• 特约专稿 •

Usefulness of animal models for tuberculosis research—A review

Isamu Sugawara

Shanghai Pulmonary Hospital，Tongji University，Shanghai 200433，China

Abstract：When *Mycobacterium tuberculosis* infects humans，about 20% of those infected actually develop tuberculosis. In Japan，the incidence of tuberculosis in 2008 was 19.4/100 000 persons（24 760 cases）and the rate has been decreasing gradually，but is still higher than that in the USA，Holland，and Belgium，for example. Histologically，experimental tuberculosis displays exudative inflammation，proliferative inflammation and productive inflammation depending on the time course. In productive inflammation，granulomatous lesions with necrotic centers are formed. The typical granulomas consist of epithelioid macrophages，Langhans' multinucleated giant cells，lymphocytes and fibroblasts，and the process of their formation involves many cytokines，chemokines and transcription factors. These findings have been derived primarily from animal experiments utilizing an airborne infection apparatus. This review focuses on the basic research aspect of tuberculosis—what has been found through animal experiments by our research group—and furthermore，indicates what we should do in tuberculosis research in the near future.

Key words：Animal model；Genome；Immunity；*Mycobacterium tuberculosis*；Specific inflammation

1　How is experimental tuberculosis induced and what immune systems are involved?

Using animal experiments and an inhalation exposure system，the pathologic condition of the infected animals was followed up for one year. Exudative inflammation was observed for the first 10 days. Thereafter，granulomas，which corresponded to foci of proliferative inflammation，were formed. Cavity formation was not recognized in animal tuberculosis，except in rabbits[1]. Dr. Arthur Dannenberg at Johns-Hopkins University was one of the pioneers of experimental tuberculosis research. Using rabbit models，he described the pathology of tuberculosis in detail[2]. There are five stages：onset，symbiosis，early stage of caseous necrosis，interplay of cell-mediated immunity and tissue-damaging delayed-type hypersensitivity，and liquefaction and cavity formation. In stage 1，tubercle bacilli are usually destroyed or inhibited by the mature resident alveolar macrophages that ingest them. If bacilli are not destroyed，they grow and eventually destroy the alveolar macrophages. In stage 2，bacilli grow logarithmically within the immature non-activated macrophages. These macrophages enter a tubercle from the bloodstream. This stage is termed symbiosis because bacilli multiply locally

without apparent damage to the host，and macrophages accumulate and divide. In stage 3，the stage at which caseous necrosis first occurs，the number of viable bacilli becomes stationary because their growth is inhibited by the immune response to tuberculin-like antigens released from bacilli. Stage 4 is the stage that usually determines whether the disease becomes clinically apparent.

Cell-mediated immunity plays a major role in this situation. The cytotoxic delayed-type hypersensitivity immune response kills these macrophages，causing enlargement of the caseous center and progression of the disease. If a decent cell-mediated immune response develops，a mantle of highly activated macrophages surrounds the caseous necrosis. In stage 5，bacilli evade host defenses. When liquefaction of the caseous center occurs，the bacilli multiply extracellularly，frequently attaining very large numbers. The high，local concentration of tuberculin-like products derived from these bacilli causes a tissue-damaging delayed-type hypersensitivity response that erodes the bronchial wall，forming a cavity. Liquefaction and cavity formation do not occur at all in mice and rats.

T cells can be divided into two subsets，T helper 1 (Th1) and Th2，on the basis of the cytokines they produce. In tuberculosis，Th1 plays a major role in defense against tuberculosis. Th1 cells suppress Th2 cells，and interferon γ (IFN-γ) down-regulates Th2 responses. We do not know the roles played by B cells in tuberculosis. When activated B cells increase the production of IFN-γ by nature killer (NK) cells，and via antibody-dependent cell-mediated cytotoxicity (ADCC) they confer NK cells the specific function of killing bacilli-laden macrophages. The exact roles of CD25 T cells and γ/δ T cells remain unknown because the cell populations in the granulomatous lesions are very low. As long as NK T cell knockout mice are used，NK T cells do not play a major role in defense against tuberculosis[3].

It goes without saying that alveolar macrophages play a protective role in tuberculosis development. However，the roles of neutrophils in the development of tuberculosis have been neglected for a long time and remain unknown. We developed a lipopolysaccharide (LPS)-induced transient neutrophilia model in the lungs. LPS (50 μg/ml) was administered intratracheally to male Fischer rats[4]，which were then infected with *Mycobacterium tuberculosis* (*M. tuberculosis*) via an airborne route. Intratracheal injection of LPS significantly blocked the development of pulmonary granulomas and significantly reduced the number of pulmonary colony-forming unit (CFU). Treatment with amphotericin B (an LPS inhibitor) or neutralizing anti-rat neutrophil antibody reversed the development of pulmonary lesions. LPS-induced transient neutrophilia prevented early mycobacterial infection. The timing of LPS administration was important. When given intratracheally，at least 10 days after aerial infection，LPS did not prevent the development of tuberculosis. Neutrophils obtained by bronchoalveolar lavage (BAL) killed *M. tuberculosis*. These results indicate clearly that neutrophils participate actively in defense against early-phase tuberculosis.

When tubercle bacilli reach alveoli，they are phagocytosed by resident alveolar macrophages. Although tubercle bacilli are killed by alveolar macrophages，tubercle bacilli can also kill macrophages through an apoptotic process. In order to determine the fate of tubercle bacilli once they enter the phagosomes of macrophages，we collected alveolar macrophages by BAL from aerially infected guinea pigs. At 12 days after infection，one out of about 10 000 alveolar macrophages of various sizes contained many tubercle bacilli. This indicates that certain alveolar macrophages permit *M. tuberculosis* to replicate in the phagosomes，although most of the tubercle bacilli are

killed by activated alveolar macrophages. It would be very interesting to examine the survival mechanism of *M. tuberculosis* at the single-cell level; however, we still do not know why macrophages targeted by tubercle bacilli cannot kill bacilli. It is also known that macrophages possess an autophagy mechanism for removal of old organelles, in this case old infected phagosomes[8]. Autophagic pathways can overcome the trafficking block imposed by *M. tuberculosis*.

2 What is significance of IFN - γ and tumor necrosis factor α in protection against tuberculosis?

When we infected IFN-γ knockout mice with avirulent H37Ra or bacillus Calmette-Guérin (BCG) Pasteur, we found multinucleated giant cells in the granulomatous lesions. The lesions also contained tubercle bacilli and consisted of multinucleated cell clusters, being immunopositive with anti-Mac-3 antibody[5]. We subsequently infected many knockout mice with *M. tuberculosis*, but no Langhans' multinucleated giant cells were recognized. Thus, it appears that formation of multinucleated giant cells requires optimal combinations and concentrations of various cytokines; furthermore, IFN-γ levels have to be significantly low. The technique of gene targeting (knockout) has swept through biomedical research. IFN-γ, tumor necrosis factor α (TNF-α), interferon regulatory factor 1 (IRF-1), nuclear factor for interleukin 6 expression (NF-IL-6), nuclear factor κB (NF-κB) p50, signal transducer and activator of transcription 1 (STAT1), and STAT4 knockout mice succumb to *M. tuberculosis* infection over time. There appears to be a cytokine and transcription factor hierarchy in experimental tuberculosis. The results indicate that these molecules play major roles in defense against the disease, IFN-γ and TNF-α being the leading players in this respect[6].

3 What is relationship between nitric oxide and apoptosis in tuberculosis?

Host T cells kill *M. tuberculosis* and *M. tuberculosis* also kills T cells. It is very important to investigate the killing mechanism. It is generally believed that nitric oxide (NO) plays a leading role in the killing of tubercle bacilli. In my experience, this is so in mice and rats infected with tuberculosis. However, it is uncertain whether NO produced by human mononuclear phagocytes can also kill tubercle bacilli. NO is regulated by inducible NO synthase (iNOS), and it has been reported that macrophages in the lungs of individuals with clinically active *M. tuberculosis* infection often express catalytically competent iNOS[7]. Of course, that does not exclude the possibility that other yet unknown molecules may be responsible for tubercle bacilli killing.

The iNOS knockout mice were not killed with *M. tuberculosis*. Thus, there is a possibility that molecule(s) other than iNOS can kill *M. tuberculosis*.

4 What is present status of new tuberculosis vaccines?

The efficacy of BCG against adult pulmonary tuberculosis still remains controversial. However, it is not easy to find *M. tuberculosis*-derived immunogenic antigens suitable for tuberculosis vaccines because only a few such antigens have been detected so far. Several tuberculosis vaccines are currently being tested using various models[9,10]. These include recombinant BCG vaccine expressing Ag85A or Ag85B, recombinant modified vaccinia virus Ankara expressing Ag85A, tuberculosis polyprotein vaccine, Mtb72f, 6 kDa early secreted antigenic target (ESAT-6) subunit vaccine, auxotrophic vaccines for tuberculosis, and recombinant BCG overexpressing major extracellular proteins (rBCG30). Several promising tuberculo-

sis vaccine candidates may become available after verification in monkey studies.

Some promising tuberculosis vaccine candidates are now in phase I and II trials. We propose conducting a global multicenter study of the tuberculosis vaccine thus chosen using the same experimental protocols，and selecting a promising one on the basis of consensus.

5 What is importance of the DNA genome of *M. tuberculosis*?

The genome of *M. tuberculosis* H37Rv has been sequenced[11]，and the length of the DNA is reported to be 4.04 Mbp. It was hoped that this would allow complete clarification of the functions of *M. tuberculosis*，but this still remains a distant goal. Seven percent of the genome encodes proteins of unknown function and 26% encodes conserved hypothetical proteins. At present，there are not many proteins that are immunogenic and thus suitable for tuberculosis vaccine design. Another important point is that *M. tuberculosis* does not possess toxins. This is in sharp contrast to *Listeria monocytogenes* （a type of intracellular pathogen） that possesses listerial toxin. Different protein antigens may be clarified in the near future. Some proteins may be good tuberculosis vaccine candidates.

6 How are high-risk factors involved in tuberculosis?

There are many high-risk factors for tuberculosis，including human immunodeficiency virus （HIV），malnutrition，aging，and poverty. Diabetes mellitus （DM） is also one such factor. Although there is a clinical association between DM and tuberculosis，no experimental evidence for this association exists. We attempted to clarify whether type 1 diabetic （KDP） and Goto Kakizaki （GK） type 2 diabetic rats were more susceptible to *M. tuberculosis* than non-diabetic wild

type （WT） rats. The infected diabetic rats developed large granulomas without central necrosis in their lungs，liver，or spleen. This was consistent with a significant increase in the number of CFU of *M. tuberculosis* in the lungs and spleen （$P<0.01$）. Insulin treatment resulted in significant reduction of tubercle bacilli in the infected KDP rats （$P<0.01$）. Pulmonary IFN-γ，TNF-α，and IL-1β mRNA levels were higher in the infected diabetic rats than in WT rats. Alveolar macrophages from KDP rats were not fully activated by *M. tuberculosis* infection because they did not secrete NO that can kill *M. tuberculosis* （$P<0.01$）. However，there was no significant difference in phagocytosis of tubercle bacilli by alveolar macrophages between KDP and WT rats. Taken together，it appears that KDP and GK rats are more susceptible to *M. tuberculosis* than WT rats[12,13]. Hypercholesterolemia and hyperlipidemia were not observed in these rats. When we investigated the relationship between DM and tuberculosis exacerbation at tuberculosis specialist hospitals，the above-stated findings are also true for human tuberculosis and DM[14].

7 Concluding remarks — What we should do in tuberculosis research in the near future?

It now seems appropriate to point out several reasons why tuberculosis belongs to the category of specific inflammation. First，as mentioned above，*M. tuberculosis* does not possess toxin，while another typical intracellular pathogen，*Listeria monocytogenes*，does. Most would consider that toxins are responsible for acute inflammation. Second，the cell wall of *M. tuberculosis* consists of various sugars，polysaccharides and lipids. Generally，immune responses induced by these moieties are low，but some mycolic acid derivatives （trehalose dimycolate and methyl ketomycolate） are granulomatogenic factors. Lastly，the doubling time of *M. tuberculosis* is around

20-24 h, and it replicates very slowly, surviving in phagosomes of specific alveolar macrophages for a long time. We are currently planning a project to examine why *M. tuberculosis* replicates in certain alveolar macrophages at the single-cell level, a phenomenon that we have termed the death escape mechanism. It would also be interesting to examine the process (termed autophagy) by which infected macrophages remove old infected phagosomes.

Mycobacterial research is one of a number of fascinating areas that challenge us as professional researchers. I hope that the next generation of researchers will tackle many of the issues that still remain unresolved.

References

[1] Enarson DA, Wang JS, Dirks JM. The incidence of active tuberculosis in a large urban area [J]. Am J Epidemiol, 1989, 129 (6): 1268-1276.

[2] Dannenberg AM. Pathogenesis of Human Pulmonary Tuberculosis. Insights from the Rabbit Model [M]. Washington, DC: ASM Press, 2006.

[3] Sugawara I, Yamada H, Mizuno S, Li C, Nakayama T, Taniguchi M. Mycobacterial infection in natural killer T cell knockout mice [J]. Tuberculosis (Edinb), 2002, 82 (2-3): 97-104.

[4] Sugawara I, Udagawa T, Yamada H. Rat neutrophils prevent the development of tuberculosis [J]. Infect Immun, 2004, 72 (3):1804-1806.

[5] Sugawara I, Yamada H, Kazumi Y, Doi N, Otomo K, Aoki T, Mizuno S, Udagawa T, Tagawa Y, Iwakura Y. Induction of granulomas in IFN-γ gene-disrupted mice by avirulent but not by virulent strains of Mycobacterium tuberculosis [J]. J Med Microbiol, 1998, 47 (10):871-877.

[6] Sugawara I, Yamada H, Shi R. Pulmonary tuberculosis in various gene knockout mice with special emphasis on roles of cytokines and transcription factors [J]. Curr Respir Med Rev, 2005, 1(1): 7-13.

[7] Nicholson S, Bonecini-Almeida Mda G, Lapa e Silva JR, Nathan C, Xie QW, Numford R, Weidner JR, Calaycay J, Geng J, Boechat N, Linhares C, Rom W, Ho JL. Inducible nitric oxide synthase in pulmonary alveolar macrophages from patients with tuberculosis [J]. J Exp Med, 1996, 183 (5):2293-2302.

[8] Gutierrez MG, Master SS, Singh SB, Taylor GA, Colombo MI, Deretic V. Autophagy is a defense mechanism inhibiting BCG and Mycobacterium tuberculosis survival in infected macrophages [J]. Cell, 2004, 119 (6):753-766.

[9] Sugawara I, Li Z, Sun L, Udagawa T, Taniyama T. Recombinant BCG Tokyo (Ag85A) protects cynomolgus monkeys (Macaca fascicularis) infected with H37Rv Mycobacterium tuberculosis [J]. Tuberculosis (Edinb), 2007, 87 (6): 518-525.

[10] Sugawara I, Sun L, Mizuno S, Taniyama T. Protective efficacy of recombinant BCG Tokyo (Ag85A) in rhesus monkeys (Macaca mulatta) infected intratracheally with H37Rv Mycobacterium tuberculosis [J]. Tuberculosis (Edinb), 2009, 89 (1):62-67.

[11] Cole ST, Brosch R, Parkhill J, Garnier T, Churcher C, Harris D, Gordon SV, Eiglmeier K, Gas S, Barry CE 3rd, Tekaia F, Badcock K, Basham D, Brown D, Chillingworth T, Connor R, Davies R, Devlin K, Feltwell T, Gentles S, Hamlin N, Holroyd S, Hornsby T, Jagels K, Krogh A, McLean J, Moule S, Murphy L, Oliver K, Osborne J, Quail MA, Rajandream MA, Rogers J, Rutter S, Seeger K, Skelton J, Squares R, Squares S, Sulston JE, Taylor K, Whitehead S, Barrell BG. Deciphering the biology of Mycobacterium tuberculosis from the complete genome sequence [J]. Nature, 1998, 393 (6685):537-544.

[12] Sugawara I, Yamada H, Mizuno S. Pulmonary tuberculosis in spontaneously diabetic goto kakizaki rats [J]. Tohoku J Exp Med, 2004, 204 (2):135-145.

[13] Sugawara I, Mizuno S. Higher susceptibility of type 1 diabetic rats to Mycobacterium tuberculosis infection [J]. Tohoku J Exp Med, 2008, 216 (4): 363-370.

[14] Zhang Q, Xiao H, Sugawara I. Tuberculosis complicated by diabetes mellitus at Shanghai pulmonary hospital, China [J]. Jpn J Infect Dis, 2009, 62 (5):390-391.