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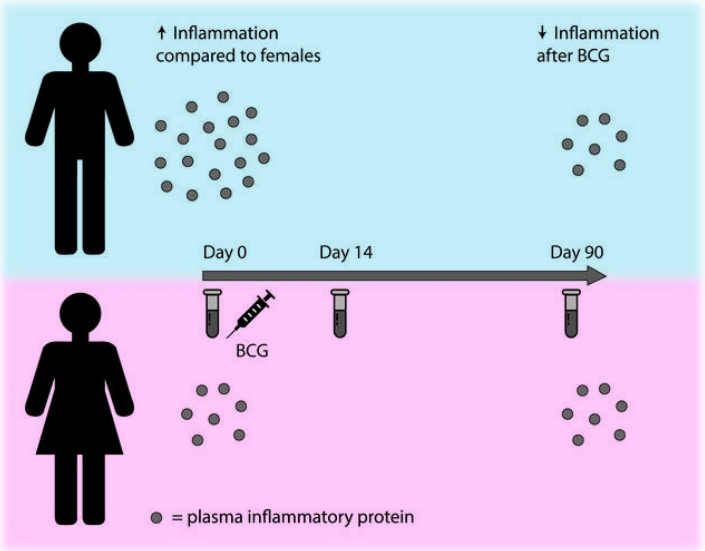
Graphical abstract



BCG vaccination in humans inhibits systemic inflammation in a sex-dependent manner

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Declaration of Interests

MGN and LABJ are scientific founders of Trained Therapeutics Discovery. All other authors declare no financial interest.

ABSTRACT

Background. Induction of innate immune memory, also termed trained immunity, by the anti-tuberculosis vaccine Bacillus Calmette-Guérin (BCG) contributes to protection against heterologous infections. However, the overall impact of BCG vaccination on the inflammatory status of an individual is not known: while induction of trained immunity may suggest increased inflammation, BCG vaccination has been epidemiologically associated with a reduced incidence of inflammatory and allergic diseases.

Methods. We investigated the impact of BCG (BCG-Bulgaria, InterVax) vaccination on systemic inflammation in a cohort of 303 healthy volunteers, as well as the effect of the inflammatory status on the response to vaccination. A targeted proteome platform was used to measure circulating inflammatory proteins before and after BCG vaccination, while ex-vivo *Mycobacterium tuberculosis* and *Staphylococcus aureus* induced cytokine responses in peripheral blood mononuclear cells were used to assess trained immunity.

Results. While BCG vaccination enhanced cytokine responses to restimulation, it reduced systemic inflammation. This effect was validated in three smaller cohorts, and was much stronger in men than in women. In addition, baseline circulating inflammatory markers were associated with *ex vivo* cytokine responses (trained immunity) after BCG vaccination.

Conclusion. The capacity of BCG to enhance microbial responsiveness while dampening systemic inflammation should be further explored for potential therapeutic applications.

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INTRODUCTION

The traditional view of vaccines is that they protect against a particular infection by induction of long-lasting specific adaptive immune memory. The discovery of the induction of non-specific innate immune memory (also termed *trained immunity*) by the anti-tuberculosis vaccine Bacillus Calmette-Guérin (BCG) has led to a paradigm shift in our understanding of our immune system (1, 2). BCG vaccination can induce epigenetic modifications and metabolic rewiring of monocytes, resulting in increased cytokine responses upon subsequent non-related pathogen challenge, up to one year after vaccination (3-6). The longevity of this effect is explained by BCG-induced reshaping of hematopoietic stem and progenitor cells within the bone marrow compartment, resulting in long-lasting transcriptional changes associated with myeloid cell development and function (3, 4). Recent BCG vaccination was shown to reduce *Plasmodium falciparum* parasitemia in a controlled human malaria infection model (5), as well as yellow fever viremia following yellow fever vaccination (6), suggesting that BCG-induced trained immunity also contributes to enhanced overall protection of infants after BCG vaccination (7-12). However, future studies are warranted to assess the duration of these effects.

While studies so far suggest that BCG vaccination elicits enhanced activation only upon subsequent reinfection (5, 6), concerns may be raised that BCG could promote a pro-inflammatory environment facilitating the development of inflammatory mediated diseases such as atherosclerosis (13-15), auto-immune and auto-inflammatory diseases (16, 17). On the other hand, epidemiological studies suggest that BCG vaccination reduces the risk of atopy, eczema and asthma (18-22), diseases in which inflammation plays an important role. The impact of BCG vaccination on the inflammatory status of an individual needs therefore to be explored. In addition, BCG-induced specific (23, 24) and non–specific (7, 25) protective effects vary across different settings and are variable between healthy volunteers (5, 6, 26). Understanding this variability may help identify individuals that will specifically benefit from BCG vaccination, or other interventions aimed at induction of trained immunity (2, 27). The pre-vaccination inflammatory status may contribute towards observed variability in immune responses

after BCG vaccination, as was recently shown for hepatitis B (28), and yellow fever vaccination (29). Therefore, we aimed to investigate the interaction between inflammation and BCG vaccination by assessing a comprehensive set of circulating inflammatory biomarkers before and after BCG vaccination in a cohort of 303 healthy volunteers from the Human Functional Genomics Project (300-BCG cohort, www.humanfunctionalgenomics.org), and we validated the findings in three independent cohorts.

RESULTS

BCG vaccination downregulates circulating inflammatory markers

A total of 307 healthy volunteers were included in the 300BCG cohort. Four participants were excluded from further analysis due to medication use, resulting in 303 volunteers who completed the first visit (**figure 1**). 56% of the participants were female, the mean age was 26 years (range 18-71), and the mean body mass index (BMI) was 22.5 (± 2.6 SD) kg/m^2. BCG scars (data available for 286 volunteers) developed in 271 individuals (95%), with a mean size of 0.42 cm (± 0.17 SD) three months after vaccination.

A targeted proteome platform was used to measure 92 inflammatory markers before (n = 301), and two weeks (n = 292) and three months (n = 277) after BCG vaccination. The quality of the measurement was high, with 99% of the samples passing quality control. Overall, 73 of the 92 (79%) proteins were detected in at least 75% of the plasma samples and included in the analysis. Numerous baseline circulating inflammatory proteins showed a positive correlation with baseline whole blood counts of immune cell subsets (**figure 2A**), as exemplified by the association of circulating oncostatin M (OSM) concentrations and neutrophil counts (**figure 2B**) or circulating IL-6 concentrations and monocyte counts (**figure 2C**). The fact that a number of inflammatory mediators are associated with cell counts is not necessarily surprising, as a significant number of the inflammatory mediators are correlated with each other, and in turn inflammation is associated with immune cell numbers in the blood. Surprisingly however, one third of the proteins (25 out of 73) showed a significant decrease two weeks after BCG vaccination (**figure 3A**). Of the 25 proteins with significantly reduced concentrations at two weeks, 10 remained lower three months after BCG vaccination (**figure 3B**), such as tumor necrosis factor ligand superfamily member 12 (TWEAK) and sirtuin 2 (SIRT2) (**figure 3C-D**)**.** No circulating protein was found to be significantly increased three months after BCG vaccination. An overview of fold changes in circulating inflammatory proteins is included in **supplementary table 1**.

As an internal validation for a true effect of BCG vaccination, fold changes of significantly changed proteins were compared between BCG scar positive volunteers (n = 271) versus volunteers who did not develop a scar (n = 15). In total five proteins differed significantly between scar positive and scar negative volunteers (**figure 4A**), with all proteins being lower in individuals that had a scar three months after vaccination. SIRT2 is given as an example of a protein which is significantly lower in scar-positive individuals three months after BCG vaccination in **figure 4B**. Only a small proportion of participants (5%) did not develop a scar. Therefore, the circulatory proteins that significantly changed after BCG vaccination were also correlated to scar size. In addition to the five proteins that showed a significant difference between scar negative and scar positive volunteers, five other proteins (ADA, MCP-2, Caspase-8, CCL23 and OPG) were also significantly associated with scar size. The direction of the effect was similar for all ten proteins: an increase in scar size was associated with a stronger reduction in the circulatory protein after BCG vaccination, further supporting the finding that BCG-induced immunological effects result in decreased systemic inflammation.

To validate our findings, this identical set of proteins was determined before and after BCG vaccination in plasma samples of 39 adult volunteers from three independent BCG vaccination trials (4-6). Even within these small cohorts, we were able to validate our findings regarding reduced concentrations of ADA, TWEAK, delta and notch-like epidermal growth factor-related receptor (DNER), and neurotrophin-3 (NT-3), and increased concentration of IL-17C after BCG vaccination (**figure 5**).

BCG vaccination affects whole blood cell counts

Considering the observed differences in inflammatory status post BCG vaccination, we evaluated whole blood counts before and after BCG vaccination. Although total white blood counts remained stable after vaccination, BCG vaccination induced a slight increase in both lymphocyte (median 1.87 × 10^9/L at baseline and 1.97 × 10^9/L at two weeks, p = 0.009) and monocyte (median 0.47 × 10^9/L at baseline and 0.48 × 10^9/L at two weeks, p = 0.003) counts, which returned to baseline between two

weeks and three months after vaccination. In addition, red blood cell counts and hemoglobin levels showed a slight reduction two weeks after vaccination ($p < 0.0001$ for both), eosinophil counts showed a slight increase up to three months ($p = 0.002$), and platelet counts a mild reduction three months after vaccination ($p = 0.0005$). An overview of median whole blood cell counts per visit and p-values is included in **supplementary table 2**.

Anti-inflammatory effect of BCG vaccination is sex-dependent

We next examined the effect of age, sex and cytomegalovirus (CMV) serostatus on the concentrations of inflammatory proteins. The median age of our study population was 23 (range 18-71), and 80% of the cohort was 25 or younger. Of the 73 proteins analyzed in our cohort, 6 had a negative correlation with age, and 19 a positive correlation with age (FDR < 0.05). We also measured CMV IgG in the plasma of our study participants. Five (CXCL9, FGF-19, TRAIL, CXCL10, and CXCL11) out of the 73 proteins analyzed in our study were higher in the CMV IgG positive individuals (24%) compared to the CMV IgG negative individuals ($p<0.05$), but none of these differences where statistically significant after correction for multiple testing.

Stratified by sex (males n = 132, females n = 171), concentrations of inflammatory markers were significantly different between males and females at baseline (**figure 6A**). Pre-vaccination concentrations of 41 inflammatory markers were significantly higher in males compared to females, and 6 proteins were found to be significantly lower in males compared to females. These sex-dependent differences in inflammatory markers were validated in a second set of healthy volunteers (males n = 215, females n = 278). In total, 34 proteins were significantly different between males and females in both healthy cohorts (**figure 6B**). Of these 34, only 3 proteins were higher in females, while 31 were significantly higher in males.

Strikingly, the effect of BCG on systemic inflammation appeared to be much stronger in males, with 25 proteins showing a significant lower concentration two weeks after BCG vaccination after correction for multiple testing and none a higher concentration (**figure 6C**). Three months after BCG vaccination, 16 of the 25 downregulated proteins at the two weeks timepoint remained significantly lower compared to baseline in males (**figure 6D**). In contrast, no significant changes were found at two weeks or three months after BCG vaccination in females after correction for multiple testing. An overview of fold changes in circulating inflammatory proteins can be found in **supplementary table 1**.

Baseline characteristics such as age and BMI did not differ between males and females, but there were minor significant differences in red blood cell, platelet, monocyte and eosinophil counts (**supplementary table 3**). In order to try to explain the sex-differential effects, we correlated circulating concentrations of adipokines (adiponectin, resistin and leptin; higher in females), and testosterone levels (higher in males), with concentrations of the inflammatory proteins that were downregulated after BCG vaccination in males but not in females. Interestingly, baseline plasma testosterone showed a negative association with fold increase of several circulating inflammatory proteins after BCG vaccination in males (**figure 7A**). This relation is given as an example for CXCL1 in **figure 7B**.

Correlation between inflammatory proteins and ex vivo cytokine production is sex-dependent

We next examined if inflammatory protein profiles predicted ex-vivo PBMC-derived cytokine production before and after BCG vaccination. Two weeks and three months after BCG vaccination, both *M. tuberculosis* and *S. aureus*-induced production of innate cytokines were upregulated, *S. aureus* induced TNF-α given as an example in **figure 8A**, in both men and women (sex-specific data not shown). Specific adaptive immune memory responses, as assessed by specific stimulation of IFN-γ production with *M. tuberculosis*, were also upregulated by BCG vaccination (**figure 8B**). At two weeks after vaccination, the increase in IFN-γ in response to *M. tuberculosis* was significantly higher in females compared to males ($p = 0.025$, **figure 8C**). No changes in lymphocyte or monocyte percentages within

the PBMC fraction could be observed after BCG vaccination (**supplementary table 4**)**.** Numerous pre-vaccination inflammatory proteins correlated with the increase in innate cytokine production capacity following BCG vaccination (**figure 8D**). Circulating inflammatory proteins predominantly correlated with trained immunity responses two weeks after vaccination, and less with longer term responses three months after vaccination. Interestingly, females mostly showed positive correlations whereas males only showed negative correlations between baseline circulating proteins and trained immunity responses following BCG vaccination, as for instance clearly shown for the relations between plasma ADA, CD5, CD8a, IL-12B, TNFRSF9 and increase in *S. aureus*-induced IL-6. Especially in males, lower baseline concentrations of several inflammatory proteins were associated with increased *M. tuberculosis*-induced IL-1β, IL-6 and TNF-α responses after BCG vaccination. In contrast, higher baseline concentrations of several other inflammatory proteins were associated with enhanced *M. tuberculosis*-induced IFN-γ responses. Interestingly, circulating IL-10 showed a strong positive correlation with increased *M. tuberculosis*-induced IFN-γ production after BCG vaccination in both males and females.

DISCUSSION

BCG vaccination has important non-specific effects by protecting against heterologous infections (7-12), being effective against bladder cancer (30), and reducing the risk of developing allergic and auto-immune diseases (18-22). However, while earlier studies have reported an upregulation of cytokine responses to non-specific *ex vivo* restimulation (trained immunity), the impact of BCG vaccination on steady-state levels of inflammation was largely unknown. In this large cohort of healthy volunteers, we show that BCG vaccination enhances the capability of innate immune cells to respond with an anti-microbial response (assessed by cytokine production capacity), but at the same time downregulates the systemic inflammation as measured by decreased concentrations of pro-inflammatory proteins in the circulation of healthy volunteers.

This modulatory effect on systemic inflammation may explain some of the beneficial effects of BCG vaccination in inflammatory diseases. Induction of trained immunity by endogenous stimuli is believed to contribute to the development of atherosclerosis (13-15, 31), auto-immune and auto-inflammatory diseases (16, 17), and due to the induction of trained immunity by BCG, one might hypothesize that BCG vaccination is a risk factor for these conditions. However, our results argue against a potentiating effect of BCG vaccination on inflammatory diseases such as atherosclerosis, and rather suggest that it may actually protect against inflammatory conditions. In support of this hypothesis, BCG vaccination in mice reduced the levels of circulating pro-inflammatory cytokines (32), lowered plasma cholesterol, and delayed the formation (33) and size of atherosclerotic lesions (32). Prospective studies have demonstrated beneficial effects of BCG vaccination in patients with auto-immune diseases such as multiple sclerosis (34-36) and type 1 diabetes mellitus (37, 38). Other studies have shown that BCG can prevent the development of diabetes in mice (39, 40). The reduction in circulating inflammatory markers following BCG vaccination might as well contribute to the lower reported incidence of atopy and allergy after BCG vaccination (18, 19). Although a large prospective BCG vaccination trial in Danish newborns showed no effect on the incidence of atopy on the age of 13 months (41), a protective effect

against atopic dermatitis was observed (22), in line with previous findings from a Dutch trial (21). Lastly, a recent report suggested a beneficial effect of BCG on the development of Alzheimer's disease (42) in which downregulating inflammatory processes may play a role (43).

It remains unknown how BCG reduces overall inflammation while at the same time improving myeloid, NK- and heterologous T-cell responsiveness to microbial challenges. Earlier studies have focused on the gene sets that are upregulated during BCG vaccination in human myeloid cells. It is imperative that future studies extend these investigations in two directions: to evaluate the genes sets that are eventually silenced by BCG vaccination on the one hand, and to investigate the effects of BCG vaccination on non-immune cells, which could also contribute to the release of inflammatory mediators on the other hand. In earlier studies from our group we have shown that after induction of trained immunity markers of both M1 and M2 macrophages are upregulated: we therefore proposed that the trained immunity phenotype is distinct from the classical M1/M2 dichotomy (44). In addition, earlier studies have shown that BCG vaccination tips the balance towards a more Th1-type response (45). A comprehensive assessment of these processes in follow-up studies is warranted.

Interestingly, we found a strong sex-differential effect, with significant reductions of inflammatory proteins after BCG vaccination in males only. Several studies have shown that both protective as well as detrimental non-specific effects of vaccines are sex-dependent (10, 46-51). Most of these consisted of observational studies, but three randomized trials have shown a sex-specific effect in all cause morbidity and mortality after neonatal BCG vaccination (10). Within the first week after vaccination, strong protective effects were detected in boys, whereas girls did benefit beyond the first week after vaccination (10). We found several negative correlations between baseline testosterone concentrations and changes in circulating proteins after BCG vaccination in males, suggesting a possible role for testosterone in our observed sex-differential effects after BCG vaccination. In line with these findings, previous *in vitro* studies have demonstrated that high dihydrotestosterone concentrations reduced monocyte-derived IL-6 and TNF-α production after 24-hour stimulation with

BCG (52). It remains to be investigated how our findings link to the observed sex-differences in epidemiological studies, considering that BCG is mainly administered in neonates in tuberculosis endemic countries, which is different from our study. It might seem evident that the mechanisms responsible for sex-differences are different in young infants compared to adults due to the differences in sex hormones. Interestingly however, sex hormone levels peak shortly after birth, the so-called 'mini puberty' (53), which is at the same time as neonates normally receive their BCG vaccination, indicating that sex hormones could modulate BCG-induced effects in the neonatal period as well as later in life. Still, there might be different mechanisms operating in these different age groups, which is why future research should investigate these effects in other relevant populations.

Another important finding from this study is that the pre-vaccination inflammatory status alters both specific and non-specific immune responses after BCG vaccination. Circulating inflammatory proteins mostly seem to potentiate short-term non-specific effects of BCG vaccination (trained immunity responses two weeks post-vaccination), and in a lesser extend the longer-term induction of trained immunity. A sex-specificity was detected in the effect of baseline inflammation on induction of trained immunity. The lower the baseline inflammation in males, the better the training responses, whereas higher baseline inflammation in females was associated with an enhanced training phenotype. Considering the differences in concentrations of baseline inflammatory status between males and females, our results suggest a certain optimum in pre-vaccination circulating inflammatory markers facilitates induction of trained immunity after BCG vaccination. In addition, baseline inflammation also impacts the longer-term effects of BCG vaccination on the induction of specific *M. tuberculosis*-induced cytokine responses. In males, we found a clear dichotomy in the associations between baseline inflammatory status and *M. tuberculosis*-induced cytokine responses after BCG vaccination: a predominantly negative association between several circulating inflammatory proteins and innate cytokine responses, and exclusively positive associations between circulating inflammatory markers and IFN-γ responses. For specific adaptive responses, higher baseline concentrations of IL-10, IL-12B and CXCL10 (also known as interferon-gamma-induced protein 10), which has previously shown to be

important in mycobacterial outgrowth and was identified as possible novel marker of trained immunity (54), resulted in increased *M. tuberculosis*-induced IFN-γ responses. Our data are partially in line with previous observations that lower pre-vaccination inflammation enhances vaccine immunogenicity (28, 29), which in our case holds specifically true for induction of innate immune memory responses in males. Effects of pre-vaccination inflammatory status have recently been found for hepatitis B vaccination (28): a higher frequency of activated innate immune cells and pro-inflammatory cytokines both correlated with lower neutralizing antibody titers following HBV vaccination (28). Similarly, after yellow fever vaccination, baseline numbers of activated $CD8^+$ T cells and B cells and pro-inflammatory monocytes resulted in lower neutralizing antibody titers following vaccination (29).

Our study is limited by the fact that we have used a focused proteomics platform. Future studies should expand these investigations by studying the effect of BCG vaccination at a broader level by untargeted proteomics. Moreover, longitudinal studies should focus on the risk of developing inflammatory diseases after BCG vaccination. Also, our validation cohorts were too small to be analyzed when stratified by sex. Finally, BCG-Denmark was used in the third validation cohort, while BCG-Bulgaria was used in our study and the other two validation cohorts. Considering the differences in the immune response induced by different BCG strains (55, 56), this might explain some of the discrepancies between the cohort studies.

The findings from this study confirm the immunomodulatory properties of BCG vaccination, but also demonstrate a clear effect of inflammation on (non-)specific immunogenicity of BCG vaccination. Our findings are likely to explain at least some of the multiple examples from the literature in which BCG improved or protected against inflammatory, allergic or autoimmune diseases. More studies are needed to increase our understanding of the interaction between inflammation, epigenetic reprogramming and cellular metabolism of innate immune cells during induction of trained immunity, as well as possible sex-specificity of these effects. Multiple tuberculosis vaccine candidates have now entered clinical trials to potentially replace BCG in the future. These vaccines should also be tested for

their ability to induce trained immunity and affect systemic inflammation, as well as for potential sex-differences. A better understanding of these effects may help optimize vaccine efficacy and explore novel applications of BCG vaccination.

METHODS

Study design and patient cohorts

To study the immunological effects of BCG vaccination, 307 healthy (male and female) adult volunteers of Western-European ancestry were included in the 300BCG cohort between April 2017 and June 2018 in the Radboud University Medical Center, the Netherlands. Healthy volunteers were recruited using local advertisement and flyers in Nijmegen, and were compensated for participation. After written informed consent was obtained, blood was collected, followed by administration of a standard dose of 0.1 mL BCG (BCG-Bulgaria, InterVax) intradermally in the left upper arm by a medical doctor. Vaccination of study participants was organized in batches of 6-16 subjects per day. Two weeks and three months after BCG vaccination, additional blood samples were collected. Exclusion criteria were use of systemic medication other than oral contraceptives or acetaminophen, use of antibiotics three months before inclusion, previous BCG vaccination, history of tuberculosis, any febrile illness four weeks before participation, any vaccination three months before participation, and a medical history of immunodeficiency.

A healthy cohort of 493 individuals of Western-European descent was used as an independent validation cohort (500FG cohort, see www.humanfunctionalgenomics.org). The participants were recruited between August 2013 and December 2014 at the Radboud University Medical Center, the Netherlands (57). In addition, three BCG studies conducted at the Radboud university medical center, all in BCG-naïve participants, were used as independent validation cohorts. In validation cohort 1, fifteen subjects (67% male, age 18–50 years) received a standard dose of BCG vaccination (BCG-Bulgaria, InterVax) between January 2017 and April 2017 (4). Blood was drawn before, two weeks, and three months after vaccination. In validation cohort 2, nine healthy volunteers (33% male, age 18–35 years) received a standard dose of BCG vaccination (BCG-Bulgaria, InterVax) in August 2016 (5). Blood was drawn at baseline and five weeks after vaccination. In validation cohort 3, fifteen male volunteers

(age 19-37 years) received a standard dose of BCG vaccination (BCG-Denmark, SSI) between February 2015 and November 2015 (6). Blood for was drawn at baseline and four weeks after vaccination.

Peripheral blood mononuclear cell isolation and stimulation

Peripheral blood mononuclear cells (PBMCs) were isolated from EDTA whole blood with Ficoll-Paque (GE healthcare, UK) density gradient separation. PBMCs were washed twice with phosphate buffered saline (PBS) and counted with a Sysmex hematology analyzer (XN-450). Complete blood counts were performed on EDTA whole blood and PBMC fractions after Ficoll isolation on a Sysmex XN-450 hematology analyzer. Cells were suspended in Dutch modified RPMI 1640 medium (Roswell Park Memorial Institute, Invitrogen, CA, USA), supplemented with 50 µg/mL gentamycin, 2 mM Glutamax (GIBCO) and 1 mM pyruvate (GIBCO). 5×10^5 PBMCs were cultured in a final volume of 200 µL/well in round bottom 96-well plates (Greiner) and stimulated with RPMI (medium control), heat-killed *Mycobacterium tuberculosis* (*M. tuberculosis*) HR37v (5 µg/mL, specific stimulus), or heat-killed *Staphylococcus aureus* (*S. aureus*) (10^6 CFU/mL, non-specific stimulus). Supernatants were collected after 24 hours and 7 days of incubation at 37˚C and stored at -20˚C until analysis. Cytokine levels were measured in 24 hours (IL-1β, IL-6, and TNF-α) and 7 days (IFN-γ) supernatants. Supernatant samples from one participant from different timepoints were measured on the same plate to ensure that variation between plates would not affect the calculated fold changes.

Protein and hormone measurements

Circulating plasma inflammatory markers were measured before, two weeks, and three months after BCG vaccination using the commercially available Olink Proteomics AB (Uppsala Sweden) Inflammation Panel (92 inflammatory proteins), using a Proceek © Multiplex proximity extension assay (58). In this assay proteins are recognized by pairs of antibodies coupled to cDNA strands, which bind when they

are in close proximity and extend by a polymerase reaction. A pooled plasma sample and an interplate control were included on each plate in triplicate to correct for batch differences. Plasma samples from one participant from different timepoints were measured on the same plate to ensure that variation between plates would not affect the calculated fold changes. Detected proteins are normalized and measured on a log_2-scale as normalized protein expression values.

In addition, adiponectin, resistin and leptin were measured in EDTA plasma at baseline using the R&D Systems DuoSet ELISA kits following the manufacturer's protocol. Testosterone was also measured in plasma at baseline by LCMSMS after protein precipitation and solid-phase extraction as described previously (57). Also, IgG class antibodies to CMV were measured in EDTA plasma using the Genway Biotech ELISA according to the manufacturer's protocol.

Statistics

All computational analyses were performed in R 3.3.3. Proteins were excluded from the analysis when the target protein was detected in less than 75% of the samples. Protein concentrations under the detection threshold were replaced with the proteins lower limit of detection. Protein circulation concentrations were then correlated with blood counts using Spearman's Rank-Order correlation. Protein concentrations were compared between baseline and two weeks as well as three months after BCG vaccination using Wilcoxon matched-pairs signed rank tests. A false discovery rate (FDR) based on Benjamini-80 Hochberg procedure of less than 0.05 was considered significant. Subsequently, blood counts were compared between baseline and two weeks as well as three months after BCG vaccination using the Wilcoxon matched-pairs signed rank test. Baseline testosterone, adiponectin, resistin and leptin levels were correlated with fold change circulating inflammatory markers using Spearman's Rank-Order correlation. And finally, raw cytokine values from the PBMC stimulation experiments were first log-transformed, and then corrected for batch effects using a linear regression model. Corrected cytokine concentrations were compared between baseline and two weeks as well as three months

after BCG vaccination using Wilcoxon matched-pairs signed rank tests. Corrected cytokine production was converted to fold changes from baseline. These fold changes were thereafter correlated with baseline inflammatory markers using Spearman's Rank-Order correlation separated by sex. A two-sided p value of less than 0.05 was considered statistically significant.

Study approval

The 300BCG (NL58553.091.16) and 500FG (NL42561.091.12) studies were approved by the Arnhem-Nijmegen Medical Ethical Committee. Validation cohorts 1 (NL55825.091.15) and 3 (NL50160.092.24) were also approved by the Arnhem-Nijmegen Medical Ethical Committee, and validation cohort 2 was approved by the Central Committee on Research Involving Human Subjects (CCMO NL56222.091.15). Written informed consent was obtained before any research procedure was initiated. All studies were performed in accordance with the declaration of Helsinki.

Author contributions

MN, LCJdB and VACMK designed the study. VACMK, LCJdB, VPM, SJCFMM, HL and HD conducted the cohort study and performed the experiments. LCJdB, JW, BC and RJWA conducted the trials used as validation cohorts. VACMK and LCJdB analyzed the data. MN, RvC, LABJ and CB supervised the analysis and interpretation of results. LCJdB and VACMK wrote the manuscript which was critically reviewed and approved by all authors.

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SUPPLEMENTARY MATERIAL

Supplementary table 1. Overview of circulating inflammatory proteins and fold changes (FC) after BCG vaccination

Supplementary table 2. Changes in whole blood counts after BCG vaccination

Supplementary table 3. Differences in age, BMI and whole blood counts at baseline between males and females

Supplementary table 4. Changes in lymphocyte, monocyte and neutrophil percentages within the PBMC fraction after BCG vaccination

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Figure 1.

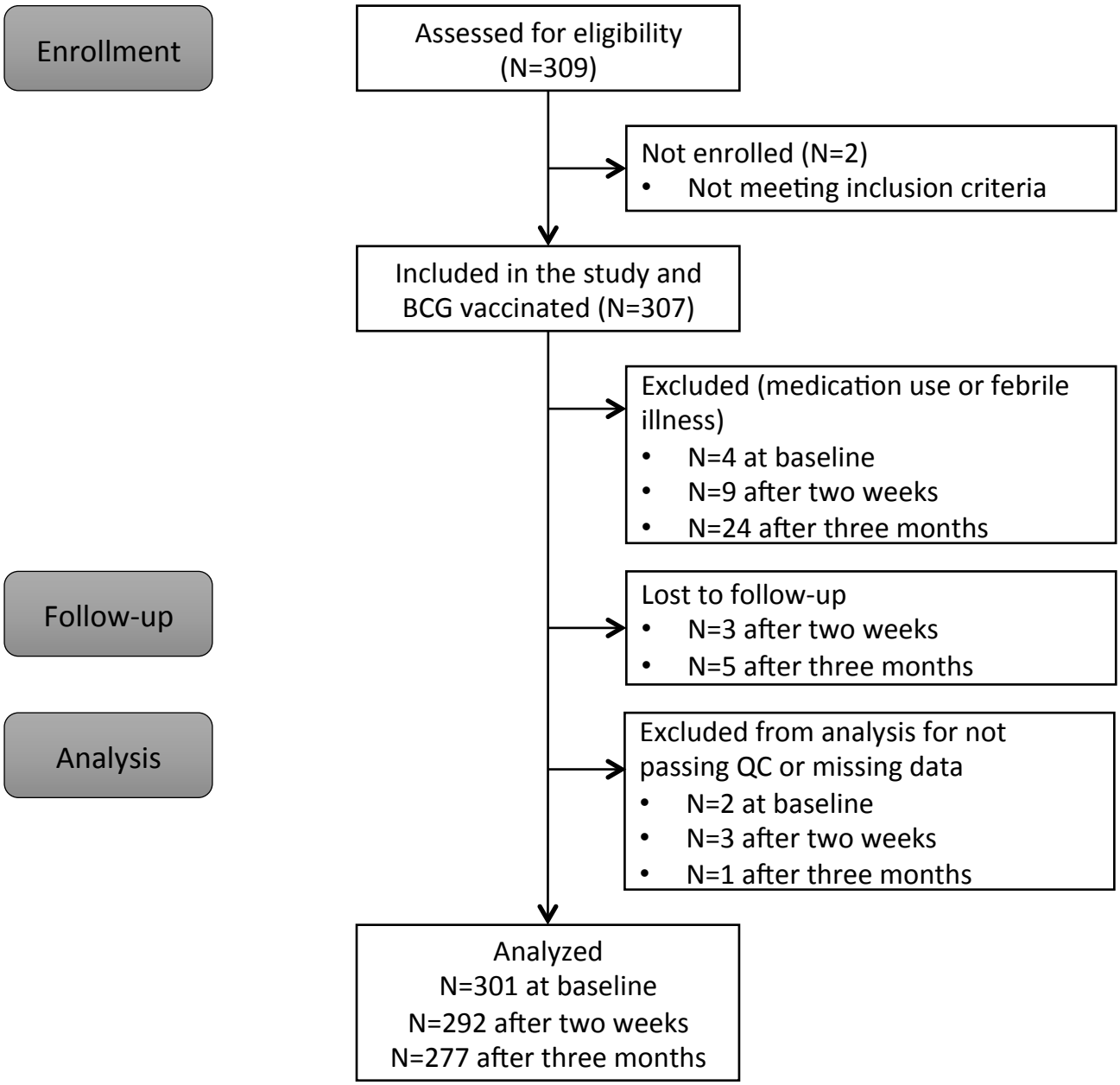


Figure 1. Flow chart of the study
Flow diagram describing the number of participants who were enrolled in the study, who were excluded or dropped out of the study, or excluded from further analysis.

Figure 2.

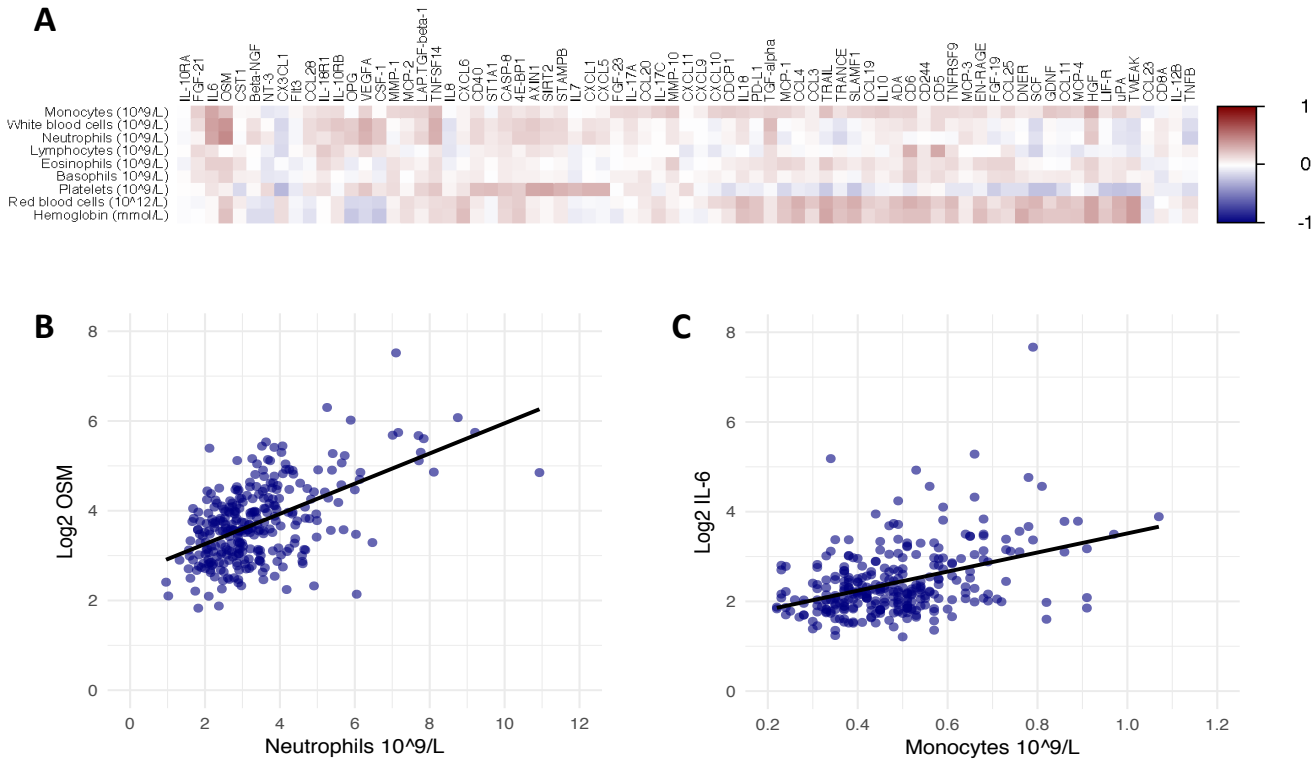


Figure 2. Correlations between baseline inflammatory markers and baseline whole blood counts
Spearman correlations between absolute whole blood counts (monocytes, total white blood cells, neutrophils, lymphocytes, eosinophils, basophils, platelets, red blood cells and hemoglobin) and circulating inflammatory markers at baseline (before BCG vaccination). Positive correlations are depicted in red, negative correlations in blue (n = 302) (**A**). Spearman correlations between whole blood neutrophil counts and circulating oncostatin M (OSM) (**B**), and between whole blood monocyte counts and circulating IL-6 (**C**) are shown as examples of positive correlations (n = 300).

Figure 3.

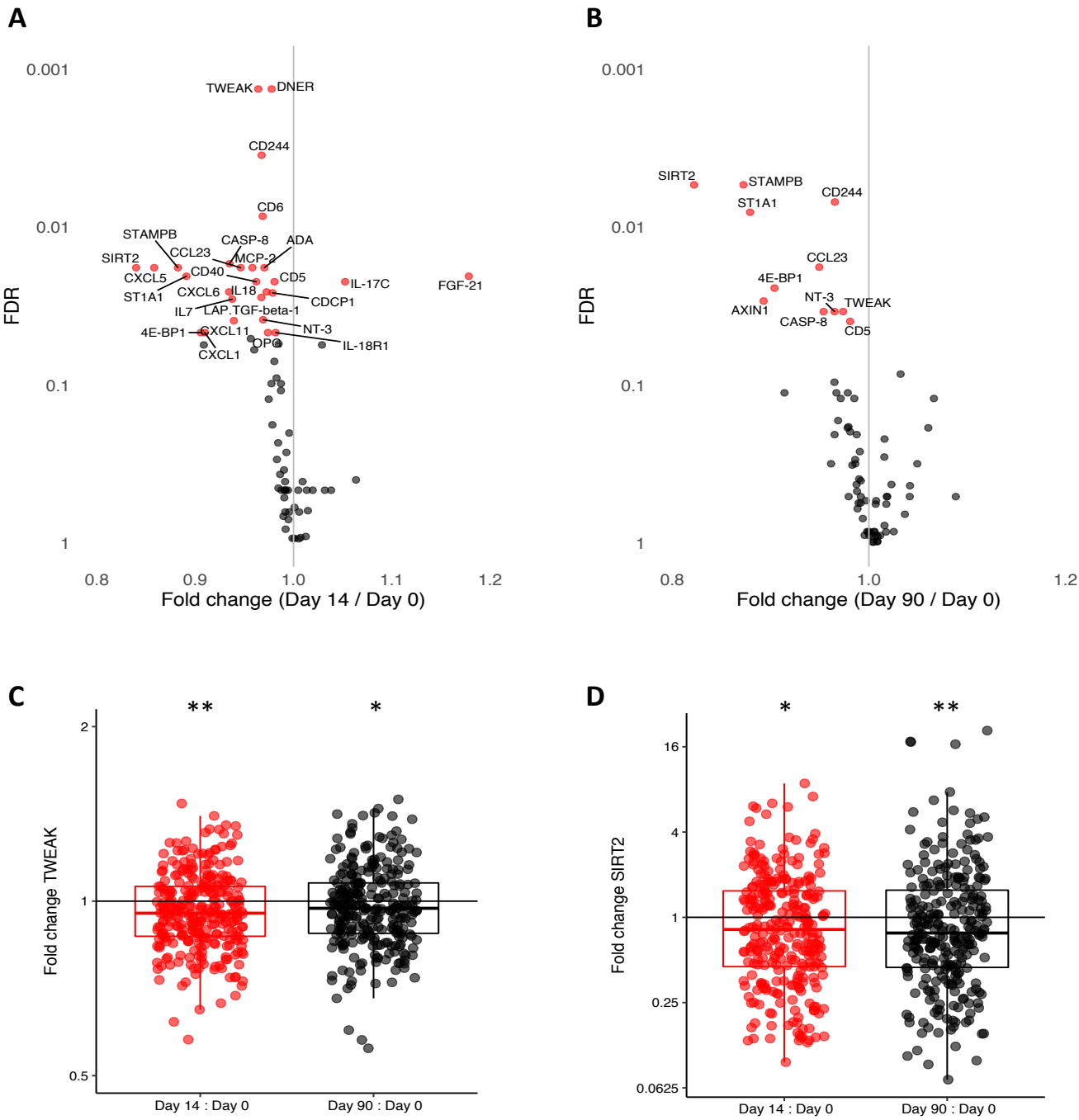


Figure 3. Inflammation after BCG vaccination

Fold changes of circulating inflammatory markers of day 14 versus baseline (**A**) and day 90 versus baseline (**B**). Significant changes compared to baseline are depicted in red, non-significant changes are depicted in gray (n = 290-fold change day 14 versus baseline, n = 275 fold change day 90 versus baseline, FDR < 0.05 is considered significant). Fold changes of TWEAK (**C**) and sirtuin 2 (**D**) are depicted as examples of significantly decreased circulating inflammatory markers after BCG vaccination (* FDR < 0.05, ** FDR < 0.01).

Figure 4.

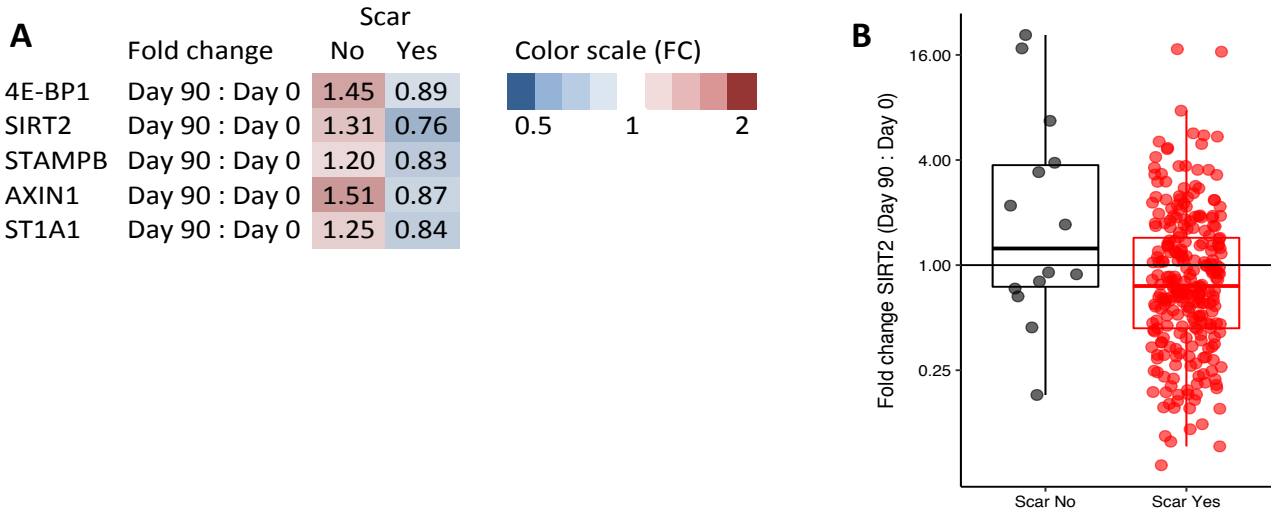


Figure 4. Differences in fold change circulating proteins between scar positive and scar negative individuals

Significant differences in fold changes of circulating proteins (significantly different in the entire cohort after FDR < 0.05 correction) between scar positive (n = 290) and scar negative (n = 15) individuals 90 days after vaccination (**A**), fold changes higher than 1 depicted on a red scale, fold changes lower than 1 depicted on a blue scale, Wilcoxon matched-pairs signed rank test, $p < 0.05$. Sirtuin 2 (SIRT2) plotted as an example of a protein which is significantly lower in scar positive individuals three months after BCG vaccination (**B**).

Figure 5.

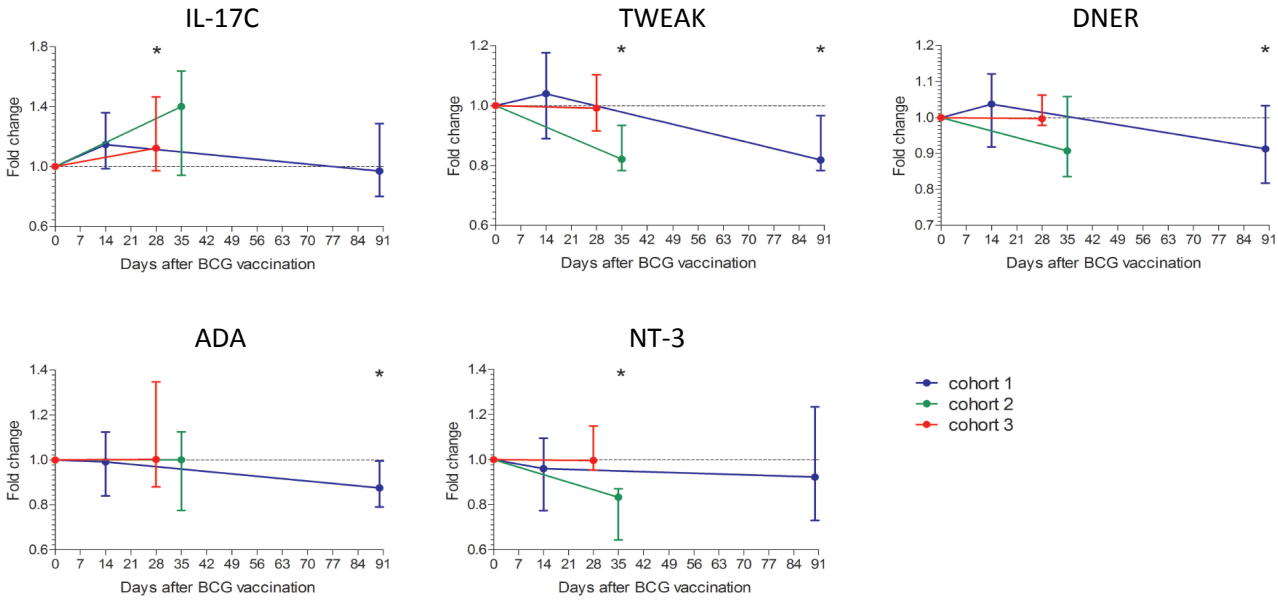


Figure 5. Validation of changes in circulating proteins after BCG vaccination
Fold changes of circulating inflammatory markers (IL-17C, TWEAK, DNER, ADA and NT-3) after BCG vaccination compared to baseline, validated in at least one of the three validation cohorts. The blue line represents cohort 1 (n = 15), the green line cohort 2 (n = 9) and the red line cohort 3 (n = 15) (Wilcoxon matched-pairs signed rank test, * $p < 0.05$). Median ± range is depicted per timepoint.

Figure 6.

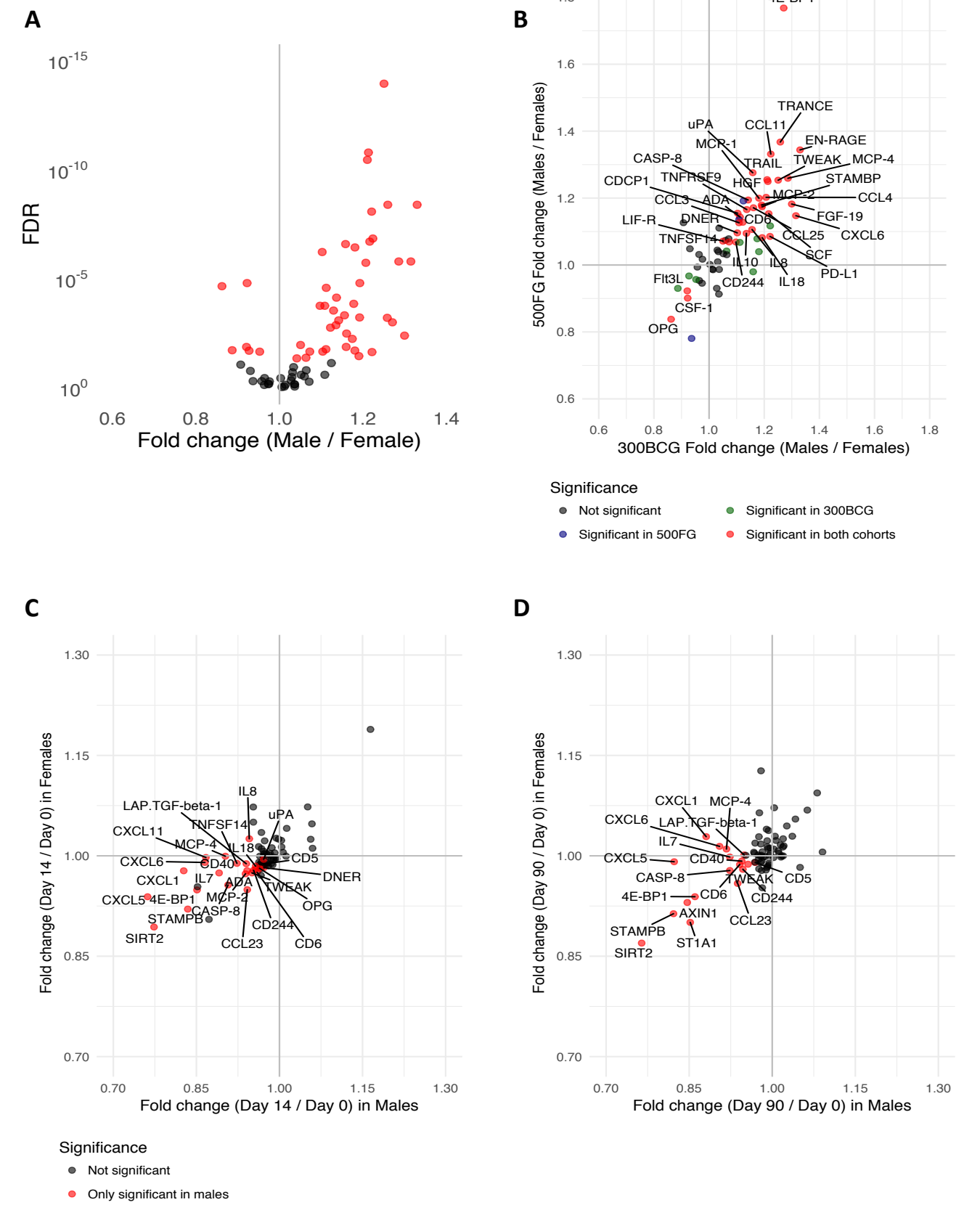

A
FDR
Fold change (Male / Female)
B
500FG Fold change (Males / Females)
300BCG Fold change (Males / Females)
Significance
Not significant
Significant in 300BCG
Significant in 500FG
Significant in both cohorts
C
Fold change (Day 14 / Day 0) in Females
Fold change (Day 14 / Day 0) in Males
Significance
Not significant
Only significant in males
D
Fold change (Day 90 / Day 0) in Females
Fold change (Day 90 / Day 0) in Males

Figure 6. Sex-specific effect of BCG vaccination on systemic inflammation

Comparison of baseline circulating inflammatory proteins plotted as fold changes between males (n = 132) and females (n = 171). Significant changes between sexes are depicted in red (FDR < 0.05) (**A**). Comparison of inflammatory proteins between males and females from the discovery cohort (300BCG) were plotted against the comparison between males (n = 215) and females (n = 278) from the validation cohort (500FG). Proteins that were only significantly different in the 300BCG cohort are depicted in green (n = 9), the ones that were only significant in the 500FG cohort are depicted in blue (n = 3), and the proteins significantly different between males and females in both cohorts are depicted in red (n = 34) and are labelled with their name (FDR < 0.05) (**B**). Fold changes of circulating inflammatory markers of day 14 versus baseline (**C)** and day 90 versus baseline (**D**) in the males only (n = 132) versus the females only (n = 171) subset. Significant changes compared to baseline in the males only subset are depicted in red (FDR < 0.05), and proteins that did not significantly change after BCG vaccination in either the males only or the females only subset are depicted in gray. There were no proteins significantly different in the females only subset.

Figure 7.

A

	Fold change	Testosteron	Adiponectin	Leptin	Resistin
4EBP1	Day 14 : Day 0	-0.20*	-0.05	0.06	-0.09
CASP8	Day 14 : Day 0	-0.22*	-0.1	0.04	-0.02
CD40	Day 14 : Day 0	-0.22*	-0.03	0.00	-0.05
CD5	Day 90 : Day 0	0.08	0.19*	0.02	0.06
CXCL1	Day 14 : Day 0	-0.29***	-0.06	0.09	0.00
CXCL5	Day 14 : Day 0	-0.26**	-0.05	0.03	0.00
CXCL6	Day 14 : Day 0	-0.21*	-0.02	0.06	0.03
IL-7	Day 14 : Day 0	-0.22*	-0.07	0.02	0.00
OPG	Day 14 : Day 0	0.22*	-0.01	-0.07	0.01
SIRT2	Day 14 : Day 0	-0.27*	-0.08	0.06	-0.03
ST1A1	Day 90 : Day 0	-0.21*	0.1	-0.03	-0.07
STAMPB	Day 14 : Day 0	-0.28**	-0.05	0.07	-0.03

B

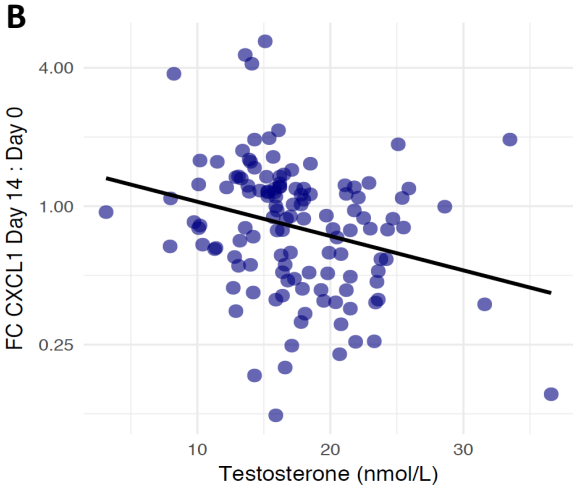
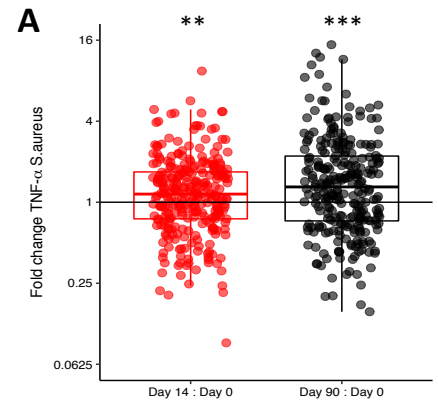
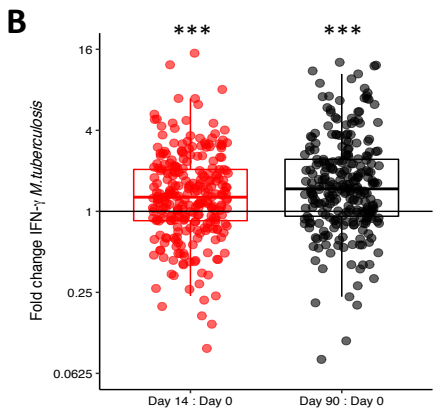
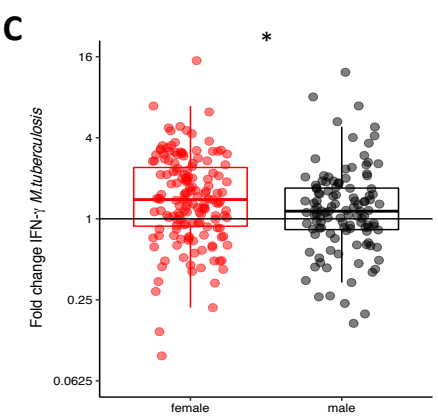


Figure 7. Correlations between circulating hormones and inflammatory proteins
Fold changes of proteins that significantly changed after BCG vaccination in males were correlated to baseline testosterone, adiponectin, leptin and resistin concentrations. Only proteins with a significant correlation with one of the hormones are depicted in this figure (Spearman correlation, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). The color represents the strength and the direction of the correlation (**A**). Spearman correlation between testosterone at baseline and fold change in CXCL1 two weeks after vaccination is shown as an example (**B**).

Figure 8.


A
**
***
16
4
1
0.25
0.0625
Fold change TNF-α S.aureus
Day 14 : Day 0
Day 90 : Day 0


B
***
***
16
4
1
0.25
0.0625
Fold change IFN-γ M.tuberculosis
Day 14 : Day 0
Day 90 : Day 0


C
*
16
4
1
0.25
0.0625
Fold change IFN-γ M.tuberculosis
female
male

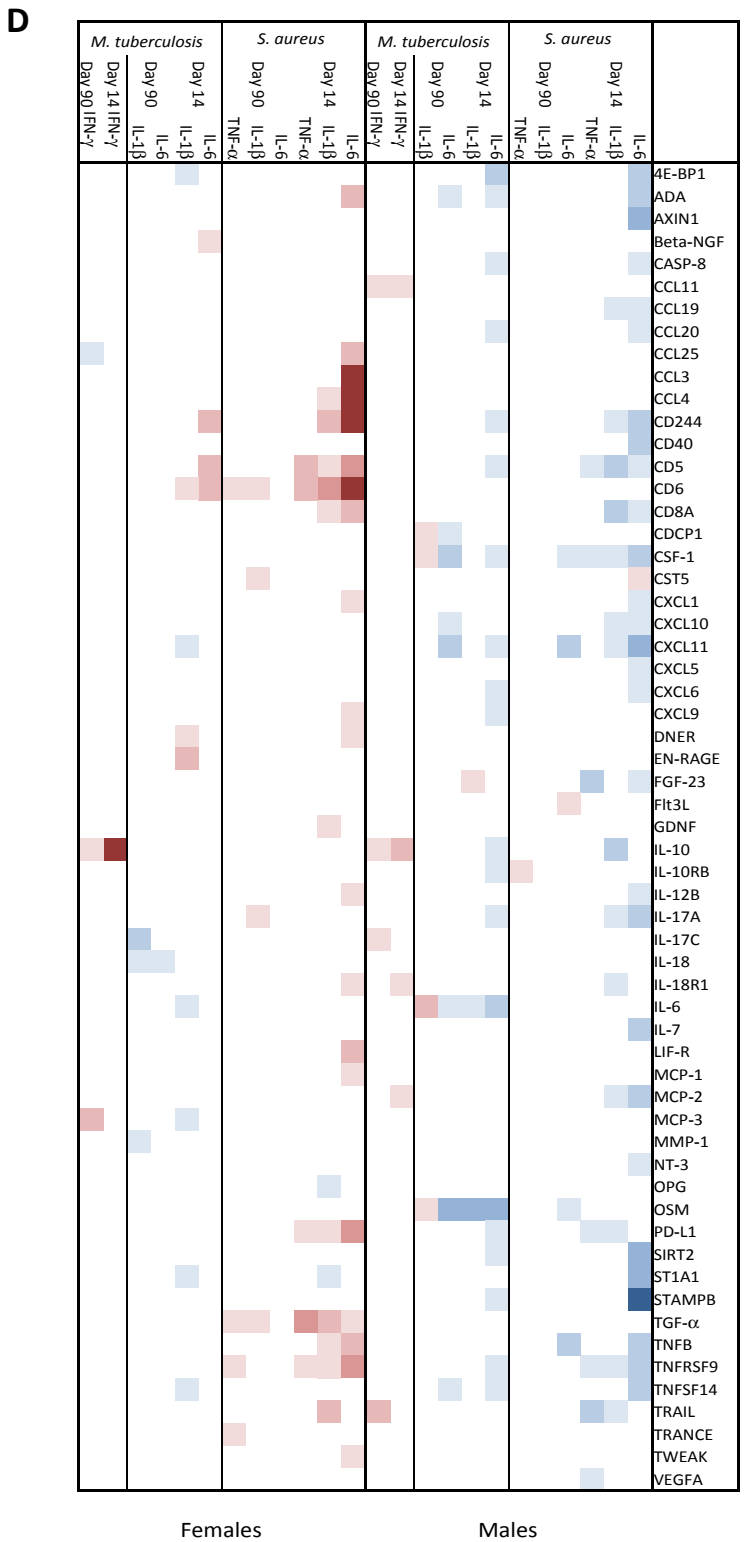

D
M. tuberculosis
S. aureus
M. tuberculosis
S. aureus
Day 90 IFN-γ
Day 14 IFN-γ
Day 90 IL-1β
Day 90 IL-6
Day 14 IL-1β
Day 14 IL-6
Day 90 TNF-α
Day 90 IL-1β
Day 90 IL-6
Day 14 TNF-α
Day 14 IL-1β
Day 14 IL-6
Day 90 IFN-γ
Day 14 IFN-γ
Day 90 IL-1β
Day 90 IL-6
Day 14 IL-1β
Day 14 IL-6
Day 90 TNF-α
Day 90 IL-1β
Day 90 IL-6
Day 14 TNF-α
Day 14 IL-1β
Day 14 IL-6
4E-BP1
ADA
AXIN1
Beta-NGF
CASP-8
CCL11
CCL19
CCL20
CCL25
CCL3
CCL4
CD244
CD40
CD5
CD6
CD8A
CDCP1
CSF-1
CST5
CXCL1
CXCL10
CXCL11
CXCL5
CXCL6
CXCL9
DNER
EN-RAGE
FGF-23
Flt3L
GDNF
IL-10
IL-10RB
IL-12B
IL-17A
IL-17C
IL-18
IL-18R1
IL-6
IL-7
LIF-R
MCP-1
MCP-2
MCP-3
MMP-1
NT-3
OPG
OSM
PD-L1
SIRT2
ST1A1
STAMPB
TGF-α
TNFB
TNFRSF9
TNFSF14
TRAIL
TRANCE
TWEAK
VEGFA
Females
Males

Figure 8. Ex vivo PBMC-derived cytokine production and associations with baseline circulating inflammatory proteins

Fold changes compared to baseline of ex vivo PBMC-derived S. aureus-induced TNF-α responses (**A**) and M. tuberculosis-induced IFN-γ responses (**B**) of day 14 versus baseline and day 90 and baseline as examples of upregulated cytokine responses after BCG vaccination (fold change day 14 versus baseline n = 289, fold change day 90 versus baseline n = 275, Wilcoxon matched-pairs signed rank test, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). Fold changes of IFN-γ in response to M. tuberculosis of day 14 versus baseline separated by sex (**C**). Spearman correlations between baseline inflammatory proteins and fold changes of PBMC-derived S. aureus-induced IL-1β, IL-6 and TNF-α responses and M. tuberculosis-induced IFN-γ responses separated by sex (**D**). Significant, positive correlations (rho > 0) are depicted in red, significant negative correlations (rho < 0) in blue, and non-significant correlations in white. Only proteins with a significant correlation with at least one of the ex vivo cytokine responses are depicted in this figure.