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Immunological Aspects

A new model for chronic and reactivation tuberculosis: Infection with genetically attenuated *Mycobacterium tuberculosis* in mice with polar susceptibility

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ABSTRACT

TB infection in mice develops relatively rapidly which interferes with experimental dissection of immune responses and lung pathology features that differ between genetically susceptible and resistant hosts. Earlier we have shown that the *M. tuberculosis* strain lacking four of five *Rpf* genes (ΔACDE) is seriously attenuated for growth *in vivo*. Using this strain, we assessed key parameters of lung pathology, immune and inflammatory responses in chronic and reactivation TB infections in highly susceptible I/St and more resistant B6 mice. ΔACDE mycobacteria progressively multiplied only in I/St lungs, whilst in B6 lung CFU counts decreased with time. Condensed TB foci apeared in B6 lungs at week 4 of infection, whilst in I/St their formation was delayed. At the late phase of infection, in I/St lungs TB foci fused resulting in extensive pneumonia, whereas in B6 lungs pathology was limited to condensed foci. Macrophage and neutrophil populations characteristically differed between I/St and B6 mice at early and late stages of infection: more neutrophils accumulated in I/St and more macrophages in B6 lungs. The expression level of chemokine genes involved in neutrophil influx was higher in I/St compared to B6 lungs. B6 lung cells produced more IFN-γ, IL-6 and IL-11 at the early and late phases of infection. Overall, using a new mouse model of slow TB progression, we demonstrate two important features of ineffective infection control underlined by shifts in lung inflammation: delay in early granuloma formation and fusion of granulomas resulting in consolidated pneumonia late in the infectious course.

1. Introduction

It is generally considered that in the absence of overt dysfunctions in the immune system not more than 3–10% of individuals infected with *M. tuberculosis* eventually develop clinical disease [1]. About 90% of infected individuals without clinical manifestations comprise an enormous reservoir of latent tuberculosis infection (LTBI). Apparently, LTBI may last asymptomatically for a very long time and represents the most common variant of tuberculosis (TB) infection [2]. In some of these latently infected individuals, infection progresses to the active state, becomes contagious and seriously affects the epidemiological situation [3]. Presently, little is known about the mechanisms of protective immunity to and pathogenesis of LTBI. Despite serious attention to the problem of TB latency and reactivation during recent decades, we still do not understand the biology of chronic mycobacterial containment, LTBI and its transition to overt infection [4].

Immune responses against *M. tuberculosis* have been characterized in considerable detail (reviewed in Refs. [5–7]). Nevertheless, it is not clear what combination of immune reactions restricts mycobacterial growth and protects the lung from tissue damage [8,9], nor what local immune reactions in the lungs and lymphoid organs contain the latent state of infection [10,11]. In mice, immune responses during prolonged TB infection have been mostly studied in two settings: chronic disease after a low-dose aerosol challenge in B6 mice [12,13], and reactivation disease after antibiotic treatment withdrawal (Cornell model) in a few mouse strains [14–17]. Although these experiments provided many interesting observations (reviewed in Ref. [18]), intrinsic limitations of models themselves raise questions about the relevance of these observations to the problem of TB latency in humans [19,20]. Nevertheless, by combining genetically susceptible mice, low-dose aerosol

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challenge and prolonged chemotherapy with two antibiotics in the Cornell-like TB model, we observed indefinite resolution of lung disease without total eradication of mycobacterial cells and DNA from the lung tissue [15] – the phenotype closely resembling LTBI in humans.

All chronic TB mouse models applied so far have provided infection dynamics that ended up with inevitable death of the host caused by lung pathology. Even the most often used "low" doses of infection (50–200 CFU per mouse) kill relatively resistant B6 mice within as few as 7–10 months post challenge [13]. In our hands, aerosol infection of B6 mice with ~100 CFU of *M. tuberculosis* H37Rv resulted in a classical picture: after logarithmic growth for 1 month, lung CFU counts dropped ~1-log during month 2 of infection, returned to the 1st month level at weeks 9–10 and thereafter grew slowly. All mice died before day 300 post challenge [18]. Thus, in our model, the type of immune response was not sufficient for effective control of mycobacterial growth in the lungs for a substantial period, and the duration of survival was far too short to study long-term TB containment followed by late relapse associated with aging.

Analogously, serious complications accompany the modeling of TB reactivation in Cornell-like models. In early studies, all research teams reported reactivation of infection after cessation of antibiotic treatment only in a proportion of mice, although genetically identical inbred animals were used in most experiments [21]. Later, we demonstrated that the disease reactivation differs profoundly between genetically susceptible I/St mice, which display reactivation disease in all animals, and relatively resistant B6 mice demonstrating slow reactivation in only some of the animals. However, although I/St mice might be a more reliable tool for modeling TB relapse after chemotherapy withdrawal than mice of more resistant strains, resuscitation of progressive growth in organs of highly virulent *M. tuberculosis* occurred too rapidly in these hyper-susceptible animals and all mice died before month 6 after chemotherapy withdrawal [15]. Again, the total duration of the experiment (~9 months) was not sufficient for evaluating late reactivation events. Attempts to apply longer (3-mo) combined rifampicin (RIF) plus isoniazid (INH) treatment of I/St mice resulted in an interesting picture of a life-long mycobacterial persistence *in vivo*, but reactivation disease did not develop at all [15]. In contrast, a more sophisticated model of infection based upon transient failure of $CD4^+$ T cell-depleted B6 mice to control infection after subcutaneous challenge demonstrated relatively rapid (< 6-mo) relapse of mycobacterial burden and lung pathology [22].

Considering the incompleteness and discrepancies of these results, we decided to develop a new approach for establishing extremely slow, but progressive, TB infection: infect mice with a mycobacterial strain highly attenuated due to knockout mutations in genes involved in mycobacterial growth control *in vivo*. For many years, the TB scientific community has been studying experimental infections with attenuated mycobacteria for modeling several aspects of mycobacterial pathogenesis, dissemination, vaccination and treatment. However, we designed our model to meet requirements that often have not been taken into account. We decided not to use mycobacteria belonging to the species other than *M. tuberculosis*. Several low virulent (e.g., *M. avium*), or non-virulent (e.g., *M. bovis* BCG) mycobacteria display completely different mechanisms of genetic and immunological host control compared to *M. tuberculosis* (reviewed in Ref. [23] and, thus, do not adequately mimic TB infection. In addition, these mycobacteria lack the RD1 secretion system [24] essential for the intracellular life style of the parasite [25,26]. The latter is applicable also to several *M. tuberculosis* strains attenuated due to genetic disruption of virulence factors encoded in RD1, such as ESAT-6 and cfp10 [27]. An interesting exception is the mutant non-virulent *M. tuberculosis* strain 18b, but its attenuation *in vitro* and *in vivo* is totally streptomycin-dependent and complete [28], making usage of 18b strain for chronic, let alone reactivation, experiments under non-SPF conditions problematic. Thus, we decided to use a mycobacterial strain, which is markedly attenuated, possesses residual virulence and is not deficient for genes contained within the RD1 system.

Earlier we have shown that *M. tuberculosis* strains lacking three of five genes belonging to the *Rpf* family are seriously attenuated for growth *in vivo*, and that the quadruple *Rpf* deletion mutant ΔACDE displays defective growth in mouse lungs after aerosol infection [29,30]. Thus, the *M. tuberculosis*-ΔACDE strain (hereafter, ΔACDE) meets the requirements described above: it is profoundly attenuated and retains the RD1 secretion system. Using this strain, we performed experiments to assess key parameters of mycobacterial multiplication, mouse mortality, lung pathology, and immune and inflammatory responses in chronic and reactivation TB infection in highly susceptible I/St and relatively resistant C57BL/6 mice [18].

2. Materials and methods

Mice of inbred strains I/StSnEgYCit (I/St) and C57BL/6JCit (B6) were bred and maintained under conventional conditions at the Animal Facilities of the Central Institute for Tuberculosis (CIT, Moscow, Russia) in accordance with guidelines from the Russian Ministry of Health # 755, and under the NIH Office of Laboratory Animal Welfare (OLAW) Assurance #A5502-11. Water and food were provided *ad libitum*. Female mice of 10–12 week of age in the beginning of experiments were used. All experimental procedures were approved by the CIT animal care committee (IACUC protocols #2, 7, 8, 11, approved on March 6, 2016).

2.1. Mycobacteria and infection

Attenuated phenotypes of multiple Rpf-KO *M. tuberculosis* strains, including ΔACDE quadruple mutant, as well as complementation data, were described previously [31]. The ΔACDE strain was used in our previous studies [30]. Preparations of clump-free mid-log-phase suspensions of mycobacteria used for challenge were prepared as described elsewhere [32,33]. The general experimental scheme is displayed in Supplementary Fig. 1. Mice in groups of 36 (experiment 1: chronic infection model, duration 400 days) and 45 (experiment 2: chronic infection + Cornell models, duration 550 days) of each strain were infected with a calculated dose of $\sim 10^3$ CFU of *M. tuberculosis*-ΔACDE using the Inhalation Exposure System (Glas-Col, Terre Haute, IN) as described previously [15]. Assessment of the inhaled dose by the lung CFU counting one day after challenge provided the actual infectious dose of $2.8 \pm 0.2 \times 10^3$ CFU per mouse with no significant differences between the two mouse strains. At different time points post challenge, mice were euthanized by a thiopental (Biochemie GmbH, Vienna, Austria) overdose. Lungs were aseptically removed, and individual lobes of the right lungs were either enzymatically digested for cell isolation or homogenized for CFU counting and RNA extraction. Left lungs were used for histological assessments.

2.2. Lung cell suspensions

Lungs were enzymatically digested as described previously [33,34]. Briefly, blood vessels were washed out by heart perfusion via cut vena cava with 0.02% EDTA-PBS, lungs removed, sliced into 1–2 mm^3 pieces and incubated at 37 °C for 90 min in supplemented RPMI-1640 containing 200 U/ml collagenase and 50 U/ml DNase-I (Sigma, MO). Single cell suspensions from 4 mice were obtained individually, washed twice in HBSS containing 2% FCS and antibiotics. 3×10^5 cells were used for assessment of surface phenotypes and the remaining sample was used for cell culturing.

2.3. CFU counts, survival time and lung pathology

At indicated time points following infection, spleens and identical lobes of the right lungs from individual mice were homogenized in 2.0 ml of sterile saline, and 10-fold serial dilutions of 0.2 ml samples