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PRECISION IMMUNE MONITORING

Response of SARS-CoV-2 Spike-Specific B Cells to Vaccination

Detection and phenotypic characterization of spike-specific B-cell populations from peripheral blood mononuclear cell (PBMC) samples using Klickmer® reagents and flow cytometry.

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Highlights

- We examined the frequency of spike-specific B cells in healthy individuals before and after vaccination against SARS-CoV-2, as well as after a breakthrough infection.
- Klickmer® reagents were loaded with recombinant SARS-CoV-2 spike protein. The spike-Klickmer® reagents allowed sensitive detection of spike-specific B cells and subsequent phenotypic characterization based on antibody staining.
- Vaccination elicited a spike-specific B-cell response in sample donors. That cell subset was enriched with $CD27^{+}IgD^{-}$ memory B cells. The frequency of spike-specific B cells increased further in response to breakthrough infection.
- Long-term monitoring of B-cell populations in response to vaccination supports vaccination development and evaluation.

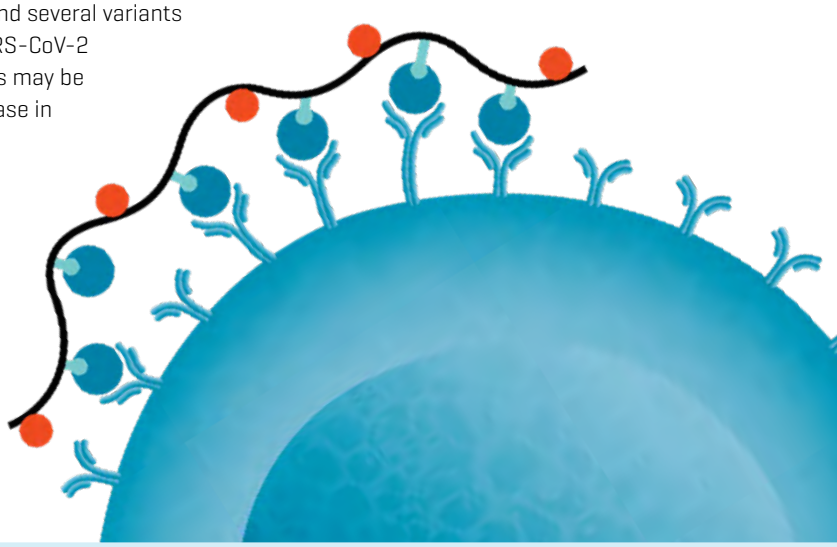
Introduction

The emergence of the coronavirus SARS-CoV-2 at the end of 2019 marked not only the beginning of a global pandemic that would infect over 630 million people around the world[1], but also a shift in the type of vaccines that can be deployed as countermeasures. A focus of much research during the last years has been understanding the role of the immune system in the progression of the disease and in mounting defenses to the novel virus. B cells are integral to the interplay that characterizes the adaptive immune response. Their response to infection and vaccination is dynamic and durable, ranging in effects from cytokine production and antigen presentation to antibody secretion.[2]

B Cell and Antibody Responses to SARS-CoV-2 Vaccination

Numerous vaccines were developed during the SARS-CoV-2 pandemic to control the spread and mitigate the virulence of the virus, based on inactivated virus, viral protein subunits or mRNA. In general, SARS-CoV-2 vaccinations activate and expand SARS-CoV-2–specific memory B cells. The rise in this subset of the B-cell population in blood persists into the sixth month after vaccination, albeit with a small but noticeable decline. Vaccinations also produce a robust increase in antibodies – neutralizing, against the spike protein, in particular against the receptor binding domain – that lasts six months with some decline.[3]

Spike-specific memory B cells generated in response to vaccination also show resilience to viral variants. In fact, they were better able to cross-bind several variants of concern compared to memory B cells arising from SARS-CoV-2 infection.[3] Thus, these durable and versatile memory cells may be responsible for continued protection against severe disease in vaccinated individuals.



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Klickmer® is a High-Avidity Reagent that is Tailorable to Antigen-Specific B Cells

Based on the Immudex Dextramer® technology, Klickmer® is a multipurpose reagent that arranges numerous acceptor sites along a flexible dextran backbone coupled to PE, FITC, APC or no fluorescent label (Fig. 1). The acceptor sites bind a biotinylated molecule of choice. Klickmer® reagents are commonly used for the sensitive detection and isolation of rare or low-affinity, antigen-specific B cells without the need for upstream stimulation, enrichment, or expansion.

Did you know?

Spike-Klickmer® can be readily combined with dCODE Klickmer® technology which is equipped with unique DNA barcodes to investigate for instance spike+ B cell specificities by NGS or single-cell multi-omics, adding information on B Cell Receptor (BCR) sequences, surface marker and gene expression.

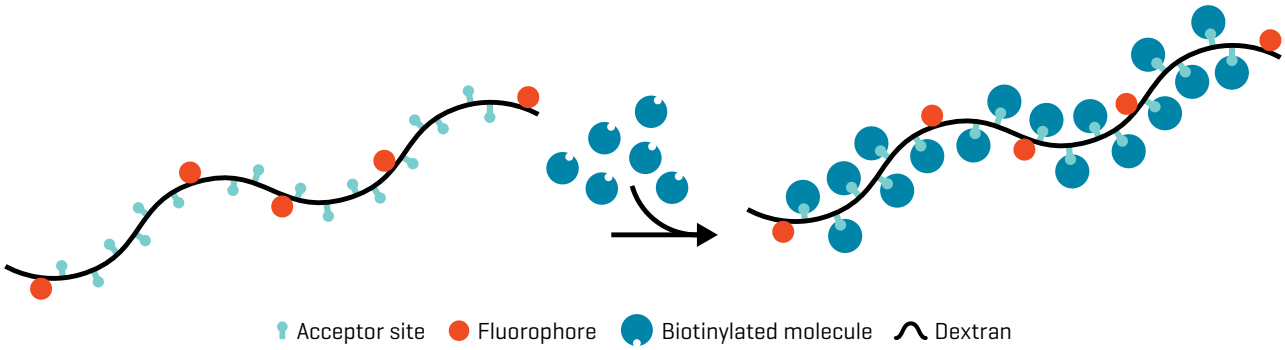

Fig. 1: General structure of a Klickmer® reagent.

We used the customizability of Klickmer® reagents to assemble a reagent capable of detecting and characterizing SARS-CoV-2 spike-specific B cells in PBMC samples taken from donors before and after two doses of a SARS-CoV-2 vaccine. At 140 kDa, the spike protein is quite large. Still, the full-sized mono-biotinylated recombinant protein was successfully loaded on two Klickmer® reagents, one with the fluorochrome PE and the other with APC. The two spike-Klickmer® reagents were combined into the same staining tube and used together in staining cells to minimize signal noise via double discrimination. Optimizing the antigen loading ratio resulted in two high-quality spike-Klickmer® reagents with low background.

Methods

Klickmer® reagents

- Klickmer®-PE, DX01K-PE (160 nM; Cat. No. DX01K-PE)
- Klickmer®-APC, DX01K-APC (160 nM; Cat. No. DX01K-APC)

Tip:

To exclude B cells that bind non-specifically to fluorochromes, we recommend double discrimination by staining with two Klickmer® reagents, one with PE and the other with APC.

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Additional reagents

- Mono-biotinylated recombinant SARS-CoV-2 spike protein (ACRO Biosystems, cat. no. SPN-C82E9) in aqueous buffer (pH 7.0-7.5) with a biotinylation level > 75% and no excess of free biotin
- Mono-biotinylated recombinant protein known to be absent in donor samples as negative control
- Human Fc block reagent, BD Biosciences
- d-Biotin solution 100 µM, Avidity, BIO200
- Fixable Viability Stain 575V, BD Biosciences
- Antibodies for staining of other surface markers, BV421 mouse anti-human CD19, V500 mouse anti-human CD3, PerCP-Cy5.5 mouse anti-human CD14, BV786 mouse anti-human CD27, FITC mouse anti-human IgD, BD Biosciences
- PBB buffer: Phosphate-buffered saline (PBS), 1% bovine serum albumin (BSA), pH 7.0
- Wash buffer: PBS, 5% fetal calf serum (FCS), pH 7.4
- Brilliant blue stain buffer, BD Biosciences

Sample collection

PBMC samples were prepared from whole blood collected from healthy subjects (n = 4) before receiving two doses of a spike-specific vaccine against SARS-CoV-2. The same sample preparation was done with whole blood drawn from the same donors 4 to 6 weeks after administration of the second dose (Fig. 2). An additional sample was collected from one donor after a breakthrough infection.

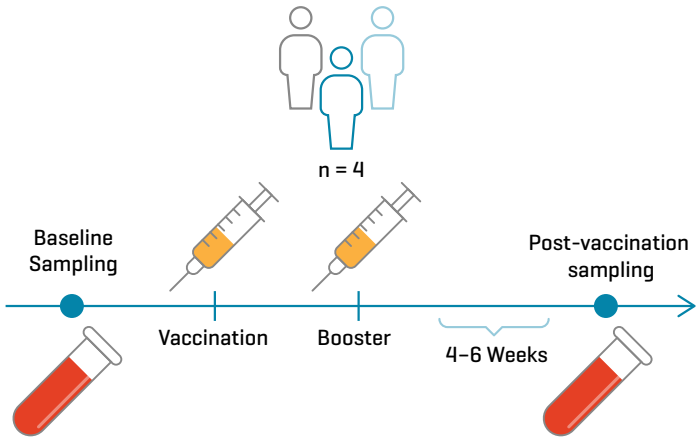

Fig. 2: Sample collection scheduled from four donors before and after SARS-CoV-2 vaccination.

Preparation of spike-Klickmer® reagents

This protocol describes the preparation of 20 µL spike-Klickmer®-PE and spike-Klickmer®-APC reagent solutions at a ratio of 7 spike molecules per Klickmer® molecule. For this purpose, the concentration of recombinant spike protein used was 2000 nM. However, volumes and concentrations can be adjusted to your experiment using the formula below. The preparation of the negative control Klickmer® reagent is not described but follows the same procedure.

$$\textit{Spike protein volume [µL]} = \frac{\textit{[desired loaded Klickmer volume [µL]*stock Klickmer concentration [nM]*number ligands per Klickmer]}}{\textit{[spike protein concentration [nM]]}}$$

Additionally, the volume of PBB buffer needed can be calculated with the following formula. Table 1 illustrates volumes of spike protein, Klickmer® reagent stock solution, and PPB buffer needed for a given number of staining tests.

Spike protein volume [µL] = desired loaded Klickmer volume [µL]-spike protein volume [µL]-stock Klickmer volume [µL]

Table 1: **Volumes of spike-Klickmer® reagents**

No. of tests	Recombinant spike protein (2000 nM)	Klickmer stock solution (PE or APC) (160 nM)	PBB buffer
1	6 µL	2 µL	2 µL
5	28 µL	10 µL	12 µL
10	56 µL	20 µL	24 µL
20	112 µL	40 µL	48 µL

Source: Each test corresponds to staining 1-3 x 10^6 PBMCs.

1. Add the calculated volume of spike protein into two light-protected reaction tubes, labelled spike-Klickmer®-PE and spike-Klickmer®-APC, respectively.
2. Add Klickmer®-PE or Klickmer®-APC to their corresponding tubes to a final concentration of 32 nM. This corresponds to 1/5 of the total volume of the desired spike-Klickmer® reagent solution.
3. Gently pipette the contents of each tube 5 times to mix.
4. Add the calculated volume of PBB buffer and gently pipette the contents 5 times to mix.
5. Incubate for 30 min in the dark at room temperature.
6. Protected from light, the spike-Klickmer® solutions can be stored for up to 1 week at 2–8 °C.

Tip:

In principle, any mono-biotinylated ligand can be loaded onto a Klickmer reagent. If you are working with a different antigen than the spike protein, we recommend testing at least 3 different ratios of mono-biotinylated ligand per Klickmer® (low, medium, and high loading capacity) to optimize the ligand/Klickmer® reagent ratio for your specific application. Too low valency may result in too low avidity to detect the target while too high valency may generate unwanted background staining. The size of the ligand you are loading may also influence the optimal ratio. Please note that the different colored Klickmer® products have different loading capacities.

Preparation of PBMC sample for staining of spike-specific B cells

PBMCs prepared from donated samples underwent a series of stainings to first ascertain their viability, then detect spike-specific B cells, and lastly, characterize the cells phenotypically based on antibody binding **(Fig. 3)**.

Tip:

We recommend staining with Klickmer® before antibody staining, to avoid that binding of antibodies could result in loss of sensitivity.

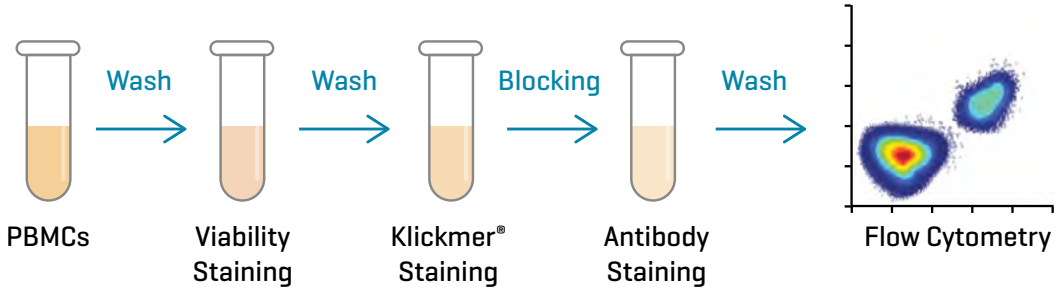

Fig. 3: Overview of Klickmer® staining procedure to analyze spike-specific B cells before and after SARS-CoV-2 vaccination.

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1. If frozen, thaw sample PBMCs (up to 1–3 x 10^6) and resuspend in 10 mL wash buffer.
2. Centrifuge at 300 x g for 10 mins and remove supernatant. Repeat washing for a total of 2 washes.
3. Resuspend the final cell pellet in 1 mL wash buffer and incubate with 2 µL of BD Fixable Viability Stain 575V stock solution for 15 min in the dark at room temperature.
4. Add 10 mL wash buffer, centrifuge at 300 x g for 10 min and remove the supernatant.
5. Resuspend the cell pellet in a suitable volume of BD brilliant blue stain buffer.

Staining of spike-specific B cells in PBMC sample

1. Spin the spike-Klickmer® solutions generated above at 10,000 x g for 1 min.
2. Add 0.4 µL 100 µM d-Biotin to an empty flow tube. Introduce 10 µL spike-Klickmer®-PE and 10 µL spike-Klickmer®-APC solutions into the same flow tube and vortex briefly.
3. Add 50 µL cell suspension to the mixture and vortex briefly.
4. Incubate for 20 min. in the dark at room temperature.
5. Follow manufacturer's instructions to add Fc block reagent (0.5 mg/mL) to the cells for a final amount of 2.5 µg. Incubate for 10 min. at room temperature.
6. Add 5 µL of each fluorochrome-conjugated antibody and gently pipette 5 times to mix.
7. Incubate for 20 min. in the dark at room temperature.
8. Add 2 mL wash buffer and centrifuge at 300 x g for 10 minutes and remove supernatant. Repeat washing for a total of 2 washes.
9. Resuspend the cell pellet in 100 µL wash buffer.
10. Proceed to analyze the samples on a flow cytometer and make sure to acquire a minimum of 50,000 to 100,000 B cells to detect a sufficient number of spike-specific B cells.

Tip:

To perform a phenotypic analysis of the spike-specific B cells, after staining the PBMCs can be subjected to a cocktail of antibodies for example against CD3, CD14, CD19, CD27, IgM, IgD, CD38, and CD138.

Flow cytometry analysis

1. Gate on the lymphocyte population in the forward scatter (FSC)/side scatter (SSC) plot.
2. Gate on singlets in the FSC-A/FSC-H and SSC-A/SSC-H plots.
3. Gate on viable CD19+ cells in the CD19/viability stain plot to identify living B cells.
4. Exclude CD3+ and CD14+ cells by gating on CD3– and CD14– cells in the CD3/CD14 plot.
5. Finally, to exclude false-positive cells, use double discrimination in a two-parameter plot with spike-Klickmer®-PE and spike-Klickmer®-APC to gate on spike-specific B cells on the diagonal of the dot plot **(Fig. 4)**.
6. Optionally, use a CD27/IgD plot to investigate the memory status of the B cells **(Fig. 5)**.

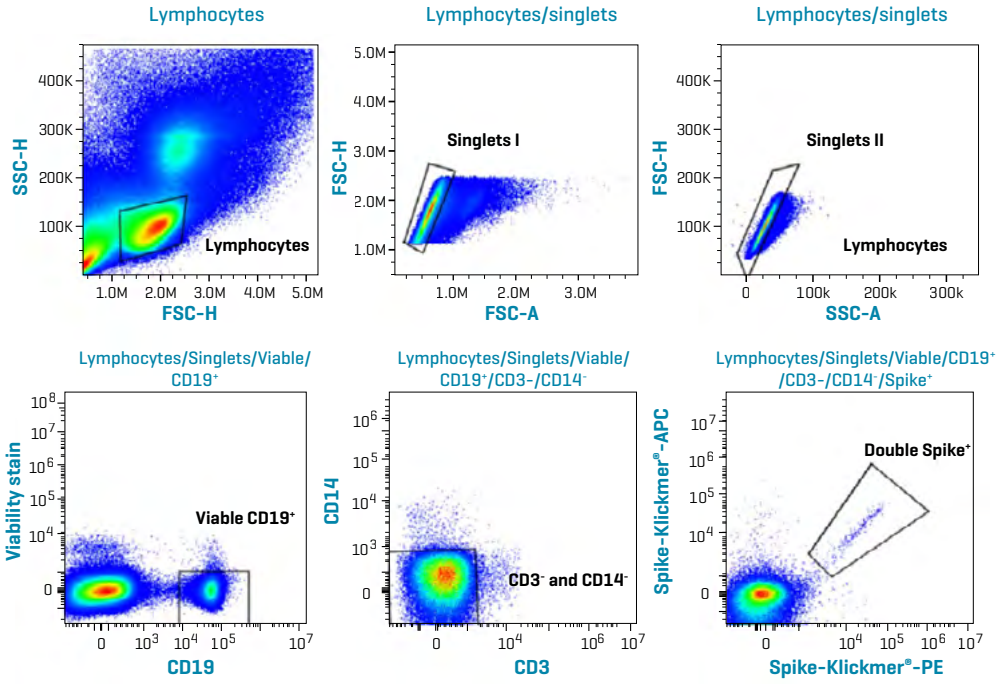


Fig. 4: Gating strategy to identify spike-specific B cells using double discrimination.

Results

A spike-specific B cell response was detected using two Klickmer® reagents with different fluorophores coupled to recombinant spike protein. The spike response was investigated in four vaccinated individuals in samples collected before and after dual vaccination.

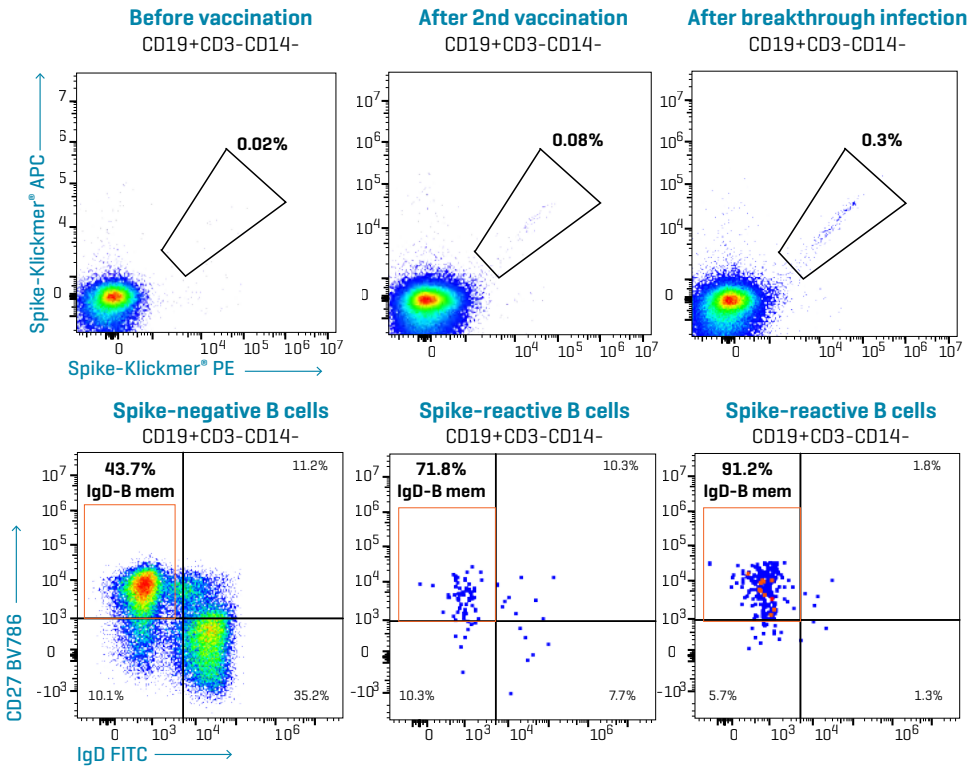
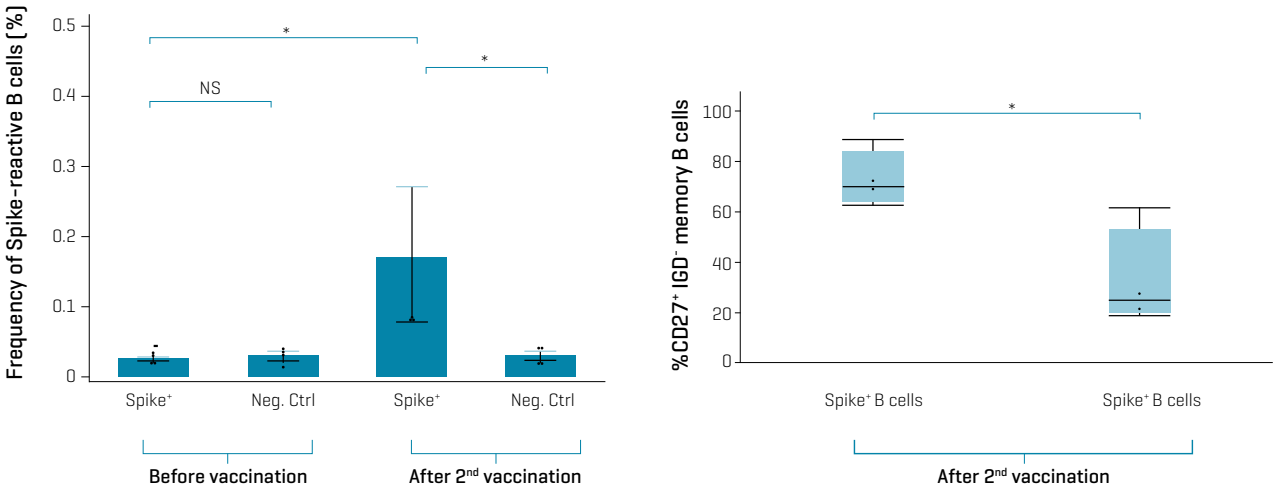


Fig. 5: Detection of spike-specific B cells in a subject at baseline, after vaccination, and after breakthrough infection using spike-Klickmer® reagents and flow cytometry analysis.



The Wilcoxon matched pairs signed-rank test was used to compare matched pairs of samples (*p≤0.05).

Fig. 6: Frequency of specific B-cell population subsets from four sample donors. Left: Comparative proportion of spike-specific B cells before and after two doses of SARS-CoV-2 vaccinations. As a reference, PBMCs were stained with a negative control Klickmer® reagent. Right: Proportion of CD27+IgD– memory B cells in the subset of spike-specific B cells compared to the total B-cell population. Matched pairs were analyzed with the non-parametric Wilcoxon matched pairs signed rank test. Significant differences ($p < 0.05$) are depicted with asterisks.

Vaccination triggered a rise in spike-specific B cells in all four sample donors. The frequency of spike-reactive B cells was significantly higher post-vaccination compared to negative controls and to pre-vaccination samples **(Fig. 6, left)**. The negative controls were PE- and APC-Klickmer coupled with a recombinant protein known to be absent in the donors. The proportion of spike-specific B cells was not significantly different to negative controls in donors prior to vaccination. The post-vaccination spike-specific B-cell population was additionally enriched with IgD^- memory B cells compared to the total population of B cells **(Fig. 6, right)**.

Interestingly, the proportion of spike-specific B cells jumped almost four-old over post-vaccination levels in the individual that experienced a breakthrough infection **(Fig. 5)**. That rise in spike-specific B cells corroborates the observation that the vaccinated immune system can mount a rapid recall response upon infection.

The memory status of the spike-specific B cells was examined by looking at CD27 and IgD staining. B cells that were reactive to the spike protein predominantly had a $CD27^+IgD^-$ class-switched memory phenotype (71.8% versus 43.7% of spike-negative B cells; **Fig. 5**). The increase in IgD^- memory B cells confirmed that the detected subpopulation was, in fact, reactive to the spike protein, and not an artifact. Additionally, the proportion of $CD27^+IgD^-$ memory B cells increased further in the subject who experienced a breakthrough infection.

Discussion

Antigen-specific memory B cells play an instrumental role in the durability of vaccine-induced immunity in the fight against SARS-CoV-2 and likely other future pathogens of concern. In this study, a spike-specific memory B-cell population subset showed a robust expansion in response to SARS-CoV-2 vaccination and likely accounted for the strong and fast recall response observed in the sample donor who experienced a breakthrough infection.

Longitudinal monitoring of antigen-specific B-cell populations is a key component in characterizing disease progression and the temporal profile of vaccine action. To that end, it will be important to use versatile and robust methods that sensitively detect the diversity of B cell subsets responsible for orchestrating plasma cells and memory-based responses to SARS-CoV-2 and its emerging variants. This study demonstrates the use of Klickmer® reagents as a sensitive tool to detect and characterize target B cell-populations.

Future perspectives

Monitoring the SARS-CoV-2-specific B-cell response is important to understand the durability of the immune response and investigate the role that memory cells play in protection against severe disease. Further studies are needed to characterize the B-cell populations involved in long-term protection against SARS-CoV-2. Such studies will include precise measurements of B cell subsets and their persistence, along with an in-depth analysis of their functional phenotype. Such longitudinal profiles can help evaluate new vaccine candidates and guide development strategies as the scientific community anticipates the emergence of variants. The Klickmer® reagents loaded with spike protein and other disease-relevant antigens will be instrumental to these investigations. Furthermore, they can be readily combined with the Immudex dCODE® technology to expand datasets with information on B cell receptor sequences and gene expression via next-generation sequencing and single-cell multi-omics.

Conclusion

- Klickmer® reagents loaded with the full-sized SARS-CoV-2 spike protein enable reliable detection of spike-specific B cells by flow cytometry without upstream stimulation, enrichment, or expansion.
- The frequency of spike-specific B cells increased significantly after vaccination and spikes prominently after breakthrough infection.
- The spike-specific B-cell population generated after a two-dose SARS-CoV-2 vaccination is enriched with $CD27^{+}IgD^{-}$ memory B cells.

Resources

Klickmer® Technology: https://www.immudex.com/products/basic-research/klickmer/

dCODE Klickmer® Technology: https://www.immudex.com/products/basic-research/dcode-dextramer-ngsmulti-omics/dcode-klickmer/

Monitoring Cellular Immunity in COVID-19: https://www.immudex.com/applications/insights/covid-19/

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