The Journal of Microbiology, October 2009, p. 633-640 Vol. 47, No. 5
DOI 10.1007/s12275-009-0139-1

Comparative Analysis of Immune Responses to *Mycobacterium abscessus* Infection and Its Antigens in Two Murine Models

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(Received April 28, 2009 / Accepted June 1, 2009)

***Mycobacterium abscessus* has been identified as an emerging pulmonary pathogen in humans. Because little is known regarding immune responses elicited by *M. abscessus* or its antigens, immunological responses were studied in two murine models subjected to intravenous (high-dose or systemic infection) or pulmonary (low-dose or local infection) inoculation with *M. abscessus* ATCC 19977. An overall comparison between the two models showed similar patterns of bacterial survival and host immune responses. The colonization of *M. abscessus* was the highest at 5 days post-infection (dpi) and its elimination was positively correlated with cell-mediated immunity in both challenges. However, an inverse relationship was observed between progressive inflammation and mycobacterial colonization levels in mice infected with a high dose at 14 dpi. Regarding antigens, culture filtrate (CF) of *M. abscessus* strongly induced IFN-γ secretion, whereas cellular extract (CE) antigen elicited strong antibody responses. The antibody response to *M. abscessus* antigens in mice subjected to low-dose infection increased when the cellular immune response decreased over 14 dpi. However, the antibody response for the high-dose infection increased promptly after the infection. In comparison of cytokine expression in lung homogenates after *M. abscessus* infection, Th1 and Th2 cytokines increased simultaneously in the high-dose infection, whereas only cell-mediated immunity developed in the low-dose pulmonary infection. These findings not only enhance our understanding of the immune response to *M. abscessus* infection according to systemic or pulmonary infection, but may also aid in immunological diagnosis and vaccine development.**

***Keywords*:** *M. abscessus*, murine infection model, immune response, antigens, cytokines

Mycobacterium abscessus belongs to a group of rapidly growing mycobacteria (RGM) that has emerged as a significant pathogen in humans (Byrd and Lyons, 1999; Wallace *et al*., 2002; Griffith *et al*., 2007; Glassroth, 2008). *M. abscessus* is ubiquitous in the environment and is particularly prevalent in natural and municipal water sources, soil, and hospital equipment (Brown-Elliot and Wallace, 2002; De Groote and Huitt, 2006; Ramesh and Sundar, 2008). This pathogen is capable of causing skin and soft tissue infections, as well as pulmonary infections (Primm *et al*., 2004). In fact, *M. abscessus* is increasingly recovered from patients with cystic fibrosis (Maiz-Carro and Navas-Elorza, 2002; Olivier *et al*., 2003). Several studies have identified strains of *M. abscessus* that are more pathogenic than other RGM (Wallace *et al*., 2002). *M. abscessus* infections are notoriously difficult to treat; long-term treatment with multiple antimicrobial agents is usually required (Razvi and Saiman, 2007).

Disease may result from immune responses that interplay with the organism or antigens. The clinical manifestations of the infection depend on many variables, including the host's genetic inheritance, immune status, and prior exposure to cross-reacting antigens from other mycobacteria. Many studies on mycobacterium-host interaction have been performed by infecting mice with doses of mycobacteria ranging from 10^6 to 10^8 colony forming units (CFUs) (Casadevall and Pirofski, 2000). However, these approaches do not reflect the immune response generated by natural exposure to the pathogen, in which small bacterial loads infect the host via various routes, such as water, aerosols, and skin lesions (Falkinham, 2002).

Currently, the host immune response to *M. abscessus* or its antigens is not completely understood, although the importance of the cell-mediated immune response has been documented in murine models (Rottman *et al*., 2007). In a recent study, TNF-α was also identified as an important factor in the control of disseminated *M. abscessus* infection in C57BL/6 mice (Rottman *et al*., 2007). Although both TNF-α and IFN-γ play critical roles in protective immunity against mycobacterial infection and immunopathology (Sampaio *et al*., 2008), the function of the humoral immune response requires further characterization in *M. abscessus* infections for the future development of diagnostic tools and vaccines (Jung *et al*., 2008). Furthermore, distinguishing the functions of cellular compartments and extracellular antigens in-

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volved in these immune responses will allow the advancement of antigen studies. Very little information is currently available regarding *M. abscessus* antigen types that are involved in the host immune response.

In addition, it is important to note that the route of mycobacterial infection is extremely important (Gupta and Katoch, 2005; Rottman *et al*., 2007) to understand immunological events in host. The intravenous and aerosol routes of infection are most commonly used in studies of mycobacteria-induced immune responses. For example, *M. abscessus* infection in the lung is clinically resistant to most antibiotics and is rarely cured, whereas *M. abscessus* infections in the skin and soft tissues are relatively treatable (Herdman and Steele, 2004). Because *M. abscessus* can cause both disseminated and pulmonary diseases, it is extremely important to compare the immune responses elicited by both intravenous and aerosol *M. abscessus* infection.

Therefore, the aim of this study was to characterize host immune responses elicited by *M. abscessus* and its antigens in two models: an aerosolized model using relatively low concentrations of *M. abscessus* to examine lung infection, and an intravenous model with high-dose induction to examine the systemic response.

Materials and Methods

Bacterial strains and antigen preparation

M. abscessus ATCC 19977 (Moore and Frerichs, 1953) was obtained from the American Type Culture Collection (ATCC, USA) and initially cultured in 7H9 broth supplemented with 10% (v/v) oleic acid-albumin-dextrose-catalase (OADC, Becton Dickinson, USA) for one week at 37°C. Single cell suspensions were homogenized, filtered, prepared, and stored for inoculation as previously described (Shin *et al*., 2007).

To prepare culture filtrate (CF) and cellular extract (CE) antigens from mycobacteria, *M. abscessus* ATCC 19977 and other mycobacterial reference strains were cultivated in modified Watson-Reid medium (mWR, pH 6.0) at 37°C for 2 or 6 weeks, as previously described (Shin *et al*., 2008). Slow-growing mycobacteria (SGM), such as *M. avium* ATCC 35712, *M. tuberculosis* ATCC 27294, and *M. bovis* ATCC 19210, were cultivated in 7H9-OADC broth for 6 weeks at 37°C. RGM, such as *M. abscessus* ATCC 19977, *M. chelonae* ATCC 35749, *M. fortuitum* ATCC 49404, *M. terrae* ATCC, and *M. phlei* ATCC 11758, were grown in 7H9-OADC broth at 37°C for 2 weeks.

CF and CE antigen concentrations were determined using the bicinchoninic acid (BCA) protein assay kit (Pierce, USA). These antigens were used to stimulate lymphocyte and study the antibody responsesin murine infection models of *M. abscessus*.

Animals, infection, bacterial counts, and histopathological analysis

Specific pathogen-free female C57BL/6 mice at 5~6 weeks of age were purchased from Japan SLC, Inc. (Shijuoka, Japan) and maintained under barrier conditions in a BL-3 biohazard animal room at Yonsei University Medical Research Center. The animals were fed a sterile commercial mouse diet and provided with water *ad libitum*. All animal experiments were done according to the regulation of Institutional Animal Care and Use Committee, Yonesi University Health System.

Immune responses induced by pulmonary infection (i.e., local infection via direct aerosol exposure) and those induced by intravenous infection (i.e., systemic infection via high-dose induction) were analyzed and compared. Two groups of C57BL/6 mice (n=40) were infected with *M. abscessus* ATCC 19977 via intravenous injection (high-dose) or aerosol challenge (low-dose). Briefly, mice were challenged with 1×10^7 CFUs of *M. abscessus* intravenously via the tail vein (Rottman *et al*., 2007). For the aerosol challenge, mice were exposed using an inhalation device (Glas-Col, USA) calibrated to deliver approximately 400 bacteria into the lungs. Five mice per group were euthanized at 1, 2, 5, 14, 28, and 56 days post-infection (dpi), and their livers, spleens, and lungs were collected for both histopathological and bacteriological examinations. The numbers of viable bacteria in the lung, liver, and spleen were determined by plating serial dilutions of the organ homogenates onto Middlebrook 7H11 agar (Difco, USA). Colonies were counted after 3~4 days incubation at 37°C. The resultant values were reported as the means of $\log_{10}$CFU±the standard deviation (SD) per group of mice.

Tissue samples collected for histopathology were preserved in 10% neutral-buffered formalin, embedded in paraffin, cut into 4- to 5-μm sections, and stained with hematoxylin and eosin or Ziehl-Neelsen acid-fast stain. A pathologist examined tissue sections from infected animals at 2, 5, 14, 28, and 56 dpi. The severity of the inflammatory response was scored on a scale of 0 to 5, based on lesion size and number of lesions per field. Tissues with more than three fields containing multiple and large-sized lesions were assigned a score of 5.

ELISPOT assays for IFN-γ detection

The cell-mediated immune response was challenged using various antigens produced from several *Mycobacterium* species as described above. Mice were euthanized with CO_2 and splenocytes were obtained by preparing single-cell suspensions from spleen tissue dispersed with sterilized glass slides. Erythrocytes were lysed in a solution containing 155 mM ammonium chloride and 10 mM potassium bicarbonate buffer, and thoroughly washed.

The enzyme-linked immunospot (ELISPOT) assay was modified to detect CF antigens and IFN-γ secreting cells in the CE fraction (Irwin *et al*., 2008). Briefly, 96-well multiscreen filter plates (Multiscreen MAHA; Millipore, France) were coated with 10 μg/ml rat anti-mouse IFN-γ antibody (Clone R4-6A2; BD Pharmingen, USA) in 50 μl of phosphate-buffered saline (PBS). After overnight incubation at 4°C, the wells were washed and blocked for 2 h with 200 μl of RPMI medium containing 10% fetal bovine serum (FBS; Bio-Whittaker, USA). Splenocytes were re-suspended to 2×10^5 cells/well and plated in triplicate. Cells were incubated at 37°C for 24 h, with or without antigen. After incubation, the plate was washed six times with PBS containing 1% Tween 20 and incubated 2 h at room temperature with 5 μg/ml biotinylated rat anti-mouse IFN-γ antibody (clone XMG1.2; BD Pharmingen) in 50 μl of PBS containing 10% FBS, followed by incubation at room temperature for 1 h.

Spots were developed by adding 50 μl of a 5-bromo-4-chloro-3,3-indolyl phosphate/nitroblue tetrazolium solution (Boehringer Mannheim, USA) and incubated at room temperature for 10 min. Spots were counted electronically using Zeiss KS-ELISPOT software (version 4.5.21; Carl Zeiss, Germany). Naive C57BL/6 splenocytes (2×10^5 cells/well) cultured in the presence or absence of 2.5 μg/ml concanavalin A (Sigma) were used for positive and negative controls, respectively.

Antibody response to *M. abcessus* antigens

To investigate antibody responses at various time points, enzyme-linked immunosorbent assay (ELISA) was performed using CF antigens and CE obtained from *M. abscessus*. Antigens (2 μg/ml) were diluted in coating buffer (14.2 mM Na_2CO_3, 34.9 mM $NaHCO_3$, 3.1 mM NaN_3, pH 9.6), and 100 μl of each antigen preparation was used to coat 96-well plates (Maxisorp; Nalge Nunc International, USA) at 4°C overnight. After rinsing with washing buffer (0.05% Tween 20 in PBS), the samples were blocked with 10% normal goat serum (Sigma) at room temperature for 2 h. Serum diluted in 10 mM PBS (100 μl, pH 7.4) was added to each well and incubated for 30 min at room temperature. The wells were then rinsed three times with washing buffer (0.05% Tween 20 in PBS), and 100 μl of goat anti-mouse IgG antibody conjugated with horse radish peroxidase (HRP, Sigma) at dilutions of 1:10,000 were added to all wells and incubated for 1 h. One hundred microliters of 3,3,5,5-tetramethylbenzidine (TMB) substrate (Sigma) were then added to each well followed by incubation at room temperature for 2 min; the reaction was quenched with the addition of 100 μl of stop solution (Sigma). The optical density (OD) of the final sample in each well was measured at 450 nm using a spectrometer (Molecular Devices, USA). The experiment was duplicated and sera from two *M. abscessus*-immunized mice were used as positive controls, while sera from two non-infected mice were used as negative controls.

Cytokine profiles in lung homogenates

Changes in cytokine protein expression after *M. abscessus* infection were examined in murine lung tissue homogenates. Lung homogenates were obtained by homogenizing lung tissues at 10,000 rpm (Polytron PT 3000; Kinematica, Inc., USA) and bead beating as previously described with slight modifications (Shin *et al*., 2005). The samples were centrifuged at 13,000×g for 10 min at 4°C and the supernatants were carefully collected for subsequent analysis. Total protein concentrations of each sample were normalized to 1.0 mg/ml using the BCA Protein Assay kit (Pierce) prior to performing assays.

Murine IFN-γ, TNF-α, interleukin (IL)-1β, IL-10, and IL-12 were analyzed in lung homogenates via ELISA (Endogen, USA), according to the manufacturer's instructions. The amount of each cytokine was calculated by comparison with a standard curve generated by serial dilutions of recombinant murine cytokine preparations; a standard curve was generated for each assay plate. The induction levels for each cytokine were expressed as fold-changes in comparison to those from non-infected murine lung homogenates.

Statistical analysis

Comparison between groups was performed using a Wilcoxon signed-rank test and values were considered significantly different at $P<0.05$. Statistical analyses were performed using statistical software (GraphPad Prism Software, version 4.03; GraphPad Software, USA).

Results

Bacterial survival in murine infection models

M. abscessus survival in two murine infection models was compared by quantifying CFUs in the lungs, liver, and spleen after 8 weeks. Mice subjected to a low-dose aerosol challenge with *M. abscessus* developed infections restricted to the lung; however, high-dose intravenous infection resulted in a systemic infection that persisted for 8 weeks. At 6 weeks post-infection, the aerosol-induced infection had decreased to undetectable levels and CFUs were not recovered from the spleen or liver (Fig. 1A). In the intravenous model, a relatively large number of *M. abscessus* CFUs survived in the lung and liver however, bacterial clearance occurred

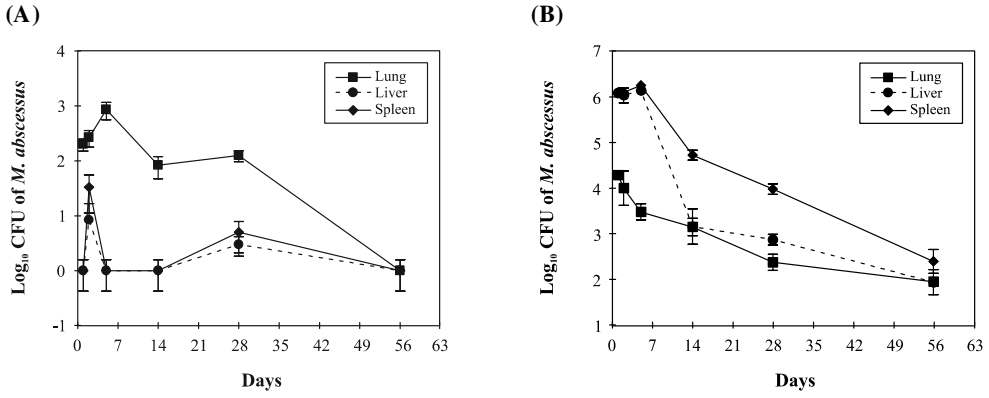

Fig. 1. Bacterial survival of *M. abscessus* in murine organs. Two groups of mice (5~6 mice) were infected via aerosol challenge (A) low dose of 4×10^2 CFU/mouse, restricted to lungs or intravenous infection (B) high dose of 1×10^7 CFU/mouse, eliciting systemic infection. Bars represent the standard errors calculated from the mean estimated CFUs obtained from organ samples.

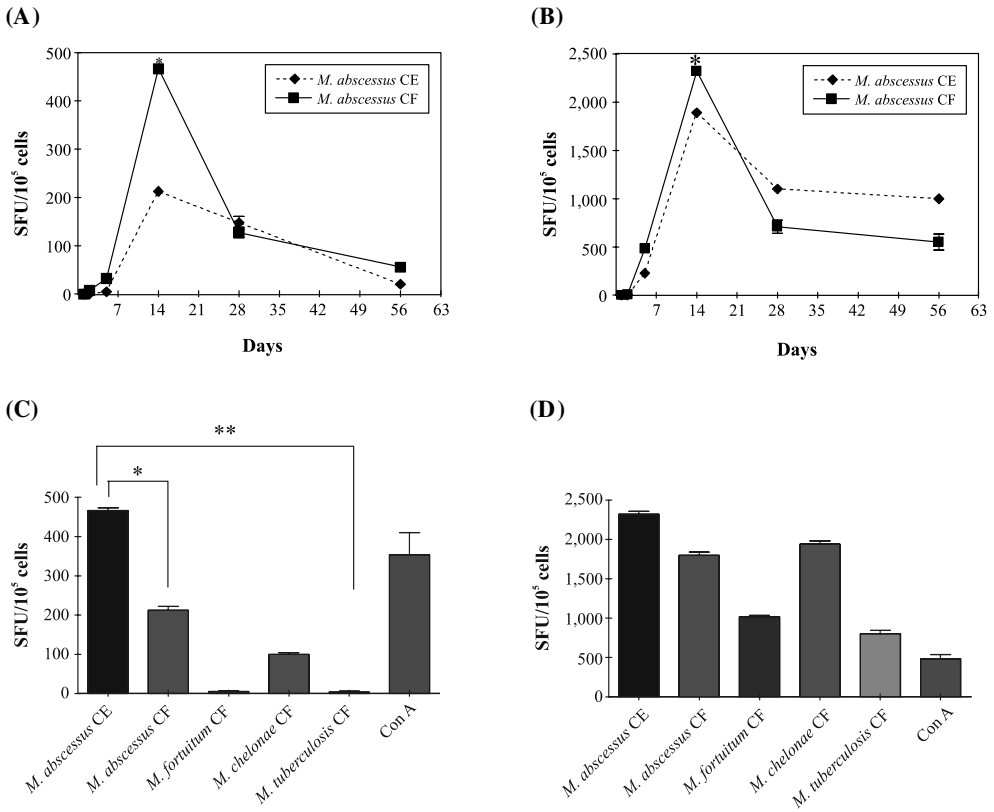

Fig. 2. Measurement of IFN-γ secreting cells after stimulation with the antigens derived from *M. abscessus* ATCC 19977 in time-dependent manner (A) and a variety of mycobacterial antigens at 14 days post-infection (B) in the low-dose aerosol (left panel) and high dose intravenous (right panel) infection models. (* $P<0.05$; ** $P<0.01$)

rapidly in the liver and very slowly in the lung (Fig. 1B). The highest infection rate was observed at 5 dpi in both models. After this time point, bacterial survival decreased rapidly, regardless of the infection route (Fig. 1).

Mycobacterial antigen-induced stimulation of IFN-γ production

A variety of mycobacterial antigens were tested for the ability to elicit cell-mediated immune responses after *M. abscessus* infection. Overall, similar time-dependent patterns of IFN-γ production were observed in both infection models and the highest level of IFN-γ production was observed at 14 dpi in both models (Fig. 2). Interestingly, IFN-γ-producing cells were significantly more elicited in responses to *M. abscessus* and *M. chelonae* CF antigens than those produced by the other antigens in the low-dose aerosol infection (Fig.

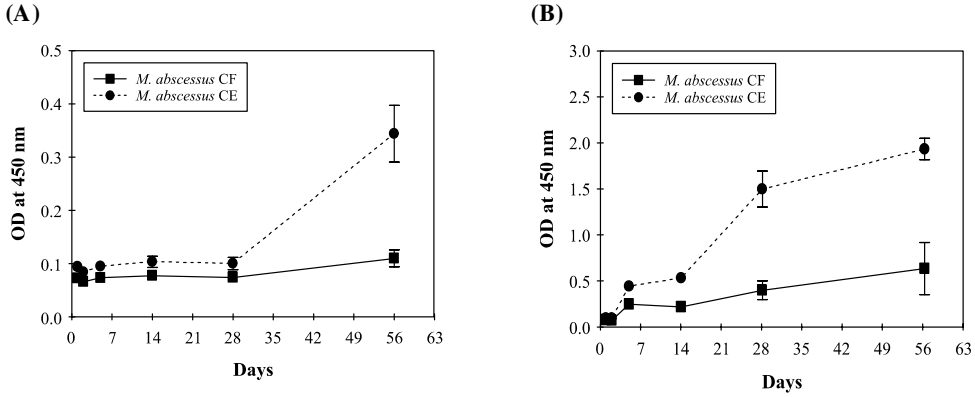

Fig. 3. Antibody responses to *M. abscessus* antigens from culture filtrate (CF) or cellular extract (CE) in the low-dose aerosol (A) and high-dose intravenous (B) infection models. ELISA results for sera obtained from mouse infected with *M. abscessus* are expressed in the mean optical density (OD at 450 nm) of 5~6 mice.

2A). In contrast, IFN-γ-producing cells increased significantly in response to all tested antigens in the high-dose infection (Fig. 2B). The constant number of IFN-γ secreting cells was counted in the stimulation with Con A and no significant IFN-γ secreting cells increased in the absence of the stimulants (data not shown).

Antibody responses to *M. abscessus* antigens

Antibody responses induced by *M. abscessus* infection were compared after low-dose aerosol and high-dose intravenous infections according to the type of *M. abscessus* antigen (i.e., CF *versus* CE antigens) and time period. The low-dose aerosol infection did not induce significant antibody responses (Fig. 3A) by 28 dpi, whereas the high-dose infection resulted in a potent humoral response to *M. abscessus* CE antigens (Fig. 3B). In both infections, antibody responses to the CE antigen were superior to those of CF antigens (Fig. 3). Interestingly, in sera obtained from mice subjected to aerosol challenge, the antibody response to CE antigens began to increase at 28 dpi, when IFN-γ production decreased. In

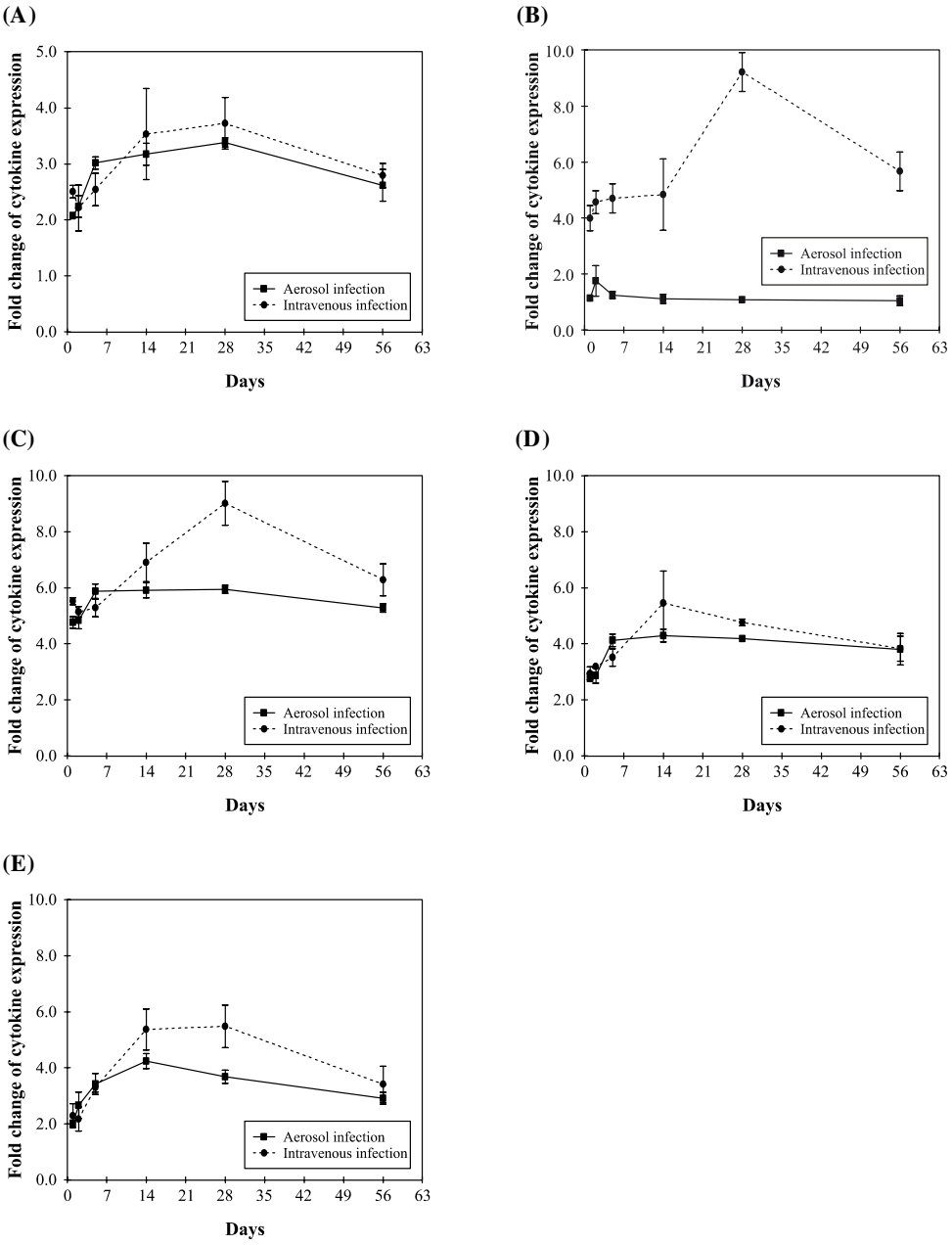

Fig. 4. Cytokine expression in lung homogenates from C57BL/6 mice infected with *M. abscessus* ATCC 19977 via aerosol or intravenous administration. ELISA-based cytokine assays were performed to determine the ratio of cytokine expression in the infected *versus* control groups. Each time point represents data from 5∼6 samples and were performed in triplicate. (A) IL-1, (B) IL-10, (C) IL-12, (D) TNF-α, and (E) IFN-γ.

addition, CF antigens from *M. abscessus* were the strongest inducers of IFN-γ production, whereas no significant antibody response to these antigens was detected in the low-dose infection (Fig. 3A).

Pulmonary cytokine profiles

Cytokine production of IL-1, IL-10, IL-12, IFN-γ, and TNF-α were measured in lung homogenates and compared between the two murine infection models. The protein concentration of each sample was normalized to 1 μg/ml before assaying. Cytokine expression was reported as a ratio to cytokine expression in the control (non-infected mice). Significant pro-inflammatory cytokine expression was observed in both models at similar levels (Fig. 4). However, pro-inflammatory cytokines were over-expressed at an early timepoint in aerosol-infected animals, whereas intravenously infected animals showed peak cytokine expression between 14 and 28 dpi. Interestingly, the anti-inflammatory cytokine IL-10 increased at 28 dpi as a result of intravenous challenge (Fig. 4B).

Histopathology

Two groups of C57BL/6 mice (n=20) were infected with *M. abscessus* ATCC 19977 via low-dose aerosol challenge or high-dose intravenous injection as mentioned above. As described above, no significant inflammation developed in mice subjected to low-dose aerosol challenge (data not shown), whereas, multifocal inflammation was observed at 8 weeks in mice subjected to high-dose intravenous infection (Fig. 5). Liver sections showed the most obvious response to *M. abscessus* infection, consisting of a granulomatous response with macrophage aggregation and admixed lymphocytes occasionally accompanied by hepatocellular death (Fig. 5).

Animals subjected to high-dose intravenous infection showed granuloma formation in liver sections. Both the size and number of inflammatory foci, or granulomas, increased over time (over 14 days), indicating disease progression (Fig. 5A and B). During the early stage of infection (1∼5 dpi), most animals infected with the high dose displayed primarily active immune responses with lymphocytic inflammation, whereas granulomatous inflammation and granuloma formation were typically observed during later stages (14∼28 dpi). Additionally, the severity of the inflammation reached a level of 3 out of 5 at 8 weeks post-infection for mice challenged with the high-dose infection. In contrast, the granulomatous response of mice directly infected via low-dose aerosol challenge ranged between the levels of 0 and 1. In mice subjected to the high-dose infection, the granulomatous response showed progressive severity (i.e., larger in size and surrounded by fibrous tissues) over the first 14 dpi (Fig. 5B). On the other hand, the low-dose infection induced relatively minor lesions at 2 weeks post-infection and remained at this level as time progressed.

Discussion

Although intensive studies on mycobacterial infection have been carried out, the early immune responses induced by *M. abscessus* are not completely understood. As previously reported, the virulence of *M. tuberculosis* depends extensively on experimental conditions, such as the route of infection and inoculum size (Hernandez-Pando *et al*., 1997). Our results confirm that bacterial inoculum size and infection route influence bacterial distribution in the organs and the host immune response.

IFN-γ is a central cytokine that inhibits mycobacterial infection, as demonstrated by the high mycobacterial susceptibility of mice with a defective IFN-γ gene (Flynn *et al*., 1993; Doherty and Sher, 1997; Hartmann and Plum, 1999). Increases in the number of IFN-γ-secreting cells were positively correlated with decreased bacterial load in the lungs. However, an inverse relationship between progressive inflammation and the number of IFN-γ-secreting cells was observed in mice infected with a high dose on 14 dpi. It is possible that the low levels of surviving *M. abscessus* at 14 dpi may reflect the initiation of a strong immune response, as evidenced by the observed increase in granuloma formation.

A significant antibody response to *M. abscessus* antigens did not develop until 28 dpi in the low-dose pulmonary infection model, whereas an elevated antibody response was detected in the high-dose intravenous infection model at a very early time point. It may be that a Th2 immune response was not evoked in the low-dose pulmonary infection because it was unnecessary, whereas it may in fact play a

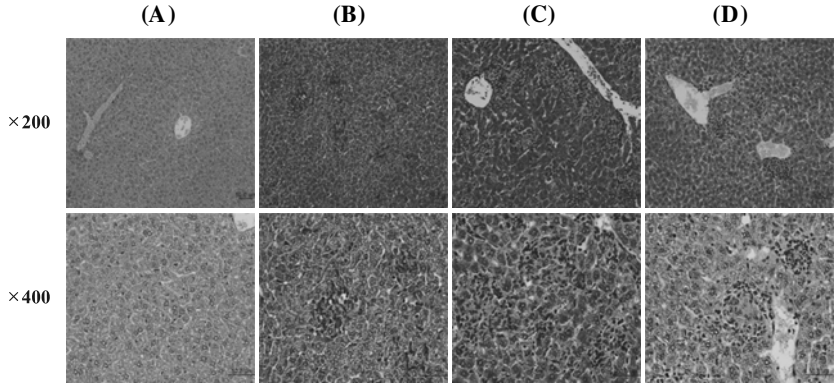

Fig. 5. Histopathological analysis of mice infected with *M. abscessus* ATCC 19977. Liver tissue from mice before infection (A) and subjected to high-dose infection at 5 (B), 14 (C), and 56 (D) days post-infection (dpi) were harvested and stained with hematoxylin and eosin.

role in controlling *M. abscessus* infection at high doses. The increased antibody responses in the high dose intravenous model may be involved in aiding bacterial clearance, for example, by opsonizing bacteria and enhancing phagocytosis.

Another significant finding in this study is that mice show differential immune responses against CE *versus* CF antigens from *M. abscessus*. To our knowledge, this is the first description of the immune profile of *M. abscessus* antigen-induced responses in mice. CF antigens strongly stimulated IFN-γ-secreting cells, whereas CE antigens appeared to induce primarily antibody responses. This may be explained by the availability of secreted proteins for interaction between antigen-presenting cells and T lymphocytes, leading to cell-mediated immune responses during the early stages of infection. However, the Th2 response drives antibody production by accumulating antigens from bacteria killed during the Th1 response. A number of studies have focused on identifying the antigens responsible for the cellular immune responses observed in the early stages of infection (Harboe *et al*., 1986; Wiker *et al.*, 1986; Nagai *et al.*, 1991; Romain *et al*., 1993; Sonnenberg and Belisle, 1997; Weldingh *et al.*, 1998; Rosenkrands *et al.*, 2000). Proteins secreted by the bacteria during growth may be important in this respect. These antigens have also received much attention in attempts to improve diagnostic tests and in the design of new vaccines against tuberculosis. Thus, it is likely that some of these antigens are also potential candidates for use in diagnostic assays that distinguish between bacterial infections based on the induction of cellular *versus* humoral immune responses.

Cytokine expression is generally lower in peripheral blood than at local sites of infection (Begara-McGorum *et al.*, 1998). The expression of cytokines is also uniquely associated with the polarity of disease states in mycobacterial infections (Stabel, 2000). Thus, pulmonary cytokine profiles after *M. abscessus* infection were studied and compared in both challenges. Overall, a similar pattern of cytokine expression was observed in both models, with the exception of IL-10. The promptly enhanced production of TNF-α and IFN-γ implies a prominent, immediate T-cell response induced by *M. abscessus* in both infections. Cytokines associated with inflammation, such as IL-1 and IL-12, also increased rapidly in the lungs of both models. During the early stages of mycobacterial infection, macrophages and T-cells actively interact to control the infection. In addition, these cytokines have a variety of immunoregulatory effects, which may influence mycobacterial killing and generate disordered inflammation within the lungs.

By combining histopathology, colonization data, and changes in immune response, we were able to assess the overall relationship between *M. abscessus* and host immunity. Low-dose *M. abscessus* infection via aerosol exposure was unable to efficiently colonize other organs (particularly, the spleen and liver). However, high-dose intravenous infection led to significantly decreased levels of colonization during the first 2 weeks, followed by slight decreases at laterstages. However, lesions that formed in mice infected with the high dose showed progressive inflammation until 14 dpi. An inverse relationship between inflammatory response and mycobacterial colonization was observed for samples collected at 14 dpi. It is possible that decreasing *M. abscessus* survival at 14 dpi is attributable to a peak in the strong immune response, as evidenced by an increase in granuloma formation and IFN-γ secretion.

Airborne infection is thought to be the primary route of *M. abscessus* infection. C57BL/6 mice were able to efficiently clear *M. abscessus* from the lungs when the mycobacterium was delivered via aerosol at a low dose; in contrast, clearance was much less efficient in mice subjected to a high intravenous dose. In order to eliminate the mycobacterium from the lungs, an early immune response characterized by IFN-γ production for both infections and an antibody response for the high-dose infection were required. Although there is still no evidence of animal-to-human or human-to-human transmission of *M. abscessus* (Meissner and Anz, 1977; Von Reyn *et al*., 1993; Ahrens *et al*., 1995; Guerrero *et al*., 1995; Tanaka *et al*., 2000), latent, reactivated, or persistent models to examine a variety of immunologic factors that modulate acute infections are required to further understand the complexity of *M. abscessus* infection.

Acknowledgements

This work was funded by a grant from the Korea Science and Engineering Foundation (KOSEF) through the Infection Signaling Network Research Center (R13-2007-020-01000-0) at Chungnam National University, and by an additional KOSEF grant through the Ministry of Science and Technology (MOST; Grant# R01-2007-000-10702-0).

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