Cellular & Molecular Immunology (2010) 7, 355–360

www.nature.com/cmi

RESEARCH ARTICLE

Type 1 T-cell responses in chlamydial lung infections are associated with local MIP-1α response

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Chemokines and their receptors are important mediators of leukocyte trafficking and recruitment and sometimes work as modulators of T-cell responses during infections and inflammation. Modulating the biological activity of chemokines has been found to influence the course of diseases. However, little is known about the role of chemokine responses during chlamydial lung infections. We therefore analyzed the dynamics of multiple chemokines, which are frequently associated with type 1 (Th1) T cell immune responses, and their receptors for their expression in the lungs during *Chlamydia muridarum* (*Cm*) infections. We also examined the relationship between chemokine responses and the development of Th1 responses as well as the clearance of infection. Our results showed that in parallel with the high levels of gamma interferon (IFN-γ) and IL-12 production in the lungs and draining lymph nodes, and the expansion of IFN-γ-producing CD4 and $CD8^+$ T cells, the production of the cell-related chemokines RANTES, IFN-γ-inducible protein-10 (IP-10) and macrophage inflammatory protein-1α (MIP-1α) and their receptor CCR1 was elevated in the lung tissues after infection. Interestingly, in a later phase of infection, the expression of RANTES and IP-10 remained elevated but the expression of MIP-1α and CCR1 decreased to a low level, which suggests a closer association with the pattern of Th1 cytokine responses in the process of infection. These results suggest a close association between the MIP-1α response and the Th1-type T-cell responses in chlamydial lung infections.
Cellular & Molecular Immunology (2010) **7,** 355–360; doi:10.1038/cmi.2010.32; published online 12 July 2010

Keywords: *Chlamydial pneumonia*; MIP-1α; RANTES; Th1 response

INTRODUCTION

Chlamydia is an obligate intracellular bacterial pathogen that is estimated to cause at least one infection during the lifetime of nearly every person.[1] Although most infections are mild or subclinical, *Chlamydia* is a common cause of community-acquired pneumonia, bronchitis, pharyngitis and sinusitis.[2] It has also been reported to be associated with chronic diseases, including asthma, chronic obstructive pulmonary disease and multiple sclerosis.[3–5] It causes significant human morbidity despite the availability of effective antimicrobial treatments.[6,7]

Recent studies have clearly shown that clearance of chlamydial infections requires high levels of cell-mediated immune responses, especially $CD4^+$ T helper 1 (Th1) cells and $CD8^+$ cytotoxic T cells, which produce gamma interferon (IFN-γ).[8,9] In animals lacking IFN-γ signaling, bacterial loads are higher and the clearance of organisms is greatly hampered.[10] Moreover, in the absence of $CD4^+$ or $CD8^+$ T cells, *Chlamydia*-infected mice show increased bacterial burdens and disease severity.[11] Current vaccine candidates only show limited protection against *Chlamydial* infections because they lack the ability to induce and retain a Th1 response and cannot foster long-term protective immunity in the host. The proper selection and application of chemokines, which can be used as possible mucosal adjuvants that promote lymphocyte activation and recruitment,[12,13] are expected to enhance protective T-cell responses and minimize the pathological changes caused by *Chlamydial* infections.

Chemokines are members of a large superfamily of small, secretory proteins that are structurally and functionally related. Based on the spacing of the first two conserved cysteine residues at the N-terminus, chemokines are subdivided into the CXC, CC, CX3C and C chemokine subfamilies.[14] As a group, these proteins modulate multiple aspects of the host inflammatory response against infection, such as leukocyte adhesion and migration and inflammatory cell activation, and even contribute to the balance of type 1 and type 2 T-cell responses. However, little is known about their response in chlamydial lung infections. Some recent reports suggest that IFN-γ-inducible protein-10 (IP-10) (CXC chemokine), RANTES and macrophage inflammatory protein-1α (MIP-1α) (CC chemokine) are associated with the regulation of T-cell differentiation and polarize more for Th1 than for Th2 responses.[15–17] It is important to note, however, that

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Received 13 January 2010; revised 3 May 2010; accepted 14 May 2010

inconsistent findings have also been reported.[18,19] Therefore, we propose that these chemokines contribute to the Th1 responses needed for the clearance of *Chlamydial* infection.

To determine whether the chemokines IP-10, RANTES and MIP-1α and the receptor CCR1 are produced during the development of *Chlamydial* pneumonia and to examine the relationships of these proteins with Th1 *Chlamydia*-specific immune responses and the disease process, we analyzed the expression of these chemokines in the lungs of mice exposed to *Chlamydia muridarum* (*Cm*) at different times post-infection (p.i.). Our results demonstrate that the expression of all of the tested chemokines, RANTES, IP-10 and MIP-1α, and their receptor CCR1 were upregulated during the early phase of *Chlamydial* lung infection in conjunction with the enhancement of Th1-related cytokine responses. However, we found that the expression of MIP-1α and CCR1 are better associated with the Th1-related T-cell responses because in the later phase of infection, MIP-1α and CCR1 were decreased in parallel with the decrease of Th1 responses, while the levels of IP-10 and RANTES remained elevated. These data suggest that MIP-1α is likely an important chemokine for the protective Th1 responses in *Chlamydial* lung infection.

MATERIALS AND METHODS

Organism and infection of mice

Cm, formally called mouse pneumonitis biovar of *Chlamydia trachomatis* (MoPn), was grown in HeLa 229 cells and purified by discontinuous density gradient centrifugation as described previously.[17] The purified organisms were resuspended in sucrose–phosphate–glutamic acid buffer and stored at −80 °C until use. Female C57BL/6 mice, 6–8 weeks old, were purchased from the Beijing Academy of Military Medical Sciences Center for Laboratory Animals (Beijing, China) and maintained at a pathogen-free animal care facility at Tianjin Medical University (Tianjin, China). Mice were inoculated intranasally with 3×10^3 inclusion-forming units (IFUs) *Cm* in a volume of 40 μl and monitored daily for body weight changes and viability following infection.

The *in vivo* growth of the organism

To determine the *in vivo* growth of the organism, the mice were killed at designed days p.i. The lung tissues were homogenized in 3 ml sucrose–phosphate–glutamic acid buffer. After a brief centrifugation, the tissue supernatants were kept at −80 °C until use. Chlamydial infectivity in the lung homogenates was assayed by infection of HeLa 229 cell monolayers for 48 h followed by enumeration of inclusions that were stained by *Chlamydia*-specific murine monoclonal antibody and horseradish peroxidase-conjugated goat antimouse immunoglobulin G secondary antibodies as described previously.[20] The lung tissue supernatant was also used for determining the levels of MIP-1α by ELISA.

Reverse transcription-PCR

Lung tissues were frozen immediately in liquid nitrogen and stored at −80 °C until use. Total lung RNA was isolated using TRIzol Reagent (Invitrogen, San Diego, CA, USA) according to the manufacturer's protocol. Reverse transcription of 1 μg total RNA was performed using Moloney murine leukemia virus reverse transcriptase (TaKaRa, Dalian, China). The cDNA products were used as a template for PCR. The expression of chemokine and cytokine mRNA is presented as a percentage of β-actin. The primers used in the PCR analysis are as followed: RANTES, 270 bp, P1 5′-GAA GAT CTC TGC AGC TGC CCT-3′, P2 5′-GCT CAT CTC CAA ATA GTT GA-3′; IP-10, 431 bp, P1 5′-CCT ATC CTG CCC ACG TGT TG-3′, P2 5′-CGC ACC TCC ACA TAG CTT ACA-3′; MIP-1α, 257 bp, P1 5′-GCC CTT GCT GTT CTT CTC TGT-3′; P2 5′-GGC ATT CAG TTC CAG GTC AGT-3′; IFN-γ, 380 bp, P1 5′-AAC GCT ACA CAC TGC ATC T-3′; P2 5′-TGC TC ATT GTA ATG CT TGG-3′; IL-12, 411 bp, P1 5′-CTC ACC TGT GAC ACG CCT GA-3′; P2 5′-CAG GAC ACT GAA TAC TTC TC-3′; β-actin, 550 bp, P1 5′-GTG GGG CGC CCC AGG CAC CA-3′; P2 5′-CTC CTT AAT GTC ACG CAC GAT TTC-3′. PCR products were run on 1.2–1.8% agarose gels, and the bands were analyzed for density on scion image software.

Isolation of splenocytes and lymph node cells and cytokine analysis

Spleens and mediastinal and hilar lymph nodes were harvested from the mice at selected times after infection and processed into single-cell suspensions. Briefly, the spleens and mediastinal and hilar lymph nodes were digested in 2 mg/ml collagenase D (Roche Diagnostics, Meylan, France) in RPMI-1640 at 37 °C for 30 min. After lysis of red blood cells by adding Red Blood Cell Lysis Buffer (150 mM NH_4Cl, 10 mM $KHCO_3$ and 0.1 mM EDTA), the single-cell suspensions of spleen and lymph node cells were cultured at 7.5×10^6 and 5×10^6 cells/well, respectively, with complete RPMI medium in the presence or absence of UV-inactivated *Cm* (1×10^5 IFUs/ml) in 48 well plates at 37 °C in 5% CO_2 for 72 h. Culture supernatants were assayed to determine the levels of IFN-γ and IL-12 by ELISA as previously described.[21]

Intracellular cytokine analysis

For intracellular cytokine staining, splenocytes were stimulated with phorbyl myristate acetate (50 ng/ml; BD PharMingen, San Jose, CA, USA) and ionomycin (1 μg/ml; BD PharMingen) and incubated for 6 h in complete RPMI-1640 medium with 10% fetal bovine serum at 37 °C. Monensin (eBioscence, San Diego, CA, USA) was added to accumulate cytokines intracellularly during the last 3 h of incubation. The cells were then stained with FITC-antimouse CD4 and FITC-antimouse CD8. After fixation and permeabilization, intracellular cytokine staining was performed using phycoerythrin-antimouse-IFN-γ. The cells were washed and analyzed by flow cytometry as described previously.[20]

Histopathological analysis of the lung

The lung tissues from the naive and infected mice were fixed in 10% formalin, and tissue sections were stained with hematoxylin and eosin. Histological changes were observed under light microscopy as previously described.[20]

Statistical analysis

The Student's *t*-test was performed to determine statistical differences between the groups. One-way ANOVA analysis was used for analyzing data for multiple groups. Values were considered to be significantly different when the *P* value was <0.05.

RESULTS

Cm infection induced Th1 cytokine production in local tissue and peripheral immune organs

Th1 cytokine production is thought to be the major mechanism for the clearance of *Chlamydial* infections. We therefore investigated the gene expression and protein production of the Th1 cytokine IFN-γ and the Th1-related cytokine IL-12 in the lung tissues and peripheral immune organs at selected time points p.i. Both IFN-γ and IL-12 mRNA expressions were significantly increased following infection, but the increase of IL-12 appeared earlier (day 2 p.i.) than that of IFN-γ (day 3 p.i.; Figure 1). The mRNA for the Th2 cytokines IL-4 and IL-5 was not

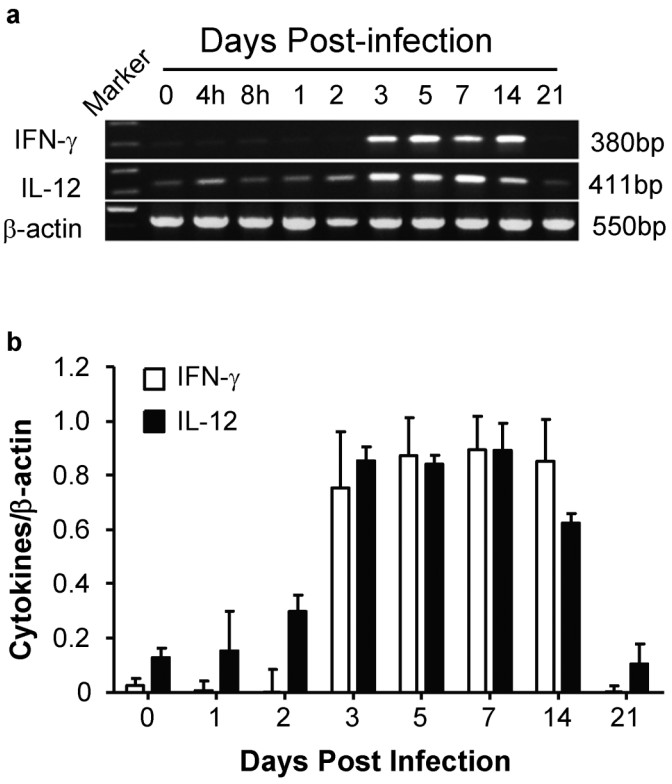

Figure 1 The mRNA expression of IFN-γ and IL-12 in the lungs during *Cm* infection. Mice were inoculated intranasally with 3×10^3 IFUs *Cm*. Total RNA was extracted from fresh lung tissues and assayed for IFN-γ and IL-12 mRNA expression by RT-PCR using the specific primers described in the 'Materials and methods' section. (**a**) Representative image of three independent experiments with similar results; (**b**) the ratios of IFN-γ/β-actin and IL-12/β-actin band densities are shown. *Cm*, *Chlamydia muridarum*; IFN-γ, gamma interferon; IFU, inclusion-forming unit; RT, reverse transcription.

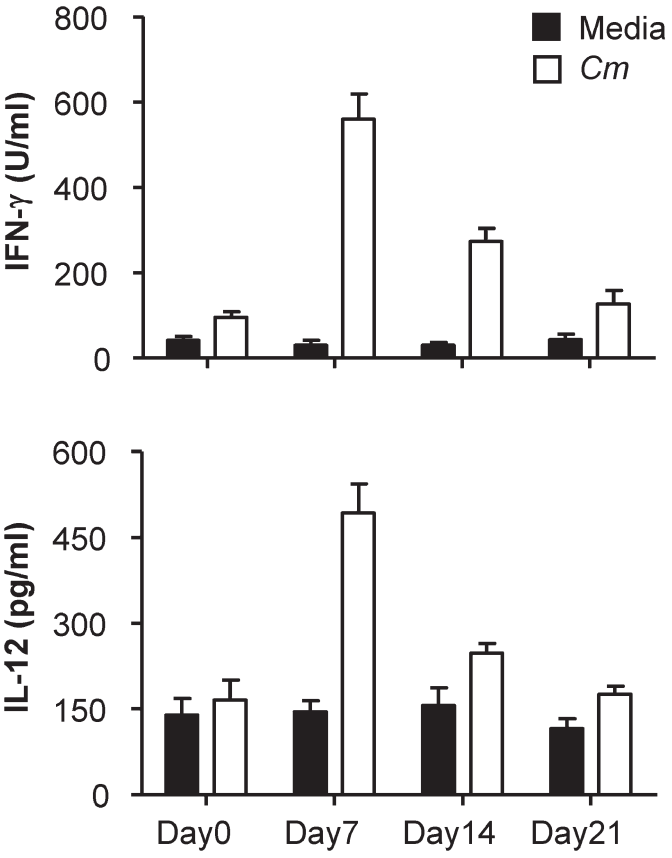

Figure 2 Production of the Th1-related cytokines IFN-γ and IL-12 in the mediastinal and hilar lymph node cells during *Cm* infection. Mice were infected intranasally with *Cm* as described in the legend for Figure 1 and killed at day 7, 14 or 21 post-infection. Mediastinal and hilar lymph node cells were cultured with UV-inactivated *Cm* or control media. IFN-γ and IL-12 levels in 48-h culture supernatants were measured by ELISA as described in the 'Materials and methods' section. Statistical analysis was performed using the Student's *t*-test. Data represent mean±SD from three independent experiments with four mice per group. *Cm*, *Chlamydia muridarum*; IFN-γ, gamma interferon; Th1, T helper 1.

detectable in the lungs at the tested time points during *Chlamydial* infection (data not shown).

The antigen-driven production of IFN-γ and IL-12 by the mediastinal and hilar lymph nodes was further analyzed by *in vitro* culture. The results showed that lymph node cells cultured with UV-inactivated *Chlamydial* antigen produced much higher levels of IFN-γ and IL-12 p.i. compared to control media (Figure 2). Intracellular cytokine staining analyses also showed significantly increased IFN-γ-producing $CD4^+$ and $CD8^+$ T cells in the spleen at days 7 and 14 p.i. compared to that in the naive control mice (day 0; Figure 3a and b). Both the *in vitro* culture and flow cytometry tests showed that production of Th1 cytokines was higher at day 7 p.i. than at day 14 p.i. These results demonstrated that mice exposed to *Chlamydia* through the airway induced a characteristic Th1-type immune response, which has been shown to be critical for protection against *Chlamydial* infection.

Cm infection-induced production of chemokines and chemokine receptors in the lung

The mRNA expression levels of several chemokines, RANTES, IP-10 and MIP-1α, and the receptor CCR1 in the lungs were further analyzed following intranasal *Cm* infection. As shown in Figure 4a and b, the expression of RANTES, IP-10, MIP-1α and CCR1 were significantly upregulated beginning at day 3 p.i. after intranasal inoculation with *Cm*, which correlated with the elevation of Th1 responses. Interestingly, the expression of MIP-1α and CCR1 decreased to a low level at day 14 p.i., in parallel with the production pattern of IFN-γ and IL-12, while the expression of RANTES and IP-10 remained high at day 14 p.i. In contrast with MIP-1α and CCR1, there was obvious gene expression of RANTES and IP-10 in normal lung tissue (day 0) and at day 21 after infection, suggesting constitutive expression of these chemokines in the lung tissues. The protein secretion of the chief chemokine MIP-1α in the lung tissue supernatants showed the same dynamics as its mRNA expression (Figure 4c). These results suggest that MIP-1α expression has a closer association with the type 1 T-cell response in chlamydial lung infections than RANTES and IP-10. Therefore, the combination of the expression of MIP-1α and its receptor in the T cells is likely a more significant mechanism of attracting Th1 cells to infection sites for the clearance of *Chlamydial* lung infections.

Clearance of chlamydial infection was associated with Th1-type cytokine and chemokine responses

To elucidate the relationship between cytokine and chemokine production and the clearance of chlamydial infections, we assessed body weight changes and chlamydial growth and performed histological analyses of the lungs following intranasal *Cm* infection. Body weight loss started from day 3 p.i., decreased to the lowest level on day 7 and

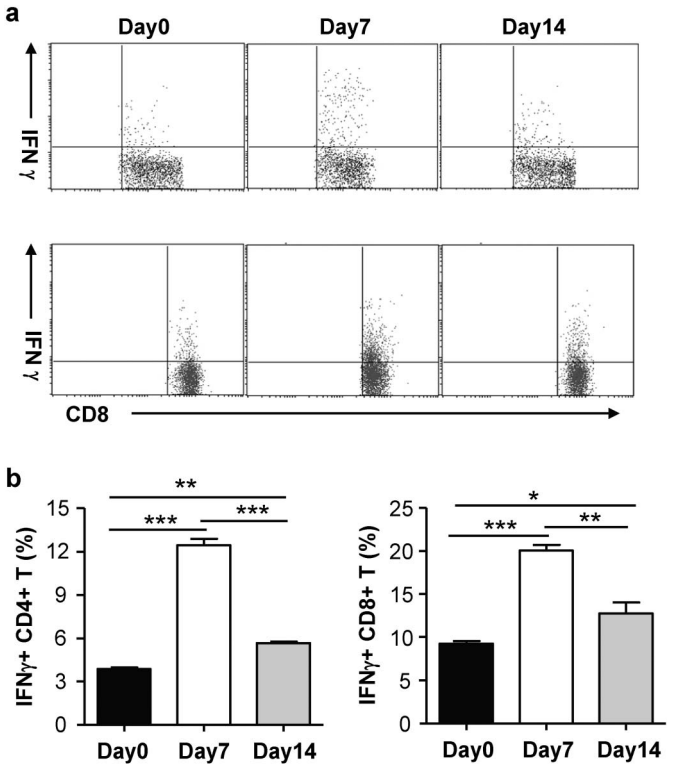

Figure 3 IFN-γ-producing CD4 and CD8$^+$ T cells in the spleen after intranasal *Cm* infection. Mice were infected with *Cm* as described in the legend for Figure 1. Spleen cells obtained at days 7 and 14 post-infection were analyzed for IFN-γ production by intracellular cytokine staining as described in the 'Materials and methods' section. (**a**) IFN-γ-producing CD4 and CD8$^+$ T cells; (**b**) summary graphs representing IFN-γ production by CD4$^+$ and CD8$^+$ T cells, respectively. Data represent mean±SD from three independent experiments with four mice per group. Statistical analysis was performed using the Student's *t*-test. $^{**}P<0.05$, $^{***}P<0.01$, comparing the infected and control mice. *Cm*, *Chlamydia muridarum*; IFN-γ, gamma interferon.

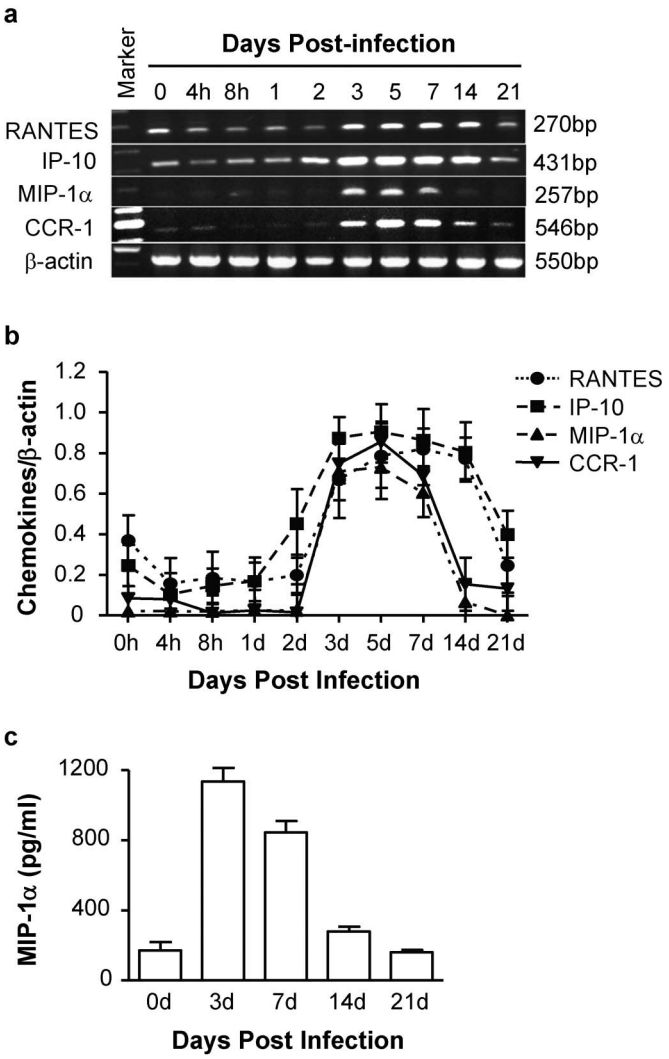

Figure 4 Chemokines and chemokine receptor production in the lungs during *Cm* infections. Mice were inoculated with *Cm* as described in the legend for Figure 1. Fresh lung tissues were collected at selected times post-infection (four mice per group at each time point), and total cellular RNA was isolated. The expression levels of RANTES, IP-10, MIP-1α and CCR1 were evaluated by RT-PCR. The lung tissue supernatants were collected at days 3, 7, 14 and 21 post-infection and analyzed for MIP-1α production by ELISA. (**a**) Representative image of three independent experiments with similar results; (**b**) the ratios of chemokines/β-actin and receptor/β-actin band densities are shown; (**c**) MIP-1α levels in lung supernatants were determined by ELISA. *Cm*, *Chlamydia muridarum*; IP-10, IFN-γ-inducible protein-10; MIP-1α, macrophage inflammatory protein-1α; RT, reverse transcription.

gradually recovered to the original weight by day 14 (Figure 5a). Tests of the *in vivo* growth of the *Chlamydia* in the lungs showed that *Cm* growth was detected from day 3 p.i., peaked at day 7 and became hardly detectable at day 21 p.i. (Figure 5b). Histopathological examination of the lungs showed significant tissue damage and pathological changes at day 7 in *Cm*-infected mice relative to naive control mice, and these effects gradually decreased to lower levels by day 14 (Figure 5c). The course of the *Chlamydial* infection was correlated with the kinetics of the Th1-type cytokine and chemokine response, especially with MIP-1α expression.

DISCUSSION

Previous studies of chlamydial airway infections in mice by both our group and others have shown that the type 1 CD4 and CD8 T-cell immune responses are associated with protection against chlamydial infection.[6,21–25] These studies were primarily done by analyzing spleen cells. Our data in this study further confirm this conclusion by detecting type 1 immune responses in both local infected tissues and draining lymph nodes during respiratory tract *Cm* infections and the relationship between *Cm* clearance from the lung and Th1 immune responses in mice. Our results show that the expression of IL-12 and IFN-γ in the lungs was significantly upregulated starting at day 3 following *Cm* infection. The earlier expression of Th1 cytokines in the lungs is likely of great significance because *Cm* was reported to be largely restricted to the epithelium mucosa.[26] *Cm* respiratory infections induced high levels of both IFN-γ and IL-12 production in the lungs and in draining lymph nodes. More importantly, the kinetics of *Cm* growth in the lungs and the body weight loss following infection were highly correlated with the pattern of Th1 responses. These results suggest that the Th1 response plays a critical role in the protective immunity against *Cm* infection.

The expression of chemokines is reported to be inducible and upregulated in inflammatory lesions, thus playing an important role in attracting effector T cells.[27] Chemokines also play a role in directing different types of immune response following infection. Some recent studies suggested that the chemokines RANTES, MIP-1α and IP-10,

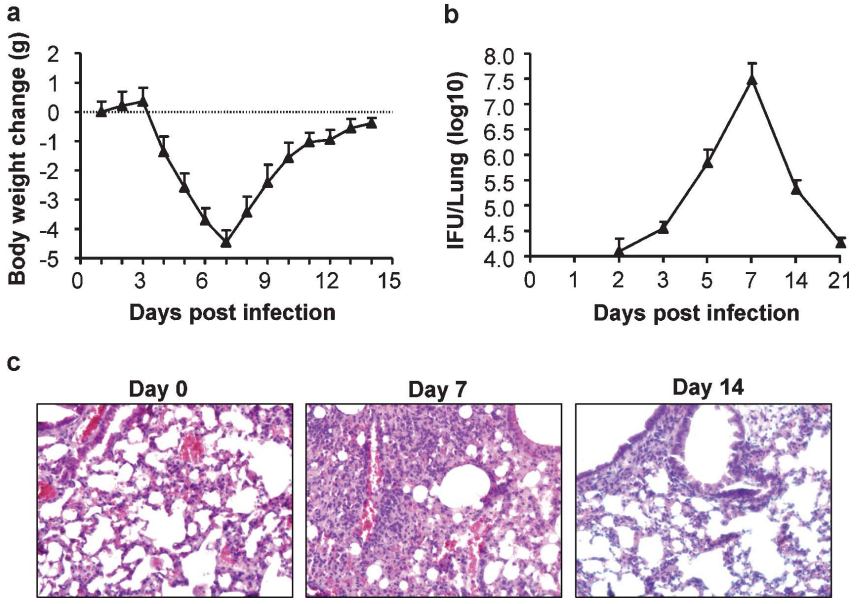

Figure 5 Body weight changes and lung clearance of *Cm* in C57 mice after intranasal infection. Mice were inoculated with *Cm* as described in the legend for Figure 1. (**a**) Mice were monitored daily for body weight changes. (**b**) Mice were killed on day 3, 5, 7, 14 or 21 post-infection, and the lungs were analyzed for *in vivo* chlamydial growth as described in the 'Materials and methods' section. (**c**) Lung sections were stained by H&E for histological analysis at ×400 magnification under light microscopy at days 0, 7 and 14 post-infection. *Cm*, *Chlamydia muridarum*; H&E, hematoxylin and eosin; IFU, inclusion-forming unit.

and one of their receptors, CCR1, are responsible for recruiting the relevant Th1 cells and that MIP-1α is a specific promoter of the Th1 response.[28] During *Chlamydial* genital tract infections, increased MIP-1α levels are associated with a stronger Th1 response.[29] Upregulation of MIP-1α mRNA in dendritic cells pulsed with non-viable *Chlamydia* generated a potent protective Th1 immune response following adoptive transfer.[30] We show here an enhancement of the chemokines RANTES, MIP-1α and IP-10 and one of their receptors, CCR1, in local tissues following chlamydial lung infection and that this enhancement is associated with the development of Th1 responses.

Chlamydia, as intracellular bacteria, differs from extracellular bacteria in that it can survive and replicate within cells. It may therefore take more time for the infected cells to respond. As shown in Figure 4, the expression of the chemokines MIP-1α, RENTES and IP-10 following chlamydial infection was increased at day 3 p.i., which corresponds with the amount of time needed for *Chlamydia* to finish its first intracellular life cycle. In contrast, cytokine and chemokine secretion following infection with *Salmonella* or invasive bacteria increases within 90 min after infection, reaching maximal levels at 3 h p.i. and decreasing rapidly thereafter,[31] suggesting that different mechanisms are involved. Moreover, all of the tested chemokines (MIP-1α, RENTES and IP-10) are highly expressed in parallel with the increased Th1 responses, which begin to increase at day 3 p.i. However, in the late stage of infection (day 14), the expression of RANTES and IP-10 remained high while the expression of MIP-1α and CCR1 dramatically decreased to much lower levels as the Th1 responses were reduced and the *Chlamydia* was cleared. These results demonstrate a close association between MIP-1α and Th1 cell trafficking during *Cm* lung infections. In contrast, IP-10 and RANTES showed less association with Th1 responses, particularly in the late stage of infection. This is consistent with previous reports that showed inconsistent correlations of these chemokines with T-cell responses.[18,19] Aside from Th1-driven protection, RANTES can also induce Th2-mediated immunopathology.[18] Furthermore, IP-10 has been shown to be upregulated in pulmonary allergic inflammations, which contributes to airway hyper-reactivity and Th2-type inflammations in asthma models.[19]

Although our data suggest a close relationship between MIP-1α and Th1 responses in *Chlamydial* infections, the potential mechanisms for the effects of MIP-1α on Th1 cells are unknown. Previous studies have suggested that MIP-1α is able to polarize T cells toward a Th1 response by reducing the number of IL-4- and IL-10-producing CD4 T cells[32] and that it enhances the type 1 cytokine production of $CD8^+$ T cells.[33] Moreover, it has been reported that MIP-1α can drive T-cell antigen receptor-transgenic Th0 cells to differentiate into Th1 cells *in vitro*,[34] and mice lacking MIP-1α are unable to generate a protective Th1 response.[35] Therefore, MIP-1α may promote Th1 responses through direct and indirect means.

In summary, our data show that *Cm* lung infections induce a series of chemokine responses that are associated with the development of Th1 responses and the clearance of local infection. Moreover, we found that although the expression levels of all of the tested chemokines (RANTES, MIP-1α and IP-10) were associated with Th1 responses in the early stage of *Cm* infection, the expression levels of MIP-1α showed a better correlation with the Th1 cytokine response, which suggests the critical importance of MIP-1α in the host defense against chlamydial lung infections.

ACKNOWLEDGEMENTS

This work was supported by a fund from the Tianjin Municipal Science and Technology Commission (07JCYBJC10600).

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