages and recruit monocytes from the blood to the site of infection (28). It is generally accepted that the initial wave of protective immunity involves the generation of $CD4^+$ T cells of the T-helper type 1 (Th1) phenotype which secrete interleukin 2 (IL-2) and gamma interferon (IFN-γ) (4, 28, 29). In addition to these T-cell-associated cytokines, a variety of other cytokines and chemokines are also produced during the emergence of the acquired response to mycobacterial infection. These include proinflammatory cytokines such as IL-1 and tumor necrosis factor alpha (TNF-α) (16, 47, 48) as well as chemokines (2, 10, 11, 13) with selective chemotactic and haptotactic properties (1, 20, 38, 42) for leukocytes.

Chemokines are 8- to 14-kDa peptides which are currently classified into two subfamilies by virtue of a conserved cysteine residue arrangement near the NH_2 terminus (3, 26). Members of the α subfamily contain a -C-X-C- motif and include the proteins KC, macrophage inflammatory protein 2 (MIP-2), and 10-kDa, interferon-inducible protein (IP-10) in the murine system, while those of the β subfamily possess a -C-C- arrangement and include macrophage chemotactic protein 1 (MCP-1), MIP-1α/β, and RANTES. Murine KC and MIP-2, the putative homologs of human GROα/β/γ (50), are potent neutrophil-recruiting chemokines in mice which likely serve a compensatory function for IL-8, which has not been found in mice to date. IP-10, which may be involved in delayed-type hypersensitivity (13), is an unusual member of the α subfamily which attracts monocytes and activated T lymphocytes rather than polymorphonuclear leukocytes (8). MCP-1, originally characterized as the product of the early competence gene JE (57), is a potent chemoattractant of both monocytes and activated T lymphocytes (22). MIP-1α/β, originally described as a peptide doublet (52), consists of the products of two separate genes, those encoding MIP-1α and MIP-1β (33). Though human MIP-1 recruits human monocytes (45), as do all other β chemokines, murine MIP-1 recruits and activates polymorphonuclear leukocytes (2, 52) while activating only specific functions in murine monocytes (9). It has also been shown that in humans MIP-1α preferentially attracts $CD8^+$ T cells whereas MIP-1β attracts mainly $CD4^+$ T cells (43). Chemokines which recruit monocytes and T lymphocytes are secreted not only by immune cells but also by nonimmune cells, including fibroblasts (21, 56), keratinocytes (13), smooth muscle cells (46), vascular endothelial cells (36, 40), and parenchymal epithelial cells (39), in response to traditional immune mediators at inflammatory sites including IL-1 and TNF (21, 24, 36).

As all of the β chemokines and the α chemokine IP-10 have been shown to recruit or activate T cells and monocytes at inflammatory foci, it is possible that these molecules would be expressed following exposure of the host to infection with *M. tuberculosis*. In this report, we show that this is indeed the case by demonstrating that three *M. tuberculosis* strains induced various levels of expression of multiple chemokine and cytokine genes both in infected macrophages and in the lungs following low-dose aerosol infection of mice. The results of this study indicate that infected mice mobilize a substantial chemokine response following exposure to virulent strains of *M. tuberculosis*, although the levels induced clearly vary among the different isolates.

MATERIALS AND METHODS

Mice. Experiments were performed with 8- to 10-week-old female specific-pathogen-free C57BL/6 mice purchased from The Jackson Laboratory (Bar Harbor, Maine).

Bacteria. Virulent *M. tuberculosis* strains Erdman (TMCC 107), CSU 22, and CSU 46 were grown to mid-log phase in Proskauer-Beck or glycerine alanine salts medium containing 0.01% Tween 80 and stored in ampoules frozen at −70°C until use. Each strain was passaged in media fewer than three times since

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original collection. CSU 22, a catalase-negative strain, is multidrug resistant and was previously described as a "fast-growing tuberculosis strain" (27). CSU 46 is a streptomycin-resistant strain which has virulence similar to that of Erdman (see Fig. 1).

Media and reagents. Bone marrow-derived macrophages were cultured in Dulbecco's minimal essential medium (DMEM) containing 10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES), 2 mM L-glutamine, 0.05 mM 2-mercaptoethanol, 100 U of penicillin per ml, 100 μg of streptomycin per ml, 250 ng of amphotericin B per ml, and MEM nonessential amino acids supplemented with 10% heat-inactivated, endotoxin-low fetal calf serum (Summit Biotechnologies, Inc., Fort Collins, Colo.) and 10% L-929 fibroblast-conditioned medium (sDMEM). Lipopolysaccharide (LPS) from *Escherichia coli* O55:B5 and other tissue culture reagents were purchased from Sigma Chemical Co. (St. Louis, Mo.). Macrophage monolayers were primed with murine recombinant IFN-γ (rIFN-γ) (Genzyme, Boston, Mass.). RNA was extracted by using RNAzol B (Biotecx Laboratories, Inc., Houston, Tex.).

Bone marrow-derived macrophage culture. Mice were killed by cervical dislocation, and the femurs were aseptically removed. The marrow was flushed out of the femurs with ice-cold sDMEM. Bone marrow cells were plated at 6.5×10^6 to 8×10^6 per 90-mm^2 tissue culture-treated petri dish (Falcon 3003; Becton Dickinson Labware, Lincoln Park, N.J.) in 8 ml of sDMEM and incubated at 37°C in 5% CO_2. After 24 h, 4 ml of sDMEM was added, and on day 6 nonadherent cells were removed by replacement of half of the medium with an equal volume of sDMEM. Cell cultures were incubated for another 2 days before half of the medium was replaced with sDMEM lacking 2-mercaptoethanol, antibiotics and antimycotics, and L-929-conditioned medium. This was repeated after an additional 24 h, and bone marrow-derived macrophages were used 24 h later, on day 10.

Experimental infections. For in vitro infections, bone marrow-derived macrophages were primed by the addition of murine recombinant IFN-γ (100 U/ml) and control macrophages received an equal volume of medium alone. Macrophages were stimulated with LPS (1 μg/ml) or infected at a 1:3 ratio with *M. tuberculosis* 18 to 24 h after priming. Culture supernatants were collected and frozen at −70°C at various times after infection, and macrophage RNA was collected by addition of RNAzol B to control and infected monolayers.

For in vivo infection, mice were infected aerogenically with *M. tuberculosis*, and the course of infection in target organs was monitored against time by harvesting lungs and enumerating viable bacilli as described elsewhere (27). Briefly, mice were placed in the exposure chamber of a Middlebrook Aerosol Generation device (Glas-col Inc., Terre Haute, Ind.) and given an infectious dose of approximately 50 to 100 viable bacilli within the lungs by use of a venturi nebulizer loaded with 10 ml of a suspension of *M. tuberculosis* at a concentration previously calibrated to provide the desired uptake over a 30-min period. Serial dilutions of individual whole organ homogenates were plated on nutrient 7H11 agar, and bacterial colony formation was counted 2 to 3 weeks later after incubation at 37°C. The data were expressed as the $\log_{10}$ value of the mean number of bacteria recovered from each organ ($n = 4$). For detection of cytokine gene expression in the lungs of infected mice, two lobes were separately homogenized in 1 ml of RNAzol B by using a tissue polytron and frozen at −70°C.

Cytokine ELISA. TNF-α secretion in the supernatents from triplicate macrophage monolayers was detected by using a sandwich enzyme-linked immunosorbent assay (ELISA) antibody pair (KM-TNFa; Endogen, Cambridge, Mass.) in Immunoplate Maxisorb 96-well plates (Nunc, Naperville, Ill.). Each sample was analyzed in triplicate according to the manufacturer's directions.

For MCP-1 detection, a sandwich ELISA antibody pair (capture no., 18241D; detection no., 18272D) (Pharmingen, San Diego, Calif.) was used in Corning 96-well ELISA plates (Corning, Newark, Calif.). Avidin-peroxidase (Sigma) and 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) substrate were used in the detection steps. The concentrations of MCP-1 in picograms per milliliter in triplicate samples of monolayer supernatants were calculated from a standard curve for murine recombinant MCP-1 from duplicate wells on each plate. Plates were read at 405 nm.

Preparation of cDNA. Total macrophage culture monolayer and lung RNA was collected and reverse transcribed into cDNA as described previously (54). Briefly, cell or tissue lysate total RNA was isolated from RNAzol by chloroform-phenol extraction and precipitated with cold isopropanol (32). cDNA was synthesized by adding 1 μg of RNA to a final reaction volume of 25 μl containing 0.25 μM deoxynucleotide triphosphate (dNTP) mixture (Boehringer Mannheim, Indianapolis, Ind.), 8 mM dithiothreitol (Gibco BRL Life Technologies, Gaithersburg, Md.), 1× reverse transcriptase buffer (50 mM Tris-HCl [pH 8.3], 75 mM KCl, 3 mM $MgCl_2$), 0.08 A_{260} unit of random hexamers (Boehringer Mannheim), and 200 U of Moloney murine leukemia virus reverse transcriptase (Gibco BRL Life Technologies). The reaction mixture was incubated at 37°C for 1 h, heated to 90°C for 5 min to denature the reverse transcriptase, and cooled on ice for 5 min. cDNA was diluted 1:9 in DNase- and RNase-free water and stored at −70°C.

PCR amplification of cDNA. Aliquots of cDNA were used as a template for amplification by PCR with programs of 23 to 40 cycles each consisting of denaturation at 95°C for 1 min, annealing at 54°C for 1 min, and extension at 72°C for 2 min. The PCR product was subjected to a final extension period of 7 min at 72°C and stored at −20°C. Twenty-three cycles were used to amplify TNF-α, IL-1β, and hypoxanthine phosphoribosyl transferase (HPRT) reactions; 30 cycles were used for IFN-γ; 35 cycles were used for IL-2 and MCP-1; 32 were used for IP-10; and 34 were used for MIP-1α and MIP-2. Primer pairs for murine TNF-α and IL-1β (53); IL-2, IFN-γ, and HPRT (41); and IP-10 and MCP-1 (37) were published previously. Sense and antisense primer oligonucleotides were selected from published sequence data for MIP-1α (51) and MIP-2 (44) based upon greater than 50% GC content and locations at corresponding 5′ and 3′ ends of the coding region for the mature proteins. Primer sequences were GCAGCAG CGAGTACCAGTCCC and GGCATTCAGTTCCAGGTCAG for MIP-1α and GCCCCTCCCACCTGCCGGCTCC and CTGAACCAGGGGGGCTTCA GGG for MIP-2. All primers were used at a final concentration of 0.2 μM in a 50-μl PCR mixture containing 0.20 mM dNTP mix, 1× PCR buffer (Promega, Madison, Wis.) containing $MgCl_2$ for a final concentration of 1.5 mM, and 1 U of *Taq* polymerase (Promega).

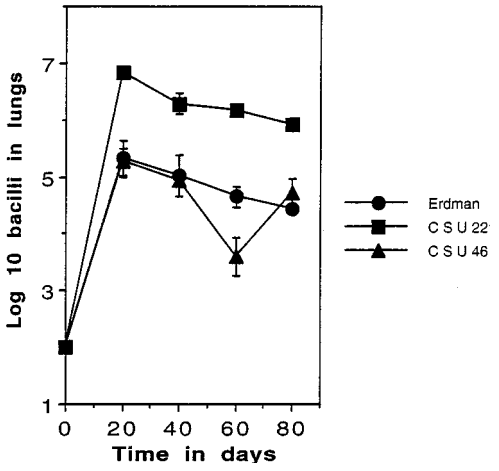

FIG. 1. Growth of three strains of *M. tuberculosis* in the lungs of C57BL/6 mice following exposure to low-dose aerosol infections. The growth pattern for CSU 22 is representative of a fast grower ($P \leq 0.0025$ for CSU 22 versus CSU 46 or Erdman), while the growth of CSU 46 closely follows that of the virulent laboratory Erdman strain. Data shown are mean values for four mice ± standard errors of the means.

Detection of PCR product. The PCR product was subjected to electrophoresis in 1% agarose, Southern blotting, and hybridization with fluorescein-conjugated specific probes which were visualized with a commercial chemiluminescent detection kit (ECL detection system; Amersham Life Sciences, Arlington Heights, Ill.) as described elsewhere (53). Oligonucleotide probes for TNF-α and IL-1β (53) and IL-2, IFN-γ, and HPRT (41) were published previously. Published DNA sequences for IP-10 (25), MCP-1 (31), MIP-1α (51), and MIP-2 (44) were used to generate specific probes with high GC content and the following sequences located between the corresponding primer pair sequences: 5′-CCCT GCGAGCCTATCCTGCCC-3′ (IP-10), 5′-CCAGATGCAGTTAACGCCCC-3′ (MCP-1), 5′-GCCTGCTGCTTCTCCTACAG-3′ (MIP-1α), and 5′-GTGTGAC GCCCCCAGGACCCC-3′ (MIP-2). Densities of the signals on chemiluminescent detection film were measured in pixels with a flatbed scanner (Microtek Scanmaker IIXE) and quantified with Image software (shareware; National Institutes of Health, Bethesda, Md.). Each sample was normalized to the corresponding HPRT pixel value to correct any discrepancies in the amount of initial cDNA that was PCR amplified. The amount of PCR product was determined by comparison of pixel values with a standard curve of twofold dilutions of PCR-amplified cDNA from LPS-stimulated bone marrow-derived macrophage monolayers. A long-linear relationship between chemiluminescent signal and fold dilution of control RNA was indicated as described elsewhere (6).

Statistical analysis. Statistical analysis of data was performed by using Student's *t* test, and differences were considered significant if *P* values were ≤0.05.

RESULTS

Growth of strains of *M. tuberculosis* following aerosol exposure of mice. In order to establish the relative virulence of each *M. tuberculosis* strain, we infected mice by means of a low-dose aerosol exposure. The growth of the three strains of *M. tuberculosis* following deposition in the lungs of approximately 50 to 100 bacilli is shown in Fig. 1. Strain CSU 46, an organism that we have not previously characterized, grew similarly to the well-known Erdman strain in that the experimental infection grew about 3 logs in the lungs over the first 20 days, after which

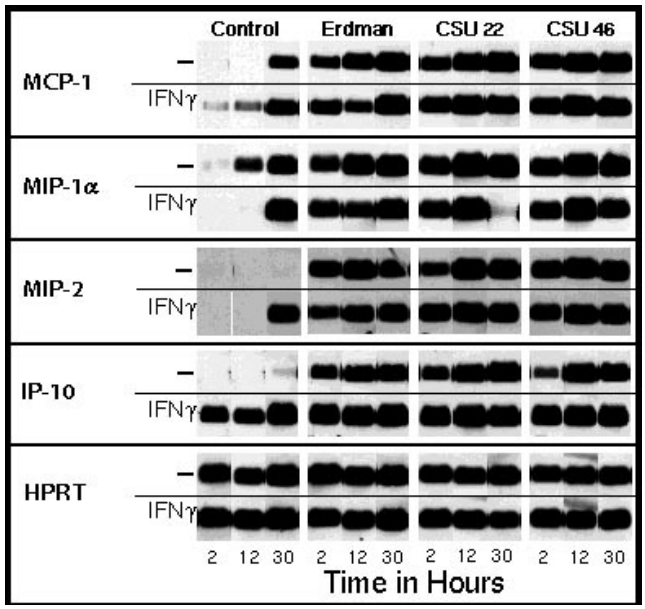

FIG. 2. Expression of chemokine genes in bone marrow-derived macrophages from C57BL/6 mice following infection with *M. tuberculosis*. RT-PCR product was generated from infected monolayers by using primers for chemokines MCP-1, MIP-1α, MIP-2, and IP-10 and could be detected at 2, 6, and 30 h after infection with Erdman, CSU 22, or CSU 46 alone (−) or in addition to priming with 100 U of murine rIFN-γ (IFNγ) per ml. Each experiment utilized triplicate wells of infected macrophage monolayers, and the mRNA from each well was harvested and analyzed separately. RT-PCR product from uninfected monolayers (Control) ($n = 3$) was also detected for each chemokine to show the basal levels of gene expression in macrophages, and RT-PCR product for the housekeeping gene *HPRT* from the same cells was measured to ensure that equal amounts of RNA were analyzed. Data shown are a representative result from two separate experiments.

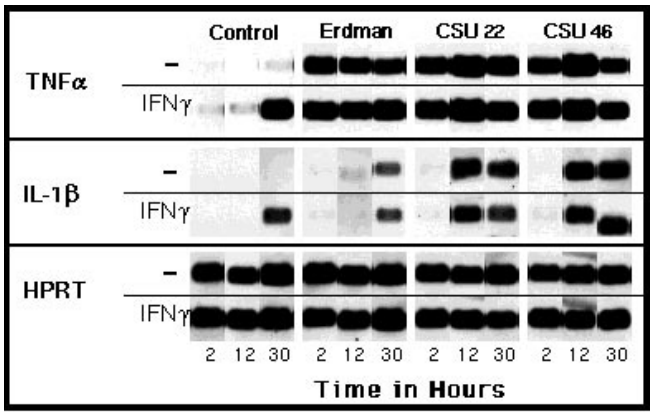

FIG. 3. Expression of proinflammatory cytokines in bone marrow-derived macrophages from C57BL/6 mice following infection with *M. tuberculosis*. RT-PCR product generated by using primers specific for TNF-α and IL-1β could be detected at 2, 12, and 30 h after infection with Erdman, CSU 22, or CSU 46 alone (−) or in addition to priming with 100 U of murine rIFN-γ (IFNγ) per ml. Basal levels of expression in uninfected macrophages (Control) were also measured, and all values were compared with levels of expression of HPRT to ensure that equal amounts of cDNA were analyzed. Data shown are a representative result from two separate experiments.

some clearance of the bacterial load by host immunity ensued. In contrast, strain CSU 22, a "fast-growing" strain (27), grew nearly 2 logs higher in the lungs before containment by the host occurred.

Induction of chemokine, TNF-α, and IL-1β gene expression following infection of murine bone marrow-derived macrophages with *M. tuberculosis*. Chemokine and other cytokine gene expression following infection with various isolates of *M. tuberculosis* was determined in experiments using cultures of murine bone marrow-derived macrophages (BMMØ). Expression of chemokines MIP-2 and IP-10 of the α subfamily in this system was compared with expression of MCP-1 and MIP-1α of the β subfamily by semiquantitative reverse transcriptase PCR (RT-PCR). MCP-1, MIP-1α, MIP-2, and IP-10 gene expression could be detected as early as 2 h postinfection, with maximal levels reached by 12 to 30 h as shown in Fig. 2. Similar amounts of message were induced by all three *M. tuberculosis* strains by 12 h postinfection for each chemokine. Priming of macrophages with murine rIFN-γ for 18 to 24 h prior to infection failed to significantly affect the levels of expression of the genes analyzed with the exception of IP-10, for which mRNA production was accelerated. Priming with rIFN-γ also increased levels of IP-10 message in uninfected macrophages at all time points.

Expression of the proinflammatory cytokines TNF-α and IL-1β was also detected in the infected cells, but with different kinetics of induction as shown in Fig. 3. TNF-α message was similarly induced by all three strains by 2 h. However, levels of IL-1β message did not peak until 12 h postinfection, and for Erdman-infected macrophages, IL-1β mRNA expression did not reach the levels observed in CSU 22- or CSU 46-infected macrophages by the end of the 30-h experimental period.

Secretion of MCP-1 and TNF-α from macrophages infected with *M. tuberculosis*. To determine whether secretion of chemokines accompanied gene expression in the infected macrophages, supernatants were analyzed by ELISA for the presence of MCP-1 and TNF-α coincident with RNA analysis. As depicted in Fig. 4, all three strains of *M. tuberculosis* induced

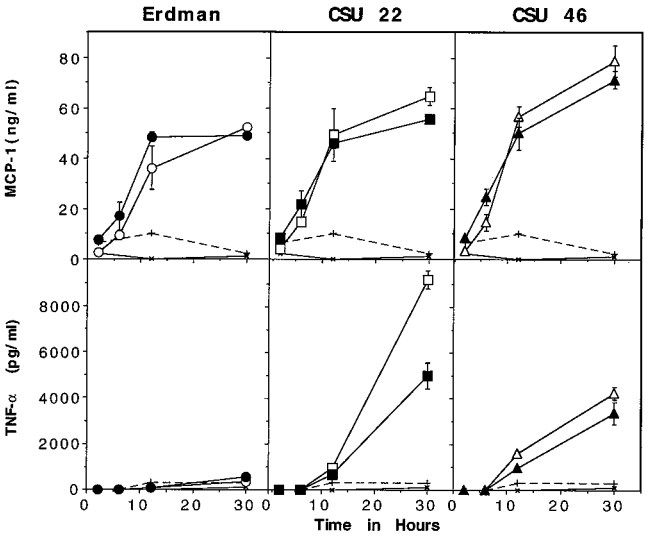

FIG. 4. Secretion of MCP-1 and TNF-α following infection of bone marrow-derived macrophages from C57BL/6 mice with *M. tuberculosis*. Culture supernatants from triplicate macrophage monolayers used to generate PCR product for Fig. 2 and 3 were individually analyzed by sandwich ELISA for MCP-1 and TNF-α to quantitate the secretion of antigenic proteins within 30 h after infection with the Erdman (circles), CSU 22 (squares), or CSU 46 (triangles) strain. Exposing infected macrophages (open symbols) to rIFN-γ (closed symbols) failed to consistently alter the secretion of MCP-1 or TNF-α significantly ($P \geq 0.05$). Negligible secretion of cytokines was detected for uninfected macrophages (x) or such cells exposed to rIFN-γ (+). Data are expressed as the mean values for triplicate wells ± standard deviations. Data shown are a representative result from three separate experiments.

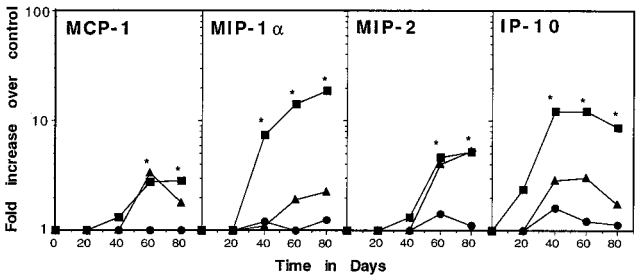

FIG. 5. Expression of chemokine message in the lungs of C57BL/6 mice following exposure to low-dose aerosols of *M. tuberculosis*. Mice ($n = 3$) were infected with Erdman (●), CSU 22 (■), or CSU 46 (▲), and RT-PCR product was generated individually from the cDNA of an identical lung lobe from each mouse by using primers specific for MCP-1, MIP-1α, MIP-2, and IP-10. Product was detected by chemiluminescence and quantitated as pixel values by using a scanner device. Values were normalized for the amount of mRNA by factoring in the HPRT value for each sample, and the fold increase over control values for uninfected lungs was calculated for each mouse. Data are expressed as the mean values for triplicate samples at each time point [*, $P \leq 0.05$ for clinical isolate(s) versus Erdman]. Data shown are a representative result from two separate experiments.

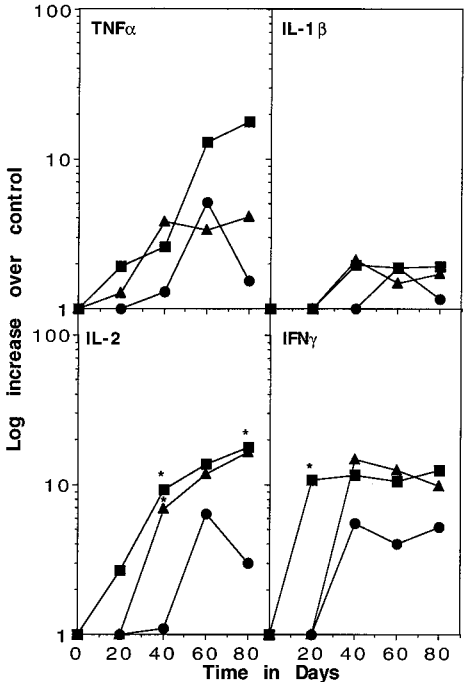

FIG. 6. Expression of cytokine message in the lungs of C57BL/6 mice following exposure to low-dose aerosols of three strains of *M. tuberculosis*. Samples of cDNA from Erdman (●)-, CSU 22 (■)-, and CSU 46 (▲)-infected lung lobes ($n = 3$) were amplified individually by using primers specific for TNF-α, IL-1β, IL-2, and IFN-γ and quantitated as described in the legend to Fig. 5. Data are expressed as the mean values for triplicate samples at each time point [*, $P \leq 0.015$ for clinical isolate(s) versus Erdman]. Data shown are a representative result from two separate experiments.

secretion of antigenic MCP-1 detectable as early as 6 h after infection, and all strains induced MCP-1 secretion at similar levels. Addition of IFN-γ did not influence the level of secretion, nor did this cytokine induce any secretion in uninfected cells.

In terms of TNF-α secretion, differences in cytokine levels among the three infections were observed, with CSU 22-infected macrophages producing 2.5-fold more antigenic TNF-α ($P \leq 0.002$) than cells infected with the CSU 46 strain by 30 h. The Erdman strain induced negligible levels of TNF-α secretion from infected macrophages over the same period. This difference did not appear to be attributable to differences in the levels of growth of infections in the host macrophages, as these levels were not appreciably different over the first 30 h of the culture; by 48 h, Erdman infections had grown less than 0.1 log, while CSU 22 infections had grown by less than 0.2 log (data not shown).

Induction of chemokine and cytokine gene expression in the lungs of mice infected aerogenically with *M. tuberculosis*. Having demonstrated the expression of the chemokine genes in vitro, we then performed aerosol infection experiments in vivo in order to determine the kinetics of the emergence of these chemokines in the lungs of mice exposed to the different strains of *M. tuberculosis*. Basal levels of expression of MCP-1, MIP-1α, and MIP-2 were nearly undetectable in the lungs of uninfected mice, whereas low levels of IP-10 were seen (data not shown). Following infection, CSU 22-infected lungs were the first to show significantly greater expression ($P \leq 0.05$) of MIP-1α (7-fold) and IP-10 (10-fold) than the Erdman-infected lungs (by day 40) as depicted in Fig. 5. For MCP-1 and MIP-2, similar levels of chemokine response were seen with CSU 22 and CSU 46, as both strains induced similar levels of expression of MCP-1 (twofold) and MIP-2 (fourfold) by day 60. For mice exposed to the Erdman strain, all four chemokine responses of the lungs were not significantly different from the expression levels of uninfected lungs.

We also characterized expression of proinflammatory and T-cell-derived cytokines in the same infected lungs as shown in Fig. 6. Expression of TNF-α and IL-1β in the lungs was induced similarly in all three experimental infections. For IL-2, CSU 22- and CSU 46-infected lungs produced responses significantly greater (nine- and sevenfold, respectively) than levels induced by the Erdman strain by day 40. Infection with CSU 22 induced the earliest IFN-γ response by day 20, and yet all three strains of *M. tuberculosis* induced similar responses by day 40.

DISCUSSION

The results of this study show that infection with different strains of *M. tuberculosis* induced the expression of genes encoding four known chemokines. Representative members of both the α chemokine subfamily (MIP-2 and IP-10) and the β subfamily (MCP-1 and MIP-1α) were induced in BMMØ monolayers infected with *M. tuberculosis* CSU 22, CSU 46, or Erdman. The isolates CSU 22 and CSU 46 also stimulated expression of all four chemokine genes in the lungs of mice infected by a low-dose aerosol challenge with the bacilli. Thus, mycobacterial infection induces the expression of genes encoding chemokines capable of attracting not only monocytes and activated T lymphocytes, but also those known to preferentially attract polymorphonuclear leukocytes.

The finding that the *M. tuberculosis* strains all induced the α chemokine MIP-2, a known preferential recruiter of neutrophils, is both interesting and surprising in view of the nature of the T-cell–monocyte granulomatous response to the infection. However, it has been previously noted (2) that intraperitoneal injection of mice with *Mycobacterium bovis* BCG induces a significant influx of neutrophils, an event that can be prevented by the administration of neutralizing antibodies to MIP-1 and MIP-2. In the present study, however, we observed few neutrophils accumulating in the lung tissues of infected mice (data not shown), leading us to conclude that although MIP-2 may well be produced, the presence of other chemokines or cyto-

kines may interfere with or counteract the neutrophil attractant properties of this and other α subfamily peptides.

In this regard, it is in fact quite likely that regulatory cytokine networks in the lung modulate the production of chemokines in response to *M. tuberculosis* infection. While IL-1 and TNF have been shown to directly up-regulate the production of both α and β chemokines (21, 24, 36), production of these cytokines could, in turn, be influenced by the local production of macrophage-deactivating factors, including IL-10 (19), transforming growth factor β (5, 15, 34), and prostaglandin E_2 (30); this suggests that considerable cross-regulation of the production of chemokines may occur. It is also likely that IL-12 can modulate the chemokine response, since recent observations have linked this cytokine to promotion of granuloma formation in mice infected with *M. tuberculosis* (6). These include the observations of substantial accumulation of macrophages in severe combined immunodeficient (SCID) mice treated with IL-12 and the formation of diffuse granulomas in normal infected mice treated with neutralizing antibodies to IL-12. Hence, these data collectively suggest that the recruitment of monocytes into infected lesions is also heavily influenced by the presence of IL-12.

We found that IFN-γ did not play a major role in the earliest in vitro regulation of any chemokine analyzed, except for IP-10. IFN-γ was capable of inducing transcription of IP-10 in uninfected cells as well as enhancing the rate of transcription in mycobacterium-infected cells. Interestingly, in vivo expression of IP-10 always occurred after expression of the gene for IFN-γ in all infected lungs, and the levels of expression of this chemokine correlated with the relative amounts of IFN-γ gene expression induced by each mycobacterial strain. It seems likely, therefore, that IFN-γ plays only a minor role in the regulation of the early chemokine response; in fact, if IFN-γ were present to any extent during the early phase of the course of the infection, then it would probably come from a non-T-cell source such as natural killer cells. It also follows that if IFN-γ influenced chemokine activity, it would likely involve those chemokines contributing to chronic inflammation, such as IP-10 (26), rather than chemokines involved in an acute response.

In order to determine whether increased steady-state levels of chemokine gene expression correlated with increased secretion of mature protein, we measured levels of MCP-1 secreted from infected BMMØ monolayers. We found that increased transcription was indeed accompanied by increased levels of secretion of MCP-1 within the experimental period. All three strains of *M. tuberculosis* induced similar levels of MCP-1 transcription and secretion, and priming of macrophages with rIFN-γ did not affect expression of MCP-1. We also noted that MCP-1 secretion was independent of the virulence of the strain of *M. tuberculosis*, an observation that is similar to the demonstration of similar levels of IL-8 secretion from human monocytic lines in response to infection with the H37Rv or H37Ra strain of *M. tuberculosis* (11). Our finding that MCP-1 secretion was detected earlier than secretion of TNF-α from the same macrophage monolayers supports the hypothesis that MCP-1, originally characterized as an early response protein of platelet-derived growth factor-activated fibroblasts (56), is an early component of the macrophage response to mycobacterial infection.

We also observed, however, that the pattern of TNF-α secretion was different from that of MCP-1 secretion. Although all three strains tested here were shown to be virulent in the mouse model, CSU 22 and CSU 46 induced significant amounts of TNF-α secretion by macrophages while the Erdman strain did not, even though mRNA for TNF-α was present in cells infected with this particular strain. This failure to secrete TNF-α was not due to general inhibition of secretory events, since MCP-1 secretion occurred at similar levels for all cells. It has been demonstrated that cytoplasmic as well as nuclear mechanisms contribute to the regulation of TNF-α release (14, 23, 49). It is possible to detect message in the absence of the production of protein, given that repeated AU-rich motifs in the 3′ untranslated region of TNF-α message have been shown to confer instability as an important post-transcriptional regulatory mechanism (12, 17, 18). A similar discrepancy between the production of mRNA and secretion of TNF-α protein was observed in human monocytes infected with isogenic variants of a strain of *Mycobacterium avium* (35); however, this group demonstrated that the stability of the TNF-α mRNA did not vary among *M. avium*-infected human monocytes. Our findings are reminiscent of the observation elsewhere (7) that purified mannose-capped lipoarabinomannan from the Erdman strain induces transient gene expression and low levels of TNF-α secretion in comparison with levels induced by lipoarabinomannan which is not capped by mannan in human monocytes, hence suggesting that some form of post-translational block may be involved. One possibility might be interference by the Erdman-derived lipoarabinomannan molecules themselves, given their ability to leave the phagosome and traffic into other cellular compartments (55).

We then turned to the in vivo situation to analyze the abilities of the three mycobacterial strains to induce a chemokine response in lungs of mice infected with a low-dose aerosol challenge of bacilli. It was found that strains CSU 22 and CSU 46 induced expression of all four chemokine genes tested while the Erdman strain did not appreciably induce production of message for these chemokines, an observation thus differing from our demonstration of similar in vitro levels of chemokine gene expression in the BMMØ cultures.

The observation that the Erdman strain failed to stimulate chemokine expression at levels comparable to those induced by CSU 46 was interesting in light of the fact that the two strains grew similarly in the lungs of infected mice. One explanation might be related to the possible lack of TNF-α secretion in the lungs of the Erdman-infected mice. Hence, our current working hypothesis is that in mice infected with the isolates CSU 22 and CSU 46, the production of significant levels of TNF-α from local macrophages actively promotes the ability of surrounding lung tissue cells, including vascular endothelial cells, fibroblasts, and alveolar epithelial cells, to produce large amounts of chemokines. However, for the Erdman infection, TNF-α was not produced and chemokine levels were negligible. This in turn suggests that the chemokine response induced by macrophages may be TNF dependent, a hypothesis that we are currently testing by using specific TNF receptor inhibitors in infected mice. The fact that IL-1 shares with TNF-α the ability to induce the production of chemokines in lung parenchymal cells (16, 47, 48) suggests that IL-1 could compensate for a lack of TNF-α in Erdman-infected lungs. However, similarly low levels of IL-1β gene expression were detected in lungs infected with any of the three *M. tuberculosis* strains.

Finally, we determined the kinetics of expression of the genes for the cytokines IL-2 and IFN-γ in comparison with the chemokine response. While the Erdman strain failed to induce levels of chemokine expression similar to levels induced by CSU 22 and CSU 46 strains, levels of IFN-γ and IL-2 gene expression were similar by day 60 in all three experimental infections. Given the fact that the growth of all three infections in the lungs was contained by the host by 20 days, we conclude that while the presence of infection-induced chemokine production is a facet of the murine response, expression of these

genes for chemokines does not appear to be essential to the expression of specific resistance.

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