Tuberculosis 91 (2011) 378–385

Contents lists available at ScienceDirect

Tuberculosis

journal homepage: http://intl.elsevierhealth.com/journals/tube

MECHANISMS OF PATHOGENESIS

Increased Foxp3 expression in guinea pigs infected with W-Beijing strains of *M. tuberculosis*

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ARTICLE INFO

Article history:
Received 30 November 2010
Received in revised form
28 April 2011
Accepted 6 June 2011

Keywords:
Mycobacterium tuberculosis
Guinea pig
Foxp3+ regulatory T cells
Clinical isolates
W-Beijing strains

SUMMARY

There is increasing evidence that clinical isolates of *Mycobacterium tuberculosis* that belong to the W-Beijing genotype of newly emerging strains are often of very high virulence when tested in small animal models, including the mouse and guinea pig. In this report we provide further evidence to support this contention, and show that two W-Beijing strains are of very high virulence when introduced by low dose aerosol into outbred guinea pigs. In addition to severe lung pathology, each of these infections was associated with large influxes of activated CD4 and CD8 T cells into the lungs. Large influxes of macrophages were also observed, but the fraction of these showing evidence of activation by Class-II expression was relatively low. A progressive increase in neutrophils was also seen, with highest levels accumulating in the lungs of the W-Beijing infected animals. In the case of these two infections mRNA levels for TH1 cytokines was elevated early, but these then declined, and were replaced by increasing levels of message encoding for Foxp3, IL-10, and TGFβ. These observations support the hypothesis that W-Beijing strains are potent inducers of regulatory T cells, and that this event may enhance survival and transmission of these bacilli.

Introduction

The global epidemic caused by the bacterial pathogen *Mycobacterium tuberculosis* continues unabated, with the most recent figures in 2009 estimating 9.4 million incident cases of tuberculosis, with about 1.3 million deaths.[1,2] It is becoming evident that a significant percentage of new clinical isolates of *M. tuberculosis* are of extremely high virulence.[3–5] Amongst these, the W-Beijing family of *M. tuberculosis* is globally distributed and is being increasingly documented as a cause of major outbreaks of infection worldwide that involve multidrug-resistant strains.[6–10] Increasing evidence suggests that the Beijing genotype family can induce distinctly different host immune responses compared to other *M. tuberculosis* strains, and amongst these is the newly emerging idea that this family induces the generation of regulatory T cells[11]; an event that could allow evasion of both innate[12] and acquired immunity.[11,13]

Despite the obvious high virulence of these newly emerging clinical strains, most work on screening new drugs and vaccines has used the "laboratory strains" H37Rv and Erdman.[14,15] This is of concern, because it has already been noted[11] in the mouse model that such strains are of far less potency in terms of their capacity to induce regulatory T cell responses. To date however it remains unknown if this caveat extends to the guinea pig animal model, which remains the gold standard for testing new vaccine candidates. To begin to address this question we compared three clinical isolates (two of them, W-Beijing strains) in parallel with the two laboratory strains for their ability to infect and grow in the lungs of guinea pigs after low dose aerosol infection. Using newly developed flow cytometry for this species[16] we were further able to monitor the influx of several cell populations into the lungs, including activated T cell subsets. We are unable as yet to perform intracellular staining for cytokines in this species, but were able to track TH1 cytokines, as well as cytokines associated with negative regulation of immunity, using RT-PCR.

The results of this study show that the two W-Beijing strains, as well as a multidrug-resistant "P family" isolate, grew to higher numbers in the lungs of these animals compared to the two

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doi:10.1016/j.tube.2011.06.001

laboratory strains. This was further associated with more severe lung pathology, and reduced survival. These events were associated with an initial higher expression of message encoding the TH1 cytokines IL-12p40 and gamma interferon (IFNγ) in animals infected with the clinical strains, but this was then followed by progressive increases in mRNA encoding the regulatory T cell markers Foxp3, IL-10, and TGFβ in the animals exposed to the W-Beijing isolates. These data thus lead us to hypothesize that W-Beijing isolates of *M. tuberculosis* induce potent T regulatory cell responses in the guinea pig model, a finding that has the potential to serious confound vaccine testing in this model which is routinely performed using the laboratory strains.

Methods

Guinea pigs

Female outbred Hartley guinea pigs (~500 g in weight) were purchased from the Charles River Laboratories (North Wilmington, MA, USA) and held under barrier conditions in a Biosafety Level III animal laboratory. The specific pathogen-free nature of the guinea pig colonies was demonstrated by testing sentinel animals. All experimental protocols were approved by the Animal Care and Usage Committee of Colorado State University and comply with NIH guidelines.

Experimental infections

The laboratory strains *M. tuberculosis* H37Rv and Erdman were originally obtained from the Trudeau Institute collection, Saranac Lake, NY. The clinical isolates used in this study were chosen specifically because they were all associated with outbreaks in the United States. Three strains from the Public Health Research Institute TB Center collection were selected in regard to their genetic backgrounds and their resistance phenotype. Strain TN14149 ("W10" DNA fingerprint) is a member of the W-Beijing strain family; it is drug susceptible, and has an identical fingerprint to the sequenced strain 210. Strain TN5904 is a "P" family cluster group VI MDR-TB isolate with resistance to isoniazid, rifampin, p-aminosalicylic acid, and streptomycin, that was isolated from HIV-positive patients that had undergone exogenous reinfection.[17] The W-Beijing strain SA161 is an isolate found within a cluster of cases in Arkansas.

All of the strains used in this study were grown in 7H9 broth containing 0.05% Tween-80. Thawed aliquots of frozen cultures were diluted in sterile water to the desired inoculum concentrations. A Madison chamber aerosol generation device was used to expose the animals to *M. tuberculosis*. This device was calibrated to deliver approximately 20 bacilli into the lungs. Lung bacterial counts on days 10, 30 and 60 were determined by plating serial dilutions of tissue homogenates on nutrient 7H11 agar and counting colony-forming units after 3 weeks incubation at 37 °C. In survival studies, animals showing substantial weight loss with no evidence of weight rebound were euthanized. The results shown in the survival studies are based upon 8-10 guinea pigs per group.

Histological analysis

The lung lobes, spleen and mediastinal lymph nodes from each guinea pig were fixed with 4% paraformaldehyde in phosphate buffered saline (PBS). Sections from these tissues were stained using hematoxylin and eosin. The concurrent progression of lung and lymph node lesions was evaluated using a histological grading system.[18]

Organ digestion

To prepare single cell suspensions, the lungs, lymph nodes and spleens were perfused with 20 ml of a solution containing PBS and heparin (50 U/ml; Sigma–Aldrich, St. Louis, MO) through the pulmonary artery and the caudal lobe aseptically removed from the pulmonary cavity, placed in media and dissected. The dissected lung tissue was incubated with complete DMEM (cDMEM media) containing collagenase XI (0.7 mg/ml; Sigma–Aldrich) and type IV bovine pancreatic DNase (30 μg/ml; Sigma–Aldrich) for 30 min at 37 °C. The digested lungs were further disrupted by gently pushing the tissue twice through a cell strainer (BD Biosciences, Lincoln Park, NJ). Red blood cells were lysed with ACK buffer, washed and resuspended in cDMEM. Total cell numbers were determined by flow cytometry using BD™ Liquid Counting Beads, as described by the manufacturer (BD PharMingen, San Jose, CA USA 95131).

Flow cytometric analysis of cell surface markers

Single cell suspensions from each individual guinea pig were incubated first with antibodies as previously described[16,19] to CD4, CD8, pan T cell, CD45, MIL4, B cell, macrophage and class-II antibodies at 4 °C for 30 min in the dark after washing the cells with PBS containing 0.1% sodium azide (Sigma–Aldrich). The anti-guinea pig macrophage MR-1 antibody is an intracytoplasmic antigen and therefore cell membranes were permeabilized using Leucoperm (Serotec Inc, Raleigh, NC) according to the manufacturer's instructions prior to intracellular staining. Data acquisition and analysis were done using a FACSCalibur flow cytometer (BD Biosciences, Mountain View, CA) and CellQuest software (BD Biosciences, San Jose, CA). Compensation of the spectral overlap for each fluorochrome was done using CD4 or MIL4 or CD3 antigens from cells gated in the FSC^{low} versus SSC^{low}; $FSC^{mid/high}$ versus $SSC^{mid/high}$; SSC^{low} versus $MIL4^{+}$; SSC^{high} versus $MIL4^{neg}$ and SSC^{high} versus $MIL4^{+}$ region respectively. Analyses were performed with an acquisition of at least 100,000 total events.

RT-PCR analysis

Expression of mRNA encoding the cytokines IFNγ, IL-12p40, TNFα, TGFβ, IL-10, and the regulatory T cell associated intracellular marker Foxp3, was quantified using real-time reverse transcription-polymerase chain reactions (RT-PCR). One lobe from each guinea pig ($n = 5$) lung was added to 1 ml of TRIzol RNA reagent (Invitrogen), homogenized, and frozen immediately. Total RNA was extracted according to the manufacturer's protocol. RNA samples from each group and each time point were reverse transcribed using the Reverse Transcriptase Enzyme (M-MLV RT- Invitrogene). Four μl samples of cDNA were then amplified using the iQ SYBR Green Supermix (Bio-Rad) following the manufacturer's protocol on the iQ5 iCycler amplification detection system (Bio-Rad). A negative control using ultra pure Molecular Biology water as the template and a non-template control (NTC) were ran to confirm that the signals were derived from RNA and not due to contaminating genomic DNA. In order to ensure that only the correct gene was amplified, and was not the presence of primer-dimer or non-specific secondary products, a Melt Curve was performed for each run. Fold induction of mRNA was determined by analyzing cycle threshold (C_T) values normalized for HPRT (C_T) expression. The primer sequences for guinea pig IFNγ, TNFα, TGFβ1, IL-12p40 and 18S were previously published.[20,21] The primer sequences for guinea pig Foxp3 and IL-10 were determined with assistance from Dr Anand Damodaran (Genotypic Technology, Bangalore, India). Primer sequences used for Foxp3 were forward: 5′