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The impact of interferon-γ pathway on trained immunity induction by vaccination with Bacille Calmette-Guérin

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Bacillus Calmette-Guérin (BCG) has multiple heterologous off-target effects which extend beyond tuberculosis (TB) prophylaxis, which include protection against other non-tuberculous infections, autoimmune diseases, and tumor development. These heterologous effects are at least partially mediated by induction of trained immunity. In this study, we aimed to investigate the impact of IFNγ production capacity on induction of trained immunity in human volunteers vaccinated with BCG. We evaluated inflammation and immune activation-specific cytokine responses (IFNγ, TNF, IL-1, and IL-6) in PBMCs isolated from 323 healthy volunteers vaccinated with BCG and stimulated with either *Mycobacterium tuberculosis* or *Staphylococcus aureus*. We further assessed the impact of genetic variants in genes crucial for the biological activity of IFNγ pathway on trained immunity using single nucleotide polymorphism (SNP) genotyping. We found a significant correlation between baseline IFNγ production capacity and induction of trained immunity, as assessed by the fold-change increase in IL-6 production at both day 14 and day 90 post-vaccination compared to production before vaccination. A similar correlation was found between basal IFNγ production and increased IL-1β production at day 14 after BCG. This suggests that individuals with higher IFNγ production capacity exhibit stronger trained immunity responses post-BCG vaccination. This hypothesis is supported by the finding that SNPs in genes involved in the IFNγ biological pathway significantly influence trained immunity responses in humans. IFNγ production capacity and genetic variations in the IFNγ pathway genes impact the magnitude of trained immunity response, providing insights into the regulation of innate memory responses.

Keywords BCG, Trained immunity, Interferon-γ, Cytokine response, Genetic variation

Bacillus Calmette-Guérin (BCG), which has been in use as a vaccine against tuberculosis (TB) for more than a century, has multiple heterologous off-target effects which extend beyond TB prophylaxis and include protection against non-tuberculous infections, autoimmune diseases, and tumor development[1,2]. These non-specific protective effects of BCG have been validated in experimental studies showing non-specific protection by BCG in models of bacterial, parasitic, fungal, and viral infection[3]. Accumulating evidence shows that BCG can reduce overall neonatal mortality. A combined analysis of 3 clinical trials from Guinea-Bissau showed a 38% reduction of neonatal mortality following BCG vaccination by protecting against neonatal sepsis and respiratory tract infections in addition to protection against TB[4,5]. A WHO-SAGE working group concluded in a systematic review that BCG vaccination likely has important protective effects against overall mortality in children[6]. In addition, recent clinical studies have shown similar protective heterologous effects of BCG vaccination in adults as well, which resulted in reduced incidence of respiratory tract infections[7]. In the context of COVID-19 the effects of BCG vaccination are mixed. While there are studies which states the lack of strong protective effect against the total number of COVID-19 infections [PMID 36976004, 33299492], other studies show that BCG vaccination

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may offer non-specific protection against COVID-19 by enhancing innate immune responses through trained immunity and heterologous lymphocyte activation[8]. Importantly, a meta-analysis of the randomized BCG trials during the pandemic showed that BCG vaccination significantly decreased COVID-19-related mortality[8].

The mechanisms behind the protective heterologous effects of BCG vaccination have only recently been started to be deciphered. While immune memory has been traditionally considered a defining feature of the adaptive immunity, recent studies have shown that long-term changes in innate immune responses can be induced by certain vaccines and infections as well, a process termed trained immunity[9]. In line with this, BCG vaccination can amplify the response of innate immune cells to restimulation and the antimicrobial properties of the immune cells in an antigen-agnostic manner[10]. Long-term increased innate immune responses have been extensively described for monocytes and NK cells at both functional and transcriptional level[11,12] and are mainly attributed to epigenetic reprogramming and changes in cellular metabolism following BCG vaccination[13]. The activated phenotype of trained immunity has been demonstrated to last up to at least 12 months after BCG vaccination, although epidemiological data suggest heterologous protection against infections that can last up to 60 months after vaccination[14]. However, it is noteworthy that trained immunity is a reversible and shorter-lived process than the epitope-specific immunological memory of the adaptive immune system[15,16].

While induction of trained immunity is an intrinsic property of the innate immune cells such as monocytes, macrophages or neutrophils, recent studies in animals have shown that IFNγ released from T-cells and NK-cells can strongly amplify trained immunity responses[17]. Basal IFN-γ production before BCG vaccination may influence the magnitude of trained immunity by priming innate immune cells into a more responsive state. IFN-γ has been shown to act as a modulator of monocyte function. At baseline, individuals with higher endogenous IFN-γ levels may already have partially activated monocytes or macrophages, which could facilitate or enhance their responsiveness to further stimulation—such as BCG vaccination. IFN-γ can promote chromatin remodeling and transcriptional activation of proinflammatory genes in monocytes. When this cytokine is present prior to or during BCG exposure, it may synergize with the epigenetic reprogramming induced by BCG to amplify trained immunity. Therefore, individuals with elevated basal IFN-γ may experience stronger innate memory responses upon secondary challenges with heterologous pathogens, due to an enhanced "training" signal. These data have been supported by in-vitro data showing that IFNγ pathway is important for the amplification of trained immunity[18]. Whether this is also true in-vivo in humans after trained immunity-inducing vaccination is not known.

In the present study, we aimed to investigate the impact of IFNγ production capacity on trained immunity in humans. We studied this in a cohort of 323 healthy volunteers vaccinated with BCG (300-BCG cohort), previously described[19]. We collected blood samples and isolated PBMCs at three timepoints: pre-vaccination (day 0), early post-vaccination (day 14), and a later phase when innate immune memory may persist (day 90). PBMCs were stimulated with either *Mycobacterium tuberculosis* or *Staphylococcus aureus* to evaluate cytokine responses. We defined trained immunity as the fold-change increase in cytokine production (IL-1β, IL-6, or TNF) in response to BCG vaccination at days 14 and 90 compared to baseline production at day 0.

We assessed the correlation between baseline IFNγ production capacity and the fold-change increase in IL-6 and IL-1β production at both day 14 and day 90 post-vaccination. To further validate the role of IFNγ in amplifying trained immunity, we analyzed the impact of single nucleotide polymorphisms (SNPs) in genes critical to the IFNγ pathway for the induction of trained immunity.

Materials and methods

Human volunteers: the 300-BCG cohort

The study was conducted on a cohort of 323 healthy adults from the Netherlands, vaccinated with Bacillus Calmette-Guérin (referred to as the 300BCG cohort). The cohort included 44% males and 56% females, with an age range of 18 to 71 years. Informed consent was obtained from all subjects and/or their legal guardian(s). Individuals were vaccinated with 0.1 mL of BCG (BCG vaccine strain Bulgaria; Intervax, Canada). The study was approved by the Arnhem-Nijmegen Ethical Committee (approval number N158553.091.16. All methods were performed in accordance with the Declaration of Helsinki. An overview of the study is presented in Fig. 1.

Measurement of cytokine production capacity

Blood samples were collected from each individual at three time points: day 0 (pre-vaccination), day 14 (early post-vaccination), and day 90 (later phase when innate immune memory may persist). Peripheral blood mononuclear cells (PBMCs) were isolated from EDTA blood by density centrifugation of blood diluted 1:1 in pyrogen- free PBS over Ficoll-Paque (GE Healthcare). Cells were washed twice in PBS and resuspended in Dutch modified RPMI 1640 culture medium (Invitrogen) supplemented with 50 mg/ml gentamicin (Centrafarm), 2 mM glutamax (Gibco), and 1 mM pyruvate (Gibco). Cells were counted using a Sysmex hematology analyzer (XN-450). 500,000 PBMCs were cultured in a final volume of 200 µl volume in round-bottom 96-well plates (Greiner) and incubated with control medium (negative control), heat-killed *M. tuberculosis* H37Rv (5 mg/ml) used as a specific stimulus to assess IFN-γ production or heat-killed *S. aureus* (10^6 CFU/ml, clinical isolate) used for assessment of IL-1β, IL-6, and TNF concentrations. Supernatants were collected after 24 h (for innate cytokine production) or after 7 days (for IFN-γ), and stored at − 20 C until analysis. Cytokines were measured in supernatants with ELISA (R&D Systems) according to the instructions of the manufacturer. Secretion level of cytokines measured on day 0 was considered as the baseline immunological response, serving as a reference point for quantifying cytokine induction at days 14 and 90 post-vaccination. Samples from all three time points from one individual were measured on the same plate in order to mitigate the effect of potential plate-based technical variation on calculated fold changes between time points.

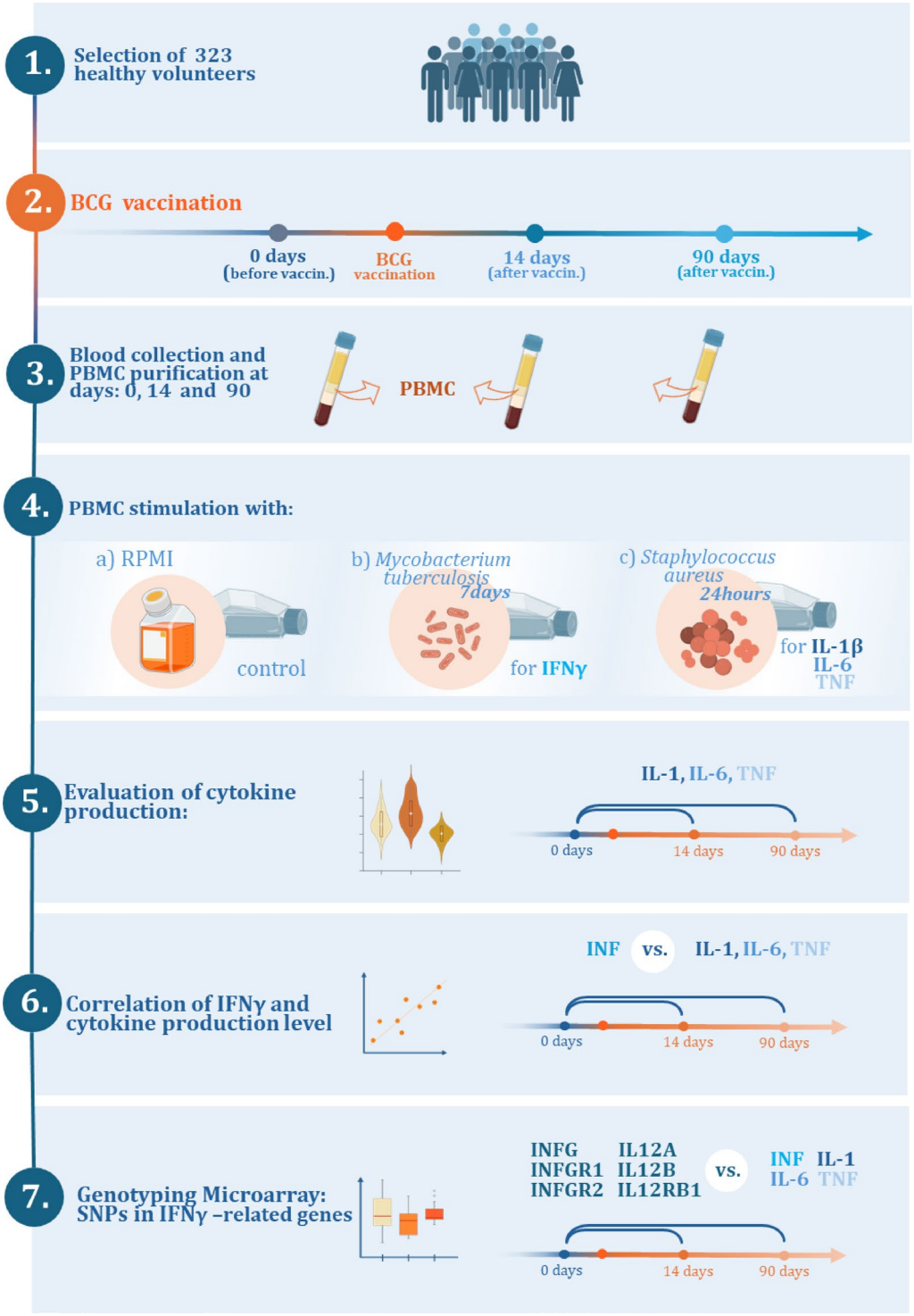

Fig. 1. Overview of the cohort and the study.

Genetic analysis

DNA was isolated from EDTA venous blood using the Gentra Pure Gene Blood kit, in accordance with the manufacturer's instructions (Qiagen, Venlo, the Netherlands). Genotype analysis was performed using the commercially available SNP chipDNA, Infinium Global Screening Array MD version 1.0 from Illumina. Genotype calling was performed using Opticall 0.7.0 with default settings. Genetic variant calling was performed using *optiCall* 0.7.0 with default settings. Samples with a call rate below 0.01 were excluded, as were variants with a Hardy-Weinberg equilibrium (HWE) below 10^{-4} or with a minor allele frequency (MAF) below 10^{-3}. Variant strands were mapped to the 1000 Genomes Project (1000G) reference panel using *Genotype Harmonizer*[20]. We excluded one sample from the dataset due to high relatedness as calculated using *Plink* v1.90b. Next, we imputed

the samples on the Michigan imputation server using the Human Reference Consortium (HRC) r1.1 2016 as the reference panel. We filtered out variants with an R2 below 0.3 for imputation quality[21]. After imputation, we further identified and excluded 17 genetic outliers. Of these outliers, five individuals showed high relatedness in pair with other individuals ($0.4 < PI\_HAT < 0.5$), and 12 samples were removed as ethnic outliers based on multidimensional scaling analysis (MDS). We finally selected 4,296,841 SNPs with MAF 5% for follow-up analysis. After quality control for both genetic and immunological assessment, genotype and cytokine data on trained immunity responses were available for a total of 267 individuals.

Statistical analysis

The individual trained immunity response was measured as the fold change in cytokine production in trained PBMCs after BCG vaccination as compared to nontrained cells isolated before vaccination. Following quality check for cytokine distribution and after excluding genetic outliers, we mapped the log-transformed fold changes of cytokine production to genotype data using a linear regression model with age and sex as covariates to correct the distributions of fold change of cytokine production. R-package Matrix-eQTL was used for cytokine QTL mapping. We used a cutoff of $P < 0.05$ to identify suggestive QTL associations in genes of the IFNγ pathway affecting trained immunity responses.

Results

Induction of trained immunity by BCG vaccination

BCG vaccination increased heterologous IL-1β production in response to *Staphylococcus aureus* stimulation of PBMCs, as illustrated in Fig. 2a. The concentrations of IL-1β produced by PBMCs upon *S. aureus* stimulation were significantly elevated at both day 14 and day 90 post-vaccination compared to pre-vaccination (day 0). The median concentration of IL-1β production after stimulation with *S, aureus* was 2250.89 pg/mL before vaccination, 2351.90 pg/mL 2 weeks after vaccination, and 2594.21pg/mL 3 months after vaccination Statistical comparisons revealed a significant difference in IL-1β production capacity between day 0 and day 14 ($p = 0.099$), day 0 and day 14 ($p = 0.00013$) as well as day 14 and day 90 ($p = 0.026$). Heterologous production of IL-6 after BCG vaccination was slightly higher after vaccination, but the difference did not reach statistical significance (Fig. 2b). TNF production by PBMCs in response to *S. aureus* stimulation did not show statistically significant changes at either day 14 or day 90 post-vaccination compared to baseline (day 0) (data not shown).

IFNγ production capacity correlated with trained immunity responses

To investigate whether trained immunity induction in humans is influenced by the IFNγ production capacity, as assessed after stimulation with *M. tuberculosis*. We performed correlation analysis between the baseline IFNγ production, and the fold-change increase in proinflammatory cytokine production after BCG vaccination. We observed a significant positive correlation between baseline IFNγ production capacity and trained immunity responses following BCG vaccination, with distinct patterns for IL-6 and IL-1β. Individuals with higher baseline IFNγ production showed a greater fold-change increase in IL-1β production from day 0 to day 14 after ex vivo stimulation of PBMCs with *S. aureus* (Fig. 3a). A similar positive correlation tended to be present for the fold-

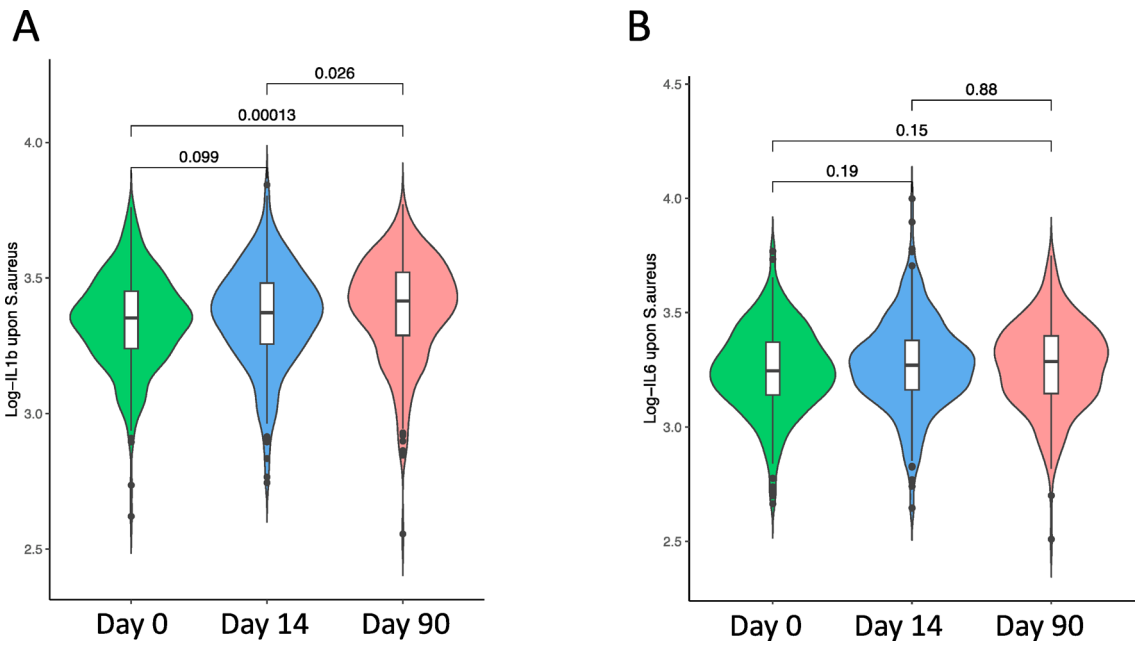

Fig. 2. The effect of BCG vaccination on heterologous induction of IL-1β (panel A) and IL-6 (panel B) by stimulation of freshly isolated PBMCs with heat-killed *S. aureus* for 24 h. Cytokine production capacity was assessed before BCG vaccination (day 0), as well as 14 and 90 days after vaccination.

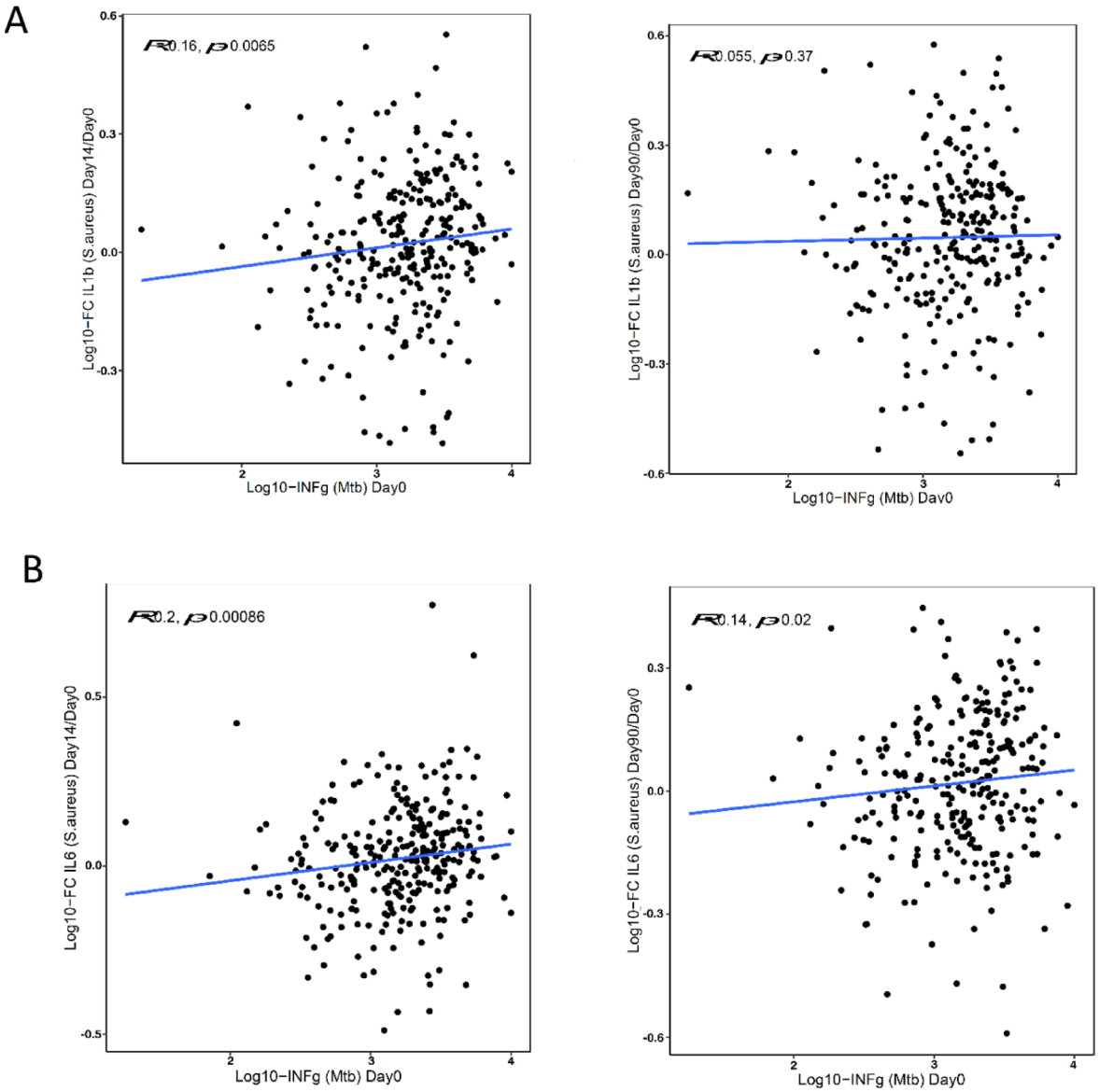

Fig. 3. Correlations between the fold-increase of IL-1β (panel A) and IL-6 (panel B) production capacity between day 14 and day 0, and between day 90 and day 0, respectively, and the baseline capacity of the PBMCs to produce IFNγ upon stimulation with *M. tuberculosis*.

increase in IL-1β between days 90 and 0, but this did not reach statistical significance. In contrast, fold increase in IL-6 production capacity at both time points (day 14 and 90) post-BCG vaccination compared with baseline production was significantly correlated with the capacity of PBMCs to produce IFNγ (Fig. 3b). No correlation was observed between baseline IFNγ concentrations and the increase in TNF production from day 0 to day 14 or from day 0 to day 90, indicating that baseline IFNγ does not significantly influence TNF responses following BCG vaccination (data not shown).

SNPs in genes of IFNγ pathway influence trained immunity after BCG vaccination

To validate the role of IFNγ for the amplification of trained immunity response in humans, we assessed the impact of genetic variants in close proximity to genes (+/-250 kb around the genes) crucial for the biological activity of IFNγ pathway on trained immunity. We extracted information on single nucleotide polymorphisms (SNPs) in genes encoding for IFNγ and its receptors (*IFNG*, *IFNGR1*, *IFNGR2)*, as well as IL-12 and its receptors (*IL12A*, *IL12B*, *IL-12RB1*, *IL-12RB2*) and assessed the impact of these SNPs on the induction of trained immunity, defined as the fold-change increase in studied cytokines. Importantly, SNPs in all seven genes significantly impacted the induction of trained immunity by BCG vaccination, demonstrating the importance of IFNγ pathway in this process. In total, 69 SNPs (p-values < 0.05) were identified in the genes associated with the IFNγ pathway that influence the induction of trained immunity (Supplementary Table S1, S2). The impact of three of the most important SNPs in *IFNG* and *IFNGR1* influencing trained immunity responses is shown in Fig. 4.

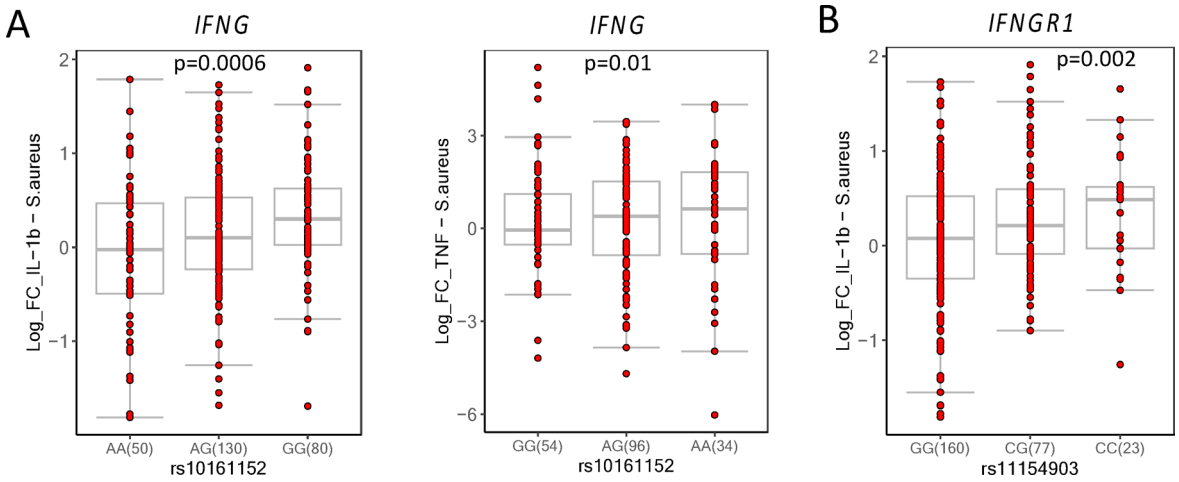

Fig. 4. The effect of single-nucleotide polymorphisms in *IFNG* (panels A) and *IFNGR1* (panel B) for the induction of trained immunity by vaccination with BCG in human volunteers, as assessed by the fold increase in cytokine production capacity between before and 90 days after BCG vaccination.

Discussions

BCG vaccination is probably the vaccine in which the heterologous protective effects have been most thoroughly studied: they extend from protection against overall mortality in children[22], to increased resistance to infections in the elderly[23] and protection against recurrence in bladder cancer[24]. Induction of trained immunity has been proposed to be one of the main mechanisms mediating the beneficial heterologous effects of vaccines in general, and BCG in particular[25]. Therefore, understanding the mechanisms that induce and modulate trained immunity is a crucial step for improving the broad protection induced by vaccines on the one hand, and the efficacy of immunotherapy in cancer. In this context, IFNγ has been recently shown to potently amplify trained immunity responses both in animal and in-vitro experimental studies[17,18,26], but it was not known whether similar amplification loops are present in vivo in humans. In the present study we demonstrate that the capacity of BCG vaccination to induce trained immunity is directly correlated with the IFNγ production capacity of the individual. We also showed that polymorphisms in genes involved in the IFNγ biological pathway influence the capacity of BCG to induce trained immunity.

First, our study validate the results of earlier studies that BCG vaccination leads to a significant and sustained increase in IL-1β production by PBMCs in response to *S. aureus* stimulation. The data show a marked enhancement in IL-1β production at both day 14 and 90 post-vaccination. The pronounced elevation in IL-1β production capacity at day 90 ($p = 0.00013$) compared to baseline suggests that BCG vaccination trains the immune system to maintain heightened readiness well beyond the immediate post-vaccination period. This sustained elevation could play a crucial role in providing extended protection against various infections, potentially explaining some of the broad, non-specific benefits observed with BCG vaccination[27]. In contrast, no statistically significant increases were observed for IL-6 or TNF production following BCG vaccination, despite earlier studies that demonstrated that the production of these cytokines can be increased as well[28]. In addition to possible experimental variation between the studies, a likely explanation for this difference lies in the BCG strain used for vaccination between the various studies. In the present study, we used BCG Bulgaria strain, which is a close variant of BCG Russia, due to a temporary lack of availability of the BCG-Denmark strain usually used in Europe, and that has been used in our earlier studies. Indeed, BCG-Russia strains have been suggested to be less immunogenic compared to BCG-Denmark and BCG-Japan strains[29]. Apart from differences in BCG strain, population characteristics may influence the magnitude of observed cytokine responses and consequently, BCG-induced trained immunity. In regions with high environmental mycobacterial exposure, immune cells may already be partially activated or desensitized, potentially altering their responsiveness to BCG vaccination and subsequent ex vivo stimulation. Conversely, in populations with limited exposure to such environmental microbes, BCG may elicit a more robust trained immunity response due to a relatively naive immune baseline. In line with this, Young and collaborators demonstrated that mice exposed to *Mycobacterium avium strains* (environmental mycobacteria) before BCG vaccination showed decreased IFN-γ production[30].

Despite the relatively milder trained immunity effects induced by the BCG vaccination in our study, this was clearly influenced by the capacity of the cells to induce IFNγ production. Interestingly, in our study IFNγ impacted more strongly the post-BCG production of IL-1β and IL-6, rather than TNF, although this needs to be validated in additional studies. IFNγ is known to be produced by classical CD4 lymphocytes, NK cells or γδ-T-cells[31], and future studies should address which of these cell populations is most important for amplification of trained immunity responses. We have also shown previously that IFNγ production capacity is strongly associated with the extent of the local scar after the BCG vaccination[32]. The relation between IFNγ and trained immunity demonstrated in this study provides thus an explanation for the association between the BCG scaring and heterologous protection against mortality[33]. It is especially basal IFNγ production which is important for induction of trained immunity, likely because the amplitude of trained immunity is defined in the first days after

vaccination (hence basal production), and not by IFNγ production capacity late after vaccination, when trained immunity is already established. The molecular mechanisms through which IFNg amplify cytokine production capacity remain to be investigated in follow-up studies. It can be hypothesized that IFN-γ can promote trained immunity by inhibition of anti-inflammatory cytokines, as it has been shown to promote the recruitment of the histone methyltransferase EZH2 to the promoters of anti-inflammatory genes such as MERTK, PPARG, and RANK, leading to the deposition of the repressive histone mark H3K27me3 and subsequent transcriptional silencing of these genes[34].

Investigating the impact of genetic polymorphisms on certain biological traits is one of the few accessible approaches in humans to demonstrate causality. To assess whether IFNγ biological pathway can indeed modulate trained immunity responses, we investigated the effect of polymorphisms in several of the genes of this pathway (including *IFNG* itself, its receptor *IFNGR1*, or IFNγ-inducing cytokine *IL12* and its receptors) on trained immunity, Our findings underscore the critical role of genetic variation in the IL-12 and IFNγ pathways in shaping cytokine responses associated with trained immunity. Specifically, we identified several SNPs around *IL12*, *IFNG* and *IFNGR1*-related genes that were significantly associated with induction of trained immunity. This demonstrates the causal effect between a potent IFNγ pathway and trained immune response induced by BCG vaccination in humans in vivo. This has important consequences for both the understanding of mechanisms responsible for trained immunity induction in humans, as well as for the design of new approaches to improve efficacy of vaccination and immunotherapy.

In conclusion, our study shows that baseline IFNγ production is a predictive biomarker for BCG vaccination-induced immunity in humans. In addition, the demonstration that polymorphisms in genes associated with IFNγ production influence trained immunity shows that this important lymphoid cell-derived cytokine has a potential causal role for the induction of trained immunity. This underscores the fact that innate and adaptive immune responses are in a continuous interaction: while innate immune cells-derived cytokines support adaptive immune responses, T-cell-derived IFNγ can in turn amplify the innate immune memory responses represented by trained immunity. This suggests that IFNγ pathway is potentially an important target for amplification of trained immunity responses and protection for both prevention of infections by vaccines and trained immunity-dependent immunotherapy in cancer.

Data availability

All data generated or analyzed during this study are included in this published article and its supplementary information files.

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Author contributions

EI, AC, and IBN wrote the main manuscript text; VACMK, JCJB and VM analysed the samples; SCFMM, and VPM processed the experimental data and performed the analysis; MGN and LABJ devised the project, the main conceptual ideas and proof outline. All authors discussed the results and contributed to the final manuscript.

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Declarations

Competing interests

MGN is a Scientific Founder of Biotrip, Lemba TX, Salvina TX and TTxD. LABJ is Scientific Founder of Lemba TX, TTxD and Salvina TX.

Additional information

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