Article

Novel Constructs of Tuberculosis Gene Vaccine and Its Immune Effect on Mice

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A novel tuberculosis (TB) gene vaccine containing mouse granulocyte macrophage-colony stimulating factor (mGM-CSF) and a TB antigen (Ag85A) was developed in this study. The genes encoding Ag85A and mGM-CSF were amplified by PCR respectively from the Ag85A-containing pBSby5 and pC-mGM-CSF. The genes were then cloned into two different polylinker sites of plasmid pIRES, forming a novel TB gene vaccine construct pI85AGM. Following transfection of pI85AGM plasmid into 7721 cell line by Lipofectamine™, the expression of Ag85A and GM-CSF proteins was identified by Western blotting or RT-PCR. Then Balb/c mice were inoculated with the recombinant pI85AGM, pI85A, pIGM or plasmid alone, respectively. The activities of CTL, NK cells and the Ag85A-stimulated proliferation of spleen cells were measured by MTT method. The serum antibody against Ag85A was detected by ELISA. The results showed that the Ag85A and GM-CSF proteins could be expressed in 7721 cell line and the activity of CTLs and the proliferation of spleen cells were significantly increased in the pI85AGM-immunized mice, indicating that the pI85AGM-immunized mice could generate specific immune responses to Ag85A. This study might provid possibility for developing novel anti-TB gene vaccine. *Cellular & Molecular Immunology*. 2005; 2(1):57-62.*Cellular & Molecular Immunology*. 2005;2(1):57-62.

Key Words: tuberculosis, vaccine, Ag85A, GM-CSF, NK, CTL

Introduction

Tuberculosis (TB) remains a major cause of mortality worldwide, with one-third of the world population infected, 8 million people developing the active disease and is responsible for 2 million deaths per year all around the world (1, 2) because of poor diagnosis, a low-efficiency TB vaccine BCG, a long-term antibacterial chemotherapy regimen (approximately 6 months), and an emergence of multiple drug resistant strains of mycobacterium TB especially in people with human immune deficiency of virus infection. For such reasons, effective short-term chemotherapy and an effective vaccine must be developed (3-5).

DNA vaccination, based on the introduction, *via i.m.*, particle bombardment, or nasal spray, of a purified DNA plasmid encoding polypeptide sequence of interest into cells of the vaccinated animals to induce an immune response to antigen protein expressed *in vivo*, is a newly developed vaccine approach. The resulting *in situ* protein synthesis involves biosynthetic processing and post-translational modifications. The effectiveness of DNA vaccines has been demonstrated in many animal models (6-8), which may serve as a promising tool for controlling TB development. However, vaccines encoding single antigen of mycobacterium did not produce protective effect as BCG did and the immunogenicity of DNA vaccine is lower in primates than in rodents (1). So a lot of investigations were focused on taking new strategies for enhancing DNA vaccine immunogenicity, such as, DNA vaccines with heterogonous priming-boosting vaccination regimens (9), nanoparticles enhancing the immunogenicity of DNA vaccine (11, 12), and plasmid-delivered cytokines as an effective adjuvant to increase the

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Abbreviations: TB, tuberculosis; Ag85A, antigen 85A; mGM-CSF, mouse granulocyte macrophage-colony stimulating factor; BCG, Bacille de Calmette-Guerin; PBS, phosphate-buffered saline; NK, nature killer; PCR, polymerase chain reaction; CTL, cytotoxic T lymphocytes; ELISA, enzyme linked immunosorbent assay; OD, optical densities; SI, stimulation index; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; *i.m.*, intramuscular injection.

protective efficacy of a single DNA vaccine against TB infection (13-15).

In this study, we have designed a novel construct of TB gene vaccine encoding mGM-CSF and Ag85A and investigated its immune effect in mice. We showed that DNA vaccine constructs expressed Ag85A protein efficiently in eukaryotic cells and mGM-CSF could enhance Ag85A immunogenicity, inducing robust immune response to Ag85A in mice.

Materials and Methods

Mice and cell lines

Balb/c mice, male, 6-7 weeks were supplied by University of Science and Technology of China. All mice were housed under pathogen-free condition. The 7721 cell line (human hepatoma cells), SP2/0 cell line (Balb/c mice myeloma cells) and YAC-1 cell line (Moloney leukemia-induced T-cell lymphoma of A/Sn mouse origin) were obtained from cellular institute in Shanghai and were cultured at 37ºC in 5% CO_2 atmosphere in RPMI 1640 supplemented with 10% fetal bovine serum, 100 U/ml penicillin G sodium and 100 μg/ml streptomycin sulfate.

Plasmids and primers

Plasmid pBSby was gifted by Professor Huygen in Pasteur Institute in Belgium. Plasmid pIRES was purchased from BD company (USA) and plasmid pc-mGM-CSF was constructed by our laboratory. The sequence of PCR sense primer for Ag85A gene was 5'-TC GCTAGC ATG CAG CTT GTT GAC AGG G-3' and that of anti-sense primer was 5'-GGA ACGCGT C TAG ATG TTG TGT CTG T-3', which contain restriction endonuclease Nhe I and Mlu I sites respectively. The sequence of PCR sense primer for mGM-CSF gene was 5'-GC TCTAGA AGA TCA CCG GCG AAG GA-3' and that of anti-sense primer was 5'-TAT GCGGCCGC TTC CTC ATT TTT GGC C-3', which contain restriction endonuclease Xba I and Not I sites respectively.

Generation of recombinant pI85AGM constructs

The cDNAs encoding Ag85A and mGM-CSF were prepared from the plasmid pBSby and plasmid pc-mGM-CSF by PCR. Then the purified Ag85A cDNA and mGM-CSF cDNA were respectively inserted into plasmid pIRES at two different polylinker sites and formed the recombinant pI85AGM gene vaccine constructs.

Transfections

Seventy percent confluent 7721 cells in complete medium were transferred to 6-well plate for 24 hours then cells were transfected with different recombinant plasmid respectively. Mixture of the Lipofectamine™ 2000 reagent (Invitrogen, USA) and serum-free medium with different recombinant plasmid were incubated for 20 minutes at room temperature and then directly put into 7721 cells after the cells medium was removed from plates. Growth medium may be replaced after incubation for 5 hours, followed by selection with 600 μg/ml of G418 (Clonch, USA) 24 hours later. The 7721 cells were transfected with blank plasmid as control. About 6-8 days, G418-resistant 7721 clones were selected, clonally isolated and screened further for protein expression analysis by Western blotting or RT-PCR.

Western blotting

Cells were lysed and the proteins were collected in protein extraction buffer (Novagen, Germany) according to manufactory's protocol. Western blot was performed using Western-Breeze Kit (Invitrogen, CA) after 12% SDS-polyacrylamide gel electrophoresis. The primary antibody (Anti-TB Ag85A) was purchased from Genway Company (USA). All steps were performed according to Kit's protocol.

RT-PCR

Total cellular RNA was extracted from 7721 cells transfected with different recombinants using Rneasy Mini Kit (Qiagen, CA) according to the manufacture's instructions. All extracts from cells transfected with different recombinants were digested with DNase I to avoid recombinants contamination and cDNA was synthesized with the reverse SuperScript Choice System (Invitrogen, CA). cDNA of GM-CSF and Ag85A was respectively amplified by PCR with primers as mentioned above.

Animal immunization

Balb/c mice were randomly divided into four groups which were immunized with recombinant pI85AGM, recombinant pI85A, recombinant pIGM, and plasmid without any gene insert fragment, respectively (8 mice in each group). Each mouse was inoculated *i.m.* with 100 μg recombinant plasmid (a total of 300 μg/each mouse was immunized for three times with an interval of two weeks between each immunization).

Cellular immune responses detection

The activities of CTL, NK cells and the Ag85A-stimulated proliferation of spleen cells were respectively detected by MTT colorimetry method (16). For NK activity, the spleen cells were extracted in sterile conditions and homogenized and incubated 15 minutes at room temperature in ACK buffer (150 mM NH_4Cl, 1 mM $KHCO_3$ and 0.1 mM EDTA, pH 7.4). Then cells were washed with RPMI and resuspended in complete medium. To assay cell viability, the cell suspension was mixed with 0.4% Trypan blue (Sigma, St Louis, MO) and counted using a Neubawer chamber (17). Target cells (5×10^4 YAC-1) and effector cells (2.5×10^6 spleen cells) were simultaneously seeded in 96-well plate at 50:1 ratios of effector to target (E:T) and the final total volume was 200 μl. After the plate was incubated at 37°C in 5% CO_2 for 20 hours, supernatant was discarded, and then each well was added with 200 μl MTT for additional 4 hours incubation. Then supernatants were discarded and the plates were measured with the plate reader (Bio-Rad) at OD_{560} after 20 μl dimethylsulfoxide was added and shaken for a while. For CTL activity, the assays were also performed using MTT colorimetry method as described as NK activity assay. The

effector cells (2.5×10^6/ml) extracted from mouse spleen were incubated for 24 hours with 5×10^4 SP2/0 cells transfected with various recombinants respectively as target cells at 50:1 ratios of E:T. All cytotoxic activity assays were performed in triplicate. The activities of CTL and NK were calculated using following formulas:

$$\text{CTL/NK activity (\%)} = (1 - \frac{\text{OD (E + T)} - \text{ODE}}{\text{ODT}}) \times 100\%$$

E, effector cells; T, target cells; ODE, effector cells mean OD value; ODT, target cells mean OD value; OD (E + T), effector cells plus target cells mean OD value.

For spleen cell proliferation assays, spleen cells were split in flat-bottom 96-well plates (5×10^6 cells/well) in the presence of the indicated quantities of antigen (0.1 mg/ml Ag85A) or not in triplicate wells. The final volume was 200 μl/well filled with the above-mentioned medium. The results were expressed as stimulation index (SI). SI was calculated using the formula: SI = [OD value (stimulated culture) − OD value (control culture)]/OD value (control culture). SI ≥ 2.1 was taken as positive or efficient proliferation response.

Antibody detecting

Blood was collected every 3 weeks from mice by retro-ocular bleeding after the second immunization and serum were immediately isolated from the clot and stored at 4ºC for antibody detecting. The antibody against Ag85A (gifted by Professor Huygen) was detected by ELISA as described previously (14). In briefly, 100 μl Ag85A (10 μg/ml) was used to coat 96-well plate overnight at 4ºC, washing three times with PBS, and blocked with 10% bovine serum albumn in PBS for 1 hour at 37ºC.The plates were then washed three times with washing buffer PBST (0.05% Tween 20 in 1 × PBS). Blocking buffer (100 μl) contained mouse serum was add to plates for 1.5 hours at 20ºC. The plates were thoroughly washed with PBST and further incubated with anti-mouse IgG-horseradish peroxidase (Sigma) at 37ºC for 1 hour. Horseradish peroxidase activity was detected by using a chromogenic substance, *o*-phenylenediamine tetrahydrochloride (Sigma), in citrate-phosphate buffer (pH 5.4) and H_2O_2 at 1 μl/ml. The reaction were terminated by using 0.5 M H_2SO_4, and the absorbance values were measured at 490 nm in ELISA reader (Bio-Rad). Each ELISA experiment was performed for at least three times.

Statistical analysis

Statistical analysis was performed using the Student's test for the difference between group experiment and group control. The p values < 0.05 was taken as statistically significant.

Results

Generation of cDNA fragments Ag85A and mGM-CSF and different recombinants identification

The cDNAs encoding Ag85A and mGM-CSF were amplified from plasmid pBSby and plasmid pc-mGM-CSF by PCR. The recombinants of pI85A and pI85AGM were primarily identified by restriction endonuclease digestion. As shown in Figures 1A and 1B, Ag85A cDNA and mGM-CSF cDNA were cut from recombinant pI85AGM and shown in lane 3 and lane 5 respectively. The digested fragments size were same as with fragment size of PCR products (in lane 2 and lane 6 respectively). The sequencing results of recombinant pI85AGM were consistent with Genbank DNA sequences of Ag85A gene and mGM-CSF gene (data not shown).

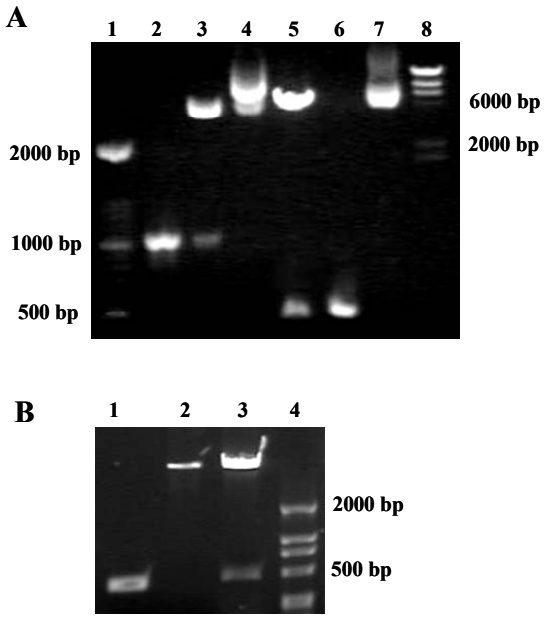

Figure 1. Amplification genes of Ag85A and mGM-CSF, identification pIGM and pI85AGM recombinants by PCR. (A) Lane 1, DNA marker 1; lane 2, PCR products of Ag85A gene; lane 3, the recombinant pI85AGM was digested with Nhe I and Mlu I restrictively; lane 4, the recombinant pI85AGM; lane 5, the recombinant pI85AGM was digested with Xba I and Not I restrictively; lane 6, PCR products of GM-CSF gene; lane 7, blank plasmid; lane 8, DNA Marker 2. (B) Lane 1, PCR products of mGM-CSF gene; lane 2, blank plasmid; lane 3, the recombinant pIGM was digested with Not I and Xba I restrictively; lane 4, DNA marker.

Analysis of protein expression by Western blotting and RT-PCR

Cells extracts were collected from 7721 cells transfected with recombinant pI85AGM and blank plasmid respectively. The mGM-CSF protein expression was analysed by RT-PCR. In Figure 2A, there is an mGM-CSF band in lane 2, whose size is the same as PCR products fragment size of mGM-CSF and it suggests that the mGM-CSF mRNA could be correctly expressed in 7721 cells. Western blot was performed using WesternBreeze Kit after 12% SDS-polyacrylamide gel electrophoresis. As shown in Figure 2B, a positive band was detected around 32 kD position in lane 3 and no bands was detected in lane 1 and lane 2, which indicates that recombinant pI85AGM can correctly express protein Ag85A in 7721 cells.

Activities of NK and CTL

As shown in Figure 3A, NK activity of group pIGM was the

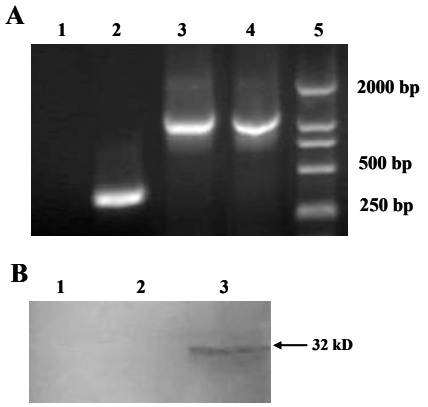

Figure 2. Analysis of GM-CSF and Ag85A protein expression in 7721 cell line. (A) RT-PCR analysis of GM-CSF and Ag85A protein expression in 7721 cell line. Total cellular RNA was extracted from 7721 cells transfected with different recombinant plamid. GM-CSF or Ag85A genes were amplified from those extractions by PCR. Lane 1, cells transfected with blank plasmid; lane 2, cells transfected with recombinant pIGM; lane 3, cells transfected with recombinant pI85AGM; lane 4, cells transfected with recombinant pI85A; lane 5, DNA marker. All extracts from cells transfected with recombinant pIGM or recombinant pI85AGM or blank plasmid digested with DNase I. (B) Western blot analysis Ag85A protein expressed in 7721 cell line. Lane 1, 7721 cell line; lane 2, cell line transfected with blank plasmid; lane 3, cell line transfected with recombinant pI85AGM.

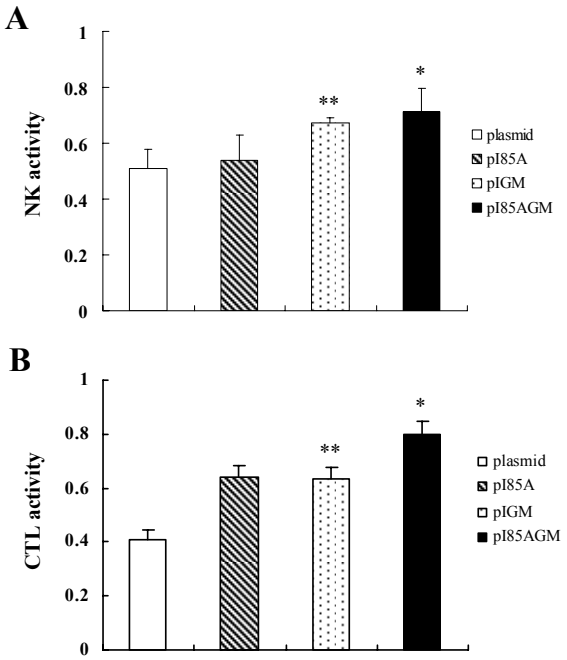

Figure 3. Detection of cytotoxic activities of CTL and NK. (A) The NK activity was measured by the MTT colorimetry assay and the data were represented the percentage of mean counts OD value and all cytotoxic assays were performed at least three times. *Compared with group pIGM, $p > 0.05$ and compared with group blank plasmid, $p < 0.05$. **Compared with group plasmid, $p < 0.05$. (B) The CTL activity was measured and the data representation are described as A. Effector cells were spleen cells and SP2/0 cells (5×10^4/ml) transfected with various recombinants respectively in 0.1 ml of RPMI 1640 were used as target cells. *Compared with groups of pI85A and pIGM, $p < 0.01$. **Compared with group blank plasmid, $p < 0.01$.

highest (71.7%) among 3 other groups and the group pI85AGM ranked the second (67.2%) and there was no significant change between this two groups ($p > 0.05$). However, the NK activity of the two groups was significantly increased compared with group blank plasmid. In Figure 3B, the CTL activity of group pI85AGM was the highest (79.81%) among 4 groups and CTL activities of other three groups were 64% in group pI85A, 63.11% in group pIGM and 40.56% in group blank plasmid respectively. The CTL activity in group pI85AGM was obviously increased compared with that of other three groups and the divergence is significant ($p < 0.01$), and yet the differences are not significant among group pI85A, group pIGM and group blank plasmid.

Spleen cell proliferation response to Ag85A

As shown in Figure 4, the spleen cell proliferation response to Ag85A in mice immunized with recombinant pI85AGM was significantly increased compared with group blank plasmid and group control with a SI of 3.92, however, the SI of the other two groups are all less than 1.5 and the divergence is obvious among three groups ($p < 0.01$).

Serum antibody levels of mice immunized with difference recombinants

The serum antibody level of mice immunized with recombinant pI85A or pI85AGM was significantly increased compared with other three groups after mice were inoculated with various recombinants for three times and the divergence is significant ($p < 0.01$, Figure 4). No significant changes were detected among group pIGM, group blank plasmid and group control. The results indicate that the Ag85A could induce mice to generate antibody response, however, GM-CSF has no enhancing effect on Ag85A stimulation antibody response because recombinant pI85AGM or pI85A induce antibody against Ag85A at the same level.

Discussion

The failure of current BCG vaccine in controlling the global TB epidemic highlights an urgent need for improved TB vaccine formulations (5, 14, 18) or the development of novel vaccine strategies to replace or supplement BCG (19, 20). Although efficient antigen presentation and IFN-γ production by mycobacterial-specific T lymphocytes are required for protection against Mycobacterium tuberculosis (19), BCG vaccine cannot induce naïve T lymphocytes to produce IFN-γ, which may explain the low efficiency of BCG as a TB vaccine. In current study, we have designed and developed a novel TB gene vaccine constructs encoding mGM-CSF and

TB Ag85A proteins respectively and investigated the effect of mGM-CSF on enhancing Ag85A immunogenicity in a mouse strain that is slightly weak responders to fusion protein of GM-CSF and Ag85A (21).

Balb/c mice were immunized *i.m.* with various recombinants for three times with an interval of two weeks between each inoculation. The CTL activity was significantly increased in mice immunized with pI85A than that of mice immunized with blank plasmid, but was lower than that of mice immunized with pI85AGM. Our previous experiment data indicated mGM-CSF was an effective immunoadjuvant for improving the levels of interleukin 2 and IFN-γ in the serum as well as enhancing the CTL activity in tumor model of mice inoculated with recombinant pc-mGM-CSF (data not shown). The current experiment results show that GM-CSF and Ag85A could be expressed simultaneously in mice immunized with recombinant pI85AGM and the immunogenicity of Ag85A was enhanced in mice with mGM-CSF expression, in which cellular immune responses to Ag85A was remarkably increased and the CTL activity was obviously enhanced. It seems evident that mGM-CSF plays a crucial role in generation of effective T cell subsets and in determining the magnitude of the response by DNA-based gene vaccines (14).

Although natural cytotoxicity activity of NK cells was remarkably higher in mice immunized with pI85AGM than that of mice immunized with blank plasmid, it was lower than that of mice immunized with pIGM, but the diversity was not significant ($p > 0.05$). Our results also revealed that the enhancement of CTL activity is more obvious than that of NK cells in mice immunized with recombinant pI85AGM. We suppose that Ag85A expressed in mice immunized with pI85AGM may be recognized as an endogenous antigen which could be presented to $CD8^+$ lymphocytes. So the CTLs could be stimulated much more easily compared with NK cell, although NK cells do not need primary stimulation. Therefore, the immunogenicity of Ag85A on CTL activity was stronger than that of NK activity in our experimental system as shown in Figures 3A and 3B. The spleen cell proliferation response to Ag85A stimulation is also stronger in mice immunized with pI85AGM than that of mice immunized with blank plasmid or that of normal mice. These results suggest that Ag85A encoded by recombinant pI85AGM can make mouse spleen cells immunized and induce spleen cells to proliferate following Ag85A secondary stimulation in immunized mice.

Mice inoculated with recombinant pI85A or pI85AGM can generate antibodies against Ag85A and the serum antibody level of mice is significantly increased compared with that of group PBS, group blank plasmid or group pIGM after secondary immunization. It indicates that the pI85AGM, a novel gene vaccine constructs, not only generate potent cellular immunity, but also induce obvious antibody immune response to Ag85A in immunized mice. However, the GM-CSF transgene-based adjuvant formulation does not increase the immunogenicity of tuberculosis Ag85A to B cells because recombinant pI85AGM or pI85A induce mice to generate antibodies a similar level in immunized mice.

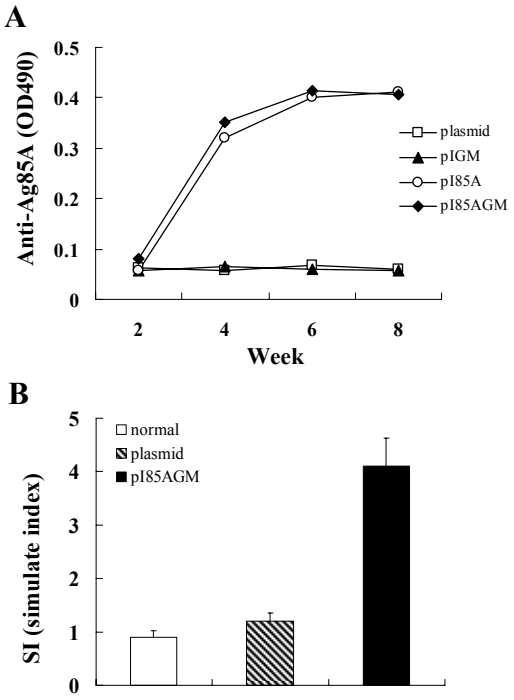

Figure 4. Serum antibody levels in dynamic state and spleen cell proliferation response to Ag85A. (A) Serum antibody against Ag85A was detected by ELISA. The results were expressed as the OD value at 490 nm. The serum antibody level of mice immunized with recombinant pI85A or pI85AGM was significantly increased in the fourth week when mice were immunized the second time and the peak of antibody arrived in the sixth week when mice were immunized the third time. But no change at the serum antibody was detected in mice immunized with recombinant pIGM or blank plasmid or PBS. The divergence was significant ($p < 0.01$) between group pI85A or group pI85AGM and other three groups. (B) Spleen cell proliferation response to Ag85A *in vitro* was detected by MTT method. The result suggested that spleen cells of mice immunized with recombinant pI85AGM appeared to significantly proliferate under the stimulation of Ag85A compared with that of blank plasmid and control ($p < 0.01$). All data were obtained from 3 or 4 independent experiments.

In conclusion, we successfully developed a novel TB gene vaccine constructs pI85AGM and it could induce mice to generate potent immune responses to AG85A. The GM-CSF as an effective adjuvant can increase Ag85A immunogenicity and enhance the activity of CTL in mice immunized with recombinant pI85AGM. Further studies are required to evaluate the protection effect of pI85AGM on mice after being challenged with TB. Following such a consideration, a better and effective TB vaccine might be developed, which may replace or supplement the traditional BCG vaccine.

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