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BCG activation of trained immunity is associated with induction of cross reactive COVID-19 antibodies in a BCG vaccinated population

Najeeha Talat Iqbal[1,2☯]*, **Kumail Ahmed**[1☯], **Tehniat Sattar**[1], **Fatima Aziz**[1], **Rabia Hussain**[3☯]

1 Department of Pediatrics and Child Health, The Aga Khan University, Karachi, Pakistan, **2** Department of Biological and Biomedical Sciences, The Aga Khan University, Karachi, Pakistan, **3** Department of Pathology and Laboratory Medicine, The Aga Khan University, Karachi, Pakistan

☯ These authors contributed equally to this work.
* najeeha.iqbal@aku.edu

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Abstract

Background

Pakistan is endemic to a diverse set of parasitic, mycobacterial and viral diseases. The recognition of BCG Trained Immunity (TI) led us to postulate that the continued presence of BCG-TI may play a protective role, previously reported for both infectious and noninfectious conditions. Most of the previous studies have addressed the issue of BCG-TI in the paediatric populations. This study addressed the key issue of maintenance of BCG-TI in a wider age range (adolescent and adults) to identify the strength and quality of the immune responses.

Objective

To assess the BCG-induced recall responses in healthy individuals by cytokines secreted from the TI network and its potential role in providing cross-protection against COVID-19 and other viral infections.

Study design

In this cross-sectional study, healthy young adults and adolescents (n = 20) were recruited from 16–40 years of age, with no prior history of TB treatment, autoimmune, or chronic inflammatory condition.

Methods

BCG-induced cytokine responses were assessed using prototypic markers for cells of the TI network [macrophages [M1 (TNFα, IFNγ), M2 (IL10)], NK (IL2), Gamma delta (γδ) T (IL17, IL4)] and SARS CoV2 IgG antibodies against RBD using short-term (12 hrs.) cultures assay.

Competing interests: The authors have declared that no competing interests exist.

Results

Significant differences were observed in the magnitude of recall responses to BCG with macrophage cytokines showing the highest mean levels of TNFα (9148 pg/ml) followed by IL10 (488 pg/ml) and IFNγ (355 pg/ml). The ratio of unstimulated vs.BCG-stimulated cytokines was 132 fold higher for TNFα, 40 fold fo r IL10, and 27 fold for IFNγ. Furthermore, SARS-CoV-2 antibodies were also detected in unstimulated plasma which showed cross reactivity with BCG.

Conclusion

The presence of cross reactive antibodies to SARS-CoV-2 and the relative ratio of pro- and anti-inflammatory cytokines secreted by activated TI cellular network may play a pivotal role in protection in the early stages of infection as observed during the COVID-19 pandemic in the younger age groups resulting in lower morbidity and mortality.

1. Introduction

Pakistan ranks among the top six highest TB burden countries, contributing approximately 56% of the total TB burden globally [1,2]. WHO introduced Expanded Vaccine Immunization in Pakistan in 1965 at the population levels to most of the prevalent infections. Pakistan still provides Bacille Calmette-Guerin (BCG) vaccine at birth (>90%). Although BCG protection against pulmonary disease was limited in adults [3–5] but in children, BCG vaccination provides protection against disseminated disease in early childhood [6,7] as well exhibits non-specific beneficial effects by decreasing mortality in children with respiratory viral illnesses [8], and sepsis [9,10]. For these reasons, BCG vaccine coverage has been continued by WHO in TB endemic countries. BCG vaccination is given at birth, so there are no reliable markers for assessing BCG vaccination status in young adults, as T cells are known to wane over time and most available markers (BCG scar, MT, or PPD) are reflecting T cell memory responses [11]. EPI schedules include BCG and Oral Polio Vaccine (OPV) at birth and other vaccines (MMR, tetanus, Pneumococcal, Pertussis, Rota virus and Diphtheria) at 6, 10 & 14 weeks, which provides protection against twelve life threatening diseases in early childhood. During the COVID-19 pandemic, Pakistan reported relatively low morbidity and mortality rates compared to the rates reported by the Western Countries where no universal vaccination policy exists or BCG has been discontinued [12]. The case fatality rate is 1.95% whereas recovery rate is 98.05% (https://www.worldometers.info/coronavirus/country/pakistan/). It was therefore, speculated that BCG may also provide cross-protection against COVID-19 disease [13–15] and may contribute to decreased morbidity and mortality in Pakistan [12]. The rationale for this speculation lies in the fact that BCG is a potent stimulator of the innate arm of the immune system which recognizes conserved antigens (PAMPs) shared across several species [16–18]. Although innate immunity was not considered to have memory, recently a concept of TI possessing memory has emerged [19,20]. TI is defined as a bridge between innate and adaptive immune response, predominantly involving innate cells. Recurrent boosting of the innate cellular network in a TB endemic area such as Pakistan may occur due to environmental exposure to TB or non-pathogenic mycobacteria resulting in remodeling and epigenetic changes in monocytes which result in a heightened immune response upon re-infection [21,22], thus providing protection to other non-related pathogens such as COVID-19 [23]. Trained immunity

concept was shown as rewiring of monocytes after exposure to BCG resulted in major shift in metabolic pathway from Glucose to Glutamine, and epigenetic changes in mTOR pathway [24]. The reprogramming of innate immune cells is not limited to BCG or infectious diseases, but has implications in auto-immune inflammatory disorders, neurological and cardiometabolic disease [25]. Although there is extensive data available with respect to recall responses in the adaptive or T cell arm of the immune response in BCG vaccinated countries including Pakistan [22], there is little information regarding recall responses in the innate arm (TI) in adults. The cells that form the TI network are cells of the monocyte/macrophages (Mf) lineage comprising of several subsets (M1, M2a, M2b, and M2c), natural killer (NK) cells, and gamma delta (γδ) T cells derived from the T cell lineage. B cells are also activated by certain antigens to secrete IgG antibodies without T cell help via BCR cross linking and thus form part of the innate network [26,27]. Innate cells are activated by PAMPs via their appropriate Pattern Recognition Receptors (PRRs) [28,29] (S1 Table). Cytokines act as growth factors, activators, modulators, and regulators of the immune response, and TI acts as a bridge between the innate and adaptive arms in orchestrating the adaptive arm. We, therefore, investigated BCG induced TI responses by assessing prototypic cytokine secreted by the cells of the innate network. The balance between pro- and anti-inflammatory cytokines may play a key role in disease outcome. Our primary objective was to identify TI activation, using BCG stimulated whole blood assay (WBA) and purified peripheral blood mononuclear cells (PBMCs) cultures [30]. Another interesting observation was the presence of BCG cross-reactive IgG antibodies to SARS-CoV-2 antigens. We hypothesize, that the presence of PAMPs like molecules in SARS-CoV-2 may activate the TI network in BCG vaccinated young adults. Both the presence of SARS-CoV-2 cross reacticve IgG antibodies and cytokine secreted by TI network may play a crucial role in determining less severe disease outcome of COVID-19 in young adults as observed during the COVID-19 pandemic in Pakistan and other countries with wide BCG coverage.

2. Materials and methods

2.1. Ethics

The research protocol was approved by the Ethical Review Committee of Aga Khan University (ERC protocol 2885–14) Pakistan. Written informed consent was obtained from the patients. For children less than 18 years of age written informed assent was taken from their guardians.

2.2. Study design

The study subjects were recruited from one of the field sites developed by The Department of Pediatrics and Child Health, Aga Khan University, Karachi, from 12/11/2014 to 25/05/2016. Subjects (n = 20) were enrolled after informed consent. The inclusion criteria were healthy adolescents, matched for gender and age (range: 16–40 years). Exclusion criteria were a history of past TB treatment or autoimmune or chronic disease, or steroid therapy for any condition. BCG scar presence was noted, and the Mantoux Test was also conducted (S2 Table).

2.3. Sample processing

Five ml of blood was used for whole blood assay (WBA) after diluting 1/4 with complete RPMI 1640, while the rest of the blood was used to isolate PBMCs by Ficoll-Isopaque density gradient centrifugation. Media was supplemented with 200mM L-Glutamine, 10X Penicillin-streptomycin mix, 1 M HEPES, and 100 mM Sodium Pyruvate (Sigma Aldrich, St. Louis MO). Cell concentration was adjusted at 2x10^6 cells/ml in 24 well cell culture plates (Corning Incorporated, NY, USA).

2.4. BCG stimulation of cell cultures

Freeze-dried Glutamate BCG was obtained from the Serum Institute India. BCG (2-8x10^6 CFU) was reconstituted in 250μl of RPMI to obtain MOI of 1.2 in 60μl of BCG as per Hanekom protocol [31]. Freshly reconstituted BCG was added to stimulate the peripheral blood mononuclear cells (PBMCs) (2x10^6) and whole blood (WB) (1/4 diluted) and incubated for 12 hrs. Supernatants were harvested and stored at −80 ˚C until further use. Additionally, WB and PBMCs were stimulated using 10μg/ml of Lipopolysaccharide (LPS) (Sigma Aldrich, St. Louis MO).

2.5. Assessment of cytokine responses in BCG induced cell cultures

BCG stimulated and un-stimulated cell cultures were assessed for secreted cytokines post 12 hrs. incubation. Cytokines were analyzed on Bio-Rad Luminex 200 platform. The bead mixed panel includes IL2, IL4, IL10, IL17A, IFNγ, and TNFα. Data were acquired using Bio-Plex Manager Software version 6.1 (Bio-Rad).

2.6. Gene expression analysis for BCG stimulated cells using real time PCR

2.6.1. RNA extraction. RNA extraction was carried out after RBC lysis from WBA and PBMCs culture using TRIzol reagent (Ambion, life technologies, Carlsbad CA, USA). Finally, the RNA pellet was re-suspended in 20μl of DNase RNase-free water (Gibco, Life Technologies, NY, USA). RNA purity and quantity were checked by Nanodrop 2000c.

2.6.2 cDNA synthesis. cDNA was synthesized using the Bio-Rad Iscript buffer (Bio-Rad Lab. Hercules, CA) as per the manufacturer's instruction. The protocol was run on the CFX 96 Bio-Rad (Hercules, CA) platform.

2.6.3. Real-time quantitative PCR. The oligonucleotide primers were designed from IDT for Granzyme (GZMA), Butyrophilin (BTN3A2), Tumor Necrosis Factor (TNFα), and 36B4 (as housekeeping) genes. qPCR was performed using Bio-Rad SYBR Green (Bio-Rad Lab. Hercules, CA) as per the manufacturer's instructions. Briefly, cDNA was used as a template with a 1:10 ratio of cDNA and SYBR green in a total of 25μl of reaction and read at Bio-Rad CFX 96 platform. All results of the test gene were normalized with a housekeeping gene (36B4 gene). ddCt was used to express gene expression after normalizing with the housekeeping gene. Primers used to analyze different genes are shown in (S3 Table).

2.7. Assessment of IgG anti-RBD (SARS-CoV-2) in plasma using ELISA assay

Immulon 4 ELISA plates were coated with receptor binding domain (RBD) antigen (Institute of Protein Design- IPD, The University of Washington Seattle USA) (2 μg/ml) in 1X PBS as coating buffer and incubated overnight at 4 ˚C. Plates were washed with 1X PBS-0.1% Tween20 (PBST) and blocked with 2% bovine serum albumin (BSA). Plasma samples were diluted 1:500 in 1X PBST-2% BSA and added to their respective wells. Plates were incubated for 1.5 hours at room temperature with shaking. Plates were subsequently washed with 1X PBST during each step. Goat Anti-human-IgG (H+L) secondary antibody conjugated with horse-reddish peroxidase was added at 1:30000 dilution and plates were incubated for 1 hour at room temperature while shaking. Plates were finally developed using o-phenylenediamine (OPD) tablets mixed with sodium perborate buffer (Sigma-Aldrich). All plates were read on a MultiSkan Sky ELISA plate reader (ThermoFisher Scientific MA USA) at 492 nm. A dose response curve was developed using a high titer of positive IgG antibodies pool from COVID-19 patient's samples. The positive pool was serially diluted from 1:1000 to 1:128000 and an

unknown concentration of IgG was read using the standard curve. R^2 was 0.9954 and the negative pool has less than 0.3 O.D. Samples were considered positive for IgG if they had higher Mean +3SD from the negative pool.

2.8. Data analysis

The data were analyzed using a non-parametric test between the subjects using the Mann-Whitney U test and Wilcoxon Sign Rank test for within subjects. Spearman rank correlation analyses were applied for a significant correlation between cytokines. Principal component analyses use factor analyses to cluster cytokines into different groups. IBM Corp. Released 2012. IBM SPSS Statistics for Windows, Version 21.0. was used for data entry and analyses. R Studio Package, Version 4.1.2, (www.r-project.org) was used for data visualization using heat maps and dot plots. Cytokine data was reported after the deduction of background signal from un-stimulated controls. The signal to Noise ratio S/N was calculated by dividing the mean of stimulated cytokine level by mean of spontaneous or background secretions. Cytokine or gene expression data was reported after normalization of the test gene by the housekeeping gene for stimulated and un-stimulated controls, the results are expressed as an arbitrary unit (AU) of the expressed gene relative to the expression of negative or un-stimulated control using the ddCt method.

3. Results

3.1. Characteristics of the study group

COVID-19 pre-pandemic healthy adults (n = 20) were recruited between 2015 to 2016. Gender distribution was similar, and the median age was 27 years. BCG scar and MT frequency were 40% and 10% respectively. This is not surprising as T cell responses wane over time. MT positivity was much lower (10%) than BCG scar probably because of the low sensitivity of MT (Table 1).

Since there is >90% BCG vaccination coverage at birth in Pakistan, neither BCG scar nor MT positivity was reflective of BCG vaccinated status. Since there was also no difference in cytokine responses between BCG vaccinated and non-vaccinated subjects (S1 Fig). We, therefore, carried out the TI analyses in the un-stratified study.

3.2. Cytokine secretion in un-stimulated and BCG stimulated whole blood assay (WBA) and PBMCs cultures post 12-hour incubation

BCG induced recall responses were observed in all cells of the TI network. Fig 1 shows the complete dynamics of cytokine secretion for both assays. Spontaneous cytokine activation is shown as a line over the shaded area representing BCG stimulation. All cytokines showed

Table 1. Characteristics of study participants.

Study Subjects	**n = 20**
Male / Female	10 / 10
TB Contact History	2 / 20
BCG Scar	8 / 20
MT positivity (> 5.0 mm)	1 / 20
Age: Median; Range (Years)	27; (16–40)
Cell count (PBMCs per ml) mean ± SE	190.023 ± 2.23

Note: All participants were enrolled pre-COVID-19 pandemic.

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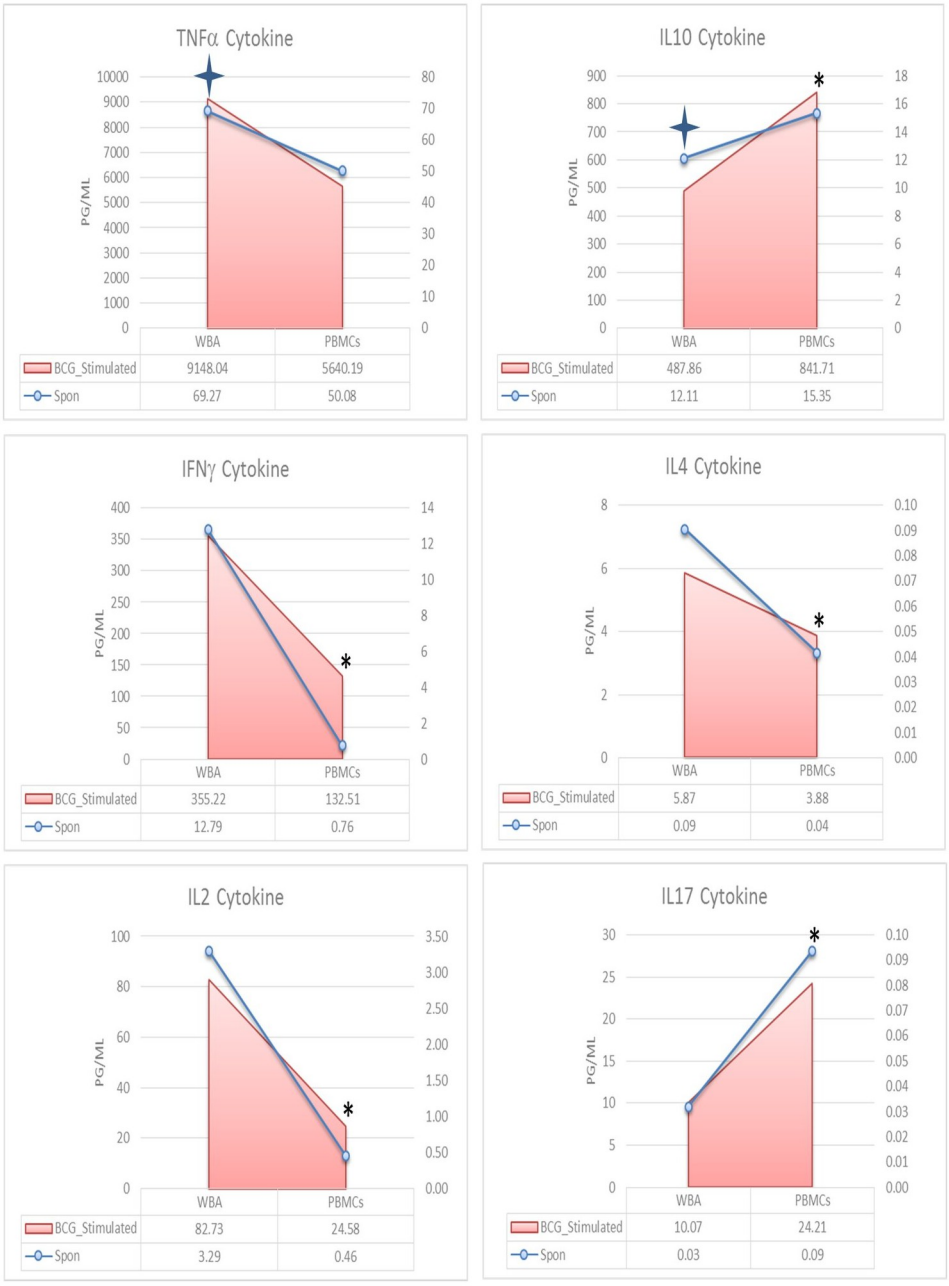

Fig 1. Cytokine secretion in spontaneous and Bacille Calmette-Guerin (BCG) stimulated Whole Blood Assay (WBA) and peripheral blood mononuclear cells (PBMCs) short-term culture supernatants. The shaded area compares secretion of WBA and PBMCs (n = 20) as the mean level of individual cytokine in BCG stimulated cultures (12 hrs.) shown on the primary axis (left side). The line graph compares the spontaneous secretion of the cytokine to be read on the secondary axis (right side). The "4-sided star" indicates a significant *p*-value for spontaneous cytokine secretion, whereas "asterisks" shows the difference in BCG stimulated cytokines in WBA and PBMCs. Wilcoxon Sign Rank test was applied for the significant difference in the cytokine level. $p < 0.05$ was considered significant.

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recall responses but there were several log differences in cytokines secreted by cells of monocyte (TNFα, IFNγ, IL10) and cells of the T cell lineage (IL2, IL4, IL17). When BCG stimulated cytokines were compared in the two assays, TNFα, IFNγ, IL2, and IL4 were up regulated in the WBA culture compared to the PBMCs culture. On the contrary, IL17

($p = 0.003$) and IL10 ($p = 0.013$) showed significantly higher secretion in PBMCs (Fig 1-shaded area).

When WBA and PBMCs cultures were compared for spontaneous cytokine secretion S4 Table), significant differences were noted for macrophage cytokines with higher responses in WBA for TNFα (MWU; $p = 0.009$) and significantly lower IL10 (MWU; $p = 0.017$) in WBA compared to PBMCs. Because of differential spontaneous secretion in the two assays, we analyzed net responses which probably reflect true recall responses. This analysis provided a different set of answers. The highest concentrations (*p*g/ml) were again observed with macrophage secreted cytokines but with more clear differences in the mean level of cytokines in two assays (WBA vs PBMCs: TNFα, 9148 vs 5640; IFNγ, 355 vs 132; IL10 488 vs 842; IL2, 83 vs 24; IL4, 6 vs 4; IL17, 10 vs 24) (S2 Fig).

We also looked at recall responses in terms of the ratio of increase post stimulation (Signal/Noise) in the two assays. Four of six cytokines showed comparable S/N ratio for WBA and PBMCs (IL2, IL4, IL-10 and IL17) while differences in S/N ratio were higher for TNFα in WBA (132 –fold) compared to PBMCs (113-fold). IFNγ on the other hand showed lower concentrations in WBA compared to PBMC with a higher ratio for PBMC (WBA; 27 v s PBMCs 132) (S5 Table). So, both the magnitude and ratio of recall response are higher for macrophage secreted cytokines in WBA compared to PBMCs indicating that WBA may provide a better window for assessing recall responses.

3.3. Effect of BCG scar on BCG stimulated cytokine secretion

We also compared cytokine responses in subjects stratified by BCG scar positivity. Although the group sizes are relatively small, no significant differences were noted in cytokine secretion in WBA for the two groups (S1 Fig). This is not surprising as the presence of BCG scar is related to adaptive T cell responses and not to innate immunity.

3.4. Effect of BCG scar on gene expression in stimulated PBMCs

We also carried out prototypic gene expression analysis associated with TNFα (macrophages), Granzyme A (GZMA; NK cells), and Butyrophilin (BTN3A2; gamma delta (γδ) T cells) in PBMCs (S3 Table). TNFα was the only gene which showed differential upregulation in the BCG scar negative subjects compared to the BCG scar positive subjects ($p = 0.038$). The implications of up regulated TNFα expression in BCG scar negative donors and its association with BCG vaccination status is unclear. There was no difference in gene expression for natural killer (NK) cells and gamma-delta (γδ) T cells (Fig 2).

3.4.1. Principal component analysis (PCA). We next addressed the issue of the relationship between different cytokines in the two assays. Principal Component Analysis (PCA) was carried out to determine cytokine clustering patterns in WBA and PBMCs (Fig 3). PCA separated data into two components PC1 which contained 5/6 cytokines and PC2 with IL10 in WBA cultures. Cytokines secreted from PBMCs are clustered as a single component. This is interesting as PC1 clusters all the pro-inflammatory cytokines which are derived predominantly from M1 macrophages, while IL10 which is an anti-inflammatory cytokine is secreted not only from M2 macrophages but several other cell sources of the TI network. Therefore, PC analysis confirms our previous contention that TNFα and IL10 are from different cell sources.

3.4.2. Correlation analysis of secreted cytokines in WBA and PBMCs cultures. To further understand the relationship between secreted cytokines in WBA and PBMC, we carried out correlation analyses. (Fig 4). Very similar patterns were observed with both WBA (4A) and PBMCs (4B).

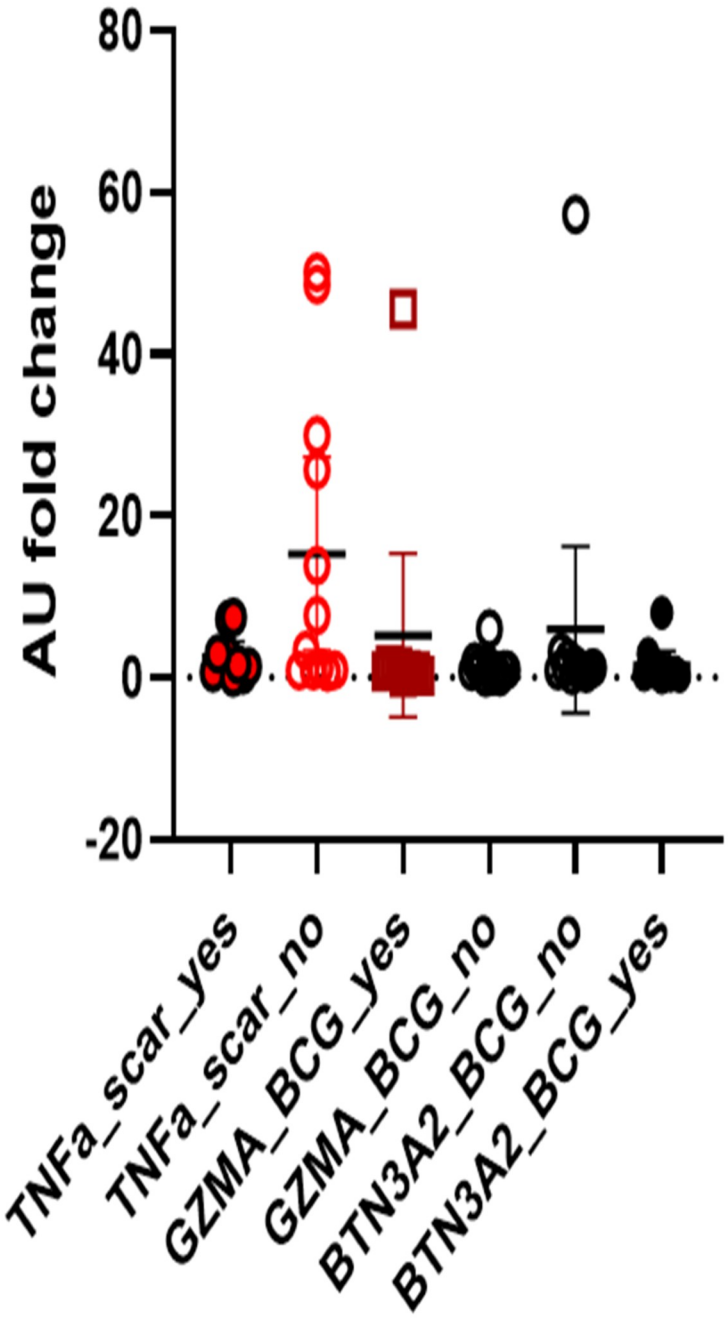

Fig 2. Expression of innate and adaptive genes in response to Bacille Calmette-Guerin (BCG). The scatter plot shows the fold changes in the expression of Tumor Necrosis Factor (TNFα), Granzyme A (GZMA), and Butyrophilin (BTN3A2) genes in response to live BCG stimulation of PBMCs. The fold changes are shown as relative expression compared to non-stimulated media control (10% FBS) using 2x10^6 cells between 12–24 hrs. Wilcoxon Sign Rank test were applied for the comparison of Scar positive and negative subjects.

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The correlation indices are shown in S6 and S7 Tables. All cytokines showed a highly significant positive correlation (>0.5), except for IL10 in WBA (highest, r = 0.515 with IL4; lowest r = 0.277 with IL2), and PBMCs (highest, r = 0.681 with IL17; lowest r = 0.455 with IFNγ). This is consistent with what we observed in PCA analysis with IL10 grouping differently than

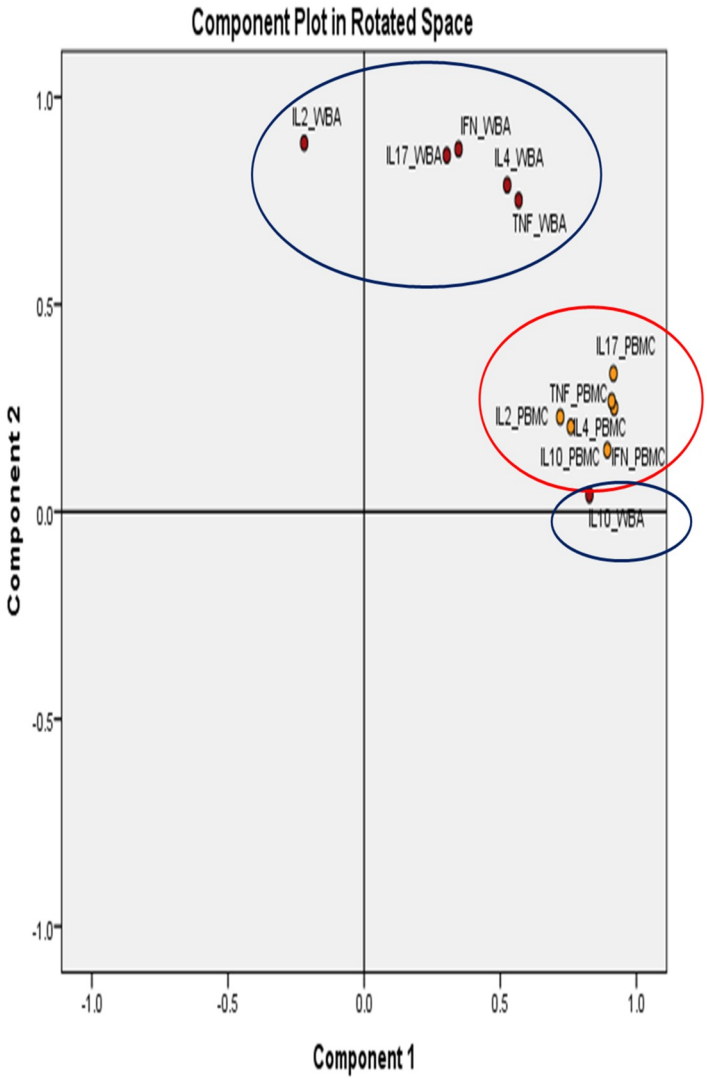

Fig 3. Assay dependent clustering pattern of BCG stimulated cytokines. The orange dots indicate cytokines analyzed through PBMCs while the red dot indicates cytokines analyzed through WBA. The PCA separated data into two major components. All variables explained 80% of the variability in the model.

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all other cytokines. Differences in PC compartments and differential correlation in WBA and PBMCs culture suggest that WBA culture may be a more appropriate window for investigating TI responses as it is better reflective of in vivo situations due to minimal processing.

3.5. Comparison of cytokine secretion in WBA cell culture in response to BCG and LPS stimulation

Lipopolysaccharide (LPS) is a potent mitogen and a strong stimulator of macrophages. We next compared BCG and LPS stimulated cytokine secretion to identify if there was any commonality in antigen recognition between those antigens. The concordance of antigen recognition with BCG and LPS is shown in Fig 5. Responses with LPS were comparable for TNFα, and IFNγ, while IL17, IL2, and IL4 showed a much lower overall magnitude of responses with

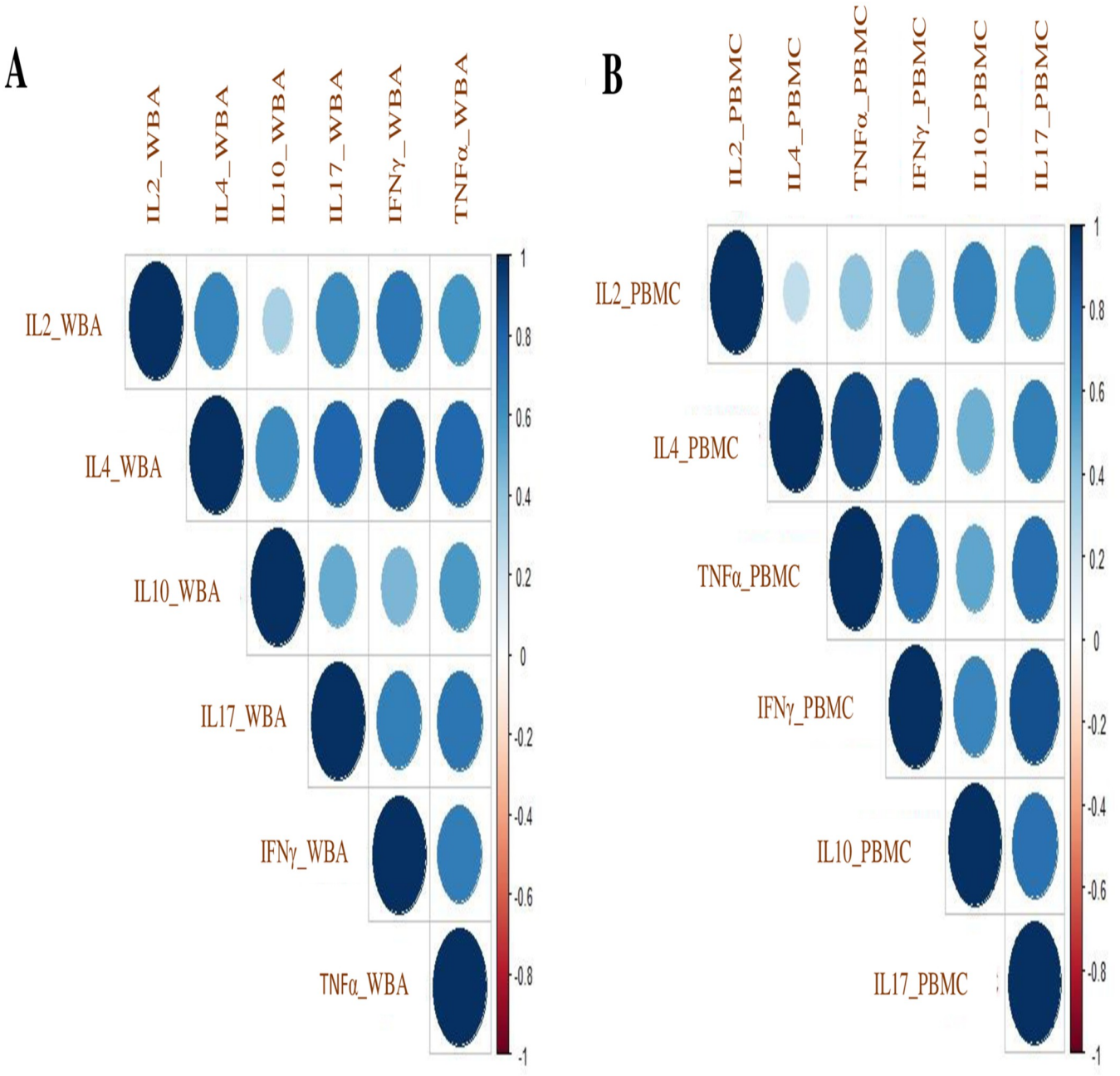

Fig 4. Cytokine correlation index in WBA and PBMCs cultures. Correlation plot showing a correlation coefficient of cytokines using the intensity of colors at the scale of -1.0 to +1.0 in WBA (**A**) and PBMCs assay (**B**).

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LPS antigen. IL10 was higher in LPS stimulated samples compared to BCG. Interestingly, BCG was a better stimulus compared to LPS with IL17, IL2, and IL4.

As expected, the highest correlation between LPS and BCG was observed with TNFα (SR rho = 0.67; $p = 0.002$) followed by IL4 (SR rho = 0.611; $p = 0.005$), IL2 (SR rho = 0.562; $p = 0.015$) and IFNγ (SR rho = 0.464; $p = 0.045$). Again, no correlation was observed with IL10 (SR rho = -0.033; $p = 0.896$) and IL17 (SR rho = 0.273; $p = 0.258$) (S8 Table). These results

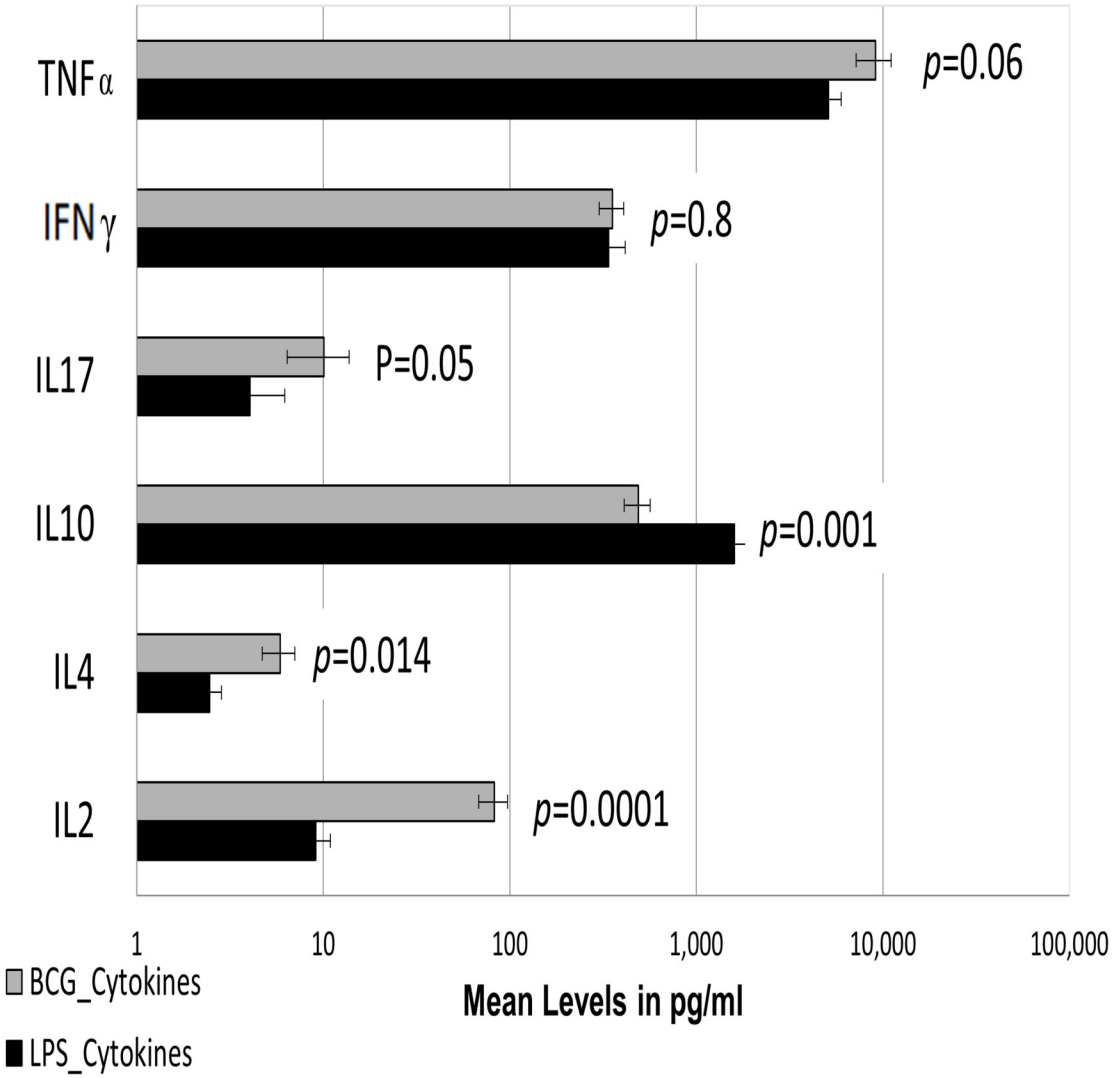

Fig 5. Comparative analysis of Bacille Calmette-Guerin (BCG) and Lipopolysaccharide (LPS) stimulated cytokine secretion in Whole Blood Assay (WBA) cell culture. Comparison of cytokine secretion in response to BCG and LPS stimulation in WBA cell cultures at 12 and 48 hrs. respectively (n = 20). Horizontal bars indicate mean levels with standard error bars around the mean. Student t-tests were carried out to assess the significance of differences between BCG and LPS.

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strongly suggest that all induced cytokines are recognized as PAMPs like molecules and are derived from cells of the TI network.

3.6. Assessment of IgG anti-RBD antibodies in the plasma of healthy donors

Since it has been reported that BCG provides non-specific immunity against other respiratory infections, we investigated if cross-reactive IgG against Receptor Binding Domain (RBD) were present. A calibration curve was generated with a pool of COVID-19 positive sera (S3 Fig) with the endpoint titer considered as 1 unit/ml.

All healthy donors showed considerable levels of SARS-CoV-2 RBD antibodies (Fig 6). The binding of antibodies to RBD was inhibited in the presence of BCG suggesting recognition of cross-reactive PAMPs like molecules in RBD.

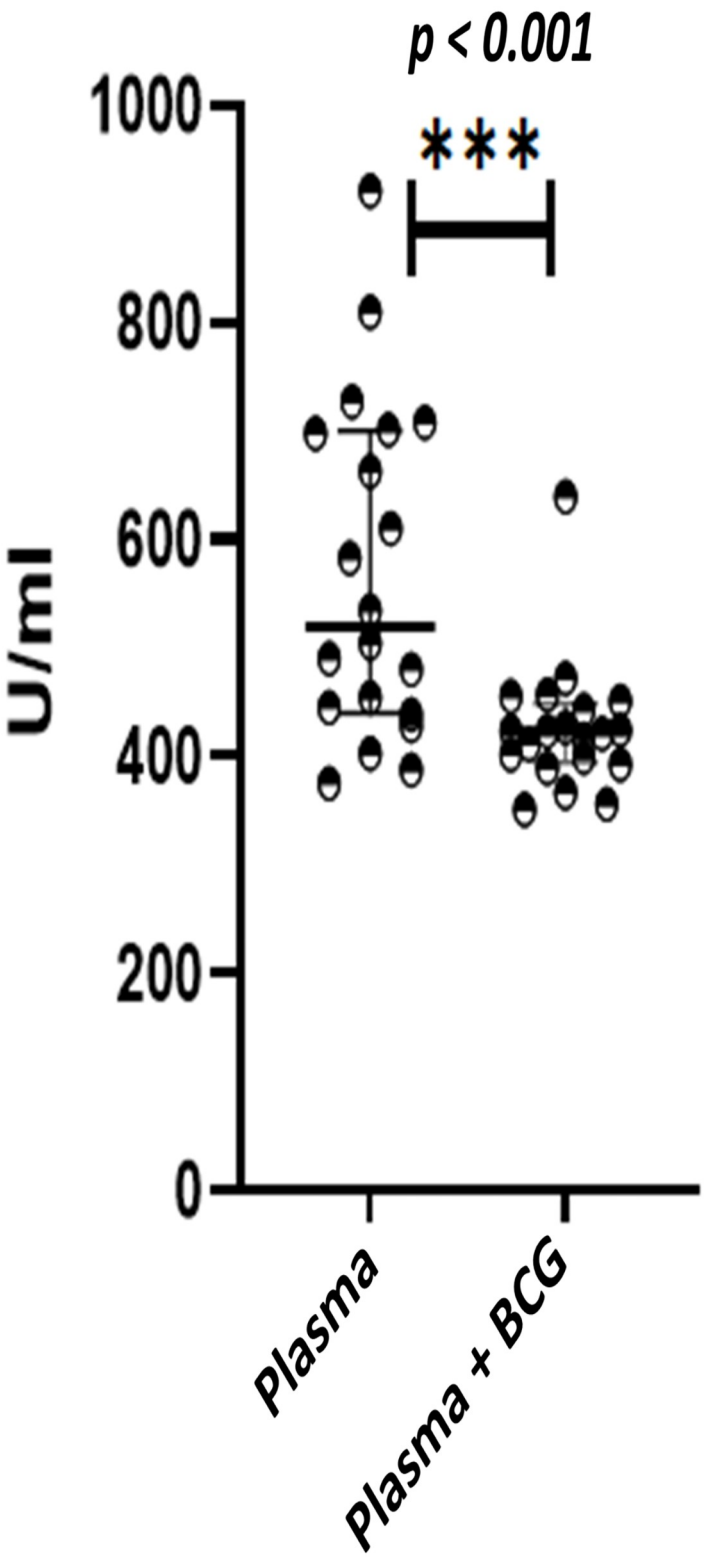

Fig 6. Assessment of cross-reactive IgG antibodies to COVID-19 Receptor-Binding Domain (RBD). Results are expressed as units /ml. Antibody assessment was carried out in plasma and BCG stimulated supernatants. All samples were collected before the COVID-19 pandemic. Mann-Whitney U tests were carried out to determine the significance of the difference.

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3.7. Relationship of IgG with cytokines

The correlation plot (Fig 7) shows the relationship of IgG anti-RBD with BCG and LPS-driven cytokines in WBA. There was no relationship between IgG with either IL10 or IL4 which are considered growth factors for B cells. However, IL17 (r = -0.791; $p = 0.001$) and IFNγ (r = -0.717; $p = 0.005$) showed a strong negative relationship with IgG anti-RBD. We believe that IgG recognition is not only T independent but recognizes different cross-reactive epitopes such as carbohydrate moieties and therefore shows a totally independent response compared to BCG and LPS induced cytokines.

4. Discussion

In this study, we show BCG-induced activation of the TI cellular network across the board in healthy adult donors in a BCG vaccinated area. This study is unique in the sense that case recruitment was conducted between 2015–2016 before the onset of COVID-19 pandemic. Secondly, while most previous studies have focussed on BCG vaccinated paediatric group, we have extended the age range to young adults to address the issue of longevity of BCG recall responses in TI network. This study therefore provides information related to BCG related longevity of TI recall responses in young adults pre COVID-19 pandemic in a TB endemic country, and is not possible to obtain post COVID-19. The importance of TI is the early activation (minutes to hours) of encountering a danger signal and can play a crucial role in stopping the infection in its track at the initial stages [25,32].

One limitation of this study was the lack of an objective marker to determine BCG vaccination status in adults. Neither BCG scar (40% positive) nor MT (10%) could provide this information as these markers are related to T cells which wanes over time [11]. Interestingly, TNFα gene expression was the only biomarker which showed differential expression in BCG Scar negative and Scar positive donors. Since kinetics of cytokine gene expression is different for different cytokines more detailed analyses are required to understand the implications of this finding in relation to BCG scar and vaccination status. However, with a >90 percent coverage of BCG vaccination at birth in Pakistan, we feel confident that the majority of our donors were vaccinated, and we are observing BCG recall responses for different cytokines in these healthy donors. This is further supported by similar cytokine secretion in BCG scar negative and BCG scar positive subjects (S1 Fig).

Cytokine activation was most marked with the classical (M1) and alternatively activated (M2) macrophages as demonstrated by the pro-inflammatory cytokines TNFα and IFNγ (M1) and immunosuppressive cytokine IL10 (M2) [33]. Previously we have shown that IFNγ / IL10 plays a critical role in TB disease progression [34]. The ratio observed in the current study is consistent with a protective ratio. IFNγ is also contributed by NK cells and NK activation may play a critical role in viral uptake and killing, thus reducing the burden of viral load at the initial stages of infection. In addition, there was also BCG activation of γδ T cells (IL4 and IL17) but lower compared to Macrophage activation (TNFα and IL10). However, limited activation was observed for NK cells (IL2) [16,35]. The redundancy in cytokine secretion from different sources makes it difficult to evaluate individual contribution of cell subtypes. We addressed this issue by analyzing clustering patterns using Principal component analysis (PCA). The fact that these cytokines are from distinct sources was confirmed by the distinct clustering patterns observed with TNFα / IFNγ clustering as one component (PC1) and IL10 as a second component (PC2) in WB. Surprisingly, PBMCs showed only one cluster. Purification of PBMCs has been shown to result in a loss of adherent cells. [36,37] The decrease in IFNγ and increase in IL10 in PBMC suggests a selective loss of M1 cells. This observation is also consistent with the

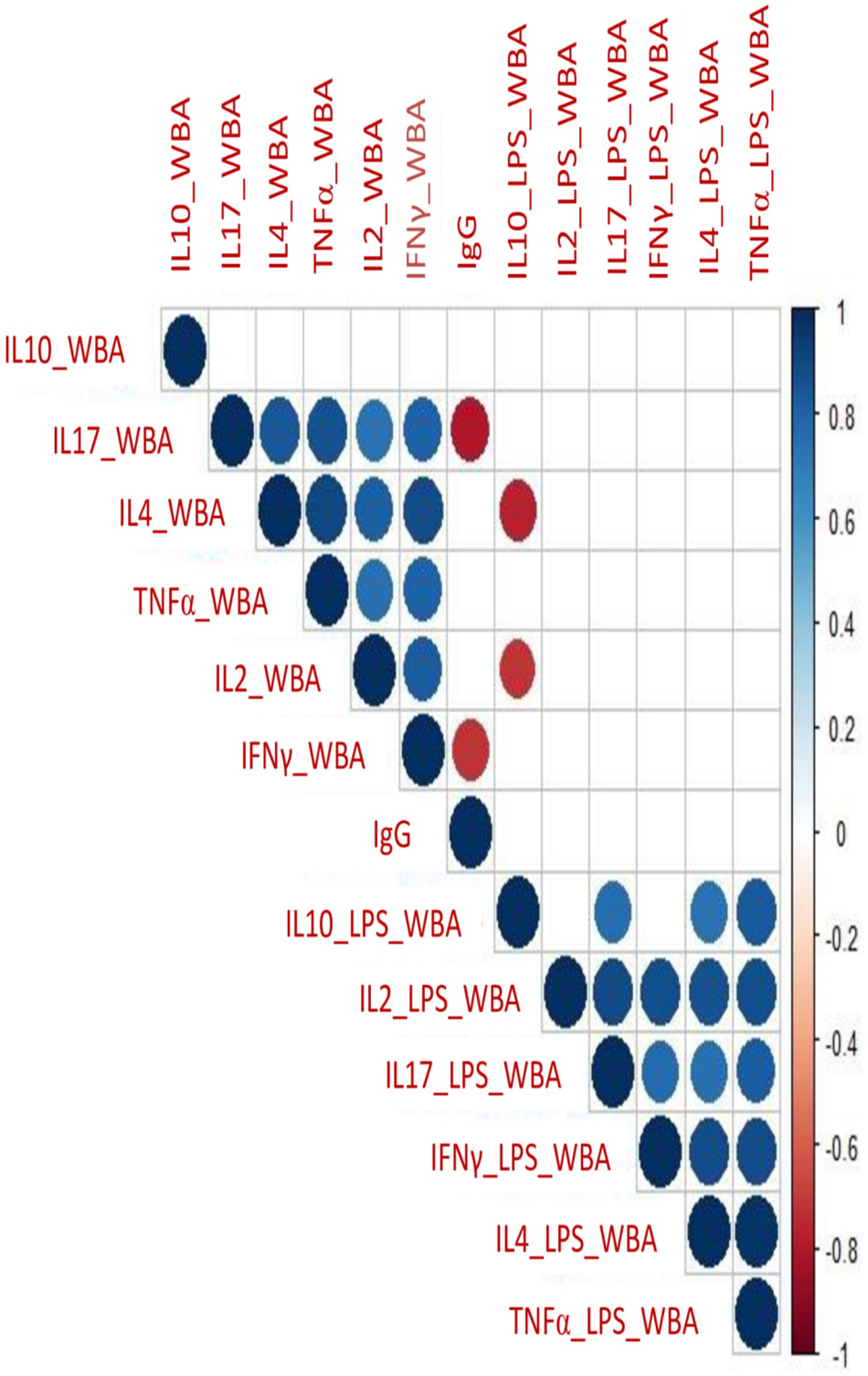

Fig 7. Relationship of IgG anti-RBD with BCG and LPS driven cytokines in Whole Blood Assay (WBA). Correlation plot of cytokines derived from WBA (BCG and LPS) and IgG. Corrplot showing a correlation coefficient of cytokines using the intensity of colors at the scale of -1.0 to +1.0 in WBA. All significant correlation at the level of $p = 0.01$ is shown on the plot as circles and $p > 0.01$ are shown as blank.

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known cross regulation of IFNγ and IL10. Again, these observations reinforce the point that WBA may be more reflective of in vivo situations.

Kleinnijenhuis et al., 2014 [38] were the first to describe TI in BCG vaccinated healthy donors to Candida albicans. The main difference between innate and adaptive immunity is that cells of the TI recognize antigens shared across pathogens and do not require a second signal as is the case with adaptive immunity. Comparison of BCG-induced responses with LPS which is present in bacterial cell wall and is similar to the lipid coat of mycobacteria showed similar responses for the two-antigen confirming strong recognition of conserved epitopes by innate network. As expected, the two cytokines associated with M1 macrophages (TNFα and IFNγ) showed a high level of correlation with BCG and LPS-induced TNFα, supporting the recognition of PAMPs like moieties in both molecules [39]. We have only analyzed prototypic cytokines, but there are other cytokines such as IL1 and IL6 which are also secreted by these macrophages and may also play a role in regulating and orchestrating the adaptive arm. However, the extended set of cytokines was not within the scope of our study.

Up-regulation of the pro- and anti-inflammatory arm of the innate system may serve a crucial role in creating an appropriate balance and dictating the evolution of the disease process. BCG recall responses assessed as a ratio of TNFα/IL10 was 3.3 and 2.0 in WBA and PBMCs respectively (S5 Table). A balanced activation of pro- and anti-inflammatory cytokines at the initial stages of the disease may play a key role in determining disease outcomes to a variety of cross-reactive pathogens including SARS-CoV-2 infections [40]. In this context, we have also observed BCG cross-reactive antibodies to SARS-CoV-2 RBD antigen. None of the cytokines showed any relationship with IgG anti-RBD although both IL4 and IL10 are B cell growth factors in the adaptive arm of the immune response [41]. Therefore, this is most likely memory responses of long term T independent memory B cells which are activated directly by cross reactive B cell epitopes [42]. Such cross-reactive IgG anti-SARS-CoV-2 RBD antibodies may be able to restrict viral replication by forming immune complexes and facilitating entry into macrophages and NK cells at the initial stages. Dissecting the nature of IgG subtypes may shed further light into protective vs pathogenic antibodies.

BCG is a cross reactive mycobacteria and BCG vaccination was given with the assumption that the cross-reactive response will be sufficient for protection against M. tuberculosis. BCG, however, is not effective against pulmonary tuberculosis, but was shown to provide protection against disseminated and miliary disease in children and was therefore continued in TB endemic countries. Protection against disseminated tuberculosis is a function of the innate arm and may be responsible for providing cross protection to other pathogens at entry. This may be one explanation for the protection against non-related pathogens in BCG vaccinated children. There are widely different policies for BCG vaccination in different countries and therefore may result in variable immune responses and protection. Although there is wide coverage globally with BCG [2], the main differences may lie in the community exposure to not only TB but to nonrelated pathogens in endemic countries which may play a key role in re-stimulation of the innate arm of the immune system in healthy donors.

The strongest immune response is shown for Heat Shock Proteins (HSPs) which are conserved across species [43]. HSP activates cells of the trained immunity network as well as T cells of the adaptive arm. It is not surprising that immune responses to HSPs have been shown to correlate with a favorable outcome of COVID-19 disease [44].

Further work needs to be done to evaluate the key cells and epitopes which are driving the cytokine TI network particularly as it relates to macrophage subsets and B cells and its implications in cross protection to other non-related pathogens.

5. Conclusion

In conclusion, our study clearly shows that there is a strong recall of BCG induced TI responses in the entire cellular network, indicating that trained immunity in high disease burden countries may be maintained well beyond the recently vaccinated pediatric population. Innate immunity plays an important role in immune surveillance due to its recognition of conserved epitopes across various microbial species. It was also clear that TI network was recognizing conserved molecules as indicated by similar recognition of LPS by the cellular network and recognition of SARS-CoV-2 antigens by IgG antibodies. Such TI recognition may play an important role in reducing the viral loads at the early stages of infection. Identification of conserved epitopes shared across species could help in designing new vaccines.

Supporting information

S1 Fig. Comparison of cytokine secretion in relation to Bacille Calmette-Guerin (BCG) status in whole blood assay (WBA).
(DOCX)

S2 Fig. Comparison of cytokine secretion in short term BCG stimulated whole blood assay (WBA) and peripheral blood mononuclear cells (PBMCs) cultures.
(DOCX)

S3 Fig. Calibration curve with IgG antibodies to COVID-19 Receptor binding domain (RBD).
(DOCX)

S1 Table. Cells and inducers of cytokines in the Innate arm of the immune system.
(DOCX)

S2 Table. Age-wise (minimum to maximum) BCG scar status, Mantoux Test (MT) readings, and gender distribution of study participants.
(DOCX)

S3 Table. Primers for detecting gene expression in BCG activated cells of the innate immune system.
(DOCX)

S4 Table. Comparison of spontaneous secretion of cytokines in whole blood assay (WBA) and peripheral blood mononuclear cells (PBMCs) cultures.
(DOCX)

S5 Table. The magnitude of BCG recall responses in Trained Immunity (TI).
(DOCX)

S6 Table. Correlation between cytokines secretions in response to Bacille Calmette-Guerin in Whole Blood Assay (WBA) culture.
(DOCX)

S7 Table. Correlation between cytokine secretions in response to Bacille Calmette-Guerin in Peripheral Blood Mononuclear Cells (PBMCs) culture.
(DOCX)

S8 Table. Spearman rank correlation between BCG and LPS stimulated cytokines secreted in whole blood assay (WBA) culture.
(DOCX)

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Author Contributions

Writing – original draft: Najeeha Talat Iqbal, Kumail Ahmed, Tehniat Sattar, Fatima Aziz, Rabia Hussain.

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