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Tissue plasminogen activator (tPA) signal sequence enhances immunogenicity of MVA-based vaccine against tuberculosis

Yiming Kou[a], Yongqing Xu[a], Zhilei Zhao[a], Jie Liu[a], Yang Wu[a], Qingrui You[a], Linli Wang[a], Feng Gao[a,b], Linjun Cai[a,b,*], Chunlai Jiang[a,b,*]

[a] National Engineering Laboratory for AIDS Vaccine, School of Life Science, Jilin University, Changchun 130012, PR China
[b] Key Laboratory for Molecular Enzymology and Engineering of the Ministry of Education, School of Life Science, Jilin University, Changchun 130012, PR China

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ABSTRACT

Tuberculosis (TB) remains a serious health problem worldwide, and the only available vaccine, bacillus Calmette-Guérin (BCG), has shown highly variable efficacy in adults against TB. New vaccines are urgently needed, and the modified vaccinia virus Ankara (MVA)-based vaccine has emerged as one of the most promising candidates based on many preclinical and early clinical studies over the past few years. However, the maximum tolerable dose and strength of induced immune responses have limited the protective effect of MVA-based prophylactic vaccines. To improve the immunogenicity of MVA-based vaccines, we introduced the tPA signal sequence in order to increase the antigen expression and secretion. Two recombinant MVA vectors expressing the Ag85B-TB10.4 fusion protein with or without tPA signal sequence were constructed and verified. Following the homologous prime-boost administration regimen in mice, levels of antigen-specific antibodies and cytokines (*e.g.*, IFN-γ, TNF-α, IL-5, IL-6) and the percent of activated T cells were found to be significantly increased by the tPA signal sequence. However, the mean IgG2a/IgG1 ratios in the two recombinant MVA immunization groups were similar. Our present study demonstrated that the tPA signal sequence could enhance the immunogenicity of an MVA-based vaccine against TB without changing the balance of Th1 and Th2 immune responses. Thus, the tPA signal sequence may be applied to MVA-vector based vaccines for providing a better immune effect.

1. Introduction

Tuberculosis (TB), one of the most of threatening infectious diseases worldwide, is caused by *Mycobacterium tuberculosis* (*M.tb*). According to a recent report by the World Health Organization, TB caused illness in 10.4 million people and accounted for 1.5 million deaths in 2015 [1]. Bacilli Calmette-Guérin (BCG) is the only licensed vaccine against TB. Although it provides effective protection against *M.tb* in children, BCG has highly variable efficacy (0–80%) against adult pulmonary TB [2]. With the emergence and increased prevalence of drug-resistant and multidrug-resistant TB, the development of new vaccines that can either replace BCG or prolong efficacy is urgently needed.

Several TB vaccine candidates are currently undergoing clinical trials. MVA85A, a modified vaccinia virus Ankara (MVA) expressing the *M.tb* antigen 85A, was the first TB vaccine candidate to be tested in a clinical trial since BCG [3]. With the loss of 12% of the genome [4], MVA is incapable of causing disseminated infection even in severely immunocompromised animals, but it is still capable of inducing strong T cell responses [5]. Although MVA is a good viral vector, it still has some shortcomings. Through examination of the testing of different doses of recombinant MVA in many clinical trials, an apparent threshold effect has emerged with regard to the maximum tolerable dose for prophylactic vaccines, thus limiting the protective effect to some extent [6]. Several DNA vaccines against TB have been developed with the tissue plasminogen activator (tPA) signal sequence as a heterologous leader sequence to drive a target protein into the cellular secretion pathway with higher expression [7–10]. Some researchers also have constructed replication-deficient adenoviruses expressing *M.tb* antigens with the tPA signal peptide sequence replacing native signal sequences [8,11,12]. However, to our knowledge, the tPA signal peptide has not been applied in an MVA-vectored TB vaccine.

In the present study, we investigated whether the tPA signal peptide could improve antigen expression in a recombinant MVA and induce a stronger immune response. A recombinant MVA expressing Ag85B and TB10.4 (previously tested as vaccine candidates in the fusion protein H4 [7]) was developed without and with the tPA signal peptide sequence and named MVAH4 and MVATH4, respectively. In order to compare the immunogenicity between MVAH4 and MVATH4, we

* Corresponding authors at: School of Life Science, Jilin University, No. 2699 Qianjin Street, Changchun 130012, PR China.
E-mail addresses: linjun_cai@jlu.edu.cn (L. Cai), jiangcl@jlu.edu.cn (C. Jiang).

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administrated the vaccines using a homologous prime-boost strategy in a mouse model. By using multiparameter flow cytometry, we demonstrated that the tPA signal peptide is a potential candidate for improving the immunogenicity of an MVA vector vaccine.

2. Materials and methods

2.1. Construction of plasmids and recombinant MVA

Genes encoding Ag85B and TB10.4 were amplified by PCR from the genome of BCG (GenBank accession no. AM408590) and fused with a 45-bp linker encoding $(Gly_4Ser)_3$. The original signal peptide of Ag85B was deleted or replaced by human tPA signal peptide (GenBank accession no. E04506). Both sequences were inserted into pcDNA3.1(−) for confirming the expression and secretion of heterologous proteins in mammalian cells.

For constructing the recombinant MVA virus, the shuttle vector pSC11M1 was used, and the antigen genes were driven by the P7.5 promotor. Recombinant MVA was then generated by standard procedures [13–15]. Briefly, BHK-21 tk-ts13 cells were infected with wild-type MVA and then transfected with the pSC11M1-vector plasmid using Lipofectamine 2000. Recombinant MVA was selected by blue plaque purification, and 5-bromodeoxyuridine was used for thymidine kinase (TK) selection and recombinant MVA amplification. Purification of recombinant MVA was performed using zonal sucrose gradient centrifugation. Viral stocks were stored in aliquots at −80 °C until use. Titers were determined by immunostaining with a polyclonal antibody against vaccinia virus (20-VR69, Fitzgerald).

2.2. Western blot

For confirming the expression and secretion of Ag85B-TB10.4, a series of recombinant pcDNA3.1(−) plasmids were transfected into HEK293T cells using Lipofectamine 2000 following the manufacturer's guidelines. Briefly, 2 μg of each plasmid was mixed with 6 μL Lipofectamine 2000 and added to cells at 80% confluency. Four hours later, the culture media was replaced with DMEM containing 2% fetal calf serum, and part of the cultures were treated with 5 μg/mL Brefeldin A (BFA). Cells were maintained in a humidified air-5% CO_2 atmosphere at 37 °C for 48 h. The cell lysates and culture supernatant were then harvested and assayed by Western blot with rabbit polyclonal antibodies. Horseradish peroxidase (HRP)-conjugated anti-rabbit antibodies were used as the secondary antibody.

To confirm the expression of Ag85B-TB10.4, BHK-21 tk-ts13 cells were innoculated with recombinant MVA (negative control), MVAH4 or MVATH4. Two days later, the cells were harvested, and the expression of Ag85B-TB10.4 was confirmed by Western blot with Ag85B and TB10.4 polyclonal antibodies.

2.3. Animals and vaccination

This study was performed with specific pathogen free (SPF) female BALB/c mice (6–8 weeks old), which were divided into three groups (n = 5) and kept in individual ventilated cages. The animals were immunized with MVAH4 and MVATH4 two times at two-week intervals subcutaneously at a dose of 1×10^7 pfu [16]. MVA was immunized subcutaneously at the same dose in the control group. Two weeks after the final vaccination, the mice were killed by dislocation of the cervical vertebra, which was performed under sodium pentobarbital anesthesia. All efforts were made to minimize suffering of the animals. Spleens and serum were collected for subsequent assays.

2.4. Antigen proteins and peptides

Ag85B and TB10.4 were prepared for expression as recombinant C-terminal 6His-tagged proteins from *Escherichia coli* and for purification using the HisTrap HP column (GE Healthcare) under denaturing conditions. Based on epitope mapping of Ag85B, H-2^d-restricted class I peptides (9-2 peptide, MPVGGQSSF; 9-1 peptide, IYAGSLSAL) and class II peptides (18-1 peptide, FLTSELPQWLSANRAVKP; 18-2 peptide, HSWEYWGAQLNAMKGDLQ) were used [17].

2.5. Enzyme-linked immunosorbent assays (ELISA)

Serum samples were collected two weeks after the final vaccination, and antigen-specific antibodies were detected by ELISA. The ELISA plates were coated with 0.5 μg/well Ag85B or TB10.4 overnight. The sera were 50-fold diluted in PBS supplemented with 1% BSA, and primary antibodies (goat anti-mouse IgG, IgG1, IgG2a, Sigma) diluted at 1:1000 were used. After using the HRP-conjugated rabbit anti-goat secondary antibody, the enzyme reaction was developed with TMB substrate for 15 min and stopped with 2 M H_2SO_4. Optical density (OD) was determined with a microplate reader at a wavelength of 450 nm.

2.6. Spleen cell isolation and IFN-γ ELISpot assays

Spleens were removed aseptically and homogenized by rubbing through a fine-mesh stainless steel sieve into complete RPMI-1640 medium containing 10% fetal bovine serum (FBS). ACK buffer was used to lyse erythrocytes, and splenocytes were counted and resuspended at 1×10^7 cells/mL.

IFN-γ ELISPOT assays were carried out according to the manufacturer's recommended protocol (BD Biosciences) to detect and enumerate IFN-γ-secreting T cells. Briefly, 96-well filter plates were coated with 5 μg/mL of the purified anti-mouse IFN-γ mAb. After overnight incubation at 4 °C, the wells were washed and blocked for 2 h at room temperature with RPMI-1640 containing 10% FBS. Thereafter, splenocytes were added to the wells (1×10^6 cells/well) with or without peptides and proteins (final concentration: 5 μg/mL). Each condition was performed in duplicate. Cells were then incubated for 24 h at 37 °C, and plates were washed with deionized water and PBS containing 0.05% Tween-20. Plates were then incubated for 2 h with 2 μg/mL biotinylated anti-mouse IFN-γ mAb, washed and incubated for 1 h with streptavidin-HRP. Spots were developed by adding a final substrate solution to each well. Results were expressed as the number of spot-forming cells (SFC) per 10^6 splenocytes.

2.7. Cytokine analyses

The lymphocytes were plated at a concentration of 5×10^6 cells/well in 24-well plates in RPMI-1640 medium containing 10% fetal calf serum. The cells were stimulated of Ag85B or TB10.4 protein for 48 h. After stimulation, the supernatants were harvested, and IFN-γ, TNF-α, IL-2, IL-4, IL-5, IL-6 and IL-10 were analyzed using the mouse Th1/Th2 Panel Multianalyte Flow Assay Kit (Biolegend) according to the manufacturer's instructions.

2.8. Flow cytometry

Splenocytes (1×10^6) were stimulated with 5 μg/mL Ag85B and TB10.4 protein overnight. Thereafter, the cells were washed and blocked with mouse CD16/CD32 and stained for surface markers using CD3-FITC, CD4-APC, CD8-PerCP and CD69-PE (Biolegend) diluted in cell staining buffer (Biolegend) on ice. The stained cells were then analyzed on the BD Accuri™ C6, collecting about 30,000 events per sample.

2.9. Statistical analysis

Data are presented as the mean ± SD, and *T*-tests were performed for independent samples. For the above statistical analysis, Medcalc 13 was used. *P* values of < 0.05 were considered significant.

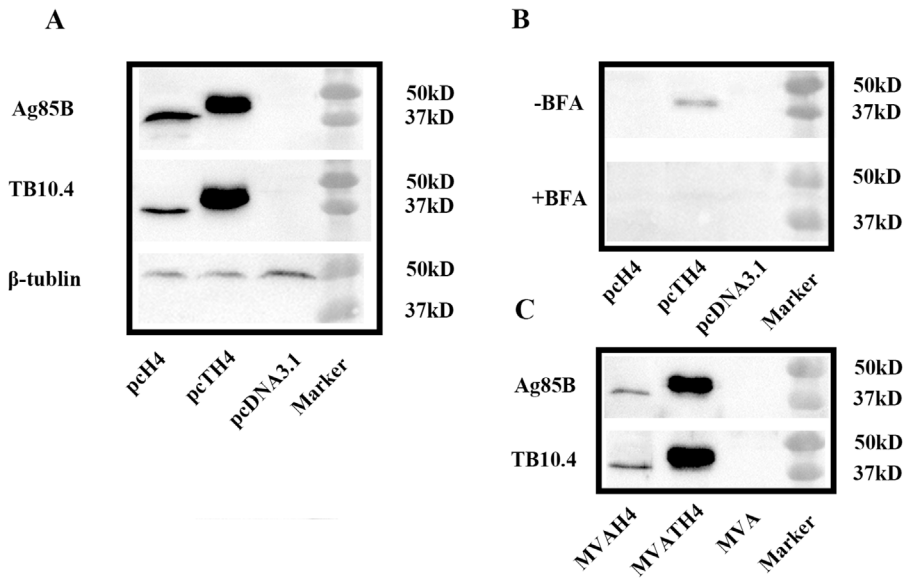

Fig. 1. Western blots of plasmid-transfected or virus-infected cells. A, Western blots of cell lysates transfected by recombinant pcDNA3.1 plasmid. Expression of Ag85B-TB10.4 was probed using rabbit anti-Ag85B and anti-TB10.4 polyclonal antibodies, and mouse anti-β-tublin monoclonal antibody is used as a control. B, HEK293T cells were transfected with recombinant pcDNA3.1 and cultured with or without BFA (5 μg/mL). The culture supernatant was detected by an anti-TB10.4 polyclonal antibodies. C, Western blots of recombinant MVA-infected BHK-21 tk-ts13 cell lysates.

3. Results

3.1. Expression of antigen gene in transfected cells and infected cells

To confirm the expression and secretion of antigen proteins in mammalian cells, Ag85B-TB10.4 genes in the pcDNA3.1(−) vector were designed without and with the tPA signal sequence fused at the N-terminal and named pcH4 and pcTH4, respectively. Recombinant plasmids were transfected into 293T cells, and the cell lysate and supernatant were assayed by Western blot with different antibodies.

When detected with anti-Ag85B and anti-TB10.4 polyclonal antibodies, the pcH4 group demonstrated bands consistent with the predicted molecular weight, while the pcTH4 group showed a higher molecular weight by the addition of the tPA signal peptide. The pcTH4 group showed a remarkably enhanced expression of approximately 5–10 fold in the cell lysate when compared with the non-tPA group (grayscale analysis) (Fig. 1A). The fusion protein in the culture supernatant was only detected in the pcTH4 group. When treated with BFA, which inhibited intracellular protein transport, the fusion protein could no longer be detected in the supernatant (Fig. 1B). These results indicated that the tPA signal sequence could effectively increase the expression and secretion of Ag85B-TB10.4.

Confirmation of recombinant MVA was obtained by Western blotting of recombinant MVA infected cell lysates. The expected band was detected in the cell lysate of BHK-21 tk-ts13 cells infected with MVAH4 and MVATH4, but not from those infected with the empty MVA vector (Fig. 1C). These observations demonstrated that recombinant MVA could express Ag85B-TB10.4, and use of the tPA signal sequence resulted in the expected effect in recombinant MVA.

3.2. Antigen-specific antibody responses elicited by recombinant MVA

The Th1-associated IgG2a, Th2-associated IgG1 and total IgG in sera obtained from recombinant MVA immunized mice were measured. As shown in Fig. 2, levels of anti-Ag85B and anti-TB10.4 IgG in MVATH4 immunized mice were significantly higher than those in MVAH4 immunized mice, which indicated that MVATH4 could induce stronger humoral responses than MVAH4. Additionally, levels of antigen-specific IgG2a and IgG1 in MVATH4 immunized mice were markedly higher than those in MVAH4 immunized mice, while the mean IgG2a/IgG1 ratios were similar in both groups (1.12 and 1.08, respectively) (Fig. 2). These data implied that MVATH4 and MVAH4 elicited a balanced Th1 and Th2 immune response.

3.3. IFN-γ responses induced by recombinant MVA

Spleen cells isolated from immunized mice were co-cultured with antigens (Ag85B, TB10.4, 9-1p, 9-2p, 18-1p and 18-2p), and then the IFN-γ-secreting cells were measured by ELISpot assay. As compared with the MVAH4 immunized group, significantly stronger IFN-γ responses upon stimulation with either proteins or peptides were found in the MVATH4 immunized group (Fig. 3). This result indicated that tPA could increase the ratio of IFN-γ-secreting cells, which may be conducive to the prevention of TB. Ag85B stimulation resulted in a slightly higher spot count when compared with TB10.4, indicating that Ag85B might be a stronger immunogen than TB10.4.

Next, we wanted to know whether the added tPA signal sequence in the recombinant MVA would have any effect on IFN-γ-producing T cell subsets. Splenocytes were stimulated with various immunodominant T-cell epitope peptides of Ag85B. Stimulation with either MHC-I-specific (9-1p and 9-2p) or MHC-II-specific (18-1p and 18-2p) peptides induced a similar number of spot counts, indicating that antigen-specific IFN-γ-producing CD4 and CD8T cells were generated by recombinant MVA immunization.

These results suggested that MVATH4 induced more IFN-γ-producing cells than MVAH4, and the IFN-γ-producing cells consisted of both CD4 and CD8T cells.

3.4. Th1/Th2 cytokine profiles

To characterize the tendency of Th1/Th2 polarization, type 1-associated cytokines (IFN-γ, TNF-α and IL-2) and type 2-associated cytokines (IL-4, IL-5, IL-6 and IL-10) in the splenocyte culture supernatants were examined. The splenocytes were stimulated with Ag85B and TB10.4 (5 μg/mL) for 48 h. The cytokines in the culture supernatants were then measured with a mouse Th1/Th2 Panel Multi-analyte Flow Assay Kit (Biolegend). As shown in Table 1, concentrations of all studied cytokines in the recombinant MVA immunized groups were higher than those in the MVA immunized group, and the MVATH4 immunization induced much greater cytokine production than MVAH4. IFN-γ, TNF-α, IL-5 and IL-6 were significantly increased in MVAH4 groups when compared to MVA groups, and these cytokines were significantly increased in MVATH4 groups when compared to MVAH4 groups ($P < 0.05$). These data suggested that MVAH4 and MVATH4 exerted immunomodulatory activities *via* both Th1 and Th2 pathways. As compared with MVAH4 immunization, MVATH4 immunization could induce a 5–10-fold increase of IFN-γ production, implying that

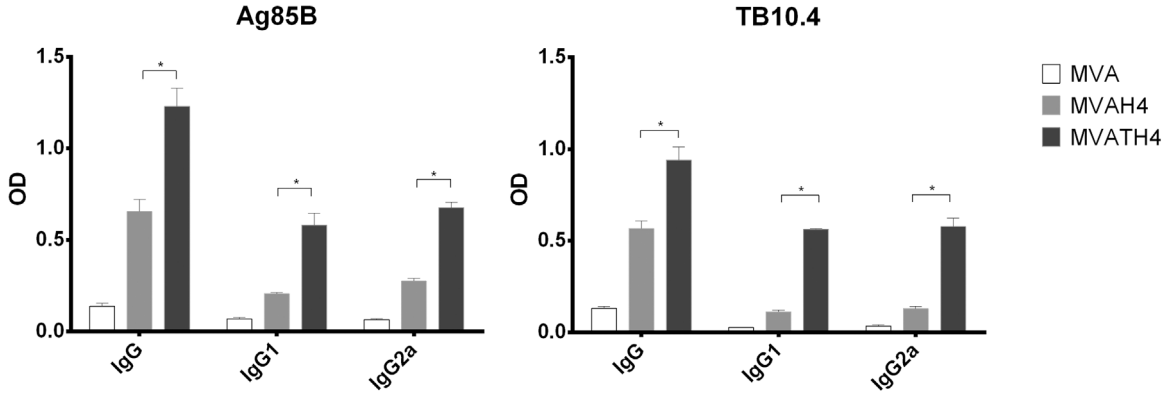

Fig. 2. Induction of antigen-specific antibodies in recombinant MVA vaccinated mice. Serum samples were collected two weeks after the last vaccination and analyzed by ELISA for the presence of anti-Ag85B and anti-TB10.4 IgG, IgG1 and IgG2a. The serum samples were 50-fold diluted with assay buffer. *$P < 0.05$.

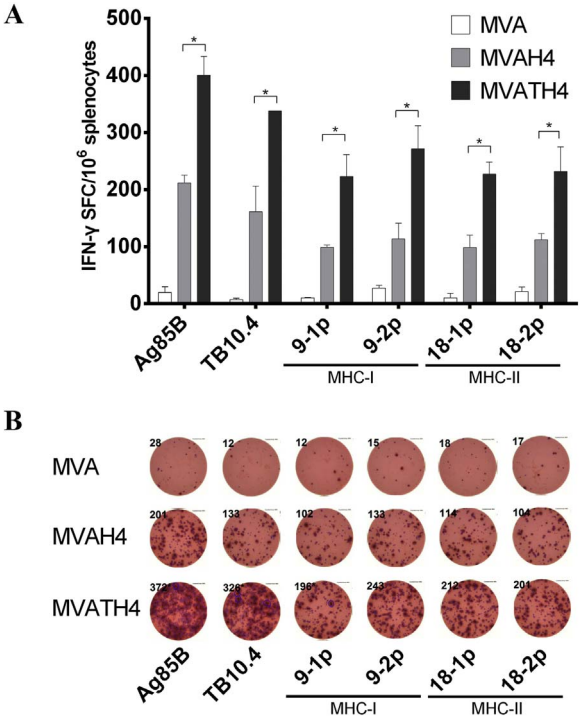

Fig. 3. ELISPOT IFN-γ. A, Frequencies of antigen-specific IFN-γ secreting cells were assayed *ex vivo via* IFN-γ ELISPOT using freshly isolated splenocytes two weeks after boost immunization. Data shown are mean standard deviations for two independent assays. *$P < 0.05$. B, A representative raw ELISPOT data.

the tPA signal sequence may have the ability to enhance the immunogenicity of MVA-based vaccine against TB.

3.5. CD69 surface expression on T cells

To characterize the phenotype of antigen-specific activated T cells, splenocytes were stimulated *in vitro* with or without antigen and analyzed by flow cytometry for CD3, CD4, CD8 and CD69 expression. The proportions of $CD8^+$ or $CD4^+$ T cells with CD69 surface expression in each group without stimulation were not significantly different, while the percentages of $CD69^+CD4^+$ T cells and $CD69^+CD8^+$ T cells in MVATH4 groups were slightly higher (Fig. 4). Ag85B and TB10.4 stimulations could significantly increase the populations of $CD69^+CD4^+$ T cells and $CD69^+CD8^+$ T cells in recombinant MVA groups, and these cell populations in MVATH4 immunized groups were higher than those of MVAH4 immunized groups.

4. Discussion

Due to the increasing numbers of new cases of extensively drug-resistant and multidrug-resistant TB and enhanced susceptibility to TB in HIV-infected individuals, the widely used attenuated BCG vaccine has been unable to meet the needs of TB prevention and control. Several TB vaccine candidates are currently undergoing clinical trials. These clinical candidates are based on a variety of vaccine approaches, such as recombinant or attenuated BCG, viral-vectored candidates and fusion protein subunits [18]. Among them, viral vector-based vaccines can enhance immunogenicity without an adjuvant and induce a robust cytotoxic T lymphocyte (CTL) response to eliminate infected cells. The most advanced vaccines are MVA85A [3], AERAS-402 [19] and AdHu5.85A [20]. In addition, a replication-deficient chimpanzee adenovirus vector expressing Ag85A is at the stage of clinical phase I trials

Table 1
Th1/Th2 cytokine profiles.

Cytokines	Ag85B			TB10.4		
	MVA	MVAH4	MVATH4	MVA	MVAH4	MVATH4
IFN-γ	36.3 ± 8.51	**366.04 ± 39.68****	**1770.54 ± 41.52*****	36.11 ± 20.1	**326.08 ± 59.94***	**3176.93 ± 835.63***
TNF-α	1.79 ± 0.21	**19.18 ± 0.36*****	**46.83 ± 2.96****	1.41 ± 0.71	**16.05 ± 1.35****	**62.12 ± 13.65***
IL-2	27.66 ± 3.06	32.05 ± 5.71	64.26 ± 17.11	24.01 ± 6.77	32.19 ± 8.88	**122.68 ± 16.92***
IL-4	21.24 ± 5.2	31.25 ± 0.81	**97.38 ± 20.49***	35.42 ± 7.57	42.13 ± 16.95	179.09 ± 89.23
IL-5	18.51 ± 1.98	**137.76 ± 11.48****	**467.33 ± 10.62****	16.28 ± 10.69	**118.4 ± 3.02****	**863.26 ± 172.73***
IL-6	64.16 ± 6.74	**200.21 ± 13.44****	**259.83 ± 5.28***	53.64 ± 15.82	**136.4 ± 13.92***	**392.42 ± 44.46***
IL-10	27.2 ± 17.77	63.63 ± 6.82	76.42 ± 1.91	27.54 ± 4.55	33.97 ± 6.62	**110.04 ± 0.08****

Abbreviations: MVA, modified vaccinia virus Ankara; MVAH4, MVA express the Ag85B-TB10.4; MVATH4, MVA express the Ag85B-TB10.4 with tPA signal sequence; IFN-γ, interferon-γ; IL, interleukin; TNF-α, tumour necrosis factor-α. Unit: pg/mL. Results represent the mean and SD of five animals per group. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ for MVA versus MVAH4 or MVAH4 versus MVATH4, Student t test.

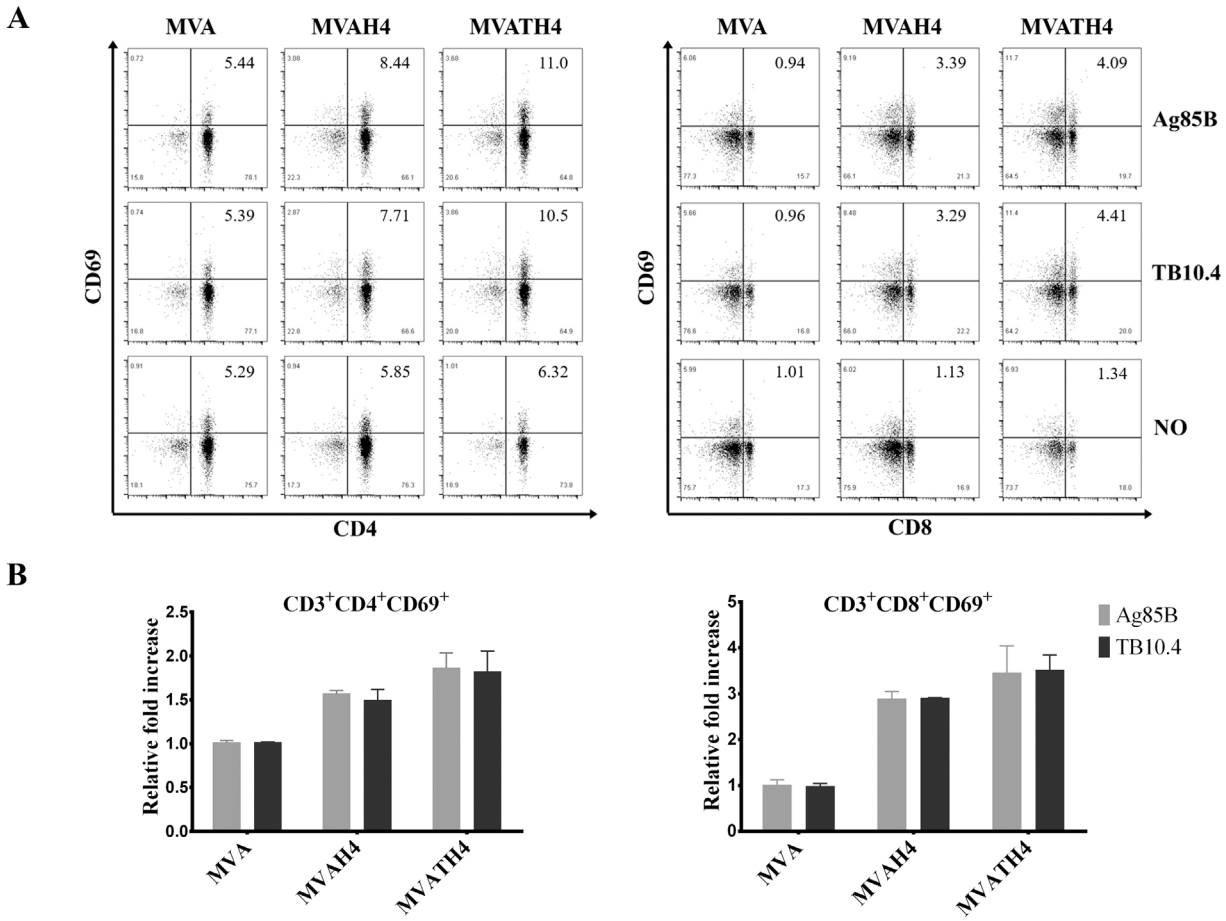

Fig. 4. CD69 expression on T cells. A, A representative flow cytometry analysis of CD69 expression on T cells. Splenocytes were stimulated *in vitro* with (5 μg/mL Ag85 B or TB10.4 protein) or without a specific antigen overnight, and measured by flow cytometry. Values represent the percentages of $CD69^+CD4^+$ cells and $CD69^+CD8^+$ cells in T cells. B, The relative fold increase of $CD69^+CD4^+$ cells (left panel) or $CD69^+CD8^+$ cells (right panel) is the ratio of the percentages of $CD69^+$ T cells in antigen (Ag85B or TB10.4 protein)-treated T cells to the percentages of these cells in control solvent-treated T cells. Data are representative of two independent experiments.

in combination with MVA85A [21]. Based by the analysis using open-source epitope binding prediction programs, Ag85B-TB10.4 was suggested to provide the most consistent protection [22], and a protein subunit adjuvanted vaccine (HyVac4, H4) containing the antigen Ag85B-TB10.4 is being evaluated in phase II studies in Africa [23,24]. Similarly, a vaccine strategy involving the adjuvanted fusion protein H28 (consisting of Ag85B-TB10.4-Rv2660c) and MVA expressing H28 has been shown to induce different T cell subset combinations and strong protection in both a mouse and a non-human primate TB model [25]. However, MVA28 alone showed the lowest protection in this study, perhaps partly due to the poor strength of the immune response. Indeed, the administration of MVA has a maximum tolerable dose for prophylactic vaccines, which impedes the development of MVA-based TB vaccines.

To date, several DNA vaccines against TB have been developed with tPA as a heterologous leader sequence to drive a target protein into the cellular secretion pathway with higher expression [7–10]. Some replication-deficient adenovirus expressing *M.tb* antigens have also been constructed with the tPA signal peptide sequence in place of native signal sequences [8,11,12]. The tPA signal sequence is efficient in facilitating transport of protein from the endoplasmic reticulum (ER) to the Golgi apparatus, which can increase the expression and secretion of antigen. Besides the proteasome-dependent antigen-presenting pathway, an endogenous antigen containing the tPA signal sequence can be processed and presented *via* a furin-based antigen presenting pathway [26,27].

In the present study, in order to improve the immunogenicity of MVA-based vaccines, we constructed a recombinant MVA expressing the Ag85B-TB10.4 fusion protein with a tPA signal sequence. Fig. 1 shows that the tPA signal sequence could enhance the expression and secretion of Ag85B-TB10.4. We also found that humoral and cellular immune responses were enhanced by recombinant MVA containing the tPA signal sequence.

Levels of IgG and the studied cytokines were increased in MVATH4 immunized groups. The ELISpot assay and cytokine profile results showed that the MVATH4 significantly increased the number of IFN-γ-producing splenocytes and the production of IFN-γ. Although IFN-γ has recently been considered as necessary but not sufficient for bacterial control [28], it is still known to play an important role in the fight against *M.tb*. In the current study, TNF-α and IL-6 were increased significantly in MVAH4 immunized groups, and the tPA signal sequence could augment the levels of these two cytokines. TNF-α is required in the protective immune response against *M.tb* in mice [29]. It was also shown to promote IL-17 production by inducing dendritic cells to direct the differentiation of Th cells towards the Th17 phenotype [30], as well as reduce the number of FoxP3-positive Treg cells in the draining lymph nodes [31]. The production of IL-6, a key pleotropic pro-inflammatory cytokine, is critical for controlling the early phases of mycobacterial multiplication and granuloma formation [32–35]. IL-6 knockout mice were shown to be highly susceptible to *M.tb*, and IL-6 could contribute to vaccine-induced protective immunity in mice. These results indicated that the tPA signal sequence could enhance the immunogenicity of the MVA-based TB vaccine, and the MVATH4 vaccine showed the ability to induce a better protective immune response against TB than the

MVAH4 vaccine.

Although the levels of antigen-specific antibodies in MVATH4 immunized mice were markedly higher than those in MVAH4 immunized mice, the mean IgG2a/IgG1 ratios in both groups were similar (Fig. 2). These data implied that the tPA signal sequence could enhance the strength of the immune response, but it did not change the balance of Th1 and Th2 immune responses.

Furthermore, the activated T cell numbers were increased in MVATH4 immunized groups (Fig. 4). CD69 is a membrane molecule transiently expressed on activated lymphocytes. It has been found to be selectively expressed in inflammatory infiltrates, suggesting that it plays a role in the pathogenesis of inflammatory diseases [36]. As T cells that produce multiple factors are termed polyfuctional T cells after stimulation, percentages of polyfunctional $CD8^+$ T cells within $CD8^+CD69^+$ fractions were significantly higher than that in the total $CD8^+$ fraction. Previous studies have also demonstrated that both $CD4^+CD69^+$ T cells and $CD8^+CD69^+$ T cells represented effector or effector memory cells and quickly produced large amounts of cytokines or killing molecules following short-term stimulation [37,38]. Therefore, the expression of $CD4^+CD69^+$ and $CD8^+CD69^+$ may be a useful marker for protection against TB induced by a vaccine. Our study shows that MVAH4 could induce the antigen-specific response of $CD4^+CD69^+$ and $CD8^+CD69^+$ T cells, and the tPA signal sequence increased the percentages of those cells. We speculate that the increased frequencies of $CD4^+CD69^+$ and $CD8^+CD69^+$ T cells by the tPA signal sequence may be associated with protection against TB.

In summary, the results showed that the tPA signal sequence could enhance MVA vaccine induced immune responses. MVATH4 elicited stronger humoral and cellular immune responses, but did not change the Th1/Th2 response pattern. The study suggests that the tPA signal sequence may be applied to MVA-vectored vaccines for improving the immune response. Thus, the protective efficacy of MVATH4 will need to be investigated in the future.

Conflicts of interest

The authors declare that they have no competing interests.

Acknowledgments

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