

Chlamydia trachomatis Mouse Pneumonitis Lung Infection in IL-18 and IL-12 Knockout Mice: IL-12 Is Dominant over IL-18 for Protective Immunity

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Abstract

Background: Interferon (IFN)- γ is a key to protective immunity against a variety of intracellular bacterial infections, including *Chlamydia trachomatis*. Interleukin (IL)-18, a recently identified Th1 cytokine, together with IL-12 is a strong stimulator for IFN- γ production. We investigated the relative roles of IL-18 and IL-12 in protective immunity to *C. trachomatis* mouse pneumonitis (MoPn) infection using gene knockout (KO) and wild-type (WT) mice.

Materials and Methods: Mice were intranasally infected with *C. trachomatis* MoPn and protective immunity was assessed among groups of mice by daily body weight changes, lung growth of MoPn, and histopathological appearances at day 10 postinfection. The corresponding immune responses for each group of mice at the same postinfection time point were evaluated by measuring antigen-specific antibody isotype responses and cytokine profiles.

Results: Our results showed that IL-18 deficiency had little or no influence on clearance of MoPn from the lung, although KO mice exhibited slightly more severe inflammatory reactions in lung tissues, as well as reduced systemic and local IFN- γ production, com-

pared with WT mice. Results with IL-18 KO mice were in sharp contrast to those observed with IL-12 KO mice that showed substantially reduced clearance of MoPn from the lungs, substantial reductions of antigen-specific systemic and lung IFN- γ production, decreased ratio of MoPn-specific immunoglobulin G (IgG)2a/IgG1, and severe pathological changes in the lung with extensive polymorphonuclear, instead of mononuclear, cell infiltration. Exogenous IL-12 or IL-18 was able to increase IFN- γ production in IL-18 KO mice; whereas, only exogenous IL-12, but not IL-18, enhanced IFN- γ production in IL-12 KO mice. Caspase-1 is the key protease for activation of IL-18 precursor into the bioactive form, and caspase-1 KO mice also displayed similar bacterial clearance and body weight loss to that in WT mice at early stages of MoPn infection. This further confirmed that IL-18 was not essential for host defense against chlamydia infection. **Conclusions:** These results suggest that IL-12, rather than IL-18, plays the dominant role in the development of protective immunity against chlamydia lung infection, although both cytokines are involved in the in vivo regulation of IFN- γ production.

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Introduction

Chlamydia trachomatis is an obligate intracellular bacterium that causes a variety of human diseases, including sexually transmitted diseases, trachoma, and infant pneumonia. Several cytokines are known to play critical roles in host resistance or susceptibility to chlamydial infec-

tion, including interferon (IFN)- γ , interleukin (IL)-12, and IL-10. IL-12 is a powerful factor for the generation of T helper-1 (Th1) cell-mediated immune responses and is required for the eradication of chlamydial genital infection (1,2). Administration of exogenous IL-12 early after infection significantly alleviated the severity of chlamydial pneumonia, abolished mortality, and decreased chlamydia growth in vivo (3). IFN- γ is essential for preventing systemic dissemination and/or eliminating chronic persistence of chlamydia infection (1,2,4,5). Recently, we reported that IFN- γ knockout (KO) mice showed Th2-associated inflammation and failed to control *C. trachomatis* mouse pneumonitis (MoPn) infection in the lung (6). In contrast to IFN- γ KO mice, mice with genetic deficiency of endogenous IL-10 showed accelerated pulmonary clearance of MoPn and significantly enhanced delayed-type hypersensitivity (DTH) responses, which could be inhibited by local administration of recombinant interleukin (rIL)-10 (7). Th2-biased immune responses related to the overproduction of IL-10 in inbred mouse strains were associated with increased susceptibility to chlamydia infection (8,9). Overall, these results suggest that Th1 cells are required for chlamydia immunity and that IL-10 inhibits the priming and expansion of Th1 immune responses.

IL-18 is a recently characterized cytokine that acts as a costimulatory factor for the production of IFN- γ . It has been demonstrated that IL-18 can be produced in response to bacterial and inflammatory stimuli by various cell types, including activated mononuclear cells (10), macrophages (11), keratinocytes (12), and dendritic cells (13). IL-18 is synthesized in a precursor form and converted to a biologically active form by the protease, caspase-1 (14,15). IL-18 has multiple biological functions, including induction of IFN- γ production by natural killer (NK) cells and activated Th1 cells (16), enhancement of Fas ligand-mediated cytotoxicity of murine Th1 cells (17,18), augmentation of NK cell cytotoxicity (16,19), and up-regulation of intercellular adhesion molecular (ICAM)-1 expression on epidermal cells (20). Because of its ability to induce IFN- γ production, IL-18 is classified in the Th1 cytokine family, together with IL-2, IL-12, IL-15, and IFN- γ . The major role for IL-18 appears to be to interact synergistically with IL-12 to enhance IFN- γ production. The combination of IL-18 and IL-12 is far more

effective at inducing IFN- γ production than either cytokine alone (21,22).

Previous studies demonstrated the importance of IL-18 in host defense against microbial infection. IL-18 KO mice show significant defects in NK cell activity and impaired Th1 immune response following the intravenous infection with *Propionibacterium acnes* or *Mycobacterium bovis* (16). IL-18 also contributes to host resistance in mice to infection with *Mycobacterium* (23), *Leishmania major*, *Staphylococcus aureus* (24), *Salmonella typhimurium* (25), and *Herpes simplex virus* (26). In the present study, we investigated the role of endogenous IL-18 in mediating protection against *C. trachomatis* MoPn lung infection using IL-18 KO mice, and compared the relative importance of IL-18 to IL-12 in host defense to MoPn infection.

Materials and Methods

Mice

Eight- to twelve-week-old C57BL/6 mice were obtained from Charles River Canada (St. Constant, Quebec, Canada). Age and sex-matched homozygous IL-18 and IL-12 p40 KO mice with C57BL/6 background were previously described (24) or purchased from Jackson Laboratories (Bar Harbor, ME), respectively. Homozygous caspase-1 KO and matched heterozygous mice were kindly provided by Dr. Winnie Wong from BASF Bioresearch Inc. (Worcester, MA). Mice were housed at the University of Manitoba animal facility in filter-top cages.

Chlamydia Organism and Intranasal Inoculation

Chlamydia MoPn was grown in Hela 229 cells and elementary bodies (EBs) were purified on discontinuous density gradients of centrifugation as previously described (27). The infectivity of purified EBs was titrated by infection of Hela cell monolayers for 36 hr, followed by the fixation of cells with methanol and enumeration of inclusions. The cells having chlamydial inclusions were detected by the staining with anti-chlamydial lipopolysaccharide monoclonal antibody (LPS mAb) (ViroStat, Portland, ME) as previously described (27). For in vitro stimulation, EBs were heat inactivated at 70°C for 30 min.

Mice were intranasally inoculated with 1×10^4 inclusion forming unit (IFU) of MoPn and monitored daily for body weight changes. Mice

were sacrificed at 10 days after inoculation. The lungs, kidneys and livers were aseptically removed and homogenized with a cell grinder in 3 ml of sucrose phosphate glutamic acid buffer. Tissue suspensions were centrifuged at $500 \times g$ for 10 min at 4°C to remove coarse tissue debris and were frozen at -80°C until tested. For quantitation of MoPn in the homogenates of various organs, Hela 229 monolayer was inoculated with serially diluted tissue supernatants for 2 hr and followed by culture for 36 hr. The cells having chlamydial inclusions were detected as those in the titration of purified EBs.

Determination of MoPn-specific Antibody Levels

Mice were sacrificed at 10 days postinfection. Mice sera were collected for the determination of MoPn-specific antibody (Ab) responses by using ELISA as previously described (27). Briefly, mice sera were added to 96-well plate coated with 10^5 IFUs of MoPn organisms. Abs bound to the coated antigens were detected using biotin-conjugated anti-mouse immunoglobulin G (IgG)2a and IgG1 (Caltag, Burlingame, CA) and followed by the addition of streptavidin and substrate (Sigma Inc., St. Louis, MI). Results were expressed as ELISA titers at 30 min using the endpoint [cutoff at optical density (O.D.) 405 nm, 0.5] of the titration curves.

Spleen Cell Culture and Cytokine Determination

Spleens were collected at 10 days postinfection. Spleen cells were cultured at 7.5×10^6 cells/ml with or without heat-killed EBs (HK-EBs; 5×10^4 IFU/ml) in the presence or absence of 2 ng/ml of rIL-12 (PharMingen, San Diego, CA), 20 ng/ml of rIL-18 (Research Diagnostic Inc., Flanders, NJ) or anti-mouse CD4 mAb (YTS-191, kindly provided by Dr. Waldmann, University of Oxford, Oxford, U.K.) at $5 \mu\text{g/ml}$ for 3 days, as indicated. Culture supernatants were harvested for IFN- γ , IL-12, and IL-10 analysis by sandwich ELISAs. Ab pairs for determination of IFN- γ , IL-12, and IL-10 were purchased from PharMingen. Ab pairs for IL-18 were obtained from Research Diagnostic Inc. Cytokines in the lung homogenate supernatants were also tested using these ELISAs.

Histopathological Analysis

The lungs of MoPn-infected mice were isolated at day 10 postinfection and fixed in 10% buffered formalin and embedded in paraffin as previously described (6). Tissue sections ($7 \mu\text{m}$) were then stained with hematoxylin

and eosin and examined under light microscopy by a person blinded to the identification of mouse groups.

Statistical Analysis

Student's *t*-test was used for analysis of statistical significance (*p*-value).

Results

Chlamydia Clearance in IL-18 KO and Caspase-1 KO Mice

To define the role of endogenous IL-18 on *C. trachomatis* MoPn lung infection, we examined in vivo chlamydial growth and disease severity in IL-18 KO mice following intranasal infection with a sublethal dose of MoPn. As shown in Fig. 1A, IL-18 KO mice showed slightly greater body weight loss than wild type (WT) mice, but the

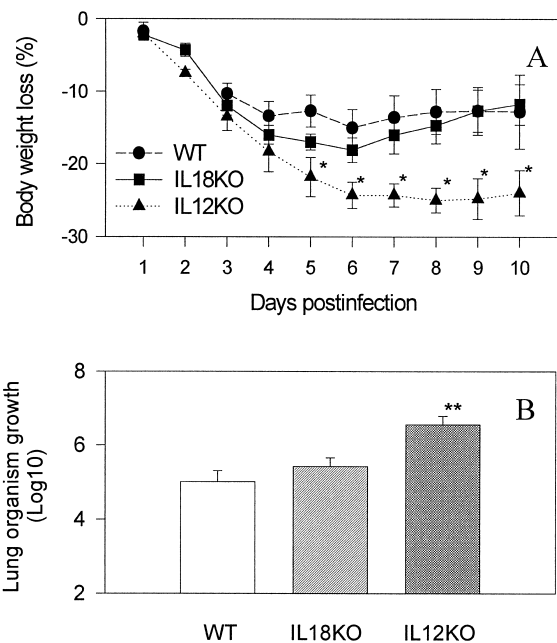


Fig. 1. Lung clearance and body weight changes after intranasal chlamydial infection. Three to four age- and sex-matched interleukin (IL)-18 knockout (KO), IL-12 KO and wild type (WT) mice were intranasally infected with 10^4 inclusion forming units (IFU) of *C. trachomatis* mouse pneumonitis (MoPn) biovar and monitored daily for body weight changes. The mice were sacrificed at 10 days postinfection and the lung homogenates were analyzed for in vivo chlamydia growth as described in "Materials and Methods." The data represent the mean \pm standard error of the mean (SEM) of the percentages of body weight loss (A) or chlamydial IFU (\log_{10}) per lung (B) from one of three separated experiments with similar results. **p* < 0.05 or ***p* < 0.01, compared with those in WT mice.

recovery of lost body weight was similar for both groups of mice. IL-18 KO mice exhibited similar lung clearance as WT mice at 10 days following MoPn infection (Fig. 1B). Unlike IL-18 KO mice, IL-12 KO mice displayed severe disease with reduced ability to clear lung infection, as well as failure to recover lost body weight (Fig. 1A, B). Viable organisms were not recovered from livers and kidneys in all mouse groups when tested at day 10 following intranasal infection (data not shown), the time of peak in vivo chlamydial growth (7). This suggested that no significant dissemination of the infection had occurred in either IL-18 or IL-12 KO mice, at least during the early stages of infection. This finding was consistent with a previous report showing severe, but not disseminated, genital tract infection in IL-12-deleted mice (2). Since caspase-1 is a cysteine protease that is required for the proteolytic activation of the IL-18 precursor into a bioactive form (14,15), we took an alternative approach to evaluating the role of IL-18 in the development of anti-chlamydia immunity by using caspase-1 KO mice. Consistent with the observations in IL-18 KO mice, there was no significant difference in body weight loss (data not shown) and chlamydia growth in the lungs of caspase-1 KO and WT mice at 10 days following lung infection [6.8 ± 0.6 (KO) vs. 6.3 ± 0.5 (WT), \log_{10} IFU/lung, $n = 5$, $p > 0.05$]. The results indicated that, unlike IL-12, endogenous IL-18 played a limited role in host defense against chlamydial infection.

Immune Responses in IL-18 KO Mice Following Chlamydia Lung Infection

It has been established that Th₁ cell-mediated immune responses play a dominant role in protection against chlamydial infection and that Th1 or Th2 immune response can be inferred by the measurement of antibody isotypes and

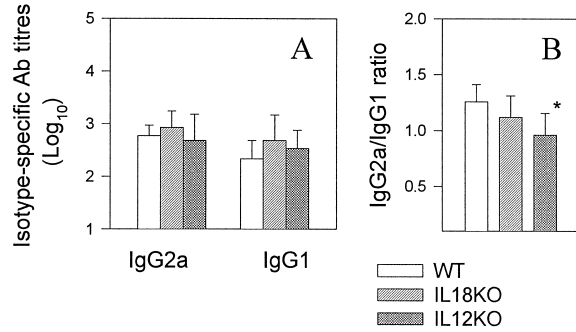


Fig. 2. MoPn-specific IgG2a and IgG1 responses following chlamydial infection. Age- and sex-matched interleukin (IL)-18 knockout (KO), IL-12 KO and wild type (WT) mice were intranasally infected with mouse pneumonitis (MoPn) (1×10^4 inclusion forming units (IFU)/mouse). Sera were collected at 10 days postinfection for determination of MoPn-specific immunoglobulin G (IgG)2a and IgG1 using ELISA assays described in “Materials and Methods.” The data are expressed as mean \pm standard error of the mean (SEM) of the titers (\log_{10} transformed) of all the sera in the same mouse group (A). The ratio of MoPn-specific IgG2a/IgG1 is shown in (B). * $p < 0.05$, compared with those in WT and IL-18 KO mice.

cytokine profile. We, therefore, examined the type of immune responses in KO and WT mice by measuring MoPn-specific serum IgG2a and IgG1 Ab production and cytokine profiles after infection. Figure 2A shows that there were no significant differences in Ab isotype levels among mouse groups tested. However, the ratio of MoPn-specific serum IgG2a/IgG1 in IL-12 KO, but not in IL-18 KO mice, was significantly lower than those in WT mice postinfection (Fig. 2B), indicating impaired Th1 immune responses in IL-12KO mice.

Th1 (IFN- γ and IL-12) and Th2 (IL-10) cytokine production by spleen cells from infected IL-18 KO, IL-12 KO, and WT mice was also examined. As shown in Table 1, there were a

Table 1. Splenic interferon (IFN)- γ production from mice intranasally infected with mouse pneumonitis (MoPn) (u/ml)^a

	Medium	HK-EB	HK-EB + rIL-18	HK-EB + rIL-12	HK-EB + anti-CD4
Wild type	<10	426 \pm 85	3075 \pm 1511	3345 \pm 1511	16 \pm 8
IL-18 KO	<10	139 \pm 32 ^b	2014 \pm 446	2116 \pm 742	18 \pm 12
IL-12 KO	<10	16 \pm 6 ^b	30 \pm 13 ^b	2316 \pm 1142	<10

^aValues are means \pm standard deviations from 4 to 9 mice each group at 10 days postinfection. ^bSignificantly different ($p < 0.05$), compared with those in wild type (WT) mice. HK-EB, heat-killed elementary bodies; recombinant interleukin (rIL); IL, interleukin; KO, knockout.

moderate but significant decrease in the production of IFN- γ following antigen specific re-stimulation of spleen cells from infected IL-18 KO mice. IFN- γ production by antigen stimulated spleen cells was compromised much more severely in IL-12 KO mice, compared with IL-18 KO and WT mice. Interestingly, although the addition of either exogenous IL-18 or IL-12 significantly enhanced IFN- γ production in IL-18 KO mice, only IL-12, and not IL-18, significantly increased IFN- γ production by spleen cells from IL-12 KO mice. There was no significant change in IL-12 or IL-10 production in IL-18 KO mice, and there was no significant change in IL-10 production in IL-12 KO mice (Fig. 3). CD4 T cells were the major source of IFN- γ and IL-10 in the tested model system, because the addition of anti-CD4 mAb abolished most IFN- γ and IL-10, but not IL-12, production (Table 1 and data not shown). The results indicated that, although both IL-12 and IL-18 play a role in Th1 cell activation, exogenous IL-12 can replace the effect of IL-18, but not vice versa. This suggests that the critical role for IL-12 is in the initiation of Th1 response following chlamydial infection.

Pathological Changes and Local Cytokine Profiles in the Lungs of WT and KO Mice Following Chlamydia Infection

We next analyzed the histopathological features and local cytokine profile in the lung after intranasal MoPn infection in WT and KO mice. The histological appearance of chlamydia-infected lung in WT mice was considerably different from that

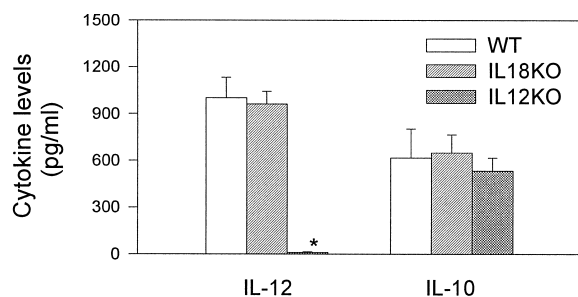


Fig. 3. Antigen-driven cytokine responses by spleen cells. Spleen cells from mouse pneumonitis (MoPn)-infected interleukin (IL)-18 knockout (KO), IL-12 KO and wild type (WT) mice were cultured at 7.5×10^6 cells/ml with stimulation by heat-killed elementary bodies (EBs). Supernatants were harvested for IL-12 and IL-10 assays by ELISA. Data represented the mean \pm standard deviation (SD) of results from 4 to 9 mice each group after 3 days in culture. * represents undetectable level of IL-12.

in either IL-18 KO or IL-12 KO mice (Fig. 4). The severity of inflammation and consolidation was more profound in KO mice, especially in IL-12 KO mice, compared with WT mice. The infiltrating cell types also appeared different among different groups of mice. In WT mice, the inflammatory infiltrates were composed of entirely mononuclear cells (Fig. 4C, D). In IL-18 KO mice, lung sections showed inflammation characterized by areas of consolidation with inflammatory exudate rich in both mononuclear cells and polymorphonuclear cells (Fig. 4E, F). Polymorphonuclear infiltrates were more numerous in IL-12 KO mice (Fig. 4G, H). We also measured local IFN- γ production by testing lung homogenates (Table 2). Consistent with observations using spleen cell cultures, IFN- γ levels were significantly lower in the lung homogenates of infected IL-18 KO and IL-12 KO mice than in WT mice.

Discussion

We characterized the in vivo role of endogenous IL-18 in the resolution of lung MoPn infection using gene knockout mice. The deficiency of IL-18 was associated with a moderate reduction in systemic and lung IFN- γ production, slightly more severe inflammatory reaction and body weight loss, but without measurably affecting MoPn lung clearance and the ratio of MoPn-specific IgG2a/IgG1. Since IL-18 requires caspase-1 activation for its biological function, we repeated these experiments using caspase-1 KO mice and, again, noted no significant differences in lung MoPn clearance between caspase-1 KO and WT mice at early stages of infection. In contrast, IL-12 KO mice showed significantly increased susceptibility to MoPn infection, characterized by severe body weight loss and organism growth in the lung, as well as impaired local and systemic IFN- γ production and decreased MoPn-specific IgG2a/IgG1 ratio. The results suggest that, although both IL-18 and IL-12 are involved in the regulation of IFN- γ production in vivo, IL-12, rather than IL-18, plays a more critical role in protective immunity against *C. trachomatis* MoPn lung infection.

Previous studies demonstrated that clearance of MoPn infection was exquisitely dependent on major histocompatibility complex (MHC) class II-mediated pathway, thus, indicating the critical role of CD4 T cell-mediated

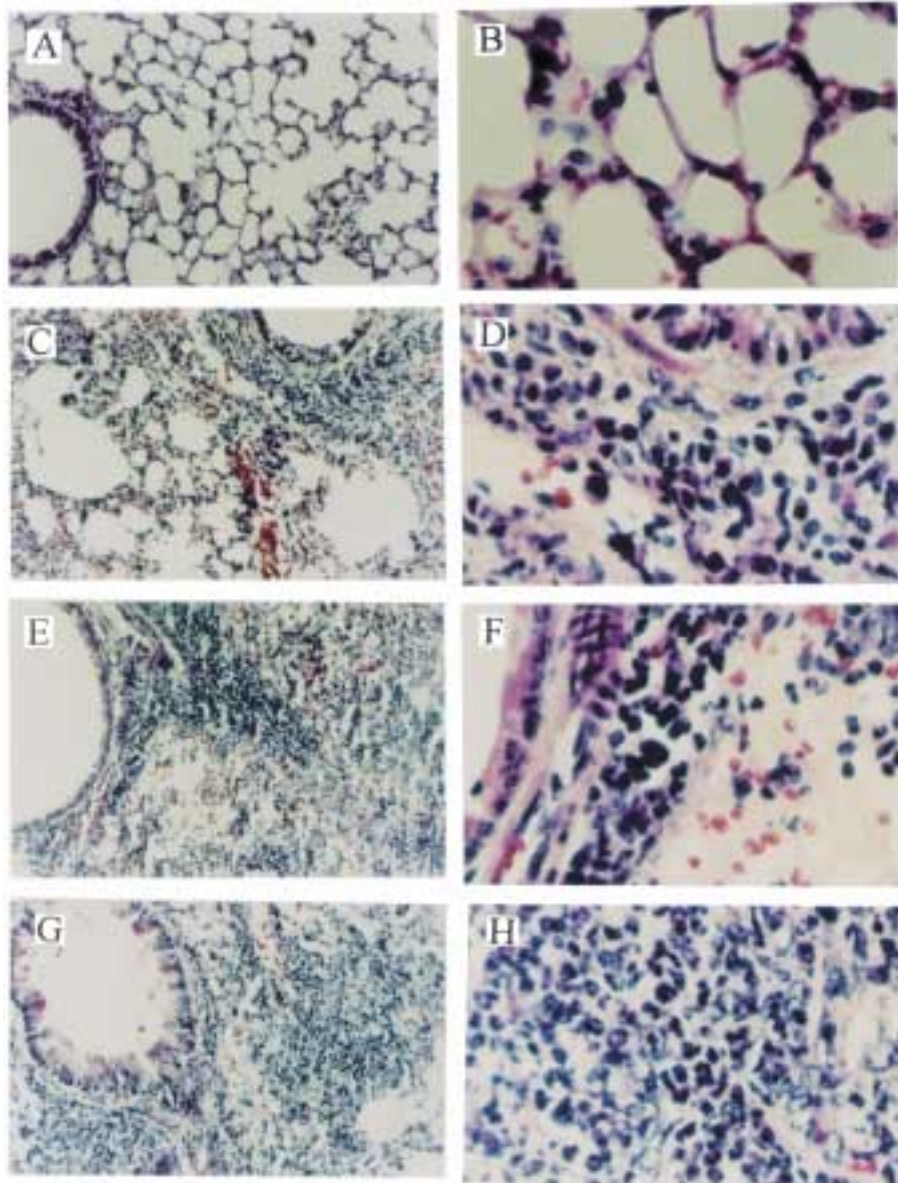


Fig. 4. Histological analysis of lung inflammatory reactions in mice following lung *C. trachomatis* mouse pneumonitis (MoPn) infection. Lung tissues were collected on day 10 postinfection and fixed by formalin and embedded in paraffin, followed by staining with hematoxylin and eosin. (A) and (B) show normal structure from naïve mice. Mice had bronchiolar-interstitial pneumonia with extensive infiltrates

immune responses in anti-chlamydial immunity (1,4,5). The relative contribution of individual cytokines related to CD4 T-cell responses has been extensively studied in mouse models of chlamydial infection. In particular, recent studies suggest that IFN- γ , a typical Th1 cytokine, is required for the prevention of systemic dissemination of MoPn infection following genital tract (2) and lung

mainly composed of mononuclear cells in wild type (WT) mice (C and D) and mixed polymorphonuclear and mononuclear cells in interleukin (IL)-18 knockout (KO) mice (E and F). IL-12 KO mice showed more severe inflammation with infiltration, predominately by polymorphonuclear cells (G and H). Magnification, 200 \times (A, C, E and G); 400 \times (B, D, F and H).

infection (6), as well as for resistance to rechallenge with *C. trachomatis* serovar D (1,5). Johansson et al. (1,5) compared the susceptibility to chlamydia infection of mice with either IFN- γ receptor or IL-12 deficiency. They demonstrated that IFN- γ receptor KO mice, but not IL-12 KO mice, remained susceptible to reinfection after recovered from primary infection although both kinds of KO mice

Table 2. Cytokine levels in lung homogenates from mice intranasally infected with mouse pneumonitis (MoPn)^a

	IFN γ (u/ml)	IL-12 (pg/ml)	IL-18 (pg/ml)	IL-10 (pg/ml)
Wild type	43 \pm 11	3910 \pm 890	3650 \pm 680	415 \pm 41
IL-12KO	13 \pm 1 ^b	ND ^c	3300 \pm 810	544 \pm 84
IL-18KO	17 \pm 2 ^b	3618 \pm 879	ND ^c	433 \pm 37

^aValues are means \pm standard deviations from 4 to 9 mice each group at day 10 postinfection. ^bSignificantly different ($p < 0.05$), compared with those in wild type (WT) mice. ^cnot detectable. IFN- γ , interferon- γ ; IL, interleukin; KO, knockout.

showed significantly more severe primary genital tract infection, compared with WT mice. Thus, they concluded that IFN- γ production by Th1 cells was the particular important mechanism in immune protection against chlamydial infection.

Like IL-12, IL-18 has been shown to activate NK cells and induce IFN- γ production by Th1 cells. IL-18 has been reported to protect mice against pulmonary and systemic infection with *Cryptococcus neoformans* (28) and *Yersinia enterocolitica* (29), mainly through enhancing IFN- γ production. IL-18 KO mice showed marked granulomatous lesions and increased mycobacterial growth in the lung, which was partially inhibited by the treatment with exogenous IL-18 (23). In the present study, the lung clearance of MoPn in IL-18 KO mice appears comparable to that in WT mice, although the former show relatively more severe signs of systemic illness, as manifested by slightly greater body weight loss and slightly more severe lung inflammatory reaction. Both IL-18 KO and WT mice recovered from primary infection and were highly resistant to secondary infection (data not shown). It has been reported that Th1 and NK cells from IL-18 KO mice are still able to produce IFN- γ , although the levels are low (16). We also note less, but still significant, levels of IFN- γ production in IL-18 KO mice following MoPn infection. It is possible, therefore, that the extent of IFN- γ decrease in IL-18 KO mice is not severe enough to damage greatly the protective immunity to chlamydial infection. Another possibility is that MoPn can be partially cleared by IFN- γ -independent pathway, as suggested by Perry et al. (2), especially since IL-18 KO mice show normal levels of IL-12 production, which may mediate protection via both IFN- γ dependent and independent manners.

IL-18 and IL-12 can work synergistically in IFN- γ production (21). IL-12 up-regulates the

expression of IL-18 receptor on cells producing IFN- γ (30). It has been demonstrated that IL-12-deleted mice or IL-12 KO mice display poor MoPn clearance and significantly reduced IFN- γ production following genital tract chlamydial infection (1,2). Our studies using a lung-infected model shows that, although IL-12 can enhance IFN- γ production in IL-18 KO mice, exogenous IL-18 fails to induce antigen-specific IFN- γ production in MoPn-infected IL-12 KO mice. This suggests an essential role for IL-12 in Th1 cell development following chlamydia infection. Collectively, the present and previous studies indicate that IL-12 is crucial for development of Th1 response and MoPn clearance in both genital tract and lung infection models. The data showing that exogenous IL-12 greatly increases IFN- γ production in both IL-18 KO and IL-12 KO mice suggest that IL-12 can also regulate IFN- γ production through an IL-18 independent mechanism.

In conclusion, our study shows that, although both IL-12 and IL-18 play a role in IFN- γ production following lung chlamydial infection, IL-12 is the dominant cytokine regulating the Th1 response that mediates protective immunity. The role of IL-18 is mainly ancillary and is not essential for organism clearance during chlamydial infection.

Acknowledgments

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