# Title: Immune correlates of early clearance of *Mycobacterium tuberculosis* among tuberculosis household contacts in Indonesia

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**One Sentence Summary:** Absence of IGRA conversion among heavily exposed TB contacts is associated with BCG vaccination and altered innate immune cell phenotype and function.

**Abstract:** Some individuals, even when heavily exposed to an infectious tuberculosis patient, do not develop a specific T-cell response as measured by interferon-gamma release assay (IGRA). This could be explained by an IFN-γ-independent adaptive immune response, or an effective innate host response clearing *Mycobacterium tuberculosis (Mtb)* without adaptive immunity. In heavily exposed Indonesian tuberculosis household contacts (n=1347), a persistently IGRA negative status was associated with presence of a BCG scar, and - especially among BCG-vaccinated individuals - with altered innate immune cells dynamics, higher heterologous (*Escherichia coli*-induced) proinflammatory cytokine production, and higher inflammatory proteins in the IGRA mitogen tube. Neither circulating concentrations of *Mtb*-specific antibodies nor functional antibody activity associated with IGRA status at baseline or follow-up. In a cohort of adults in a low tuberculosis incidence setting, BCG vaccination induced heterologous innate cytokine production, but only marginally affected *Mtb*-specific antibody profiles. Our findings suggest that a more efficient host innate immune response, rather than a humoral response, mediates early clearance of *Mtb*. The protective effect of BCG vaccination against *Mtb* infection may be linked to innate immune priming, also termed 'trained immunity'.

**Main Text:**

## INTRODUCTION

Some people who are heavily exposed to an infectious tuberculosis patient do not develop evidence of an antigen-specific T-cell response, as measured with an interferon gamma release assay (IGRA). We have previously found that approximately one quarter of heavily exposed tuberculosis household contacts in Indonesia do not develop a positive IGRA during three months follow-up *(1)*. One might argue that these individuals either clear inhaled *Mycobacterium tuberculosis (Mtb)* through a protective innate host response, or that they develop an IFN-γ-independent adaptive immune response.

Interestingly, tuberculosis household contacts with a BCG-scar showed a ~50% lower risk of IGRA conversion compared to unvaccinated individuals *(1)*. BCG protection decreased with increasing *Mtb* exposure, and correlated with the heterologous innate immune response *(2)*. These data suggest that BCG-induced innate immune priming (also termed 'trained immunity'), which has shown to protect against *Mtb* in experimental models *(3–5)*, may clear inhaled *Mtb* before an adaptive immune response (as measured with an IGRA) can develop.

Rather than reflecting protective innate immune clearance, a persistently negative IGRA-status among heavily exposed household contacts might also be explained by an IFN-γ-independent adaptive immune response. In Uganda, contacts who had tested IGRA- and tuberculin skin test (TST)-negative over several years (so-called 'resisters'), had detectable IFN-γ-negative T-cell responses to ESAT6/CFP10, the antigens used for IGRA-testing and absent in BCG *(6)*. They also had similar concentrations of IgG, IgM and IgA antibodies to different *Mtb* antigens as IGRA-positive contacts *(6)*. Other studies, in humans *(7)* as well as primates *(8)*, have also found in anti-*Mtb* antibodies, and suggested that they may protect against *Mtb* infection as well TB disease *(9)* in an IFN-γ-independent way.

To improve our understanding of the correlates of protection against *Mtb* infection, we examined innate immune cell phenotype and function, and a broad range of anti-*Mtb* specific antibody features in heavily exposed tuberculosis household contacts in Indonesia, as well as in BCG-vaccinated adults in a low-TB incidence setting.

# RESULTS

## Subhead 1: Characteristics of tuberculosis household contacts in Indonesia

Among 1347 heavily exposed tuberculosis household contacts, after exclusion of individuals with active TB, 780 (57.9%) had a positive and 433 (32.1%) had a negative IGRA result at baseline. Baseline IGRA positive individuals had spent more time with the index patient, and more often slept in the same room as them (**Table 1**). Among household contacts with a negative IGRA at baseline, 116 (26%) converted to a positive IGRA at 14 weeks. IGRA conversion was associated with higher exposure, while a persistently IGRA negative status was associated with the presence of a BCG scar (RR 0.56 [95% CI, 0.40 - 0.77]; *P*<0.001, **Table S1**). To strengthen the phenotypes, a strict cut-off value was used for negative IGRA results (<0.15 IU/mL) and conversion to a positive IGRA result at 14 weeks (>0.7 IU/mL). Using these stricter criteria, we compared 51 participants classified as IGRA converters and 237 as persistently IGRA-negative individuals (**Fig. S1, Table S1**). Using these IGRA cut-offs, differences between IGRA converters and persistently IGRA-negative individuals in the level of exposure to the index patient, and in the proportion of individuals with a BCG scar (RR 0.35 [95%CI, 0.21 - 0.58]; *P*<0.001, **Table S1**) were more pronounced. Also, IGRA conversion was more among HHCs of index patients with *Mtb* Beijing genotype strains isolated from sputum compared to those infected with other genotype strains, and BCG vaccination appeared less protective against infection by Beijing strains *(10)*. Using stricter IGRA criteria, we saw a stronger relative risk (RR) for infection after exposure to Beijing versus other genotype strains (RR 1.84 [95% CI, 1.11-2.97], *P*=0.015 with strict criteria vs RR 1.44 [95% CI, 0.98-2.10], *P*<0.001 with the manufacturer IGRA criteria, **Table S2A**). Similarly, the genotype-dependent difference in protection conferred by BCG vaccination was stronger with stricter IGRA cut-offs (**Table S2B)**.

## Subhead 2: Different dynamics of innate immune cells in IGRA negative contacts

Among a subset of household contacts with a negative IGRA at baseline that had given informed consent for an additional blood draw at week 2 and week 14 (N=102), 16 different innate immune cell subsets were measured using flow cytometry. For further analysis we included participants who had data for both time points, including 22 IGRA converters and 48 persistently IGRA-negative individuals. At week 2, there were no statistically significant differences in innate immune cell numbers between groups (**Fig. S2**). When results at week 2 and week 14 were compared, innate immune cell numbers showed no statistically significant change in IGRA converters, while persistently IGRA-negative individuals showed a significant reduction in the numbers of $CD14^{hi}CD16^{-}$ classical monocytes, $CD14^{hi}CD16^{+}$ intermediate monocytes, $CD14^{low}CD16^{+}$ non-classical monocytes, $CD16^{+}$ mature granulocytes, $CD16^{dim}$ immature granulocytes, and $V\delta2^{-}$ γδ T cells (**Fig. 1B**). When analysis was restricted to persistently IGRA-negatives contacts, the decrease in numbers of total monocytes, classical monocytes, intermediate monocytes, non-classical monocytes, mature granulocytes, and $V\delta2^{-}$ γδ T cells was more pronounced among individuals with a BCG scar (N=38) compared to those without (N=10) (**Fig. 1C**),while this subgroup of persistently IGRA-negative individuals with a BCG scar also showed a significant reduction in $CD56^{dim}$ NK cells (**Fig. S3, Fig. 1C**).

## Subhead 3: Association of innate cytokine production with IGRA status

We next examined how innate immune markers correlated with IGRA status (**Fig. 2A**). First, we compared baseline production of TNF, IL-8, IL-6, IL-1β, IL-1Ra, and IL-10 upon stimulation with *Mtb,* BCG, and with *E. coli* as a heterologous stimulus. As expected, baseline

IGRA-positive individuals (N=145) showed higher cytokine production upon *Mtb* and BCG stimulation compared to baseline IGRA-negative individuals (N=328) (**Fig. 2B, 2C**). Also, logistic regression showed a strong association of the innate cytokine production after both *Mtb* and BCG stimulation with IGRA positivity at baseline. (**Fig. 2D**). Among baseline IGRA-negative individuals, those who remained IGRA-negative after 14 weeks (N=237) showed higher innate cytokine production upon *E. coli* stimulation compared to those whose IGRA –converted to positive (N=91) (**Fig. 2B, 2C, Table S3**), and logistic regression showed IL-6 and IL-8 production upon *E. coli* stimulation to be associated with persistently IGRA-negativity at follow-up (**Fig. 2E**). Interestingly, the association of *E.coli*-induced production and persistently IGRA-negativity at follow-up was stronger in contacts with a BCG scar compared to those without for IL-8, TNF and IL-6 (**Fig. 2F**).

**Subhead 4: Associations of baseline IGRA supernatant inflammatory proteins with IGRA status at follow-up**

Building on the ex-vivo cytokine production data, we measured inflammatory proteins in supernatants of baseline IGRA nil and mitogen tubes. Several proinflammatory proteins (ADA, MCP-3 [CCL7], TWEAK, IL-17C, and IL-18) showed significantly higher concentrations (logistic regression with adjustment for age, sex, BMI, and exposure risk score) in baseline IGRA supernatants of contacts whose IGRA remained negative compared to those whose IGRA converted to positive at 14 weeks (**Fig 3A**). Differentially abundant proteins showed consistent results in nil and mitogen tubes (**Fig 3B**). Besides the aforementioned proteins, 5 additional inflammatory proteins in mitogen-stimulated IGRA supernatants (CSF-1, CD244, DNER, CD6, and VEGFA) correlated with IFN-γ (TBAg – Nil) levels at 14 weeks after adjustment for age, sex, BMI, and exposure risk score (**Fig 3D**).

**Subhead 5: Antibodies and antibody function in relation to IGRA status**

Antibodies were measured at baseline in randomly selected IGRA-positive (n=100) and all IGRA-negative contacts (N=433). Similar to the larger cohort, IGRA-positive individuals had higher exposure to the index case, and (by definition) higher quantitative IGRA results (**Table S2**). After filtering for antibodies with concentrations higher than those measured in PBS, 25 out of 55 *Mtb*-antigen specific antibody isotypes were selected for analysis (**Fig. S4**). Antibodies showed a moderate association with age, sex and BMI (**Fig. S5A)**. No antibodies measured at baseline were significantly different between IGRA-positive and IGRA-negative individuals (**Fig. 4A**). Partial least squares – discriminant analysis (PLS-DA) showed overlapping clusters of IGRA-positive and IGRA-negative individuals (**Fig. 4B**). Also, no antibody levels were associated with IGRA status at baseline based on logistic regression analysis adjusting for age, sex, and BMI, and correction for multiple testing (**Fig. 4C**).

We next examined if antibodies against *Mtb* measured at baseline were associated with risk of IGRA-conversion, using strict IGRA cut-off criteria. No antibodies were significantly different between persistently IGRA-negative individuals (N=237) and IGRA converters (N=51; **Fig. 4D**). PLS-DA showed no differences between the groups (**Fig. 4E**). In addition, no antibodies were associated with the risk of IGRA conversion in logistic regression (**Fig. 4F**). Moreover, when analysis was limited to household contacts with a BCG-scar, no differences between groups were found in antibody concentrations (data not shown).

Antibodies can exert their function through lysis of infected cells by complement activation, or promote cellular or neutrophil phagocytosis, which might add to clearance of *Mtb* upon exposure. Focusing on LAM-specific antibodies which had the highest variable of importance projection scores in the PLS-DA (**Fig. S6**), we examined if antibody-dependent complement deposition (ADCD), antibody-dependent cellular phagocytosis (ADCP), and antibody-dependent neutrophil phagocytosis (ADNP) were associated with IGRA conversion. Using our stricter IGRA criteria and a subset of individuals matched for age and sex, IGRA converters (N=50) had higher MFI for LAM-dependent ADCD than persistently IGRA-negative individuals (N=50), while ADCP and ADNP showed no difference based on univariate testing (**Fig. S7A**). However, in logistic regression adjusting for age, sex, and BMI, there was no association between ADCD, ADCP, or ADNP with IGRA status during follow-up (**Fig. S7B**).

### Subhead 6: Effect of BCG vaccination on cytokine production and anti-*Mtb* antibodies

To further investigate the induction of innate immune responses and antibody production after mycobacterial stimulation in vivo, we next used a cohort of healthy volunteers vaccinated with BCG in a low-TB incidence setting *(11)*. We purposely selected a low burden setting, to look at the effect of BCG vaccination – which had shown strong relations with immune markers in the high burden setting – avoiding confounding by exposure to *M. tuberculosis*.

As expected, BCG vaccination led to an increase in ex vivo *Mtb*-induced IFN-γ production, but also to an increase in innate cytokines (**Fig. 5A**). As previously shown, BCG vaccination also led to increased heterologous cytokine production, although not in all individuals, as depicted for stimulation with *Staphylococcus aureus* in **Fig. 5B**. To examine a possible effect of BCG vaccination on anti-*Mtb* antibodies, we measured concentrations of 5 antibody isotypes and binding level of 2 Fc-receptors, to 9 *Mtb* antigens standardized to HA. After 90 days, when corrected for multiple testing, several *Mtb*-specific IgG3 showed a statistically significant, albeit minimal increase, while several *Mtb*-specific IgM antibodies showed a minimal decrease (**Fig. 5C/D, Fig. S8**).

## DISCUSSION

In a tuberculosis household study in Indonesia, approximately one fourth of heavily-exposed contacts still had a negative IGRA three months after tuberculosis diagnosis of the index case. Examining their innate immune response as a possible mechanism to remain uninfected, individuals with a persistently negative IGRA showed a stronger reduction of innate immune cells over time compared to IGRA converters, and higher heterologous production of cytokines and inflammatory proteins at baseline. No differences were found in baseline concentration or function of anti-*Mtb* antibodies, as a possible marker of an IFN-γ independent adaptive immune response. Among contacts with a BCG scar, which was associated with a persistently negative IGRA status, more pronounced differences were seen in innate immune cell numbers and function between IGRA converters and persistently IGRA-negative individuals. Furthermore, in a low-incidence setting, adult BCG vaccination induced heterologous cytokine production, but did not lead to significant changes in anti-*Mtb* antibodies.

A T cell-mediated IFN-γ response is important, but not sufficient for protection against tuberculosis *(12)*. T-cell mediated interferon-γ (IFN-γ) responses against *Mycobacterium tuberculosis* (*Mtb*) antigens are used for diagnosis of *Mtb* infection, with IFN-γ release assays (IGRAs) *(13)*. T-cell immunity is crucial for protection against tuberculosis, as shown by the fact

that among people with HIV, loss of CD4 T-cells correlates with the risk of tuberculosis *(14)*. In addition, rare genetic defects have demonstrated the crucial role of IFN-γ-signaling in mycobacterial infections *(15)*. Nevertheless, high IGRA IFN-γ production, as a mirror of T cell-mediated immunoreactivity against *Mtb*, increases rather than reduces an individual's likelihood of developing TB disease *(16)*. Also, *Mtb* seems to benefit from T cell recognition, as evidenced by the hyper-conserved T cell epitope sequences in the *Mtb* genome *(17)*. In addition, the MVA85A vaccine, which induces robust secretion of IFN-γ by CD4+ T cells, showed no protection against TB disease in clinical trials *(18, 19)*. As such, these studies strongly argue that innate or other CD4/IFN-γ-independent mechanisms are also required for protection against tuberculosis. It should be noted that the correlates of protection against *Mtb* infection and TB disease are not necessarily the same.

Determining why some individuals do not develop a positive T cell dependent TST or IGRA despite heavy exposure to *Mtb* can help identify novel correlates of protection against *Mtb* infection. The terms 'early clearance' *(20)* and 'resisters' have been used to label this clinical phenotype *(21)*. We studied early clearance in tuberculosis contacts in the context of a well-defined exposure within a household, with a relative short follow-up, while so-called resisters are tuberculosis contacts with negative TSTs and IGRAs despite living in a high-incidence setting for years. Early clearance can be defined as a relative, or dynamic, measure of protection against *Mtb* infection *(22)*, as we and others have shown that it is less common with heavier *Mtb* exposure *(1)*, or exposure to more virulent Beijing genotype strains *(10)*. In contrast, resisters can be seen as individuals who do not establish *Mtb* infection despite repeated tuberculosis exposure of varying intensity over a long period of time *(21)*.

Our study on early clearance in tuberculosis household contacts in Indonesia points to a significant role for innate immunity in the early protective response against *Mtb*. This hypothesis is supported by the elevated heterologous production of proinflammatory cytokines and inflammatory proteins, both produced mainly by innate immune cells, in persistently IGRA-negative individuals. In addition, the reduction in innate cell numbers which was found among contacts with a repeatedly negative IGRA at follow-up likely reflects the resolution of a protective innate inflammatory resolution after early clearance of *Mtb,* similar to the decreasing monocyte to lymphocyte ratio which has been reported during treatment of tuberculosis patients *(23)* and after TB preventive therapy of *Mtb* infected individuals *(24)*.

The different innate immune cell numbers and function in 'early clearers' in our study likely reflects a trained immunity *(22)* endotype associated with rapid elimination of the mycobacteria. This is further supported by the observation that the differences in innate immune cell phenotype and heterologous cytokine production between IGRA converters and persistently IGRA-negative individuals were more pronounced when analysis was restricted to BCG-vaccinated individuals. These findings mimic those of studies focusing on BCG-induced trained immunity in tuberculosis. In mice, BCG vaccination induces trained immunity in hematopoietic stem cells, which upon adoptive transfer conferred protection against *Mtb* in non-vaccinated mice *(3)*. Similarly, in a macaque model with repeated limiting-dose of *Mtb* challenge, pulmonary mucosal BCG vaccination induced a stronger trained immunity response *(4)* and longer delay of IGRA-conversion compared to intradermal BCG *(5)*. In mice, induction of trained immunity through beta-glucan administration also protected against *Mtb (25).* Collectively, this suggests that induction of trained immunity may protect tuberculosis contacts against *Mtb* infection, and might help development of other interventions to prevent tuberculosis.

New vaccines preferably should strengthen innate immune protection that can withstand intense *Mtb* exposure.

There is renewed interest in the possible protective role of antibodies against tuberculosis. In one study, compared to tuberculosis patients, individuals with latent *Mtb* infection showed a higher abundance, higher Fc receptor binding, and higher antibody-dependent cellular cytotoxicity for several *Mtb*-specific antibodies *(26)*. In another study, circulating anti-*Mtb* antibodies that conferred protection against tuberculosis in mice were found in a proportion of healthcare workers, but not in tuberculosis patients *(27)*. Also, 40 tuberculosis household contacts in Uganda who had remained TST and IGRA-negative for several years (so-called 'resisters') were found to have detectable levels of *Mtb*-specific antibodies, similar to 39 *Mtb* IGRA/TST-positive individuals *(6)*. While in a study in South Africa, 30 TST/IGRA-negative miners showed lower levels of *Mtb*-specific IgG and lower binding of *Mtb*-specific FcγR2B and FcγR3A compared to 37 TST/IGRA positive individuals *(7)*.

In our large study in heavily exposed contacts, *Mtb*-specific antibody features (both abundance and functionality) were not different when we compared 100 IGRA-positive and 433 IGRA-negative household contacts at time of diagnosis of the index patient. Also, no differences in baseline antibody features were seen between 51 IGRA converters and 237 persistently IGRA-negative individuals. The difference between our data and previous studies from the literature investigating the impact of antibodies could be due to several causes. Differences in the phenotypes of the participants ('early clearance' versus 'resisters'), our use of stricter IGRA-criteria to avoid possible misclassification , or our adjustment of antibody concentrations to control measurements, may provide some explanation. Of note, the presence of a BCG-scar was associated with protection against IGRA-conversion, and BCG vaccination status interacted with innate immune correlates in household contacts, but no such relation was found between BCG vaccination and antibody profiles. Finally, intradermal BCG vaccination of adults in a low-incidence setting, which has been shown to induce trained immunity and associated with an enhanced capacity to control mycobacterial growth *(28, 29),* did not significantly alter titers of *Mtb*-specific antibodies. This is in line with older studies on BCG vaccination from Sweden, which showed protection against tuberculosis, but no significant increase in *Mtb*-specific antibodies *(30)*.

Our study has several limitations. Our definition of *Mtb* infection was based on IGRA, which cannot distinguish mere immunoreactivity from actual infection. However, our primary comparison was between contacts who remain IGRA-negative after 3 months, and those who convert to a positive IGRA, likely reflecting new *Mtb* infection from their recent exposure. IGRA measurements, especially with results around the standard cut-off, also show variation which could lead to misclassification, but this is unlikely with our stricter cut-offs for a negative and positive IGRA. Finally, future studies could investigate the kinetics of the immune responses over a longer period of time.

Our study also has clear strengths that allow studying correlates of protection against *Mtb* infection. We used a large cohort specifically recruited to study early clearance with follow-up of baseline IGRA-negative household contacts, we had precise estimates of *Mtb* exposure that were strongly associated with IGRA conversion and protection from BCG, and we examined both innate immune correlates and antibody features. Our findings on associations with BCG were reproduced in an independent study on BCG vaccination in a low-incidence setting. Other strengths include our optimization of signal to noise ratio in antibody measurements through

proper filtering of antibody measurements and standardization against the positive control hemagglutinin, and correction for multiple testing in all analyses.

In conclusion, our findings suggest that a more efficient host innate immune response, rather than a humoral response, mediates early clearance of *Mtb*. The protective effect of BCG vaccination against *Mtb* infection may be linked to induction of a trained immunity phenotype. Future studies should examine if induction of trained immunity can help prevention of tuberculosis in highly-exposed individuals, including in the evaluation of new TB vaccines that may offer improved protection over BCG.

## MATERIALS AND METHODS

### Study design and participants

This study was embedded within a large household contact study (INFECT) which was conducted in Bandung, Indonesia, between 2014 and 2018 *(1)*. In short, household contacts of sputum smear-positive TB patients (0.5% of whom were HIV-infected) were eligible if they were older than 5 years and had had no previous TB. They were screened for active TB using a symptoms screen, chest X-ray and sputum microscopy and culture. Sociodemographic data and risk factors for *Mtb* infection were collected, including the level of exposure *(1)*, as measured by sleeping proximity, time spent with the index patient, and presence of cavities, and sputum mycobacterial load in the index patient. *Mtb* infection status of contacts was assessed by QuantiFERON-TB Gold In-Tube (QFT-GIT) IGRA, which was repeated at 14 weeks in those who were initially IGRA-negative. Based on IGRA results, contacts were first classified as persistently IGRA-negative individuals and IGRA converters using the manufacturer's cut-off value for the TB antigen (TBAg) tube (0.35 IU/mL). To strengthen the phenotypes, we applied stricter definitions of low-negative and high-positive IGRA results, only including individuals whose baseline IFN-γ result (TBAg – nil tube) was <0.15 IU/mL, and whose follow-up IGRA (TBAg – nil) was either <0.15 IU/mL (persistently IGRA-negative individuals) or >0.7 IU/mL (IGRA converters). The INFECT study was approved by the Health Research Ethics Committee of Universitas Padjadjaran Indonesia (14/UN6.C2.1.2/KEPK/PN/2014) and the Southern Health and Disability Ethics Committee New Zealand (13/STH/132).

The BCG vaccination cohort (300BCG) recruited volunteers of Western European ancestry between April 2017 and June 2018 at the Radboud University Medical Center *(11)*. Following the acquisition of written informed consent, participants underwent blood collection and then received a standard 0.1 mL dose of BCG (BCG-Bulgaria, InterVax) administered intradermally in the left upper arm by a medical doctor. The vaccination process for the study participants was conducted in groups ranging from 6 to 16 individuals each day. Blood samples were obtained two weeks and three months post-vaccination with BCG. Participants were excluded if they had been using systemic medications (excluding oral contraceptives or acetaminophen), antibiotics within three months prior to the study, a previous BCG vaccination, a history of tuberculosis, any feverish illness in the four weeks preceding the study, any vaccinations in the three months before the study, or had a medical history indicating immunodeficiency. The 300BCG (NL58553.091.16) study was approved by the Arnhem-Nijmegen Medical Ethical Committee.

### Innate immune cell phenotyping and cytokine production

Innate immune cell phenotyping with gating strategy and whole blood cytokine assays from INFECT cohort were performed as previously described *(2)*. In short, we mixed heparinized blood with 123Count eBeads, followed by staining with one of three antibody panels designed to identify monocytes (Panel 1), granulocytes (Panel 1), innate αβ T-cells (Panel 2), natural killer (NK) cells (Panel 2), NK T cells (Panel 3), and γδ T-cells subsets (Panel 3). Data were collected using a FACSCalibur flow cytometer and analyzed using FlowJo software. For whole blood cytokines, samples were incubated with BCG (Danish strain 1331) $1 \times 10^5$ CFU/mL (Statens Serum Institut), *Mtb* 5 μg/mL, *Streptococcus pneumoniae* (ATCC 49619) $1 \times 10^6$ CFU/mL, *Escherichia coli* $1 \times 10^6$ CFU/mL, or culture medium for 24 hours at 37°C. Supernatants were stored at –80°C until batchwise enzyme linked immunosorbent assay (ELISA) measurement of tumor necrosis factor (TNF), interleukin (IL) 1β, IL-1Ra, and IL-10 (R&D Systems), IL-6, and IL-8 (Sanquin).

In the 300BCG cohort, PBMC ex vivo stimulation assays were performed as previously described *(11)*. PBMCs were isolated from EDTA whole blood with Ficoll-Paque (GE Healthcare) density gradient separation. PBMCs ($5 \times 10^5$) were cultured in a final volume of 200 μL/well in round-bottom 96-well plates (Greiner) and stimulated with RPMI 1640 (medium control), heat-killed *M. tuberculosis* H37Rv (5 μg/mL, specific stimulus), or heat-killed *S. aureus* ($1 \times 10^6$ CFU/mL, nonspecific 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 at 24 hours (IL-1β, IL-6, and TNF) and 7 days (IFN-γ). Supernatant samples from all time points for a participant were measured on the same plate to ensure that variation between plates would not affect the calculated fold changes.

## IGRA supernatant inflammatory marker measurements

Inflammatory proteins from IGRA supernatant nil and mitogen tube (PHA stimulation) were measured using the commercially available Olink Proteomics AB Inflammation Panel (92 inflammatory proteins) (Uppsala Sweden). In this assay, proteins are recognized by antibody pairs coupled to cDNA strands which bind in close proximity, followed by extension by a polymerase reaction. Quality control was performed by Olink Proteomics with 8% samples not passing the quality control and subsequently excluded from the analysis. We only analyzed proteins detected in 75% of individuals. Overall, 67 of the 92 (81.5%) proteins were detected in at least 75% of the plasma samples and included in the analysis.

## Antibody measurements

For antibody assays, *Mtb* antigens tested were: purified protein derivative (PPD) (Statens Serum Institute), Ag85A and B in a 1:1 ratio (BEI Resources Cat#NR-49427 and #NR-53526), recombinant ESAT-6 (BEI Resources Cat#NR-49424) and CFP-10 (BEI Resources Cat#NR-49425) in a 1:1 ratio, HspX (BEI Resources Cat#NR-49428), and lipoarabinomannan (LAM) (BEI Resources Cat#NR-14848). An equal mixture of influenza antigens from HA1(B/Brisbane/60/2008) and HA1 (A/New Caledonia/20/99) (Immune Technology Corp ITIT-003-001p and IT-003-B3p) was used as a positive assay control.

A Luminex assay was used to quantify the relative levels of antigen-specific antibody isotypes and subclasses and their ability to bind Fc receptors. Luminex Magplex carboxylated microspheres (Luminex Corporation) were coupled to proteins/antigens via covalent N-hydroxysuccinimide (NHS)–ester linkages by 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydro-chloride (EDC) and sulfo-NHS per manufacturer recommendations. LAM was modified

by 4-(4,6-dimethoxy [1,3,5]triazin-2-yl)-4-methyl-morpholinium (DMTMM) prior to conjugation. Individual microsphere with unique fluorescence regions allowed for multiplexed flow cytometry-based quantifications *(31)*.

Diluted serum samples were incubated with pooled microspheres for 16 h at room temperature then washed three times with 0.1% bovine serum albumin (BSA)/0.05% Tween-20 in PBS. Secondary incubations were performed for 2 h at room temperature. Then, samples were washed three times prior to acquisition. For each assay, median fluorescence intensity (MFI) for each bead region was measured using an iQue Plus Screener (Intellicyt). For detection of FcγR-binding antibodies, diluted serum samples were incubated with the antigen-coated beads as above. For detection, PE-labeled Strepavidin was coupled to biotinylated, purified FcγRs (Duke Human Vaccine Institute). Excess D-desthiobiotin was used to saturate unbound Strep-PE. The Strep-FcγR was then diluted in 0.1 % BSA, 0.05 % Tween-20, and 1X PBS. The blocked detection reagent was then added as a secondary step similar to above and MFI for each bead region was quantified using an iQue Plus Screener (Intellicyt)

**Data analysis and statistics**

All computational analyses were performed in R 4.2.3 with Rstudio integrated development environment *(32, 33)*. Figures were generated using the R package 'ggplot2' or 'ggpubr' unless stated otherwise *(34)*. Tables were made using the R package 'gtsummary' *(35)*.

To compare cell subpopulations in INFECT cohort across different time periods, the $\log_{10}$-transformed cell counts at 2 weeks and 14 weeks were calculated for each participant. Unpaired Mann-Whitney U tests were used to compare cell subpopulation between groups at week 2 *(34)*. Paired Wilcoxon signed rank tests was used for paired significance comparisons of transformed cell counts in week 2 and week 14*(34)*. Both calculations were adjusted using Benjamini-Hochberg (false discovery rate). The median fold change and 95% confidence interval calculated on untransformed cell counts are also presented. The fold change between IGRA converters and persistently IGRA-negatives was compared using unpaired Mann-Whitney U test. The same was done to show the median fold change and 95% confidence interval between persistently IGRA-negative with BCG scar and without BCG scar. A decrease in cell count is indicated by a fold change of less than 1. The median fold change and confidence interval were calculated using MedianCI function from `DescTools` R package *(36)*. Paired Wilcoxon signed rank tests were used and the *P*-value were adjusted for multiple testing using Benjamini-Hochberg.

For cytokine measurements in the INFECT cohort, the level of cytokine that fell below the detection limit were substituted with the lowest detectable limit for each cytokine (39 pg/mL for TNF, 19.5 pg/mL for IL-1β, 195 pg/mL for IL-1Ra, 312 pg/mL for both IL-6 and IL-8, and 4.68 pg/mL for IL-10); the highest number for which this was done was for *Mtb* induced TNF production (3%). Contaminated samples, defined as samples with detectable IL-6 in unstimulated samples, were removed from the analysis. Cytokine data were $\log_{10}$ transformed. Batch effects were removed using the RemoveBatchEffect function from 'limma' *(37)*, and analyses were carried out on the residuals from this model fit. Heatmaps were created using the 'ComplexHeatmap' package *(38)* visualizing the median Z-score of the batch-adjusted cytokine variables. Unpaired Mann-Whitney U tests were used to compare adjusted cytokine levels between groups. Logistic regression was used to estimate the associations between cytokine production and IGRA status at baseline and follow-up. In the regression model to find the

association of cytokine with IGRA status at baseline, we used uncorrected $log_{10}$ transformed cytokine measurements and adjusted for age, sex, BMI, blood monocyte count, blood lymphocyte count and batch in the formula. While for the association of cytokine with IGRA status at follow-up, we added exposure risk score as a covariate Odds ratios were calculated from the beta estimates and adjusted for multiple testing using Benjamini-Hochberg.

For inflammatory proteins, only samples and proteins that passed quality control were used for the analysis. As protein measurements, especially in low concentration, can be affected by hemolysis, we excluded proteins that might be impacted by hemolysis of less than 3.8g/L based on the Olink Inflammatory Protein validation data sheet. We also excluded samples that had hemolysis more than 15g/L (as determined by two researchers blinded to IGRA status independently visually matching the sample to the hemolysis concentration reference in the Olink validation data sheet). The inflammatory protein relative levels (NPX) were $log_2$ transformed. Logistic regression models were used to estimate the association between NPX measurement of each inflammatory proteins at baseline and IGRA status at follow-up adjusting for age, sex, BMI, and exposure score. In addition, linear regression was used to find the correlation between inflammatory protein level with quantitative IGRA IFN-γ (TBAg – Nil) levels at follow-up.

For analysis of antibody profiles, for each individual anti-*Mtb* antibody levels were divided by the level of hemagglutinin (HA)-specific antibody as a positive control, and the resulting ratio was $log_{10}$ transformed. We established a lower limit of quantification for each antigen as the mean MFI + 6SD (standard deviation) in the PBS control. For statistical comparisons of antibody profiles by IGRA status, we used unpaired Mann-Whitney U tests, corrected for multiple testing by a Benjamini-Hochberg, and showed the fold change in the heatmap. Supervised clustering using partial least squares discriminant analysis (PLS-DA) using 'mixOmics' package on Z-scored data was used to discriminate the antibody profile explained by IGRA status, both at baselines and at follow-up *(39)*. Logistic regression models adjusting for age, sex, and BMI were used to find the associations between baseline antibody levels and IGRA status at baseline and at follow-up. Functional antibody variables (antibody dependent complement deposition, antibody-dependent cellular phagocytosis, and antibody-dependent neutrophil phagocytosis) specific for LAM were compared using the unpaired Mann-Whitney U tests. In addition, logistic regression adjusting for age, sex, and BMI was used to estimate associations between antibody functionality and IGRA status at follow-up.

In the 300BCG cohort, ex vivo cytokine measurements were $log_{10}$ transformed and corrected for batch effect using linear regression *(40)*. The heatmap of fold change between pre-vaccination and day 90 post vaccination were shown. Paired Wilcoxon signed rank tests were used for statistical comparisons of the pre-vaccination and 90-days post vaccination ex vivo cytokine levels. Antibody MFI were standardized to the MFI of HA-specific antibody as above. The ratios were then $log_{10}$ transformed. The heatmap of fold change of antibody level between pre-vaccination and 90 days post-vaccination were shown. Paired Wilcoxon signed rank tests were used for statistical comparisons of the pre-vaccination and 90-days post vaccination.

## List of Supplementary Materials

Fig. S1 to S8

Tables S1 to S5

Data file S1 (Excel file)

## References and Notes

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# Figures

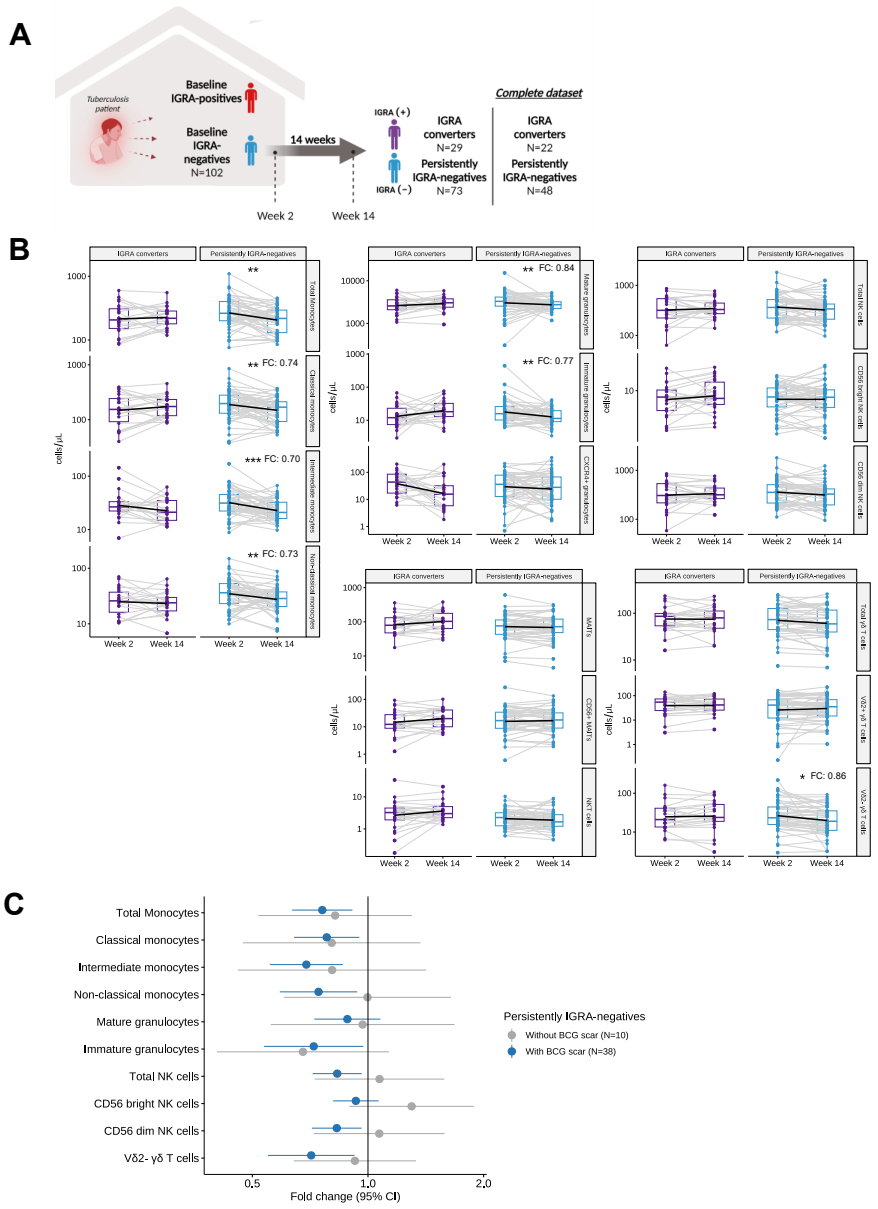
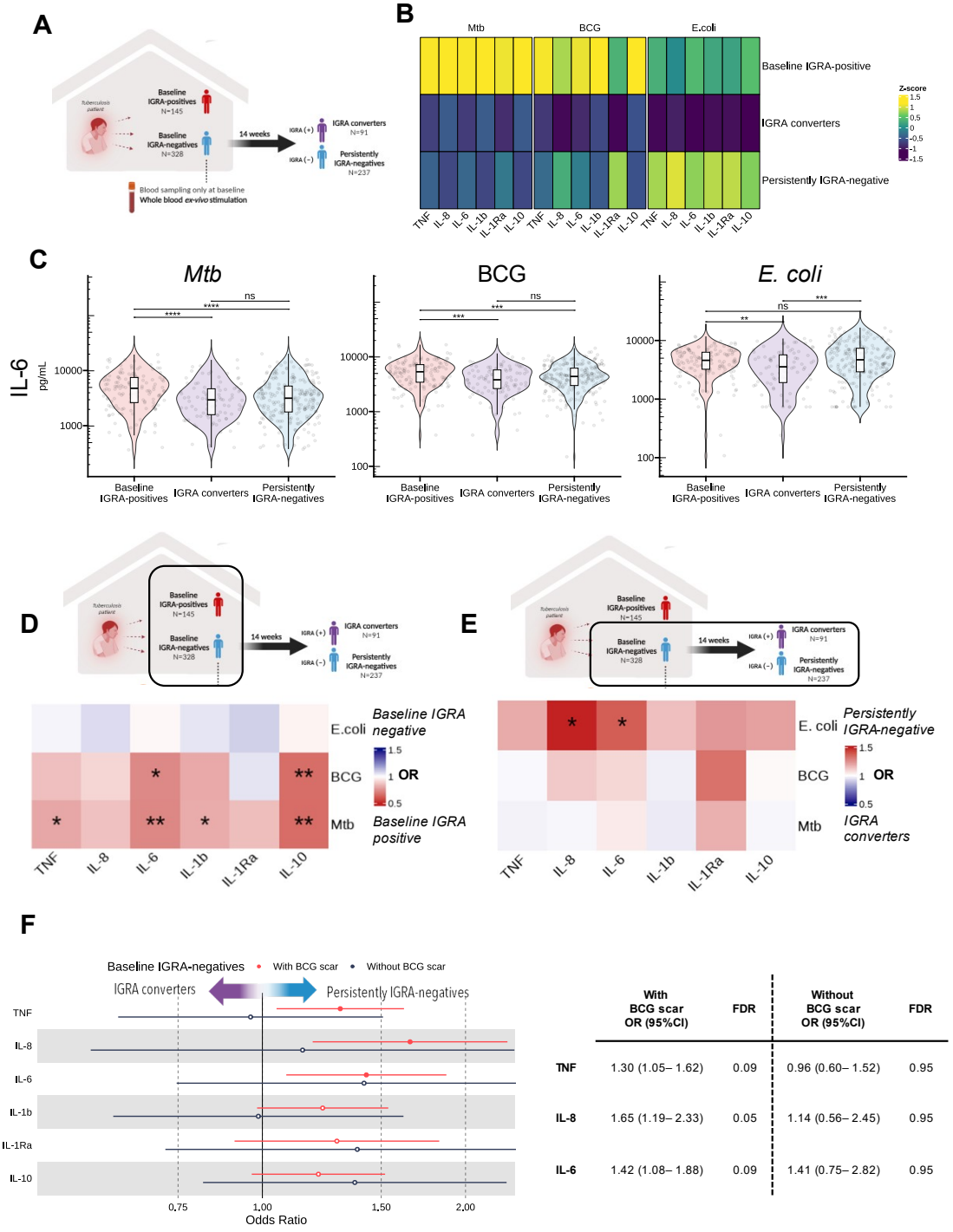



**Fig. 1. The dynamics of innate immune cells in IGRA-converters and persistently IGRA-negative individuals.** (**A**) Overview of the flow cytometry dataset. (**B**) Frequencies of circulating innate immune cells (numbers / µL blood) were compared between week 2 and week 14 in IGRA converters (N=22), and persistently IGRA-negative individuals (N=48, [FDR<0.1, <0.05, <0.01; *, **, ***; FC = median fold change]). (**C**) Persistently IGRA-negatives with BCG scar (N=38) showed a larger decrease in cell numbers than participants without BCG scar (N=10) in the innate circulating immune cells from week 2 to week 14.




| | With BCG scar OR (95%CI) | FDR | Without BCG scar OR (95%CI) | FDR |
|---|---|---|---|---|
| TNF | 1.30 (1.05– 1.62) | 0.09 | 0.96 (0.60– 1.52) | 0.95 |
| IL-8 | 1.65 (1.19– 2.33) | 0.05 | 1.14 (0.56– 2.45) | 0.95 |
| IL-6 | 1.42 (1.08– 1.88) | 0.09 | 1.41 (0.75– 2.82) | 0.95 |

**Fig. 2**. **Study outline and ex vivo cytokine production.** (**A**) Baseline whole blood ex vivo cytokine production, compared between baseline IGRA-negative (N=328) and IGRA-positive individuals (N=145), and between IGRA converters (N=91) and persistently IGRA-negative individuals (N=237). (**B**) Cytokine production following stimulation with *Mtb*, BCG, and *E.coli,* with higher *Mtb*-induced cytokine production in baseline IGRA-positive individuals, and higher *E. coli*-induced production in persistently IGRA-negative individuals. (**C**) *Mtb*, BCG, and *E.coli*-induced IL-6 production (as a representative), stratified for IGRA-status (Mann-Whitney U test after correction for multiple testing). Association between cytokine production and IGRA status at baseline (**D**) and 14 weeks (**E**), expressed as odds ratio (using logistic regression adjusting for age, sex, BMI, exposure score, blood monocyte count, blood lymphocyte count, and batch). (**F**) Relation between baseline ex-vivo cytokine production (in IGRA-negative individuals) and IGRA status at 14 weeks, shown as odds ratios, stratified for BCG vaccination status. All models corrected for multiple testing (Benjamini-Hochberg). (FDR<0.1, <0.05, <0.01, <0.001; closed circle & *, **, ***, ****)

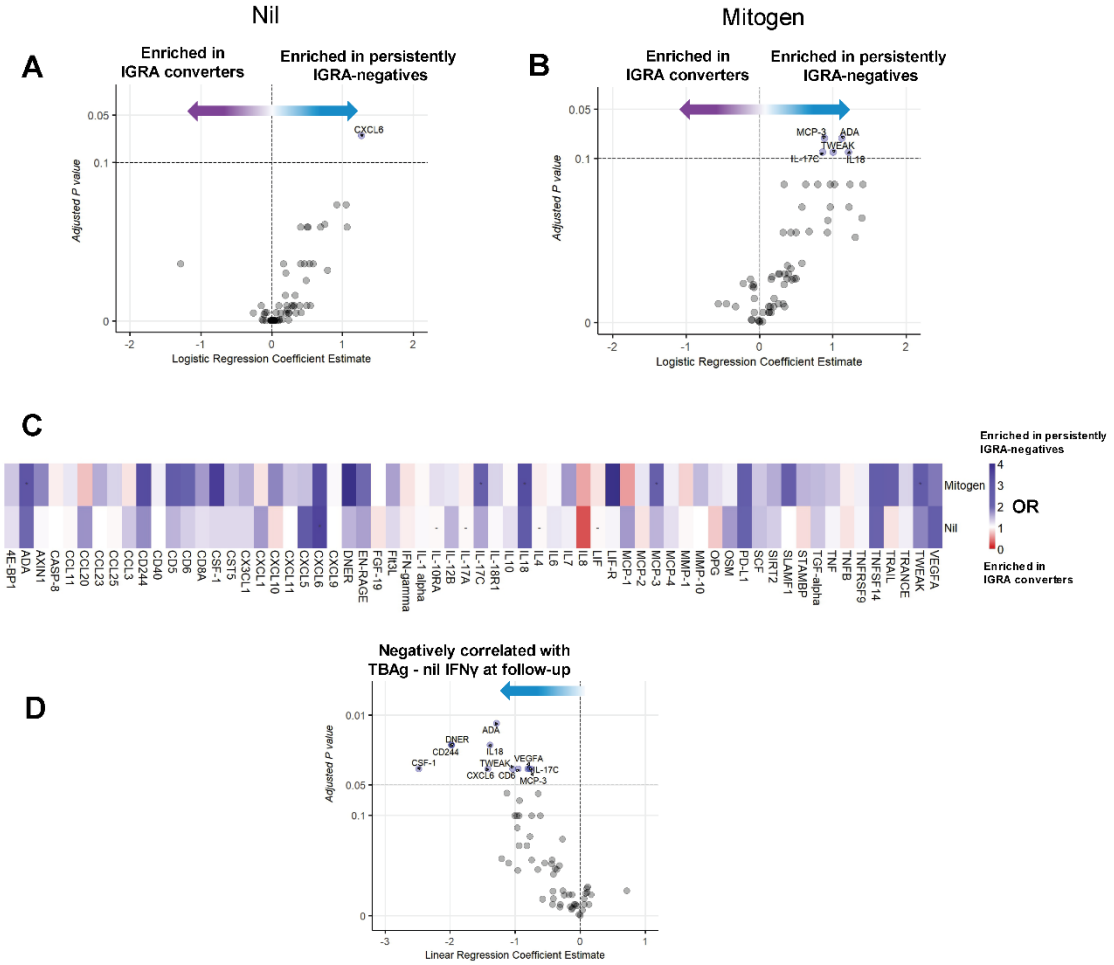



**Fig. 3**. **Inflammatory proteins in IGRA supernatants.** Inflammatory proteins relative concentrations (NPX unit, $\log_2$ transformed) in IGRA supernatants (nil and mitogen) at baseline were compared between IGRA converters (N=48) and persistently IGRA-negative individuals (N=128). (**A**) A persistently IGRA-negative status was associated with higher CXCL6 in the baseline IGRA nil tube, and (**B**) with ADA, MCP-3 (CCL7), TWEAK, IL-17C, and IL-18 in the baseline mitogen tube (logistic regression adjusted for age, sex, BMI, and exposure score; FDR<0.1). (**C**) Associations between persistently IGRA-negative status and concentrations of all proteins measured in IGRA nil and mitogen tube (Odds ratios, adjusted for age, sex, BMI, and exposure score). (**D**) In the mitogen tubes, the same proteins, as well as CSF-1, DNER, CD244, and VEGFA, showed a correlation with quantitative TBAg - nil IFNγ IGRA results after correction for multiple testing with lower FDR cutoff of 0.05.

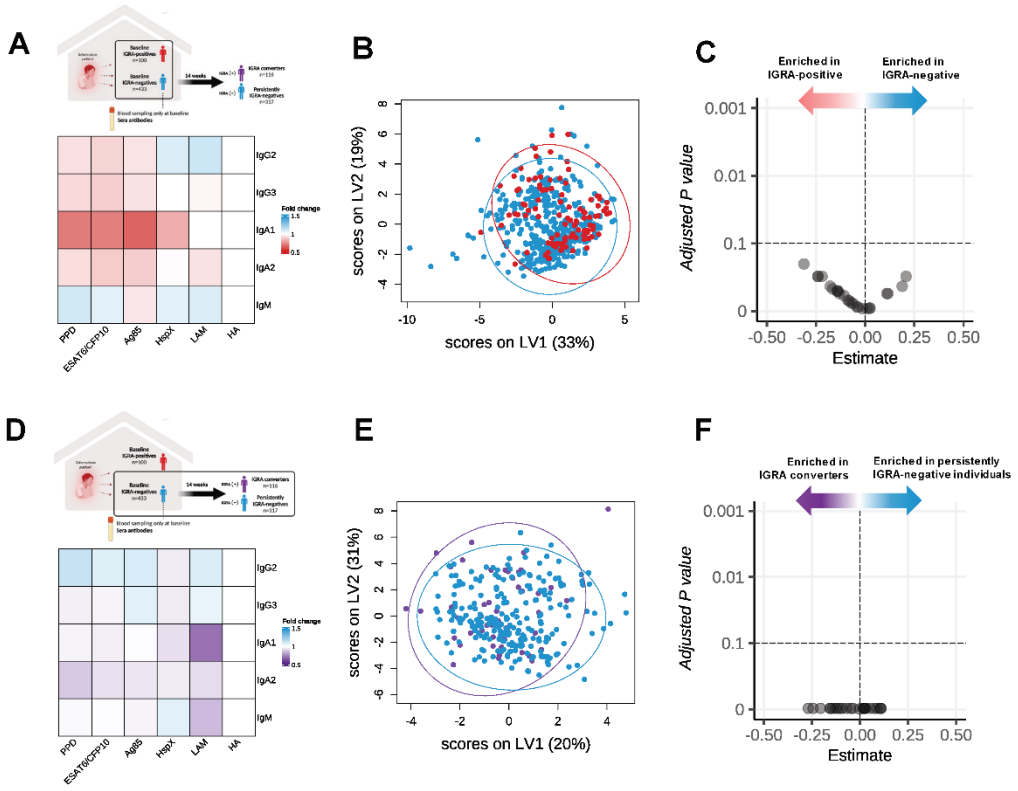
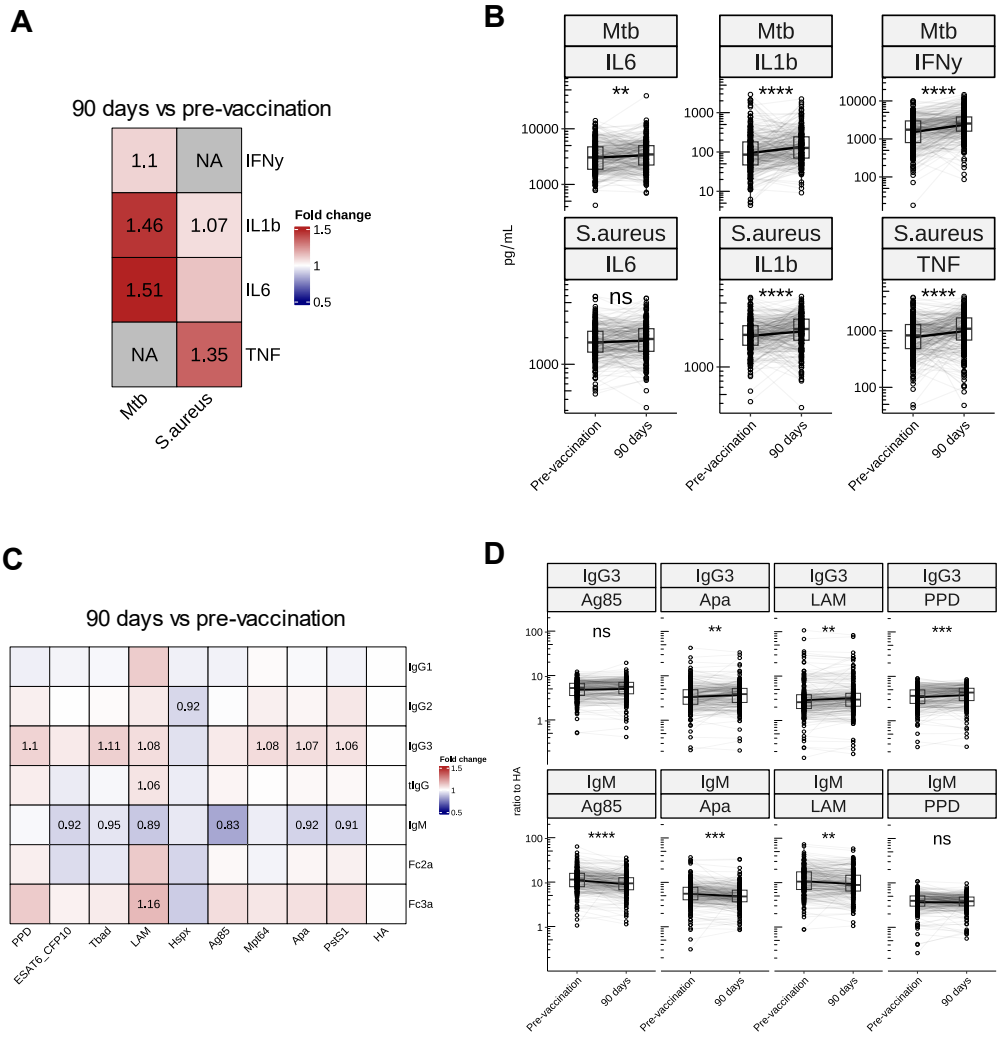



**Fig. 4. Antibody profiles according to IGRA-status at baseline and follow-up.** Antibody profiles were compared between IGRA-positive (N=100) and IGRA-negative (N=433) tuberculosis household contacts; and between IGRA converters (N=51) and persistently IGRA-negative individuals (N=237), based on strict IGRA criteria (<0.15 IU/mL for negative and >0.70 IU/mL for positive)**.** Fold differences in antibody levels (shown as ratio of antibodies corrected for the positive control hemagglutinin [HA]), are shown according to IGRA status at baseline (**A;** red: higher antibody levels in IGRA-positive individuals) and follow-up (**D;** purple: higher antibody levels in IGRA converters). No difference reached statistical significance, thus, numbers not shown in the heatmap (Mann-Whitney U test; FDR <0.1). (**B**) Partial least squares discriminant analysis (PLS-DA) using the selected 25 antibodies was used to visualize differences in antibody levels between baseline IGRA-positive (red) and -negative (blue) individuals, and (**E**) between IGRA converters (purple) and persistently IGRA-negative individuals (blue). (**C**) In logistic regression, no antibody was associated with IGRA status at baseline or follow-up (**F**), after adjustment for age, BMI and exposure.

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739 **Fig. 5. Effect of BCG vaccination on cytokine production and Mtb-specific antibodies.**
740 Heatmap showing fold change (**A**) and the paired boxplot (**B**) of ex vivo cytokine
741 production in Dutch healthy adults (N=298) before and 90 days after BCG vaccination.
742 Shown are 24-hour stimulation of PBMCs with *Mtb* and *S. aureus,* and 7-day stimulation
743 of *Mtb* for IFN-γ**.** Heatmap showing fold change of hemaglutinnin-standardized anti-
744 *Mtb* antibody levels at day 90 compared to the pre-vaccination, with statistically
745 significant fold changes shown in numbers (**C**)**.** Changes in IgM and IgG3 antibody
746 against different *Mtb* antigens (**D**). FDR<0.1, <0.05, <0.01, <0.001; *, **, ***, ****.

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749 **Table 1. Characteristics of tuberculosis household contacts according to baseline IGRA-**
750 **status**
751
752

| | Baseline IGRA-positive[a] N = 780 | Baseline IGRA-negative[a] N = 433 | *P* value[b] |
|---|---|---|---|
| **Case contact characteristics** | | | |
| Age | 31 (17 – 47) | 22 (12 – 39) | <0.001 |
| Female sex | 58% | 53% | 0.089 |
| Presence of BCG scar | 78% | 84% | 0.013 |
| Current and previous smoking | 35% | 31% | 0.27 |
| BMI, kg/m$^2$ | 21.6 (18.0 – 25.4) | 20.2 (16.8 – 24.4) | 0.001 |
| Diabetes[c] | 3.2% | 3.9% | 0.41 |
| Exposure to the index case | | | |
| Sleeping in the same room as the index case | 30% | 20% | <0.001 |
| Waking hours spent with the index case a day before enrollment | 5 (2 – 10) | 4 (1 – 8) | 0.001 |
| Index case highest smear grade | | | <0.001 |
| *Scanty* | 5.4% | 8.3% | |
| *1+* | 18% | 28% | |
| *2+* | 26% | 25% | |
| *3+* | 50% | 38% | |
| Presence of cavities on chest x-ray of index | 56% | 44% | <0.001 |
| Extent of x-ray abnormalities | 50 (25 – 71) | 40 (25 – 59) | <0.001 |
| *M. tuberculosis* Beijing genotype in the index case | 35% | 28% | 0.022 |
| Blood count parameters at baseline | | | |
| Hemoglobin g/dL | 13.70 (12.80 – 14.90) | 13.70 (12.80 – 15.00) | 0.56 |
| Platelets 1,000/mm$^3$ | 298 (257 – 351) | 305 (258 – 360) | 0.17 |
| Leukocytes 1,000/mm$^3$ | 7.50 (6.50 – 8.90) | 7.40 (6.20 – 8.60) | 0.077 |
| Lymphocytes 1,000/µL | 2.60 (2.15 – 3.20) | 2.60 (2.12 – 3.07) | 0.20 |
| Neutrophiles 1,000/µL | 4.12 (3.35 – 5.07) | 4.03 (3.20 – 5.02) | 0.23 |
| Monocytes 1,000/µL | 0.46 (0.35 – 0.60) | 0.43 (0.32 – 0.56) | 0.003 |
| Quantitative IFNγ release assay result | | | |
| IFNγ Nil tube IU/L | 0.15 (0.09 – 0.29) | 0.14 (0.08 – 0.28) | 0.042 |
| IFNγ TB-Nil tube IU/L | 2.8 (1.1 – 6.7) | 0.0 (0.0 – 0.1) | <0.001 |
| IFNγ Mitogen-Nil tube IU/L | 9.32 (3.72 – 10.00) | 8.68 (3.41 – 10.00) | 0.53 |

Abbreviations: BCG, Bacillus Calmette-Guerin; BMI, body mass index; IQR, interquartile range.

[a] Median (IQR); %

[b] Mann-Whitney U test; Pearson's Chi-squared test; Fisher's exact test

[c] Diabetes defined as follows: no diabetes, random capillary blood glucose >101 mg/dL or hemoglobin A1c (HbA1c) <5.7%; prediabetes, HbA1c 5.7%–6.4%; diabetes, HbA1c ≥6.5.

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