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BCG vaccination induces enhanced frequencies of dendritic cells and altered plasma levels of type I and type III interferons in elderly individuals

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ABSTRACT

Objective: BCG can improve the response to vaccines directed against viral infections, and also, BCG vaccination reduces all-cause mortality, most likely by protecting against unrelated infections. However, the effect of BCG vaccination on dendritic cell (DC) subsets is not well characterized.
Methods: We investigated the impact of BCG vaccination on the frequencies of DC subsets and type I and III interferons (IFNs) using whole blood and plasma samples in a group of elderly individuals (age 60-80 years) at one-month post-vaccination as part of our clinical study to examine the effect of BCG on COVID-19.
Results: Our results demonstrate that BCG vaccination induced enhanced frequencies of plasmacytoid DC (pDC) and myeloid DC (mDC). BCG vaccination also induced diminished plasma levels of type I IFNs, IFNα and IFNβ but increased levels of type III IFNs, IL-28A and IL-29.
Conclusions: Thus, BCG vaccination was associated with enhanced DC subsets and IL-28A/IL-29 in elderly individuals, suggesting its ability to induce non-specific innate immune responses.

Introduction

Bacillus Calmette–Guérin (BCG) is a live-attenuated vaccine primarily established to protect against childhood meningitis and disseminated tuberculosis (TB) (Foster et al., 2021). Several epidemiological findings suggest that BCG may increase the capacity of the immune system to fight against pathogens other than TB (Leentjens et al., 2015) and such non-specific responses augment both T cell-mediated adaptive and innate immune memory in a process called trained immunity; this could have important implications for improving vaccination strategies. (Netea and van Crevel, 2014). Bearing in mind the high morbidity and mortality due to COVID-19 in the elderly population, a possible protective effect through BCG in this group would be of clinical significance (O'Neill and Netea, 2020). However, the effect of BCG vaccination in protecting against heterologous infections in elderly individuals is still unclear as not many biological studies have been conducted so far to support this hypothesis.

Dendritic cells (DC) are the most effective antigen-presenting cells (APC), playing essential roles in bridging the innate and adaptive immune responses (Banchereau and Steinman, 1998). DCs are more efficient than other APCs, B cells and monocytes, in inducing T-cell proliferation (Inaba et al., 1989, Ludewig et al., 1999). Furthermore, DCs also play a significant role in establishing immunologic memory (Ludewig et al., 1999). Type I interferons (IFN-α/β) have a wide range of antiviral activities, which stimulate an antiviral response across various cell types (Mantlo et al., 2020). Like type 1 interferons, type III IFNs termed IFN lambda-1(IL-29), IFN lambda-2 (IL-28A), and IFN lambda-3 (IL-28B) also play important roles in antiviral immune activities (Zhou et al., 2018). Studies have also shown that type I and type III IFNs are able to inhibit SARS–

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CoV-2 replication (Felgenhauer et al., 2020), but the effect of BCG vaccination in inducing type I and III IFNs is not well studied.

Hence, we examined the induction of DC subsets in response to BCG vaccination in elderly individuals at baseline and one month post-vaccination along with baseline frequencies in unvaccinated individuals. We also examined the circulating levels of type I and type III IFNs following vaccination. We demonstrate that BCG vaccination induces significantly enhanced frequencies of DC subsets and altered type I and type III IFNs, suggesting that BCG can potentially boost immune responses in a non -specific or off-target manner in these elderly individuals.

Materials and Methods

Ethics statement

The study was approved by the Ethics Committees of NIRT (NIRT-INo:2020010). Informed written consent was obtained from all participants. The study is part of the clinical study entitled, Study to evaluate the effectiveness of the BCG vaccine in reducing morbidity and mortality in elderly individuals in COVID-19 hotspots in India (NCT04475302).

Study Population

Elderly individuals between 60 - 80 years of age residing in hotspots for SARS-Cov2 infection were included in the study between July 2020 and September 2020 in Chennai, India, after obtaining informed written consent from the study participants. The elderly population positive for SARS-Cov2 infection by either antibody (serology) or PCR test, HIV-infected or individuals with malignancy or on immunosuppressive drugs or transplant recipients and those on dialysis or anti-psychiatric medications or hypersensitivity to vaccinations, were not included in the study. Also, those who had been diagnosed with tuberculosis (TB) in the previous 6-months or were currently on anti-TB treatment were not included in the study.

Fifty four participants received a single dose of BCG vaccine (Freeze-dried) manufactured by Serum Institute of India, Pune. The adult dose of BCG vaccine was 0.1 mL injected intradermally over the distal insertion of the deltoid muscle onto the left humerus (approximately one-third down the left upper arm). In case of a previous vaccination scar or ulcer/injury, or tattoo on the left upper arm, vaccination was given in the right upper arm. Thirty two elderly individuals from the same hotspot area were not vaccinated and were considered as controls. Blood was drawn from the vaccinated participants at baseline (before vaccination) and one month following vaccination. Blood was drawn from the controls only at baseline.

Ex vivo analysis

All antibodies used in the study were from BD Biosciences (San Jose, CA), BD Pharmingen (San Diego, CA), eBioscience (San Diego, CA), or R&D Systems (Minneapolis, MN). Whole blood was used for ex vivo phenotyping, and it was performed on all \86 individuals. Briefly, to 250μl aliquots of whole blood, a cocktail of monoclonal antibodies specific for various immune cell types was added. Plasmacytoid DCs were classified as (Lin– HLA-DR$^+$ CD123$^+$) and myeloid DCs (Lin– HLA-DR$^+$ CD11c$^+$). Monocyte phenotyping was performed using antibodies directed against CD45-PerCP, CD14-Pacific Blue, HLA-DR-PE-Cy7 (clone L243; BD), and CD16-APC- Cy7. Eight-color flow cytometry was performed on a FACS Canto II flow cytometer with FACSDIVA software, version 6 (Becton Dickinson). The gating was set by forward and side scatter, and 100 000 gated events were acquired. Data were collected and analyzed using FLOW JO software (TreeStar, Ashland, OR).

Table I
Demographics of the study population.

Subjects Enrolled	Vaccinated n=54	Non-Vaccinated n=32
Age (Median)	65 (60 -78)	63 (60 -80)
Gender (M/F)	34/20	15/17
Height (Median)	160 cm	155 cm
Weight (Median)	62 Kg	63 Kg
Pulse rate (Median)	86	88
Systolic Blood Pressure (Median)	132	140
Diastolic Blood Pressure (Median)	81	80
SPOS% (Median)	98	98
Diabetes Mellitus no. (%)	14 (26 %)	5 (15 %)
Smoking, no. (%)	2 (4 %)	2 (6 %)
Alcoholism, no. (%)	3 (6 %)	2 (6 %)
Cardiovascular Disease, no. (%)	8 (15 %)	3 (9 %)
Respiratory Diseases, no. (%)	5 (9 %)	2 (6%)

ELISA

Circulating levels of IFNα and IFNβ were measured using Luminex Human Magnetic multiplex assay kit (R&D Systems). IL-28A, IL-28B, and IL-29 were measured using the DuoSet ELISA kit (R&D Systems). The lowest detection limits were as follows: IFNα, 3.9 pg/mL; IFNβ, 3.25 pg/ml; IL-28A, 62.5 pg/mL; IL-28B, 62.5 pg/mL and IL-29, 62.5 pg/mL. The lowest standard value was assigned to the samples that were below the threshold of detection.

Statistical analysis

Geometric means (GM) were used for measurements of central tendency. Wilcoxon signed-rank test was used to compare frequencies of immune subsets and type I and III interferons in the BCG vaccinated group at month 0 (M0) and month 1 (M1). Statistically significant differences between unvaccinated and BCG vaccinated M1 groups were analyzed using the Mann-Whitney test. Analyses were performed using Graph-Pad PRISM Version 9.0. Correlation matrix analysis was done using statistical software JMP 14.0 (SAS, Cary, NC, USA).

Results

Study population

The demographics of the study population are shown in Table I. From July 2020 through September 2020, 86 individuals were enrolled in the study, 54 in the vaccinated arm and 32 in the unvaccinated arm. All the vaccinated individuals were followed up at month 1 post-vaccination with no loss to follow-up. The median age was 65 (Range: 60-78) years in BCG vaccinated group and 63 years (Range: 60-80) in the unvaccinated group. There were 34 males and 20 females in the BCG vaccinated and 15 males and 17 females in the unvaccinated group. In the enrolled population, 26% of BCG vaccinated and 15% of unvaccinated individuals had diabetes mellitus, while 15% and 9% had cardiovascular disease, respectively. In our cohort, 4%-6% were current smokers, and 6% were alcoholics. Other baseline characteristics were similar between the two arms.

BCG vaccination induces enhanced frequencies of myeloid and plasmacytoid DCs

To assess the ex vivo phenotype of DC subsets following BCG vaccination, we compared the subsets at baseline or before BCG

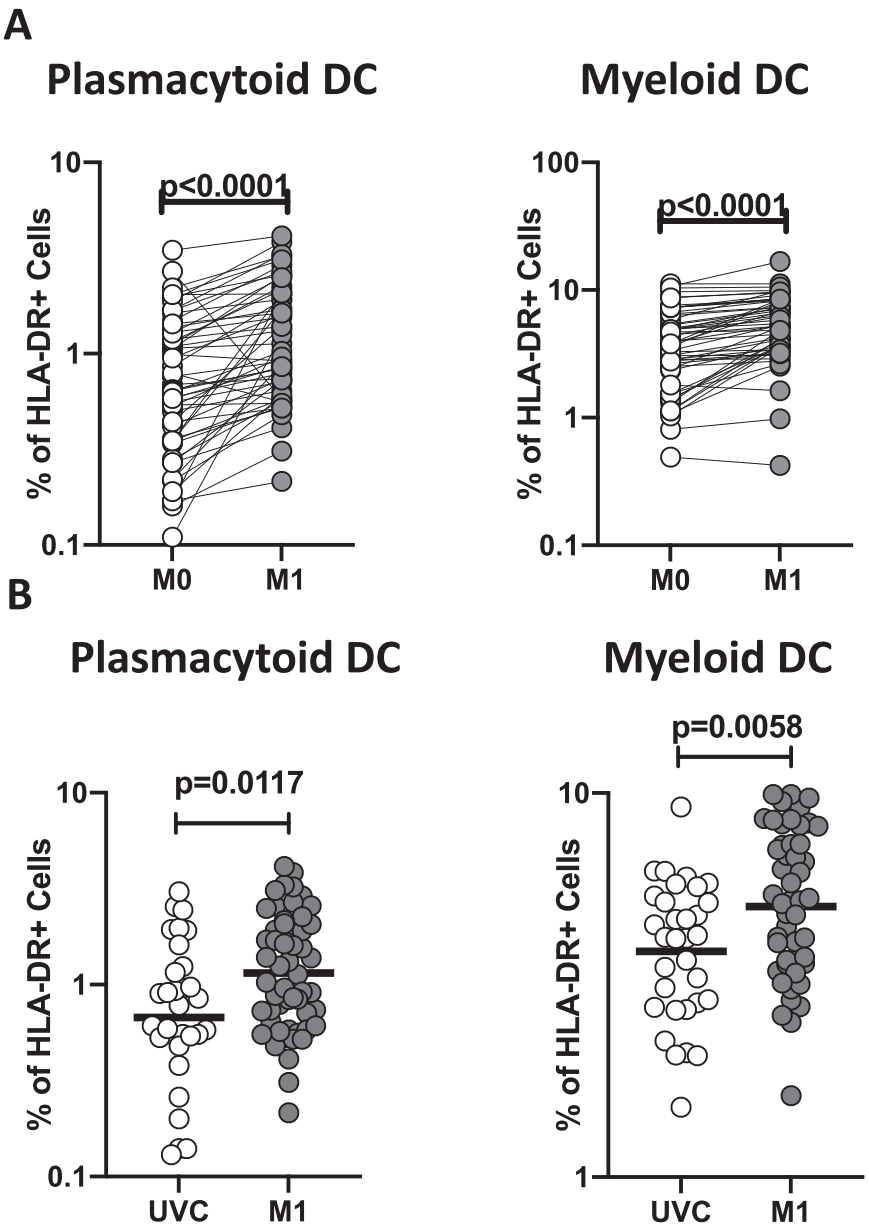

Figure 1. BCG vaccination is associated with heightened frequencies of dendritic cell subsets
(A) Frequencies of dendritic cell (DC) subsets (plasmacytoid DC and myeloid DC) in BCG pre-vaccinated [M0] (n = 54) and month 1 following vaccination [M1] (n = 54). Data are shown in line diagrams, with each line representing a single individual. P values were calculated using the Wilcoxon matched pair tests with Holms correction for multiple comparisons. (B) Frequencies of dendritic cell (DC) subsets (plasmacytoid DC and myeloid DC) in BCG unvaccinated (UVC) (n = 32) and post vaccinated [M1] (n = 54) individuals. The data are represented as scatter plots, with each circle representing a single individual. P values were calculated using the Mann-Whitney test with Holm's correction for multiple comparisons.

vaccination (M0) and at month 1 (M1) post-vaccination. As shown in Fig 1A, the frequencies of myeloid and plasmacytoid DCs had significantly increased at M1 compared to M0 in BCG-vaccinated individuals. Next, we compared the frequencies of DC subsets in post-vaccinated individuals to unvaccinated controls. As shown in Fig 1B, BCG vaccinated individuals exhibited increased frequencies of both myeloid and plasmacytoid DCs compared to unvaccinated controls. A representative flow cytometry plot showing the gating strategy for DC subsets is shown in Sup. Fig. Thus, BCG vaccination induces enhanced frequencies of DC subsets in elderly individuals.

BCG vaccination induces diminished plasma levels of type I interferons

To study the plasma levels of type I IFNs following BCG vaccination, we compared the plasma levels of IFNα and IFNβ at baseline or before BCG vaccination (M0) and at month 1 (M1) post-vaccination. As shown in Figure 2A, IFNα ($p<0.0001$) and IFNβ ($p=0.0001$) showed significantly diminished levels at M1 compared to M0. Next, we compared the plasma levels of type I IFNs in post-vaccinated individuals to unvaccinated controls. As shown in Fig 2B, BCG vaccinated individuals exhibited decreased circulating

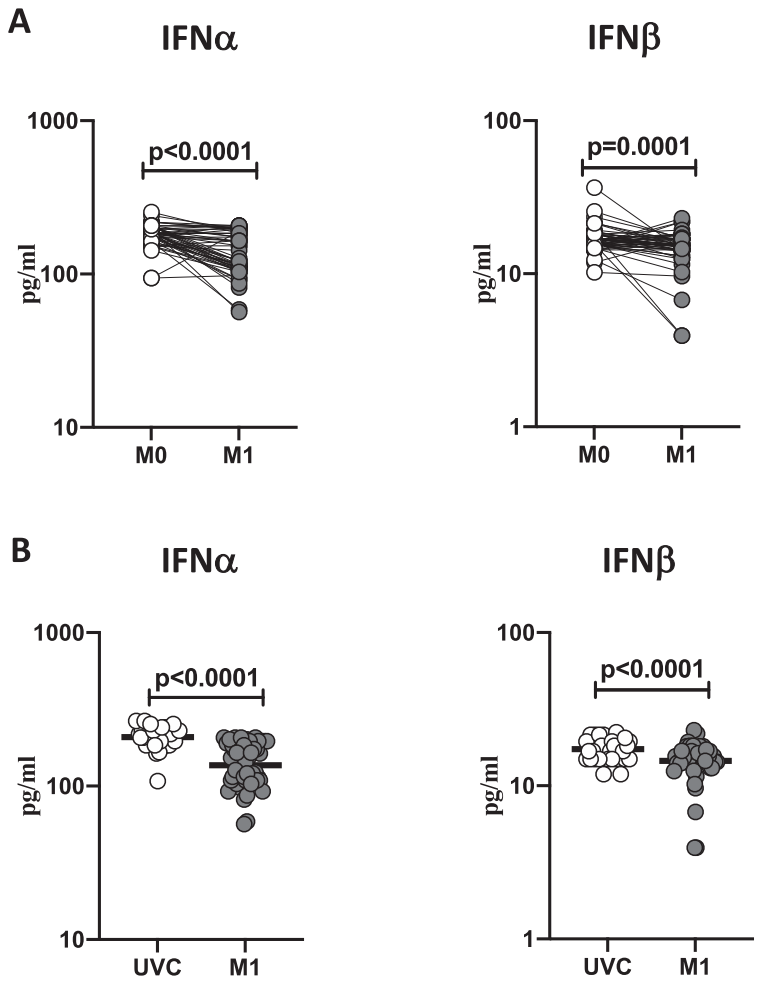

Figure 2. BCG vaccination is associated with decreased circulating levels of type I interferons
(A) The plasma levels of type I IFNs like IFNα and IFNβ were measured in BCG pre-vaccinated [M0] (n = 54) and month 1 following vaccination [M1] (n = 54). Data are shown as line diagrams, with each line representing a single individual. p values were calculated using the Wilcoxon matched pair tests with Holms correction for multiple comparisons. **(B)** The plasma levels of type I IFNs like IFNα and IFNβ in BCG unvaccinated (UVC) (n = 32) and post vaccinated [M1] (n = 54) individuals. The data are represented as scatter plots, with each circle representing a single individual. p values were calculated using the Mann-Whitney test with Holm's correction for multiple comparisons

levels of IFNα ($p<0.0001$) and IFNβ ($p<0.0001$). Thus, BCG vaccination induces diminished systemic levels of type I IFNs in elderly individuals.

BCG vaccination induces enhanced plasma levels of type III interferons

To study the plasma levels of type III IFNs following BCG vaccination, we compared the plasma levels of IL-28A, IL-28B, and IL-29 at baseline or before BCG vaccination (M0) and at month 1 (M1) post-vaccination. As shown in Figure 3A, IL-28A ($p<0.0001$) and IL-29 ($p<0.0001$) showed significantly increased levels at M1 compared to M0. Next, we compared the plasma levels of type III IFNs in post-vaccinated individuals to unvaccinated controls. As shown in Fig 3B, BCG vaccinated individuals exhibited significantly increased circulating levels of IL-28A ($p=0.0059$) and IL-29 ($p=0.0006$). Thus, BCG vaccination induces increased systemic levels of type III interferons in elderly individuals.

Associations between DC subsets and type I and III interferons

We wanted to identify correlations between frequencies of DC subsets (pDC and mDC) and type I (IFNα and IFNβ) and III IFNs (IL-28A, IL-28 B, and IL-29) in BCG vaccinated individuals. As shown in Fig. 4, a multiparametric matrix correlation plot showed no significant correlation between the DC subsets and type I IFNs. However, a strong positive correlation between plasma levels of IL-29 with the frequencies of pDC was observed. No significant correlation was seen between IL-28A and IL-28B and DC subsets. Our results overall indicate a partial association between the DC subsets and type I and III IFNs.

Discussion

Naturally, the elderly population is at a greater risk for infection against new infectious episodes. We have chosen to investigate the elderly population residing in COVID-19 hotspots as it is known that this population is at a high risk of developing infections. Various clinical trials have shown that BCG vaccination limits the number of infections of all causes, especially respiratory tract infections, arguing for a protective effect (Giamarellos-Bourboulis et al., 2020, Madsen et al., 2020). Few other studies have determined the protective effect of BCG vaccination against SARS-CoV2 in elderly individuals (Ten Doesschate et al., 2020). Various findings over the years have reported that BCG vaccines can induce a dominant non-

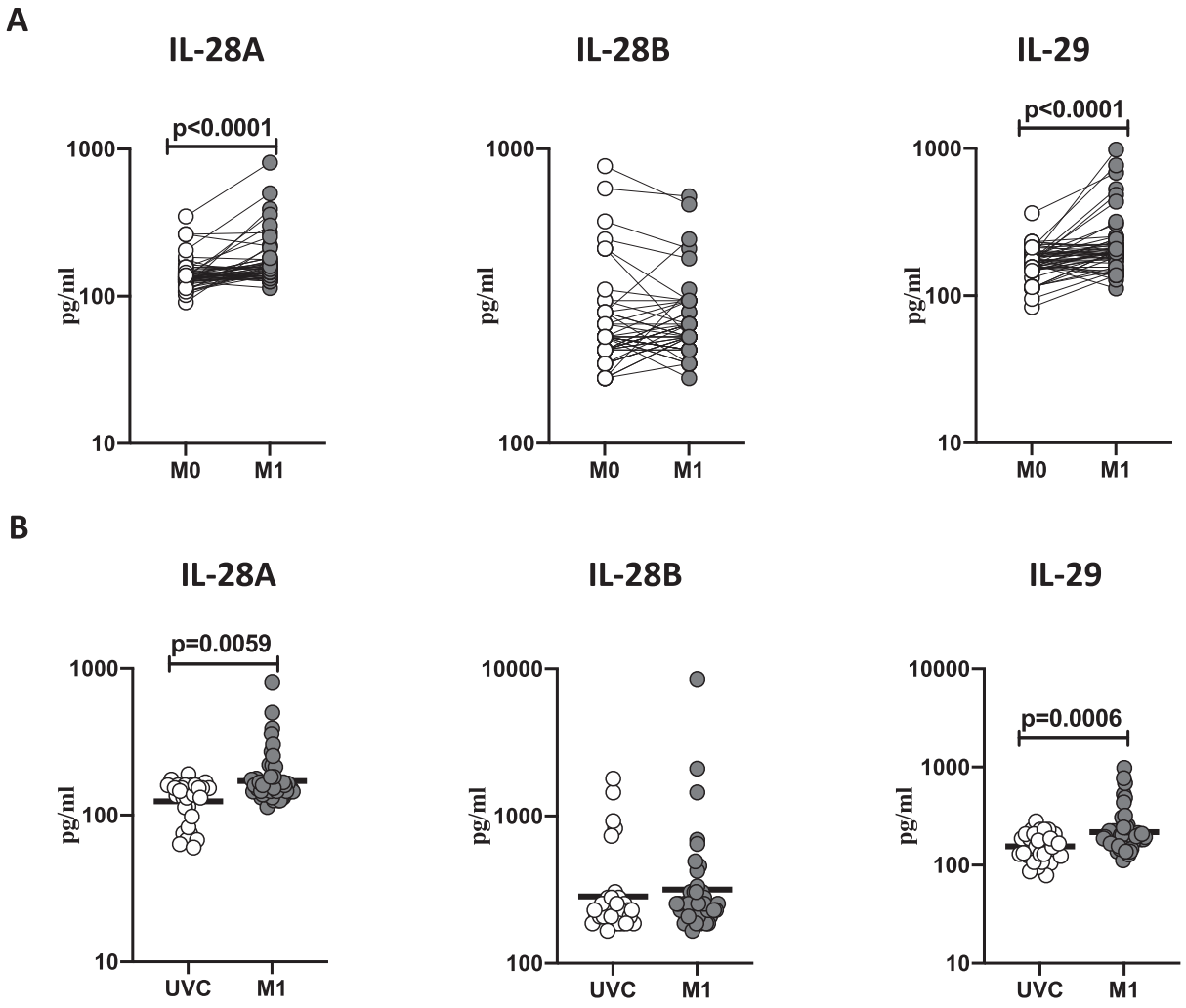

Figure 3. BCG vaccination is associated with increased circulating levels of type III interferons
(A) The plasma levels of type III IFNs like IL-28A, IL-28B, and IL-29 were measured in BCG pre-vaccinated [M0] (n = 54) and month 1 following vaccination [M1] (n = 54). Data are shown as line diagrams, with each line representing a single individual; p values were calculated using the Wilcoxon matched pair tests with Holms correction for multiple comparisons. **(B)** The plasma levels of type III IFNs like IL-28A, IL-28B, and IL-29 in BCG unvaccinated (UVC) (n = 32) and post vaccinated [M1] (n = 54) individuals. The data are represented as scatter plots, with each circle representing a single individual. p values were calculated using the Mann-Whitney test with Holm's correction for multiple comparisons

specific immune response, but it is still unclear if the BCG vaccine can offer meaningful protection against diseases like COVID-19. (Aspatwar et al., 2021). Actually, numerous clinical trials are ongoing to estimate the capacity of the BCG vaccine to modulate immunity against COVID-19. The main goal of these clinical trials is to determine if BCG vaccination lessens the incidence and severity of the SARS-CoV-2 infection. These studies will eventually help us to understand whether and to what extent BCG offers protection against SARS-CoV-2 (Aspatwar et al., 2021). In addition, studies have also reported that BCG vaccination in the elderly population resulted in decreased risk of pneumonia in tuberculin-positive individuals, indicating that administering BCG is one of the effective strategies for the prevention of pneumonia (Ohrui et al., 2005). In this current study, we wanted to evaluate the impact of BCG on DC subsets, type I and III interferons in elderly individuals residing in COVID-19 hotspots. As part of the study protocol, we studied the dendritic cells, Type 1 and III immune responses generated by BCG vaccination in a group of elderly individuals.

DCs encompass a diverse population of cells that play an important role in initiating, directing, and regulating adaptive immune responses (Soto et al., 2020). Activating the adaptive immune response requires the presentation of antigens to T cells by DC and macrophages (Hilligan and Ronchese, 2020). Delays in the activation of DCs often results in a delay in the induction of the adaptive immune response to pathogens. Thus, methods to either increase the frequencies of DCs or activate DCs would improve the protective efficacy of vaccines (Fucikova et al., 2019). Our data showing elevated frequencies of both mDC and pDC thus clearly illustrate a critical effect of BCG vaccination in possibly enhancing the innate immune response to specific and non-specific pathogens in elderly individuals. Moreover, while mDC is mainly involved in the process of antigen–presentation (Macri et al., 2018), pDC is the primary source of Type 1 interferons in the host (Leylek and Idoyaga, 2019). Thus, it is potentially likely that increased frequencies of pDC might heighten the propensity of pDC to mount Type I IFN responses against encountered pathogens.

Interferons constitute the first line of defense against microbial infections, particularly against viruses (Sa Ribero et al., 2020). Type I interferons and DCs share an overlying history; studies have been well reported that expression of type 1 IFNs by DC and their

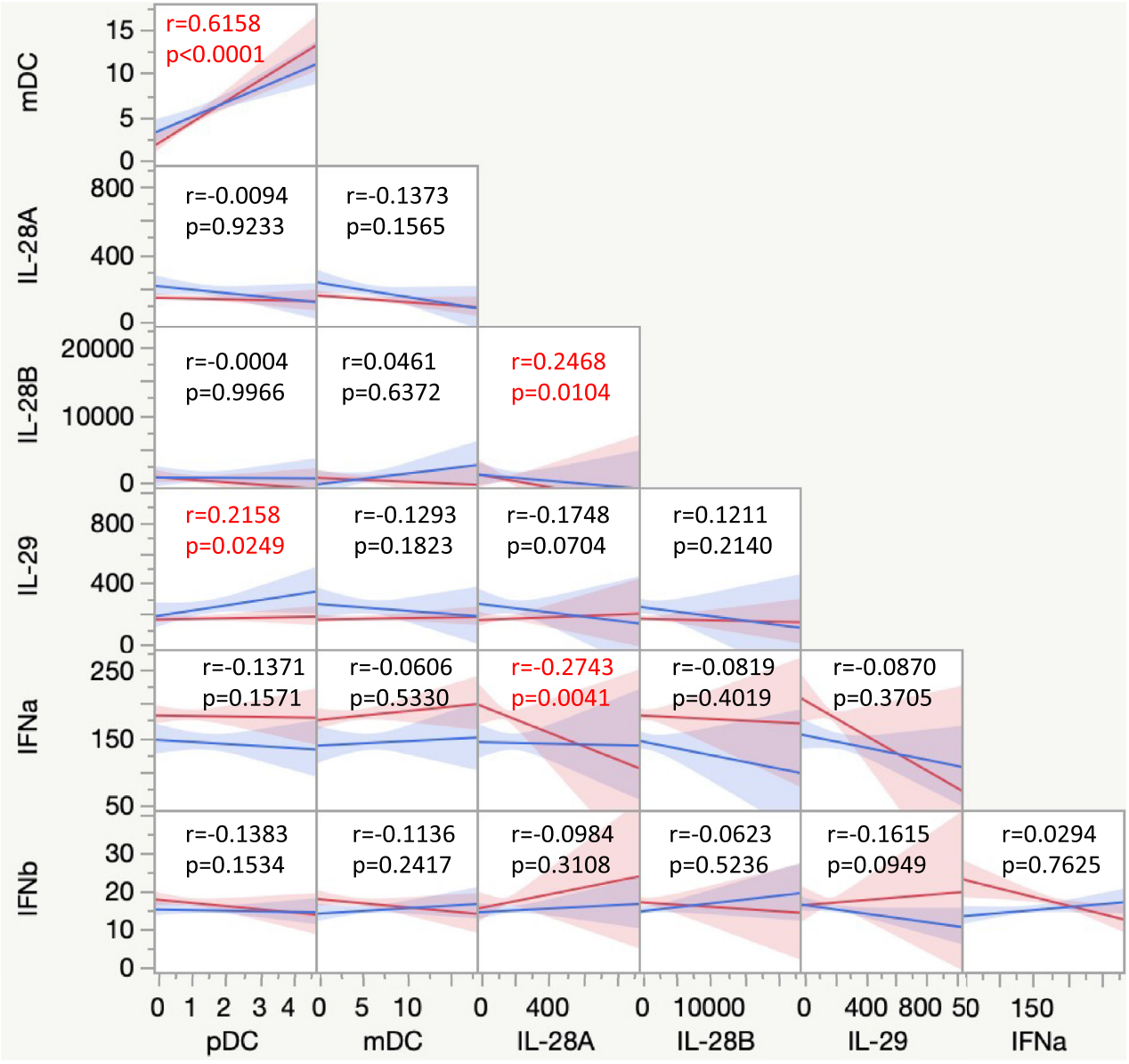

Figure 4. Relationship between DC subsets and type I and III interferons
Multiparametric matrix correlation plot of DC subsets and type I and III interferons in all individuals with BCG pre-vaccinated and month 1 following vaccination. Spearman's correlation coefficients are visualized. The blue line represents the x-axis parameter, and the red line represents the y-axis parameter.

interface are one of the essential components of the innate and adaptive immune responses (Fitzgerald-Bocarsly and Feng, 2007). It is also known that cross-talk between pDC and mDC via type I IFNs has been involved in some pathological situations (Fitzgerald-Bocarsly and Feng, 2007). Even in our study, we observed a significant increase in the frequencies of DC subsets, whereas there was a significant decrease in the circulating level of IFNα and IFNβ one month post BCG vaccination, indicating that this inverse relationship may be involved in the containment of infection.

Type III IFNs typically act on epithelial cells in response to viral infection (Sommereyns et al., 2008). Plasmacytoid dendritic cells are the foremost producers of Type III IFNs (Yin et al., 2012, Zhang et al., 2013). Published data suggest that the impact of Type III IFNs on host immunity extends beyond its effect at the mucosal level to effects on systemic immune responses, specifically the innate and adaptive arms of the immune response (Zanoni et al., 2017). Thus, Type III IFNs are known to play a crucial role in adaptive immune responses to viral and bacterial infection, alter anti-tumor responses, and affect immunity (Lasfar et al., 2016, Lazear et al., 2015, Wack et al., 2015). Some studies have reported that Type III IFNs are the prominent IFNs produced after viral infection (Andreakos et al., 2017). In our study, we observed elevated circulating levels of IL-28A and IL-29 after one month of BCG vaccination, which in turn was correlated with the elevated levels of dendritic cells. However, their role in SAR-CoV-2 infections has not, to our knowledge, been explored.

In summary, our study highlights the effect of BCG vaccination in modulating the frequencies of DC subsets. Study limitations are that samples were collected only during the baseline visit and not at follow-up in control individuals and that all the measured data are reported only in percentages, not absolute numbers. Our study also reveals an effect of BCG in inducing a positive correlation with type III IFNs and DC subsets. The possible cellular mechanism is that type I or III IFNs may contribute to DC maturation, which is promoted by BCG vaccination. Although our study did not examine the mechanical changes in the immune system, our data reveal a

vital role for BCG vaccination in boosting immune responses in the elderly population.

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Author Contributions

Designed the study (SB, CP); conducted experiments (NPK, RA, AN, NS, RMR, VV); acquired data (NPK, RA, AN); analyzed data (NPK, RA); contributed reagents and also revised subsequent drafts of the manuscript (SB, CP); responsible for the enrolment of the participants and also contributed to acquisition and interpretation of clinical data (CP, BPK, BJ, DK, ST); wrote the manuscript (SB, NPK, CP). All authors read and approved the final manuscript.

Conflict of Interest Statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Data and materials availability

All the reported data are available within the manuscript.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ijid.2021.07.041.

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