

Function of ssDNA aptamer and aptamer pool against *Mycobacterium tuberculosis* in a mouse model

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Abstract. Novel antibacterial agents against *Mycobacterium tuberculosis* (MTB) are crucial due to the high infection and mortality rates associated with the disease. Our previous study confirmed that aptamers from a whole bacterium obtained by the Systematic Evolution of Ligands by Exponential Enrichment method specifically bound to the MTB virulent strain (H37Rv). In the present study, the function of aptamers against MTB in a mouse model was further determined. It was demonstrated that the NK2 aptamer has marked inhibitory effects on the adhesion/invasion of H37Rv to macrophages *in vitro* and stimulates intracellular IFN- γ production in CD4⁺ T-cells. The aptamer pool exhibited the strongest inhibitory effect on H37Rv adhesion/invasion to CD8⁺ T-cells *in vitro* compared with all aptamers-treated and control groups. Histopathological examination of lung biopsy specimens revealed a correlation between aptamer presence and lower pulmonary alveoli fusion, swelling and more prominent air spaces. Acid-fast staining of biopsy specimens from the lungs of the mice demonstrated parallel treatment effects. Results of the present study indicate that the 10th pool aptamers and NK2 may play an active role against H37Rv, however, the effect was different *in vivo* and *in vitro*. The treatment effect of 10th pool aptamers was found to be better in comparison to NK2 *in vivo*. Additional target sites involved in pathogenicity of H37Rv were also revealed and the NK2 binding site and aptamers, including the 10th pool aptamers, may antagonize these sites. Further studies are required to screen for other

valuable aptamers which may be used as therapeutic drugs in combination with NK2.

Introduction

Tuberculosis (TB) caused by *Mycobacterium tuberculosis* (MTB) has the highest mortality rate worldwide of any infectious disease and has been declared a global health emergency by the World Health Organization (1,2). The attenuated *M. bovis* Bacillus Calmette Guerin (BCG) is the only available vaccine against TB. However, BCG exhibits varying efficacy (0-80%) against adult pulmonary TB (3). Emergence of drug-resistant isolates of MTB highlight the continued necessity for the discovery and development of drugs active against the bacterium (4-8).

Aptamers have high affinity and specificity for their targets and have been developed for use as oligonucleotide analogs of antibodies. These molecules exhibit several advantages over antibodies and antibiotics. Aptamers are smaller than antibodies and therefore exhibit improved cell penetration, blood clearance and chemical modification. They are also nonimmunogenic and readily synthesized and therefore do not induce an immune host response, which may cause harmful side-effects (9,10). Single-stranded DNA (ssDNA) aptamers exhibit more variable structures, longer relative temperature stability and shelf-life than antibodies and therefore demonstrate significant potential for *in vivo* use as therapeutics (11,12).

In previous years the Systematic Evolution of Ligands by Exponential Enrichment (SELEX) technique has become increasingly important for the study of protein function, as well as in drug discovery and identification of antagonists against a number of functional proteins (13,14). However, little is known regarding the *in vitro* SELEX selection strategy utilized for the generation of inhibitors against whole bacteria. Our previous study extracted special aptamers (NK2) and aptamer pools (10th pool) from a whole bacterial SELEX strategy (15) and demonstrated that aptamers inhibit MBT H37Rv invasion of macrophages *in vitro* (16). In the present study, we further evaluated the function of the 10th pool and NK2 against MBT H37Rv in a mouse model. This investiga-

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tion may aid the development of a new antitubercular agent based on an aptamer species.

Materials and methods

Bacterial strain and animals. MTB H37Rv (strain ATCC 93009) was purchased from the Beijing Biological Product Institute (Beijing, China). Bacteria were maintained on Lowenstein-Jensen (L-J) medium and harvested in log phase growth. Prior to use, bacilli were washed in 0.05% Tween-80 saline and triturated to uniformity. C57BL/6 mice (from the Experimental Building of the Animal Laboratory Center, Wuhan University, Wuhan, China) of either sex were used at 5-6 weeks of age. Bacterial cultures and animal tests were performed in the Animal Biosafety Level 3 Laboratory (ABSL-III) of Wuhan University School of Medicine. The research procedures and the animal protocols in this study were approved by the HPKLTM Ethics & Animal Use Committee (approval ID: HPKLTME201005).

Fluorescence microscopy and phagocytic index. C57BL/6 mouse peritoneal macrophages were collected and cultured in RPMI-1640 medium with 10% FBS and penicillin and streptomycin. Following 48 h, cell number was estimated using trypan blue staining. To analyze the effect of aptamers on H37Rv invasion of macrophages, 10^8 cfu H37Rv bacteria was incubated with aptamers (pretreated at 85°C for 15 min and then incubated on ice for 3 min) at 37°C for 15 min (control group was untreated with aptamer). Following this, centrifugation at 12,000 rpm for 5 min was performed and the supernatant was discarded. Mouse peritoneal macrophages (10^6) were mixed with 10^8 cfu H37Rv in 2 ml medium. Phagocytosis was allowed to occur for 1 h during mixing at 37°C in 6-well costar plates. A pretreated (polylysine, 0.1 mg/ml) coverslip was applied to each analytic well. The coverslip was dislodged and fixed by frigorific acetone and then stained with auramine O. The coverslip was then observed under a fluorescence microscope (CW4000, Leica, Wetzlar, Germany). Counts of 200 cells from each coverslip were performed and the phagocytic index was calculated using the formula: phagocytic index = (total MTB phagocytosed by macrophage)/(macrophage number of phagocytosed MTB).

Flow cytometry analysis of the effect of aptamers on the invasion of H37Rv to CD4⁺ and CD8⁺ T cells. H37Rv (10^8 cfu; prestained with Rhodamine B) was incubated for 1 h with 10^6 peripheral blood mononuclear cells (PBMCs) collected from the tail vein of C57BL/6 mice. Experimental groups were pretreated with the 10th pool or NK2 as previously described (15). Following this, the cells were washed twice with PBS and incubated with 5 μ l mouse anti-CD3-PE-Cy5, anti-CD4-FITC (Caltag Laboratories, Buckingham, UK) or isotype control antibodies, at the concentration recommended by the manufacturer's instructions, for 30 min at 4°C. The cells were then fixed with 70% ethanol and analyzed using a fluorescence-activated cell sorter (FACS; Epics Altra II, Beckman Coulter, Miami, FL, USA).

Effect of aptamers on the intracellular expression of IFN- γ of CD4⁺ and CD8⁺ T cells in H37Rv-infected splenocytes. Murine splenocytes (2×10^6) were incubated with 10^8 cfu H37Rv

or pretreated with the 10th pool and NK2 for 1 h. Following this, 1 μ g/ml monensin (eBioscience, San Diego, CA, USA) was added and incubated at 37°C for 2 h in a 5% (v/v) CO₂ atmosphere. Cells were washed twice with PBS, resuspended in 100 μ l cold PBS and incubated with 5 μ l mouse anti-CD3-PE-Cy5 and anti-CD4-FITC antibodies (Caltag Laboratories) at 4°C for 30 min. The cells were fixed using 70% ethanol and incubated with 5 μ l PE-conjugated mouse anti-IFN- γ (Caltag Laboratories) at 37°C for 30 min in the dark. Stained cells were washed with PBS and intracellular cytokine expression of IFN- γ in CD3⁺ CD4⁺ and CD3⁺ CD8⁺ T cells was analyzed by FACS.

Challenge infection and analysis of survival rate. C57BL/6 female mice (18-22 g) were used in the study. H37Rv bacteria used for *in vivo* experiments underwent several passages in C57BL/6 mice to enhance virulence (17). Mice were injected intravenously with 10^7 cfu H37Rv/mouse in 0.4 ml saline. Equal MTB was pretreated with 8 μ g of the 10th pool or NK2 aptamers, the supernatant was then discarded, and pellets were centrifuged prior to injection with 0.4 ml saline. Mice were fed with standard pelleted food and water for 20 days and each group was composed of 8 mice. Mortality was monitored daily. Survival analysis was analyzed by Kaplan-Meier (SPSS 13.0, Chicago, IL, USA).

Histopathology and acid-fast stain. Lungs or spleens from mice sacrificed 20 days post-infection were homogenized in saline and plated on L-J medium. Following 4-6 weeks incubation at 37°C, the number of viable organisms in the lungs or spleens was determined. Sections of the lungs and spleens were soaked in 10% paraformaldehyde for a minimum of 12 h and examined with hematoxylin and eosin stain and acid-fast stain, respectively.

Statistical analysis. Data were presented as the mean \pm SEM and were analyzed using one-way analysis of variance followed by the Student-Newman-Keuls post-hoc test. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Inhibition of MTB invasion of mouse peritoneal macrophages. Compared with untreated, groups pretreated with aptamers (NK2 or 10th pool) inhibited MTB invasion of mouse peritoneal macrophages (Fig. 1A). Similar results were obtained by comparing the phagocytic index with fluorescence microscope observations (Fig. 1B). MTB invasion of mouse peritoneal macrophages was higher in NK2 than the 10th pool.

Inhibition of H37Rv invasion to CD3⁺ CD8⁺ T cells. H37Rv invasion to CD3⁺ CD8⁺ T cells was inhibited by the aptamers. Invasion to CD3⁺ CD4⁺ T cells was not inhibited. H37Rv invasion to CD3⁺ CD8⁺ T cells was found to be significantly decreased from 50.4% (without aptamers) to 33.9, 28, 21 and 20% with NK2 aptamer, 10th, 7th and 3rd aptamer pool, respectively (Fig. 2A). These data indicate that aptamer pools protect CD8⁺ T cells against H37Rv infection.

Aptamers increase intracellular IFN- γ secretion of CD3⁺ CD4⁺ T cells. Intracellular IFN- γ levels in CD3⁺ CD4⁺ and

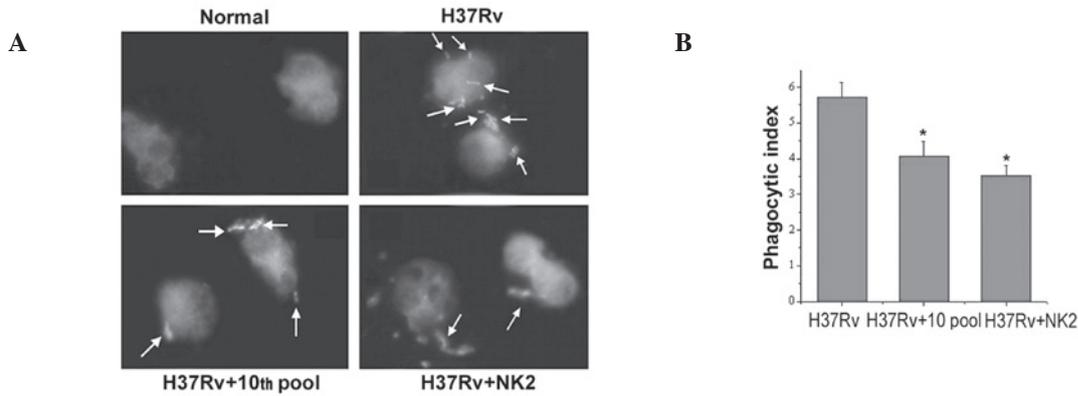


Figure 1. Inhibitory effects of aptamers on H37Rv invasion of murine peritoneal macrophages. (A) Fluorescence microscope images (x1,000), MTB bacteria are labeled with arrows. (B) Quantity analysis of phagocytic index. Data are presented as the mean \pm standard error of the mean (SEM) and were analyzed using one-way analysis of variance followed by the Student-Newman-Keuls post hoc test. *P<0.05, vs. H37Rv. MTB, *Mycobacterium tuberculosis*.

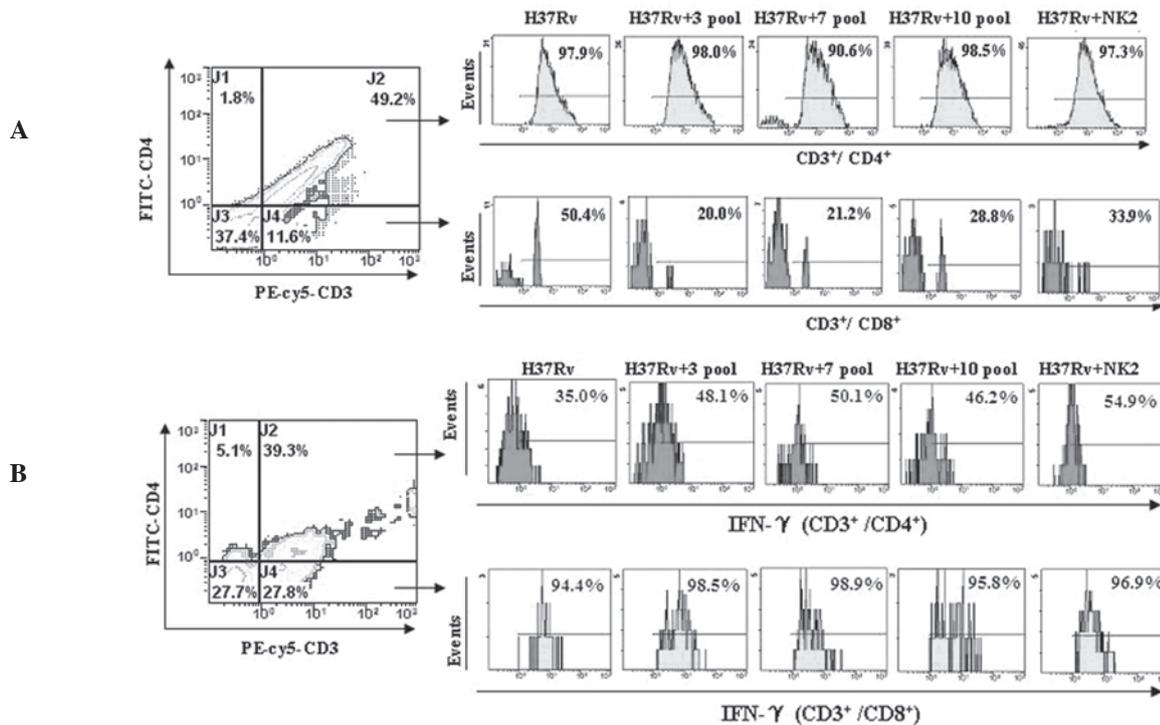


Figure 2. Flow cytometry analysis of (A) inhibitory effects of aptamers on H37Rv invasion to CD8⁺ T cells and (B) intracellular IFN- γ levels in CD4⁺ T cells.

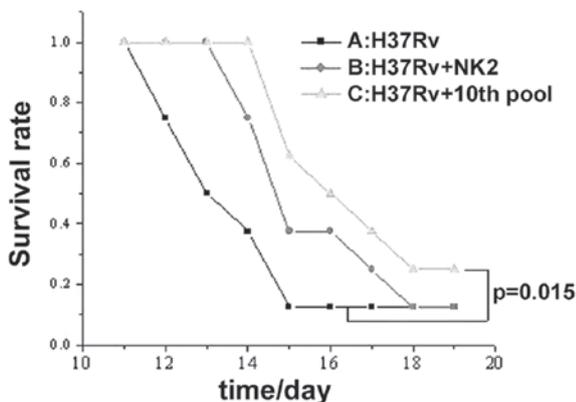


Figure 3. Survival rate following 20-day infection. Kaplan-Meier methods were used to calculate the survival analyses.

CD3⁺ CD8⁺ T cells were detected by flow cytometry. When PBMCs from mice were infected by H37Rv *in vitro*, increased levels of intracellular IFN- γ were observed in the presence of aptamers in CD3⁺ CD4⁺ T cells, but not in CD3⁺/CD8⁺ T cells. Intracellular IFN- γ levels in CD3⁺ CD4⁺ T cells were increased from 35% (without aptamers) to 48.1, 50.1, 46.2, 53.6 and 54.9% with 3rd, 7th, 10th and 12th aptamer pools and NK2 aptamer, respectively. The NK2 aptamer had the strongest stimulatory effect on intracellular IFN- γ levels in CD3⁺ CD4⁺ T cells (Fig. 2B). These data indicate that aptamers, particularly the NK2 aptamer, stimulate IFN- γ production and decrease the infection efficiency of MTB.

Treatment of H37Rv in vivo. Effect of 10th round aptamer pool or NK2 aptamer on acute tuberculosis in mice was

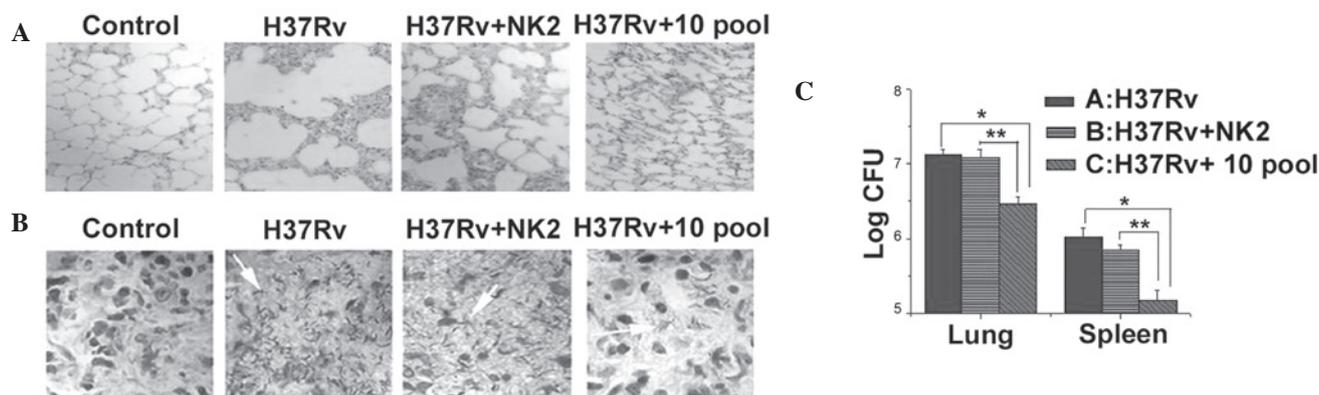


Figure 4. (A) Histopathological examination of biopsy specimens from lungs of mice (HE, x40) and (B) acid fast stain of biopsy specimens from lungs of mice (x1,000), MTB bacteria are labeled with arrows. (C) Determination of CFU from lung and spleen of infected mice with or without aptamers treatment. Colony counts were determined after 4-weekly incubation at 37°C and presented in log cfu. Data are presented as the mean \pm SEM and were analyzed using one-way analysis of variance (ANOVA) followed by the Student-Newman-Keuls post hoc test. * $P < 0.05$ vs. H37Rv; ** $P < 0.01$, vs. H37Rv + NK2. MTB, *Mycobacterium tuberculosis*.

examined. In the control group, H37Rv injection for 13 days, resulted in 50% mortality. Survival rate of the mice was prolonged for 2 days with a single injection of aptamer NK2 and 3 days with a single injection of aptamer pool (10th round selection; $P < 0.05$; Fig. 3). Histopathological examination of lungs from mice injected with H37Rv revealed a marked acute inflammatory reaction compared with normal mice. NK2- or aptamer pool treated-mice demonstrated decreased pulmonary alveoli fusion and swelling and more prominent air spaces, similar to lungs of normal mice (Fig. 4A). Furthermore, compared with normal mice the density of acid fast bacillus in the lung was higher in control mice than mice that had received a single injection of aptamer NK2 or 10th pool aptamers (Fig. 4B). Number of H37Rv colonies was higher in control mice than in the 10th aptamer pool or NK2 groups (Fig. 4C). The present study revealed that, *in vivo*, the 10th pool of aptamers and NK2 had an active therapeutic effect on H37Rv infection and the 10th pool of aptamers was more effective than NK2.

Discussion

The use of aptamers as therapeutic drugs has been reported in numerous research fields, including HIV (18) and cancer (19). Aptamers may inhibit MTB infection through blockage of virulence components or epitopes of H37Rv. *In vitro*, inhibition of H37Rv macrophage infection by NK2 aptamers was higher when compared with the 10th aptamer pool (Fig. 1), however, the 10th aptamer pool prolonged the survival rate of mice, enhancing clearance of the bacterium *in vivo* to a more significant degree (Figs. 3 and 4). This may be due to the complex surface of MTB and the degradation of DNA aptamers by nuclease enzymes (20). The 10th aptamer pool contained various non-special aptamers that may aid resistance to nucleases and function in synergy with the NK2 aptamer. Thus, the 10th aptamer pool was more effective than the NK2 aptamer *in vivo*. Previously, modified aptamers with longer half-lives were developed to resist nuclease digestion. However, disadvantages have been identified, including an

increased rate of integration into the chromosomal DNA of host T cells. There are multiple binding sites and antigen epitopes on the surface of H37Rv and synergism of several aptamers is required to inhibit H37Rv infection. In the present study, we noted that inhibition of H37Rv invasion to CD8⁺ T cells decreased as the screening process progressed (Fig. 2A). This observation may be due to inhibitory aptamers being missed through SELEX. This may be explained by important components in the reverse target (BCG) which stimulate the cytotoxic T-cell effect. Currently, BCG is the only vaccine against tuberculosis, however, its immune protective effects are not always effective. A possible explanation for reduced efficacy may be due to the effect of aptamers on CD4⁺ and CD8⁺ T cells (Fig. 2).

The present study demonstrates that the aptamer pool and NK2 aptamer exhibit protection against tuberculosis, however, their mechanisms of action are different. NK2 revealed improved inhibition of H37Rv invasion to mice peritoneal macrophages and stimulation of CD4⁺ T-cell INF- γ secretion compared with the 10th pool. However, survival rate and histological analysis revealed that the 10th pool has a better therapeutic effect compared with NK2. The results demonstrated we should not only pay attention to those aptamers in the majority (including NK2), but also investigate the role of early deserted aptamers. The present study demonstrates the limitations of ssDNA aptamers, highlighting a number of factors which must be considered to avoid available aptamer loss.

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