INTRODUCTION

The major focus of current vaccinology studies has become the regulation of Th1 and Th2 polarization of T helper cells. Th1 and Th2 lymphocytes develop from the same Th0 precursor and differentiate into two functionally distinct subsets (Rengarajan et al., 2000). The balance of cytokines produced by these subsets is a key factor influencing the character of an immune response. Th1 cells secrete cytokines IL-2 and IFN-γ, which are required for cell-mediated reactions. Th2 cells produce IL-4, IL-5, IL-10 and IL-13, which mediate B-cell activation and antibody production. In general, an efficient clearance of intracellular pathogens is based on cellular immunity, while antibody responses are best suited for extracellular pathogens. The selective differentiation of naive Th0 lymphocytes into effector Th1 and Th2 cells has important consequences in the development of an immune response to invasive pathogens. These two Th subsets cross-regulate each other through antagonistic activity of their cytokines (Cottrez et al., 2000). The divergence of Th1 and Th2 differentiation provides a mechanism whereby first-line innate immune responses guide appropriate effector T cell activities that allow clearing pathogens and establishing long-lived memory. Inappropriate effector T cells fail to control the infection. In this case, persistent effector T cell responses drive chronic inflammatory disorders such as autoimmunity and allergy.

Today, more than three billion people have been vaccinated with the only currently available vaccine against tuberculosis (TB), which is the attenuated M. bovis Bacillus Calmette Guérin (BCG) strain (WHO, Global TB Report, 2014). However, TB still remains the leading cause of mortality due to bacterial pathogens. It is estimated that approximately 60 million people all over the world suffer from TB and annually about 3 million people die of the disease. Nevertheless, it is clear that the neonatal BCG vaccination is associated with a significantly lower incidence of TB in children (Tala-Heikkila et al., 1998; Kelly et al., 1997). Moreover, BCG protects against severe forms of childhood TB, especially meningeal and military TB in the areas of the world with a high prevalence of the disease (Powell et al., 2006). On the other hand, numerous controlled clinical trials yielded diverse and often contradictory results on protecting adults from TB by BCG vaccination. There is a general consensus that BCG is at best credited with a 50% overall protective efficacy (Brewer et al., 1995). It has become apparent that the immune responses to BCG bacilli are greatly influenced by the immanent host cellular immune system and involve both innate and adaptive cell-mediated components. However, the exact role of effector mechanisms in the cellular response to the BCG vaccine is complex and remains to be fully established. Skin test-
ing with PPD is the only currently available method of detecting BCG-induced cell-mediated immunity, in vivo. Several years of our studies on the skin tuberculin test in BCG vaccinated individuals have shown that only just over 50% of them develop the delayed type hypersensitivity (DTH) to PPD. The individual variability in development of the BCG-driven DTH to tuberculin prompted us to address a question as to whether Th1/Th2 polarization is involved in the lack of skin responsiveness to PPD. We performed our study to evaluate the rate for tuberculin skin sensitivity among healthy students who received neonatal BCG vaccination and usually BCG revaccination(s) at school age. Because our students had also been given diphtheria-tetanus toxoids-whole cell pertussis antigen (DTwP) vaccine in their childhood, we evaluated the relation between skin PPD-driven responses and the levels of serum IgG to pertussis toxin and diphtheria toxin. To assess the possible importance of Th1/Th2 polarization in responses to BCG and DTwP vaccines, we measured the concentration of IFN-γ as a prototypic Th1 cytokine and IL-10 as a representative of Th2 cytokine in the cultures of peripheral blood mononuclear lymphocytes (PBML) after the stimulation with PPD. Critical inducers of the Th helper subset development are dendritic cells (DCs), which educate T helper cells by direct cell-cell contacts and by released cytokines (Bedford et al., 2008). CD4(+) T helper cells recognise M.tb antigens that are presented via MHC (major histocompatibility complex) class II molecules on the surface of DCs. We performed comparative studies in the individuals with positive and negative skin tuberculosis tests, taking into account: a) the expression of DC surface signaling receptors (CD86, CD80 and HLA-DR), which are indispensable in the formation of an immune synapse between DCs and T helper cells, b) the production of IL-10 and IL-23, which provide the differentiation of Th2 and Th1 responding cells, respectively, by DCs, c) the efficient induction of mycobacterial antigen specific IFN-γ production, that plays a key role in the resistance to TB in co-cultures of DCs with lymphocytes.

### MATERIALS AND METHODS

#### Study participants

A total study population consisted of 118 healthy students at the Faculty of Biology and Environmental Protection, University of Lodz, Poland, age range 18–30 years, who had been subjected to BCG vaccination according to the state policy. All participants had been subjected to neonatal BCG vaccination. BCG revaccinations were given to those who were tuberculin-negative after Mantoux testing with PPD. The rate for tuberculin skin sensitivity among healthy students who received neonatal BCG vaccination and usually BCG revaccination(s) at school age was determined using a Victor 2 multifunctional counter (Wallac, Turku, Finland) set at 450 nm.

#### Lymphocyte cultures

PBML (peripheral blood mononuclear leukocytes) were isolated from heparinised blood by density gradient centrifugation (1500 rpm, 30 min, 21°C) on Histopaque (Sigma). The mononuclear cells at the interface were harvested, washed twice, suspended in complete culture medium (RPMI-1640, 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, 5 µg/ml gentamicin), and incubated in plastic culture plates for 1.5 h at 37°C in 5% CO₂ to remove adherent cells from the suspension. Nonadherent cells, lymphocytes and a very small proportion of dendritic cells were harvested and washed twice with the culture medium (Balfour et al., 1990). Viable cells were counted by trypan blue exclusion and the number of cells was then adjusted to 4×10⁶ cells/ml in complete RPMI-1640 medium. Cells were cultured in 96-well tissue culture plates (Nunc, Kastrup, Denmark) with or without PPD (20 µg/ml; Statens Serum Institute, Copenhagen, Denmark) or phytohemagglutinin (PHA) (10 µg/ml; Sigma). Supernatants were then obtained from each culture well at 24 and 96 h, centrifuged twice (at 1000 rpm and 4000 rpm) and stored at −70°C until analysed for IFN-γ and IL-10.

#### Monocyte-derived DC preparations

Peripheral blood was obtained using vacutainer tubes with spray-coated heparin (Becton Dickinson). Monocytes were separated from PBMC by immunomagnetic positive separation using CD14⁺ Microbeads (Miltenyi Biotec, Germany) into CD14⁺ and CD14⁻ fractions. CD14⁺ lymphocytes were frozen until use (Thurner et al., 1999). The purity of CD14⁺ monocytes was determined to be 96% to 98% on the basis of forward and side scatter parameters in conjunction with CD14 staining using standard flow cytometry (data not shown). Monocytes were suspended in complete culture medium at density of 1×10⁶/ml and the cells were placed into 6-well flat-bottomed culture plates and allowed to differentiate into DCs by incubation for 6 days in RPMI-1640 supplemented with 1% antibiotics and 10% FCS in the presence of 25 ng/ml human GM-CSF and 10 ng/ml human recombinant IL-4 (R&D Systems, USA). Immature DCs at density of 1×10⁶ cells/ml were incubated for 24 h (37°C, 5% CO₂) with live BCG bacteria at a multiplicity of infection (MOI) of 1:1 or 10 µg/ml PPD. Lipopolysaccharide (LPS) from E. coli O55:B5 (1 µg/ml; Sigma) was used as a positive control of DC activity and DCs in medium alone (unpulsed DCs) represented a negative control. Supernatants of the cultures were tested by ELISA (Eli-pair Diaclone test) for the presence of IL-10 and IL-23 (detection sensitivity: 5 pg/ml for IL-10 and 20 pg/ml for IL-23 (Szpakowski et al., 2014).

#### Estimation of CD86, CD80 and HLA-DR expression on DC surface by flow cytometry

Stimulated and unstimulated DCs were collected from the 6-well plate by using PBS/2mM EDTA, then washed in PBS and in-
cubated for 30 min. at 4°C with following mAb (Becton Dickinson): fluorescein isothiocyanate (FITC)-conjugated anti-CD86, -anti-HLA-DR or phycoerythrin (PE)-conjugated anti-CD80, or an irrelevant isotype-matched mAb as control. Data were analyzed using the FACs LSRII (BD) and FlowJo software, a minimum 10 000 events were collected. The calculated mean fluorescence intensity represents the molecular density on the cell surface and the percentage of positive DC for each marker.

**DC-lymphocyte co-cultures.** The preserved lymphocytes at 1×10⁶ cells/ml were thawed and co-cultured for 24 h (37°C, 5% CO₂) with autologous unpulsed DCs or DCs, which were primed with PPD, BCG or LPS, at a ratio of 10 lymphocytes per one DC. Supernatants were tested for IFN-γ by ELISA using Diaclon’s kit. The limit of detection was 5 pg/ml. To measure intracellular IFN-γ, 8 hours before the cell culture, 0.67 µl BD Golgi stop was added to DC-lymphocyte co-cultures. The cells were collected by using PBS/2 mM EDTA, washed in PBS and then incubated for 30 min. at 4°C with BD Biosciences; Pharmingen: phycoerythrin (PE)-conjugated anti-human CD4, allophycocyanin (APC)-conjugated anti-human CD8, AlexaFluor 700 anti-human CD56, or an irrelevant isotype-matched mAb as control. The cells were washed with PBS and suspended in Fixation/Permeabilization solution (BD Cytofix/Cytoperm). After 20 min. at 4°C, the cells were washed with Perm/Wash buffer and incubated for 30 min. at 4°C with Becton Dickinson (BD) fluorescein isothiocyanate (FITC)-conjugated anti-human IFN-γ or an irrelevant isotype-matched mAb. Data were analyzed using the FACs LSRII (BD) and FlowJo software, a minimum of 10 000 events were collected. The proportion of CD4+ and CD8+ T lymphocytes and CD56+ NK cells among the cells producing IFN-γ was calculated.

**Estimation of mycobacterial antigen driven IFN-γ and IL-10 production.** The levels of IFN-γ in supernatants of mycobacterial antigen stimulated lymphocyte cultures were determined by ELISA. Maxisorp plates (Nunc, Kastrup, Denmark) were coated with the mouse monoclonal anti-human IFN-γ antibody (R&D, Minneapolis, MN, USA) at 5 µg/ml, overnight at room temperature. After blocking with phosphate-buffered saline (PBS) buffer containing 1% bovine serum albumin and 5% sucrose, 100 µl of supernatants were added to wells and incubated for 1.5 h at 37°C. The wells were washed three times with PBS containing 0.05% Tween 80 and incubated for 1.5 h at 37°C with the biotinylated goat anti-human IFN-γ antibody (R&D) at 400 ng/ml. After washing, avidin coupled to peroxidase was added to the wells (0.25 µg/ml), and the plates were incubated for 20 min at 37°C. Final three washes were followed by the addition of OPD substrate (Sigma, St. Louis, Michigan, USA). The optical density was measured after 20 min at 450 nm on a Victor 2 spectrophotometric reader (Wallac, Turku, Finland). The cytokine was quantified with reference to standard curves generated using human recombinant IFN-γ (R&D). The sensitivity of the assay was 80 pg/ml. Concentrations of IL-10 in supernatants of lymphocyte cultures were determined with the use of a commercial ELISA kit (Diaclon, Bençancon Cedex, France) following the instructions of the manufacturer. The detection limit of the cytokine was 5 pg/ml.

**Statistical analysis.** The Statistica software (Statsoft) was used for all analyses of the data. The Mann-Whitney U test was used to determine the differences in IgG and cytokine levels. The Fisher’s exact two-tailed test and odds ratios (OR) were calculated to assess the significance of PPD-driven IL-10 and IFN-γ responses. The 95% confidence intervals (95% CIs) were also determined. The P value used for significance in statistical tests was 0.05. A correlation between the investigated parameters was analysed with the Pearson’s correlation r test.

**RESULTS**

**BCG-vaccinated students differ in their tuberculin skin test reactions**

A total of 118 BCG vaccinated students were skin tested with PPD. Out of those, 63 (53%) responded to PPD with ≥10 mm induration and they were classified as tuberculin-positive (TST+) (Table 1). The remaining 55 (47%) had induration of less than 10 mm and they were considered tuberculin-negative (TST-). None of the participants had a history of TB. BCG revaccination of tuberculin-negatives has been an integral part of the Polish TB program. In the group of TST+ students, 6 (10%) received a BCG vaccine only once, at birth, 43 (68%) participants were BCG revaccinated at the age of 6, and 14 (22%) students were additionally revaccinated at the age of 12. The skin test reaction sizes to PPD were 14.7 ± 3.5 mm, 13.8 ± 3.4 mm and 12.1 ± 1.9 mm, respectively, in tuberculin-positive students who received one, two or three BCG inoculations. However, these differences were not significant (p > 0.05). Among tuberculin-negative participants, 8 (14%) received BCG vaccine at birth only, 28 (51%) students were revaccinated at the age of 6, and 13 (24%) received the third dose of BCG vaccine at the age of 12. Six (11%) tuberculin-negative students received two additional BCG revaccinations; the fourth and fifth doses of the BCG vaccine were administered to them when they were 18 years old but this was at least 5 years before participation in this study. We conclude that the lack of DTH to PPD in about 47% of BCG-vaccinated students was not a transient phenomenon but was persistent after repeated intradermal BCG boosters. In order to understand the cause(s) of lack of DTH to PPD in persistently tuberculin-negative participants vaccinated with BCG, we studied blood samples from 55 randomly selected students to determine PPD-driven production of IFN-γ and IL-10 by peripheral blood lymphocytes of prototypic Th1 and Th2 cytokines, respectively.

<table>
<thead>
<tr>
<th>Table 1. Characteristics of the study groups</th>
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<tbody>
<tr>
<td><strong>Participant Group</strong></td>
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<td><strong>Age</strong> (range)</td>
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<td><strong>Sex</strong></td>
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<td>Female</td>
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<td>Male</td>
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<td><strong>BCG vaccination</strong></td>
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<td><strong>Ethnicity</strong></td>
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<td><strong>BCG inoculations</strong></td>
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<td>1</td>
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<td>2</td>
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**Table 2. IFN-γ and IL-10 release by PPD-stimulated lymphocytes from tuberculin-positive (TST+) and tuberculin-negative (TST-) volunteers**

<table>
<thead>
<tr>
<th>Participants</th>
<th>48 h</th>
<th>96 h</th>
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<tbody>
<tr>
<td></td>
<td>Level (pg/ml)</td>
<td>OR (95%CI)</td>
</tr>
<tr>
<td><strong>TST+</strong></td>
<td>23/35 (66%)</td>
<td>3.8</td>
</tr>
<tr>
<td><strong>TST-</strong></td>
<td>7/20 (35%)</td>
<td>0.04 (1.2; 12.1)</td>
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</table>

<table>
<thead>
<tr>
<th>Participants</th>
<th>24 h</th>
<th>48 h</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Level (pg/ml)</td>
<td>OR (95%CI)</td>
</tr>
<tr>
<td><strong>TST+</strong></td>
<td>26/35 (74%)</td>
<td>0.3</td>
</tr>
<tr>
<td><strong>TST-</strong></td>
<td>18/20 (90%)</td>
<td>0.3 (0.1, 1.7)</td>
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</table>

**IFN-γ responses to PPD**

Peripheral lymphocytes from TST+ and TST- participants were stimulated with PPD or PHA for 4 days *in vitro* in four parallel cultures. The concentration of IFN-γ was determined at time points between 24-96 h and the level of this cytokine increased with culturing time. The negative tuberculin skin responses were associated with significantly delayed and decreased levels of IFN-γ in PPD-stimulated lymphocyte cultures. As shown in Table 2, this cytokine was detected in two-day PPD-stimulated cultures of lymphocytes from 66% of TST+ versus 35% of TST- participants (p = 0.04; OR = 3.8; 95% CI, 1.2, 12.1). Then, PPD-driven IFN-γ production was assessed at 96 h when lymphocytes from 97% of skin test positive and from 70% of skin test negative students responded by IFN-γ secretion (p = 0.01; OR = 14.6; 95% CI, 1.6, 132.3). Comparing the average levels of PPD-stimulated IFN-γ production, we observed that lymphocytes from TST+ participants produced significantly more cytokines than the cells from TST- volunteers (7396±4806 pg/ml versus 3264±3526 pg/ml; U = 60, p = 0.001) (Table 2). In addition, the intensity of PPD-driven *in vitro* production of IFN-γ by peripheral lymphocytes was correlated with the skin test reaction sizes to PPD *in vivo* (r = 0.49; p = 0.002). High levels of IFN-γ were also detected in the culture supernatants of lymphocytes stimulated with PHA (data not shown). However, lymphocytes from tuberculin-positive and negative students produced similar levels of IFN-γ when stimulated with PHA (6761±4472 pg/ml versus 6598±3410 pg/ml).

**IL-10 responses to PPD**

The IL-10 production was assessed at 24 h and 48 h of peripheral lymphocyte cultures *in vitro* in the medium with or without PPD or PHA. At time points between 24-48 h, we observed no difference in the rate of IL-10 production by the PPD-stimulated lymphocytes from TST+ and TST- students (Table 2). At 24 h, PPD driven IL-10 production was detected in 74% and 90% of the cultures from TST+ and TST- participants, respectively (p = 0.3; OR = 0.3; 95% CI, 0.1, 1.7) (Table 2). The negative tuberculin skin responses were associated with significantly higher levels of IL-10 in PPD-stimulated lymphocyte cultures. The concentration of IL-10 produced in response to PPD in the cultures of lymphocytes from TST+ students was significantly lower than in the cultures of lymphocytes from TST- participants (281±306 pg/ml versus 589±438 pg/ml; U = 94; p = 0.03). Moreover, there was a negative correlation between the size of skin reactions to PPD and the production of IL-10 in PPD-stimulated lymphocyte cultures (r = 0.36; p = 0.03). There was no significant difference in the PHA-driven production of IL-10 by lymphocytes from TST+ and TST- students (813±497 pg/ml versus 994±581 pg/ml).

**Serum levels of anti-pertussis toxin IgG and anti-diphtheria toxin IgG**

Our results indicated that type 1 cytokine response to PPD was positively associated with the generation of tuberculin sensitivity in the students subjected to BCG vaccination. However, these students had also been given the DTwP (diphtheria-tetanus toxoids-whole cell pertussis antigens) vaccine in the childhood. This has prompted us to address a question as to whether the type 1 cytokine response and cellular sensitivity to PPD have an implication for the students’ humoral responses to pertussis vaccine. Using the ELISA test, the levels of IgG antibodies against pertussis toxin in the sera from 55 students under study were measured. Data in Table 3 show that the average levels of serum anti-pertussis toxin IgG were significantly higher in TST+ students (45.9±20.5 NovaTecUnits NTU/ml) than in TST- (30.7±15.86 NTU/ml) participants (U = 18; p = 0.0002). Moreover, the negative correlation was noticed between the levels of IgG to pertussis

**Table 3. IgG anti-pertussis toxin and IgG anti-diphtheria toxin in sera from tuberculin-positive (TST+) and tuberculin-negative (TST-) volunteers**

<table>
<thead>
<tr>
<th>Participants</th>
<th>IgG anti-pertussis toxin</th>
<th>IgG anti-diphtheria toxin</th>
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<tr>
<td></td>
<td>NTU/ml</td>
<td>p</td>
</tr>
<tr>
<td><strong>TST+</strong></td>
<td>30.7±15.86</td>
<td>0.0002</td>
</tr>
<tr>
<td><strong>TST-</strong></td>
<td>45.9±20.5</td>
<td>0.37±0.29</td>
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A significant association was observed between the concentration of anti-pertussis IgG in the participants’ sera and the ability of their lymphocytes to respond to PPD by IL-10 production ($r=0.47; p=0.005$). In contrast, no differences in the levels of anti-diphtheria toxin IgG in the sera from TST+ (0.4±0.32 NTU/ml) and TST− (0.37±0.29 NTU/ml) individuals were found (Table 3).

**Activation markers of mycobacterial antigen pulsed dendritic cells**

To better explore the molecular mechanisms of Th1 dependent IFN-γ expression in DTH reaction to PPD in young people vaccinated with BCG, we investigated surface expression of activation markers of monocyte derived dendritic cells in TST+ and TST− participants. DCs were pulsed with PPD, BCG or LPS (a positive control) and the expression of co-stimulation markers CD86 and CD80 as well as the HLA-DR molecule levels were compared to control conditions, i.e. unstimulated DCs as a negative control. Dendritic cell incubation with BCG but not PPD slightly increased the medium expression of CD86 on DCs from TST− and TST+ individuals (Fig. 1).

In contrast, both BCG and PPD induced an up-regulation of CD80 on DCs from the majority of TST+ and TST− individuals (Fig. 2). However, more individuals
whose DCs had not responded to PPD or BCG stimulation with the up-regulation of CD80, were in the group of TST −. Similarly more individuals, whose DCs have not responded to PPD or BCG with increased HLA-DR expression, were in the TST − group (Fig. 3). At the same time, the median value of HLA-DR expression on PPD-pulsed DCs in TST − individuals was significantly lower (p = 0.05) as compared to TST + subjects (Fig. 4). Next, the cytokine profile of mycobacterial antigen pulsed DCs which might affect the T-cell polarization was analyzed. IL-10 and IL-23 production was measured in DC cultures incubated with PPD, BCG or LPS. In almost all individuals, LPS-primed DCs produced IL-10 and IL-23 (Table 4). The proportion of individuals whose DCs produced IL-10 in response to BCG was significantly higher in TST − than TST + volunteers. However, there were no intergroup differences in PPD driven IL-10 production. DC incubation with PPD or BCG increased the proportion of IL-23 producers twice as much in TST + and by about 3–4 times in TST − individuals. The differences did not reach statistical significance.

**IFN-γ production in co-cultures of lymphocytes responding to mycobacterial antigens presented by DCs**

The potency of peripheral blood lymphocytes to respond to PPD and BCG presented by DCs in TST − versus TST + individuals was analyzed. DCs were stimulated with PPD or BCG or they were left unstimulated. Then, co-cultures of lymphocytes, free of CD14(+) macrophages, with autologous DCs were conducted for 24 hours and the phenotype of cells containing intracellular IFN-γ as well as the levels of released IFN-γ were analyzed. As shown in Fig. 5, T CD4(+) and T CD8(+) lymphocytes and CD56(+) NK cells expressed intracellular IFN-γ in responses to mycobacterial antigens presented by DCs. The proportion of T CD4(+) cells synthesizing IFN-γ

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**Table 4. IL-10 and IL-23 release by antigen-stimulated dendritic cells from tuberculin-positive (TST +) and tuberculin-negative (TST −) volunteers**

<table>
<thead>
<tr>
<th>Participants</th>
<th>IL-10</th>
<th>DC</th>
<th>Responders n/N (%)</th>
<th>p</th>
<th>OR (95%CI)</th>
<th>PPD</th>
<th>Responders n/N (%)</th>
<th>p</th>
<th>OR (95%CI)</th>
<th>BCG</th>
<th>Responders n/N (%)</th>
<th>p</th>
<th>OR (95%CI)</th>
<th>LPS</th>
<th>Responders n/N (%)</th>
<th>p</th>
<th>OR (95%CI)</th>
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<td>TST −/TST +</td>
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<tr>
<td>12/32</td>
<td>0.96</td>
<td>0.9</td>
<td>19/31</td>
<td>0.97</td>
<td>0.89</td>
<td>16/31</td>
<td>0.01</td>
<td>29/30</td>
<td>0.92 ND</td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>36%</td>
<td>(36%)</td>
<td>(0.3; 2.4)</td>
<td>61%</td>
<td>(33%</td>
<td>(0.3;2.4)</td>
<td>52%</td>
<td>(0.3;2.4)</td>
<td>97%</td>
<td>(0.3;2.4)</td>
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<td>14/35</td>
<td>0.35</td>
<td>(39%)</td>
<td>23/36</td>
<td>0.89</td>
<td>(0.3;2.4)</td>
<td>30/36</td>
<td>0.21</td>
<td>36/36</td>
<td>(100%)</td>
<td></td>
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<tr>
<td>1.0</td>
<td>ND</td>
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<td>0.21</td>
<td>(1.0;0.7)</td>
<td>0.21</td>
<td>(0.3;2.4)</td>
<td>0.21</td>
<td>(0.3;2.4)</td>
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<table>
<thead>
<tr>
<th>Participants</th>
<th>IL-23</th>
<th>DC</th>
<th>Responders n/N (%)</th>
<th>p</th>
<th>OR (95%CI)</th>
<th>PPD</th>
<th>Responders n/N (%)</th>
<th>p</th>
<th>OR (95%CI)</th>
<th>BCG</th>
<th>Responders n/N (%)</th>
<th>p</th>
<th>OR (95%CI)</th>
<th>LPS</th>
<th>Responders n/N (%)</th>
<th>p</th>
<th>OR (95%CI)</th>
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<td>TST −/TST +</td>
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<tr>
<td>6/18</td>
<td>0.15</td>
<td>4.75</td>
<td>9/16</td>
<td>0.31</td>
<td>2.57</td>
<td>16/31</td>
<td>0.85</td>
<td>17/18</td>
<td>1.0 ND</td>
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<tr>
<td>33%</td>
<td>(33%)</td>
<td>(0.8; 27.5)</td>
<td>56%</td>
<td>(33%</td>
<td>(0.6;10.3)</td>
<td>52%</td>
<td>(0.6;10.3)</td>
<td>94%</td>
<td>(0.6;10.3)</td>
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<tr>
<td>2/21</td>
<td>0.10</td>
<td>2.57</td>
<td>6/18</td>
<td>0.85</td>
<td>1.33</td>
<td>8/18</td>
<td>1.33</td>
<td>18/18</td>
<td>(100%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>0.15</td>
<td>ND</td>
<td></td>
<td>0.15</td>
<td>(0.4;4.3)</td>
<td>0.15</td>
<td>(0.4;4.3)</td>
<td>0.15</td>
<td>(0.4;4.3)</td>
<td></td>
<td></td>
<td></td>
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</tbody>
</table>

Abbreviations: DC, unprimed dendritic cells; PPD (purified protein derivative)/BCG (M. bovis BCG)/LPS (lipopolysaccharide), pulsed dendritic cells; ND, not determined because of no values in one or more groups.

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**Figure 5.** The percentage of CD4+ and CD8+ T cells and CD56+ NK cells amount IFN-γ producing lymphocytes responding to PPD or BCG pulsed dendritic cells or unstimulated DC in DC-lymphocyte co-cultures performed for tuberculin-negative (TST −) and tuberculin-positive (TST +) volunteers.
Table 5. IFN-γ release by lymphocytes responding to PPD or BCG-pulsed dendritic cells in co-cultures for tuberculosis-positive (TST⁺) and tuberculosis-negative (TST⁻) volunteers

<table>
<thead>
<tr>
<th>Participants</th>
<th>IFN-γ (pg/ml)</th>
<th>p</th>
<th>BCG (pg/ml)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>TST⁺</td>
<td>7102 ± 1590</td>
<td>0.03</td>
<td>5653 ± 2166</td>
<td>0.03</td>
</tr>
<tr>
<td>TST⁻</td>
<td>1053 ± 517</td>
<td></td>
<td>1307 ± 1030</td>
<td></td>
</tr>
</tbody>
</table>

exceeded significantly the proportion of T CD8(+) containing this cytokine. Lymphocytes isolated from peripheral blood of TST⁺ individuals produced significantly more IFN-γ in response to PPD or BCG presented by DCs (7102.3 ± 1589.9 and 5653.0 ± 2458.6 pg/ml, respectively) than identically educated blood lymphocytes from TST⁻ volunteers (1053.6 ± 517.1 and 1307.6 ± 1030.3 pg/ml; p = 0.03) (Table 5).

DISCUSSION

Tuberculosis (TB) has been annotated as a global emergency disease despite being treatable. The HIV epidemic and the emergence of multidrug-resistant strains of Mycobacterium tuberculosis have increased the spread of TB. Although the current M. bovis BCG vaccine has been central to the control of TB, particularly in children, the protection offered by vaccination is insufficient and is not life-long (Kauffmann et al., 2013). The tuberculin skin test provides a useful in vitro model for studying the effector side of immune response to mycobacteria. TST allows measuring DTH to purified PPD, which usually arises concomitantly with the development of protective immunity in individuals exposed to M. tuberculosis or the BCG vaccine. However, PPD by itself does not activate antimycobacterial primary immune responses in individuals lacking pre-existing immunity (Mollenkopf et al., 2004). The sequence of events leading to the local reactivity to PPD in a positive TST is incompletely defined but is likely initiated by tuberculin protein uptake by dendritic cells (DC) which present antigens to recirculating T cells. These cells then generate cytokine and chemokine signals that attract blood monocytes and lymphocytes, leading to skin induration. Recently, Bond at al. concluded that multiple DCs of the myeloid lineage infiltrated the PPD driven skin inductions in healthy TST positive individuals (Bond et al., 2012). In our study, 118 healthy students were skin tested with PPD. All of them had been subjected to neonatal BCG vaccination and often revaccinated at school age. During our study, only 53% of the participants exhibited the DTH responses to PPD. The remaining students were persistently tuberculin-negative including those who received 4–5 inoculations of the BCG vaccine. These findings correspond with the studies examining the impact of BCG revaccination of tuberculosis-negative school children in TB prevention (Tala-Heikkila et al., 1998). The efficacy of BCG revaccination was found to be low or nonexistent in Finland, a country with low TB incidence. Our data do not allow answering the question if BCG revaccinations induced protective responses to tuberculosis in the participants who were not able to develop DTH to PPD. Tuberculin sensitivity is not a marker for protection and so the lack of skin reaction to PPD does not necessarily signify the lack of adaptive resistance to mycobacteria (Jepson et al., 2001).

However, our data clearly show the variations in the cell-mediated immune responses of adolescents to BCG vaccination.

The mycobacterial antigen(s) — specific T cells are required for the control of infections with M. tuberculosis. The efficiency of protective anti-mycobacterial immunity depends on type 1 CD4⁺ T helper cells producing IFN-γ, which is the most effective activator of the bactericidal activity of macrophages. The absence of strong type 1-mediated immunity correlates most closely with TB progression characterized by exacerbated bacterial growth, significant tissue necrosis and premature death (Flynn et al., 2001). We extended our study by analyzing the cytokine responses induced by PPD in peripheral blood lymphocytes from healthy students subjected to BCG vaccination. An important aspect of our findings is that the occurrence of PPD-driven skin reactions was significantly associated with the high levels of Th1 cytokine — IFN-γ. BCG vaccination induced the development of DTH to tuberculin only in the participants whose lymphocytes rapidly produced high levels of IFN-γ (Table 2) when stimulated with PPD in vitro.

With regard to the inadequate protective effect of BCG vaccination against TB, it is interesting to note that in this study 47% of BCG-vaccinated/revaccinated healthy students responded to PPD with <10 mm of induration. Thus, this group of participants displayed persistent incapability of developing tuberculin sensitivity in response to attenuated M. bovis BCG bacilli. Interestingly, Delgado and coworkers showed that 37% out of the total 364 pulmonary tuberculosis patients had induration of less than 10 mm during screening for PPD sensitivity (Delgado et al., 2002). Furthermore, the inability to develop tuberculin sensitivity in response to attenuated M. bovis BCG bacilli observed by us and to virulent M. tuberculosis reported by Delgado et al. was significantly associated with the enhanced production of immunosuppressive IL-10 and compromised release of the effector IFN-γ by peripheral lymphocytes stimulated with PPD in vitro (Delgado et al., 2002) (Table 2).

An immanent inability to develop Th1-dependent DTH response to PPD, which was observed in tuberculosis-negative students, prompted us to extend our study to the characterisation of Th2-dependent humoral response to the whole cell pertussis antigen and diphtheria toxins. All the participants had been given 4 doses of the DTwP vaccine in their childhood. Pertussis and diphtheria are acute contagious diseases caused by Bordetella pertussis or Corynebacterium diphtheriae bacteria. The main virulence factors of these pathogens are secreted toxins that allow them to cause specific symptoms of the disease. The DTwP vaccine and a natural infection with B. pertussis or C. diphtheriae induce the development of specific Th2-type response ensuring the anti-bacterial protection. The protective immunity against the diseases depends on the presence of specific IgG antibodies directed against bacterial toxins. Although the number of reported pertussis cases declined dramatically following the introduction of universal childhood pertussis vaccination, the disease is still endemic worldwide (Gzyl et al., 2004). Moreover, in some countries with high levels of immunization with the whole cell pertussis vaccine a re-emergence of pertussis has been reported (de Melker et al., 1997; Hardwick et al., 2002; de Serres et al., 1995; Olin et al., 2003; Tan et al., 2005; Fingerman et al., 2006).

The re-emergence of pertussis in children and adults has been explained by the changes in the circulating bacterial populations, allowing bacteria to evade the immune control (Hardwick et al., 2002; Fingerman et al., 2006,
Fiett et al., 2003). Our results, however, suggest that individual variations in humoral response to whole killed 
*B. pertussis* bacteria can have implications for inadequate resistance to pertussis in the vaccinated individuals. In 
the group of healthy students subjected to childhood DTWP vaccination we found a significant negative 
correlation between the levels of serum anti-pertussis IgG and PPD-driven production of IFN-γ *in vitro*, and skin 
tuberculin reactivity *in vivo*. High levels of anti-pertussis IgG were detected in the sera from the participants who were 
persistently tuberculin negative and whose lymphocytes produced high levels of Th2 cytokine — IL-10 in the 
cultures stimulated with PPD. Moreover, the PPD-driven IFN-γ production in these TST- participants was 
significantly lower in comparison to the INF-γ levels detected in the group of TST+ students (Table 2). No 
association between DTH to PPD and the levels of IgG against diphtheria toxin was noticed in the study groups. In a 
previous study we also observed no differences in the levels of IgG specific to PPD or mycobacterial Hs65 
antigen between the groups of TST- and TST+ healthy BCG vaccinated volunteers (Paziak-Domańska et al., 2002). It is possible that soluble protein antigens, such as diphtheria toxoid, PPD or Hs65, induce humoral 
response, which is more dependent on Th2 cytokines other than IL-10, e.g. IL-4 or IL-5. In fact, peripheral blood 
lymphocytes from healthy BCG vaccinated volunteers produced IL-5 in the PPD stimulated cultures (Kowalewicz-Kulbat et al., 2008). The whole cell pertussis vaccine carries a variety of antigens, T-dependent and T-independent 
and this vaccine induces significantly more proinflammatory cytokines, such as IL-6, IL-β, TNF-α, 
than does the diphtheria vaccine (Blood-Siegfried et al., 1998). A possibility that in certain individuals a vigorous 
BCG — specific response of Th1 type may cause a down-regulation of Th2 response to pertussis bacteria and 
decrease the production of anti-pertussis IgG should not be excluded. In summary, the results of this study 
provide the evidence that variation in Th1/Th2 lymphocyte balance might be an important determinant of the 
immune response to bacterial vaccines.

To more deeply explore the molecular mechanisms determining the development or lack of development of 
DTH to the BCG vaccine, our research was focused on dendritic cells. The efficiency of DC to present mycobacterial 
antigens to T lymphocytes, which are also known to infiltrate the positive TST reaction, depends on both recruitment of DC to the skin and their antigen processing and regulatory capacity. Dendritic cells, being the most potent antigen presenting cells, play a key role in 
triggering the adaptive response to virulent as well as attenuated mycobacteria. To define the relative role of 
DC in the maintenance of TST positivity, in the present study we investigated the responses of monocyte-derived 
DC to live *M. bovis* BCG bacteria and PPD. Simultaneous investigations of changes in DC phenotypic markers, 
expression of cytokines and eliciting specific cellular response of lymphocytes shed new light on the role of 
mycobacterial derived products as compared with the whole *M. bovis* BCG bacilli in modulating DC functions 
allowing them to promote or stop DTH response.

Interaction of DCs with live *M. bovis* BCG but not with PPD, resulted in cell maturation manifested by a 
similarly slight increase in the expression of CD86 in the group of tuberculin-positive and tuberculin-negative 
students. In contrast, both stimuli, PPD and BCG, caused an increase in the expression of the DC CD80 
co-stimulatory receptor (Fig. 2). This increase occurred in a slightly higher proportion of volunteers that were

TST+ than TST-, however, it is worth mentioning that the increase in the co-stimulatory receptors on DCs is 
indispensable for the formation of the immune synapse between DC and T lymphocytes and, in this way, it is 
mostly important in the antigen presentation process and has a significant impact on generating efficient T cell 
responses (Henderson et al., 1996).

The significant difference in the expression of HLA-DR molecules on PPD stimulated DCs in the group of 
TST- and TST+ participants merits utmost consideration (Fig. 4). The median value of the HLA-DR expression 
on PPD-pulsed DCs in TST- individuals was significantly lower as compared to TST+ students. The expression 
of MHC class II molecules including HLA-DR characterizes the degree of functional maturity of DCs as well as 
its potential in presenting antigens to antigen-specific T helper cells (Steinman et al., 2008). The results 
of the study clearly suggest that a diminished ability of DCs from TST- volunteers to increase the expression of 
MHC-class II antigen and co-stimulatory CD86 in the milieu of PPD contributes to the persistent incapability 
of developing DTH to the attenuated *M. bovis* BCG vaccine. Such a property of the immune system of TST- 
students was accompanied by a significant enhancement of IL-10 production by PPD-stimulated lymphocytes 
(Table 3), the cell fraction which contained both T and B lymphocytes as well as a small proportion of dendritic 
cells and natural killer (NK) cells. Additionally, BCG-driven IL-10 production, but not IL-23 release by DCs 
in the group of TST+ group was significantly more frequent than in the TST- volunteers.

The reported findings provide a possible explanation of the disadvantage of tuberculin-negative BCG 
vaccinated students to develop DTH to PPD, as a consequence of the IL-10 dependent Th2 polarization of T 
cells responding to mycobacterial antigens. On the other hand, development of DTH to PPD in BCG vaccinated 
volunteers appears to be a consequence of the strength of HLA-DR and co-stimulatory receptors expression on 
DCs enabling a polarized Th1 response to mycobacterial antigens (Table 5). Peripheral blood lymphocytes in 
TST+ participants produced significantly more IFN-γ in a direct response to PPD, but also in response to 
PPD or live BCG bacteria presented by the autologous DCs (Table 2 and 5). In view of the reported data, the 
IFN-γ driven IL-10 production, but not IL-23 release by DCs in the group of TST+ group was significantly more 
frequent than in the TST- volunteers.

Taken together, our data suggest that the expression of delayed type hypersensitivity to PPD in young people 
who had been subjected to neonatal BCG vaccination and revaccinated at school age, depends on their 
immanent tendency of Th1/Th2 polarization response to mycobacterial antigens. Differences among the types 
of dendritic cell activities contribute to development of tuberculin reactivity.

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