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MODEL SYSTEMS

A new murine model to study musculoskeletal tuberculosis (short communication)

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SUMMARY

Musculoskeletal tuberculosis (TB) is a severe extrapulmonary manifestation of chronic *Mycobacterium (M.) tuberculosis* infection. Considering increasing incidence, multi-drug resistance and associated treatment difficulties, more preclinical research is needed. In this study we developed a murine model for musculoskeletal TB. Mice, intranasally infected with *M. tuberculosis*, were sacrificed after ten months. Mycobacterial growth was detected in lung and femur homogenates. Ziehl–Neelsen staining of paraffin-embedded femurs showed acid-fast rods in the myelum and Magnetic Resonance Imaging demonstrated osteomyelitis and macronodular tuberculomas. This new murine model of musculoskeletal TB might be of value to further investigate immunologic and radiologic responses.

1. Introduction

Tuberculosis (TB) is one of the most devastating infectious diseases worldwide, with one-third of the world population being infected with the causative agent *Mycobacterium (M.) tuberculosis* [1]. In 2012, 8.7 million new TB cases and 1.4 million deaths were registered [1,2]. TB is characterized by well-known caseating lung lesions but might have extrapulmonary disease manifestations upon either lymphogenous or hematogenous dissemination of bacteria, which are present with a rising incidence in 20%–40% of all TB patients [1–4]. Musculoskeletal TB, representing approximately 11% of all extrapulmonary TB cases [3–5], most frequently affects the spine, hip and knee resulting in osteomyelitis, paraspinal abscesses, TB arthritis and granulomas in the bone [5]. Spinal TB in particular, also referred to as Pott's disease, represents approximately half of all cases of musculoskeletal TB [6].

Musculoskeletal TB results from hematogenous spread of *M. tuberculosis* from other sites, most often the lungs. Once mycobacteria have infected the bone marrow, granulomatous lesions are formed that attract multiple inflammatory cells, including foamy macrophages and T-cells [5,7]. As the lesion grows caseation occurs, with destruction of the bone trabeculae and, in a later stadium, the bone cortex [7,8]. The predilection of mycobacteria for the spine and large joints may be explained by the rich vascular supply of vertebra and growth plates of the long bones [5,8]. In spinal TB the infection is thought to spread from two adjacent vertebrae into the adjoining intervertebral space. When two vertebrae are affected, the disc, which is avascular in adults, cannot receive nutrients and becomes necrotic which leads to vertebral narrowing and eventually to vertebral collapse and spinal damage. Spinal TB is indolent, slow-growing and, if left untreated, may cause complications such as (paraspinal) abscesses, spinal cord compression, spine deformations and neurological deficits, including radicular pain and even severe paraplegia [6–8].

Thus far, Magnetic Resonance Imaging (MRI) is the best modality to diagnose musculoskeletal involvement of TB, with a 100%

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sensitivity and 88% specificity [6,8]. Due to the increasing incidence of HIV-associated and multi-drug resistant TB, the incidence of musculoskeletal TB is also increasing [3–5]. Evidently, more research is needed to study the clinical and immunological features of this disease and mouse models of TB have proven to be of great value. No murine models of musculoskeletal TB have been described so far. Therefore, in this study we aimed to develop a mouse model suitable for investigation of immunologic and radiologic responses during musculoskeletal TB.

2. Materials and methods

Fourteen 8-week old female wild-type (WT) C57BL/6 mice (Charles River, Maastricht, The Netherlands) were inoculated intranasally with 150 colony forming units (CFU) of *M. tuberculosis* (Erdman strain) in 50 μl NaCl0.9%, as previously described [9]. Five control mice were inoculated with 50 μl NaCl0.9% only. Mice were maintained at the animal care facility of the Academic Medical Center (University of Amsterdam), according to national guidelines with free access to food and water. The Committee on Use and Care of Animals of the University of Amsterdam approved all experiments. Ten months after instillation of *M. tuberculosis* or saline, 10 mice were sacrificed under intraperitoneal anesthesia containing ketamin (Eurovet Animal Health, Bladel, The Netherlands) and medetomidin (Pfizer Animal Health Care, Capelle aan den IJssel, The Netherlands). From these mice organs were processed as previously described [9]. Briefly, left lungs, liver, spleen and left femurs were removed aseptically and homogenized at 4 °C in 4 volumes of sterile saline using a tissue homogenizer (Biospec Products, Bartlesville, OK). CFU were determined from serial dilutions of organ homogenates that were plated in tenfold dilutions on Middlebrook 7H11 plates and incubated at 37 °C 5% CO_2 for 21 days before colonies were counted. Additionally, from these 10 infected and from 3 control mice right femurs and lumbar spines were removed and fixed in 4% formalin for 24 h before embedding them in paraffin. Four μm-sections were stained for H&E and Ziehl–Neelsen (ZN) to detect acid-fast rods. For ZN-staining, after deparafinization, slides were flooded with carbofuchsin (Merck, Darmstadt, Germany) and heated for 10 min until steam appeared. Next, slides were flooded with 1% hydrochloric acid (25%) in alcohol (70%), rinsed with tap water, stained with 0.1% methylene blue for 10 s, which was then rinsed off with tap water. Slides were scored by a well-qualified pathologist.

For cytokine measurements, organ homogenates were diluted 1:2 in lysis buffer containing 300 mM NaCl, 30 mM Tris, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 1% Triton X-100 and protease inhibitor cocktail (Roche, Indianapolis, IN). Homogenates were centrifuged at 1500*g* at 4 °C for 15 min, supernatants were sterilized using a 0.22 μm pore-size filter (Corning Inc., Corning, NY) and stored at −20 °C until analysis. Levels of interferon (IFN)-γ, tumor necrosis factor (TNF)-α, interleukin (IL)-4, IL-6, IL-10, keratinocyte-derived chemokine (KC) and macrophage inflammatory protein (MIP)-2 were measured by ELISA according to the manufacturer's instructions (all from R&D Systems, Minneapolis, MO).

Ten months after instillation of *M. tuberculosis* ($n = 4$) or saline ($n = 2$) separate sets of mice were euthanized by CO_2 inhalation, placed in an airtight plastic bag to prevent contamination and immediately thereafter scanned on a clinical, whole body 3 T MRI-scanner (Intera, Philips Medical Systems, Best, the Netherlands) using a circular receive-only surface radiofrequency-coil (ø 5 cm) for signal reception. MRI consisted of localizers followed by a T2*-weighted sagittal fast gradient echo ($TR/TE_1/\Delta TE$ 1500/2.8/4 ms, FA 36°, slice thickness 1.6 mm, voxel size 0.39 × 0.39 mm) and a 3D T2-weighted sagittal turbo spin echo (TR/TE 2000/332 ms, FA 90°, isotropic voxels of 0.45 × 0.45 × 0.45 mm) with coronal reconstructions. Mice were placed prone in the scanner, perpendicular to the bore axis. Total acquisition time including positioning per mouse was 20 min. For each mouse a standardized set of items was evaluated including: presence of granuloma, infiltration of inflammatory cells and osteomyelitis in both femur and spinal column. With respect to the spinal column, collapse of vertebral bodies, paraspinal abscesses, herniation of intervertebral discs, erosions and angular kyfosis were assessed. Presence of abscesses in the supraclavicular, gluteal, groin and psoas regions was noted. Additionally the lungs were evaluated for granuloma formation. The primary read was performed independently by 2 research trainees. After a consensus reading, the combined results were (re-) evaluated by an experienced musculoskeletal radiologist and a MR physicist. Based on this meeting, a new set of coronal slices were reconstructed to improve evaluation of the spleen. In a second and final consensus meeting, the abovementioned items as well as lung and spleen were (re-)evaluated. Besides this qualitative assessment, T2* times of femoral and spinal bone marrow and the spleen were assessed using a mono-exponential signal decay function. Regions-of-Interest (ROIs) were drawn on the first echo of the T2*-weighted dataset and copied to later echoes. Mean signal intensities inside the ROIs at each echo time (TE) were fit against the TE to obtain the T2* times in ms.

Data are expressed as dots plots with medians. Comparisons between groups were conducted using the Mann–Whitney *U* test. For differences between T2*-times an unpaired *t*-test was used. All analyses were done using GraphPad Prism® version 5.01 (San Diego, CA). *P*-values <0.05 were considered statistically significant.

3. Results

Ten months after infection *M. tuberculosis* could be cultured from lungs, spleen, liver and femur, indicating dissemination from the primary site of infection (Figure 1A). There was no significant correlation between bacterial loads in the lungs and femur (Figure 1B). Both in lung and femur homogenates, significantly increased IFN-γ and KC levels were measured after infection with *M. tuberculosis* when compared with uninfected controls Figure 1(C) and (D), Online Table 1; both $P < 0.05$). TB-infection was also associated with increased levels of IL-4, IL-6 and MIP-2 in lung homogenates when compared to uninfected controls. These significant differences could not be detected in femur homogenates (Table 1). Cross-sections of right femurs and lumbar spines of TB-infected mice did not show an overt tissue reaction to infection with *M. tuberculosis*, as no granuloma, no osteomyelitis and no paraspinal abscesses could be detected (Figure 1(E)–(J)). Nonetheless, ZN-staining of these tissues showed acid-fast rods in the myelum of femurs of TB-infected mice, fitting with mycobacterial infiltration of the bone (Figure 1(K) and (L)).

MR features of TB-associated osteomyelitis, reflected as an increased signal intensity (SI) [10], were seen in the vertebral bodies of all TB-infected mice and were absent in control mice (Figure 2(A) and (B)). Femurs showed increased SI in all TB-infected mice when compared to uninfected mice (Figure 2(C) and (D)). Additionally, focal areas of increased spleen SI were seen on coronal reconstructions in all 4 TB-infected mice. These were absent in the uninfected mice, whereas liver SI was comparable between both groups (data not shown). No other items of the evaluation set were noted in any of the mice. In addition, a clear SI difference between lung tissue of TB-infected and control mice was seen (not shown). Mean T2*-times of the spinal column and femur but not of spleen were lower in TB-infected compared with uninfected mice (Supplementary Table 1). This may reflect the replacement of fatty bone marrow (with longer T2*) by osteomyelitis-related water content, hence lowering T2*-times in the spine and femur. This