Perturbations in spike specific peripheral T follicular helper cells in SARS-CoV2 breakthrough convalescent individuals immunized by BBV152 vaccine.

Satish Devadas[1], Soumya Sengupta[1], Shubham Shaw K[1], Sanchari Chatterjee[1], Gargee Bhattacharya[1], Prakash Barik K[1], and Soma Chattopadhyay[1]

[1]Institute of Life Sciences

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Abstract

SARS-CoV2 infection has caused an increase in mortality and morbidity but with vaccination, the disease severity has significantly reduced. But with emergence of various variants of concerns (VOCs), the vaccine breakthrough infection has also increased. Here, we studied circulating spike specific T follicular response (cTfh) in infection naïve vaccinees and convalescent vaccinees (individuals who got delta breakthrough infection after 2 doses of BBV152 vaccine) to understand their response as they are the most crucial cells that are involve in vaccine mediated protection by helping in B-cell maturation. Our results indicated that cTfh cells in both the groups recognized wild type and delta spike protein but memory response to wild type spike was superior in infection naïve than in convalescent group. The cytokine response particularly IL-21 from cTfh cells was also higher in infection naïve than convalescent vaccines indicating a dampened cTfh response in convalescent vaccines after breakthrough infection. Also, there was positive correlation between IL-21 from cTfh cells and neutralizing antibodies of infection naïve vaccinees. Multiple cytokine analysis also revealed higher inflammation in convalescent vaccinees. Our data indicated necessity of third booster dose may be individual specific depending in the steady state functional phenotype of immune cells.

Short Communication

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Soumya Sengupta[1,2,3+] , Shubham K Shaw[1,2,3+] , Sanchari Chatterjee[1,2,4], Gargee Bhattacharya[1,2,3], Prakash K Barik[1,3], Soma Chattopadhyay[1,2,4], Satish Devadas[1,2,3*]

1 Institute of Life Sciences, Nalco Square, Bhubaneswar, Odisha, India.

2 Regional Centre for Biotechnology (RCB), 3rd Milestone, Faridabad-Gurgaon, Haryana, India

3 T cell and Immune Response Lab, Institute of Life Sciences, Nalco Square, Bhubaneswar, Odisha, India.

4 Molecular Virology Lab, Institute of Life Sciences, Nalco Square, Bhubaneswar, Odisha, India.

+These authors contributed equally

*Address of Corresponding author:

Dr. Satish Devadas (SD)

T cell and Immune Response Lab,

Infectious Disease Biology,

Institute of Life Sciences Institute of Life Sciences,

(Autonomous Institute of Dept of Biotechnology, Govt. of India),

Nalco Square, Bhubaneswar-751023. Odisha, India

Phone No: 0091 674 2300701; Fax No: 0091 674 2300728 E-mail: satdevs@ils.res.in

Running Title- Peripheral T cell response in Convalescent vaccinees.

Abstract

SARS-CoV2 infection has caused an increase in mortality and morbidity but with vaccination, the disease severity has significantly reduced. But with emergence of various variants of concerns (VOCs), the vaccine breakthrough infection has also increased. Here, we studied circulating spike specific T follicular response (cTfh) in infection naïve vaccinees and convalescent vaccinees (individuals who got delta breakthrough infection after 2 doses of BBV152 vaccine) to understand their response as they are the most crucial cells that are involve in vaccine mediated protection by helping in B-cell maturation. Our results indicated that cTfh cells in both the groups recognized wild type and delta spike protein but memory response to wild type spike was superior in infection naïve than in convalescent group. The cytokine response particularly IL-21 from cTfh cells was also higher in infection naïve than convalescent vaccines indicating a dampened cTfh response in convalescent vaccines after breakthrough infection. Also, there was positive correlation between IL-21 from cTfh cells and neutralizing antibodies of infection naïve vaccinees. Multiple cytokine analysis also revealed higher inflammation in convalescent vaccinees. Our data indicated necessity of third booster dose may be individual specific depending in the steady state functional phenotype of immune cells.

Keywords

Adaptive immune response, T follicular cells, neutralizing antibodies, inactivated vaccines, SARS-CoV2 viral variants.

1. Introduction

The constant mutations in SARS-CoV2 the Spike protein have given rise to number of VOCs all of which had potential to evade the memory immune cells arising due to vaccination[1]. In particularly the second wave caused by Delta variant wreaked havoc all throughout the world. In India also the wave caused by Delta variant was most destructive in terms of loss of life[2]. In India 10 vaccines have been approved ChAdOx-1 S (AstraZeneca/Serum Institute of India), BBV152 (Bharat Biotech), Ad26.COV2. S (Johnson and Johnson), BECOV2D (Biologicals E Ltd.), Gam-COVID-Vac (Gamaleya Research Institute of Epidemiology and Microbiology.), mRNA-1273(Moderna), NVX-CoV2373 (Serum Institute of India), ZyCoV-D (Zydus Cadilla), Sputnik light (Gamaleya Research Institute of Epidemiology and Microbiology), Lyophilized mRNA Vaccine for Injection (COVID-19) [HGCO-19] (Gennova Biopharmaceuticals), BBV154 - Adenovirus vectored, intranasal vaccine (iNCOVACC) (Bharat Biotech).

The exact extent of protection by vaccines given against different variants is varied according to different reports. BBV152 (Covaxin) is an inactivated vaccine developed in India, which have been approved by the WHO and have been widely used in several countries. It is still a matter of scientific debate that whether inactivated vaccinees are still working against variants of concern. Most of the studies looked into neutralization antibodies to understand the protection conferred by these vaccines but the role of $CD4^+$T cells is still not clear. An earlier study from India revealed that the BBV152 vaccine displayed low neutralization reductions against variants of concerns but elicited a robust T cell response[3]. But even after vaccinations a large number of people were infected with different variants of concerns in successive Covid-19 waves indicating that complexity of immunological protection imparted by vaccination is still illusive in case of Covid-19 vaccines. $CD4^+$ T cells are major players that impart protection against viral infections and within the $CD4^+$ T cell subset the role $CD4^+$T follicular cells (Tfh) have been instrumental in helping B cells to produce different isotypes of immunoglobulins and for their affinity maturation[4]. But their role in SARS-CoV2 vaccination induced protection and how their phenotype is affected after breakthrough infection is

still not clear. So, to understand Tfh response in this study we investigated the phenotype and response of Tfh cells in healthy volunteers who were vaccinated with BBV154 (inactivated whole virus vaccine) and had breakthrough infection along with volunteers who were also vaccinated but did not contract the virus during the second wave in India which was mainly driven by Delta variant of SARS-CoV2.

2. Materials and Methods

2.1 Study Design and Sample Collection

Peripheral venous blood was collected from 40 donors, all of them were vaccinated with double dose of inactivated SARS-CoV2 vaccine and out of 40 donors, 20 donors experienced breakthrough infection during delta wave (convalescent vaccines), the rest 20 donors did not have any prior history of SARS-CoV2 infection or did not have any breakthrough infection (naïve vaccinees). The blood collection was performed after 4-6 months of the completion of the second dose. To understand the immune response after SARS-CoV2 infection blood was collected from 20 convalescent patients at least after 1 month of their testing negative of SARS-CoV2. The details of the donors are given in Table 1. These convalescent patients were also vaccinated and tested positive during the delta wave of the SARS-CoV2 virus. The details of the sequence deposited at the time of infection is available at the following link https://inda.rcb.ac.in/insacog/statreportzonelineagegraph. PBMC was isolated from blood, resuspended in CS10 media (Stem cell Technologies), and stored in liquid nitrogen. Plasma was stored at -80 till further use.

2.2 Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Institutional Human Ethics Committee, Institute of Life Sciences. The Institutional Ethics Committee (IEC)/Institutional Review Board (IRB) reference number is 106/HEC/2021.

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

2.3 Flow cytometry detection of SARS-CoV2 specific Tfh cells

Cryopreserved human PBMC were thawed and rested for 5h in 37. Then $1x10^6$ cells per well in duplicate were stimulated with 2µg/ml of SARS-CoV2 S peptides of both wild type strain and delta strain (Milteni Biotech) for 20h. After stimulation, cells were washed and stained with Zombie Dye Aqua (Biolegend) followed by blocking by Trustain Fcx (Biolegend). Then they were stained with a cocktail of monoclonal antibodies CD3 BUV 737(BD Biosciences), CD4 PE (Biolegend), CCR7 APC (Biolegend), CD45RA BV 786 (Biolegend), OX-40 BV421(Biolegend), CCR6 BV711 (Biolegend), CXCR3 BV605 (Biolegend), CD25 BV650 (BD Biosciences), CXCR5 BUV 395 (BD Biosciences) and Zombie Aqua dye (Biolegend) for live and dead cell discrimination. Next, the cells were washed, fixed with 1% formaldehyde, and acquired on a BD LSR Fortessa using BD FACS Diva. For intracellular cytokine assay 1×10^6 cells per well in duplicate were stimulated with 2µg/ml of SARS-CoV2 S peptides of both wild type strain and delta strain (Milteni Biotech) for 20h with Brefeldin A being added in the last 4hrs. Cells were then stained cocktail of monoclonal antibodies CD3 BUV 737(BD Biosciences), CD4 PE (Biolegend), CCR7 APC (Biolegend), CD45RA BV 786 (Biolegend), CXCR5 BUV 395 (BD Biosciences), IFN-γ BV650(Biolegend), IL-17 BV711 (Biolegend), IL-21 BV 421 (BD Biosciences), IL4 BV605 (Biolegend) and Zombie Aqua dye (Biolegend) for live dead and dead cell discrimination. For positive controls cells from some volunteers were treated with α-CD3 and α-CD28 for AIM assay and for ICS they were stimulated with PMA/Ionomycin. Unstimulated cells were used as negative controls.

2.4 Plasma Cytokine Detection Assay

Neat plasma from naïve vaccinees and convalescent vaccinees were run in duplicates to measure 20 T cell cytokines using human Milliplex map cytokine assay kit (Millipore, Billerica, MA). The samples were acquired in a Bio-Plex 200 system (Bio-Rad, Hercules, CA) and cytokine concentrations were calculated using Bio-Plex manager software with a five-parameter (5PL) curve-fitting algorithm applied for standard curve calculation.

2.5 Neutralization assay

The pseudo-neutralization assay was performed by using the SARS-CoV2 Neutralization Antibody Elisa Kit (Invitrogen) as per manufacturers protocol. In brief, plasma samples were diluted 1:50 and added to wells coated with SARS-CoV2 RBD (Receptor Binding Domain) antigen followed by incubation and addition of Biotin conjugate. After required incubation period Streptavidin-HRP conjugate was added followed by addition of Substrate solution (TMB) and Stop solution after successive incubation periods. The plate was read on iMark microplate absorbance reader (Bio-Rad) using 450nm wavelength. Neutralization % was calculated by the following formula:

Neutralization (%) = 1- (Absorbance of unknown sample/Absorbance of negative control) $\times$ 100

$>$ 20% = Positive

$<$ 20% = Negative

2.6 Statistics

Statistical analysis was performed using the GraphPad Prism software, version 8.0.1. Data was presented as Mean±Standard Error of Mean (SEM). Wilcoxon Test was used to determine statistical significance of immunological response to wild type and delta spike proteins between same individuals of naïve vaccinees and convalescent vaccinees groups. Mann-Whitney U test was used to compare statistics between naïve vaccinees and convalescent vaccinees including cytokine profile from multiplexing and neutralisation assay. Spearman's rank correlation coefficient was used to measure the correlation between two different variables. P values less than 0.05 were considered significant ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$).

3. Results

The Activation Induced Marker (AIM) assay revealed circulating memory Tfh (cTfh) cells responded better to the spike proteins of wild type SARS-CoV2 virus compared to the delta strain in naïve vaccinees**(Fig 1B-C, G)** . There was no difference in cTfh response between the spike proteins of the wild and delta strain in convalescent vaccinees **(Fig 1D-E, H)** . The breadth of AIM response of cTfh cells to delta spike protein was higher in convalescent vaccinees compared to naïve vaccinees **(Fig 1J)** . The magnitude of response to wild type spike protein did not show difference between the two groups but both the groups responded to the wild type spike protein**(Fig 1I)** . We also observed an increase in central memory compartment ($CCR7^{+}CD45RA^{-}$) in $CD4^{+}$ T cells in response to the Spike wild type protein in infection naïve vaccinees **(Fig K-L, O)** while convalescent vaccinees did not show any difference **(Fig M-N, P**). When we investigated the antigen specific cytokine response from cTfh, both naive vaccinees and convalescent vaccinees showed higher expression of IL-21 when stimulated with wild type spike protein than to delta spike protein **(Fig 2B-E, G-H)** . But expression of IL-21 was significantly higher in naïve vaccinees compared to convalescent vaccinees both for wild type and delta type, thought it was not statistically significant for delta spike **(Fig 2 I-J)** . The neuralization activity did not show any difference between naïve vaccinees or convalescent vaccinees **(Fig 2K)** but it positively correlated with IL-21 expression from cTfh cells from naïve vaccinees**(Fig 2L-M)** . Furthermore, our phenotyping of spike specific cTfh from both the groups were biased towards cTfh2 phenotype which is CXCR3-/CCR6- cells **(Fig 2N-O).**

Our multiplex data revealed that low level of inflammation was still ongoing in these convalescent vaccinees as they displayed higher levels of IL-6 and IL-1β compared to naïve vaccinees **(Fig 3H-I)** . On the other hand, Tfh promoting cytokines like IL-21, IL-4 and IL-10**(Fig 3A-C)** and anti-viral memory response inducing cytokines like IFN-γ, IL-15, IL-12**(Fig 3D-F)** were elevated in naïve vaccinees as compared to convalescent vaccines. All the above data indicated that though cTfh cells from convalescent vaccinees recognizes both wild type and delta spike proteins, their response is low compared to naïve vaccinees.

4. Discussion

The role of cellular immunity is actively evolving in case of SARS-CoV2. With vaccination the severity of SARS-CoV2 has come down[5], but still due to evolution of different VOCs, there have been uptick of cases, leading to significant morbidity and mortality. Our study here aimed to understand the phenotype of Tfh

cells in recovered individuals of breakthrough infections and how they are different from Tfh cells encountered in only vaccinated individuals Tfh are important cells for resolution of infections and are there to help B cells for affinity maturation and isotype switching[6]. Our results showed Tfh cells from both naïve vaccinees and convalescent vaccinees recognized the spike protein from both wild and delta strains showing that epitope is conserved for T cells to respond to VOCs. This is in line with previous study which showed conservation of epitopes in VOCs to which T cells can respond[3]. IL-21, is one of the signature cytokines of Tfh and is essential for B-cell maturation[7], our study revealed that IL-21 expression from cTfh were higher in naïve vaccinees compared to convalescent vaccinees. This is in observation with another study which showed a decrease in spike specific cTfh after third booster dose with CoronaVac or breakthrough infection during early time points[8]. Interestingly, we did not observe the boosting of neutralization activity after breakthrough infection, although our data revealed a strong positive correlation between IL-21 secretion from cTfh and neutralization activity only in naïve vaccinees and not in convalescent vaccinees. This could be due the timing of sampling[9]. Most of the convalescent vaccinees showed higher levels of pro-inflammatory cytokines like IL-6 and IL-1 b compared to naïve vaccines. On the other hand, Tfh and memory response promoting cytokines like IL-21, IL-10, IL-4, and IL-15 were higher in naïve vaccinees compared to convalescent vaccinees[7,10]. Our data also revealed that most of the cTfh were of cTfh2 phenotype in both naïve vaccinees and convalescent vaccinees. The role of cTfh2 in physiology and pathology is still not clear but they can promote B-cell response better than cTfh1 but less than cTfh 17 [11].

In summary our data showed that after breakthrough infections there are perturbations in memory cTfh cells and their response is dampened. This could be due to the pro- inflammatory microenvironment that existed in these individuals even after the infection is resolved or it may be the endophenotype (heterogenous immune response) in these individuals that may be the cause of these perturbations[12,13]. Since we did not do a longitudinal study, we cannot determine whether there cTfh response were already dampened before infection or it was the effect of infection and subsequent inflammation. It also may have been possible that the cTfh response may evolve with time in these individuals since the strength and duration of antigenic stimulation of TCR along with cytokine *milieu* in the microenvironment is important for differentiation of different T helper subsets[14,15]. Overall, our study shows that hybrid immunity may be time and dosage dependent as all our convalescent vaccinees had mild symptoms during infection and infection naïve vaccinees displayed stable immune response to both wild and delta strains indicating that further consideration to booster doses may be specific for individuals or population vulnerable to more severe infections.

Author Contributions: Experimental design and conceptualization- S.S and S.D. Sample collection, processing- P.K.B. Flow cytometry experiments— S.S, S.K.S. Cytokine multiplexing—S.S., and G.B Neutralisation Assay- SaC, S.S, S.K.S and S.C. Data analysis—S.S., S.K.S and S.D. All Authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: The original contributions presented in the study are included in the article. Further inquiries can be directed to the corresponding author.

Conflicts of Interest: The authors declare no conflict of interest.

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Figure legends

Fig 1. Activation Induced Marker (AIM) assay of circulating memory Tfh revealed that cTfh cells of both infection naïve vaccinees and convalescent vaccinees recognized spike proteins of both wild type and delta strain. Representative flow cytometry plot showing negative unstimulated control **(A)** . Representative flow cytometry plot showing cTfh response in infection naïve vaccinees to the Spike protein of Wild type strain of SARS-CoV2 (SWT) **(B)** . Representative flow cytometry plot showing cTfh response in infection naïve vaccinees to the Spike protein of Delta strain of SARS-CoV2 (SDT) (**C)** Representative flow cytometry plot showing cTfh response in convalescent vaccinees to the Spike protein of Wild type strain of SARS-CoV2 (SWT) **(D)** . Representative flow cytometry plot showing cTfh response in infection convalescent vaccinees to the Spike protein of Delta strain of SARS-CoV2 (SDT) (**E)** Representative flow cytometry plot showing stimulated with α-CD3 and α-CD28 **(F)** . Cumulative graphical representation of AIM assay indicated by $CD25^{+}OX40^{+}$cTfh cells responding to SWT and SDT

in infection naïve vaccinees; (n=20) **(G**) Cumulative graphical representation of AIM assay indicated by $CD25^{+}OX40^{+}$ cTfh cells responding to SWT and SDT in convalescent vaccinees; (n=20)**(H**). Cumulative graphical representation of comparison of the AIM assay indicated by $CD25^{+}OX40^{+}$ cTfh cell response to SWT between infection naïve vaccinees (n=20) and convalescent vaccinees (n=20) **(I)** Cumulative graphical representation of comparison of AIM assay indicated by $CD25^{+}OX40^{+}$cTfh cell response to SDT between infection naïve vaccinees (n=20) and convalescent vaccinees (n=20) **(J,)** . Representative flow cytometry plot showing different T helper memory compartment response in infection naïve vaccinees to the Spike protein of Wild type strain of SARS-CoV2 (SWT) **(K)** , Representative flow cytometry plot showing different T helper memory compartment response in infection naïve vaccinees to the Spike protein of Delta strain of SARS-CoV2 (SDT) (**L).** Representative flow cytometry plot showing different T helper memory compartment response in convalescent vaccinees to the Spike protein of Wild type strain of SARS-CoV2 (SWT) **(M).**Representative flow cytometry plot showing different T helper memory compartment response in convalescent vaccinees to the Spike protein of Delta strain of SARS-CoV2 (SDT**) (N).** Cumulative graphical representation of T helper central memory cells indicated by $CCR7^{+}CD45RA^{-}$ responding to SWT and SDT in naive vaccinees; (n=20) **(O).**Cumulative graphical representation of T helper central memory cells indicated by $CCR7^{+}CD45RA^{-}$ responding to SWT and SDT in convalescent vaccinees; (n=20) **(P).** Error bar indicates SEM. Wilcoxon Test was used to determine statistical significance of immunological response to the wild type and delta spike proteins between same individuals of naïve vaccinees and convalescent vaccinees groups. Mann-Whitney U test was used to compare statistics between naïve vaccinees and convalescent vaccinees. (*), p<0.01 was considered to be very significant (**), $P < 0.001$ was considered to highly significant (***), $P < 0.0001$ was considered extremely significant (****) ns, not significant.

Fig 2. Spike specific cTfh cytokine response revealed that cTfh response is damped in convalescent vaccinees as compared to infection naïve vaccinees and spike specific IL-21 secretion from cTfh positive correlated with neutralizing antibody in infection naïve vaccinees . Representative flow cytometry plot showing negative unstimulated control**(A)** . Representative flow cytometry plot showing cTfh cytokine response in infection naïve vaccinees to the Spike protein of Wild type strain of SARS-CoV2 (SWT) **(B)** . Representative flow cytometry plot showing cTfh cytokine response in infection naïve vaccinees to the Spike protein of Delta strain of SARS-CoV2 (SDT) (**C).**Representative flow cytometry plot showing cTfh cytokine response in convalescent vaccinees to the Spike protein of Wild type strain of SARS-CoV2 (SWT) **(D)** . Representative flow cytometry plot showing cTfh cytokine response in infection convalescent vaccinees to the Spike protein of Delta strain of SARS-CoV2 (SDT) (**E).** Representative flow cytometry plot showing stimulated with PMA/Ionomycin **(F)** . Cumulative graphical representation of cytokine assay indicated by $IL\text{-}21^{+}$ cTfh cells responding to SWT and SDT in infection naïve vaccinees; (n=20) **(G**). Cumulative graphical representation of cytokine assay indicated by $IL\text{-}21^{+}$ cTfh cells responding to SWT and SDT in convalescent vaccinees; (n=20)**(H**). Cumulative graphical representation of comparison of cytokine assay indicated by $IL\text{-}21^{+}$ cTfh cell response to SWT between infection naïve vaccinees(n=20) and convalescent vaccinees (n=20) **(I).** Cumulative graphical representation of comparison of the cytokine assay indicated by $IL\text{-}21^{+}$cTfh cell response to SDT between infection naïve vaccinees (n=20) and convalescent vaccinees (n=20) **(J)** . Cumulative graphical representation of % Neutralization between infection naïve vaccinees and convalescent vaccinees **(K).** Graphical representation of co-relation analysis between % $IL\text{-}21^{+}$ spike specific cTfh cells and % neutralizing activity in Infection naïve vaccinees(**L).** Graphical representation of co-relation analysis between % $IL\text{-}21^{+}$ spike specific cTfh cells and % neutralizing activity in Infection convalescent vaccinees (**M).**Representative flow cytometry plot showing different cTfh subsets based on chemokine markers CXCR3 and CCR6 in infection naïve vaccinees**(N)** Representative flow cytometry plot showing different cTfh subsets based on chemokine markers CXCR3 and CCR6 in convalescent vaccinees **(O).** Cumulative graphical representation of T helper central memory cells indicated by $CCR7^{+}CD45RA^{-}$ responding to SWT and SDT in naive vaccinees; (n=20) **(O).** Error bars indicate SEM. Wilcoxon Test was used to determine statistical significance of immunological response to wild type and delta spike proteins between same individuals of naïve vaccinees and convalescent vaccinees groups. Mann-Whitney U test was used to compare statistics between naïve vaccinees and convalescent vaccinees including the neutralisation assay. The spearman's rank correlation coefficient was used to measure the correlation between two diffe-

rent variables (*), p<0.01 was considered to be very significant (**), $P < 0.001$ was considered to highly significant (***), $P < 0.0001$ was considered extremely significant (****) and ns, not significant.

Fig 3. Cytokines analyses from plasma of and healthy controls. Representative figures from T cell cytokines analyzed in infection naïve vaccinees (n=20) and convalescent vaccinees (n=20) are represented as dot plots. cTfh promoting cytokines IL-21 **(A),** IL-10 **(B),** IL-4 **(C).** were elevated in infection naïve vaccinees as compared to convalescent vaccinees. Amongst anti-viral memory inducing cytokines IFN-γ **(D)** , IL-15**(E),** IL-12 **(F)** and IL-17 **(G)** were also elevated in infection naïve vaccinees as compared to convalescent while inflammatory cytokines IL-1β **(H)** and IL-6 **(I)** were elevated in convalescent vaccinees as compared to infection naïve vaccinees**.** Error bar indicates SEM. Mann-Whitney U Test was performed to compare between the two groups, p<0.05 was considered statistically significant(*), p<0.01 was considered to be very significant (**), $P < 0.001$ was considered to highly significant (***), $P < 0.0001$ was considered extremely significant (****) ns, not significant.

Numbers between the brackets indicate range of the data set

Characteristics	**Infection Naïve Vaccinees**	**Convalescent Vaccinees**
Number of donors	20	20
Sex,	4 females, 16 males	8 females, 12 males
Age	31 (28- 36)	33 (30-38)
Time since recovery	Not Applicable	46 days (31-65)
Vaccination History		
Number of doses (BBV152 Inactivated vaccine)	20, 2 doses	20, 2 doses
Time since last vaccine dose	160 days (122- 183)	112 days (78-142)