Vaccine xxx (2018) xxx–xxx

Contents lists available at ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

Sequential pulmonary immunization with heterologous recombinant influenza A virus tuberculosis vaccines protects against murine *M. tuberculosis* infection

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ARTICLE INFO

Article history:
Received 31 October 2017
Received in revised form 22 February 2018
Accepted 14 March 2018
Available online xxxx

Keywords:
Tuberculosis
Mucosal vaccine
Recombinant influenza A
Antigen 85B
Protection

ABSTRACT

Tuberculosis (TB) infection affects a quarter of the global population resulting in a large burden of TB disease and mortality. The long-term control of TB requires vaccines with greater efficacy and durability than the current *Mycobacterium bovis* Bacille Calmette-Guérin (BCG). Pulmonary immunization may increase and prolong immunity at the site of *Mycobacterium tuberculosis* infection. We have investigated recombinant influenza A viruses (rIAVs) expressing the p25 $CD4^+$ T cell epitope of *M. tuberculosis* $Ag85B_{240–254}$ for single and sequential immunization against *M. tuberculosis* infection. Intranasal immunization with single dose of rIAV X31 (H3N2 strain) expressing the p25 epitope (X31-p25), induced p25-specific $CD4^+$ T cells and conferred protection against aerosol delivery of *M. tuberculosis* infection in the lungs. To enhance this effect, prime-boost immunization with hetero-subtypic rIAVs was examined. Sequential immunization with X31-p25 and a second rIAV, PR8 (H1N1 strain) expressing the same epitope (PR8-p25), increased the frequency of p25-specific IFN-γ T cell responses and polyfunctional $CD4^+$ T cells producing IFN-γ, IL-2, and TNF, compared to immunization with each rIAV alone. This combination resulted in protection against *M. tuberculosis* in both the lungs and spleen. Therefore, our study revealed that rIAV is not only an efficient vector to induce protective immunity in the lungs, but also has a potential use for sequential immunization with heterologous rIAV to boost the immunogenicity and improve the protection against *M. tuberculosis*.

1. Introduction

Tuberculosis (TB) is one of the most devastating infectious diseases of humankind. The causative agent of TB *Mycobacterium tuberculosis* infects 1.7 billion individuals, about a quarter of the global population [1], causing significant morbidity and mortality. A future TB-free world relies heavily on the discovery of new preventive interventions [2]. New TB vaccines employing alternative delivery systems are required to replace or improve the current vaccine, *Mycobacterium bovis* Bacilli Calmette-Guérin (BCG), that provides limited protective efficacy against adult pulmonary disease and has not contributed to reducing the transmission of *M. tuberculosis* [3,4]. Viral vectors are an attractive platform because of their ability to induce strong immune responses. However, although intramuscular delivery of the non-replicating Modified Vaccinia Ankara (MVA)-85A vaccine induced antigen-specific T cell responses, this vaccine failed to increase protection in BCG-immunized infants [5]. The failure of BCG to protect against pulmonary TB has been associated with the lack of airway luminal antigen-specific T cells [6]. Therefore, new vaccines triggering a strong T cell response at the site of *M. tuberculosis* infection are urgently needed.

Pulmonary immunization with an influenza A virus (IAV) vector provides an excellent delivery platform for promoting desirable immunity in the lungs. The vector has cell tropism for the respiratory mucosa [7,8], and rIAV is amenable to manipulation by reverse genetics [9] and is already licensed as live attenuated vaccine for influenza [10]. Another advantage of rIAV over other

Abbreviations: i.n, intra-nasal; MLN, mediastinal lymph node; rIAV, recombinant influenza A virus; s.c, subcutaneous; TB, tuberculosis; Th1, T-helper type 1; WHO, World Health Organisation; MVA, Modified Vaccinia Ankara.

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https://doi.org/10.1016/j.vaccine.2018.03.037
0264-410X/

viral-vectors, such as MVA or adenovirus, as TB vaccines is that it is possible to use different viral backbones for periodic boosting, thus avoiding pre-existent viral-specific immunity [11]. Serial intraperitoneal (i.p.) immunization with H1N1 and H3N2 rIAVs expressing a protein antigen from *Leishmania* resulted in stable and long lived $CD4^+$ memory T cells in spleen [12]. $CD4^+$ T cell responses are a major contributor to protective immunity against *M. tuberculosis* infection [13,14]. Therefore, we have developed rIAVs expressing an immunodominant *M. tuberculosis* $Ag85B_{240–254}$ (p25) $CD4^+$ T cell epitope as TB vaccines. Previously we demonstrated that immunization of mice with a recombinant H1N1 PR8 virus expressing the p25 epitope (PR8-p25) generates robust p25-specific $CD4^+$ T cells responses and protects against *M. tuberculosis* challenge in the lungs [15].

Here, we generated a novel H3N1 X31 rIAV expressing the same *M. tuberculosis* p25 epitope (X31-p25) and investigated its immunogenicity and protective efficacy after intranasal delivery. Furthermore, we explored the use of sequential immunization with the PR8-p25 and X31-p25 vaccines to enhance the protection against *M. tuberculosis*.

2. Methods

2.1. Mice

Six- to eight-week old female C57BL/6 mice were purchased from the Animal Resources Centre (Perth, Australia) or Australian BioResources (Moss Vale, Australia). P25 $CD4^+$ TCR transgenic (Tg) C57BL/6 mice (CD45.1) [16] were bred at the Centenary Institute**.** Mice were maintained under specific pathogen-free conditions at the Centenary Institute Animal Facility. All murine experiments were approved by The University of Sydney and Royal Prince Alfred Hospital Animal Ethics Committees.

2.2. Recombinant influenza A viruses (rIAV)

The IAV X31 (H3N2, A/HKx31) was engineered to express the *M. tuberculosis* p25 epitope (X31-p25) using the same methodology used previously to generate the H1N1 PR8-p25 vaccine [15]. In summary, 15 amino acids of the *M. tuberculosis* $Ag85B_{240–254}$ (FQDAYNAAGGHNAVF) $CD4^+$ T cell epitope were inserted into the neuraminidase (NA) stalk of the X31 virus and a recombinant influenza A virus rescued using an eight-plasmid reverse genetics system [9,17,18]. Sequence analysis of recovered virus confirmed integrity of the insert.

2.3. Bacteria and growth conditions

M. bovis BCG Pasteur and *M. tuberculosis* strain were grown in Middlebrook 7H9 (Difco) medium supplemented with glycerol (0.2% v/v), tyloxapol (0.02% v/v) and albumin-dextrose-catalase (ADC; 10% v/v). The mycobacteria were enumerated by plating serial dilutions of organ homogenates onto 7H11 (Difco) agar supplemented with oleic-acid-albumin-dextrose-catalase (OADC; 10% v/v) and glycerol (0.5% v/v).

2.4. Immunization and infection

Mice were immunized by the intranasal (i.n.) route with 10^4 plaque forming units (PFU) of X31-p25 and X31-WT, or 20 PFU of PR8-p25 [15,19]. For adoptive transfer experiments, mice received intravenous (i.v.) injection of $5x10^4$ P25 TCR Tg splenocytes one day prior to vaccination. For sequential immunization, the two rIAVs were delivered at six-week interval. Mice were immunized with 5×10^5 colony forming units (CFU) of subcutaneous (s.c.) BCG Pasteur. Mice were challenged with aerosol *M. tuberculosis* H37Rv (100–300 CFU/mouse) using an inhalation exposure system (Glas-Col, Terre Haute, IN) [15]. Four weeks after challenge, bacterial loads in the organs of mice were enumerated.

2.5. Cell preparation

Single cell suspensions were prepared from lungs, mediastinal lymph node (MLN) and spleen. Lungs were digested by incubation with Collagenase type IV (50 U/ml, Sigma) and DNAse I (13 μg/ml, Sigma) in RPMI/10% FCS for 45 min at 37 °C. Lung, MLN and spleens were disrupted by passaging the tissues through 70 μm cell strainers, washed and incubated with ACK lysis buffer to remove red blood cells. The washed cells were resuspended in RPMI/10% FCS.

2.6. IFN-γ enzyme-linked immunospot (ELISpot) assay

IFN-γ ELISpot assay was done as previously described [15,20] using anti-IFN-γ antibody AN18 and biotinylated anti-IFN-γ antibody XMG1.2. The cells ($2x10^5$/well) were cultured at 37 °C for 18 h in the presence of the following: Influenza A $NP_{366–374}$ peptide (NP; Genscript), *M. tuberculosis* $Ag85B_{240–254}$ peptide (p25; Genscript), *M. tuberculosis* H37Rv culture filtrate protein (CFP), or BCG lysate at a final concentration of 10 μg/ml. The spots were enumerated using an automated ELISpot reader (AID, Germany).

2.7. Intra-cytoplasmic cytokine staining (ICS) for flow cytometry

Lung ($1–4 \times 10^6$), MLN or spleen (4×10^6) cells were incubated in the presence of p25 peptide (10 μg/mL) at 37 °C for 6 h with addition of Brefeldin A (10 μg/mL, Sigma) for the last 3.5 h. After blocking with anti-CD16/CD32 (BD Pharmingen), the cells were stained with anti-CD3 PerCPCy5.5 (Biolegend) and anti-CD4 AF700 (BD Pharmingen) antibodies, and live/dead fixable blue staining (Life Technologies). The cells were washed, fixed and permeabilised with Cytofix (100 μl, BD Biosciences) for 20 min on ice. The cells were stained with anti-cytokine antibodies: anti-IFN-γ FITC (BD Pharmingen), anti-IL-2 APC (Biolegend), and anti-TNF PE (Biolegend) for 30 min on ice. Cells were washed, and resuspended with FACS Wash and 10% neutral buffered formalin. At least 1 million cells were acquired using a LSR Fortessa flow cytometer (BD) and analysed with FlowJo (Tree Star), and of viable lymphocytes 10–20% (lungs) or 20–25% (MLN and spleen) were $CD4^+$ T cells. Cytokine expression was determined using the gating strategy (Supplement Fig. 1) and analysed using FlowJo Boolean gating tool.

2.8. Statistical analysis

Data analyses were performed using GraphPad Prism 7 software (GraphPad Soft-ware, La Jolla, CA). Comparison between two groups was analysed using Student's *t*-test, or for more than two groups by analysis of variance (ANOVA) with a multiple comparison test and Tukey's correction. Statistical differences with $p < 0.05$ were considered significant.

3. Results

3.1. Antigen-specific responses following immunization with rIAV X31-p25

To determine if the H3N2 X31-p25 virus retained the capacity to cause productive infection, mice were immunized intranasally

Please cite this article in press as: Muflihah H et al. Sequential pulmonary immunization with heterologous recombinant influenza A virus tuberculosis vaccines protects against murine *M. tuberculosis* infection. Vaccine (2018), https://doi.org/10.1016/j.vaccine.2018.03.037