



HAL
open science

Multiple SARS-CoV-2 immunizations of an unvaccinated population lead to complex immunity. A T cell reactivity study of blood donors in Antananarivo

Solohery Lalaina Razafimahatratra, Olifara Herinirina Andriatefy, Diary Juliannie Ny Mioramalala, Fenosoa Anita Mireille Tsatoromila, Fanirisoa Randrianarisaona, Philippe Dussart, Matthieu Schoenhals

► To cite this version:

Solohery Lalaina Razafimahatratra, Olifara Herinirina Andriatefy, Diary Juliannie Ny Mioramalala, Fenosoa Anita Mireille Tsatoromila, Fanirisoa Randrianarisaona, et al.. Multiple SARS-CoV-2 immunizations of an unvaccinated population lead to complex immunity. A T cell reactivity study of blood donors in Antananarivo. *Journal of Infection and Public Health*, 2024, 17 (1), pp.175-181. 10.1016/j.jiph.2023.11.020 . hal-04325458

HAL Id: hal-04325458

<https://hal.science/hal-04325458>

Submitted on 6 Dec 2023

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



Multiple SARS-CoV-2 immunizations of an unvaccinated population lead to complex immunity. A T cell reactivity study of blood donors in Antananarivo

Solohery Lalaina Razafimahatratra^a, Olifara Herinirina Andriatefy^a,
Diary Juliannie Ny Mioramalala^a, Fensoa Anita Mireille Tsatoromila^b,
Fanirisoa Randrianarisaona^a, Philippe Dussart^c, Matthieu Schoenhals^{a,*}

^a Immunology of Infectious Diseases Unit, Institut Pasteur of Madagascar, 101 Antananarivo, Madagascar

^b Regional Blood Transfusion Centre of Analamanga, 101 Antananarivo, Madagascar

^c Direction, Institut Pasteur of Madagascar, 101 Antananarivo, Madagascar

ARTICLE INFO

Article history:

Received 24 July 2023

Received in revised form 8 November 2023

Accepted 16 November 2023

Keywords:

SARS-CoV-2

Natural immunization

T cell response

Cross-reactivity

Madagascar

ABSTRACT

Background: Madagascar has undergone multiple and robust COVID-19 waves. The resulting immune background developed by its poorly vaccinated population has however not been described.

Methods: In this study, serological analysis and specific T cell response descriptions were used to describe the history of exposures of the capital's blood donors to SARS-CoV-2 and its VOCs. Samples were collected early 2022, and pools of multiple immunogenic peptides of SARS-CoV-2 were used in an IFN- γ secretion ELISPOT assay to characterize the specific T-cell immunity developed against these potential epitopes.

Results: Multiple epidemic waves have led to 92.1% of donors having detectable antibodies, and 94.8% having developed T-cells against SARS-CoV-2. Heterogeneous reactivities to different strain-derived peptides suggested multiple immunological backgrounds in the population including 16.1% of individuals exposed at least once to a unique strain, 27.1% to two strains, 28.5% to three strains, and 23.1% to four distinct strains.

Conclusions: Cross-reactivity increased with multiple exposures but did not decrease the risk of re-infection. These results describe the extremely complex immunological background developed following multiple natural immunizations.

© 2023 The Author(s). Published by Elsevier Ltd on behalf of King Saud Bin Abdulaziz University for Health Sciences. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

Introduction

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) was first detected in Wuhan, China, in the late of 2019 causing a disease known as coronavirus disease 19 (COVID-19) [1,2]. The disease became a pandemic and more than 625 million infections had been reported worldwide, with over 6 million deaths [3].

With waning immunity and the emergence of new variants of concern (VOCs), the world has experienced many epidemic waves. The establishment of specific immunity targeting these VOCs seems required to neutralize these new VOCs and slow the epidemics down. Successful resolution of COVID-19 was associated with the

presence of T-cells and antibodies [4]. Studies of acute, convalescent COVID-19 patients and vaccinated individuals have showed that SARS-CoV-2-specific T cell responses are significantly associated with milder disease, suggesting that T cell responses may be important for control and resolution of a primary SARS-CoV-2 infection [5–8]. Previous studies reported SARS-CoV-2-specific T cell responses against spike, nucleocapsid, and membrane protein in SARS-CoV-2 unexposed individuals, indicating the presence of pre-existing cross-reactive T-cells populations that developed after prior common cold coronavirus infections. The spike S1 of SARS-CoV-2 has low resemblance to the spike S1 of endemic coronaviruses [9]. Therefore, S1-specific T-cells of SARS-CoV-2 should represent cells having derived from SARS-CoV-2 infection.

Studies conducted in Israel have showed the impact of high vaccination coverage on the course of the epidemic, including the decline in circulation of the Alpha VOC by 2021 [10,11]. Another

* Correspondence to: Immunology of Infectious Diseases Unit, Pasteur Institut of Madagascar, Ambatofotsikely, 101 Antananarivo, Madagascar.
E-mail address: schoenhals@pasteur.mg (M. Schoenhals).

study found that vaccination increases immunity of individuals with a history of natural infection [12,13]. Studies analysing the capacity of seroneutralisation from people receiving two and three doses of vaccine showed a neutralizing activity to all the VOCs except Omicron (O) subvariant (B.1.1.529) [14,15]. A study in Israel found that natural immunity acquired after infection of SARS-CoV-2 in children aged 5–11 years and adolescents aged 12–18 years protected them for 18 months based on re-infection during the circulation of Delta (D) (B.1.617.2) VOCs but the immunity acquired didn't predict the protection against new VOCs like O [16]. No studies published to date have focused on the characterization of the immunity following multiple natural immunisations.

From March 2020 to February 2022, Madagascar was hit by three COVID-19 waves (two unimodal ones and a 3rd bimodal one). A first serological study of blood donors showed that 40% of this population was immunized during the first wave of 2020. Interestingly, this populational immunity seemed sufficient to dramatically reduce the circulation of the virus during the first epidemic wave [17]. The second epidemic wave Madagascar underwent was caused by the introduction of a Beta (B) (B.1.351) VOC, as demonstrated by our later reported B-specific serosurvey. Again, about half of the population being immunized seemed sufficient to reduce the circulation of the virus during this second wave [18]. Despite the immunity acquired during the two first waves, the country has undergone more epidemic waves. Unlike during the two first ones however, serological interpretation and association to new VOC circulation was not completely possible despite Delta and Omicron circulation identification.

Describing the complexity of immunizations to SARS-CoV-2 in the Malagasy population is helpful to determine the level of protection against the disease and future emerging VOCs, given the relatively low vaccine coverage in Madagascar and the poor knowledge of the history of infection of the population of Antananarivo.

Indeed, no study has yet investigated the outcome of multiple VOCs circulations in a poorly vaccinated population. We describe for the first time the complex immune backgrounds developed by multiple SARS-CoV-2 strain circulations in an adult population through a characterization of the T response to SARS-CoV-2 and its variants (VOCs) peptides in the population of blood donors in Antananarivo, the capital of Madagascar.

Materials and methods

Study population and blood collection

Between January and April 2022, this study was conducted at the Analamanga Blood Transfusion Centre (BTC) in Antananarivo, Madagascar. The study population is subject to biases due to blood donation inherent restrictions previously described [17]. This study enrolled 669 blood donors, but excluded 167 (25%) who had tested positive for routine blood bank tests such as HIV, hepatitis B and syphilis, which impede T-proliferation and bias the analyses [19–21]. Nevertheless, 502 individuals (75%) were compliant with the study and included after non-opposition to the study (appendix O). Individuals are representative of diverse socio-economic and socio-cultural backgrounds. Extensive discussion regarding the populational representativeness of Antananarivo blood donors is available in published articles [17,18].

Twenty ml blood samples were collected from blood bags in citrated tubes and promptly sent to the Infectious Diseases Immunology Laboratory of the Pasteur Institute of Madagascar. These samples were obtained from blood donors aged 18–67, and their demographic data, including gender, age, blood collection date, and place of residence, were recorded (see Table 1). This study is part of the SARS-CoV-2 surveillance conducted by the Pasteur Institute of

Table 1
Characteristics of the participants.

Donors	N = 669
Mean Age (min-max)	34 (18–67)
Gender	
Male	477
Female	171
N/A	21
Donor category	
FBD	607
RVBD	15
Others BD	32
others	15
Blood type	
O+	282
B+	159
A+	146
AB+	28
B-	3
O-	3
A-	1
N/A	47
Region of provenance	
Analamanga	650
Itasy	2
Haute Matsiatra	1
Vakinankaratra	1
N/A	15
Excluded from the study	167
HBV positive	19/669
Syphilis positive	15/669
VIH positive	2/669
HCV positive	1/669
No serological results	68/669
Insufficient blood volume	62/669

RVBD, regular voluntary blood donor; FBD, family blood donor

Madagascar and has received approval from the Ethics and Biomedical Research Committee of the Ministry of Public Health (CERBM: IORG0000851).

PBMC and serum isolation

For all samples collected, peripheral blood mononuclear cells (PBMCs) were isolated from whole blood by density gradient centrifugation over Ficoll and cryopreserved in -80°C within four hours after collection until ELISPOT analysis. PBMCs were stored at -80°C for no longer than three months. Plasma was stored at -20°C .

ELISPOT and serological assays

SARS-CoV-2 ELISPOTs were performed using the Human IFN- γ ELISpotPLUS kit (catalog #3420–4APW-10, Mabtech) following the manufacturer's guidelines (appendix A). PBMCs were thawed and only samples with a viability of over 70% were included in the analysis (Appendix B and F). Highly immunomodulatory peptides spanning the spike S1 protein of SARS-CoV-2 were selected on www.iedb.org (appendix C). The method based on the Expectation-Maximization (EM) algorithm was used to establishing scores corresponding to individual peptide responses (appendix D).

Multiplex serology was performed using Luminex technology targeting IgA and IgG anti-Nucleocapsid (NP) or the Receptor Binding Domain (RBD) of SARS-CoV-2 (appendix E) [22].

Statistical analysis

Data was analyzed using Prism software V 8.0.1 (GraphPad, California). A Chi-square test was used to compare distribution of relative scores from each profile, a Mann-Whitney test was used to compare medians of each measured response reflecting cross-

reactivity and an ANOVA test was used to determine any statistically significant difference between the means of the measured cross-reactive response for all profiles. The significance level was set at $p < 0.05$. HeatMap figures were made using CHM builder, available at <https://build.ngchm.net/NGCHM-web-builder/>[23]. The Alluvial plot was created using R Studio.

Results

SARS-cov-2 spike protein leads to multiple potentially immunogenic peptides

Using IEDB (www.iedb.org), we identified 81 highly immunomodulatory peptides from SARS-COV-2 Spike S1 protein referenced at least twice and corresponding to peptides specific to W strain and most common VOCs in Madagascar early 2022 (B, D, O and other VOCs). Among these 81 peptides; 22 mapped with all strains; ten mapped within Wuhan strain (W) and B mutated peptides; eight mapped within W strain and D mutated peptide; two mapped within W strain and O mutated peptides; one mapped within W strain, B and D mutated peptides and one interesting region mapped within W strain, B, D and O mutated peptides, that were apparently the four major circulating strains in Madagascar (Fig. 1). The six last peptides mapped within the W strain and other VOCs not responsible for an epidemic wave in Madagascar.

Blood donors possess T-cells activated by SARS-CoV-2 immunogenic peptides

PBMCs from individuals responded to stimulation by the peptide pools (appendix G). Each peptide was present in only two pools allowing the subsequent identification of the epitope. The average number of spots per pool varied from 14 (95% confidence interval (95%CI), 12–17) to 31 (95%CI, 25–38) and from a minimum of 0 to maximum of 2368 spots per well (appendix H). For each individual, pooled-peptide spot-numbers were acquired and scores per peptide were calculated. The score associated to individual peptides varied

from 0 to 10,287 (appendix I). Indeed, peptide-associated responses varied from one individual to another suggesting different response profiles. Almost all individuals responded to at least one of the 81 selected peptides except one individual (ID 22–5298). Individuals responded on average to 42 of the 81 peptides. These results suggest that, as for the humoral responses, there is individual T-cell response variation to SARS-CoV-2 S1 derived peptide stimulation influenced by either immunization capacity or repertoire.

One single 15-AA RBD region contains sites undergoing mutations for 4 VOCs

Given the multiple selected epitopes and potential cross-activities that may exist, we focused our analysis on a series of peptides specific to 4 strains known to have circulated in Madagascar, possibly responsible for the 4 epidemic waves the country had undergone. Peptides 4, 6, 57 and 65, corresponding to region 473–487 were therefore used to attempt to describe the history of exposures to W, B, D and O strains. This region, now on referred to as profile-defining peptides (PDP), is exposed and located in the RBD region[24]. The region has one mutation site for B (E484K), one mutation site for D (T478K) and three mutation sites for O which were, also, found in its sub-lineages (S477N, T478K and E484A) (appendix P).

Results showed a very characteristic exposure pattern that classified the donors in 5 distinct groups, 23.1% (95%CI, 19.6–27.0) with quadruple exposure to all four strains (W, B, D and O), 28.5% (95%CI, 24.7–32.6) with exposure to three strains, 27.1% (95%CI, 23.4–31.1) with double exposure to two strains, 16.1% (95%CI, 13.2–19.6) with one exposure to one strain and 5.2% (95%CI, 3.6–7.5) with no exposure (Fig. 2). Analysis of the results showed 16 responses profiles according to the exposure backgrounds of each individual to the four strains (Table 2).

Individual peptide responses within a same profile were highly correlated to one another. Indeed, Intraclass Correlation Coefficients (ICC) ranged from 0.57 to 0.88 for profiles 2–16. Only profile 1

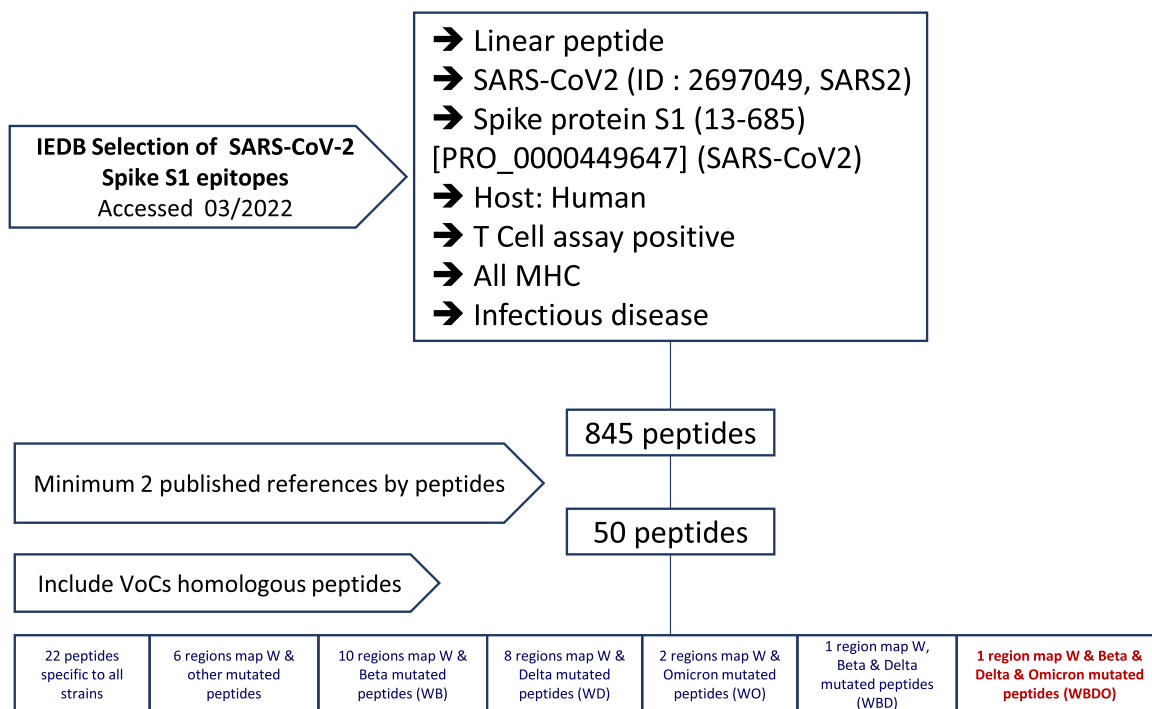


Fig. 1. Flow chart for epitope selection using www.iedb.org (march 2022) SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; MHC, major histocompatibility complex; iedb, immune epitope database and analysis resource; W, Wuhan strain; VoCs, variants of concern; B, Beta; D, Delta; O, Omicron.

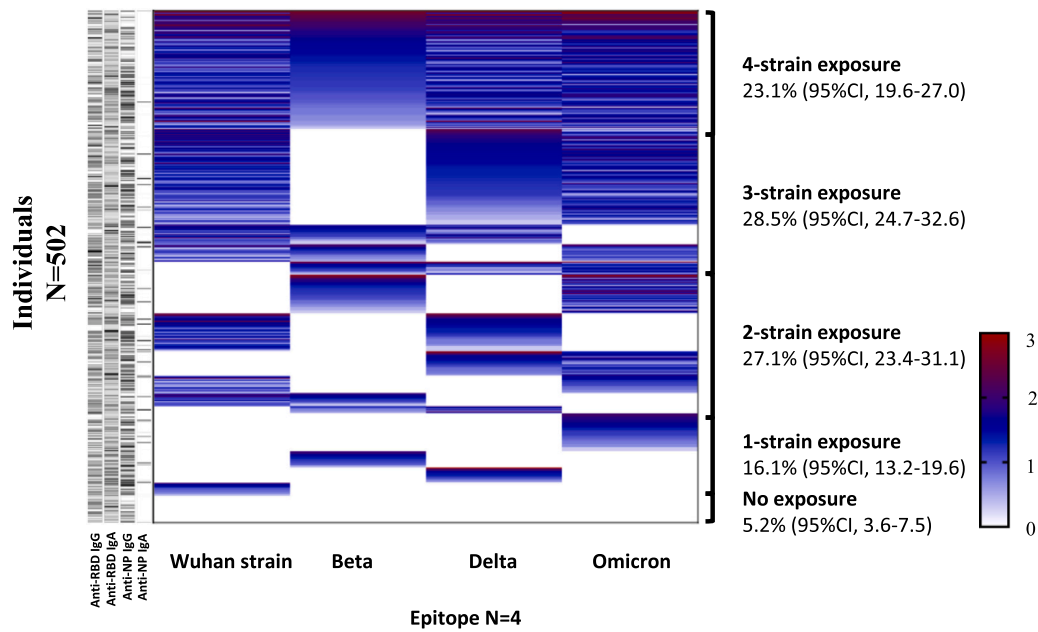


Fig. 2. Heat map of the T cell profiling of Antananarivo's blood donors. NG-CHM Builder, next-generation clustered heat map builder, <https://build.ngchm.net/NGCHM-web-builder/>. RBD, receptor-binding domain; NP, nucleocapsid protein; IgG, immunoglobulin G; IgA, immunoglobulin A; W, Wuhan strain; CI, confidence interval.

Table 2
Profiles identified by the 4 PDPs.

Profil (#)	n	% (95%CI)	n	% (95%CI)	n strain exposures
WBDO (1)	116	23.1 (19.6–27.0)	116	23.1 (19.6–27.0)	4
WDO (2)	94	18.7 (15.6–22.4)	143	28.5 (24.7–32.6)	3
WBD (3)	19	3.8 (2.4–5.8)			
WBO (4)	17	3.4 (2.1–5.4)			
BDO (5)	13	2.6 (1.5–4.4)			
BO (6)	38	7.6 (5.6–10.2)	136	27.1 (23.4–31.1)	2
WD (7)	37	7.4 (5.4–10.0)			
DO (8)	24	4.8 (3.2–7.0)			
WO (9)	17	3.4 (2.1–5.4)			
WB (10)	13	2.6 (1.5–4.4)			
BD (11)	7	1.4 (0.7–2.9)			
O (12)	37	7.4 (5.4–10.0)	81	16.1 (13.2–19.6)	1
B (13)	16	3.2 (2.0–5.1)			
D (14)	15	3.0 (1.8–4.9)			
W (15)	13	2.6 (1.5–4.4)			
Neg (16)	26	5.2 (3.6–7.5)	26	5.2 (3.6–7.5)	0

PDP, profile defining peptide; n, number of positive samples; 95%CI, 95% confidence interval; W, Wuhan strain; Variant of concern: B, Beta; D, Delta; O, Omicron.

individuals showed a relatively lower ICC (0.022) possibly due to the stringency of the test (appendix Q).

Multiple exposures increase cross reactivity

T cell responses to the 81 peptides were analysed and plotted. Hierarchical clustering grouped individuals in seemingly three groups: about 24% of individuals belonged to high responders (n = 121), 60% to medium responders (n = 299) and the last 16% to low responders (n = 82) (appendix R).

For each of the 16 previously described profiles, cross-reactivity investigation was then performed using responses to strain-specific peptides outside the 473–487 region (10 peptides for B, 8 for D, 2 for O). No peptide outside the 473–487 region was W strain-specific. Relative score was used to measure the cross-reactivity defined as the percentage of response to the above mentioned-peptides that do not belong to the strain which defines the profile of each individual.

D peptides cross-reactivity analysis was represented respecting the chronology of strain detections. There was an apparent increase in relative cross-reactivity scores for samples having been exposed most “Recently” (p < 0.0001) (Fig. 3B). Indeed, recent infections are suspected to be due to either D or O (3rd bimodal wave having occurred less than 6 months from blood collection). Older ones include W and B ones (two first waves having occurred more than 6 months from blood collection). This result although validating our immunization profiling method using PDP (as individuals profiled as having been exposed recently have higher scores than those exposed a long time ago, and are expected to show lower T cell reactivity thus scores) led us to only investigate cross-reactivity (to D and O) developed by individuals exposed to SARS-CoV-2 during the two first waves. We thus considered the time since last immunization as identical for each group: W (previous immunization with the W strain), B (previous immunization with B VOC), WB (previous immunization both with W strain and B VOC). This analysis showed a significantly increased D cross-reactivity score with two exposures (WB) versus W or B single exposures (44.3% vs 30.4% respectively, p=0.0308) and versus a single W exposure (24.9%, p=0.0106) (Fig. 3C). For O cross-reactivity, this trend was also observed when comparing cross-reactivity scores in the same profiles but did not reach significance due in part to only two peptides being associated to O only (Fig. 3D).

Cross-reactivity does not prevent new infections

We then analysed the theoretical profile evolution over the years to investigate whether increasing natural immunization, thus the number of strain exposures, would protect individuals from new emerging strain infection. Profile evolution did not seem to be influenced by previous natural immunization. In fact, we found that surprisingly, the probability of being infected by B during the second wave was actually higher but did not reach significance or individuals from the W pre-immunized group than for those from the naïve group (50.6% vs 42.0% respectively, P=0.0666). No advantage, no decrease in reinfection probability was gained through prior multiple immunizations during the D and O wave. The probability of not being infected during these waves was 7.9% for WB pre-immunized individuals, 8.1% for W pre-immunized individuals, 21.6%

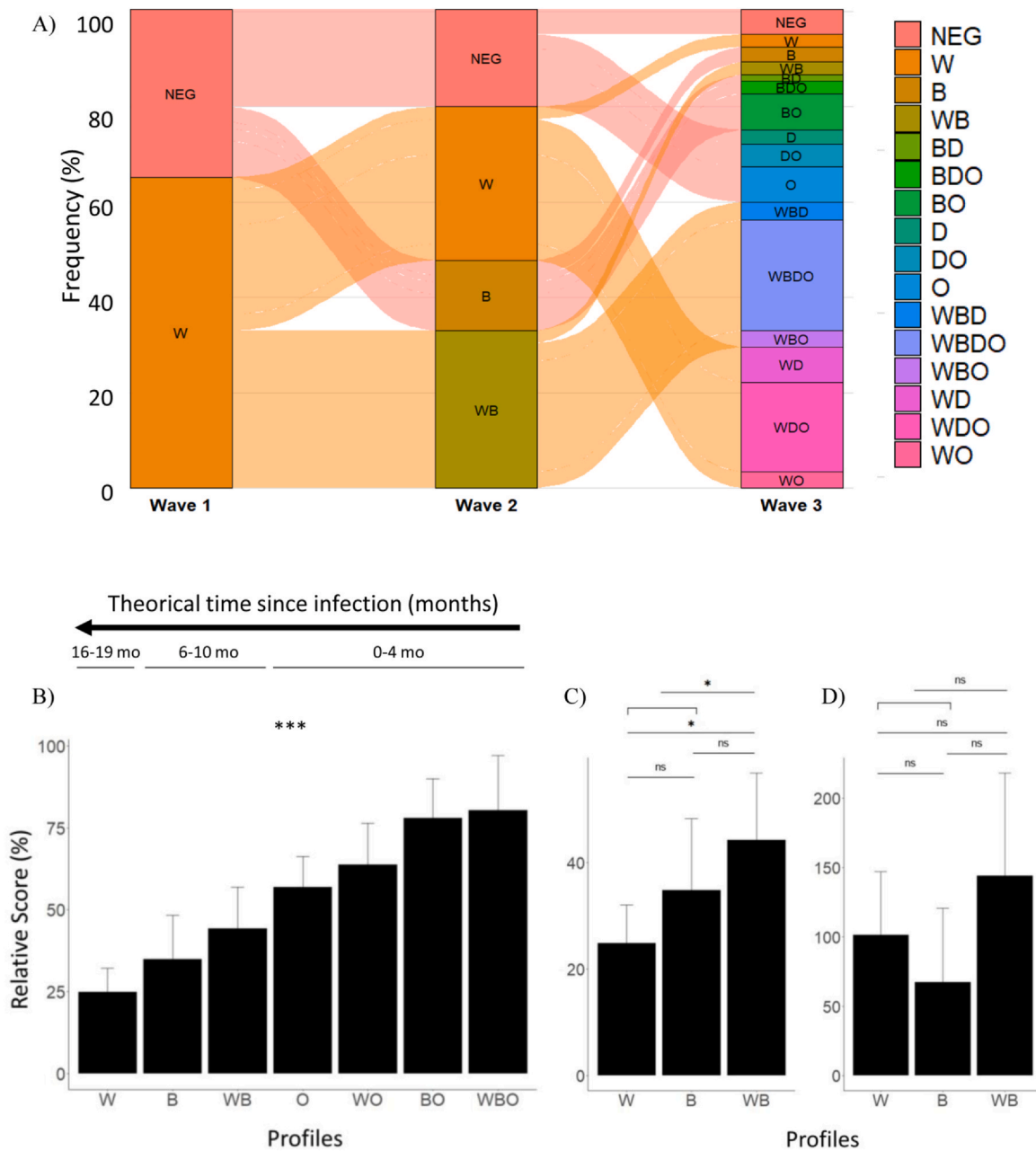


Fig. 3. Immunisation profile evolution and cross reactivity investigation. A) Profiles evolution during Madagascar's 3 epidemics waves. B) Delta cross-reactivity among the 7 profiles showed a significant decrease between recent profile (WBO) and older ones (W). Bars show the mean ± SD. Statistics calculated by one-way ANOVA (***p < 0.001). C) Delta cross-reactivity among single exposures (W or B) and double one (WB), bars show the mean ± SD. Statistics calculated by Mann-Whitney test (*p < 0.05; ns, non-significant). D) Omicron cross-reactivity among single exposures (W or B) and double one (WB), bars show the mean ± SD. Statistics calculated by Mann-Whitney test (*p < 0.05; ns, non-significant). W, Wuhan strain; B, Beta; D, Delta; O, Omicron.

for B pre-immunized individuals and 25.5% for naïve individuals (appendix J) counterintuitively suggesting W pre-immunization as a risk factor for further natural immunization.

All Antananarivo blood donors have had COVID-19

Serological analysis was performed for all samples. The results regarding anti-RBD and anti-NP IgG/IgA seropositivity are presented in the Appendix K, L. The IgG and IgA seropositivity were respectively 76.0% (95%CI, 72.1–79.6) and 16.1% (95%CI, 12.7–20.2) for anti-NP; and 82.0% (95%CI, 78.4–85.2) and 75.0% (95%CI, 70.4–79.1) for anti-RBD respectively. The seroprevalence of the more persistent antibodies, anti-RBD IgGs, added to the SARS-CoV-2 specific T-cell positivity (samples producing IFN-γ upon stimulation with one of

the 4 specific PDP) showed that 99.0% (95%CI, 97.7–99.6) of blood donors were infected at least once by SARS-CoV-2 (appendix M), whatever the circulating strain.

Discussion

BTC sampling enabled fast and efficient high quality PBMC-collections. Despite this population not being entirely representative of the general population, we have shown previously that correcting crude data from this population (over-representing young adult males) generates data extremely close to crude values and indicates that this study population is coherent for population immunity investigations in the Malagasy setting[17]. Another major limitation of the study is the absence of pre-pandemic negative control samples

and other control samples resulting from “clean”, single- and multiple- strain-described immunizations for T cell reactivity validations. These types of samples were no longer accessible in Madagascar due to COVID-19 epidemic linked liquid nitrogen shortages. Nevertheless, the T cell response-based approach used in this study utilized tools that have already been validated in several studies. Indeed, the analyses were carried out using a commercially available kit (see M&M section), which has been validated and employed in 1691 published studies (catalog #3420-4APW-10, Mabtech). Furthermore, the characterization of the exposure history was conducted on an immunomodulatory antigen, described in other studies, located in the RBD region that was selected from the Immune Epitope Database[4,25]. This region is noteworthy for containing mutation sites specific to the four SARS-CoV-2 variants analyzed in this study. Abdo A. Elfiky highlighted its significance in the interaction of the SARS-CoV-2 virus with its receptor[24].

The results provided by this study have enabled us to describe 16 distinct cellular response profiles established in the Antananarivo BTC after the three waves of COVID-19 that the country had undergone early 2022. The ELISPOT results obtained show that selecting epitopes according to our strategy highlighted specific immunogenic epitopes specific to SARS-CoV-2 strains that were able to activate collected samples.

Peptides exposed at the surface of RBD in region 473–487 and presenting signature mutations to the three main VOCs and to the W strain that have circulated in Madagascar allowed us to describe the history of exposures for each investigated sample. Indeed, these selected peptides have the specificity to present unique and characteristic mutations of each VOC and have been linked to their virulence and pathogenicity. Indeed, the E484K mutation found in B VOC allows it to escape neutralizing antibodies[26]. The D VOC associated T478K mutation, the S477N and T478K mutations have been shown to increase the affinity of O for its receptor ACE2[27] and E484A acts negatively on the recognition of pre-existing neutralizing antibodies[28]. The results obtained here allow us to gain insight into the frequencies of exposure to each of these strains and their associated characteristics.

The complex pattern of multiple exposure profiles described in this study illustrates to what extent SARS-CoV-2 and its VOCs have naturally immunized the Malagasy population of Antananarivo. These results have allowed us to describe the response profile implemented in a low vaccination population following multiple epidemic waves. These profiles suggest different protective patterns that were not evaluated in this study. A mathematical modelling-based study however, in US population found increased protection against infectivity and severity of the SARS-CoV-2 variant among a multiple exposed population (multiple infection or infection and vaccination or vaccination and boosters)[29].

The first wave in 2020, characterized by the circulation of the W strain, was ended once an immunization rate of 42% was reached (95%CI, 41–43) among blood donors[17]. This populational seroconversion was accompanied by a specific T cell response and was followed by the establishment of an immune memory, assessed by 65% (95%CI, 61–69) of samples reactive to peptide # 6 (W of PDP) in the present study. This higher value than the immediately after wave measured seroprevalence could be explained by the stealth circulation of the W strain ever since. The second wave of 2021 was triggered by the introduction of B VOC and occurred despite the close to 50% immunization rate described previously. B VOC specific serological responses were found in 63% of “recent” second wave seroconversions, or 30% in all tested donors[18]. Coherently, in this study, we have shown that 48% (95%CI, 43–52) of the blood donor population developed T-cells specific to B VOC of which 69% (63–75) were exposed to both the ancestral W strain and B VOC. The response patterns described in our analysis show specific T-cell establishment following each of these two first epidemic wave in

Madagascar. The third bimodal waves of 2022 were apparently due, at least in part, to the circulation of two highly transmissible VOCs: D and O[30]. Analysis of blood donor samples showed high T cell reactivity to both D (65%[95%CI, 60–69]) and O (71%[95%CI, 67–75]) peptides # 4 and 57 (PDP) respectively (Fig. 3 A and appendix N). The circulation of these VOCs during the third wave seems to explain the two observed epidemic peaks in Madagascar[3]. Studies in South Africa have shown similar patterns for the epidemic waves showing that the first wave is due to the ancestral W strain, the second to B VOC, the third to D VOC and the fourth to O VOC[28]. The different response patterns described in this study thus enable us to validate the history of natural infections that have led to the complex immunization the Malagasy blood donor population has acquired following these three different epidemic waves.

Cross-reactivity investigation showed an apparent increase for samples having been exposed most recently during bimodal wave D and O. This confirmed our immunization profiling method using PDP. Indeed, individuals profiled as having been exposed to D and O VOCs during the third wave showed the highest scores whereas those exposed to W strain and B VOC showed lower ones. This result shows that individuals gradually lose specific T-cells as demonstrated by others[31,32]. Our quantitative cross-reactivity analysis could thus only be performed in a context of immunizations having taken place at the same time. The quantitative results obtained with PDP, however, enabled qualitative profile definition. It is noteworthy that the profiles showing increased cross-reactivity (multiple strain exposures) did not show protection from infection during the next wave.

In Madagascar, COVID-19 epidemic waves are accompanied by low testing that underestimates the immunization undergone by the population that is in reality significant[33]. The present study highlights the importance of specific antibody testing and T cell response analysis to monitor the natural immunization undergone by the population by SARS-CoV-2. Indeed, our study showed that by April 2022, 94.8% of the collected samples produced IFN- γ upon stimulation with the 4 specific peptides from PDP and that 99.0% of blood donors had been infected at least once by SARS-CoV-2. Despite the low vaccination rate at time of collection (4%), T-cells response immunization investigation gave evidence of a complex immunization of the Malagasy population, needing to be better described in terms of aptitude to re-infection[34].

The complex populational immunization profiles found in Antananarivo have led to populational immunity. Nevertheless, a new epidemic wave hit Madagascar once again as early as June 2022. Ninety-five, 79%, 51% and 23% of individuals had however already been infected at least once, twice, three times, and four times respectively when the epidemic started, suggesting these prior-immunization profiles were not sufficient to prevent a new series of infections, that have most probably led to an even more complex pattern of immunization among which individuals have been infected at least five times by five different strains of SARS-CoV-2.

This study demonstrated a highly antigen-reactive population to SARS-CoV-2 after three epidemic waves and the circulation of four SARS-CoV-2 strains. The majority of individuals (95%) had T-cells that produced IFN- γ upon stimulation by at least one out of the four strain restricted peptides. Twenty-four percent produced IFN- γ regardless the strain-specific peptide from PDP suggesting these individuals had been infected by all four strains having circulated in Madagascar. These results show gradual enlargement of the repertoire, a priori, by natural immunizations. Our study of cross-reactivity, however, seemed to show a lack of cross-reactivity conferred protection from a later infection and would have decreased the probability of undergoing a subsequent infection by another strain, most probably due also in part to the biology of the circulating VOC itself and to its specific escape mechanisms. Although the protection conferred by these multiple immunisations was not investigated in this study, recent evidence has shown that a

previous infection highly protects against severity by 90.2% for W strain and VOCs (Alpha, B and D) previous infections and 88.9% by O BA.1 previous infection for up to 40 weeks[35].

Vaccinating a naturally immunized population establishes stronger hybrid immunity[26,36]. Yet, vaccinating a population with a complex immunity resulting from multiple prior immunizations may not be a priority. More research is needed to understand these immune profiles including the potential immune mechanisms resulting protection from severity.

Funding information

UNICEF and the French Ministry for Europe and Foreign Affairs through the REPAIR COVID-19-Africa project coordinated by the Pasteur International Network association financed this study.

Declaration of Competing Interest

The authors declare that they have no competing interest.

Acknowledgments

We gratefully acknowledge blood donors and RBTC staff.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jiph.2023.11.020.

References

- Callaway B, Cyranoski D, Mallapaty S, Stoye E, Tollefson J. Coronavirus by the numbers. *Nat [Rev En Internet]* 2018 [Acceso 6 De enero De 2021] 2020;579(1):482–3.
- Zhou P, Yang X lou, Wang XG, Hu B, Zhang L, Zhang W, et al. A pneumonia outbreak associated with a new coronavirus of probable bat origin. *Nature* 2020;579:270–3. <https://doi.org/10.1038/s41586-020-2012-7>
- World Health Organization. WHO Coronavirus Disease (COVID-19) Dashboard n. d. (<https://covid19.who.int>).
- Grifoni A, Weiskopf D, Ramirez SI, Mateus J, Dan JM, Moderbacher CR, et al. Targets of T cell responses to SARS-CoV-2 coronavirus in Humans with COVID-19 disease and unexposed individuals. *Cell* 2020;181:1489–1501.e15. <https://doi.org/10.1016/j.cell.2020.05.015>
- Liao M, Liu Y, Yuan J, Wen Y, Xu G, Zhao J, et al. Single-cell landscape of bronchoalveolar immune cells in patients with COVID-19. *Nat Med* 2020;26:842–4. <https://doi.org/10.1038/s41591-020-0901-9>
- Rydyznski Moderbacher Carolyn, Ramirez Sydney I, Dan Jennifer M, Grifoni Alba, Hastie Kathryn M, Weiskopf Daniela, Belanger Simon, Abbott Robert K, Kim Christina, Choi Jinyong, Kato Yu, Crotty Eleanor G, Kim Cheryl, Rawlings Stephen A, Mateus Jose, SC. Antigen-specific adaptive immunity to SARS-CoV-2 in acute COVID-19 and associations with age and disease severity. *Cell* 2020;183:996–1012. <https://doi.org/10.1016/j.cell.2020.09.038>
- Sekine T, Rivera-ballesteros O, Ljunggren H, Aleman S, Buggert M, Parrot T, et al. Robust T cell immunity in convalescent individuals with asymptomatic or mild COVID-19. *Cell* 2020;183:158–68.
- Zhou Runhong, To Kelvin Kai-Wang, Wong Yik-Chun, Hung K-YY Ivan Fan-Ngai, Chen Z. Acute SARS-CoV-2 Infection Impairs Dendritic Cell and T Cell Responses. *Immunity* 2020;53:864–77. <https://doi.org/10.1016/j.immuni.2020.07.026>
- Braun J, Loyal L, Frentsch M, Wendisch D, Georg P, Kurth F, et al. SARS-CoV-2-reactive T cells in healthy donors and patients with COVID-19. *Nature* 2020;587:270–4. <https://doi.org/10.1038/s41586-020-2598-9>
- Barda N, Dagan N, Cohen C, Hernán MA, Lipsitch M, Kohane IS, et al. Effectiveness of a third dose of the BNT162b2 mRNA COVID-19 vaccine for preventing severe outcomes in Israel: an observational study. *Lancet* 2021;398:2093–100. [https://doi.org/10.1016/S0140-6736\(21\)02249-2](https://doi.org/10.1016/S0140-6736(21)02249-2)
- Haas EJ, Angulo FJ, McLaughlin JM, Anis E, Singer SR, Khan F, et al. Impact and effectiveness of mRNA BNT162b2 vaccine against SARS-CoV-2 infections and COVID-19 cases, hospitalisations, and deaths following a nationwide vaccination campaign in Israel: an observational study using national surveillance data. *Lancet* 2021;397:1819–29. [https://doi.org/10.1016/S0140-6736\(21\)00947-8](https://doi.org/10.1016/S0140-6736(21)00947-8)
- Gazit S, Shlezinger R, Perez G, Lotan R, Peretz A, Ben-Tov A, et al. The Incidence of SARS-CoV-2 reinfection in persons with naturally acquired immunity with and without subsequent receipt of a single dose of BNT162b2 vaccine: a retrospective cohort study. *Ann Intern Med* 2022;175:674–81. <https://doi.org/10.7326/M21-4130>
- Nordström P, Ballin M, Nordström A. Articles Risk of SARS-CoV-2 reinfection and COVID-19 hospitalisation in individuals with natural and hybrid immunity: a retrospective, total population cohort study in Sweden. *WwwTheLancetCom/ Infect* 2022;22. [https://doi.org/10.1016/S1473-3099\(22\)00143-8](https://doi.org/10.1016/S1473-3099(22)00143-8)
- Tan C.-W., Lim B.-L., Young B.E., Ying-Yan Yeoh A., Yung C.-F., Yap W.-C., et al. Comparative neutralisation profile of SARS-CoV-2 omicron subvariants BA.2.75 and BA.5 2022. [https://doi.org/10.1016/S2666-5247\(22\)00220-8](https://doi.org/10.1016/S2666-5247(22)00220-8).
- Wang L, Kainulainen MH, Jiang N, Di H, Bonenfant G, Mills L, et al. Differential neutralization and inhibition of SARS-CoV-2 variants by antibodies elicited by COVID-19 mRNA vaccines. *Nat Commun* 2022;13. <https://doi.org/10.1038/s41467-022-31929-6>
- Patalon T, Saciuk Y, Perez G, Peretz A, Ben-Tov A, Gazit S. Dynamics of naturally-acquired immunity against SARS-CoV-2 in children and adolescents. *J Pediatr* 2023. <https://doi.org/10.1016/j.jpeds.2023.02.016>
- Schoenhals M, Rabenindrina N, Rakotontramanga JM, Dussart P, Randremanana R, Heraud J-M, et al. SARS-CoV-2 antibody seroprevalence follow-up in Malagasy blood donors during the 2020 COVID-19 Epidemic. *EBioMedicine* 2021;68:103419. <https://doi.org/10.1016/j.ebiom.2021.103419>
- Razafimahatratra SL, Diarra M, Ndiaye B, Rasoloharimanana LT, Dussart P, Sahondranirina PH, et al. Seroprevalence of ancestral and Beta SARS-CoV-2 antibodies in Malagasy blood. *Lancet Glob Health* 2020;9:e1363–4. [https://doi.org/10.1016/S2214-109X\(21\)00361-2](https://doi.org/10.1016/S2214-109X(21)00361-2)
- Das A, Hoare M, Davies N, Lopes AR, Dunn C, Kennedy PTF, et al. Functional skewing of the global CD8 T cell population in chronic hepatitis B virus infection. *J Exp Med* 2008;205:2111–24. <https://doi.org/10.1084/jem.20072076>
- Walker B, McMichael A. The T-cell response to HIV. *Cold Spring Harb Perspect Med* 2012;2. <https://doi.org/10.1101/cshperspect.a007054>
- Xia W, Zhao J, Su B, Jiao Y, Weng W, Zhang M, et al. Syphilitic infection impairs immunity by inducing both apoptosis and pyroptosis of CD4+ and CD8+ T lymphocytes. *Innate Immun* 2021;27:99–106. <https://doi.org/10.1177/1753425920952840>
- Ndiaye MDB, Rasoloharimanana LT, Razafimahatratra SL, Ratovoson R, Rasolof V, Ranaivomanana P, et al. Using a multiplex serological assay to estimate time since SARS-CoV-2 infection and past clinical presentation in malagasy patients. *Heliyon* 2023;9. <https://doi.org/10.1016/j.heliyon.2023.e17264>
- Broom BM, Ryan MC, Stucky M, Wakefield C, Melott JM, Akbari R, et al. Interactive clustered heat map builder: an easy web-based tool for creating sophisticated clustered heat maps. *F1000Res* 2020;8:1–10. <https://doi.org/10.12688/f1000research.20590.2>
- Elfiky AA. SARS-CoV-2 Spike-Heat Shock Protein A5 (GRP78) recognition may be related to the immersed human coronaviruses. *Front Pharm* 2020;11. <https://doi.org/10.3389/fphar.2020.577467>
- Zhao J, Wang L, Schank M, Dang X, Lu Z, Cao D, et al. SARS-CoV-2 specific memory T cell epitopes identified in COVID-19-recovered subjects. *Virus Res* 2021;304. <https://doi.org/10.1016/j.virusres.2021.198508>
- Soriano V, Fernández-Montero J v. New sars-cov-2 variants challenge vaccines protection. *AIDS Rev* 2021;23:57–8. <https://doi.org/10.24875/AIDSREV.M21000040>
- Jhun H, Park HY, Hisham Y, Song CS, Kim S. SARS-CoV-2 delta (B.1.617.2) variant: a unique T478K mutation in receptor binding motif (RBM) of spike gene. *Immune Netw* 2021;21. <https://doi.org/10.4110/IN.2021.21.E32>
- Viana R, Moyo S, Amoako DG, Tegally H, Scheepers C, Althaus CL, et al. Rapid epidemic expansion of the SARS-CoV-2 Omicron variant in southern Africa. *Nature* 2022;603:679–86. <https://doi.org/10.1038/s41586-022-04411-y>
- Klaassen F, Chitwood MH, Cohen T, Pitzer VE, Russi M, Swartwood NA, et al. Changes in population immunity against infection and severe disease from severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) omicron variants in the united states between december 2021 and november 2022. *Clin Infect Dis* 2023. <https://doi.org/10.1093/cid/ciad210>
- El-Shabasy RM, Nayel MA, Taher MM, Abdelmonem R, Shoueir KR, Kenawy ER. Three waves changes, new variant strains, and vaccination effect against COVID-19 pandemic. *Int J Biol Macromol* 2022;204:161–8. <https://doi.org/10.1016/j.ijbiomac.2022.01.118>
- Dan JM, Mateus J, Kato Y, Hastie KM, Yu ED, Faliti CE, et al. Immunological memory to SARS-CoV-2 assessed for up to 8 months after infection. *Science* 2021;371(1979). <https://doi.org/10.1126/science.aba4063>
- Sette A, Crotty S. Immunological memory to SARS-CoV-2 infection and COVID-19 vaccines. *Immunol Rev* 2022;310:27–46. <https://doi.org/10.1111/imr.13089>
- Evans M. v., Garchitorena A., Rakotonanahary R.J.L., Drake J.M., Andriamihaja B., Rajaonarifara E., et al. Reconciling model predictions with low reported cases of COVID-19 in Sub-Saharan Africa: insights from Madagascar. (<https://doi.org/10.1080/1654971620201816044>) 2020;13. <https://doi.org/10.1080/16549716.2020.1816044>.
- Mathieu E, Ritchie H, Ortiz-Ospina E, Roser M, Hasell J, Appel C, et al. A global database of COVID-19 vaccinations. *Nat Hum Behav* 2021;5:947–53. <https://doi.org/10.1038/s41562-021-01122-8>
- Stein C, Nassereldine H, Sorensen RJD, Amlag JO, Bisignano C, Byrne S, et al. Past SARS-CoV-2 infection protection against re-infection: a systematic review and meta-analysis. *Lancet* 2023. [https://doi.org/10.1016/S0140-6736\(22\)02465-5](https://doi.org/10.1016/S0140-6736(22)02465-5)
- Bobrovitz N, Ware H, Ma X, Li Z, Hosseini R, Cao C, et al. Protective effectiveness of previous SARS-CoV-2 infection and hybrid immunity against the omicron variant and severe disease: a systematic review and meta-regression. *Lancet Infect Dis* 2023. [https://doi.org/10.1016/S1473-3099\(22\)00801-5](https://doi.org/10.1016/S1473-3099(22)00801-5)