Protein Cell 2010, 1(4): 309–311
DOI 10.1007/s13238-010-0055-5

Protein & Cell

NEWS AND VIEWS

Zebrafishing for tuberculosis infection

Liwei Wang ✉

Institute of Biophysics, Chinese Academy of Sciences, Beijing 100101, China
✉ Correspondence: wangliwei@moon.ibp.ac.cn

Mycobacterium tuberculosis, also known as Koch's bacillus, is the causative microbe of human tuberculosis (TB) that mainly involves lungs. For centuries, pulmonary TB, known as "lao" disease in Chinese, had been a brutal killer to human beings, as no drug was available to fight it. In the second half of the twentieth century, with the discovery and clinical application of effective anti-TB drugs such as streptomycin, isoniazid, and rifamycins, people saw the dawn of eradicating this notorious disease from the world (Myers, 1963). However, the winner of this battle is not human being, but the smart microbe, *M. tuberculosis*, which evolves effective mechanisms to detoxify antibiotics. With the emergence and spread of multi-drug resistant (MDR) and extensively-drug resistant (XDR) *M. tuberculosis* strains, TB is becoming one of the most significant threats to global health. Every year, around 9 million new cases of TB are reported and 1.7 million lives are claimed by TB (Korenromp et al., 2009). Recent findings by Volkman et al. (2010) provide new insights in the fighting against TB.

M. tuberculosis is an airborne pathogen. It enters the lungs via the aerosol route and meets the host's immune surveillance at the pulmonary alveoli where it is promptly engulfed by resident macrophages and dendritic cells, and initiates a series of innate and adaptive immune responses (Bhatt and Salgame, 2007). Infected macrophages produce bacteriostatic and bactericidal molecules to suppress the growth of *M. tuberculosis* and mature the phagosomes to kill the engulfed bacteria. The interplay between the host immune system and *M. tuberculosis* is complicated by the fact that *M. tuberculosis* possesses effective mechanisms to survive and even replicate within macrophages, partly through inhibition of phagosome acidification and maturation (Frehel et al., 1986; Sturgill-Koszycki et al., 1994). Therefore, the consequences of TB infection can be very different in human population, from complete elimination to progressive disseminated diseases (Frieden et al., 2003). In most cases, TB infection develops into a latent state. It is estimated that about 2 billion people bear latent *M. tuberculosis* infection without any clinical symptoms (Lin and Ottenhoff, 2008). In latent infection, the bacilli are dormant and confined in special immune cell aggregates, called granulomas. A granuloma is an organized collection of immune cells and matrix, with a rim of lymphocytes surrounding infected macrophages (Saunders and Britton, 2007). The mycobacterium is capable of inducing necrosis of infected macrophages, which creates an acellular "caseous" space full of bacilli at the center of granulomas (Cosma et al., 2003). For a long time, it was widely believed that granulomas help the host to constrain the mycobacterial infection. However, new findings suggested that the granuloma might instead facilitate infection spread, at least increase the number of infected macrophages at the initial stage of gramuloma formation (Davis and Ramakrishnan, 2009).

To study the complex interplay between *M. tuberculosis* and human immunity, closely-related model systems have to be established. Although mouse is the most commonly used animal model for studying human TB, it has significant limitations. *M. tuberculosis* is not a natural pathogen of mice, and the pathological development of TB in mice is different from that in human. In particular, TB infection does not cause the formation of granulomas in mice. *Dictyostelium*, *Drosophila* and zebrafish have been used as model hosts to study aspects of TB that are inaccessible in mice. Studies on all of these model hosts rely on a model pathogen, *Mycobacterium marinum*, which is the closest relative to the *M. tuberculosis* complex (Stinear et al., 2008) (http://www.sanger.ac.uk/Projects/M_marinum/), and is a natural pathogen of ectotherms such as fish and amphibia (Stamm and Brown, 2004). In these hosts, *M. marinum* infection develops with key features of human TB such as granuloma formation (Prouty et al., 2003). Occasionally, *M. marinum* can infect human skins and cause a disease called "fish tank granulomas" (Lewis et al., 2003) that has all the same characteristics of *M. tuberculosis* granulomas. Other advantages of using *M. marinum* as model pathogen are that *M. marinum* grows much faster than *M. tuberculosis* and can be easily manipulated with less stringent biosafety restrictions. Compared to slim mold and fruit flies, zebrafish are particularly

relevant model hosts because they have both innate and adaptive immune systems that closely resemble those of mammals (Traver et al., 2003). Zebrafish embryos, larvae, and adults have all been used to study *Mycobacterium* infections (Davis et al., 2002; Prouty et al., 2003). Zebrafish embryos are of particular interest because they are transparent for the first three weeks of development, which provide a valuable platform for live imaging of host-pathogen interactions in real-time (Davis et al., 2002; Davis and Ramakrishnan, 2009).

In 2002, by continuously monitoring the zebrafish embryos (32 h post-fertilization) intravenously injected with *M. marinum msp12::gfp*, a constitutively fluorescent strain, L. Ramakrishnan and colleagues recorded many phenomena that nobody had seen before. They watched bacterial transferring between two macrophages through membrane tethers and uninfected tissue macrophages phogocytosing dead infected macrophages, which suggested two possible mechanisms of bacterial dissimination mediated by macrophages: direct transfer between macrophages and re-phagocytosis of bacteria within dead macrophages (Davis et al., 2002). They showed, for the first time, that the innate immunity was sufficient to initiate granuloma formation without the involvement of adaptive immunity (Davis et al., 2002). As early as 3 days post-injection (4 1/3 days post-fertilization), the infected macrophages that had extravasated into the tissues began to form granuloma-like aggregates, while T lymphocytes are not yet circulating at 4 days post-fertilization (Willett et al., 1999).

This finding challenged the old notion that granuloma formation requires lymphocytes and recruited lymphocytes help to confine mycobacterial expansion (Andersen, 1997; Saunders and Cooper, 2000). Later on, together with a couple of other lines of evidence (Volkman et al., 2004; DiGiuseppe Champion and Cox, 2007), L. Ramakrishnan and colleagues proposed that granuloma formation actually works as a bacterial tool for expanding infection. To test this hypothesis and uncover the mechanisms of mycobacterial expansion utilizing granulomas, L. Ramakrishnan and graduate student J. Muse Davis again took advantages of the powerful live imaging of zebrafish-*M.marinum* infection model and compared the embryos infected with wild-type *M. marinum* and RD1 locus-deleted *M. marinum* (Davis and Ramakrishnan, 2009). Their findings provided insights about why granulomas turn into hotbed for mycobacteria expansion. The researchers observed that uninfected macrophages moved rapidly toward and within the nascent granulomas as they were heading to chemical attractant. The newly arriving macrophages were rapidly infected by phagocytosing bacteria-containing cell remnants of dying infected macrophage. The observed infection rate was 2.3 new macrophages for each old, infected cell. They also found that some newly infected macrophages exited the primary granuloma and served as seeds for initiating new granulomas. Based on these findings, the researchers suggested that “mycobacterial expansion in early granulomas is driven by a continual cycle of death of infected macrophages and their phagocytosis by multiple newly recruited macrophages” .

Meanwhile, the Ramakrishnan and Davis determined that efficient bacterial expansion depends on the mycobacterial RD1 locus which contains genes that contribute to a secretion system called the early secreted antigen 6 kDa (ESAT-6) secretion system 1 (ESX-1). The virulent *M. marium* recruited 7-fold more uninfected macrophages to the site of infection than did RD1-deficient *M. marinum*. The RD1-deficient strain infected macrophages showed lower ratio of cell death and lower frequency of phagocytosis by uninfected cells. The RD1 locus is a well-known virulent factor of Mycobaceria (Stanley et al., 2003). This study revealed that the importance of RD1 attributed partially to its critical role in initial granuloma formation. However, the molecular basis of RD1-dependent acceleration of granuloma formation remained unclear.

To answer this question, L. Ramakrishnan and colleagues did a host gene expression survey comparing zebrafish embryos infected with wild-type *M. marinum* or RD1-deleted *M. marinum* (Volkman et al., 2010). They identified that *matrix metalloproteinase 9* (mmp9) was induced by RD1 during granuloma formation, and that inhibition of MMP9 expression in infected zebrafish embryos reduced granuloma formation and bacterial proliferation. Remarkably, they found that RD1-induced MMP9 was not expressed by macrophages within granulomas, instead it was expressed by epithelial cells surrounding the granulomas. The researchers also demonstrated that the bacterial protein ESAT6, secreted by bacteria that have been engulfed by macrophages, elicits the expression of MMP9, and that both ESAT6 and MMP9 are required for granuloma formation. Their findings showed that two secreted peptides, one from the pathogen and the other from the host, comprise a virulence axis which subverts the host’s early immune responses and benefits mycobacteria expansion.

As all profound discoveries do, the great work from Ramakrishnan and colleagues tremendously broadens our understanding about TB infection, and bring up many new questions for future studies. One of them is how ESAT6 proteins travel from the phagosomes inside macrophages to surrounding epithelial cells. Do they hijack other host factors during the long journey? Once they reach the epithelial cells, do they enter epithelial cells or initiate a signaling cascade from outside? What is the epithelial cell surface receptor for ESAT6? A great amount of effort is needed to answer these questions.

The results of Ramakrishnan and colleagues also provide the basis for developing new anti-TB strategies. Whereas traditional antibiotics kill bacteria, a new therapeutic method could be inhibiting, destroying, or neutralizing key microbial virulence factors such as ESAT6 of Mycobacteria. The second therapeutic target could be the host protease,

MMP9. The inhibitors of matrix metalloproteinases are already being developed as therapeutics for non-infectious diseases such as osteoarthritis, cirrhosis, and cancers. What makes MMP9 inhibitors more exciting for TB treatment comes from the evidence that MMP9-deficient mice may be naturally resistant to *M. tuberculosis* (Taylor et al., 2006).

Although it is still a long way to develop the efficient drug or therapeutic approach to controll TB, these recent breakthroughs from Ramakrishnan and colleagues provide insightful views for scientists and physicians to completely understand TB infection and eventually conquer this stubborn disease.

REFERENCES

Andersen, P. (1997). Host responses and antigens involved in protective immunity to Mycobacterium tuberculosis. Scand J Immunol 45, 115–131.

Bhatt, K., and Salgame, P. (2007). Host innate immune response to Mycobacterium tuberculosis. J Clin Immunol 27, 347–362.

Cosma, C.L., Sherman, D.R., and Ramakrishnan, L. (2003). The secret lives of the pathogenic mycobacteria. Annu Rev Microbiol 57, 641–676.

Davis, J.M., Clay, H., Lewis, J.L., Ghori, N., Herbomel, P., and Ramakrishnan, L. (2002). Real-time visualization of mycobacterium-macrophage interactions leading to initiation of granuloma formation in zebrafish embryos. Immunity 17, 693–702.

Davis, J.M., and Ramakrishnan, L. (2009). The role of the granuloma in expansion and dissemination of early tuberculous infection. Cell 136, 37–49.

.DiGiuseppe Champion, P.A., and Cox, J.S. (2007). Protein secretion systems in Mycobacteria. Cell Microbiol 9, 1376–1384.

Frehel, C., de Chastellier, C., Lang, T., and Rastogi, N. (1986). Evidence for inhibition of fusion of lysosomal and prelysosomal compartments with phagosomes in macrophages infected with pathogenic Mycobacterium avium. Infect Immun 52, 252–262.

.Frieden, T.R., Sterling, T.R., Munsiff, S.S., Watt, C.J., and Dye, C. (2003). Tuberculosis. Lancet 362, 887–899.

Korenromp, E.L., Bierrenbach, A.L., Williams, B.G., and Dye, C. (2009). The measurement and estimation of tuberculosis mortality. Int J Tuberc Lung Dis 13, 283–303.

Lewis, F.M., Marsh, B.J., and von Reyn, C.F. (2003). Fish tank exposure and cutaneous infections due to Mycobacterium marinum: tuberculin skin testing, treatment, and prevention. Clin Infect Dis 37, 390–397.

Lin, M.Y., and Ottenhoff, T.H. (2008). Host-pathogen interactions in latent Mycobacterium tuberculosis infection: identification of new targets for tuberculosis intervention. Endocr Metab Immune Disord Drug Targets 8, 15–29.

Myers, A. (1963). Can tuberculosis be eradicated? Chest 43, 327–329.

Prouty, M.G., Correa, N.E., Barker, L.P., Jagadeeswaran, P., and Klose, K.E. (2003). Zebrafish-Mycobacterium marinum model for mycobacterial pathogenesis. FEMS Microbiol Lett 225, 177–182.

Saunders, B.M., and Britton, W.J. (2007). Life and death in the granuloma: immunopathology of tuberculosis. Immunol Cell Biol 85, 103–111.

Saunders, B.M., and Cooper, A.M. (2000). Restraining mycobacteria: role of granulomas in mycobacterial infections. Immunol Cell Biol 78, 334–341.

Stamm, L.M., and Brown, E.J. (2004). Mycobacterium marinum: the generalization and specialization of a pathogenic mycobacterium. Microbes Infect 6, 1418–1428.

Stanley, S.A., Raghavan, S., Hwang, W.W., and Cox, J.S. (2003). Acute infection and macrophage subversion by Mycobacterium tuberculosis require a specialized secretion system. Proc Natl Acad Sci U S A 100, 13001–13006.

Stinear, T.P., Seemann, T., Harrison, P.F., Jenkin, G.A., Davies, J.K., Johnson, P.D., Abdellah, Z., Arrowsmith, C., Chillingworth, T., Churcher, C., et al. (2008). Insights from the complete genome sequence of Mycobacterium marinum on the evolution of Mycobacterium tuberculosis. Genome Res 18, 729–741.

Sturgill-Koszycki, S., Schlesinger, P.H., Chakraborty, P., Haddix, P.L., Collins, H.L., Fok, A.K., Allen, R.D., Gluck, S.L., Heuser, J., and Russell, D.G. (1994). Lack of acidification in Mycobacterium phagosomes produced by exclusion of the vesicular proton-ATPase. Science 263, 678–681.

Taylor, J.L., Hattle, J.M., Dreitz, S.A., Troudt, J.M., Izzo, L.S., Basaraba, R.J., Orme, I.M., Matrisian, L.M., and Izzo, A.A. (2006). Role for matrix metalloproteinase 9 in granuloma formation during pulmonary Mycobacterium tuberculosis infection. Infect Immun 74, 6135–6144.

Traver, D., Herbomel, P., Patton, E.E., Murphey, R.D., Yoder, J.A., Litman, G.W., Catic, A., Amemiya, C.T., Zon, L.I., and Trede, N.S. (2003). The zebrafish as a model organism to study development of the immune system. Adv Immunol 81, 253–330.

Volkman, H.E., Clay, H., Beery, D., Chang, J.C., Sherman, D.R., and Ramakrishnan, L. (2004). Tuberculous granuloma formation is enhanced by a mycobacterium virulence determinant. PLoS Biol 2, e367.

Volkman, H.E., Pozos, T.C., Zheng, J., Davis, J.M., Rawls, J.F., and Ramakrishnan, L. (2010). Tuberculous granuloma induction via interaction of a bacterial secreted protein with host epithelium. Science 327, 466–469.

Willett, C.E., Cortes, A., Zuasti, A., and Zapata, A.G. (1999). Early hematopoiesis and developing lymphoid organs in the zebrafish. Dev Dyn 214, 323–336.

