Osong Public Health Res Perspect 2012 3(2), 68–73
doi:10.1016/j.phrp.2012.01.008
pISSN 2210-9099 eISSN 2233-6052

ORIGINAL ARTICLE

Effect of Maternal Immune Status on Responsiveness of Bacillus Calmette-Guérin Vaccination in Mouse Neonates

Jong Su Choi [a,1], Ryang Yeo Kim [a,b,1], Semi Rho [a], Fanny Ewann [b], Nathalie Mielcarek [a,c], Man Ki Song [a], Cecil Czerkinsky [a], Jae-Ouk Kim [a,*]

[a]*Laboratory Science Division, International Vaccine Institute, Seoul, Korea.*
[b]*Institut Pasteur Korea, Soengnam, Korea.*
[c]*Inserm, Lille, France.*

Received: January 10, 2012
Revised: January 17, 2012
Accepted: January 20, 2012

KEYWORDS:
BCG,
maternal effect,
Mycobacterium tuberculosis,
neonates,
tuberculosis

Abstract
Objectives: Bacillus Calmette-Guérin (BCG) vaccination has proven to be efficient in immunologically naïve infants; however, it has not been investigated that maternal natural exposure to *Mycobacterium* and/or BCG vaccine could influence the characteristics of immune responses to BCG in newborns. In this study, we analyzed whether the maternal immune status to *M tuberculosis (M tb)* can affect neonatal immunity to BCG using a mouse model.
Methods: Neonates were obtained from mice that were previously exposed to live BCG, to live *M avium*, or to heat-killed *M tb* H37Rv, and from naïve control mothers. One week after birth, the neonates were divided into two subgroups: one group immunized with live BCG via the subcutaneous route and the other group of neonates sham-treated. Interferon-gamma (IFNγ) secretion in response to *in vitro* stimulation with heat-killed BCG or purified protein derivative (PPD) was examined. Protection against *M tb* infection was evaluated by challenging mice nasally with live *M tb* H37Rv followed by counting colonies from spleen and lung homogenates.
Results: BCG-immunized neonates showed increased IFNγ secretion in response to heat-killed BCG or PPD. All mice in BCG-immunized neonates subgroups showed reduced bacterial burden (colony forming unit) in the lungs when compared with control naive neonate mice. However, no statistically significant difference was observed when comparing BCG-immunized mice born from mothers previously exposed to *M avium* or immunized with either heat-killed H37Rv or live BCG and mice born from naïve mothers.
Conclusion: The maternal immune status to *M tb* does not appear to impact on the immunogenicity of BCG vaccine in their progeny in our experimental conditions.

*Corresponding author.
E-mail: jokim@ivi.int

[1]These authors contributed equally to this work.

1. Introduction

Mycobacterium tuberculosis (*M tb*) is an intracellular pathogen that causes severe infectious disease, tuberculosis (TB), which is responsible for high level of mortality and morbidity [1−3]. About 30% of the population is worldwide infected with this infectious agent [1]. Most of them are considered to have latent tuberculosis infection (LTBI) and only 10% of the infection will develop to serious pulmonary TB [2,4]. Nevertheless, new cases of this bacterial infection are still occurring mostly in developing countries, and immunologically immature infants in these regions can be easily exposed to infections with mycobacteria [5].

To prevent from TB infection, Bacillus of Calmette and Guérin (BCG) obtained from a live attenuated strain of *M bovis* (*M bovis*) has been used as an only authorized vaccine since 1921 [6]. Previous study showed outstanding effectiveness of BCG vaccination at birth in control of neonatal miliary TB and meningitis TB as well [7]. However, the vaccination with BCG shows inconsistent responsiveness and relatively low protection efficacy against predominant pulmonary TB in adults [8]. In animal and human studies, neonatal immunization with BCG showed relatively higher protection efficacy than that in adults, which is mediated by Th1-type immune responses [9,10].

Guirado and colleagues [11] reported that antibodies play an essential protective role against mycobacterial infections by passive immunization using sera obtained from mice treated with detoxified *M tb* extracts in B cell-deficient mice, while it is generally accepted that cell-mediated immune response is important in controlling *M tb* infections. While current BCG vaccine has proven to be efficient in immunologically naïve infants, a maternal history of natural exposure to mycobacteria or vaccination against TB has not been studied yet. Therefore, we hypothesized that maternal immune status to mycobacteria may play a critical role in the uptake and subsequent protective immunogenicity of BCG in newborns. In the present study, we analyzed whether the maternal immune status to *M tb* can affect the neonatal immunity to BCG in mouse neonates by assessing *in vitro* IFNγ secretion by stimulated spleen cells [11−13] and by evaluating the protective efficacy after challenge experiments. We found that neonatal BCG immunization can reduce the bacterial load in pulmonary tissues. In addition, we showed that the maternal immune history mediated by either vaccination or pre-exposure to *M tb* was insufficient to elicit the specific immune responses to BCG vaccine in their progeny. This may provide basic insights into importance of immunogenicity through neonatal vaccination and/or maternal vaccination, and it can be considered for further TB vaccine researches.

2. Materials and Methods

2.1. Animals

Six-weeks old female Balb/c mice (OrientBio or Daehan Biolink, Korea) were maintained under specific pathogen-free conditions in the animal facility, International Vaccine Institute (Seoul, Korea), where they were fed with sterilized food and water *ad libitum*. Breeding cages were checked daily for new births, and the pups were kept with the mother until weaning at 3 weeks of age. Animal infections with *M tb* were performed at the Biosafety Level 3 facility of the institute. All experiments described were approved by appropriate institutional animal care and use committees.

2.2. Bacteria

M bovis BCG Pasteur (BCG) [14], *M tuberculosis* H37Rv (*M tb* H37Rv) [15] and *M avium subsp. Hominissuis* (*M avium)*[16] were cultured in suspension in 7H9 Middlebrook media (BD Bioscience, San Diego, CA, USA) supplemented with 10% OADC (v/v, BD, Franklin Lakes, NJ, USA) and 0.05% Tween80.

2.3. Vaccination of mice

One week before mating, mice were immunized with live BCG [subcutaneously, 5×10^5 colony forming units [CFU]), live *M avium* (intranasally, 1×10^5 CFU), or heat-killed *M tb* H37Rv (intranasally, 5×10^5 CFU), respectively. Neonates obtained from individual mothers were divided into two subgroups and vaccinated 1 week after birth: one group immunized with live BCG (5×10^5 CFU) via subcutaneous route and the other group of neonates sham-treated.

2.4. Antibody determination

Four weeks after BCG vaccination, immunoglobulin G (IgG) level in serum obtained from individual mice were analyzed by enzyme-linked immunosorbent assay (ELISA). Briefly, ELISA plates were coated with heat-inactivated BCG (5×10^5 CFU/mL) in phosphate buffered saline (PBS) overnight at 4 °C. Next day, plates were washed four times with PBS-T (v/v, 0.05% Tween20, Sigma-Aldrich, St. Louis, MO, USA) and blocked with 1% bovine serum albumin (BSA) (Sigma-Aldrich, St. Louis, MO, USA) for 1 hour at room temperature. After washings, the samples were applied in serial dilutions and subsequently incubated for 2 hours at room temperature. Following extensive washings with PBS-T, horseradish peroxidase-conjugated goat anti-mouse IgG (Southern Biotech, Birmingham, AL, USA) was diluted in 1% BSA and applied. After 1-hour incubation at room temperature, the plates were washed four times, and the 3,3′, 5,5″-tetramethylbenzidine (TMB) peroxidase substrate (Moss, Inc., Pasadena, MD, USA) was subsequently added to develop the enzymatic color reaction. Optical density was detected

by using a microplate reader (molecular device, Sunnyvale, CA, USA) at 450 nm, and concentrations were given in inverse $\log_2$ at OD of 0.2.

2.5. M tb challenge of mice

Infection with *M tb* H37Rv was performed 4 weeks after BCG immunization via intranasal route with an inoculum of 200 CFU/20 μl per head. The mice were sacrificed four weeks after challenge infection, and bacterial loads in lung and spleen were determined by incubating serial dilutions of lung and spleen homogenates from individual mice on 7H11 Middlebrook agar plates. The 7H11 Middlebrook agar plates were freshly prepared with 100 μg/mL of ampicillin (Sigma-Aldrich, St. Louis, MO, USA) and 10 μg/mL of fungizone (Gibco, Grand Island, NY, USA). The plates were incubated for 3−4 weeks at 37 °C with 5% CO_2, and the numbers of bacterial colonies were counted.

2.6. Measurement of *in vitro* IFN γ production

Specific IFNγ response against heat-inactivated BCG (10 minutes at 95 °C) or purified protein derivative (PPD) (kindly provided by Institut Pasteur Korea) was measured by ELISA. It was detected after *in vitro* stimulation of splenocytes and lung cells obtained from individual mice at 3 weeks or 4 weeks after neonatal vaccination with BCG, respectively. Lung (5×10^5 cells/well) and/or spleen cells (2×10^5 cells/well) were obtained from individual mice and stimulated with either heat-killed BCG (5×10^5 CFU/mL) or PPD (5 μg/mL) for 72 hours at 37 °C with 5% CO_2. Following incubation, supernatants were harvested and stored at −80 °C until use. IFNγ level was determined by using mouse BD OptEIA IFNγ ELISA kit (BD Bioscience, San Diego, CA, USA) according to manufacturer's instructions.

2.7. Statistical analysis

All statistical analyses using the unpaired Student t-test were performed in GraphPad Prism 5 (GraphPad Software Inc., La Jolla, IL, USA). A $p < 0.05$ value was considered statistically significant.

3. Results

3.1. Determination of optimal subcutaneous BCG inoculum

Although T cell-mediated immunity plays critical roles in protection against *M tb* infection, antigen-specific antibodies induced by successful vaccination are thought to be also involved in modifying the course of *M tb* infection. We first assessed humoral immune response in response to BCG vaccination. Different doses of live BCG, 1×10^2, 5×10^2, 1×10^4, and 5×10^5 CFU were subcutaneously administered into 1-week-old neonate mice and specific anti-BCG IgG titer in serum was measured after 3 weeks of vaccination. Anti-BCG IgG production was detected solely in mice vaccinated with 5×10^5 CFU of live BCG; this dose was therefore used as a standard for further immunizations in this study (Figure 1).

3.2. Impact of maternal immune status on *in vitro* IFNγ production in neonates after BCG immunization

We analyzed cellular immune responses of neonates following BCG vaccination by measuring *in vitro* IFNγ secretion in response to BCG or PPD, since it has been described that protective immune responses to *M tb* infection is mainly mediated by T-cell immunity.

First, to compare different maternal immune backgrounds, 6-week-old female mice were administered with either live BCG subcutaneously, heat-inactivated *M tb* H37Rv intranasally, live *M avium* intranasally, or sham-treated, respectively. Mating occurred 1 week later. Neonate mice born from mothers previously exposed to above described mycobacteria were divided into two subgroups; one group consisting of neonates vaccinated with BCG at the age of 7 days and the other group of neonates sham-treated (Figure 2). After 3 weeks, spleen and lung cells from naïve and BCG immunized neonates group born from either naïve or BCG immunized mothers were analyzed for IFNγ

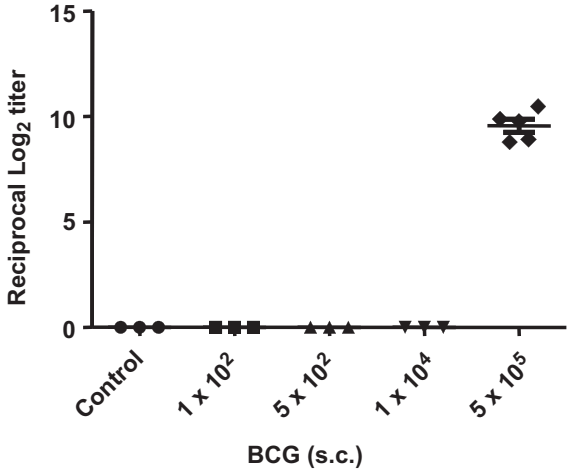

Figure 1. IgG level following vaccination with BCG. To determine the optimum dose of BCG vaccine, we first quantified the IgG level in serum in response to BCG vaccine by enzyme-linked immunosorbent assay. One week after birth, neonates ($n = 4$ to 6 heads per each group) were subcutaneously primed with different doses of live BCG. Three weeks after the BCG-priming, specific anti-BCG IgG level was measured. Anti-BCG IgG production was detected solely in mice vaccinated with 5×10^5 CFU of live BCG, Representative data are shown as reciprocal $\log_2$ Immunoglobulin G titer. Data are mean ± SD. BCG = Bacillus Calmette-Guérin; CFU = colony forming unit; IgG = immunoglobulin G; SC = subcutaneous; SD = standard deviation.

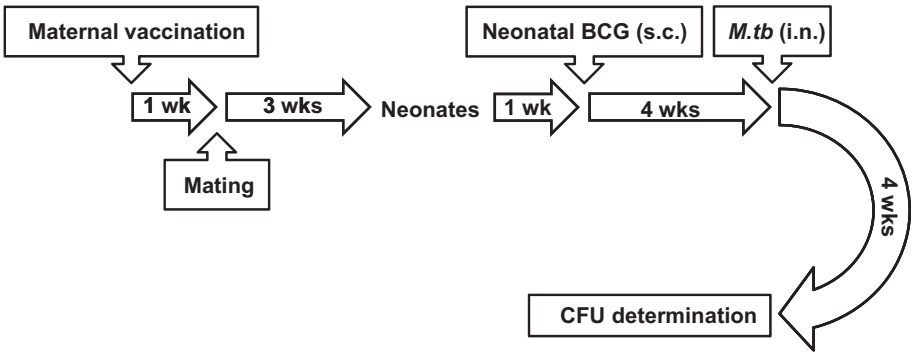

Figure 2. Schedule for *M tb* study. For this study, female mice were subcutaneously primed with live BCG (5×10^5 CFU) or heat-inactivated *M tuberculosis* H37Rv (5×10^5 CFU), respectively, while live *M avium* (1×10^5 CFU) was administered via intranasal route. The female mice were mated with naïve males a week later. When newborns obtained from each female mouse were 1-week old, they were divided into two subgroups; naïve and the BCG-vaccinated. For subsequent neonatal BCG vaccination, live BCG (5×10^5 CFU) was given subcutaneously. Three weeks after the BCG vaccination, IFNγ production by splenocytes and lung cells was analyzed after *in vitro* stimulation with heat-inactivated BCG. Four weeks after BCG vaccination, the neonates were intranasally challenged with 5×10^4 CFU of virulent *M tuberculosis* H37Rv. The mice infected with *M tb* were kept for 4 weeks at the ABSL-3 facility, until sacrifice. Bacterial loads (CFU) in lung and spleen were subsequently quantified. BCG = Bacillus Calmette-Guérin; CFU = colony forming unit; i.n. = intranasal.

secretion in response to BCG. No statistically significant difference was observed when comparing subgroups of BCG-immunized mice born from mothers previously immunized with BCG or from naive mothers in both tissues (Figure 3A and B).

Next, we examined splenic IFNγ secretion in response to PPD (Figure 3C). Statistically significant difference was detected only between BCG immunized progeny born from naïve mothers and from BCG immunized mothers (Figure 3). These results suggest that neonatal BCG vaccination can elicit cellular immune response regardless of maternal pre-exposure to mycobacteria; however, maternal pre-exposure to BCG can increase PPD-specific IFNγ production in their progeny.

3.3. Influence of maternal immunity on protective efficacy of neonatal BCG vaccination

We thereby performed *M tb* challenge experiment in order to see whether different maternal immune status can affect BCG vaccine efficacy in their progeny. Intranasal *M tb* H37Rv challenge was performed after 4 weeks of BCG vaccination, and bacterial numbers were determined from lung and spleen of individual mice after 4 weeks of challenge (Figure 2).

When compared with nonvaccinated neonates, neonates mice administered with BCG exhibit a significant decrease of the bacterial loads in the lung following *M tb* infection; however, there was no statistical significance between BCG-immunized mice born from female mice previously exposed to either heat-killed *M tb* H37Rv, live *M avium*, or live BCG, or naïve mothers. These results suggest that maternal exposure to different *M tb*-related antigens does not have impacts on neonatal immunogenicity induced by BCG vaccination. No statistically significant reduction of bacterial burden in the spleen was observed in any of the groups. These results suggest that the impact of maternal immunity on the protective immunity induced by neonatal vaccination with BCG is minimum.

4. Discussion

In this study, the effect of maternal pre-exposure to mycobacteria has been investigated on BCG vaccine efficacy in neonates using a mouse model. We found that maternal immune status to mycobacteria does not impact on the protective immunogenicity of BCG vaccine in their progeny under our experimental conditions.

The main component of immune protection transferred from mother to child is antibody. Maternal antibody is transferred to the fetus and newborns across the placenta using the neonatal Fc receptor and via breast milk, respectively [17]. Unlike pigs, horses and ruminants, which rely mainly on colostral transfer, mice allow placental transfer of maternal immunoglobulin. Therefore, neonatal mice can be applied as an alternative to human neonatal vaccination [17].

To generate enough quantity of antibodies, we first determined the optimal subcutaneous BCG inoculums in neonate and used the 5×10^5 CFU as a standard dose for immunization (Figure 1). BCG-immunized neonates showed increased IFNγ secretion in response to BCG or PPD. *In vitro* IFNγ secretion in response to PPD from splenocytes of BCG-immunized neonates born to BCG vaccinated mother is higher than BCG-immunized neonates born to naïve mothers. This statistically significant difference might indicate that maternal pre-exposure to BCG influences the neonates'

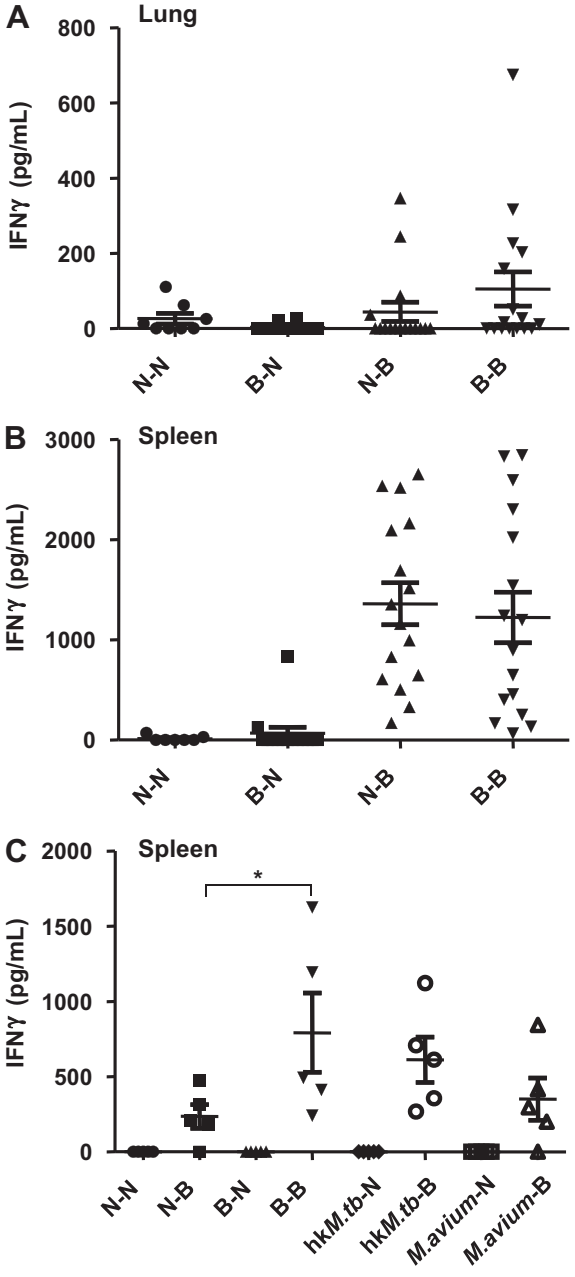

Figure 3. Impact of maternal immune status on neonatal IFNγ production in response to BCG. Specific IFNγ responses against heat-inactivated BCG (A and B) or PPD (C) were detected by *in vitro* stimulation using splenocytes and lung cells obtained from individual mice ($n = 8$ to 16 heads per each group) either at 3 weeks or 4 weeks post neonatal vaccination with BCG, respectively. There was no statistically significant difference between groups. Data are mean ± SD. *$p < 0.05$ (Student's *t*-test). B = Bacillus Calmette-Guérin (BCG)-vaccinated; hk *M tb* = heat-inactivated *M tb* H37Rv-administered; *M avium* = live *M avium*-administered; N = naïve; SD = standard deviation.

responsiveness to PPD by an immune mechanism still unknown. No statistical difference in protective efficacy against *M tb* infections was observed suggesting that the increased IFNγ production by PPD-stimulated cells was not sufficient to prevent the infection.

Protection against *M tb* infection was evaluated after intranasal challenge with *M tb* H37Rv. BCG-immunized mice were protected when compared to naive mice. However, no statistically significant difference was observed between BCG -immunized mice born from mothers previously exposed/immunized with heat-killed *M tb* H37Rv, BCG or *M avium* and mice born from naive mothers (Figure 4).

In humans, multiple factors, for instance, environmental and genetic background, nutritional and socio-economic status, may affect the BCG efficacy [18,19]. Successful neonatal immunity achieved by vaccination can effectively protect the high-risk population from *M tb* infections. This study showed that the efficacy of

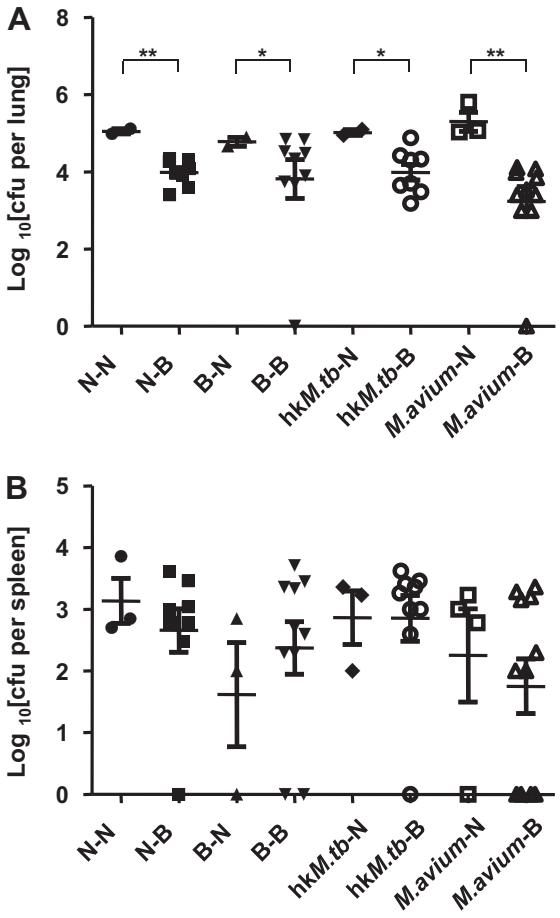

Figure 4. Bacterial burden in lung and spleen. Bacterial numbers (CFUs) were determined 4 weeks after challenge with virulent *M tb* H37Rv in lung and spleen ($n = 3$ to 11 heads per each group) of mice (Balb/c) primed with BCG vaccine Data are shown as $\log_{10}$ CFU. Data are mean ± SD. *$p < 0.05$ (Student's *t*-test) compared with the naïve and BCG-vaccinated neonates of each subgroup. **$p < 0.01$. B = Bacillus Calmette-Guérin (BCG)-vaccinated; CFU = colony forming unit; hk *M tb* = heat-inactivated *M tb* H37Rv-administered; *M avium* = live *M avium*-administered; N = naïve; SD = standard deviation.

neonatal vaccination with BCG or with recently developed new BCG-based live vaccine against tuberculosis might not be affected by maternal immunity transferred to newborns in regions endemic for *M tb* or *M avium*.

Acknowledgements

This research was supported by a grant from National Research Foundation of Korea for EU-FP collaboration program, NEWTBVAC (No.KE1002001752-11B1300-03810). The International Vaccine Institute is supported by the governments of Korea, Sweden, and the Netherlands.

References

1. Organization, W.H. WHO report: Global tuberculosis control 2011. Geneva: WHO; 2011.
2. Prevention, C.f.D.C.a. Tuberculosis. Available from: http://www.cdc.gov/tb/; 2012. [date accessed 24 February 2012].
3. Dye C. The global epidemiology of tuberculosis. Lancet 2006 Mar 18;367(9514):938−40.
4. Prevention, C.f.D.C.a. National action plan to combat multidrug-resistant tuberculosis. CDC; 1992.
5. World Health Organization. Position paper on BCG vaccination. Geneva: WHO; 2004.
6. Rodrigues LC, Wheeler JG. Protective effect of BCG against tuberculous meningitis and miliary tuberculosis: a meta-analysis. Int J Epidemiol 1993 Dec;22(6):1154−8.
7. Fine PE, Milstein J, Clements C. Issues relating to the use of BCG in immunization programs. Geneva: WHO; 1999.
8. Fine PE. Variation in protection by BCG: implications of and for heterologous immunity. Lancet 1995 Nov 18;346(8986): 1339−45.
9. Marchant A, Ota MO, Wolfe I, et al. Newborns develop a Th-1-type immune response to Mycobacterium bovis bacillus Calmette-Guérin vaccination. J Immunol 1999 Aug 15;163(4):2249−55.
10. Soares AP, Joseph S, Harbacheuski R, et al. Bacillus Calmette-Guerin vaccination of human newborns induces T cells with complex cytokine and phenotypic profiles. J Immunol 2008 Mar 1; 180(5):3569−77.
11. Guirado E, Amat I, Gil O, et al. Passive serum therapy with polyclonal antibodies against Mycobacterium tuberculosis protects against post-chemotherapy relapse of tuberculosis infection in SCID mice. Microbes Infect 2006 Apr;8(5):1252−9.
12. Cooper AM, Stewart TA, Griffin JP, et al. Disseminated tuberculosis in IFN-γ gene disrupted mice. J Exp Med 1993 Dec 1; 178(6):2243−7.
13. Bonecini-Almeida MG, Boutsikakis I, Geng J, et al. Induction of in vitro human macrophage anti-Mycobacterium tuberculosis activity: requirement for IFN-gamma and primed lymphocytes. J Immunol 1998 May 1;160(9):4490−9.
14. Ewann F, Jackson M, Pethe K, et al. Transient requirement of the PrrA-PrrB two-component system for early intracellular multiplication of Mycobacterium tuberculosis. Infect Immun 2002 May;70(5):2256−63.
15. Cole ST, Brosch R, Parkhill J, et al. Deciphering the biology of Mycobacterium tuberculosis from the complete genome sequence. Nature 1998 Jun 11;393(6685):537−44.
16. Mijs W, de Haas P, Rossau R, et al. Molecular evidence to support a proposal to reserve the designation Mycobacterium avium subsp. avium for bird-type isolates and 'M. avium subsp. hominissuis' for the human/porcine type of M. avium. Int J Syst Evol Microbiol 2002 Sep;52(Pt 5):1505−18.
17. Demirjian A, Levy O. Safety and efficacy of neonatal vaccination. Eur J Immunol 2009 Jan;39(1):36−46.
18. Dupuis S, Picard C, Fieschi C, et al. Human interferon-gamma-mediated immunity is a genetically controlled continuous trait that determines the outcome of mycobacterial invasion. Immunol Rev. 2000 Dec;178(1):129−37.
19. Dai G, McMurray DN. Nutritional modulation of host responses to mycobacteria. Front Biosci 1998 Jul 20;3:110−22.