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Immunological Aspects

Prolonged intervals during *Mycobacterium tuberculosis* subunit vaccine boosting contributes to eliciting immunity mediated by central memory-like T cells

Chunxiang Bai[a,b], Juanjuan He[a,b], Hongxia Niu[a,b], Lina Hu[c], Yanping Luo[a,d], Xun Liu[a,b], Liang Peng[e], Bingdong Zhu[a,b,*]

[a] Gansu Provincial Key Laboratory of Evidence Based Medicine and Clinical Translation &Lanzhou Center for Tuberculosis Research, School of Basic Medical Sciences, Lanzhou University, Lanzhou, China
[b] Institute of Pathogen Biology, School of Basic Medical Sciences, Lanzhou University, Lanzhou, China
[c] Lanzhou Institute of Biological Products, Lanzhou, China
[d] Department of Immunology, School of Basic Medical Sciences, Lanzhou University, Lanzhou, China
[e] School of Life Science, Lanzhou University, Lanzhou, China

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ABSTRACT

It is believed that central memory T cells (T_{CM}) provide long-term protection against tuberculosis (TB). However, the effects of TB subunit vaccine immunization schedule, especially the vaccination intervals, on T cell immune memory is still unclear. In this study, mice were immunized with fusion protein ESAT6-Ag85B-MPT64 (190–198)-Mtb8.4-Rv2626c (LT70) based subunit vaccine three times according to the following schedules: ① 0, 3rd and 6th week respectively (0-3-6w), ② 0, 4th and 12th week (0-4-12w), and ③ 0, 4th and 24th week (0-4-24w). We found that both schedules of 0-4-12w and 0-4-24w induced higher level of antigen specific IL-2, IFN-γ and TNF-α than 0-3-6w immunization. Among them, 0-4-12w induced the highest level of IL-2, which is a key cytokine mainly produced by T_{CM}. Moreover, by cultured IFN-γ ELISPOT and cell proliferation assay *etc*., we found that the vaccination schedule of 0-4-12w elicited higher numbers of T_{CM} like cells, stronger T_{CM} – mediated immune responses and higher protective efficacy against *M. bovis* BCG challenge than 0-3-6w did. It suggests that prolonging the vaccination interval of TB subunit vaccine to some extent contributes to inducing more abundant T_{CM} like cells and providing stronger immune protection against mycobacteria infection.

1. Introduction

Up to present, tuberculosis (TB) remains a major health problem worldwide. Attenuated *Mycobacterium bovis* Bacillus Calmette-Guerin (BCG) is the only licensed TB vaccine currently. It could protect children against serious TB but provide variable protection in adult, which waned with time and almost lost protection after 10–20 years [1–3]. It is reported that BCG vaccination mainly induces effector memory T cells (T_{EM}) and effector T cells (Teff) rather than long-lived central memory T cells (T_{CM}) due to persistent bacteria [4–6]. The short-lived T_{EM} confer immediate protection for incoming pathogens but cannot provide a life-long protection, while T_{CM} provide long-term recall responses [7,8]. This may explain why BCG elicits the limited duration of protection [9,10]. Therefore, it is extremely urgent to develop novel vaccines and vaccination strategies to induce sufficient quantity and quality of T_{CM} to provide host long-term protection against *Mycobacterium tuberculosis* (*M. tuberculosis*) [11].

Adjuvanted protein subunit vaccines have the capability to induce T_{CM} and provide long-term protection against *M. tuberculosis* [12–15]. This has been confirmed in several TB subunit vaccines such as H1 [12], H4 [13], H56 [14] and LT70. LT70 is composed of ESAT6, Ag85B, Mtb8.4, 190–198 peptide of MPT64 and Rv2626c, and is developed by our lab [15]. However, there is no investigation on the correlates of vaccination schedule and T cell immune memory, which is to be helpful to optimize TB subunit vaccine immunization schedule to induce ideal immune memory.

Subunit vaccines typically require multiple boosting to confer high quality and quantity of immune memory responses [16]. It is believed that an optimal time for boosting, which should be at the late stages of the effector to memory transition, is critical to induce long term

* Corresponding author. Institute of Pathogen Biology, School of Basic Medical Sciences, Lanzhou University, 199 West Donggang Road, Lanzhou, China. 730000.
E-mail addresses: baichx08@163.com (C. Bai), hejj1991@163.com (J. He), niuhongxia1985@163.com (H. Niu), hln618319@163.com (L. Hu), luoyp@lzu.edu.cn (Y. Luo), liuxun899@163.com (X. Liu), pengliang@lzu.edu.cn (L. Peng), bdzhu@lzu.edu.cn (B. Zhu).

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immune memory [7]. A short interval between the prime and boost might induce more effector T cells and less memory T cells. In HPV infection, the incidence rate ratios of condyloma were lower in an two-dose schedule with vaccination interval of 4–7 months than that in an interval of 3 months [17]. As for DTPa-HBV-IPV/Hib vaccine, the immunization schedule of 3-, 5-, and 11-month after birth had a higher level of mean anti-HBV geometric concentrations than that of 2-, 3-, and 4-month after birth [18]. These researches in HPV and HBV vaccines indicated that prolonged vaccination intervals to some extent could elicit long-lived immune memory.

As for TB adjuvanted protein-subunit vaccines, most studies traditionally applied a vaccination schedule of 3 doses at 2-week or 3-week intervals [19,20]. However, according to the principle of memory T cell development, an interval of at least 2–3 months for boosting time should be reasonable to induce more amount of T_{CM} rather than T_{EM} [7]. Therefore, it is necessary to investigate the correlates of immune memory and vaccination schedules of TB subunit vaccine. In this study, applying *M. tuberculosis* subunit vaccine LT70 [15], we explored the immune responses of three different vaccination schedules of 0-3-6w, 0-4-12w and 0-4-24w. Our study showed that the schedule of 0-4-12w tended to induce long term T_{CM} like immune responses.

2. Materials and methods

2.1. Animals

Female C57BL/6 mice aged 6–8 week were purchased from animal center of Lanzhou University (Lanzhou, China). All mice were maintained in special pathogen-free conditions in School of Basic Medical Sciences, Lanzhou University. Animals received free access to water and standard mouse chow throughout the study. Animal experiments were performed in compliance with the guidelines of China Council on Animal Care and Use.

2.2. Subunit vaccines and mycobacterial antigen preparation

The fusion protein LT70 was prepared as previously reported [15] and was suspended in phosphate-buffered saline (PBS) (0.2 mg/ml). The purified LT70 (10 μg/dose) was emulsified in an adjuvant being composed of N, *N′*-dimethyl-N, *N′*-dioctadecylammonium bromide (DDA) (250 μg/dose) and polyinosinic-polycytidylic acid [Poly (I:C)] (50 μg/dose) to construct subunit vaccine. Single mycobacterial protein Ag85B, ESAT6 and Rv2626c were purified by Ni-NTA His column (Novagen) as previously described [21].

2.3. Vaccine immunization

Mice were divided into five groups and were immunized according to the following schedules: those receiving PBS; those receiving 5×10^5 CFU of BCG Danish 1331; those receiving LT70 at 0, 4th and 24th week respectively; those receiving LT70 at 0, 4th and 12th week respectively; those receiving LT70 at 0, 3rd and 6th week respectively. All the vaccines were given subcutaneously in a total volume of 200 μl/mice. Vaccines immunization of different groups started at different time, and the last immunization time of each group was consistent.

2.4. Enzyme-linked immunosorbent assay (ELISA) for IL-2 and IFN-γ production

Spleens of mice were aseptically removed and gently ground through a 200-μm nylon cell strainer. Then single-cell suspensions were prepared with Lympholyte-M density gradient centrifugation (Dakewe Biotech Company Ltd., Shenzhen, China). Freshly isolated lymphocytes were plated in duplicate in 24-well plates at 2.5×10^6 cells per well in 500 μl of RPMI 1640 (HyClone, USA) supplemented with 100 U/ml penicillin-streptomycin and 10% fetal calf serum (Sangon Biotech, Shanghai, China) and stimulated with Ag85B (5 μg/ml) for 68 h at 37 °C, 5% CO_2. After that, cell culture supernatant was harvested from lymphocyte cultures and the concentrations of interleukin-2 (IL-2) and interferon-γ (IFN-γ) in culture supernatant were detected by ELISA as described previously [22].

2.5. Flow cytometry for intracellular cytokine (ICC) analysis

Intracellular cytokine staining of T cells was done as described previously [23]. Briefly, lymphocytes were isolated and stimulated with Ag85B (5 μg/ml) for 4 h and subsequently incubated for 5–6 h with BD GolgiPlug™ (containing brefeldin A) at 37 °C. Samples were stained with anti-CD4-FITC (RM4-5) and anti-CD8-PerCP-Cy5.5 (53–6.7). Subsequently cells were permeabilized using the BD Cytofix/Cytoperm kit according to the manufacturer's instructions and stained with anti-IFN-γ (XMG1.2), anti-TNF-α-PE-Cy7 (MP6-XT22) and anti-IL-2-PE (JES6-5H4). All samples were run on a BD FACS Cantoflow cytometer or ACEA NoveCyte. The results were obtained by analysis in NovoExpress software. All Abs were from BD Pharmingen unless noted.

2.6. Cultured IFN-γ ELISPOT assay in vitro

Lymphocytes were suspended in RPMI-1640 medium supplemented with 100 U/ml penicillin-streptomycin, 2 mM L-glutamine, 25 mM HEPES buffer, 1% sodium pyruvate, 50 mM 2-mercaptoethanol and 10% (v/v) fetal calf serum. Cells (2×10^6 cells/ml/well) were plated in quadruplicate for each animal in a 24-well plate and stimulated with a cocktail of ESAT-6, Ag85B, Rv2626c (2 μg/ml of each protein) and PPD (4 μg/ml). Cells were incubated at 37 °C and 5% CO_2 with half culture media containing IL-2 100 U/ml, which were replaced at days 3 and 7, allowing expansion of antigen-specific T cells [24]. At day 9, long-term cultured cells were harvested and antigen presenting cells (APCs) were isolated by adherence incubating freshly lymphocytes in complete medium at 37 °C, 5% CO_2 for 2 h. After this initial culture period, cultured cells were plated (1×10^6 cells/well) and re-stimulated for an additional 20 h in the presence of APCs (1.5×10^5 cells/well) in anti-IFN-γ coated ELISPOT plates (Dakewe Biotech Company Limited, Shenzhen, China). Then the standard ELISPOT assay protocol was performed as previously described. The spot forming cells (SCFs) were counted by an ELISPOT reader (Bio-sys, GmbH, Karben, Germany) [22,25].

2.7. IFN-γ secretion following twice-stimulation with antigens

In cultured IFN-γ ELISPOT assay, those T cell populations were expanded *in vitro*. Furthermore, according to the principle of long-term cultured ELISPOT assay, we set up a novel method to detect T_{CM} like cells through stimulating T cells twice, first *in vivo* and then *in vitro*. First, at 16 weeks after the final immunization, mice were treated with BCG (1×10^6 CFU/mice) by intraperitoneal injection (*i.p.*). It is supposed that the central memory T cells would consequently be activated and developed into effector memory T cells or effector T cells *in vivo*. Then, lymphocytes were isolated at 9 days later and stimulated for 4 h with mixed antigens of ESAT-6, Ag85B, Rv2626c (2 μg/ml of each protein) and PPD (4 μg/ml) *in vitro*, during that time the T_{EM} would develop into Teff and secrete cytokine IFN-γ. Intracellular cytokine (ICC) analysis were performed to analyze the IFN-γ secretion in T cells in accordance with the method mentioned above.

2.8. EdU incorporation and proliferation assay

For EdU incorporation experiments, lymphocytes (5×10^6 cells/well) were stimulated with PPD (5 μg/ml) for 7 days in 24-well plates. Three days after antigen stimulation, EdU (Click-iT™ EdU Flow Cytometry Assay Kit, Invitrogen™, OR, USA) was added at a final concentration of 30 μM and continued to culture for 4 days [26]. At the 7th