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MECHANISMS OF PATHOGENESIS

Relapse of tuberculosis versus primary tuberculosis; course, pathogenesis and therapy in mice

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SUMMARY

Relapse of tuberculosis (TB) is defined as re-emergence of clinical symptoms after stopping anti-TB treatment, while this treatment appeared effective initially. Relapse of TB can occur in patients that are therapy-compliant, but the risk of relapse is dramatically increased when patients are non-compliant. Additionally, the probability of antibiotic resistance is higher in those patients who have a relapse of TB and thus longer treatment is recommended. Further insight in the pathogenesis of relapsing TB could provide a basis for future treatment improvement. In the present study, using a murine TB model, we assessed the differences between primary TB and relapse of TB in terms of mycobacterial load in infected organs, (immuno-) histopathology, and plasma cytokine concentrations. Compared to primary TB, in relapse of TB we observed a lower mycobacterial load in lung, spleen and liver at the phase of established infection. Also the levels of TNF-α, IFN-γ, IL-6, MIG/CXCL9, IP-10/CXCL10 and IL-17 were significantly lower. It was observed that in relapse of TB memory Th-1 cells were locally and systemically expanded and congregated in the lung, permitting an efficient control of Mtb growth. Treatment response in relapse of TB is as good as the treatment response in primary TB; thereby no supportive evidence could be given for the recommended longer treatment duration in case of relapse of TB.

1. Introduction

Tuberculosis (TB) remains an infectious disease that requires extremely lengthy treatment.[1] A number of studies are ongoing to reduce treatment duration using new TB drugs while preserving treatment efficacy.[2] However, the current (minimal) six-month therapy leads to many problems, among which the high proportion of patients failing to adhere to the TB treatment.[3] As a result of this non-compliance (non-adherence) of patients, especially in TB-HIV co-infected patients there seems to be an increased risk of therapy failure, resulting in relapse of infection and/or development of resistance.[3–6] These potential problems were among the driving forces to enrol patients in Directly Observed Therapy (DOT) programmes.[7]

Recurrent TB infection should be divided into relapse of infection and re-infection, which are two fundamentally independent forms of TB infection.[8] Re-infection occurs in endemic areas where there is high incidence of TB in a crowded population (such as prisons and townships), with limited coughing hygiene and high vulnerability to infection due to malnutrition and HIV infection.[3,4] Thus, re-infection is an infection caused by a new (exogenous) strain of *Mycobacterium tuberculosis* (Mtb). In contrast, relapse of TB infection is recurrence of an untreated or inadequately treated TB infection, with the same (endogenous) Mtb strain.

Regarding the therapy of re-infection versus relapse of infection a different approach is needed. Re-infection of TB can be considered as a new primary infection allowing standard regimens to be started. This re-infection occurs often, partly because patients that had TB once have a strongly increased risk of developing TB when they are re-exposed.[9] In contrast to re-infection with TB, relapse of TB infection carries an increased risk of infection due a resistant variant of their original mycobacterial strain persisting after inadequately-treated primary TB infection, and re-growing during the relapse period.[10] As a consequence, the recommended treatment for relapsing TB infection is an 8-month re-treatment regimen, consisting of 2 months of isoniazid, rifampin, pyrazinamide, ethambutol and streptomycin followed by 1 month of isoniazid, rifampin, pyrazinamide and ethambutol and finally 5 months of isoniazid, rifampin and ethambutol.[7]

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The present study was performed to investigate the course and pathogenesis of relapse of TB versus primary TB. In our mouse TB model we induced relapse of TB by administration of suboptimal TB therapy to mice with primary TB infection, in this respect closely mimicking non-compliance of patients.[11] Parameters to characterize the course of TB infection were quantitative mycobacterial cultures from infected organs, histopathology in infected organs and cytokine profile in blood. In addition, we investigated differences in therapy response of mice with primary TB versus mice with relapse of TB.

2. Materials and methods

2.1. Bacterial culture

Mtb strain H37Rv (ATCC 27294) was used. Bacterial suspensions were cultured in Middlebrook 7H9 broth (Difco Laboratories, Detroit, MI, USA), supplemented with 10% oleic acid-albumin-dextrose-catalase enrichment (OADC, Baltimore Biological Laboratories, Baltimore, MD, USA), 0.5% glycerol (Scharlau Chemie S.A, Sentmenat, Spain) and 0.05% Tween 20 (Sigma Chemical Co, St. Louis, MO, USA), under shaking conditions at 96 rpm at 37 °C. Mtb suspensions were stored at −80 °C. Cultures on solid media were grown on Middlebrook 7H10 agar (Difco), supplemented with 10% OADC for 21 days at 37 °C with 5% CO_2.

2.2. Primary TB, relapse of TB, and treatment with anti-TB drugs

Experimental TB in mice was established via infection using the respiratory route, as described previously.[11] In short, TB drugs dosage and schedules used were derived from current clinical guidelines.[12,13] Isoniazid, rifampin and pyrazinamide, in human pharmacokinetic-equivalent doses[14–16] were administered, as described previously.[11] Treatment of primary TB was started at 4 weeks after Mtb inoculation, when patches of pneumonic lesions were observed and established infection was confirmed by stabilization of the Mtb load in infected organs and TB-characteristic histopathological findings.[11] Treatment consisted of a 9-week initial phase followed by a 4- or 17-week continuation phase. During the initial phase, animals received a combination of isoniazid [25 mg/kg], rifampin [10 mg/kg] and pyrazinamide [150 mg/kg]. In the continuation phase, animals continued with isoniazid and rifampin. Agents were administered subcutaneously once daily, 5 days a week. In mice with primary TB, treated for 13 weeks only, relapse of TB infection occurred in all mice at the end of the 13-weeks post-treatment period. At that time point the treatment of relapse of TB was started and continued for 26 weeks.

2.3. Determination of viable Mtb counts in infected organs and blood

At indicated time points mice ($n = 4$ per time point) were sacrificed by CO_2 exposure. The lung, spleen and liver were removed aseptically and processed as described previously.[11] Blood samples were taken via cardiac puncture and the entire organs were removed and homogenized in 2 mL PBS. From the undiluted tissue homogenate and the 10-fold serial dilutions of the homogenate, samples of 200 μl were plated onto solid medium for CFU counting after 3 weeks of incubation of the subculture plates.

2.4. Selection of drug-resistant Mtb

In order to detect the presence of drug-resistant Mtb mutants, samples from infected tissue homogenates were cultured on rifampin-containing and isoniazid-containing solid media. The concentrations of rifampin and isoniazid in the subculture plates were 4-fold the “critical concentration”, and were 4 mg/L rifampin, 0.8 mg/L isoniazid.

2.5. Assessment of relapse of TB infection

The number of CFU in lung, spleen and liver of mice ($n = 4$) was assessed 13 weeks after termination of TB treatment. Relapse of was defined as Mtb-positive organ cultures, while immediately after termination of treatment organs were Mtb culture-negative.

2.6. Histopathological examination of infected organs

Histopathological changes in lung, liver and spleen during the course of the (un-)treated infection were determined by sacrificing the animals ($n = 3$) at indicated time points, as described previously.[11] Paraffin-embedded tissues were cut into 4 μm sections from which one in every 7 cuts was used for haematoxylin-eosin staining. A pathologist, blinded to the experimental conditions, examined 4 slides of each tissue. The same paraffin-embedded material prepared for the histopathological studies was used to determine the local cytokine production by immunohistochemistry. Lung sections from infected mice were de-paraffinized and maintained in HCN buffer (Hepes, NaCl and $CaCl_2$). Sections were washed with HCN + 0.05% Tween 20, and the endogenous peroxidase activity was blocked with 6% H_2O_2 dissolved in PBS + 0.1% sodium azide and incubated for 1 h. After blocking with normal swine sera, tissue sections were incubated with primary antibodies overnight at 4 °C at optimal dilutions, which had been determined previously. We used primary antibodies against TNF-α (rabbit polyclonal IgG, 281 clone H-156, sc-8301, Santa Cruz Biotechnology), IFN-γ (goat polyclonal IgG, clone D-17, sc-9344, Santa Cruz Biotechnology), and IL-4 (goat polyclonal IgG, Santa Cruz Biotechnology). Secondary biotinylated antibodies (anti-rabbit-biotin IgG or anti-goat-biotin IgG) were used to detect the binding of the primary antibodies. Finally, HPR-conjugated avidin and 3,3-diaminobenzidine (DAB)/hydrogen peroxide were used to develop the reaction. Tissue sections were counterstained with haematoxylin.

2.7. Cytokine-/chemokine concentrations in blood

At week 4, 9 and 17 of primary TB and relapse of TB blood was obtained from mice, and from EDTA-blood plasma samples were prepared. Quantification of cytokines/chemokines was performed using a bead-based flow cytometry technique (xMap; Luminex Corporation, Austin, TX, USA). Mouse soluble cytokine receptor multiplex panel used (Millipore Corporation, Billerica, MA, USA), consisted of bead-labelled cytokine receptor against following biomarkers; granulocyte colony-stimulated factor (G-CSF), interferon gamma (IFN-γ), interleukin (IL)-1β, IL-2, IL-4, IL-6, IL-10, IL-12 (p40), IL-13, IL-17, IFN-γ-induced protein-10 (IP-10/CXCL10), macrophage colony-stimulated factor (M-CSF), monocyte chemoattractant protein-1 (MCP-1/CCL2), macrophage induced by IFN-γ (MIG/CXCL9), murine macrophage inflammatory protein-1α (MIP-1α), MIP-1β and tumour necrosis factor alpha (TNF-α). Tests were performed according to manufacturer’s protocol. Samples were tested in duplicates. Results in median fluorescence intensity (MFI) values were converted to pg/mL using MILLIPLEX Analyst software (Millipore) and subsequently averaged.

2.8. Statistical analysis

CFU counts were log10 transformed before analysis. The CFU counts and serum cytokine levels of the primary TB groups of mice