IL-12 Administered During *Chlamydia psittaci* Lung Infection in Mice Confers Immediate and Long-Term Protection and Reduces Macrophage Inflammatory Protein-2 Level and Neutrophil Infiltration in Lung Tissue[1]

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Protection against infections with the intracellular bacterium *Chlamydia* spp. requires Th1-polarized CD4$^+$ T cell immunity. In BALB/c mouse lung infections, immediate innate and nascent *Chlamydia*-specific immune responses following intranasal inoculation of *Chlamydia psittaci* strain B577 were modulated by 7-day i.p. administration of murine rIL-12, the initiation cytokine for Th1 immunity. Treatment with IL-12 reduced the severity of chlamydial pneumonia, abolished mortality (37.5% in untreated mice), and significantly reduced numbers of chlamydial organisms in lungs. On day 4 after inoculation, the neutrophil:macrophage ratio in bronchointerstitial pneumonias was 1.96 in untreated mice and 0.51 in IL-12-treated mice. This immediate, IL-12-mediated shift in innate inflammatory phenotype was correlated with a significant reduction of lung concentrations of the neutrophil chemoattractant macrophage inflammatory protein (MIP)-2 (putative murine homologue of human IL-8), monocyte chemotactic protein-1, and TNF-α; and a reduction in MIP-1α and IFN-γ, at high-dose infection only, and IL-12-independent IL-10 levels. *Chlamydia*-specific Ab titers and Ig isotype ratios indicated an IL-12-dependent Th1 shift. Recall responses of IL-12-primed mice to secondary chlamydial lung infection eliminated chlamydiae more effectively and generated a lung cytokine profile conducive to perpetuation of the Th1 memory population. These data support the hypothesis that genetic differences in endogenous IL-12 production and response pathways could determine disease outcomes characterized by poor chlamydial clearance and a purulent inflammatory infiltrate vs effective elimination of chlamydiae in a macrophage-dominated response. ***The Journal of Immunology,*** **1999, 162: 2217–2226.**

Similar to many other diseases caused by intracellular pathogens, cell-mediated immune responses, in particular of CD4$^+$ T cells, are crucial for resolution of infections with the obligately intracellular bacterium *Chlamydia* (1–5). Several lines of evidence have shown that among the polarized effector phenotypes of Th cells, as functionally characterized through the patterns of their cytokine secretion (6), Th1 cells mediate protection, while Th2 cells appear to be inefficient or even detrimental against chlamydial infection (7–10). Igietseme et al. (10) were first to demonstrate that adoptive transfer of a *Chlamydia*-specific Th1 lymphocyte clone mediated resolution of murine chlamydial genital infection. Subsequently, Yang et al. (7) provided evidence for genetic regulation of the *Chlamydia*-specific Th phenotype. In their murine model of chlamydial lung infection, BALB/c mice were consistently less resistant than C57BL/6 mice, and mounted Th2-biased adaptive responses to *Chlamydia* as opposed to the Th1 response produced by C57BL/6 mice. More recently, Perry et al. (8), in a mouse model of *Chlamydia trachomatis* genital infection, showed that neutralization of IL-12 significantly delayed chlamydial clearance.

Our laboratory addresses the mechanisms involved in chlamydial pathogenesis in the model of murine chlamydial lung infection, which allows for precise association of quantitative parameters in lung tissue, including chlamydial titer, with disease as expressed in the weight increase of lungs from infected mice over those of mock-infected animals. In the present study, we examined the influence of exogenous IL-12, the initiating cytokine for Th1 cell-mediated immunity (11), on the disease outcome in BALB/c mice. This mouse strain typically is highly susceptible to infections with intracellular protozoa and bacteria, including *Chlamydia*, and responds with a Th2 bias of the adaptive immune response (7, 12, 13). We asked whether IL-12 administration early in primary infection rendered BALB/c mice more resistant to chlamydial infection, and if it shifted the immune response to a Th1 type.

The chlamydial strain used in the experiments, *Chlamydia psittaci* strain B577, was originally isolated from a case of abortion in a ewe (14). This strain is highly prevalent in ruminants and is found in respiratory, intestinal, and urogenital infections in these host species (15). Occasionally, it may also cause abortion in occupationally exposed humans (16). This chlamydial strain very effectively infects the respiratory tract of mice and is closely related to avian chlamydial isolates that are the cause of a disease termed psittacosis or ornithosis, a severe, sometimes fatal, human respiratory infection with *C. psittaci* (5, 17).

We report here that administration of IL-12 early after inoculation provided immediate protection against lethality associated with *C. psittaci* lung infection, alleviated the disease severity, and elicited an Ab response consistent with a shift toward Th1 immunity against *C. psittaci* B577. Furthermore, the recall immune response in secondary infection of mice treated with IL-12 in a primary infection eliminated chlamydiae more effectively than the

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recall response of untreated controls. Cytokine profiles of the infected lung tissues suggested that, deviating from the paradigm of Th1 effector cytokines, the protective effects of IL-12 were mediated by down-modulation of expression of the chemokines macrophage inflammatory protein (MIP)[3]-2 and monocyte chemotactic protein (MCP)-1, and TNF-α, and of IFN-γ and MIP-1α at high-level chlamydial infection.

Materials and Methods

Animals

Female BALB/c (H-2^d) mice were purchased from Harlan, Sprague and Dawley (Indianapolis, IN) and were 6–8 wk old at the initiation of the experiments. The mice were housed under barrier conditions throughout the experiments and were provided standard rodent chow and water ad libitum in accordance with Auburn University and National Institutes of Health guidelines.

C. psittaci *B577*

C. psittaci strain B577 (VR-656; American Type Culture Club, Manassas, VA), isolated from ovine abortion (14), was cultured in buffalo green monkey kidney (BGMK) and NCI-H292 cells (BioWhittaker, Walkersville, MD) (18, 19). The cells were maintained in MEM (Life Technologies, Gaithersburg, MD) containing 5% FBS and 25 μg/ml of gentamicin and 2 μg/ml of amphotericin B. Confluent monolayers were inoculated with chlamydiae by centrifugation at 1200 $\times$ *g* for 1 h at 37°C, and the medium was changed to Iscove's modified Dulbecco's medium (Life Technologies) supplemented with gentamicin, amphotericin B, and 10% FBS. One week after infection, 0.25% glucose was added, and the culture medium containing released chlamydial elementary body (EB) was harvested 3–4 days later when the medium turned opaque.

Chlamydial EB were partially purified by a method following modified protocols of Caldwell et al. (20) and Miyashita and Matsumoto (21). Briefly, the harvested cell culture medium was sonicated on ice, cell nuclei were removed by centrifugation at 1,000 $\times$ *g* for 10 min at 4°C, and the chlamydial EB in the supernatant were sedimented at 12,000 $\times$ *g* for 30 min at 4°C. The EB pellet was resuspended in sucrose-phosphate-glutamate (SPG) buffer (22) and briefly sonicated, and the EB were further purified by centrifugation at 8000 $\times$ *g* for 1 h at 4°C on a step gradient of 50% sucrose and 30% renografin-76 (Squibb, Princeton, NJ), both in 30 mM Tris-HCl (pH 7.3). The sedimented EB were washed twice in SPG and frozen at −85°C until used. By using the same procedures described above, control inocula were prepared from uninfected cell culture supernatants.

Infection of mice and administration of IL-12

Mice were infected intranasally with BGMK cell-derived chlamydial EBs or control inocula. Under light metofane anesthesia, 30 μl of SPG containing chlamydial EBs or control inoculum was applied onto the nostrils with a pipette. From day 1 preinoculation to day 6 postinoculation, selected groups of mice received single daily i.p. injections of 150 ng of murine rIL-12 (Genetics Institute, Andover, MA) in 0.5 ml of PBS containing 0.5% normal serum from female BALB/c mice. Control groups were injected with PBS containing 0.5% female BALB/c serum.

At specified time points after inoculation, the mice were sacrificed by cervical dislocation, and lungs were removed under aseptic conditions. Lung weights were determined as a quantitative parameter of disease severity, and the lungs were snap frozen in liquid nitrogen and stored at −85°C until further processing.

Lung histopathology

Lungs from selected mice and infection groups were fixed in toto by intratracheal gravity infusion of 4% paraformaldehyde in PBS, further fixed by 24-h immersion in 4% paraformaldehyde and processed to paraffin by standard procedures. From some groups of mice, transverse 1-mm-thick callots of lungs were fixed by immersion in 4% paraformaldehyde in PBS and then processed to paraffin. Sections (5-μm-thick) were stained with hematoxylin-eosin for histopathological evaluation of qualitative aspects of chlamydial lung infection. Neutrophil to macrophage ratios were determined in lesions of selected lungs by counting both cell types in five or more distinct, infiltrated areas per tissue section.

[3] Abbreviations used in this paper: MIP, macrophage inflammatory protein; EB, elementary body; DTH, delayed-type hypersensitivity; IFU, inclusion forming unit; MCP, monocyte chemotactic protein; SPG, sucrose-phosphate-glutamate buffer; p.i., post-inoculation; BGMK, buffalo green monkey kidney.

Chlamydial lung burden

For cell culture quantitation of chlamydiae and cytokine determination in lung tissue, 10% tissue suspensions in SPG were prepared by homogenizing the lungs on ice in disposable tissue grinders (Sage, Crystal Lake, IL). Coarse tissue debris was removed from the homogenates by centrifugation at 1000 $\times$ *g* for 10 min at 4°C. Portions (10 and 100 μl) of the clarified suspensions were immediately inoculated by centrifugation at 37°C into shell vials containing confluent monolayers of BGMK cells grown on 12-mm round coverslips with 1 ml and 0.9 ml of Iscove's modified Dulbecco's medium, respectively (18). The remaining lung tissue suspension was stored in aliquots at −85°C for subsequent cytokine determination. Twenty-eight hours postinoculation (p.i.), the infected cell cultures were washed with PBS and fixed with 100% methanol. For visualization of chlamydial inclusions, the coverslip cultures were stained with FITC-conjugated mAb against chlamydial LPS (Sanofi Pasteur, Chaska, MN), and inclusions in at least 30 eyefields were counted in those cultures of the inoculum dilutions in which the inclusion density was 0.5–5 inclusions per eyefield of a ×40 objective. Chlamydial inclusion forming units (IFUs) in lung tissue were calculated by multiplying the mean number of inclusions per eyefield with the ratio of coverslip area to eyefield area and the dilution factor.

Cytokine levels in lung tissue

Cytokine concentrations in lung tissue were determined by two-mAb sandwich ELISAs purchased from R&D Systems (Minneapolis, MN) (MIP-1α, MIP-2, TNF-α) or Biosource International (Camarillo, CA) (MCP-1, IFN-γ, IL-10, IL-12p70). The clarified lung homogenates (50–100 μl) were used in each ELISA, and procedures followed the instructions of the manufacturers.

IgG1 and IgG2a serum Ab titers against C. psittaci *B577*

Flat-bottom polystyrene 96-well microtiter plates (Maxisorb Immunoplates, Nunc Nalge International, Rochester, NY) were coated by overnight incubation with 50 μl per well of UV-inactivated (30 min at 20 cm distance from 8W, 254 nm UV light source) NCI-H292-cultivated *C. psittaci* B577 EB suspended at 6×10^7 IFU/ml in 0.05 M $NaHCO_3$ (pH 9.6) or with appropriately diluted NCI-H292 cell control Ag. The plates were washed four times with PBS containing 0.05% Tween 20 (PBST) and blocked with 50 μl of 1% BSA in PBST. Fifty microliters of 1:200, 1:800, 1:3,200, and 1:12,800 dilutions of each serum in PBST were incubated in duplicates for 2 h at room temperature. Serum dilutions were aspirated, the plates were washed four times, and 50 μl of biotinylated goat anti-murine IgG1- or IgG2a-specific antisera (Southern Biotechnology Associates, Birmingham, AL), diluted 1:2,500 in PBST, were incubated for 2 h at room temperature. After aspiration and four washes with PBST, bound biotinylated Abs were detected by incubation with 50 μl of avidin-complexed biotinylated horseradish peroxidase (Vectastain *Elite* ABC; Vector Laboratories, Burlingame, CA), washing, reaction with tetramethylbenzidine substrate followed by stop solution (Kirkegaard and Perry Laboratories, Gaithersburg, MD), and spectrophotometric absorption determined at 450 nm. The OD of negative control plus 3 SDs was subtracted from each sample OD value, and a linear regression between corrected sample OD values and log-transformed dilution was calculated for the data from serum samples that were in the linear range of the ELISA ($0.2 \leq OD \leq 2.0$). The regression lines of each ELISA were adjusted to a reference ELISA by multiplication with the slope and intercept ratios of regression equations of the internal standard run on the reference and the respective microtiter plate. The intercept of the regression line of each serum sample with the baseline indicated the endpoint titer.

Delayed-type hypersensitivity (DTH) reaction against C. psittaci *B577*

DTH responses were assessed by injecting the left hind footpad of infected or naive mice with UV-inactivated *C. psittaci* B577 EB suspended at 3×10^7 IFU in 25 μl PBS (23). A 28.5-gauge insulin syringe was used for injection. Footpad swelling was measured 24 h after Ag injection by use of a spring-equipped dial thickness gauge (Swiss Precision Instruments, Los Angeles, CA), comparing thicknesses between injected and noninjected footpads. Measurable reactions were not observed after injection of control Ag in infected mice or of chlamydial Ag in naive mice.

Statistical analysis

Differences in mortality were analyzed by the Fisher Exact test, and all other results were analyzed by the two-tailed Mann-Whitney *U* test. The data shown are means ± SD of pooled results of duplicate or triplicate

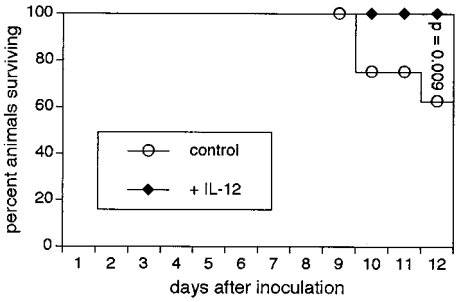

FIGURE 1. Effect of IL-12 administration on the survival of BALB/c mice after intranasal chlamydial infection. Female mice (6–8 wk old) did or did not receive single daily i.p. applications of 150 ng IL-12 from 1 day before to 6 days after intranasal inoculation of 1.9×10^6 IFU of *C. psittaci* B577. Throughout the 12-day experiment, IL-12-treated animals ($n = 16$) showed normal behavior, and all animals survived, while untreated control mice ($n = 16$) revealed clinical signs of disease, and 37.5% of the animals had died by day 12 after inoculation.

experiments with four to six mice and identical protocols, or of representative experiments of repeated experiments with minor variations of the protocol.

Results

Exogenous IL-12 administered early in C. psittaci *B577 lung infection in BALB/c mice eliminates mortality and reduces morbidity*

We first examined whether exogenous IL-12 had immediate effects on the pneumonia resulting from intranasal inoculation of chlamydiae. The chlamydial dose administered in the initial experiments, 1.9×10^6 IFU of *C. psittaci* B577, was lethal within 12 days for 37.5% of inoculated BALB/c mice. These mice exhibited, most prominently on days 3 and 4 p.i., severe clinical signs of acute disease that included: emaciated appearance, rough fur, lack of alertness, and huddling together. These signs typically became less severe on days 5–6 p.i. Beginning on day 7 p.i., many mice developed progressive signs of chronic pneumonia: emaciation and rough fur, labored respiration, and immobility. As shown in Fig. 1, mice with the most severe signs of disease typically succumbed on days 8–11 p.i. In contrast, BALB/c mice treated with IL-12 showed almost no signs of acute or chronic disease, and all animals survived to the termination of the experiment at day 12 p.i. The protective effect of IL-12 was also significantly evident in the lung weights of *Chlamydia*-infected mice compared with mock-infected control BALB/c mice (mean lung weight = 105.5 mg) and in increases of lung weights, which are reliable indicators of disease intensity. Table I shows that on day 12 p.i., the lung weights of IL-12-treated animals were 182.1 mg (72.6% increase), as compared with 273.1 mg (158.9% increase) for untreated mice. Lungs of IL-12-treated mice showed little or no macroscopic lesions, while the lungs of all untreated mice revealed major areas of consolidation. Thus, early treatment with IL-12 greatly reduced the severity of pneumonia and completely abolished mortality in BALB/c mice.

Reduced early C. psittaci *B577 pneumonia is associated with lower neutrophil:macrophage ratio in lung lesions*

To determine whether the protective effect of exogenous IL-12 influenced the nature of the chlamydial pneumonia, we analyzed the histopathological lesions in lungs from IL-12-treated vs untreated animals on days 4 and 12 after intranasal inoculation of 1.9×10^6 IFU of *C. psittaci* B577. As evident in Fig. 2*A*, on day 4 p.i., untreated animals exhibited a bronchointerstitial pneumonia with extensive interstitial and alveolar infiltrates composed predominantly of neutrophils with relatively few macrophages. In striking contrast, Fig. 2*B* demonstrates that the bronchopneumonia was much less severe in IL-12-treated mice, and the cellular infiltrates consisted predominantly of macrophages. Histological enumeration of neutrophils and macrophages revealed a neutrophil:macrophage ratio of 1.96 in untreated animals (Table I), significantly higher than the ratio of 0.51 in IL-12-treated animals. We observed that the reduced ratio in IL-12-treated mice resulted from a reduction in neutrophil numbers (32.5/eyefield in IL-12-treated mice vs 51.3 in controls) accompanied by an increase in macrophage numbers (60.5/eyefield in IL-12-treated mice vs 30.5 in controls).

Lungs of both IL-12-treated and untreated mice showed similar lesions of interstitial pneumonia on day 12 p.i. with infiltrates composed predominantly of macrophages, some lymphocytes, and few neutrophils. The neutrophil:macrophage ratios of treated and untreated animals were low and virtually identical (0.33 vs 0.25; 17.5 and 14.5 neutrophils/eyefield, 53 and 59.5 macrophages/eyefield in IL-12-treated mice and controls, respectively). Thus, IL-12 treatment had a striking effect on the early, but not the late, inflammatory lung infiltrate by reducing the neutrophil and increasing the macrophage component.

IL-12 enhances the lung clearance of C. psittaci *B577*

Next, we asked if IL-12-mediated protection against chlamydial pneumonia was associated with more effective elimination of the agent, and thus resulted in a lower antigenic stimulus. To avoid possible saturation effects at very high chlamydial doses and to more closely approximate natural chlamydial infection, we used a lower chlamydial inocula in these and subsequent experiments for analysis of cytokine profiles in lung tissue. On day 12 p.i. of 2.7×10^5 IFU of *C. psittaci* B577, the total chlamydial lung burden was 2.89×10^5 IFU in IL-12-treated animals, significantly lower than

Table I. *Effect of exogenous IL-12 on lung weight, disease severity (lung weight increase), neutrophil:macrophage ratios in lung lesions, and chlamydial lung burden after intranasal inoculation of* C. psittaci *B577*

	Lung Weight Day 12 (mg)[a]	% Lung Weight Increase Day 12[a]	Neutrophil:Macrophage Ratio Day 4[a]	Neutrophil:Macrophage Ratio Day 12[a]	*C. psittaci* B577 (IFU/lung) Day 12[b]
Control	273.1 ± 109.5^c ($n = 16$)	158.9 ± 103.8 ($n = 16$)	1.96 ± 1.40 ($n = 4$)	0.25 ± 0.11 ($n = 4$)	$11.00 \pm 4.13 \times 10^{5c}$ ($n = 4$)
+IL-12	182.1 ± 43.0* ($n = 16$)	72.6 ± 40.7* ($n = 16$)	0.51 ± 0.12** ($n = 4$)	0.33 ± 0.16 ($n = 4$)	$2.89 \pm 2.92 \times 10^5$** ($n = 4$)

[a] After inoculation of 1.9×10^6 IFU *C. psittaci* B577.
[b] After inoculation of 2.7×10^5 IFU *C. psittaci* B577.
[c] Values are mean ± SD from a representative experiment; lung weights of mock-infected BALB/c mice are 105.5 ± 6.5 mg.
* $p = 0.016$
** $p = 0.021$

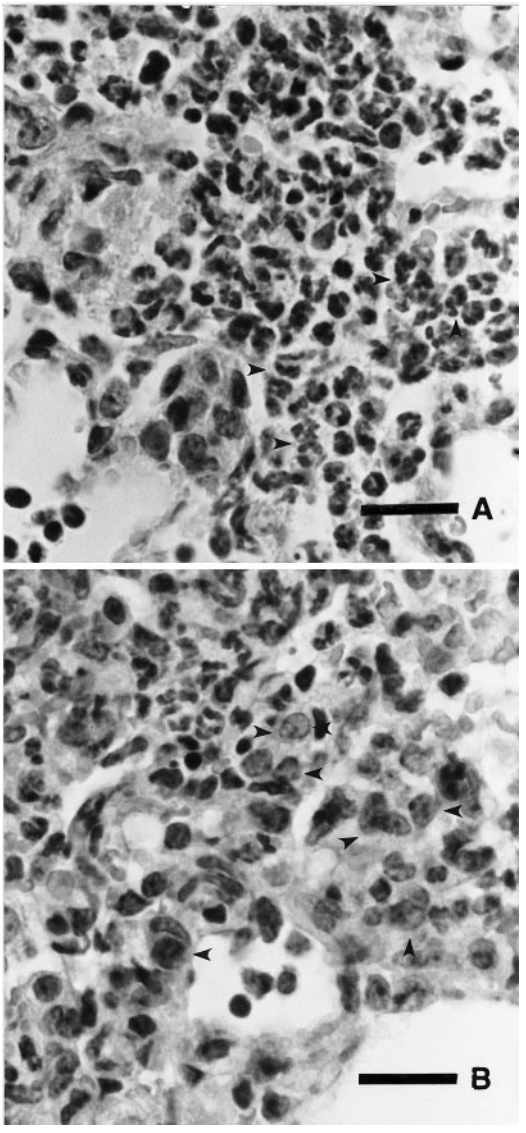

FIGURE 2. Lung sections of BALB/c mice on day 4 after intranasal inoculation of 1.9×10^6 IFU of *C. psittaci* B577, untreated controls (*A*) or with 7-day i.p. application of 150 ng IL-12 (*B*). Alveoli of untreated mice are filled with neutrophils (*A*, arrowheads); alveoli and alveolar septa in IL-12-treated mice are infiltrated predominantly by macrophages (*B*, arrowheads). Bar = 20 μm. Untreated mice have bronchointerstitial pneumonia with extensive interstitial and alveolar infiltrates composed predominantly of neutrophils and few macrophages. IL-12-treated mice have a much less severe bronchopneumonia with interstitial, and less alveolar, infiltrates composed predominantly of macrophages and fewer neutrophils.

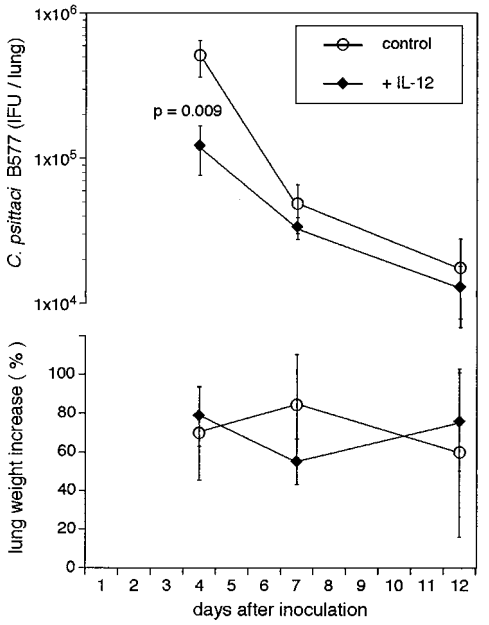

FIGURE 3. Time course of chlamydial lung load and disease severity (lung weight increase) after intranasal inoculation of mice with 8.2×10^4 IFU *C. psittaci* B577, with (+IL-12) or without (controls) 7-day IL-12 treatment. IL-12-treated mice ($n = 5$) have a substantially lower chlamydial lung load on day 4 than untreated mice ($n = 5$). The differences in chlamydial IFUs/lung are reduced on days 7 and 12, but IL-12-treated mice always have the lower chlamydial burden. Lung weights do not differ significantly between the groups ($n = 9$) and reflect low-level chlamydial pneumonitis without macroscopic lesions.

the 11×10^5 IFU in untreated control mice (Table I). Fig. 3 shows the results of a time-course experiment after intranasal inoculation of 8.2×10^4 IFU of *C. psittaci* B577. At this low chlamydial dose, the disease intensity, expressed as percent increase in lung weight over mock-infected BALB/c mice, did not differ between the groups and remained low (55–84%) throughout days 4–12 p.i. These values are typical for low-level chlamydial pneumonitis without macroscopic lesions. However, untreated controls on day 4 p.i. had a significantly higher total chlamydial lung load (5.04×10^5 IFU) than IL-12-treated mice (1.22×10^5 IFU). These differences narrowed to 4.8×10^4 vs 3.33×10^4 IFU on day 7 p.i. and to 1.73×10^4 vs 1.28×10^4 IFU on day 12 p.i., but IL-12-treated mice always had lower chlamydial burdens. These data demonstrated that IL-12 directly affected the early innate immune response to chlamydial lung infection in naive mice and that this influence resulted in significantly enhanced elimination of chlamydiae.

Lower lung tissue levels of MIP-2, MCP-1, and TNF-α are correlated with the IL-12-mediated reduction of lung neutrophils and chlamydial burden in C. psittaci *B577 pneumonia*

Based on the previous observations of IL-12-associated effects on the lung inflammatory infiltrate and chlamydial burden, we sought to identify regulatory chemokine and cytokine interactions. We determined the lung tissue profiles of major chemokines directing the recruitment and activation of neutrophils and macrophages in inflammation (24–26) and of important cytokines regulating inflammatory responses and directing antichlamydial innate and adaptive immunity (7–9). The murine C-X-C chemokine, MIP-2, is the putative functional homologue of human IL-8 and is considered to be the principal neutrophil attracting and activating chemokine in mice (24, 27, 28). Paralleling the histopathological appearance of lung lesions, we found (Fig. 4) a significantly reduced lung tissue concentration of MIP-2 in IL-12-treated mice on day 4 p.i., as compared with untreated animals (258 pg/100 mg vs 626 pg/100 mg). On day 7 p.i., the MIP-2 concentration was lower than on day 4 p.i., and differences between the groups were not significant (126 pg/100 mg vs 144 pg/100 mg). On day 12 p.i., MIP-2 concentrations in lung tissue were further reduced and virtually identical in both IL-12-treated and untreated mice (28 pg/100 mg vs 26 pg/100 mg).

MIP-1α is a proinflammatory C-C chemokine involved in macrophage and neutrophil recruitment and activation, and it potentiates the actions of IFN-γ on monocytes (24, 29–31). At the low-dose infection, we did not observe evidence for a regulatory role of

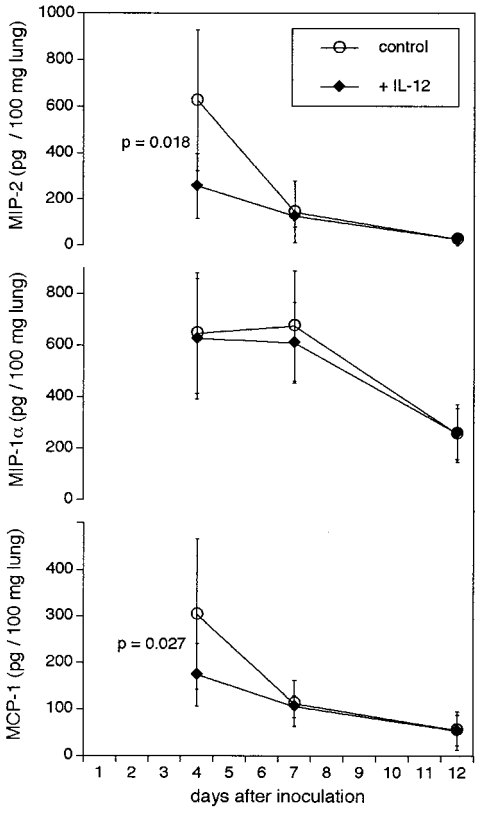

FIGURE 4. Effect of exogenous IL-12 on chemokine levels in lung tissue after intranasal inoculation of 8.2×10^4 IFU *C. psittaci* B577. The mice did (+IL-12; $n = 9$) or did not (control; $n = 9$) receive daily i.p. injections of 150 ng rmIL-12 between 1 day before and 6 days after inoculation of chlamydiae. MIP-2 and MCP-1 levels on day 4 p.i. are significantly reduced in IL-12-treated animals as compared with untreated controls. MIP-1α levels are similar in both groups. All chemokine levels declined substantially from day 4 to day 12 p.i.

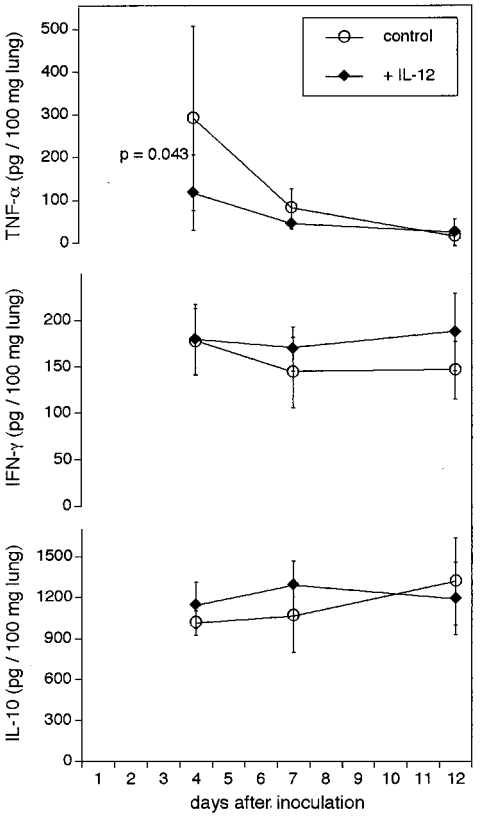

FIGURE 5. Cytokine levels in lung tissue after intranasal inoculation of 8.2×10^4 IFU *C. psittaci* B577 in the presence or absence of exogenous IL-12 (150 ng i.p./day from days -1 to $+6$). TNF-α on day 4 p.i. is significantly lowered under IL-12 administration, and the concentrations decline from day 4 to day 12 p.i. Concentrations of IFN-γ and IL-10 in lung tissue do not differ significantly between the groups ($n = 9$), and both remain constant or rise marginally from day 4 to day 12 p.i.

IL-12 on the expression of MIP-1α; similar concentrations of this chemokine were measured in IL-12-treated and untreated mice at all time points (Fig. 4). MIP-1α concentrations on days 4 and 7 p.i. remained steady at ~650 pg/100 mg lung tissue, but were reduced to ~260 pg/100 mg on day 12 p.i.

The C-C chemokine MCP-1 has central importance in monocyte recruitment, but not activation, and appears to have regulatory anti-inflammatory properties (26, 31, 32). It is required for development of oral immunological tolerance through its inhibition of Th1-related cytokines, and it promotes Th2 differentiation of $CD4^+$ T cells (31, 33). As evident in Fig. 4, IL-12 significantly down-regulated MCP-1 expression in *Chlamydia*-infected lung tissue on day 4 p.i. (174 pg/100 mg lung in IL-12-treated mice vs 304 pg/100 mg in untreated mice). On days 7 and 12 p.i., MCP-1 levels were lower and virtually identical in IL-12-treated and untreated animals (~110 pg/100 mg on day 7 p.i. and ~55 pg/100 mg on day 12 p.i.).

TNF-α, IFN-γ, and IL-10 are prominently involved in orchestrating and effecting antichlamydial immune defenses. TNF-α and IFN-γ increase resistance, and IL-10 decreases resistance (7–9). Both TNF-α and IFN-γ activate and prime macrophages for IL-12 production upon microbial stimulation, and, subsequently, IL-12 typically enhances IFN-γ output from NK cells early in infection with intracellular pathogens (11, 34, 35). IL-10 typically acts in this feedback loop as a negative regulator in addition to its anti-inflammatory and Th2-inducing properties (36, 37). We observed that under the influence of exogenous IL-12, the TNF-α level in lung tissue was significantly suppressed on day 4 after chlamydial inoculation (117 pg/100 mg lung in IL-12-treated mice vs 291 pg/100 mg in untreated mice; Fig. 5). The TNF-α lung levels were lower on days 7 and 12 p.i. and did not differ significantly between IL-12-treated and untreated groups (45 and 25 pg/100 mg lung on days 7 and 12 p.i., respectively, in IL-12-treated mice vs 82 and 15 pg/100 mg in untreated mice).

Unexpectedly, lung tissue levels of IFN-γ appeared to be independent of regulation by IL-12, and were essentially identical on day 4 p.i. in both IL-12-treated and untreated groups (180 pg/100 mg lung in IL-12-treated mice vs 177 pg/100 mg in untreated mice; Fig. 5), at a time point when IL-12 was still being administered to the treatment group. On day 7 p.i., the IFN-γ lung tissue level had declined somewhat in untreated mice (169 pg/100 mg lung in IL-12-treated mice vs 144 pg/100 mg in untreated mice); on day 12 p.i., the level remained the same in untreated mice, but had increased marginally in IL-12-treated animals (187 pg/100 mg lung in IL-12-treated mice vs 146 pg/100 mg in untreated mice). However, these differences were not significant at any time point.

IL-10 levels were higher in lung tissue of IL-12-treated mice on days 4 and 7 p.i. (1143 and 1291 pg/100 mg lung on days 4 and 7 p.i., respectively, in IL-12-treated mice vs 1014 and 1065 pg/100 mg in untreated mice). This relationship was reversed on day 12 p.i., when the IL-10 tissue concentration was higher in untreated mice (1191 pg/100 mg lung in IL-12-treated mice vs 1318

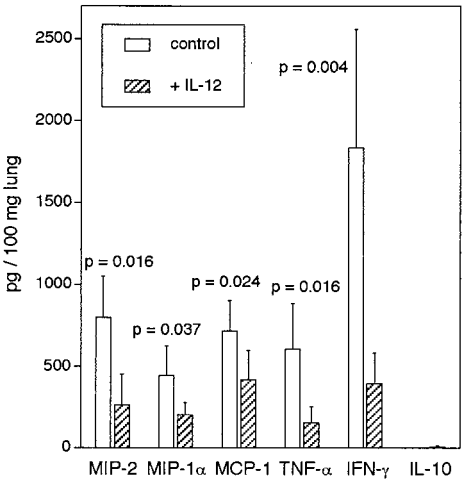

FIGURE 6. Cytokine levels in lung tissue 4 days after high-dose intranasal inoculation of 1.9×10^6 IFU *C. psittaci* B577 in the presence or absence of exogenous IL-12. MIP-2, MIP-1α, MCP-1, TNF-α, and IFN-γ concentrations are significantly reduced in mice receiving IL-12 ($n = 6$), as compared with untreated controls ($n = 6$). IL-10 in lung tissue is marginally above the detection limit in IL-12-treated mice and undetectable in controls.

pg/100 mg in untreated mice). None of the differences in IL-10 concentrations were significant.

Based on these findings, we asked whether similar changes in chemokine lung concentrations were associated with IL-12-mediated protection early in high-level chlamydial lung infection. Mice were infected with 1.9×10^6 IFU *C. psittaci* B577, treated or not treated with IL-12, and sacrificed 4 days after inoculation. As evident in Fig. 6, lung tissue levels of MIP-2 were significantly lower in IL-12-treated mice than in controls (265 pg/100 mg lung vs 797 pg/100 mg). Likewise, significant reductions in tissue concentrations were also observed for MIP-1α (205 pg/100 mg vs 445 pg/100 mg), MCP-1 (419 pg/100 mg vs 717 pg/100 mg), TNF-α (156 pg/100 mg vs 606 pg/100 mg), and unexpectedly for IFN-γ (397 pg/100 mg vs 1834 pg/100 mg). IL-10 in lung tissue was at the detection limit in IL-12-treated mice (6 pg/100 mg lung) and was undetectable in the controls.

In summary, MIP-2, MCP-1, and TNF-α lung tissue concentrations were substantially reduced as a result of exogenous IL-12 administered early in *C. psittaci* murine lung infection. MIP-1α and IFN-γ were independent of IL-12 regulation at low infectious dose, but were significantly down-regulated by IL-12 at high-level chlamydial inoculation. IL-10 lung tissue expression at low chlamydial infection was not modulated by IL-12, but essentially completely and independently of IL-12 repressed at high chlamydial inoculation. The IL-12-mediated down-regulation of MIP-2, MCP-1, TNF-α, MIP-1α, and IFN-γ was accompanied by a major shift in the lung inflammatory phenotype characterized by fewer neutrophils, enhanced clearance of chlamydial organisms, lower morbidity, and elimination of mortality.

IL-12 induces Th1-like Ab patterns against C. psittaci *B577*

To assess the phenotype of the Th cell response to *C. psittaci* lung infection elicited in the presence or absence of exogenous IL-12, we determined the levels of Ig isotypes G1 and G2a against *C. psittaci* B577, their ratios, and the DTH response to the agent. Th1-released cytokines (typically IFN-γ) evoke lower Ig production by B cells than Th2 cytokines (typically IL-4), but the difference is greater for IgG1 than for IgG2a, resulting in a lower IgG1:

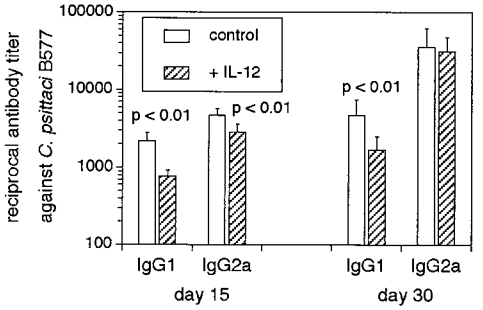

FIGURE 7. IgG1 and IgG2a Abs against *C. psittaci* B577 on days 15 and 30 after intranasal inoculation of 8.2×10^5 IFU *C. psittaci* B577, with or without IL-12 treatment. IgG1 titers in untreated controls (day 15, $n = 6$; day 30, $n = 12$) are higher at both time points than in IL-12-treated BALB/c mice (day 15, $n = 8$; day 30, $n = 12$). IgG2a titers are significantly elevated in controls on day 15 but are essentially identical in controls and IL-12-treated mice on day 30.

IgG2a ratio for Th1-polarized responses than for Th2 responses (38). DTH responses are elicited by Th1 cells, but may be suppressed by Th2 cells (6), and thus reflect the absolute level of Th1 cells as well as their ratio to Th2 cells of the same antigenic specificity.

Mice were intranasally infected with 8.2×10^5 IFU *C. psittaci* B577 with or without IL-12 treatment, and serum Ab titers and DTH responses in separate groups were determined on days 15 and 30 p.i. We found the IgG1 Ab titers on days 15 and 30 p.i. consistently and significantly higher in control mice than in IL-12-treated animals. IgG2a titers on day 15 were also significantly higher in controls, but were essentially identical on day 30 in both controls and IL-12-treated mice (Fig. 7). Thus, the IgG1:IgG2a ratios at both time points were lower in IL-12-treated animals (Fig. 8). These data clearly support the notion of a Th1 shift in the Th cell phenotype after IL-12 administration during the early phase of *C. psittaci* lung infection in BALB/c mice.

Measurement of DTH responses to UV-inactivated *C. psittaci* B577 revealed the unexpected result of lower DTH responses on both day 15 and day 30 p.i. in IL-12-treated mice vs untreated control animals (Fig. 8). While it failed to reach significance due to relatively high variation among individuals within the groups, the reduced DTH after IL-12 stimulation was consistently reproduced in several experiments and after different doses of intranasal chlamydial inocula. While we rule out suppression of the DTH in

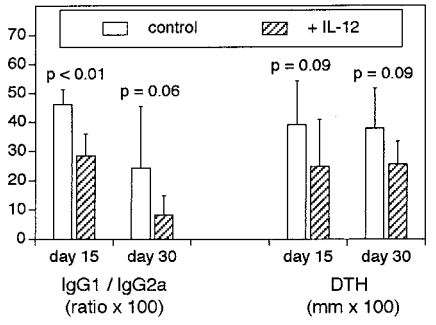

FIGURE 8. IgG1 to IgG2a Ab ratios and DTH responses to *C. psittaci* B577 on days 15 and 30 after intranasal inoculation of 8.2×10^5 IFU *C. psittaci* B577, with or without IL-12 treatment. The IgG1/IgG2a ratios of IL-12-treated animals are lower at both time points than those of untreated controls, indicating a Th1 shift in the immune response of IL-12-treated mice. DTH responses in IL-12-treated animals ($n = 8$) are lower than those of untreated controls ($n = 8$), but the differences fail to reach significance.

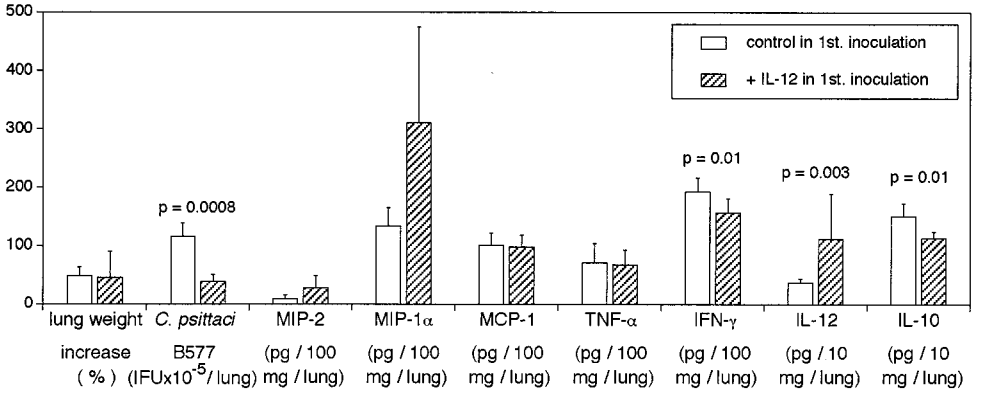

FIGURE 9. Intranasal reinfection of BALB/c mice with 1.4×10^7 IFU *C. psittaci* B577. BALB/c mice received a primary intranasal infection of 8.2×10^5 IFU *C. psittaci* B577, with or without IL-12 treatment. They were reinfected 7 wk later and sacrificed after 7 days. As evidenced by the marginal lung weight increases, both IL-12-treated ($n = 8$) and untreated animals ($n = 8$) were fully protected against disease caused by the reinfection. The chlamydial lung burden was, however, significantly lower in IL-12-primed animals. The cytokine profile suggests that the additional protective effect in IL-12-primed mice was mainly mediated by elevated levels of MIP-1α and IL-12.

IL-12-treated animals by Th2 cells, the low DTH might be the result of lower absolute numbers of Th1 cells in IL-12-treated mice than in control animals. The Ab data suggest that such a reduced amount of Th1 cells would be accompanied by a proportionally greater reduction in Th2 cell numbers, thus shifting the polarity of the Th response to Th1 after IL-12 treatment.

IL-12 treatment in primary infection is associated with enhanced resolution of, and a Th1-polarizing lung cytokine profile in, secondary C. psittaci *B577 lung infection*

We performed secondary intranasal infection experiments to determine whether the beneficial immune response generated by IL-12 administration during primary infection was maintained and resulted in a more effective recall response to chlamydial infection than in untreated BALB/c mice. Seven weeks after primary infection with 8.2×10^5 IFU *C. psittaci* B577 with or without IL-12 treatment, the mice were intranasally reinoculated with 1.4×10^7 IFU *C. psittaci* B577 and sacrificed on day 7 p.i. Evident from the marginal lung weight increases shown in Fig. 9, both groups were protected against induction of disease. The lung weight increases of 45–50% over those recorded for mock-infected mice were not accompanied by any clinical or gross signs of lung disease. Typical for lungs of protected animals following a recent reinfection, the histological appearance of lung tissue was characterized by peribronchiolar and perivascular mononuclear infiltrates composed mostly of lymphocytes and few macrophages. However, the total chlamydial lung burden in IL-12-primed animals was significantly lower (3.82×10^6 IFU) than in untreated controls (11.5×10^6 IFU). Thus, administration of IL-12 in the early phase of the primary chlamydial lung infection mediated priming of a *Chlamydia*-specific immune cell population that in the recall response resulted in a substantially more efficient elimination of chlamydiae than the immune cells of non-IL-12-primed mice, without increasing disease.

The chemokine and cytokine profiles of the lung tissue on day 7 after reinfection revealed substantial differences between IL-12-primed animals and nonprimed controls (Fig. 9). The concentration of IL-12p70 in IL-12-primed mice was significantly elevated ($p = 0.003$) over the controls (1112 pg/100 mg lung tissue of IL-12-primed mice vs 369 pg/100 mg of controls). MIP-1α levels in IL-12-primed mice were almost 3-fold greater, but the elevated levels failed to reach significance (311 pg/100 mg lung tissue of IL-12-primed mice vs 133 pg/100 mg of controls). Interestingly, IFN-γ was reduced in IL-12-primed mice (156 pg/100 mg lung vs 193 pg/100 mg in controls), as was IL-10 (1131 pg/100 mg lung vs 1506 pg/100 mg in controls). TNF-α and MCP-1 concentrations were essentially identical in both IL-12-primed and nonprimed mice, and MIP-2 levels were marginal in both groups. The lack of an enhanced IFN-γ expression in IL-12-primed mice suggests that a highly polarized *Chlamydia*-specific Th1 effector cell population was not created in these mice, just as highly polarized *Chlamydia*-specific Th2 effector cells were not elicited in the unprimed controls during primary infection (6, 39). However, the highly elevated levels of MIP-1α, IL-12p70, and the reduced level of IL-10 indicate a cytokine environment in IL-12-primed mice that would activate macrophages more effectively than that of non-IL-12-primed mice (29, 31, 36, 40). Enhanced levels of MIP-1α would potentiate macrophage activation induced by IFN-γ, while reduced levels of IL-10 would result in less inhibition of macrophages. Both of these effects could help to explain the strongly elevated IL-12p70 levels in IL-12-primed mice, presumably produced by activated macrophages. Thus, the functional properties of a Th1-polarized recall response might be accomplished in IL-12-primed mice by chemokine and cytokine regulation deviating from the classical Th1 paradigm of IFN-γ up-regulation. In addition to activating phagocytic effector cells, high levels of MIP-1α and the ratio of IL-10 to IL-12p70 concentrations in IL-12-primed mice (1.32 vs 4.26 in non-IL-12-primed animals, $p = 0.029$) both favor Th1 differentiation (31, 36). Therefore, the lung tissue cytokine profile is also highly conducive for maintenance of the existing Th1 bias in the *Chlamydia*-specific $CD4^+$ T cell population and further enhancing it.

Discussion

Recent research efforts have conclusively established that MHC class II-restricted immune responses by $CD4^+$ T cells are indispensible for immune clearance of chlamydial infections (4). Among the distinct $CD4^+$ T cell subsets (6), predominantly the Th1 type is associated with resolution of chlamydial infections (7, 8, 10). These results were derived from comparative analyses of the resistance of mouse strains to chlamydial infection, the effects of neutralization of immune molecules, or the adoptive transfer of *Chlamydia*-specific Th1 cells. In this study, we asked the direct

question if biasing the nascent *Chlamydia*-specific immune response toward Th1 immunity by administration of IL-12, the initiation cytokine for Th1 cell-mediated immunity (41), protects against, and enhances resolution of, chlamydial lung infection. Our data indicate the induction of substantial immediate and long-term protection and suggest the enhancement of an existing Th1 polarity during the recall response against *Chlamydia*.

It is not surprising that parenteral IL-12 administration induced a Th1-biased adaptive response against *C. psittaci* B577, which resulted in improved resolution of primary as well as secondary chlamydial infection (42, 43). However, the striking immediate protective effect during the innate phase of the immune response to chlamydial infection, and the mechanisms of this protection, as suggested by histopathological analyses and cytokine profiles, were unexpected. Early during infections with intracellular pathogens, IL-12 is produced mainly by macrophages stimulated by microbial products but also by dendritic cells and polymorphonuclear granulocytes (36, 44–46). IL-12 is thought to activate NK cell cytolytic activity and IFN-γ production (11, 40, 47, 48). NK cell-produced IFN-γ along with macrophage-produced TNF-α further up-regulates macrophages in a positive feedback loop to produce more IL-12 (12, 34, 49). This regulatory interaction is a necessary prerequisite for successful resolution of infections with many intracellular pathogens (35, 50–52). Analogous to these observations, we expected an up-regulation of IFN-γ and TNF-α expression in response to exogenous IL-12 during primary *C. psittaci* infection. However, quite unexpectedly, IFN-γ production in lung tissue was independent of IL-12 regulation at low chlamydial infection and strongly down-regulated at high infection. Likewise, TNF-α expression was significantly reduced rather than enhanced. Expression of IL-10, which antagonizes many functions of IL-12 (36, 37), was unchanged by IL-12 at low infection and virtually completely repressed in the early phase of high-dose chlamydial lung infection. Guler et al. (53) implicated a loss of IL-12 responsiveness of BALB/c-derived T cells during in vitro culture as the cause of the inability of BALB/c mice to generate Th1 responses to *Leishmania*. Such a genetically determined lack of IL-12 responsiveness could be invoked to explain the unexpected cytokine profile in our experiments. It would, however, also have abolished effects that we actually observed, i.e., the Th1 bias in the adaptive immune response to *Chlamydia*.

As a result of IL-12 administration, we observed in contrast to the paradigm of enhanced IFN-γ levels striking changes in chemokine expression, accompanied by a major shift in the inflammatory infiltrate toward a relative abundance of macrophages over neutrophils early in chlamydial lung infection. The beneficial down-regulation of the neutrophil influx may be attributed to the reduced level of MIP-2, which appears to have nonredundant functions in neutrophil recruitment in mice. Murine MIP-2 is the only high-affinity ligand known of the murine homologue of the two human IL-8 receptors that mediate chemotaxis and activation of neutrophils (27, 28). Another component in a possible differential regulation network of the protective effects of IL-12 in murine chlamydial pneumonitis appears to be MCP-1, which was significantly reduced as a result of IL-12 treatment. MCP-1 is a potent macrophage chemoattractant important for recruitment, but not for activation, of macrophages (26, 54). High levels of MCP-1 are associated with increased susceptibility to intracellular pathogens and enhanced Th2 cell maturation and function (32, 55). MCP-1 also inhibits IL-12 production by inflammatory macrophages, and in tandem with MIP-1α, acts as a chemokine regulating Th1/Th2 lymphocyte differentiation (31, 33). In the chlamydial lung infection, the IL-12-mediated down-regulation of MCP-1, combined with no or minor reduction in MIP-1α lung tissue levels, suggests an enhanced effect of MIP-1α in macrophage activation and directing Th1 commitment of *Chlamydia*-specific proliferating $CD4^+$ T cells (30, 31, 56). Collectively, our data indicate the anticipated overall beneficial effect of exogenous IL-12 on chlamydial infection, but suggest a regulatory pathway different from the conventional macrophage-IL-12 NK cell-IFN-γ axis. In this alternative pathway, down-modulation of the levels of the chemokines MIP-2 and MCP-1, along with reduction of the proinflammatory cytokine TNF-α, and at high infection levels also of IFN-γ and MIP-1α, accomplishes a shift to a macrophage-dominated inflammatory phenotype that is more effective at eliminating chlamydiae than the purulent inflammation observed in untreated mice. In the scenario at low-level infection, the relative excess of MIP-1α over MCP-1 would enhance macrophage activation. Furthermore, this relative excess of MIP-1α in IL-12-treated mice, extending well into the priming phase of the adaptive immune response, also favors Th1 commitment of the *Chlamydia*-specific Th cell immunity.

Among the effects of IL-12 on the adaptive response to chlamydial lung infection, the consistent reduction of DTH against chlamydial Ag is contrary to the anticipated increase (6, 7). Of the two most likely explanations for this observation, we favor the hypothesis that the rapid reduction in chlamydial load during infection under the influence of exogenous IL-12 provides a reduced antigenic stimulus and results in a smaller burst size of the T cell response than in untreated animals. Several parameters, however, suggest a Th1 bias of the IL-12-influenced Th immunity, including: reduced ratios of IgG1 to IgG2a Abs against *C. psittaci* B577, and of IL-10 to IFN-γ concentrations in lung tissue on day 12 after primary infection (6.36 in untreated mice vs 9.02 in IL-12-treated mice, $p = 0.23$); and the Th1-inducing cytokine profile in secondary chlamydial lung infection. Thus, while smaller in magnitude, as indicated by the reduced DTH, the *Chlamydia*-specific $CD4^+$ T cell population in IL-12-treated animals would be polarized toward the Th1 phenotype. The other, more unlikely explanation for the reduced DTH would be suppression of a substantial Th1 response by a *Chlamydia*-specific Th2 cell population. A conclusive distinction between these alternatives will require evaluation of the functional properties of the *Chlamydia*-specific T helper cell population by intracellular cytokine staining and flow-cytometric enumeration (57).

In secondary chlamydial infection, we found a prolonged effect of IL-12 administered early in primary infection as evidenced by the significantly accelerated elimination of chlamydiae and the Th1-inducing lung cytokine profile. Thus, our data strongly suggest that the Th1 bias created during primary infection is maintained in the memory T cell population. This is in contrast to other systems, in which an initial polarization of the Th response reverts over time to the genetically determined Th-type predisposition of a particular mouse strain (58). Furthermore, the lung cytokine profile of IL-12-primed mice 7 days after secondary infection is characterized by elevated IL-12p70 and MIP-1α, unchanged TNF-α and MCP-1, and reduced IFN-γ and IL-10 levels, as compared with untreated controls. While this profile, similar to the primary infection profile, is not entirely compatible with the paradigm of Th1 cytokine expression, it strongly implies perpetuation of the Th1 bias of T memory cells during a recall response. These results suggest that it might be rational to use IL-12 as an adjuvant in future vaccination against chlamydial infection, since the beneficial Th1 immune phenotype introduced might be maintained in boosting cycles of natural infection or vaccination.

Previous studies have demonstrated that IL-12-induced Th1 immunity is necessary for protection against, and clearance of, chlamydial infections. Collectively, our results extend this observation by demonstrating that early in chlamydial infection, IL-12 has a

direct protective effect. Surprisingly, the infected lung cytokine levels argue against a mechanism for protection based on up-regulation of IFN-γ but instead indicate a major involvement of chemokines. We are not aware of previous reports that address the observations reported here concerning the IL-12-associated reduction in MIP-2 expression and tissue recruitment of neutrophils. Our data suggest that endogenous IL-12 and IL-12 response pathways exert a major influence not only on the development of adaptive immunity but also on the phenotype of chlamydial disease via chemokine regulatory pathways. Genetically determined differences in endogenous IL-12 production and responses of individuals in outbred populations could be determinants of disease outcomes characterized by poor chlamydial clearance and a purulent inflammatory infiltrate vs effective elimination of chlamydiae in a macrophage-dominated response.

Acknowledgments

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