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**Author:** Boer, Mardi C.

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BCG-vaccination induces divergent pro-inflammatory or regulatory T-cell responses in adults

Mardi C. Boer¹, Corine Prins¹, Krista E. van Meijgaarden¹, Jaap T. van Dissel¹,², Tom H.M. Ottenhoff³*, Simone A. Joosten¹*

¹Department of Infectious Diseases, Leiden University Medical Center, Leiden, the Netherlands
²National Institute for Public Health and the Environment, RIVM, Bilthoven, the Netherlands
³These authors contributed equally to this work

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Abstract

*Mycobacterium bovis* bacillus Calmette-Guérin (BCG), the only currently available vaccine against tuberculosis, induces variable protection in adults. Immune correlates of protection are lacking, and analyses on cytokine-producing T-cell subsets in protected vs. non-protected cohorts have yielded inconsistent results. We studied the primary T-cell response, both pro-inflammatory and regulatory T-cell responses, induced by BCG-vaccination in adults. Twelve healthy adult volunteers, who were tuberculin skin test (TST)-negative, QuantiFERON test (QFT)-negative, and BCG-naive, were vaccinated with BCG and followed up prospectively. BCG-vaccination induced an unexpectedly dichotomous immune response in this small, BCG-naive young adult cohort: BCG-vaccination induced either gamma interferon-positive (IFNγ⁺) interleukin 2-positive (IL2⁺) tumor necrosis factor α-positive (TNFα⁺) polyfunctional CD4⁺ T-cells concurrent with CD4⁻IL17A⁺ and CD8⁻IFNγ⁺ T-cells, or, in contrast, virtually absent cytokine responses with induction of CD8⁺ regulatory T-cells. Significant induction of polyfunctional CD4⁺IFNγ⁺IL2⁺TNFα⁺ T-cells and IFNγ production by peripheral blood mononuclear cells (PBMCs) was confined to individuals with strong immunization-induced local skin inflammation and increased serum C-reactive protein (CRP). Conversely, in individuals with mild inflammation, regulatory-like CD8⁺ T-cells were uniquely induced. Thus, BCG-vaccination either induced a broad pro-inflammatory T-cell response with local inflammatory reactogenicity or, in contrast, a predominant CD8⁺ regulatory T-cell response with mild local inflammation, poor cytokine induction, and absent polyfunctional CD4⁺ T-cells. Further detailed fine mapping of the heterogeneous host response to BCG-vaccination using classical and non-classical immune markers will enhance our understanding of the mechanisms and determinants that underlie the induction of apparently opposite immune responses, and how these impact the ability of BCG to induce protective immunity to TB.
Introduction

Tuberculosis (TB), caused by *Mycobacterium tuberculosis* (Mtb), is the second greatest infectious cause of death worldwide after HIV, accounting for 1.3 million deaths in 2012 [1]. The only available vaccine, *Mycobacterium bovis* bacillus Calmette-Guérin (BCG), protects infants from disseminated forms of TB, but has insufficient and inconsistent efficacy in protecting adults from pulmonary TB [1;2]. A vaccine preventing active pulmonary TB, the contagious form of the disease, would greatly impact the epidemic [3], and a better understanding of vaccine-induced mechanisms of protection is essential in developing new surrogate endpoints [4].

Both CD4+ Th1 (IFNγ+) cells and CD8+ T-cells are critical for protection against TB [5]. Specifically, CD4+ IFNγ+IL2+TNFα+ polyfunctional T-cells have been proposed as correlate of vaccine-induced protective immunity in murine infection models [6]. In infants, BCG-vaccination induced specific cytokine expression in CD4+ and CD8+ T-cells [7-9], including IFNγ+IL2+TNFα+ polyfunctional CD4+ T-cells [10]. However, there was no relation between the presence of such cells and the development of TB during follow-up [11].

In adults, BCG-vaccination induced CD4+ IFNγ+ responses [12-14] as well as IFNγ- and TNFα-secreting CD8+ T-cells with cytotoxic activity [15]. However, data on induction of polyfunctional T-cells by BCG-vaccination in adults have been conflicting [16;17]. In one report, the induction of polyfunctional CD4+ T cells was similar in magnitude in BCG-vaccinated infants and adults; however, when induction was analyzed as the proportion of polyfunctional versus single-cytokine-producing T-cells, the proportion of polyfunctional CD4+ T-cells was larger in children than in adults [16]. Further, studies on latent (controlled) versus active TB in adults yielded variable results on changes in mono- and triple-cytokine producing T-cell subsets [18;19], such that it was suggested that polyfunctional T-cells are also present in active TB disease and that these cells are not a surrogate marker of protection against TB in humans [19;20].

Another explanation for the inconsistent protection induced by BCG against TB in adults is induction of regulatory T-cells (Tregs) by mycobacteria, which can dampen pro-inflammatory responses [21]. In that context, we reported that live BCG triggers the specific activation of CD8+ (but not CD4+) Tregs from peripheral blood mononuclear cells.
(PBMCs) of mycobacterial purified protein derivative (PPD)-responsive adults [22], while
others found that BCG-vaccination induced CD4+ Tregs in newborns [23] and adults [24].
Here, in a small, well-defined cohort of previously BCG-naive adults, we studied the
induction of multiple cytokine-producing as well as regulatory T-cell subsets following
BCG-vaccination.

Materials and Methods

Participants. Dutch volunteers were recruited via posters in the university library. All
volunteers were screened for tuberculosis by anamnesis (history of TB disease or
treatment), by a tuberculin skin test (TST; negative < 5mm), and by the QuantiFERON-TB
gold in-tube test, according to the manufacturer’s specifications. Included volunteers (n = 6
males, n = 6 females; median age 24 years (interquartile range (IQR) 23-25 years); median
weight 70 kg (IQR 67-80 kg); all Dutch, all Caucasian) had not been vaccinated with BCG
at any time prior to entering the trial (anamnestic, presence of scar, or on a vaccination
card), were never treated for TB disease and had negative TST and QuantiFERON test
results. In addition, they did not receive any live vaccination at < 4 weeks prior to BCG-
vaccination. Volunteers were excluded who were pregnant or not generally healthy, who
had fever or received antibiotic treatment < 2 weeks prior to enrollment, or who were
treated with immune modulating drugs < 3 months prior to enrollment; all volunteers tested
negative for HIV at screening.

Procedures. Participants were vaccinated with the live-attenuated BCG Danish strain 1331
(Statens Serum Institute, Denmark) by intradermal injection in the upper arm and were
followed up prospectively: at 2 weeks prior to vaccination, at day of vaccination, at 1, 3 and
7 days after vaccination, at 4, 8 and 12 weeks after vaccination and at 1 year after
vaccination. During follow up the injection site was inspected and photographed, and
adverse events were recorded using a standardized case report form. Venous blood samples
were collected in heparin-containing vacutainers for whole-blood stimulation assays and for
PBMC isolation and cryopreservation according to standard operating procedures. Serum
was collected from serum tubes after blood coagulation and stored at -80ºC.
Calculation of skin inflammation score. Signs of inflammation by visual inspection of the vaccination site and symptoms recorded in volunteer diaries were documented on standardized case report forms and photographed at 4, 8 and 12 weeks after vaccination. The local reaction was scored by two researchers (M.C.B. and C.P.) independently, with one point per sign of inflammation: redness of ≥ 1 cm, swelling of ≥ 1 cm, pus discharge and ulceration, pain, and regional lymph node enlargement (> 90% consensus; disagreements were solved by mutual reexamination of case report forms, photographs and volunteer diaries). The inflammation score was calculated as the cumulative scores of weeks 4, 8, and 12 after vaccination.

C-reactive protein enzyme-linked immunosorbent assay. The serum samples of all pre-vaccination and post-vaccination visits were thawed, and the C-reactive protein (CRP) concentration was measured using a standardized, highly sensitive, CRP human enzyme-linked immunosorbent assay (ELISA) according to the instructions of the manufacturer (Abnova, Heidelberg, Germany).

Whole-blood live BCG-stimulation. Bacillus Calmette-Guérin (Pasteur) was grown in 7H9 plus ADC, frozen in 25% glycerol and stored at -80°C. Before use, bacteria were thawed and washed in phosphate-buffered saline (PBS)/0.05% Tween 80 (Sigma-Aldrich). Then, 1 ml of heparinized blood was added within 1h of blood collection to Sarstedt microtubes (Sarstedt B.V., Etten-Leur, the Netherlands), containing 0.9 x 10⁶ CFU (calculated multiplicity of infection (MOI) of 3), and anti-CD28 and anti-CD49d antibodies as co-stimulants (1 μg/ml, BD Biosciences, Eerembodegem, Belgium) [25] and immediately incubated at 37°C. Staphylococcal enterotoxin B (SEB) (final concentration 5 μg/ml; Toxin Technology, Sarasota, FL, USA) and unstimulated samples were used as controls. After 3 h, Brefeldin A (10 μg/ml; Sigma-Aldrich, Zwijndrecht, the Netherlands) and Monensin (1:1000; BD Biosciences) were added and samples were transferred to a water bath set at 37°C and programmed to switch off after 12h. Samples were harvested the next morning, using EDTA (2mM; Sigma-Aldrich), fixed and erythrocyte-lysed using a fluorescence-activated cell sorter (FACS)-lysing solution (BD Biosciences), and cryopreserved in fetal calf serum with 10% dimethyl sulfoxide (DMSO) [25].
Cell cultures and BCG infection. PBMCs were thawed, and cells were counted using the CASY cell counter (Roche, Woerden, the Netherlands). Infections were done at an MOI of 1.5. SEB (final concentration 2 μg/ml; Toxin Technology) and unstimulated samples were used as controls. PBMCs were cultured in 24-well plates (2 x 10^6/w) for 6 days in Iscove's modified Dulbecco's medium (Life Technologies-Invitrogen, Bleiswijk, the Netherlands) with 10% pooled human serum. For flow cytometric analysis PBMCs were incubated for the last 16h with αCD3/28 beads (Invitrogen) and Brefeldin A (3 μg/ml; Sigma-Aldrich). Lymphocyte stimulation assays were performed using PBMCs (0.5 x 10^6/w in 48-well plates) and stimulation with 5 μg/ml PPD (Statens Serum Institute, Copenhagen, Denmark) at 37°C, 5% CO_2. Phytohemagglutinin (PHA) (final concentration 2 μg/ml; Remel Europe) and unstimulated samples were used as controls. After 7 days, supernatants were collected and an IFN-γ-ELISA (U-CyTech, Utrecht, the Netherlands) was performed. Direct IFN-γ-enzyme-linked immunosorbent spot (ELISpot) assays were performed at 1 year post-vaccination: 250,000 freshly isolated PBMCs were added in AIMV (synthetic non-human serum supplemented) medium (Invitrogen) to 96-well ELISpot plates (Millipore, Bedford, MA, USA), that were pre-coated with anti-IFN-γ-antibody (1-D1K; 5 μg/ml; Mabtech, Stockholm, Sweden) and blocked with AIMV. PBMCs were stimulated overnight with PHA (2 μg/ml), PPD (5 μg/ml) or an antigen 85B (Ag85B) peptide pool (1 μg/ml) in triplicate [26]. For detection, biotinylated anti-IFN-γ-antibody (0.5 μg/ml; Mabtech), streptavidin-alkalic phospatase conjugate (1:1000 dilution in 1% bovine serum albumin (BSA)-PBS; Mabtech IFN-γ-ELISpot kit reagent) and a SigmaFast NBT/BCIP substrate (Sigma-Aldrich) were used. Positivity for vaccine take [27] was defined as an increase of > 100% of the average count in PPD-stimulated wells compared to unstimulated controls and at least 5 spots more than in unstimulated controls [28].

Flow cytometry. Fixed whole-blood samples were thawed and stained in batches. Surface staining included CD3-Brilliant Violet 570 (clone UCHT1), CD19-Pacific Blue (clone HIB19), CD56-Brilliant Violet 421 (clone HCD56) (all Biolegend, London, U.K.), CD14-Pacific Blue (clone TüK4) and CD4-PE-Texas Red (clone S3.5) (both Life Technologies-Invitrogen); and CD8-HorizonV500 (clone RPA-T8), CD45RA-allo-phycocyanin (APC)-H7 (clone HI100) and CD62L-Brilliant Violet 605 (clone DREG-56) (all BD Biosciences). For intracellular staining IL17A-FITC (clone eBio64DEC17; eBioscience, Hatfield, UK),
IFNγ-Alexa Fluor 700 (clone B27), TNFα-APC (clone 6401.1111), IL4-PE (clone 3010.211), and CD69-PeCy5 (clone FN50) (all BD Biosciences); and IL2-Brilliant Violet 650 (clone MQ1-17H12), IL10-Pe-Cy7 (clone JES3-9D7) and IL13-PE (clone JES10-5A2) (all Biolegend) were used in permeabilization solution (Fix&Perm cell permeabilization kit, An Der Grub BioResearch GMBH, Susteren, the Netherlands).

The stimulated PBMCs were labelled with violet LIVE/DEAD stain (Vivid, Invitrogen) and surface stained with CD3-Brilliant Violet 570 (clone UCHT1), CD19-Pacific Blue (clone HIB19), CD56-Brilliant Violet 421 (clone HCD56), and CD39-PE (clone A1) (all Biolegend, London, U.K.); CD14-Pacific Blue (clone TüK4) and CD4-PE-Texas Red (clone S3.5) (both Life Technologies-Invitrogen); and CD8-HorizonV500 (clone RPA-T8; BD Biosciences). Cells were fixed and permeabilized using the Fix&Perm cell permeabilization kit. For intracellular staining the following antibodies were used: CC chemokine ligand 4 (CCL4)-fluorescein isothiocyanate (clone 24006; R&D Systems, Abingdon, UK), Foxp3-Alexa Fluor 700 (clone PCH101; eBioscience), lymphocyte activation gene (LAG)-3-atto 647N (clone 17B4; ENZO Life Sciences, Antwerp, Belgium), and CD25-allophycocyanin-H7 (clone M-A251; BD Biosciences).

Samples were acquired on a BD LSRFortessa using FACSDiva software (version 6.2, BD Biosciences) with compensated parameters. Analysis was performed using FlowJo software (version 9.5.3, Treestar, Ashland, OR, USA) and gates were synchronized per donor for all visits and for both CD4+ and CD8+ T-cell subsets, using the comparison with unstimulated samples and SEB as controls.

**Statistical analyses.** GraphPad Prism (version 6, GraphPad Software, La Jolla, CA, USA) and SPSS statistical software (version 20, SPSS IBM, Armonk, NY, USA) were used for Wilcoxon signed-rank tests and Mann-Whitney tests. To correct for paired resp. unpaired multiple testing, Friedman tests followed by Dunn’s multiple comparisons tests, and Kruskal-Wallis tests followed by Dunn’s multiple comparisons tests, respectively, were used. Only values significant after multiple testing correction are demonstrated.

**Study approval.** Approval was obtained from the Medical Ethical Committee (registration number P 12.87) of the Leiden University Medical Center, the Netherlands. Each participant signed written informed consent prior to inclusion.
Results

Participants
Twelve healthy adults (TST-negative, QuantiFERON-negative) were vaccinated with BCG ($n = 6$ males, $n = 6$ females; median age 24 years (IQR 23 - 25 years); all Caucasian). *Ex vivo* IFNγ-ELISpot assays were performed 1 year post-vaccination to determine the *Mycobacterium*-specific immunity [29]: all recipients tested positive except for one, who nevertheless demonstrated high BCG-induced IFNγ-responses in flow cytometric analysis (Supplementary figure S1).

Local reactogenicity is variable and corresponds with serum CRP and PPD-induced IFNγ production
T-cell responses as well as BCG-vaccine induced local skin reactions varied strongly between individuals. At 4, 8 and 12 weeks post-vaccination, inflammatory symptoms were scored for redness, swelling, ulceration and pus discharge, pain and regional lymph node enlargement. A cumulative skin inflammation score was then calculated (further described in the Materials and Methods). Representative photographs of low- and high-inflammation skin lesions are shown in figure 1A. Participants were subdivided into a low ($n = 6$) and a high ($n = 6$) responder group (referred to as low and high skin inflammation responders, respectively) based on skin inflammation scores using the median cumulative skin inflammation score of 7 as a cut-off (figure 1B).

Since serum CRP has been used previously to study vaccine-induced inflammation [30-32], we determined CRP concentrations by a highly sensitive ELISA. Serum CRP concentrations 3 and 7 days post-vaccination correlated with the cumulative skin inflammation score ($R^2 = 0.76$ at both visits using nonlinear regression, day 7 results shown in figure 1C, left). In high skin inflammation responders, serum CRP was significantly higher 7 days post-vaccination, compared to low skin inflammation responders ($p = 0.03$; figure 1C, right). CRP concentrations at baseline were not different between high and low inflammation responders.
We assessed the *in vitro* IFNγ production by ELISA after PPD stimulation of PBMCs for 7 days. The PBMCs of high skin inflammation responders produced more IFNγ than low skin inflammation responders 4 and 8 weeks post-vaccination (*p* = 0.026 and 0.002, resp.). IFNγ production was significantly induced at 8 and 12 weeks post-vaccination compared to pre-vaccination in high skin inflammation responders (both *p* = 0.031), but not in low skin inflammation responders (figure 1D). Prior to BCG-vaccination, IFNγ production was not significantly different between high vs. low skin inflammation responders.

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**Figure 1.** BCG-vaccination induces highly variable local inflammation that corresponds with serum CRP and IFNγ production. A: Photographs of low versus high degrees of skin inflammation at the vaccination site 12 weeks after BCG-vaccination (left photograph, cumulative inflammation score of 3; right photograph, cumulative inflammation score of 11). B: Signs of inflammation of the vaccination lesion were recorded, and a skin inflammation score was calculated as the cumulative scores of 4, 8, and 12 weeks after vaccination. This divided recipients into 6 low and 6 high responders around a median skin inflammation score of 7.
Figure 1 (continued). BCG-vaccination induces highly variable local inflammation that corresponds with serum CRP and IFNγ production. C: Serum samples of volunteers were collected at all visits for CRP ELISA. The serum CRP concentrations at day 7 after vaccination correlated with the cumulative skin inflammation scores ($R^2 = 0.76$ using nonlinear regression; left); high skin inflammation responders had significantly higher CRP values than low responders 7 days after vaccination (6 individuals in each group, with line at median; $p = 0.03$, Mann-Whitney test; right). D: PBMCs were stimulated with PPD, supernatants were harvested after 7 days, and IFNγ production was measured by ELISA. IFNγ production by PBMCs of high, compared to low skin inflammation responders was significantly higher at 4 and 8 weeks post-vaccination, and IFNγ production was significantly induced only in comparison to that at pre-vaccination in high skin inflammation responders (6 recipients in each group, line at median; Mann-Whitney test for comparison between groups, Wilcoxon signed-rank test for within-group testing).

CD4⁺IFNγ⁺IL2⁺TNFα⁺ T-cells and CD8⁺IFNγ⁺ T-cells are induced in high inflammation responders

Whole-blood samples from pre-vaccination and at 4, 8, 12 weeks and 1 year after vaccination were stimulated directly ex vivo with live BCG for 16h. The gating strategy is shown in Supplementary figure S2A, and the representative flow cytometric analyses of co-
expression of cytokines in CD4+ T-cells with negative and positive controls are shown in Supplementary figure S2B, compliant with MIATA (minimal information about T-cell assays)-guidelines [33].

BCG-vaccination induced significant IFNγ-expression in CD4+ T-cells, but only in high inflammation responders (p = 0.031, at 4 and 12 weeks post-vaccination) and not in low inflammation responders (figure 2A, left graphs). Polyfunctional CD4+IFNγ+IL2+TNFα+ T-cells were significantly induced in BCG-recipients, but a division of the high versus low inflammation responders revealed that the proportion of CD4+IFNγ+IL2+TNFα+ T-cells was significantly increased in the high versus low inflammation responders (p = 0.015, p = 0.048, and p = 0.041 at 4, 8, and 12 weeks after vaccination, resp.), but it was almost absent in the latter group. Thus, significant induction of CD4+IFNγ+IL2+TNFα+ T-cells was confined to high inflammation responders (figure 2A, right graphs).

The majority of IFNγ, TNFα and/or IL2-expressing CD4+ T-cell subsets of high inflammation responders peaked at 8 weeks post-vaccination (Supplementary figure S3A). Single-, double- and triple-cytokine-producing CD4+ T-cell subsets consisted predominantly of effector cells at 4, 8 and 12 weeks post-vaccination (Supplementary figure S3B).

CD4+IL17A+ T-cells were induced significantly post-vaccination, but only in high inflammation responders (figure 2B). Similarly, significant induction of IFNγ-expression in CD8+ T-cells was confined to high inflammation responders (figure 2B). No polyfunctional CD8+ T-cell responses could be detected (data not shown). CD4+ T-cells did not co-express IL17A and IFNγ (representative graph in Supplementary figure S2B). IL17A-expression correlated with IFNγ-expression in CD4+ T-cells at 4, 8 and 12 weeks post-vaccination (figure 2C upper graphs; R² 0.86, 0.94, and 0.83, resp., using nonlinear regression). IFNγ-expression in CD8+ T-cells highly correlated with IFNγ-expression in CD4+ T-cells (figure 2C lower graphs; R² 0.98, 0.85, and 0.99 at 4, 8, and 12 weeks post-vaccination, resp., using nonlinear regression), thus revealing a broad pro-inflammatory response induced by BCG-vaccination in high inflammation responders. No induction of IL10- or IL4/IL13-expression was observed in CD4+ T-cells in low or in high inflammation responders (figure 2D).

The association between skin inflammation score and induction of CD4+ cytokine co-expression was further substantiated by segregating vaccinees based on induction versus no
induction of polyfunctional CD4⁺IFNγ⁺IL2⁺TNFα⁺ T-cells instead of local inflammation scores. This revealed an increased total skin inflammation score in vaccinees with induction of CD4⁺ polyfunctional T-cells, compared to vaccinees with no polyfunctional CD4⁺ T-cell induction (n = 7 cytokine-responders versus n = 5 non-responders, including in the latter group a low skin inflammation responder with a skin inflammation score of 6; p = 0.037, Mann-Whitney test; Supplementary figure S4A). The differences in the CRP concentration 7 days after vaccination did not reach statistical significance in cytokine-responders vs. non-responders (Supplementary figure S4B; p = 0.078, Mann-Whitney test).

Figure 2. BCG-vaccination induces a pro-inflammatory cytokine response, predominantly in high skin inflammation responders. A: BCG-vaccination induces IFNγ⁺ and IFNγ⁺IL2⁺TNFα⁺ CD4⁺ T-cells (upper graphs) in whole blood following BCG-stimulation for 16h, but the frequency of polyfunctional CD4⁺ IFNγ⁺IL2⁺TNFα⁺ T-cells is significantly increased in high, compared to low, skin inflammation responders, and the induction of CD4⁺ IFNγ⁺ and polyfunctional CD4⁺ IFNγ⁺IL2⁺TNFα⁺ T-cells is only significant in high, not in low, skin inflammation responders (lower graphs); n = 6 recipients in each group. Horizontal lines indicate median; Mann-Whitney test for comparison between groups, Wilcoxon signed-rank test for within-group testing.
Figure 2 (continued). BCG-vaccination induces a pro-inflammatory cytokine response, predominantly in high skin inflammation responders. B: CD4+IL17A+ T-cells and CD8+IFNγ+ T-cells are significantly induced in high skin inflammation responders. C: Expression of the pro-inflammatory cytokines IL17A in CD4+ T-cells and of IFNγ in CD8+ T-cells both highly correlate with IFNγ-expression in CD4+ T-cells, as determined by nonlinear regression. (Data are shown for all recipients at 4, 8, and 12 weeks after vaccination; whole-blood BCG-stimulation for 16h). Values of 0 were not plotted. D: In CD4+ T-cells, neither IL4/IL13 nor IL10 was significantly induced in any responder group following whole-blood BCG stimulation for 16h.
Figure 3. Treg markers on CD8$^+$ T-cells, but not on CD4$^+$ T-cells, are activated by \textit{in vitro} live BCG-stimulation of PBMCs of vaccinees 8 weeks after vaccination. A: Flow cytometric analysis of (co-)expression of Treg markers CD25, CD39, and Foxp3 on CD4$^+$ and CD8$^+$ T-cells in unstimulated PBMCs and PBMCs stimulated for 6 days with live BCG. The gating strategy was similar as that described in Supplementary figure S2A, with the addition of a LIVE/DEAD vivid stain. B: Treg markers on CD8$^+$ but not CD4$^+$ T-cells were significantly activated 6 days after \textit{in vitro} live BCG stimulation, compared to unstimulated PBMCs, at 8 weeks post-vaccination. On CD4$^+$ T-cells, only expression of CD25 was significantly different from unstimulated samples (box-whiskers of all individuals, with line at median; whiskers minimum (min) to maximum (max)). C: Co-expression of Treg markers is significantly activated by live BCG on CD8$^+$ but not CD4$^+$ T-cells 8 weeks after BCG-vaccination (box-whiskers of all individuals, with line at median; whiskers min to max) (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; Wilcoxon signed-rank test).
CD8\(^+\), but not CD4\(^+\) T-cells express regulatory markers after live BCG stimulation \textit{in vitro}

PBMCs were stimulated for 6 days with live BCG to assess the expression of the regulatory markers CD25, Foxp3, CD39, lymphocyte activation gene-3 (LAG-3) and CCL4 (macrophage inflammatory protein-1\(\beta\)) by FACS-analysis [22;34;35] (gating strategy in Supplementary figure S2A). BCG-induced expression of Treg markers on CD4\(^+\) and CD8\(^+\) T-cells was then compared to that on unstimulated control samples at 8 weeks post-vaccination, the peak response (figure 3A). Expression of the single Treg markers CD25, Foxp3, CD39, LAG-3 and CCL4 was significantly induced on CD8\(^+\) T-cells by \textit{in vitro} live BCG stimulation 8 weeks post-vaccination (\(p < 0.01\) for CD25, Foxp3 and CCL4; \(p < 0.001\) for CD39 and LAG-3). In contrast, on CD4\(^+\) T-cells only CD25 was significantly induced by BCG stimulation (\(p < 0.05\)) (figure 3B).

We then analysed co-expression of Treg markers using Boolean gating with synchronized gates on a per-donor basis for both CD4\(^+\) and CD8\(^+\) T-cells [22]. Co-expression of Treg markers was significantly induced on CD8\(^+\) but not CD4\(^+\) T-cells by \textit{in vitro} live BCG stimulation 8 weeks post-vaccination (figure 3C; \(p < 0.001\)). On CD4\(^+\) T-cells, no expression of CD25\(^+\)Foxp3\(^+\)CD39\(^+\) was induced in either high or low inflammation responders (Supplementary figure S5).

CD8\(^+\)CD25\(^+\)CD39\(^+\)Foxp3\(^+\) T-cells increase post-vaccination only in low inflammation responders

We then compared Treg markers on CD8\(^+\) T-cells post- and pre-vaccination; figure 4A displays a representative flow cytometric analysis of induction of CD25\(^+\)CD39\(^+\) co-expression and expression of Foxp3 on CD25\(^+\)CD39\(^+\) CD8\(^+\) T-cells from a low skin inflammation responder. Compared to pre-vaccination, BCG-vaccination significantly induced CD8\(^+\)CD25\(^+\)Foxp3\(^+\)CD39\(^+\) T-cells as well as CD8\(^+\)CD25\(^+\)Foxp3\(^+\)CD39\(^+\)LAG-3\(^+\)CCL4\(^+\) T-cells, but only in low inflammation responders (figure 4B; \(p = 0.031\) at 8 weeks post-vaccination; \(p = 0.031\) at 4 and 8 weeks post-vaccination, resp.). In contrast, in high inflammation responders, the expression of CD25\(^+\)Foxp3\(^+\)CD39\(^+\) or CD25\(^+\)Foxp3\(^+\)CD39\(^+\)LAG-3\(^+\)CCL4\(^+\) on CD8\(^+\) T-cells post-vaccination did not differ from the patterns pre-vaccination (figure 4B).
Figure 4. BCG-vaccination induces CD8\(^+\)CD25\(^+\)CD39\(^+\)Foxp3\(^+\) T-cells in low inflammation responders.

A: Representative flow cytometric analysis of co-expression of the Treg markers CD25 and CD39 on CD8\(^+\) T-cells of a recipient with a low degree of skin inflammation (upper graphs), and the percentage of CD8\(^+\)CD25\(^+\)CD39\(^+\) T-cells expressing Foxp3 (lower graphs; black overlay of CD8\(^+\)CD25\(^+\)CD39\(^+\) T-cells over total CD8\(^+\) T-cells), 6 days after live BCG stimulation of PBMCs. B: Treg markers are significantly induced by BCG-vaccination on PBMCs stimulated with live BCG for 6 days, but only on CD8\(^+\) T-cells from low inflammation responders. The frequency of Treg markers remained constant pre- and post-vaccination on CD8\(^+\) T-cells from high inflammation responders. Depicted are CD25\(^+\)Foxp3\(^+\)CD39\(^+\) co-expression (upper graphs) and CD25\(^+\)Foxp3\(^+\)CD39\(^+\)LAG-3\(^+\)CCL4\(^+\) co-expression (lower graphs) on CD8\(^+\) T-cells of low responders (left) versus high responders (right) (box-whiskers of 6 individuals in each group, with line at median; whiskers min to max; Wilcoxon signed-rank test).
Of interest, CD8⁺CD25⁺Foxp3⁺CD39⁺ T-cells and CD8⁺CD25⁺Foxp3⁺CD39⁺LAG-3⁺CCL4⁺ T-cells were still significantly increased 1 year post-vaccination in low inflammation responders (for both \( p = 0.031 \)), suggesting that BCG-vaccination can induce long-term imprinting of CD8⁺ Treg phenotypes in individuals with low inflammation following vaccination.

**Discussion**

In this study, we describe high inter-individual variability in T-cell cytokine and regulatory responses following BCG-vaccination of BCG-naive healthy young adults in a setting where TB is not endemic. The unexpectedly dichotomous T-cell response consisted of either concurrent induction of IL2⁺, TNFα⁻ and IFNγ⁻ co-expressing polyfunctional CD4⁺ T-cell subsets, CD4⁺IL17A⁺ T-cells, and CD8⁺IFNγ⁺ T-cells in high inflammation responders, or an almost absent cytokine response accompanied by the induction of CD8⁺ regulatory T-cells in low inflammation responders. We quantified local reactivity by classical clinical symptoms of inflammation and found that the total skin inflammation score correlated with serum CRP early post-vaccination. Significant induction of IFNγ⁺IL2⁺TNFα⁺-polyfunctional CD4⁺ T-cells was confined to high inflammation responders, while the induction of regulatory-phenotype CD8⁺CD25⁺CD39⁺Foxp3⁺ and CD8⁺CD25⁺Foxp3⁺CD39⁺LAG-3⁺CCL4⁺ T-cells was confined to low inflammation responders.

In theory, this study could have been limited by the description of T-cell responses based on the skin inflammation score, since dividing high and low skin inflammation groups using the median as cut off dichotomizes the described response. However, the dichotomy was also based on the induction of polyfunctional CD4⁺IFNγ⁺IL2⁺TNFα⁺ T-cells, and this revealed a significantly increased total skin inflammation score in vaccinees with CD4⁺ polyfunctional T-cells, compared to vaccinees with no polyfunctional CD4⁺ T-cell induction. Thus, it is unlikely that the described variability in responses is caused by a dichotomized representation, and this further affirms the relation between skin reactivity.
and cytokine responses. Further, the opposing immune responses and phenotypes were observed within a relatively small cohort. Variability in BCG-immunogenicity has been ascribed to differences in pre-existing anti-mycobacterial responses in endemic vs. non-endemic settings [36;37], the presence of helminth infections [38], variations in the BCG-vaccine strain [39;40], and host genetic factors [41]. In addition, timing of sampling and technical variability may influence detection of cytokines [42;43]. This cohort, though small, was uniform in terms of age, genetic background, BCG-vaccine strain (Danish strain 1331), sampling and testing, in a setting not endemic for TB or helminth infections. This excludes the above-mentioned possible confounders, and points to an unexpectedly large variation in adult human primary BCG-vaccine induced immune responses. Importantly, we confirmed vaccine take at 1 year post-vaccination by IFNγ-ELISpot, which was positive for both high and low inflammation responders. IFNγ-ELISpot has been described as the most sensitive assay for detecting long-term vaccine responses [29] and is used in TB-vaccine trials to describe the magnitude of vaccine-induced immunity. However, a sole reliance on IFNγ-ELISpot would disregard variability in other assays, thereby not fully capturing possible correlations between variation of the human immune response and vaccine-induced protection. The etiology of this variation remains unknown, but its unravelling could contribute significantly to a better understanding of BCG and related TB-vaccine induced immunity.

The height of the in vitro cytokine response in BCG-vaccinated infants was associated with scarring of the BCG-vaccination site, but only in response to mycobacterial antigens, not unrelated antigens [44]. Also, cell-mediated immunity, as assessed by a leukocyte migration inhibition test, correlated with infant local skin reactivity 8 weeks after BCG-vaccination, but not with TST-conversion after vaccination [45]. The absence of an association between BCG-induced TST-conversion and immunity against TB has been confirmed in various populations [46]. Here, induction of cytokine responses was confined to recipients with high skin reactivity, suggesting that a simple phenotype like vaccine-induced skin inflammation might be used as a marker of strong pro-inflammatory T-cell induction in adults. The skin inflammation score was associated with serum CRP concentration 7 days post-vaccination, thus the absence of an increase in CRP early post-vaccination might be used as an indicator of absent pro-inflammatory T-cell responses at later time-points.
Interference of CD4+ Tregs with effector immunity has been described in active TB [47;48]. Following MVA-85A-vaccination circulating CD4+CD25+Foxp3+ T-cells were increased in recipients with low antigen 85A-specific IFNγ-responses compared to high IFNγ-responders [28], and MVA-85A-induced CD4+ Tregs inhibited IL17A-production in vitro [49]. Interestingly, IL10-producing CD8+ Tregs were described in TB-patients anergic to intradermally injected PPD [50]. Thus, Tregs can interfere with inflammatory and specific antigen-induced cytokine responses. We previously reported in vitro activation of CD8+ (but not CD4+) Tregs by live BCG, both phenotypically and functionally, in mycobacterially-sensitized but not PPD-unresponsive donors [22;34;35]. These BCG-activated CD8+ Tregs expressed CD25 and LAG-3 and inhibited Th1-responses through secretion of CCL4 [35]; in addition, we reported CD8+CD39+ Tregs which utilized CD39 to suppress Th1 proliferation [34]. Here, we found that CD8+CD25+CD39+Foxp3+ and CD8+CD25+Foxp3+CD39+LAG-3+CCL4+ T-cells were induced following BCG-vaccination. Interestingly, the frequency of CD8+ T-cells with these Treg phenotypes was significantly increased only in comparison to that at pre-vaccination in low inflammation responders with low to absent cytokine responses, suggesting an inverse relation between the induction of CD8+ Tregs and BCG-induced skin inflammation with T-cell cytokine production.

In murine leishmaniasis, cytokine-producing polyfunctional T-cells were inversely correlated with lesion size after (dermal) challenge [6]. In dermal BCG-challenge models in humans, vaccination-induced IFNγ-ELISpot-responses were inversely correlated with PCR quantification of BCG-load in biopsy specimens of the challenge site [51]. The PCR quantification method was suggested as a measure of pathogen clearance, possibly reflecting some degree of protective immunity, which might be used in human TB-vaccine trials. Based on the current study, it will also be relevant to assess the presence of pro-inflammatory vs. regulatory T-cells in skin vaccine or challenge lesions and to further validate the modulation of skin inflammation and/or pathogen clearance by CD8+ Tregs in relevant models. Of note, in low inflammation responders CD8+CD25+Foxp3+CD39+ T-cells were still significantly increased at 1 year post-vaccination, suggesting that BCG-vaccination can induce long-term imprinting of a CD8+ Treg phenotype with a significant memory component. Further work is needed to assess their precise longevity.
In conclusion, our results show an unexpectedly dichotomous host response to BCG-vaccination in a cohort of BCG-naive adults. It will be important to assess these divergent outcomes in settings where TB is endemic in order to determine the impact of these highly variable outcomes on protective efficacy against TB. The use of classical inflammation markers as non-classical indicators of vaccine-induced pro-inflammatory responses might be a simple means to assist in assessing BCG-induced phenotypes, even in small cohorts. Further detailed fine mapping of the heterogeneous host response to BCG-vaccination using classical and non-classical immune markers will enhance our understanding of the mechanisms and determinants that underlie the induction of apparently opposite immune responses and how these impact the ability of BCG to induce protective immunity to TB.

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Supplementary figures

Supplementary figure S1. IFNγ-ELISpot results at 1 year after vaccination. Ex vivo IFNγ-ELISpot assays using freshly isolated PBMCs were performed 1 year after vaccination to verify vaccine-induced immunity. The person that lacked positive response by ELISpot demonstrated high IFNγ-responses in flow cytometric analysis, indicating vaccine take for this person.
Supplementary figure S2. Gating strategy and cytokine expression. S2A, gating strategy: for flow cytometric analysis of fixed whole blood samples, cells were gated on single cells, lymphocytes, CD3$^+$ vs. CD14-CD19-CD56-negative, and CD8$^+$CD4$^-$ vs. CD4$^+$CD8$. For PBMCs six days after live BCG-stimulation, a violet-live/dead-stain was added prior to extracellular staining and cells were gated on CD3$^+$ vs. (live/)dead-CD14-CD19-CD56-negative. S2B, expression of cytokines in CD4$^+$ T-cells: cytokine production by CD4$^+$ T-cells after overnight live BCG-stimulation of whole blood; also depicted are unstimulated and SEB controls. No co-expression was observed of IFN$\gamma$ and IL17A.
Supplementary figure S3A. CD4⁺ T cells of high skin inflammation responders. Whole-blood samples stimulated with live BCG for 16 hours and subsequent intracellular cytokine staining: almost all BCG-induced CD4⁺ cytokine-producing subsets peaked at 8 weeks after vaccination. CD4⁺ cytokine-producing subsets of high skin inflammation responders at 4, 8, and 12 weeks after vaccination were compared with pre-vaccination (*p < 0.05 in Friedman with Dunn’s multiple comparison test).
Supplementary figure S3 (continued). CD4⁺ cytokine-producing subsets and memory compartment in high skin inflammation responders. Supplementary S3B, memory compartments of CD4⁺ cytokine-producing subsets: the majority of single-, double- and triple-cytokine producing T-cells are effector cells, in whole-blood samples stimulated with live BCG for 16 hours. Pie chart representation of the proportion of effector (CD69⁺), effector memory (CD69⁺CD45RA⁻CD62L⁻), central memory (CD69⁻CD45RA⁻CD62L⁺) and naïve (CD69⁻CD45RA⁺CD62L⁺) CD4⁺ T-cells for different cytokine-producing subsets as derived by Boolean gate analysis. Only populations of > 50 cells were included for analysis with a minimum of 3 donors per visit. Effector cell proportions were not significantly different between cytokine-producing subsets or between visits (p < 0.05 deemed significant, Kruskal-Wallis with Dunn’s multiple comparisons test).
S4A

Supplementary figure S4. Higher total skin inflammation score in vaccinees with CD4\(^+\) polyfunctional T-cell induction. Vaccinees were divided by IFN\(\gamma\)+IL2+TNF\(\alpha\) CD4\(^+\) T-cell induction, and (cumulative) skin inflammation scores (A) and CRP concentration at day 7 (B) were compared. The skin inflammation score was significantly higher in vaccinees with induction of CD4\(^+\) polyfunctional T-cells, compared to vaccinees with no induction of CD4\(^+\) polyfunctional T-cells (threshold for CD4\(^+\) T-cell induction set at change from pre-vaccination larger than the highest pre-vaccination value; 7 responders vs. 5 non-responders in each graph with line at median; Mann-Whitney test).

S5

Supplementary figure S5. CD25\(^+\)CD39\(^+\)Foxp3\(^+\) expression on CD4\(^+\) T-cells as ratio BCG-stimulated : non-stimulated. Co-expression of CD25, Foxp3 and CD39 on CD4\(^+\) T-cells (on day 6 following PBMC stimulation with or without live BCG) was analyzed as the ratio BCG-stimulated : non-stimulated. BCG-vaccination did not induce significant induction compared to pre-vaccination of CD4\(^+\)CD25\(^+\)Foxp3\(^+\)CD39\(^+\) T-cells (A); also, dividing recipients in high vs. low inflammation scores did not reveal any significant induction of Treg marker expression on CD4\(^+\) T-cells as compared to pre-vaccination (B) (dot plots with line at median for all recipients (A; \(n = 12\)) and recipients divided by skin inflammation score (B; 6 individuals in each group); significance considered as \(p < 0.05\) in Friedman with Dunn’s multiple comparisons test).