Immunopathology and Infectious Diseases

Initiation of Acquired Immunity in the Lungs of Mice Lacking Lymph Nodes after Infection with Aerosolized *Mycobacterium tuberculosis*

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Recent evidence points to lung draining lymph nodes as the site that initiates the immune response in mice infected with aerosolized *Mycobacterium tuberculosis*. Here we expanded these studies and showed that infection of mice that lack lymph nodes with aerosolized *M. tuberculosis* results in a massive mononuclear cell infiltrate in the lungs within 14 days postinfection. This infiltration clearly resembles an expansion of the bronchus-associated lymphoid tissue. As expected, no bronchus-associated lymphoid tissue was observed in *M. tuberculosis*-infected wild-type control mice. Importantly, acquired specific immune response to *M. tuberculosis* antigens could be detected in lung lymphocytes harvested from mice lacking lymph nodes as early as 14 days postinfection. In addition, the bacterial burden in these mice was indistinguishable from that observed in wild-type C57BL/6 control mice. These results indicate that in the absence of lymph nodes, priming of the immune response occurs in the lung tissues after infection of mice with aerosolized *M. tuberculosis* and clearly illustrate the enormous plasticity of the immune system to develop resistance to foreign pathogens. (Am J Pathol 2010; 176:198–204; DOI: 10.2353/ajpath.2010.090446)

Natural resistance to tuberculosis develops in ~90% of immunocompetent individuals.¹ In these persons, infection with *Mycobacterium tuberculosis* results in the development of the so-called primary complex, or Ghon complex.^{2,3} This complex comprises a focal multiplication of

the mycobacterium in the lung tissue (most commonly localized in lower lung zones) that is associated with infection and enlargement of the corresponding hilar draining lymph node(s) (lymphangitis). Most of the times this inflammatory reaction heals spontaneously and often calcifies and persists for the remainder of the person's life, a condition known as latent tuberculosis infection.^{2,3} However, reactivation of latent tuberculosis leading to chronic pulmonary tuberculosis or adult tuberculosis may occur afterwards, even decades later because of intervening events such as malnutrition or immunosuppression.^{4–6} Curiously, lung lesions during adult tuberculosis are most frequently localized in upper lung zones. These observations have stimulated intense debate about the route of *M. tuberculosis* dissemination (ie, lymphatic or hematogenous) within the host.

Using the mouse model of tuberculosis, early studies suggested a pivotal role of lymphatic circulation and lung draining lymph nodes in the initiation of immune response that follows infection of the animals with aerosolized *M. tuberculosis*.^{7,8} More recently, this suggestion was further evaluated using adoptive transfer immunity experiments with highly specific CD4⁺ TCR transgenic T cells specific for dominant epitopes of the *M. tuberculosis* antigens.^{9–11} These studies indicated that despite the intense lymphoid infiltration in the lungs immediately after aerosol challenge of the animals, initiation of the acquired specific immune response to *M. tuberculosis* does not occur in this organ, but rather in the local lung draining lymph node. In addition these studies showed that priming of

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the immune system in the lymph node is dependent of in loco antigen processing and presentation by residing antigen-presenting cells and not by migrating antigen presenting cells from the initial lung site of infection.

In this work, we addressed further the involvement of the lymphoid system in the immunopathogenesis of tuberculosis using a unique strain of mice that lack lymph nodes.^{12,13} We show that infection of these animals with aerosolized *M. tuberculosis* results in a microbial infection and systemic dissemination (spleen) that is identical to that observed in conventional mice. However, the lung pathology developed in animals that lack lymph nodes was remarkably different from that developed in conventional mice in that the mononuclear cells infiltrating the organ were massive within 14 days postinfection suggesting an expansion of the bronchus-associated lymphoid tissue (BALT). The acquired specific immune response to *M. tuberculosis* could be clearly detected locally in lung lymphocytes in the absence of lymph nodes and spleen, revealing unanticipated plasticity in the site and/or mechanism of priming.

Materials and Methods

Mice

C57BL/6 mice were obtained from Charles River Laboratories (Wilmington, MA). Lymphotoxin- α -deficient mice (LT α -KO), which are devoid of lymph nodes^{12,13} were provided by Dr. N. Ruddle (Yale University, New Haven, CT) and bred in our animal facility. All mice were maintained under specific pathogen-free conditions and used at 8 to 12 weeks of age. All experiments performed with animals were approved by the Animal Care and Use Committee of the Forsyth Institute, Colorado State University, and the University of Massachusetts Medical School.

Bacteria and Mouse Infections

Virulent *M. tuberculosis* H37Rv strain (American Type Culture Collection, Manassas, VA) bacteria were stored as frozen aliquots at -80°C . For infection of mice, bacteria were thawed, suspended in PBS/Tween 80 (0.05%) and pushed through a 26-gauge needle six times in preparation for aerosol infection. Infection was performed with ~ 100 to 200 colony-forming units (CFU)/mouse using an aerosol generation device (Inhalation Exposure System; Glas-Col, Terre Haute, IN).

Histology

Tissue sections were fixed in 10% buffered formalin and embedded in paraffin blocks. Sections (5 μm) were stained with H&E.

Immunohistochemistry

Lungs were inflated and fixed with 10% buffered formalin for ≥ 24 hours and then processed as described previ-

ously.¹⁴ Briefly, tissue sections were stained for B220 expression with a 1/20 dilution of anti-mouse CD45R/B220 antibody (550286, BD Biosciences, San Jose, CA) or with a 1/75 dilution of anti-CD3 antibody (ab5690; Abcam, Cambridge, MA). Bound antibody was detected with peroxidase-based staining according to the manufacturers' protocols. As a negative control, sections were stained in the absence of the primary antibody as well as with isotype control sera.

Colony-Forming Units

Homogenates of lungs and spleens were prepared in PBS/Tween 80 (0.05%) and plated at a 5- or 10-fold serial dilution on BBL Middlebrook 7H10 agar plates (BD Biosciences Microbiology Systems, Cockeysville, MD). CFUs were enumerated 3 to 4 weeks later.

Cell Preparation

Spleen cells were obtained using conventional procedures.¹⁵ Lung mononuclear lymphoid cells were obtained as previously described¹⁶ after lungs were perfused through the heart with PBS. Lungs were excised, minced, and digested (30 minutes, 37°C) with 1 mg/ml collagenase and 25 $\mu\text{g}/\text{ml}$ DNase (Sigma-Aldrich, St. Louis, MO) and passed through a 40- μm cell strainer. Cells were washed twice with plain RPMI 1640 and final suspension prepared in complete RPMI 1640 containing 10% fetal bovine serum (HyClone, Logan, UT), 100 $\mu\text{g}/\text{ml}$ streptomycin, 100 U/ml penicillin, 25 mM/dL HEPES, 2 mM/dL L-glutamine, and 5×10^{-2} mM/dL mercaptoethanol. Spleens were homogenized into single cells suspensions and washed twice, and splenocytes were suspended in complete RPMI 1640 as well. Number of viable cells was estimated using trypan blue.

IFN- γ ELISPOT Assay

Cytokine ELISPOT was performed in 96-well Multi-Screen-IP filter plates (Millipore, Bedford, MA) essentially as described previously.¹⁷ Assay specific reagents were obtained from BD Biosciences. Wells were prewet with 70% ethanol, immediately washed five times with PBS, and then coated with 100 μl of capture anti-IFN- γ monoclonal antibody at 5 $\mu\text{g}/\text{ml}$ in PBS, followed by incubation overnight at 4°C . Plates were washed twice with RPMI 1640 plus 10% FBS and incubated with 200 μl of complete RPMI 1640 for 3 to 4 hours at 37°C to block non-specific binding. After that, splenic cells were added at 10^5 cells/well; lung cells were used at 4×10^4 cells/well. Cells were stimulated in triplicates with 5 $\mu\text{g}/\text{ml}$ Ag85B (Colorado State University, National Institute of Allergy and Infectious Diseases/National Institutes of Health Tuberculosis Research Materials contract 1-A125174), 5 $\mu\text{g}/\text{ml}$ concanavalin A or medium alone (control) for 48 hours (37°C , 5% CO_2). Plates were washed twice with double-distilled H_2O and three times with PBS plus 0.05% Tween 20 (PBST). Biotinylated IFN- γ detection mAb at 2 $\mu\text{g}/\text{ml}$ in PBS plus 10% FBS was added and

plates incubated at room temperature for 1 hour. Wells were washed three times with PBST and streptavidin-horseradish peroxidase conjugate was added (1/100 dilution in PBS plus 10% FBS) for 1 hour at room temperature. Plates were washed again three times with PBST and twice with PBS. Substrate set (3-amino-9-ethyl-carbazole) was added and spots development was monitored protecting plates from light. The enzymatic reaction was stopped by washing wells twice with double-distilled H₂O. Plates were air-dried, and the number of spots per well was determined using the CTL ImmunoSpot Analyzer (Cellular Technology, Shaker Heights, OH). Spot-forming cell numbers were calculated by subtracting spots in presence of antigen from spots in absence of antigen (control).

Splenectomy

Mice were anesthetized with a solution of ketamine (16.5 mg/ml) and xylazine (3.5 mg/ml), and splenectomy was performed under aseptic surgical conditions. An incision of ~1 cm was performed with scissors on the mice skin at the subcostal area of the left flank. After visualizing the spleen, a small incision (~0.5 cm) was made on the abdominal muscular layer and the spleen was exposed, the vessels were dissected, and the organ was removed. The muscular wall and skin were sutured separately. Mice were allowed to recover for 10 days before infection.

Statistical Analysis

Statistical analysis was performed using unpaired *t*-test for comparisons between two groups. Values of *P* < 0.05 were considered statistically significant.

Results

Bacterial Burden and Dissemination of *M. tuberculosis* Infection in Mice with Impaired Lymphatic System

To further investigate the role of draining lymph nodes after aerosol challenge with *M. tuberculosis* we used LT α -KO mice. This mouse strain does not have lymph nodes^{12,13} and is therefore an interesting animal model to study the role of the lymphatic system on the pathogenesis of tuberculosis. The local bacterial colonization of *M. tuberculosis* in the animal's lungs and dissemination of infection after aerosol challenge of LT α -KO mice was compared with that observed in sex- and age-matched C57BL/6 mice. The LT α -KO mice used in these studies have been backcrossed into the C57BL/6 background for more than nine generations. Mice were infected via the respiratory tract using aerosolized virulent *M. tuberculosis* H37Rv strain with ~200 CFU, and dissemination of infection to systemic organs was analyzed by enumerating the bacterial load in the lungs and spleens. As control, conventional C57BL/6 mice were equally infected and ana-

Figure 1. Bacterial burden and development in LT α -KO and C57BL/6 mice infected with *M. tuberculosis*. Mice were infected with 100 to 200 aerosolized *M. tuberculosis*, and the course of infection (CFU) was determined in the lungs and spleens.

lyzed. The results (Figure 1) show that the bacterial load recovered from the lungs and spleens of both LT α -KO and C57BL/6 mice were virtually indistinguishable during the first 3 weeks after infection. Importantly, in parallel experiments to verify the overall resistance of LT α -KO mice infected with *M. tuberculosis*, we evaluated their survival in comparison with that of wild-type C57BL/6 mice. Survival in both groups of mice was 100% after at least 3 months of observation (data not shown), which clearly indicates that lack of lymph nodes does not impair the development of resistance to tuberculosis. Taken together, these results suggest a nonessential role of lymph nodes for both dissemination of *M. tuberculosis* from the primary lesions in the lungs and resistance to infection with this organism. They also indicate that the hematogenous route is an important pathway for dissemination of the infectious process within the host.

Initiation of the Immune Response in the Lungs of *M. tuberculosis*-Infected LT α -KO Mice

The clear lack of difference in bacterium burden and similar resistance between LT α -KO and C57BL/6 mice infected with *M. tuberculosis* led us to reason that this

Figure 2. Development of adaptive immune response in LT α -KO and C57BL/6 mice infected with *M. tuberculosis*. Mice (five per group/time point) were infected with 100 to 200 aerosolized *M. tuberculosis* and at the designated time points lung and spleen mononuclear lymphoid cells were obtained, stimulated for 48 hours with Ag85B (5 μ g/ml), and assayed for IFN- γ production by ELISPOT. Note that although the magnitude of the response is higher in wild-type compared with LT α -KO mice ($P < 0.05$ for lungs at 14 and 21 days and spleen at 21 days postinfection; $P > 0.05$ for spleen at 14 days postinfection), no differences are observed in the kinetics of IFN- γ production by the mononuclear cells isolated from both groups. SFC, spot-forming cells. Bars represent SE for the means of the results of five mice per group. This is one representative experiment from three experiments with essentially the same results.

model provided an interesting condition to evaluate the possibility of initiation and development of the adaptive immune response in the lungs in the absence of lung draining lymph nodes. Mice were infected with 100-200 CFU of aerosolized *M. tuberculosis* and sacrificed on days 7, 14, and 21 postinfection. Lymphocytes from both lungs and spleens were obtained, stimulated *in vitro* with Ag85B for 48 hours, and assayed by ELISPOT for production of IFN- γ . Figure 2 shows that lung lymphocytes

from LT α -KO mice clearly responded to stimulation with the *M. tuberculosis* antigen and that the kinetics of this response was identical to that of spleen lymphocytes. Moreover, this same kinetic pattern of response, although to higher magnitude, was also observed in wild-type C57BL/6 mice. The stronger response observed in the wild-type mice is likely to be, as previously proposed^{9,10} due to a more efficient initiation of the immune response in the lung draining lymph nodes, followed by recirculation of activated lymphocytes to the lungs and spleen. On the other hand, because LT α -KO mice do not have lymph nodes our results suggest that priming and initiation of the adaptive immune response following exogenous infection of the respiratory tract of mice with *M. tuberculosis* can also occur directly in the lung. However, these results do not categorically exclude the possibility that priming of the immune response in LT α -KO to *M. tuberculosis* antigens could occur initially in spleen and subsequently recirculate to the animal's lungs.

To test this possibility, LT α -KO mice were splenectomized and 10 days later were challenged with aerosolized *M. tuberculosis*. Figure 3A illustrates the extent of lung bacterial burden in these mice, which followed the same pattern of infection of that observed in conventional wild-type C57BL/6 mice and non-splenectomized LT α -KO mice (Figure 1). More importantly, lung lymphocytes obtained from the splenectomized animals at 14 and 21 days postchallenge and stimulated *in vitro* with Ag85B produced IFN- γ to similar levels as nonsplenectomized LT α -KO mice (Figure 3B). These observations clearly indicate that in the absence of lung draining lymph nodes and spleen, the priming and expression of adaptive immunity to *M. tuberculosis* antigens following aerosol infection can occur directly in the lung tissues.

Development of BALT in LT α -KO Mice Infected with *M. tuberculosis*

The histopathological examination of the lungs revealed striking differences between the lesions developed by LT α -KO mice in comparison with conventional C57BL/6 mice. Histological sections of LT α -KO mice lungs (Figure 4) at both days 14 and 21 postinfection were markedly infiltrated with mononuclear cells throughout the organ parenchyma and particularly surrounding the vessels

Figure 3. Bacterial burden and initiation of adaptive immunity in lungs of splenectomized LT α -KO mice infected with *M. tuberculosis*. LT α -KO mice were splenectomized and 10 days later infected with 100 to 200 CFU of aerosolized *M. tuberculosis* (A). In addition, at designated time points, lung mononuclear lymphoid cells were obtained, stimulated for 48 hours with Ag85B (5 μ g/ml), and assayed for IFN- γ production by ELISPOT (B). Five mice were assayed at each time point. SFC, spot-forming cell. Error bars represent the SEM.

Figure 4. Histopathology of lungs from controls and *M. tuberculosis*-infected LT α -KO and C57BL/6 mice. Mice were infected as described in Figure 1 and euthanized at the indicated time points. Lungs were removed and embedded in paraffin, and tissue sections (5 μ m) were stained with H&E. Note the absence of cellular infiltration surrounding the bronchioles of both uninfected control mice as well as in wild-type (WT) mice infected with *M. tuberculosis*. However, patches of perivascular infiltrations are seen surrounding some vessels of uninfected LT α -KO (red arrowhead). In contrast, in infected LT α -KO animals, mononuclear cell infiltrations are seen in the organ parenchyma at 14 days after infection (black arrowhead) as well as contiguously to the bronchial tree (black arrows) at both days 14 and 21 postinfection. Similar pattern is seen in the lungs of splenectomized LT α -KO. However, no inflammation is seen in C57BL/6 mice at day 14 postinfection. As expected, at day 21, granulomas are seen in the lung parenchyma of these mice (white arrowhead), but no inflammation is seen surrounding the bronchial tree. B, bronchiole; A, artery. Original magnification, $\times 100$.

and bronchial tree clearly resembling BALT. In contrast, as expected, histological sections of C57BL/6 mice lungs showed little or no inflammation at day 14 postinfection and only moderate focal infiltration of mononuclear cells at 21 days. Moreover, the tissues surrounding the bronchial tree of uninfected and age matched naïve LT α -KO had no areas of inflammation. However, in agreement with previous observations^{18,19} small patches of mononuclear cell infiltration were present around the blood vessels.

To further confirm that infection of LT α -KO with *M. tuberculosis* results in expansion of BALT, we performed immunohistochemistry analysis of lung sections using the markers B220 (B cell marker) and CD3 (T cell marker) that are generally accepted to characterize BALT in both humans and mice.^{20–22} As shown in Figure 5, these stainings identified at day 14 postinfection the presence of numerous aggregates of both B and T lymphocytes in the tissues surrounding the vessels and airways of the LT α -KO infected animals. No such staining is seen in the lung of C57BL/6 mice. The same distribution pattern of these cells was seen at 21 days postinfection of both LT α -KO and C57BL/6 mice (data not shown). These ob-

servations confirm the expansion of the BALT system in LT α -KO mice infected with *M. tuberculosis*.

Discussion

Recent observations have indicated that initiation of the adaptive immune response to *M. tuberculosis* during infection depends on antigen production and presentation in the local lung draining lymph node and not at the initial site of infection in the lungs.^{9,10} In these studies, using adoptive transfer of TCR transgenic T cells specific for either antigen Ag85B¹⁰ or ESAT-6,⁹ it was observed that the antigen-specific immune response to *M. tuberculosis* occurred initially in the lung draining lymph node preceding by 1 week the response that was detected locally in the inflamed lung tissues.

However, earlier studies have shown the lymph node less LT α -KO mice primed intranasally with soluble antigens like ovalbumin developed a potent local lung immune response.²³ These observations suggested that in the absence of lymph nodes the initiation of the immune response can occur directly in the lung tissue. In the

Figure 5. Immunohistochemistry of lungs from LT α -KO and C57BL/6 mice infected with *M. tuberculosis*. Mice were infected as described in Figure 1 and euthanized at 14 days postinfection. Lungs were removed and embedded in paraffin and tissue sections (5 μ m) were processed for immunohistochemical staining for B cells (anti-B220) and T cells (anti-CD3). Note the intense accumulation of both B and T cell aggregates (B220⁺ and CD3⁺ cells, respectively) surrounding the lung airways (black arrows) of LT α -KO mice. In contrast, no B220⁺ or CD3⁺ cells are seen in the lungs of C57BL/6 mice. B, bronchiole. Original magnification, $\times 100$.

present studies, we used this model to further evaluate the possibility of priming of the immune response in the lungs of mice challenged with aerosolized *M. tuberculosis*. The results clearly indicate that mice that lack lymph nodes respond to the infectious process similarly to conventional wild-type mice of the same major histocompatibility complex background (C57BL/6). Indeed, after infection, the subsequent bacteriological burdens that occur in the lungs and spleens in the two mouse strains were indistinguishable. In addition, the survival in both groups of mice was 100% for at least 3 months of observation. These results point to a nonessential role of lymph nodes to control the infection that follows an aerosol challenge of mice with *M. tuberculosis*. In addition, because of the indistinguishable kinetics and magnitude of the splenic bacteriological burden observed in both LT α -KO and C57BL/6 mice the results add strong support to the hematogenic dissemination concept of tuberculosis.

The unequivocal demonstration that the initiation of the adaptive immune response can occur directly in the lungs of mice infected with *M. tuberculosis* was confirmed using lymphocytes isolated from these organs harvested from both splenectomized and nonsplenectomized LT α -KO mice. On restimulation *in vitro* with the *M. tuberculosis* antigen Ag85B, the lung lymphocytes isolated from both groups of animals clearly responded with the production and secretion of high levels of IFN- γ . Although the magnitude of the immune response was higher in wild-type compared with LT α -KO mice, no differences were observed in the kinetics of the IFN- γ production by the mononuclear cells isolated from these two groups of animals. Nevertheless, these results clearly indicate that in animals that lack the conventional peripheral lymphoid organs, lymph nodes and spleen, the adaptive immune response can be initiated and developed locally in the lungs after aerosol infection with *M. tuberculosis*. Overall, we interpret these findings as evidence that mice lacking lymph nodes develop an alternative immune mechanism of defense against this important intracellular pathogen. These results are not necessarily in opposition to the

former observations that indicated that in animals with intact immune system, the initiation of the immune response to aerosol challenge with *M. tuberculosis* occurs not in the lung tissue but rather in the lung draining lymph nodes.^{9,10} Conversely, our results clearly identify an alternative mechanism used by the adaptive immune response and point to the enormous plasticity of the immune system, which is an essential feature of the host specific acquired resistance to eliminate pathogens.

Interestingly, the spleen cells harvested from LT α -KO mice at 14 days postinfection, similar to cells harvested from infected wild-type mice, strongly responded to stimulation *in vitro* with Ag85B. Curiously, at this time point postinfection the bacterial load is just beginning to be detectable in this organ. However, this kinetic of antigenic availability does not support the priming of the immune response directly in the spleen. These are intriguing results in that they suggest that lymphocytes primed in the lung tissues early on after aerosol infection could rapidly recirculate and home into a peripheral conventional lymphoid tissue. Although intriguing, this possibility needs further evidence to be substantiated.

Our results clearly indicate that the inflammation triggered by *M. tuberculosis* infection in the lungs of mice that lack lymph nodes promotes a massive expansion of BALT, a system that has been proposed to be an important component of mucosal immunity in the lung.^{24,25} This conclusion was based on the observations that indicated that 14 days after infection of LT α -KO mice with *M. tuberculosis* the tissue areas underlying the mucosal layer of the bronchioles were heavily infiltrated with mononuclear cells. Moreover, these structures were rich in both B and T cells, a strong evidence that supports the BALT nature of these infection-induced mononuclear cell infiltration in the lungs of LT α -KO mice. No such structure was seen in infected wild-type control animals as well as in uninfected controls. It is important to emphasize that although no BALT was seen in uninfected LT α -KO mice, small clusters of perivascular mononuclear cell infiltrates were present in the lung sections of these animals. These findings are in agreement with former observations that

demonstrated the presence of similar pattern of cell infiltration in lungs of LT α -KO mice that have not been intentionally infected.^{18,19} The explanation for these infiltrates in the lungs of these animals is not clearly understood. It has been postulated that aging and respiratory subclinical infection with opportunistic pathogens may result in increased lymphocyte accumulation in the lungs of LT α -KO mice.²⁵

Although circumstantial, our results support the association of BALT development with acquired resistance to *M. tuberculosis* infection in LT α -KO mice. This conclusion is based on the experiments that demonstrated that both the bacterial burden and the animal survival following infection with *M. tuberculosis* were indistinguishable between LT α -KO mice and wild-type C57BL/6 mice. Moreover, the splenectomized and infected LT α -KO mice were totally competent to developing a localized immune response in the lungs and controlling the early phase of the infection as indicated by a kinetic of bacterial burden that was not different from that observed for control-infected animals. Indeed, our results are in agreement and further support former observations that demonstrated that induced BALT in LT α -KO mice infected intranasally with influenza virus could also be associated to resistance to this virus.²⁵

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