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Neonatal BCG vaccination induces IL-10 production by $CD4^+$ $CD25^+$ T cells

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To determine the optimal time of Bacillus Calmette–Guerin (BCG) vaccination for induction of Th1 immunity, we measured the interferon (IFN)-γ and interleukin (IL)-10 secretion in purified protein derivative (PPD)-stimulated peripheral blood mononuclear cell (PBMC) cultures in newborns vaccinated at birth or 2nd month of life. Moreover, role of $CD4^+$ $CD25^+$ T cells was studied by depletion assay at 8th month. Nineteen term and healthy newborns were randomized into two groups: Group I composed of 10 newborns vaccinated with BCG at birth and the remaining 9 (group II) at 2nd month of life. PBMCs were isolated at birth, 2nd and 8th months of age, and PPD-stimulated IL-10, 5 and IFN-γ secretion were assessed. The same measurements were repeated for IL-10 and IFN-γ after the depletion of $CD4^+$ $CD25^+$ T cells at the 8th month. Children vaccinated at birth demonstrated higher PPD-stimulated IFN-γ and IL-10 levels at 2 months of age when compared to non-vaccinated ones ($p = 0.038$ and $p = 0.022$, respectively), whereas at 8 months, no significant differences were detected between the two groups. Moreover, $CD4^+$ $CD25^+$-depleted T-cell cultures resulted in lower PPD-stimulated IL-10 levels in those vaccinated at birth when compared to non-depleted condition at the 8th month ($p < 0.001$). BCG at birth upregulated PPD-stimulated IFN-γ secretion at the 2nd month and remained still detectable at 8 month after the vaccination, whereas those vaccinated at the 2nd month of life lacked that increase in IFN-γ response at the same time-point. Furthermore, depletion assays suggest that $CD4^+$ $CD25^+$ T cells are involved in PPD-stimulated IL-10 secretion in response to BCG vaccination.

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World Health Organization (WHO) (1) recommends the administration of Bacillus Calmette–Guerin (BCG) as soon as possible after birth in developing countries. In a review, Milstien et al. (2) concluded that the efficacy of modern BCG vaccines was within the range of 60–90% for disseminated tuberculosis or meningitis in young children but somewhat lower for other forms of primary tuberculosis infection. Another study in Bangkok found an adjusted protective effect for neonatal BCG vaccination around 83% (3). Several trials have shown that efficacy of BCG was highest among the youngest recipients and decreased with increasing age of vaccination (4). In a recent study, Soysal et al. (5) reported that BCG vaccine exerted a protective effect not only on dissemination of disease but also on infection of exposed children to household contacts. They also showed that having a BCG scar itself was associated with a 24% reduction in the risk of development of primary infection (6).

In some developing countries, BCG vaccination is delayed till the second month of life based on the previous studies reporting that neonatal vaccination resulted in a lower purified protein derivatives (PPD) skin response compared to vaccination at 3 months (7, 8). An important issue in this respect is that the optimal time for the induction of immunologic protection is still unknown. Another obstacle that needs to be

faced is the differentiation of mycobacterial infection vs. appropriate response to the vaccine in the presence of a positive PPD response. Considering all those facts, in newborns who have the highest risk of the development of complications and sequelae owing to disseminated tuberculosis and meningitis, it is vital that they should receive BCG vaccination at the earliest time in life.

To strengthen this clinical observation, we evaluated cytokine responses to BCG vaccination in newborns given at two different timepoints, at birth and 2 months of life. The time of upregulation of T-cell-mediated interferon (IFN)-γ and interleukin (IL)-10 secretion to PPD were tested in those two conditions. We further tested whether $CD4^+$ $CD25^+$ T cells take part in response to BCG vaccination.

Material and methods

Study design

Term and healthy newborns were enrolled between September and November 2006 in Bakirkoy Maternity and Child Hospital, Istanbul. Newborns with a family history of tuberculosis, parental atopy, congenital abnormalities, previous history of steroid, intravenous immunoglobulin therapy or neonatal sepsis were excluded from the study. Nineteen term and healthy newborns were enrolled and randomized into two groups: Group I composed of newborns vaccinated with BCG at birth, and the remaining were vaccinated at 2 months of life (group II). BCG Pasteur 1173-P2 vaccine was applied intradermally to both groups on the left shoulder by the same physician. Physical examination was performed to all participants at baseline, 2nd and 8th months. Tuberculin skin testing was performed with 0.1 ml of PPD intradermally by the Mantoux method at 8th month to all subjects. Indurations were measured 72 h later by the ballpoint-pen method. Blood was obtained for cell cultures and cytokine analyses right before the BCG vaccination and PPD testing. The study was approved by the local ethics committee of Marmara University Medical Faculty. All children's parents were informed and gave their consents for the procedures.

Immunologic analyses

Blood was drawn from all participants at baseline, 2nd and 8th month. Peripheral blood mononuclear cell (PBMC) from all subjects were isolated by Ficoll–Hypaque density gradient centrifugation and then suspended in RPMI 1640 with 2 mmol/l L-glutamine (Gibco Invitrogen, New York, USA), 100 U/ml penicillin/streptomycin, and supplemented with 10% fetal calf serum (Sigma Chem Co., St Louis, MO, USA). PBMCs (6×10^5) were incubated at baseline, 2nd and 8th months from all subjects and cultured with 1 mcg/ml PPD (Statens Serum Institute, Cophenagen, Denmark) and 5 mcg/ml phytohaemagglutinin (PHA) (Sigma) in 500 µl each in 48-well plates (Costar Corp., Cambridge, MA, USA) at 37°C with 5% CO_2 for 5 days. The supernatants were collected and stored at −20°C until tested. IFN-γ, IL-5 and IL-10 (Endogen®; Rockford, IL, USA) levels of culture supernatants were determined using commercial human ELISA kit according to the manufacturer's instructions.

At 8th months of age, $CD4^+$ $CD25^+$ T cells were depleted with negative selection of $CD4^+$ $CD25^-$ T cells with magnetic isolation (Automacs system, Miltenyi, Germany) in subjects vaccinated at birth. Isolated cells were stimulated with PPD for 5 days, and IL-10 and IFN-γ cytokine levels were determined with ELISA as mentioned earlier.

Statistical analyses

Statistical analyses were carried out by means of the Statistical Package for the Social Sciences (SPSS) program (Version 17.0; SPSS Inc., Chicago, IL, USA). Because of sample size ($n < 30$), parameters were expressed in median values and 25th–75th centiles. Mann–Whitney *U* test was used for intergroup, and Wilcoxon signed ranks test was used for intragroup comparisons. A p value < 0.05 was considered to be significant.

Results

Among the 19 newborns enrolled, 10 received BCG at birth (group I), and the remaining 9 at 2 months of age (group II). Demographic data of all participants are presented in Table 1.

PPD response at 8th month was not significantly different in newborns vaccinated at birth and 2nd month of life [induration median (25th–75th centiles); 1 (0–8) mm and 5 (1–7.5) mm, respectively, $p = 0.927$].

Baseline evaluation of cytokine levels

In the PBMC culture supernatants, PPD- and PHA-stimulated IFN-γ, IL-5 and IL-10 levels did not show statistically significant difference at baseline in either group (Tables 2 and 3).

Table 1. Demographic data of newborns vaccinated at birth (group I) and at 2nd month (group II)

	Group I	Group II	p
Gestational age (wk)	38 (38–39)	38 (38–39.5)	0.968
Gender, (n) (male/female)	6/4	5/4	
Birth weight (g)	3230 (3137.5–3807.5)	3120 (2755–3300)	0.156
Birth height (cm)	52 (50.7–53)	50 (47.5–52)	0.053
Weight at 2nd month (g)	5650 (5460–6060)	5500 (4460–5600)	0.097
Height at 2nd month (cm)	61 (58–61)	58 (56–59)	0.165
Weight at 8th month (g)	8915 (8600–9497.5)	7650 (7137.5–8101.2)	0.03
Height at 8th month (cm)	72.5 (70.3–75)	71.5 (70.7–72)	0.345
Purified protein derivative response 8th month (mm)	1 (0–8)	5 (1–7.5)	0.927

Values are expressed as median and 25th–75th centiles except gender.

Table 2. Purified protein derivative (PPD)-induced cytokine levels (pg/ml) of peripheral blood mononuclear cell (PBMC) culture supernatants at baseline, 2nd month and 8th month in group I (vaccinated at birth) and II (vaccinated at 2nd month)

	Group I	Group II	p
Baseline			
IFN-γ	61.6 (9.6–93.5)	9.7 (4.6–25.3)	0.055
IL-10	167.3 (89.1–317.3)	220.8 (67.5–334.6)	0.931
2nd month			
IFN-γ	150.1 (8.7–381.4)*	10.6 (0–33.4)†	**0.038**
IL-10	264.3 (15.6–415.9)	13.7 (4.5–18.9)†	**0.022**
8th month			
IFN-γ	419.8 (125.4–835.1)	52.8 (25.5–784.3)	0.435
IL-10	158.2 (90.2–262)	137.2 (24.4–256.1)	0.686

Values are expressed as median and 25th–75th centiles.
*PPD-induced IFN-γ levels demonstrated a statistically significant increase at 2nd month compared to baseline in group I (BCG vaccinated at birth) ($p = 0.03$).
†PPD-induced IFN- γ and IL-10 levels were significantly higher in group I compared to group II ($p = 0.038$ and $p = 0.022$, respectively).

Table 3. Phytohaemagglutinin (PHA)-induced cytokine levels (pg/ml) of peripheral blood mononuclear cell culture supernatants at baseline, 2nd month and 8th month in group I (vaccinated at birth) and II (vaccinated at 2nd month)

	Group I	Group II	p
Baseline			
IFN-γ	632.8 (539.3–798.8)	637.3 (501.0–1002.8)	0.530
IL-10	306.9 (136.9–391.1)	340.3 (191.2–359.3)	0.935
2nd month			
IFN-γ	738.0 (676.9–843.3)	451.9 (93.9–789.5)	0.345
IL-10	538.1 (225.8–799.0)	167.3 (0–185.2)	0.061
8th month			
IFN-γ	785.5 (566.2–936.4)	859.9 (102.2–993.0)	1.000
IL-10	143.4 (101.8–226.5)*	172.5 (71.5–273.7)	0.624

Values are expressed as median and 25th–75th centiles.
*PHA-induced IL-10 levels demonstrated a statistically significant increase at the 8th month compared to baseline ($p = 0.043$) and 2nd month ($p = 0.043$) in group I (BCG vaccinated at birth).

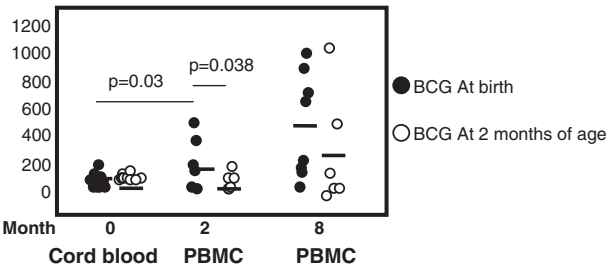

Fig. 1. Comparison of purified protein derivative (PPD)-stimulated IFN-γ levels (pg/ml) at baseline obtained from cord blood, 2nd and 8th months obtained from peripheral blood mononuclear cell (PBMC) culture supernatants in group I (BCG vaccinated at birth) and II (BCG vaccinated at 2nd month). Subjects of group I are demonstrated by dark circles and group II by white circles. Median value is indicated by black lines. Group I; baseline vs. 2 months IFN-γ levels, $p = 0.03$. Second month; group I vs. II IFN-γ, $p = 0.038$.

Between-group comparisons

Neonates vaccinated at birth (group I) demonstrated higher PPD-stimulated IFN-γ levels at 2 months of age when compared to those vaccinated at the 2nd month of life (group II) (median (25th–75th centiles); 150.1 (8.7–381.4) pg/ml vs. 10.6 (0–33.4) pg/ml respectively, $p = 0.038$). PPD-stimulated IL-10 levels were also higher in group I compared to group II at 2 months of age (median (25th–75th centiles); 264.3 (15.6–415.9) pg/ml vs. 13.7 (4.5–18.9) pg/ml, respectively, $p = 0.022$) (Figs 1 and 2).

Group I and II did not differ in PPD-stimulated IL-5 and PHA-stimulated IFN-γ, IL-5 and IL-10 levels at 2 months of age. Furthermore, there was no statistically significant difference in PPD- and PHA-stimulated IFN-γ, IL-5 and IL-10 levels at 8 months in either group. Median and 25th–75th centiles of PPD- and PHA-stimulated IFN-γ and IL-10 levels are presented in Tables 2 and 3 for both groups.

Within-group comparisons

For group I (BCG vaccinated at birth), PPD-stimulated IFN-γ levels demonstrated a

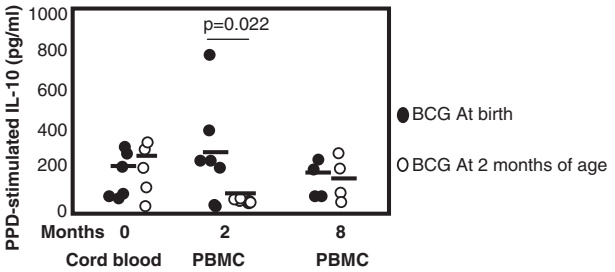

Fig. 2. Comparison of purified protein derivative (PPD)-stimulated IL-10 levels (pg/ml) at baseline obtained from cord blood, 2nd and 8th months obtained from peripheral blood mononuclear cell (PBMC) culture supernatants in group I (BCG vaccinated at birth) and II (BCG vaccinated at 2nd month). Subjects of group I are demonstrated by dark circles and group II by white circles. Median value is indicated by black lines. Second month; group I vs. II IL-10 levels, $p = 0.022$.

statistically significant increase at the 2nd month of life compared to baseline (median (25th–75th centiles); 150.1 (8.7–381.4) pg/ml vs. 61.6 (9.6–93.5) pg/ml, $p = 0.03$, respectively). No within-group differences were noted between 2nd month vs. baseline for PPD-stimulated IL-10 levels, 8th month vs. 2nd month and baseline values both for PPD-stimulated IL-10 and IFN-γ levels in group I (Table 2). PHA-stimulated IL-10 and IFN-γ levels of group I were presented in Table 3.

For group II (BCG vaccinated at 2nd month), within-group comparisons of month 2 vs. baseline, month 8 vs. 2 and baseline based on PPD- and PHA-stimulated IFN-γ, and IL-10 levels did not show statistically significant differences (Tables 2 and 3).

$CD4^+$ $CD25^+$ T cells were depleted from PPD-stimulated PBMC cultures at 8 months of age in those vaccinated at birth. PPD-stimulated IL-10 and IFN-γ levels were compared to those cytokines of non-depleted conditions at the same time-point. Results demonstrated lower levels of PPD-stimulated IL-10 ($p < 0.001$), but not IFN-γ ($p = 0.068$) in $CD4^+$ $CD25^+$ T-cell-depleted culture supernatants when compared to PBMCs (Table 4), (Fig. 3).

Table 4. 8th month assesment of IFN-γ and IL-10 levels (pg/ml) in the $CD4^+$ $CD25^+$ T-cell-depleted and non-depleted PPD-stimulated culture supernatants for the subjects vaccinated at birth

BCG vaccinated at birth	PPD-stimulated PBMC	PPD-stimulated $CD4^+$ $CD25^+$ T-cell-depleted PBMC	p
8th month			
IFN-γ	419.8 (125.4–835.1)	830.3 (681.7–1212.8)	0.068
IL-10	158.2 (90.2–262)	7.6 (4.1–11.4)	**<0.001**

Values are expressed as median and 25th–75th centiles.
PPD, purified protein derivative; PBMC, peripheral blood mononuclear cell.

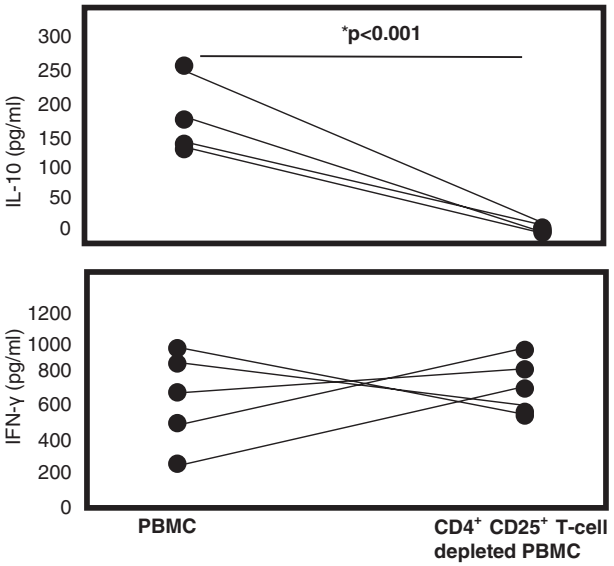

Fig. 3. Purified protein derivative (PPD)-stimulated IL-10 and IFN-γ secretion (pg/ml) of $CD4^+$ $CD25^+$ T-cell-depleted peripheral blood mononuclear cell cultures (PBMC) of group I at 8th month compared to non-depleted PBMC cultures at the same time-point. Group I; IL-10 levels of PBMC vs. $CD4^+$ $CD25^+$ T-cell-depleted PBMC, $p < 0.01$.

Discussion

In this study, we demonstrated that administration of BCG at birth resulted in higher PPD-stimulated IFN-γ and IL-10 production when compared to those vaccinated at 2 months of age. Meanwhile, comparison of the two groups based on PPD skin test response and secretion of IFN-γ, IL-10 at the 8th month revealed no significant differences. Therefore, comparable cytokine levels and PPD responses obtained at 8 months suggest that there is no benefit in delaying BCG vaccination. Moreover, depletion of $CD4^+$ $CD25^+$ T cells at 8 months revealed almost no PPD-stimulated IL-10 secretion when compared to non-depleted condition, indicating the critical role of $CD4^+$ $CD25^+$ T cells in the production of IL-10 owing to BCG vaccination.

This study is unique in that IL-10 and IFN-γ responses were evaluated in healthy newborns prospectively for 8 months. Moreover, an attempt was made to identify the source of those cytokines by depleting $CD4^+$ $CD25^+$ cells at 8th month. On the other hand, we acknowledge the fact that small number of subjects in our study group may limit the detection of differences that could be highly relevant at the population level.

A prospective controlled study by Ildırım et al. had been conducted to compare the efficacy of BCG vaccine administered to newborns within the first 3 days of life vs. third month. Efficacy was determined by PPD skin test responses, vaccine scars and the complications of the

vaccine. They reported that BCG applied at the end of the third month provided a higher rate of PPD response and fewer complications (8).

Although BCG has been used worldwide as the only vaccine for tuberculosis, its protective efficacy in human adults is controversial. There are many studies evaluating the efficacy of BCG vaccination on the basis of PPD responses at various ages or cytokine profiles. To investigate human immunologic responses to *Mycobacteria* after BCG vaccination, Nabeshimi et al. analyzed IFN-γ and IL-10 production by PBMC from health-care workers five times throughout the year post vaccination. Among the participants who had been vaccinated at infancy and rebousted at the time of study, there were 20 PPD negative and six positive subjects who were evaluated by the cytokine responses at 0, 2, 4 and 8 wk and also 12 months after vaccination. PPD-stimulated IFN-γ production reached a peak at week 8 -in accordance with our data-, and then declined until 12 months, while PPD-stimulated IL-10 production reached a peak at week 2 and then declined to its lowest point at week 8. These results indicated that BCG vaccine could induce a type I cytokine response to *Mycobacteria*, despite negative PPD responses in the majority of participants (9).

The influence of BCG given at two different time-points (birth and second month) on the peripheral blood T-cell subpopulations was also investigated by Tastan et al. T-cell counts increased significantly 2 months post vaccination in the group vaccinated at birth, but not in those at 2 months. PPD skin test responses were similar in both groups in accordance with ours (10).

In this study, we demonstrated that IFN-γ production significantly increased at 2nd month in subjects vaccinated at birth and remained still detectable at 8 months post vaccination. Our results showed that subjects vaccinated at the 2nd month lacked that increase in IFN-γ response at the same time-point. Thereby, neonatal BCG administration is sufficient in eliciting a Th1 response in comparison to later timing of vaccination. Furthermore, BCG administration at birth induced IL-10 secretion by $CD4^+$ $CD25^+$ T cells.

In conclusion, our results suggest that in developing countries, BCG vaccination should not be delayed to induce an appropriate Th1 response as soon as possible.

Acknowledgment

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Conflict of interest

None.

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