A novel recombinant DNA vaccine encoding *Mycobacterium tuberculosis* ESAT-6 and FL protects against *Mycobacterium tuberculosis* challenge in mice

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Abstract

Mycobacterium tuberculosis 6-kDa early secretory antigenic target (ESAT-6) is a dominant target antigen for cell-mediated immunity in the early phase of tuberculosis. The fms-like tyrosine kinase 3 ligand (FL) that induces potent immune response has been used as an adjuvant in vaccine development. In this study, a new recombinant plasmid (pIRES-epitope-peptides-FL) encoding three T cell epitopes of ESAT-6 and FL was constructed, and the immunogenicity of the DNA vaccine was assessed in C57BL/6 mice immunized with the plasmid DNA vaccine. Additionally, a strategy of intramuscular injection with the DNA vaccine (prime) and intranasal administration of the epitope peptides (boost) was employed to induce higher immune reaction of the mice. The results showed that mice vaccinated with the recombinant plasmid DNA vaccine and boosted with the peptides not only increased the levels of Th1 cytokines (IFN-γ and IL-12), the number of IFN-γ$^+$ T cells and activities of cytotoxic T lymphocytes as well as IgG, but also enhanced protection against *Mycobacterium tuberculosis* challenge. In conclusion, these data indicate that the novel recombinant pIRES-epitope-peptides-FL plasmid is a useful DNA vaccine for pre–venting *Mycobacterium tuberculosis* infection.

Keywords: early secretory antigenic target-6 (ESAT-6), fms-like tyrosine kinase 3 ligand (FL), *Mycobacterium tuberculosis*, recombinant plasmid, T cell epitopes

INTRODUCTION

Tuberculosis is a re-emerging disease that represents a major public health problem[1]. The World Health Organization (WHO) estimates that tuberculosis causes three million deaths and over eight million new cases annually[2,3]. At present, the only available tuberculo–sis vaccine is the attenuated strain of *Mycobacterium* (*M.*) *bovis* bacillus Calmette-Guérin (BCG). Although effective in preventing *M. tuberculosis* infection in newborns and toddlers, BCG provides poor protection in adults with variable efficacies between 0 to 80%[4].

Thus, more efficient vaccines and vaccination strate−gies against tuberculosis are urgently needed.

In order to control *M. tuberculosis* infection, nu−merous efforts have been made in preparing new vaccines, including DNA and subunit protein vac−cines[5,6]. Reportedly, *M. tuberculosis* 6-kDa early secretory antigenic target (ESAT-6) has been evalu−ated as DNA vaccines in several models[7-9]. Previous studies have demonstrated that ESAT-6 contains an−tigen epitopes recognized by T and B cells in patients and experimental animals with active tuberculo−sis[6,10-14]. Besides T cell responses, enhanced antibody response against ESAT-6 has also been displayed in *M. tuberculosis*-infected individuals[12–14]. Hence, many investigators have shown great interests in ex−ploring the roles of ESAT-6. However, comparative genomics reveal that ESAT-6 is only located in viru−lent *M. tuberculosis*[15,16], and it may also contribute to cellular invasion, phagolysosome escape and *M. tuberculosis* dissemination as a cytolytic toxin[17-22]. Therefore, when a new tuberculosis vaccine is devel−oped, it is important to avoid adverse reaction.

As we know, vaccines based on T cell antigen epitopes can result in effective immune reactions be−cause these epitopes have highly conservative sequences and relatively higher safety[23-25]. Moreover, experiments on mucosal immunization have displayed that antigen-specific T cells in the mucosa play a key role in robust immune protection[26-27]. Given that strategies involved in prime-boost have achieved variable successes against *M. tuberculosis* infections[28-30], immunization of mice with DNA vaccines containing T cell epitopes and vac−cinated with prime-boost strategy may be an ideal ap−proach to induce effective protection.

Many studies have demonstrated that DNA vac−cines encoding single or multi-T cell epitopes could obviously induce potent T cell responses[23-25], and combination of DNA vaccines and some adjuvants could enhance their immunogenicity, including ele−vating cell-mediated immunity (CMI)[31]. The fms-like tyrosine kinase 3 ligand (FL) is a growth factor that influences the development of multiple hematopoietic lineages[31]. FL has been found to promote the growth of T cells, B cells and dendritic cells (DCs)[32,33] and augment immuno-stimulatory responses to some an−tigens[34]. Therefore, co-delivery of FL for DNA vac−cine may be a feasible design.

To prepare a novel and effective recombinant DNA vaccine, in the current study, the gene fragments encoding the three T cell epitopes of *M. tuberculo−sis* ESAT-6 were selected and cloned into a pIRES plasmid together with the *FL* gene (pIRES-epitope-peptides-FL). Thereafter, the immune responses and

protective effects in C57BL/6 mice immunized with the plasmid DNA vaccine, including the effects of prime-boost strategy were evaluated.

MATERIALS AND METHODS

T cell epitope prediction and plasmid con−struction

The construction of pIRES-ESAT-6-FL plasmid containing the *ESAT-6* and *FL* genes has been previ−ously described[35]. The primary structure of ESAT-6 protein that contains potential MHC Ⅰ and MHC Ⅱ -binding T cell epitopes was analyzed using epitope prediction software (http://www.syfpeithi.com/scripts/MHCSr.dll/home.htm; http://tools.immuneepitope.org). Thereafter, the three T cell epitopes, including ESAT-6$_{4-18}$ (QQWNFAGIEAAASAI), ESAT-6$_{22-36}$ (VTSIHSLLDEGKQSL) and ESAT-6$_{56-70}$ (QKWDA−TATELNNALQ) were selected based on higher scores via the prediction software. The three epitope-peptides with HIS-tag and Ala-Ala-Tyr (AAY) linker were syn−thesized and inserted into pIRES vector or pIRES-FL plasmid, termed pIRES-epitope-peptides and pIRES-epitope-peptides-FL (*Fig. 1A*). Thereafter, these re−combinant plasmids were identified by sequencing and expressing HIS and FL proteins (*Fig. 1B*). The peptides of three T cell epitopes of ESAT-6 were synthesized by Shanghai Shangon Biological Engineering Technol−ogy and Services Co. (purity > 95%). BCG (Denmark strain 1331) was provided by the Center for Disease Control of Jiangsu Province in China.

Animal vaccination

Six to eight week-old female C57BL/6 mice (H-2^b) were purchased from the Experimental Animal Centre of Chinese Academy of Science (Shanghai, China). Animal welfare and the experimental procedures were carried out strictly in accordance with the Guide for the Care and Use of Laboratory Animals (Ministry of Health, China, 1998). The study protocol was approved by the local institutional review board at the authors´ affiliated institutions (Permit No.: 2010254). Endotox−in-free plasmids were prepared using the of EndoFree plasmid purification kit (Qiagen, USA). C57BL/6 mice were injected intramuscularly at 3-week intervals in both the quadriceps muscles with 100 μg plasmid DNA (the DNA group), including pIRES (vector), pIRES-epitope-peptides, pIRES-FL, pIRES-epitope-peptides-FL, pIRES-ESAT-6 (containing full-length ESAT-6), pIRES-ESAT-6-FL plasmids, or phosphate buffered saline (PBS), respectively. Moreover, 3 weeks after vaccination, some mice were intranasally treated with 2.5 μg mixed ESAT-6 peptides once a
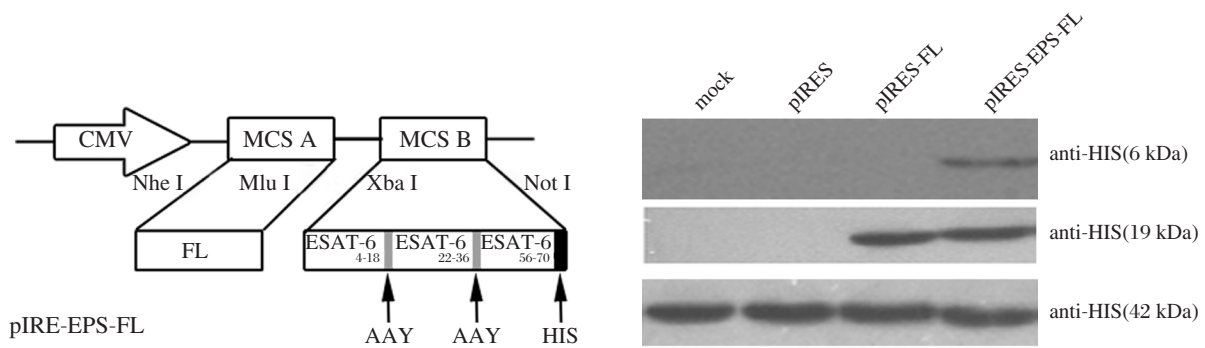
Fig. 1 **Construction and identification of pIRES-epitope-peptides-FL plasmid.** A: Schematic representation of pIRES-epitope-peptides-FL. B: The expression of different plasmids was detected by using Western blotting assays. 293T cells were trans–fected with the indicated plasmids, and the cell lysate was subjected to Western blotting assays with anti-His or anti-FL antibody. Individual experiments were conducted three times, with one representative photo for each group.

week for 2 weeks (the DNA/Ag group). All mice were sacrificed at 1 week after the last epitope-peptides boost or 3 weeks after the last plasmid DNA vacci–nation alone. In the BCG group, mice were injected subcutaneously with 10^6 colony forming units (CFUs) of freshly prepared BCG only once at equal pace. The whole schedule of vaccination is displayed in *Fig. 2*.

Western blotting assays

293T cells (American Tissue Culture Collection, USA) were transfected with pIRES-epitope-peptides or pIRES-epitope-peptides-FL using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) and cultured for 48 hours. The lysate was electrophoresed on SDS-PAGE gels. Thereafter, the transferred membrane was probed with anti-HIS or anti-FL antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA) followed by horseradish peroxidase (HRP) labeled goat anti-mouse antibody (Invitrogen, Carlsbad, CA, USA), and the signals were detected by enhanced chemilumines–cence (Pierce, Rockford, IL, USA).

ELISA

For mice immunized with plasmid DNA (the DNA group), spleen cells were isolated 3 weeks after the last treatment. For mice treated with plasmid DNA plus the epitope-peptide boost (the DNA/Ag group), spleen cells were taken 1 week after the last peptide boost. Then, the cells were cultured and stimulated with the mixture of 3 peptides (10 µg/mL) for 72 hours. The levels of IFN-γ, IL-12, IL-4 and IL-10 in the supernatants were determined by ELISA (Bio–legend, San Diego, CA, USA). Besides, for cytokine detection in vivo, bronchoalveolar lavage (BAL) flu–ids were collected, and the contents of the same cy–tokines were also detected by ELISA.

The level of ESAT-6-peptide-specific antibody in the

sera of mice was measured by ELISA. First, the micro–titer plates were coated with the mixed epitope-peptides (10 µg/mL). Then, 1:100 diluted sera were added into the coated plates. The bound antibodies were detected with HRP-conjugated goat anti-mouse IgG.

MTT and ELISPOT assays

Spleen cells from immunized mice were harvested and incubated with the epitope-peptides or me–dium. Splenocyte proliferation was detected by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays[36,37]. The results were ex–pressed as stimulation index (SI). SI was calculated using the formula: SI = [OD value (stimulated culture)-OD value (control culture)] / OD value (control cul–ture). Moreover, IFN-γ+ T cells were examined using the ELISPOT assay kit. Briefly, plates were coated with anti-IFN-γ antibodies overnight. Splenocytes (5×10^5 cells/well) were cultured upon the epitope-peptide stimulation for 48 hours. Thereafter, the bioti–nylated anti-IFN-γ antibodies were added and incu–bated for 2 hours, and the plate was supplemented with streptavidin-alkaline phosphatase for 1 hour, and then the positive spots were counted.

In vivo CTL assay

CTL target cells were prepared from the spleen of naïve mice and pulsed with the mixture of the epitope-peptides (10 µg/mL) or without the peptides overnight. The peptide-pulsed splenocytes were then labeled with a high concentration of 5-(6)-carboxyflu–orescein diacetate succinimidyl ester (CFSE) (5 µmol/L) or non-pulsed splenocytes with a low concentra–tion of CFSE (0.5 µmol/L). Then, 2.5×10^6 pulsed and 2.5×10^6 non-pulsed splenocytes were mixed and adoptively transferred to the immunized mice. The splenocytes at 18 hours after treatment were isolated
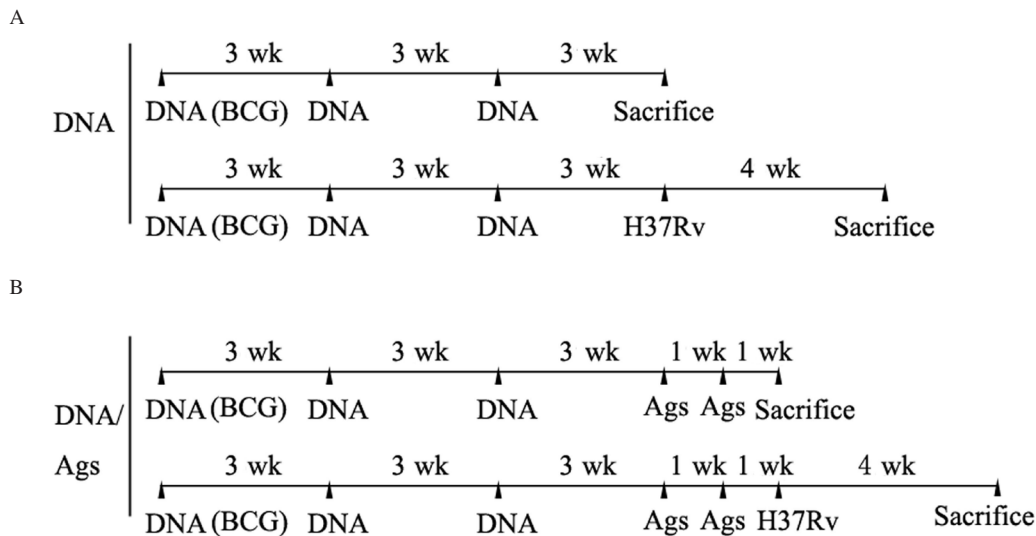
Fig. 2 **Entire immunization schedule.** A: C57BL/6 mice were injected intramuscularly three times at 3-week intervals in both quadriceps muscles with 100 μg of plasmid DNA and sacrificed at 3 weeks after the last plasmids DNA vaccination alone or at 4 weeks after infection with *M. tuberculosis* H37Rv via the airway (the DNA group). B: The mice at 3 weeks after the last plasmids DNA injection were conduced through intranasal administration of the mixed ESAT-6 epitope-peptides (Ags) once a week for 2 weeks, and then parts of the mice were sacrificed or infected with *M. tuberculosis* H37Rv via the airway the DNA/Ags group. More−over, these mice post *M. tuberculosis* H37Rv challenge for 4 weeks were sacrificed.

for acquisition on a FACScalibur instrument (BD Bi−osciences, San Jose, CA, USA). To evaluate the per−centage of specific lysis, the ratio of $CFSE_{high}/CFSE_{low}$ in the vaccinated mice was compared[37].

M. tuberculosis H37Rv challenge

Mice immunized with the plasmid DNA vaccine alone or in combination with the last epitope-peptide boost again were infected by intratracheal instillation with 5×10^5 CFUs of *M. tuberculosis* H37Rv (ATCC 27294, USA) for 4 weeks. For the bacterial burden, the lung and spleen of the mice were examined 4 weeks post *M. tuberculosis* challenge by plating serial dilu−tions of tissue homogenates onto Middlebrook 7H10 agar plates, and then the plates were cultured for 4 weeks. *M. tuberculosis* colonies were calculated and displayed as log10 CFU per organ. For histological changes, paraffin sections of lung tissues were stained with hematoxylin and eosin (H&E) and observed by a pathologist who was blind to treatment. To score lung inflammation and damage, entire lung sections were analyzed with inflammatory infiltrations, which were quantified and expressed as a percentage of the lung surface. Furthermore, to determine infiltration of T cells and macrophages, the sections were first stained with rabbit anti-mouse CD3 antibodies (Biow−orld, Dublin, OH, USA) or F4/80 antibodies (Abcam, Cambridge, MA, USA), and then incubated with HRP-

conjugated secondary antibody and counterstained with hematoxylin.

Statistical analysis

Data analyses were carried out by using the SPSS 16.0 software. All values were expressed as the mean±SEM. Analysis was performed by using two-way ANOVA. Multiple comparisons were performed with LSD test. *P* < 0.05 was considered statistically significant.

RESULTS

pIRES-epitope-peptide-FL vaccination and epitope-peptide boost potentiate Th1-biased cellular immune response

To investigate the changes of cytokine profile in−duced by vaccinations, we measured the levels of Th1/Th2 cytokines secreted by spleen cells from the mice. The levels of IFN-γ and IL-12 (***Fig. 3A***) in the supernatants of mice vaccinated with pIRES-epitope-peptides, pIRES-epitope-peptide-FL, pIRES-ESAT-6 and pIRES-ESAT-6-FL plasmids were significantly increased, compared with mice treated with PBS, pIRES, pIRES-FL and BCG, respectively. While mice immunized with pIRES-epitope-peptides-FL and pIRES-ESAT-6-FL plasmids showed markedly higher levels than those of mice treated with other plasmids, especially in mice vaccinated with the two
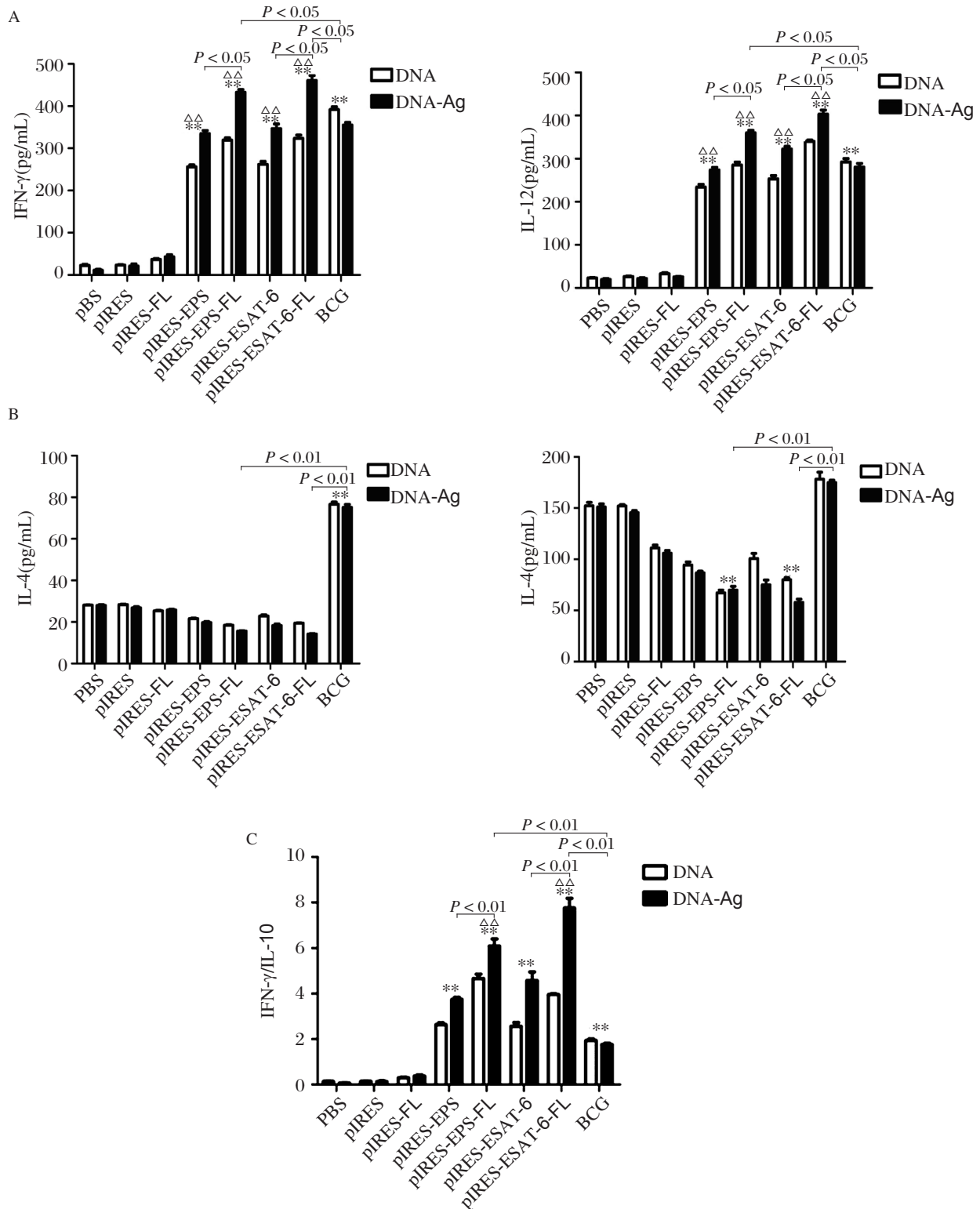
Fig. 3 **Th1 immune responses of mice immunized with different plasmids.** The concentrations of Th1/Th2 cytokines in the culture supernatant of splenocyte from the mice were measured by ELISA. The levels of IFN-γ and IL-12 represented Th1 im‒mune response A: while levels of IL-4 and IL-10 standed for Th2 immune response B: The ratio of IFN-γ/IL-10 was calculated to indicate Th1/Th2 reaction (C). [**]$P < 0.01$, indicating the levels of these cytokines in corresponding mice were markedly increased compared with those of the mice treated with PBS, pIRES or pIRES-FL; [△△] $P < 0.01$, indicating the levels of these cytokines in the mice of DNA/Ags group significantly higher than those of the mice with other corresponding DNA treatment. Other difference (such as lower than the BCG group) between the groups are presented in the figure directly. Data are shown as mean $\pm$ SEM ($n = 10$), done in triplicate.

plasmids plus the ESAT-6 peptide boost (the DNA/Ag groups). Meanwhile, the levels of IL-4 and IL-10 were relatively lower in mice treated with the same plasmids except for BCG, and a marked reduction of IL-10 was found in mice immunized with pIRES-epitope-peptide-FL and pIRES-ESAT-6-FL (**Fig. 3B**). Additionally, the ratios of IFN-γ and IL-10 average values were also calculated to represent Th1 and Th2 reaction. As shown in **Fig. 3C**, mice immunized with pIRES-epitope-peptide-FL and pIRES-ESAT-6-FL plasmids manifested obviously higher Th1/Th2 ratios than those of mice treated with other constructs. The changes of Th1 and Th2 cytokine profile mentioned

previously were more significant in the corresponding DNA/Ag treatment (**Fig. 3A-C**).

Besides, to further evaluate the boost effects of the ESAT-6 epitope-peptides (Ag) after intranasal admin–istration, we collected BAL fluids of the immunized mice and measured the levels of Th1/Th2 cytokines. The results showed a significantly enhanced IFN-γ and IL-12 production in mice vaccinated with pIRES-epitope-peptides, pIRES-epitope-peptide-FL, pIRES-ESAT-6 and pIRES-ESAT-6-FL plus the peptide boost. Furthermore, changes of cytokine profile were similar to those in the splenocyte supernatants of mice only treated with different plasmids (data not shown).
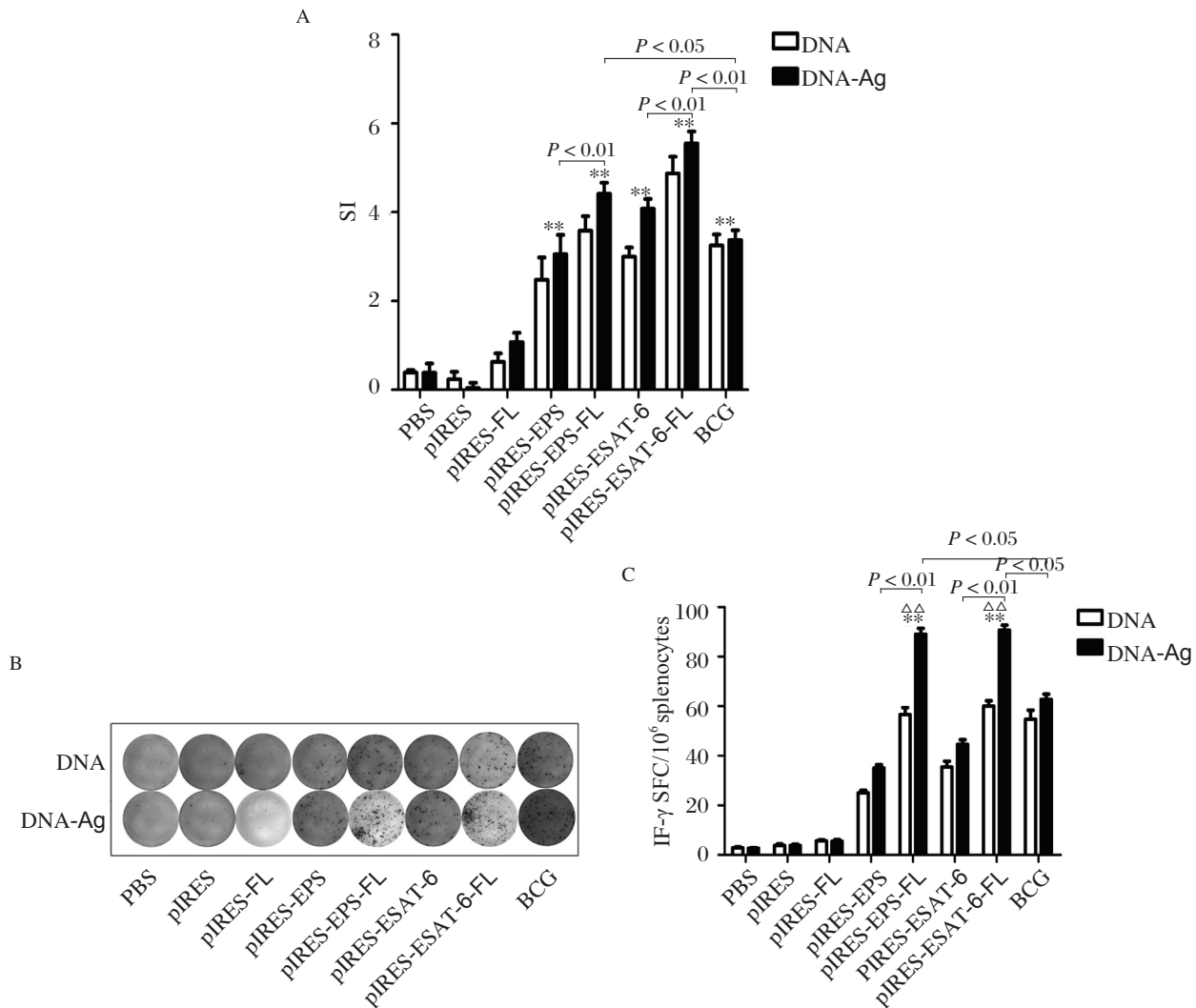

Fig. 4 The proliferation of spleen cells and numbers of IFN-γ⁺ T cells of mice vaccinated with different plasmids.
A: The spleen cells from mice at 3 weeks after the last DNA vaccination alone or at 1 week after the last epitope-peptides (Ag) boost were cultured for 72 hours upon epitope-peptides or medium stimulation alone. Then, the proliferation of spleen cells was examined using MTT, and the stimulation index (SI) in each group was calculated to determine the cell proliferation activity. B: The spleno–cytes as the above metioned time were collected and treated with the same method, and the nnubers of IFN-γ⁺T cells were quantified using ELISPOT assay. Representative images of ELISPOT response in the immunized mice and the frequency of IFN-γ⁺ T cells in the spleen were displayed. All data are shown as mean $\pm$ SEM ($n = 10$), done in triplicate. $^{**}P < 0.01$, showing much higher than that of the PBS, pIRES or pIRES-FL group, and other differences between the groups were displayed in the figure directly. $^{\triangle\triangle}P < 0.01$, meaning DNA/Ags group vs the corresponding DNA group, separately.

The results indicated that effective Th1 responses were elicited in mice immunized with plasmid DNA vaccines, especially by using the strategy of DNA prime and the peptide boost.

pIRES-epitope-peptide-FL vaccination and epitope-peptide boost increase the proliferation of spleen cells and IFN-γ⁺ T cells

As shown in *Fig. 4A*, splencytes from mice immunized with pIRES-epitope-peptides, pIRES-epitope-peptide-FL, pIRES-ESAT-6, pIRES-ESAT-6-FL and BCG displayed remarkable proliferation. Furthermore, SI increase in mice with pIRES-epitope-peptides-FL or pIRES-ESAT-6-FL treatment was more apparent relative to mice immunized with pIRES-epitope-peptides or pIRES-ESAT-6 plasmids. In addition, cell proliferation in mice treated with the corresponding plasmids plus the peptide boost (the DNA/Ag group) exhibited much higher than that in mice only treated with these plasmids (the DNA group). Meantime, as shown in *Fig. 4B*, the number of IFN-γ⁺ T cells in mice immunized with pIRES-epitope-peptides, pIRES-epitope-peptide-FL, pIRES-ESAT-6, pIRES-ESAT-6-FL and BCG were more notably increased than those of mice vaccinated with PBS, pIRE, or pIRES-FL, especially much more in mice treated with pIRES-epitope-peptide-FL and pIRES-ESAT-6-FL plasmids. Moreover, mice vaccinated with the same plasmids plus epitope-peptide boost (the DNA/Ag group) produced significantly greater IFN-γ⁺ T cells than those of mice immunized with the corresponding plasmids alone.

pIRES-epitope-peptide-FL vaccination and epitope-peptide boost markedly augment CTL activity

To assess the cytolytic function of T cells induced by pIRES-epitope-peptides-FL and other plasmid DNA vaccines, we examined epitope-specific lysis of CFSE-labeled transfected cells by flow cytometric analyses. When compared with the CTL activity of mice treated with PBS, pIRES, pIRES-FL and BCG, increased lyses were determined in mice vaccinated with the DNA vaccines of pIRES-epitope-peptides, pIRES-epitope-peptide-FL, pIRES-ESAT-6 and pIRES-ESAT-6-FL, especially much higher in mice immunized with pIRES-epitope-peptide-FL and pIRES-ESAT-6-FL (*Fig. 5*). Besides, a significantly elevated CTL activity in mice given with these plasmid vaccinations and the peptide boost were also achieved compared with mice treated with these plasmids alone.

pIRES-epitope-peptide-FL vaccination and epitope-peptide boost significantly elevate the levels of ESAT-6 peptide-specific antibody

To explore whether these plasmid DNA vaccines can induce antigen-specific humoral immune responses, we collected sera from vaccinated mice and then detected ESAT-6 peptide-specific antibody with ELISA. As shown in *Fig. 6*, the levels of specific IgG in mice vaccinated with pIRES-epitope-peptides, pIRES-epitope-peptide-FL, pIRES-ESAT-6 and pIRES-ESAT-6-FL were higher than those of mice with PBS, pIRES or pIRES-FL treatment. The levels of antibody in mice vaccinated with pIRES-epitope-peptide-FL and pIRES-ESAT-6-FL were much higher than those of mice given with other plasmids. Additionally, mice vaccinated with pIRES-epitope-peptides, pIRES-epitope-peptide-FL, pIRES-ESAT-6 and pIRES-ESAT-6-FL plasmids and boosted with the peptides (DNA/Ag) showed a notably elevated level of IgG compared with others, but not mice immunized with BCG.

pIRES-epitope-peptide-FL vaccination and epitope-peptides boost notably enhances protection from *M. tuberculosis* challenge

To further evaluate the protective potential of pIRES-epitope-peptide-FL DNA vaccine and the strategy of prime-boost vaccination, we challenged mice 3 weeks after the last plasmid immunization (the DNA group) or the plasmid injection (3 weeks) plus 1 week after the last peptide boost (the DNA/Ag group) intranasally with 5×10^5 CFUs of M. tuberculosis H37Rv (*Fig. 2*), and the bacterial burdens in the lungs or spleens including pulmonary injury of mice were observed 4 weeks post *M. tuberculosis* challenge. As shown in *Fig. 7A* and *Fig. 7B*, mice immunized with pIRES-epitope-peptides, pIRES-epitope-peptide-FL, pIRES-ESAT-6, pIRES-ESAT-6-FL and BCG displayed efficient protection from *M. tuberculosis* H37Rv infection, and mice treated with the corresponding DNA/Ag showed remarkably augmented protective effects.

For histological changes, the results revealed that the lung tissues of mice treated with PBS, pIRES, and pIRES-FL exhibited widespread and severe interstitial pneumonia, intense inflammation and diffuse granuloma, as well as increased infiltration of T cells (*Fig. 7E*) and macrophages (*Fig. 7F*) after *M. tuberculosis* challenge. However, mice immunized with pIRES-epitope-peptides, pIRES-epitope-peptide-FL, pIRES-ESAT-6 or pIRES-ESAT-6-FL plasmids showed moderate damages in the alveolar tissues accompanied
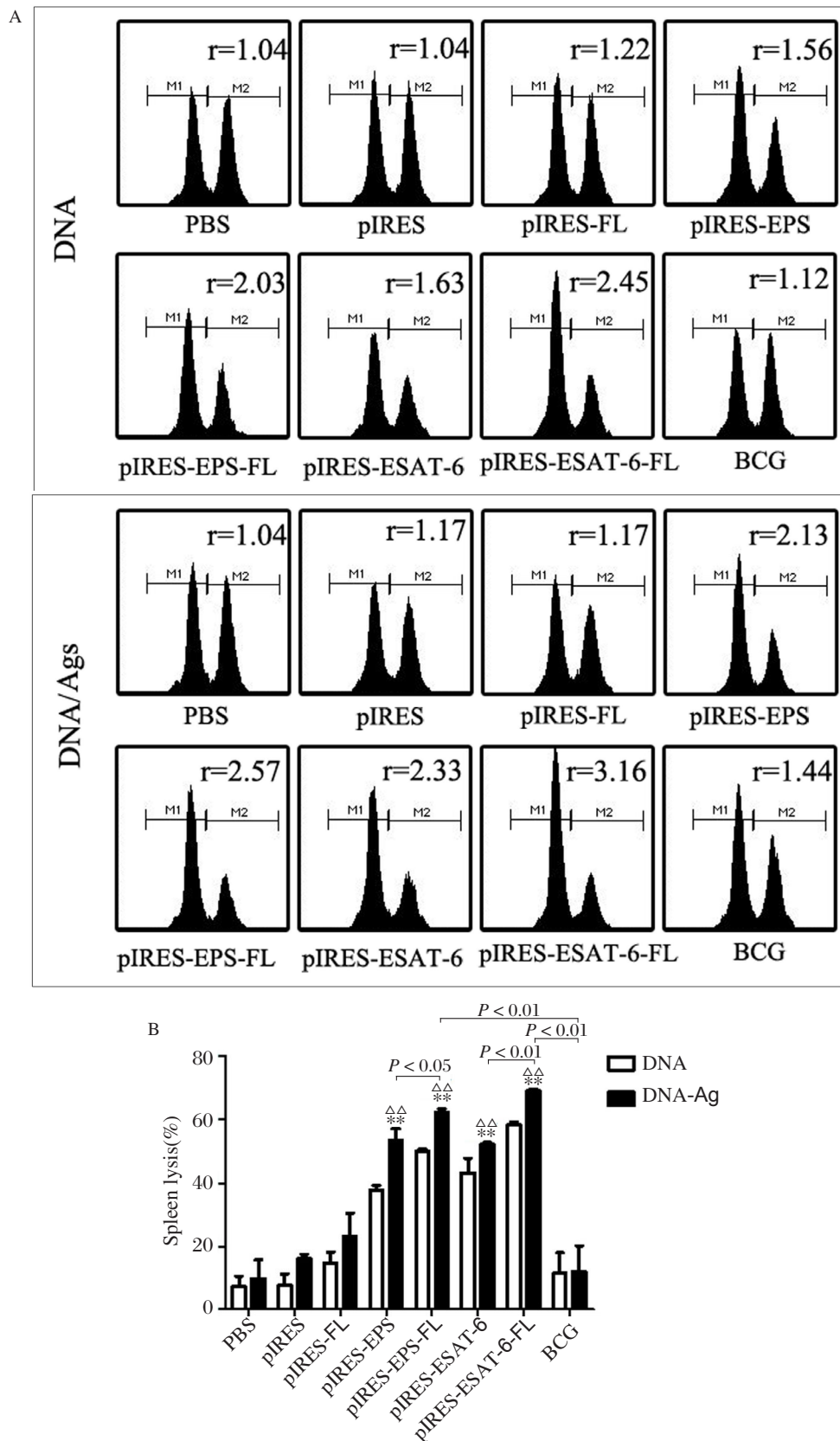
Fig. 5 The specific CTL activity of mice triggered by various plasmid DNA vaccines. Non-pulsed splenocytes (CFSE^{low}) and ESAT-6 peptides-pulsed splenocytes (CFS^{Ehigh}) from naive mice were transferred to the immunized mice. The representative histograms (A) and percentages (B) of splenic ESAT-6-specific lysis in the immunized mice were compared. $^{**}P < 0.01$, vs. the PBS, pIRES or pIRES-FL group, respectively; $^{\triangle\triangle}P < 0.01$, meaning the DNA/Ags group vs. the corresponding DNA group, respectively. Images indicate that one representative result from three experiments, and data present as mean $\pm$ SEM ($n = 10$).
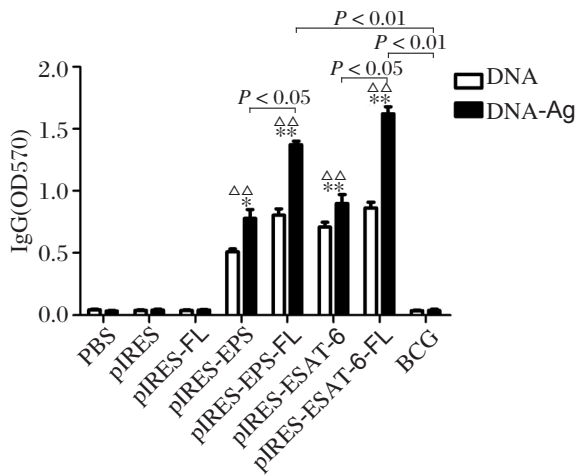
Fig. 6 **Specific antibody for epitope-peptides in the sera of mice followed by immunization procedure.** The sera samples from the mice were obtained at the indicated time (see **Fig.2**), and the levels of anti-ESAT-6 epitope-pep–tides antibody are detected by ELISA. The result is representa–tive of 3 independent experiments. The significant differences are shown in the figure directly (data showed as mean ± S.E.M., $n = 10$, $^*P < 0.05$, $^{**}P < 0.01$ indicating the level of antibody obviously higher than that in the PBS or pIRES or pIRES-FL group, respectively; $^{\triangle\triangle}P < 0.01$ showing that the level of an–tibody in the DNA/Ag group is significantly higher than that in the corresponding DNA group).

with relatively fewer numbers of infiltrated T cells and macrophages, as those of mice immunized with BCG (**Fig. 7C** and **7D**). Furthermore, mice in the DNA/Ag group displayed much stronger protection, particularly mice with pIRES-epitope-peptide-FL plus the peptides boost (**Fig. 7C** and **7D**), indicating that this novel plas–mid DNA vaccine (pIRES-epitope-peptide-FL) can indeed provide efficient protection against *M. tuber–culosis* infection.

DISCUSSION

CMI is crucial for protection against *M. tuberculo–sis* infection in both humans and animals[38-43]. The fu–ture success of DNA vaccines for tuberculosis depends on inducing stronger CMI such as producing more IFN-γ-secreting cells or Th1 cytokines. Early studies confirmed that DNA vaccines, which contained single or multiple epitopes of pathogen protein, could trig–ger efficient immune responses[44], and possess better advantages i.e. greater stability, safety and stand–ardization. Thus, the use of DNA vaccines encoding pathogen epitopes may be an effective approach to promote CMI. Reportedly, ESAT-6 is an effective an–tigen, and is strongly involved in anti-mycobacterial T cell immune reaction[10,11]. However, ESAT-6 has been found to cause apoptosis of macrophages and lysis of

erythrocytes as well as injury of epithelial cells[17,20,45]. Therefore, in order to avoid ESAT-6 toxic effects, in the study, three T cell epitopes of ESAT-6 (ESAT-6$_{4-18}$, ESAT-6$_{22-36}$ and ESAT-6$_{56-70}$) were selected based on higher scores via prediction software and ESAT-6 functions described previously[9]. Thereafter, a recombinant DNA vaccine (pIRES-epitope-peptide plasmid) was first constructed. It is worth noting that these ESAT-6 T cell epitopes not only contain domi–nant Th1 (CD$_4^+$ T cell) epitopes, but also include CTL epitopes[46].

As we know, the drawback of DNA vaccine is its relatively low immunogenicity. Antigen presenting cells (APCs), especially DCs, is an important in–ducer of immune response[31-33]. It has been demon–strated that FL can elevate CMI through increasing the number of DCs, and the maturation and function of DCs[31,33]. Since FL has been reported as a good adjuvant in DNA vaccine, in the experiment, some recombinant plasmid DNA vaccines such as pIRES-epitope-peptide-FL (encoding the three T cell epitopes of ESAT-6 and FL protein), pIRES (blank vector), pIRES-epitope-peptides (only containing the three T cell epitopes of ESAT-6), pIRES-ESAT-6 (containing full length ESAT-6) and pIRES-ESAT-6-FL (con–taining full length ESAT-6 and FL) were successfully constructed for the first time. Besides, in our previ–ous study, a DNA vaccine expressing a fusion protein of ESAT-6-FL (pIRES-ESAT-6-FL), which contains full length ESAT-6 and FL, had been developed. The vaccine could induce effective immune responses from in mice[35], but protection against *M. tuberculosis* challenge remained to be demonstrated.

Our results from the present experiment revealed that mice immunized with pIRES-epitope-peptide-FL plasmids markedly enhanced the levels of Th1 cy–tokine (IFN-γ and IL-12), the proliferation of spleen cells, and the number of IFN-γ$^+$ T cells and activity of CTLs including the content of anti-ESAT-6 anti–body. Meanwhile, mice treated with pIRES-epitope-peptide-FL plasmids displayed remarkably enhanced protection against *M. tuberculosis* challenge, e.g. reducing bacterial burden in the lung and spleen or pathological lesions of the lungs. For mice im–munized with pIRES-epitope-peptide-FL or pIRES-ESAT-6-FL plasmids, although similar effects on immunity and protection from mice were obtained, we considered the pIRES-epitope-peptides-FL plas–mid as a relatively better vaccine because the epitope vaccine could avoid the toxicity of ESAT-6 whole protein as shown by other investigators[9,16,23].

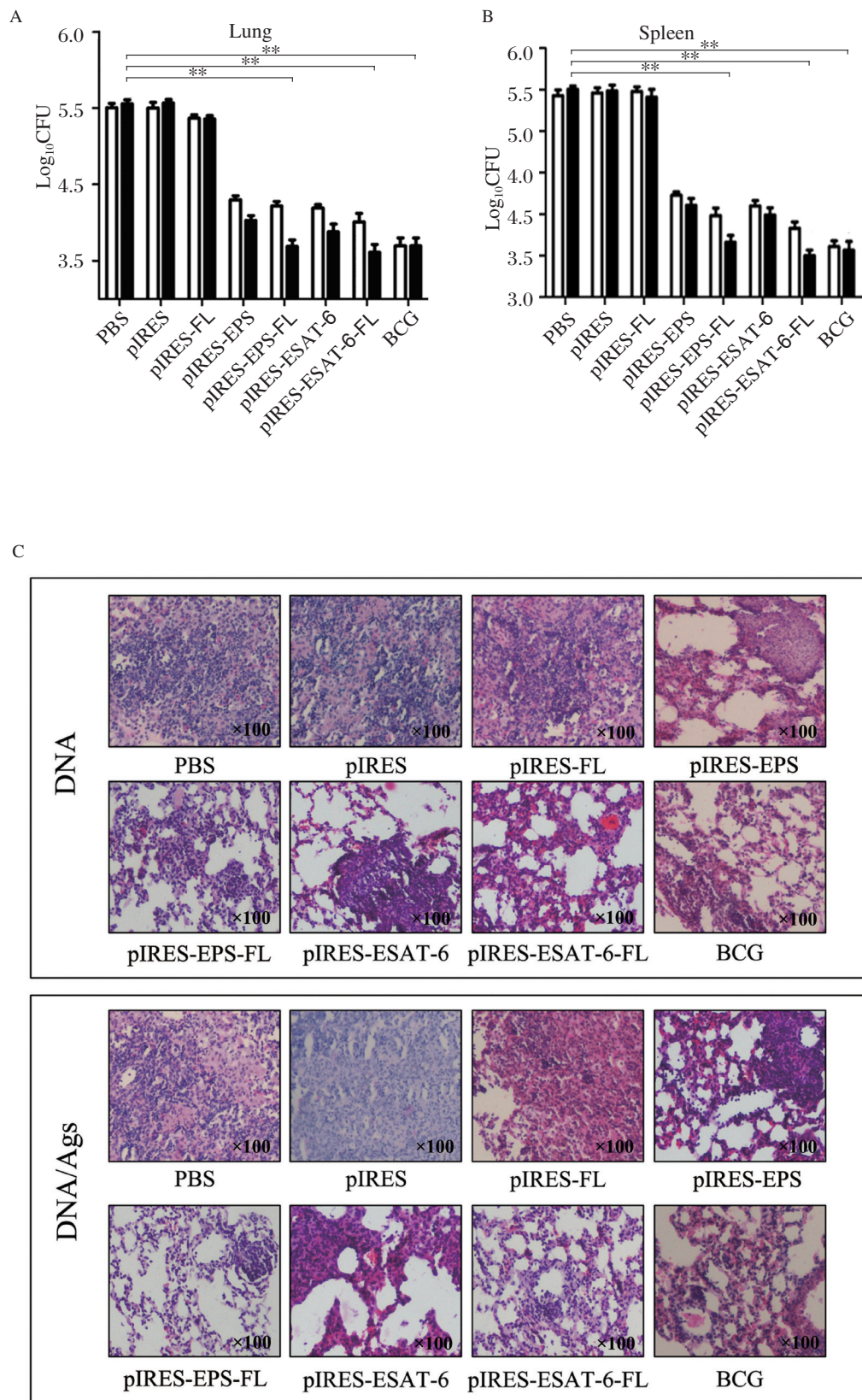Recent studies confirmed that mucosal vaccination could trigger greater protection against *M. tuberculo–*

Fig.7 Protection against M.tb H37Rv infection induced by different plasmid DNA including DNA/Ags vaccina–tion. The different vaccinated mice received an intratracheal 5×10^5 CFU of M.tb H37Rv challenge for 4 weeks, and the bacterial loads in the lungs (A) and spleens (B) of the mice were determined. Data were shown as mean $\pm$ SEM (n = 10), performed in tripli–cate. C: The sections from lung tissues were stained with HE, and evaluated for the lung inflammation (100$\times$). [**]P < 0.01, vs. PBS, pIRES or pIRES-FL group, separately. Other significant differences were displayed in the figures directly (A, B).
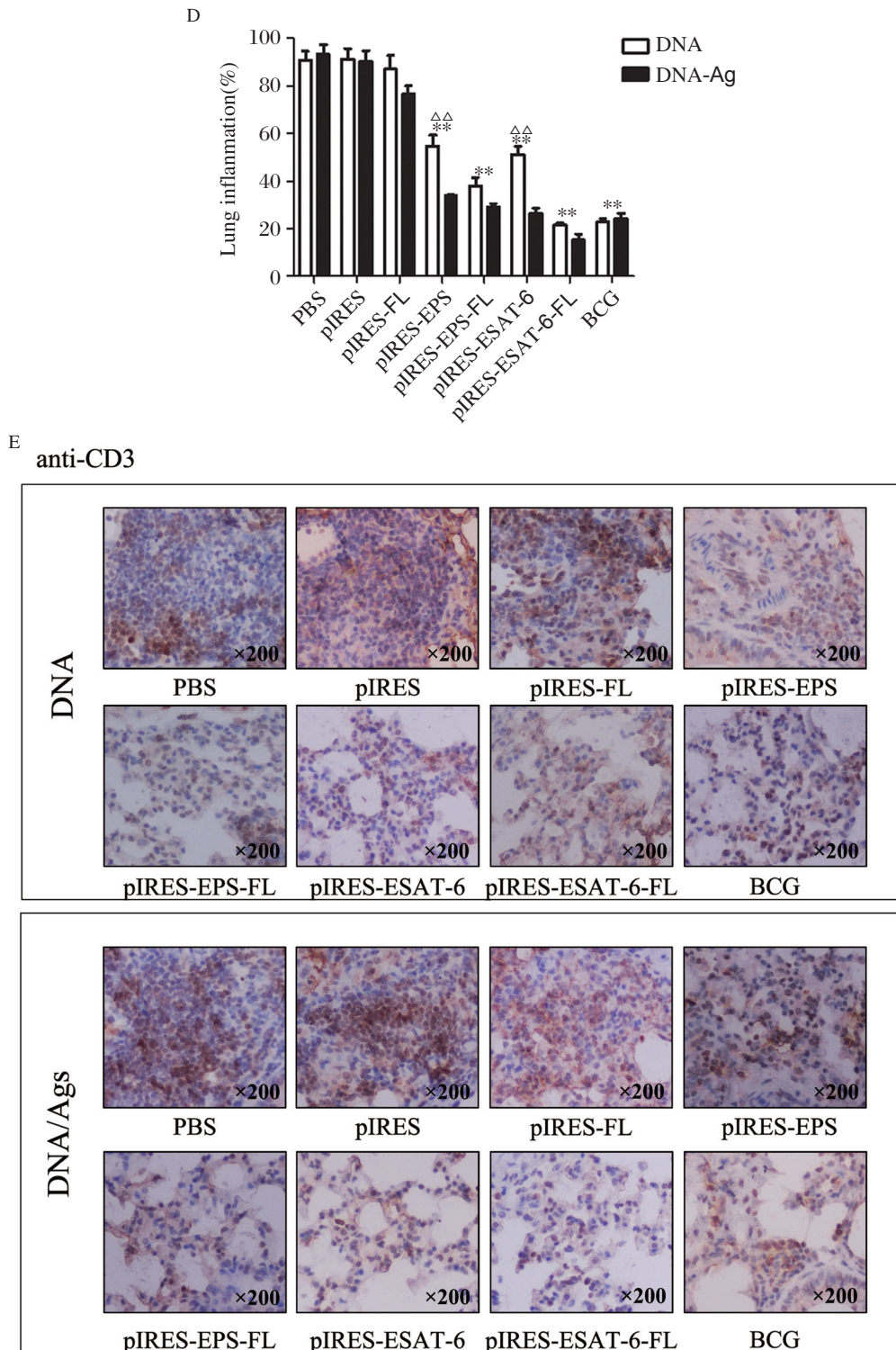
Fig. 7 (continued) Protection against M.tb H37Rv infection induced by different plasmid DNA including DNA/Ags vaccination. D: Representative sections of the lung tissue from mice immunized with different plasmids ($100\times$). E: The infiltration of CD3+ T cells was observed by immunohistochemistry staining so as to evaluate the inflammation response of the lung. **$P < 0.01$, vs. PBS, pIRES or pIRES-FL group, separately; $\triangle\triangle$ $P < 0.01$, indicating DNA/Ags vs. the corresponding DNA group.

sis infection[47-50]. Besides, airway deposition of soluble mycobacterial antigens in previously immunized mice could effectively mobilize systemic T cells into the airway lumen and provide marked immune pro-

tection upon airway *M. tuberculosis* challenge[27]. This phenomenon was also achieved from histological examination in our experiment, such as a large number of CD3 positive T cells and F4/80 positive macro-
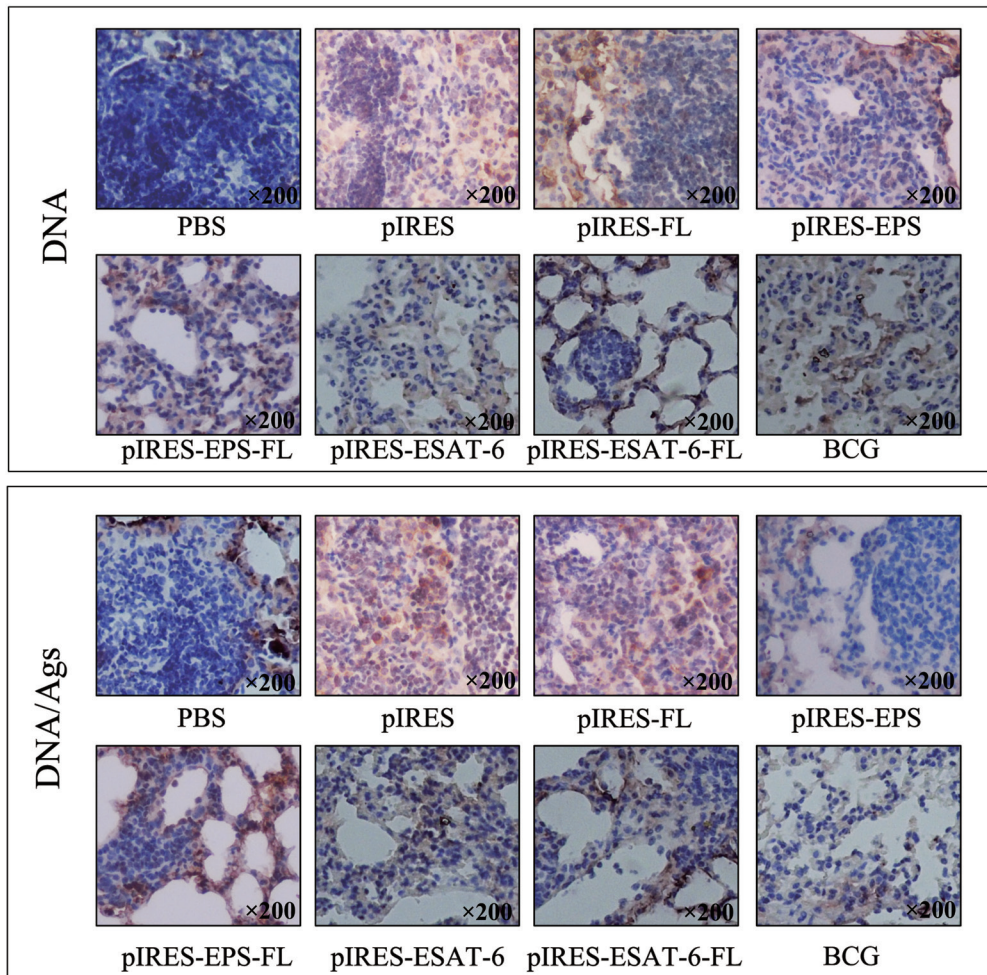
Fig. 7 **(continued) Protection against *Mycobacterium tuberculosis* H37Rv infection induced by different plasmid DNA including DNA/Ag vaccination.** F: The infiltration of F4/80+ macrophages was observed by immunohistochemistry in or– der to evaluate the inflammation response of the lung. Individual experiments were conducted 3 times, with one representation shown (200×).

phages around the bronchioles and vessels of mice treated by prime-boost vaccination.

Our data also showed that mice immunized with pIRES-epitope-peptide-FL plasmids, and then boosted with the epitope-peptides elicited significantly higher levels of IFN-γ and IL-12, which was coupled with a greater number of IFN-γ⁺ T cells and activity of CTLs, compared to mice treated with other plasmids alone. Importantly, better protection against *M. tuberculosis* H37Rv challenge, i.e. reduction of bacterial loads in the lung and spleen, and decreased pulmonary dam– age in the mice, was observed. Overall, these findings indicated that the strategy of priming with the plasmid DNA and boosting with the peptides is very efficient on improving Th1 response and CTL activity as well as immune protection against *M. tuberculosis* infection.

Notably, mice immunized with pIRES-epitope-peptide and pIRES-ESAT-6 plasmids alone or these plasmids plus the epitope-peptide boost or only BCG also exhibited a significant increase of Th1 cytokines, IFN-γ⁺ T cells, CTL activity and protective effects at different extents. However, these parameters from our study were remarkably lower than those of mice treat– ed with pIRES-epitope-peptide-FL and pIRES-ESAT-6-FL immunization, implicating that FL played a syn– ergistic role in inducing T cell response and protection against *M. tuberculosis* infection. Moreover, we noted that mice immunized with BCG could produce higher levels of Th2 cytokines (e.g. IL-4 and IL-10) besides higher levels of IFN-γ and IL-12, which resulted in a lower ratio of IFN-γ/IL-10, and the results are similar to the findings from previous studies[51,52]. Addition– ally, mice treated with BCG showed that the number of IFN-γ⁺ T cells and bacterial loads were increased, and lung injuries were reduced, but these effects were generally less than those of mice vaccinated with

pIRES-epitope-peptide-FL or pIRES-ESAT-6-FL, fur–ther implying that the two plasmid DNA vaccines may be relatively better than BCG. Although DNA vac–cines ESAT-6-epitope-peptides-FL or ESAT-6-FL and BCG also elicited an effective immune response and protective effect, and the efficacy was greater in mice treated with plasmid DNA vaccines, indicating that the DNA vaccine induces more effective protection than BCG. However, because the pIRES-epitope-peptide-FL plasmid was a DNA vaccine encoding ESAT-6 T cell epitopes and FL, and had the same effects as the vaccine containing full length of ESAT-6 plus FL (pIRES-ESAT-6-FL); therefore, the novel recombinant plasmid (pIRES-epitope-peptide-FL) may be a safer and more useful vaccine in the future.

In conclusion, the data from the present study re–vealed that mice vaccinated with the plasmid DNA vaccine (pIRES-epitope-peptide-FL) indeed induced higher CMI than the other DNA vaccines. Mice treated with the DNA vaccine or the strategy of DNA prime and the peptide boost also efficiently led to sig–nificantly augmented protection against *M. tubercu–losis* challenge, suggesting that the novel recombinant plasmid encoding the ESAT-6 T cell epitopes and FL is a useful DNA vaccine for preventing *M. tuberculo–sis* infection.

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