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The authors declare no competing interests.

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Response under pressure: deploying emerging technologies to understand B-cell-mediated immunity in COVID-19

Critical technological advances have enabled the rapid investigations into the immune responses elicited by SARS-CoV-2, the pathogen responsible for the COVID-19 pandemic. We discuss the cutting-edge methods used to deconvolve the B-cell responses against this virus and the impact they have had in the ongoing public health crisis.

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The COVID-19 pandemic is extracting an enormous toll on human populations worldwide. As overwhelming evidence has accumulated to indicate the participation of inflammatory and autoimmune responses in adverse outcomes, there is a major need to understand the immunological underpinnings of protective and pathogenic responses to a life-threatening virus for which there was little, if any, preceding immunological memory. The confluence of public health need, scientific opportunity and unparalleled technological and computational tools has provided a unique opportunity to understand the underpinnings and broad heterogeneity of the human immune response in general, and particularly in the context of primary immune responses. The study of B-cell responses in this context, responsible for antibody production in both vaccination and infection, has been a critical point of focus throughout the pandemic in understanding natural immunity development against SARS-CoV-2, vaccine longevity and memory durability against emergence viral variants.

The rapid development of technology around immunologic investigation generally, and B-cell response monitoring specifically, has resulted in a robust experimental toolset capable of extracting significant data down to the single-cell level (Fig. 1). The emergence of these tools, and their application to critical areas of human health such as vaccination[1], infection and autoimmunity[2], has allowed for the creation of a framework for B-cell response classification and development. Advances in surface phenotyping have led to an increased depth of B-cell subset identification and correlated function[3]. Next-generation sequencing has provided understanding of developmental B-cell programs[4], with single-cell technology promising to push those efforts even further. Broad antigen-specific screening technologies combined with robust monoclonal antibody (mAb) production pipelines have enabled us to understand emerging antigen-specific responses and rapidly evaluate potential therapeutics[5].

With the new technological advances, B-cell immunologists are now perfectly poised to rapidly understand mechanisms of viral clearance, disease pathogenesis and immune protection in both infection and vaccination. Furthermore, some of these novel tools have been successfully deployed to develop mAb therapeutics against SARS-CoV-2 in weeks rather than years[5]. The result has been an explosion of understanding around humoral immune development in human viral infection. Although certainly not a comprehensive list, it is important to document how these technologies have contributed to our collective investigations in dissecting the immune responses surrounding COVID-19.

High-dimensional cytometry

Since its inception, flow cytometry has served as a cornerstone technology in the identification and classification of leukocytes into increasingly refined subpopulations[6]. As such, its ability to provide increased breadth or depth of cellular characterization is a direct reflection of the number of cellular markers that can be simultaneously

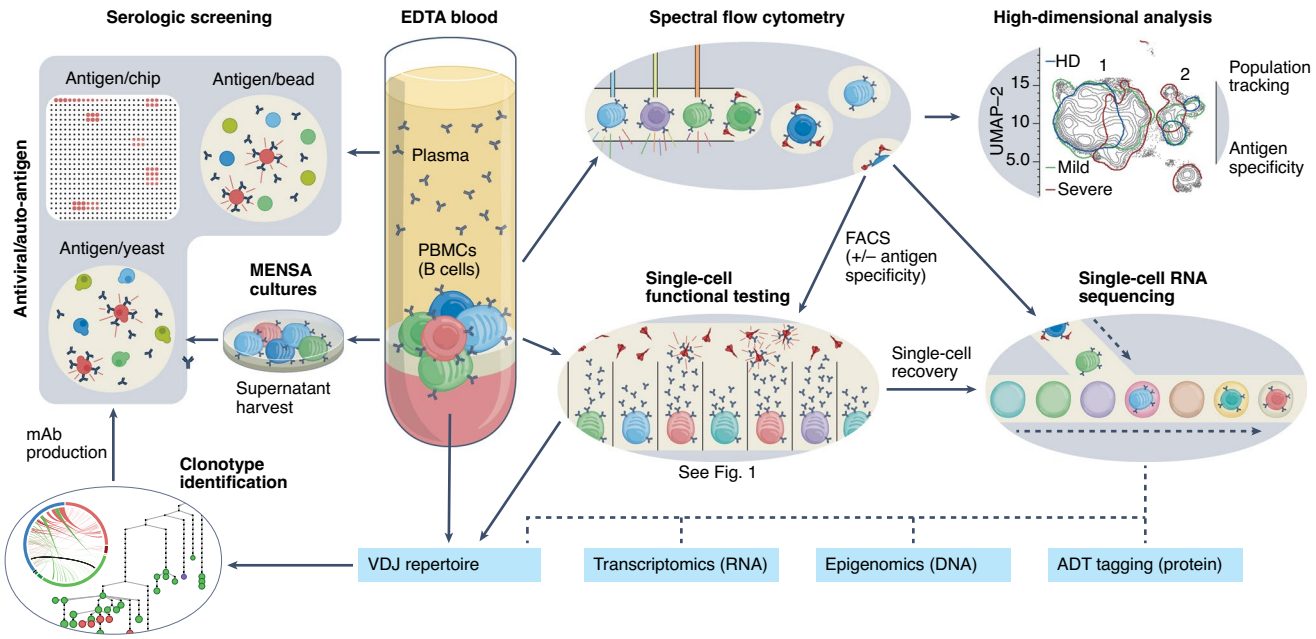

Fig. 1 | Efficient utilization of patient blood in B-cell-focused investigations of COVID-19. Routine ethylenediaminetetraacetic acid (EDTA) tube blood collection from human patients with COVID-19 can be readily processed into plasma and peripheral blood mononuclear cell (PBMC) fractions for downstream investigation. Application of high-dimensional flow cytometry panels to collected PBMCs can reveal alterations in B-cell activation pathways, antigen-specificity tracking and memory emergence and persistence, and anticipate B-cell effector functions. Cell sorting of these analysis platforms allows for single-cell functional testing of ASCs to identify clonotype specificity and neutralizing potential, and for real-time screening for therapeutic potential. Cells that are either directly sorted based on fluorescent markers or recovered from single-cell functional assays can then be shunted into single-cell sequencing applications to investigate the transcriptomic, epigenetic and repertoire features of interest from selected B-cell-derived populations. Using this multi-omics approach, single cells of interest can be identified for mAb production to screen and identify potential binding partners. Resulting mAbs, patient plasma or ex vivo ASC (MENSA) cultures can then be applied to multiplex antigen-screening tools to identify relevant viral and autoantigen reactivities across a multitude of platforms. FACS, fluorescence-activated cell sorting; UMAP, uniform manifold approximation and projection; HD, healthy donor.

and discretely identified with high confidence. In turn, the simultaneous use of multiple markers is critical to study the behavior of an increasing number of B-cell populations of different functional significance[3]. However, the foundational method for detection of those targets — immunofluorescence — makes discrete identification of high numbers of targets inherently difficult due to overlapping emission spectra of related fluorophores. Attempts to parse substantially overlapping fluorophores using traditional technology require careful signal compensation and an unavoidable loss of signal strength.

As a core methodology in immunological labs, several approaches have been tested in bypassing the traditional limitations of fluorescence-based subtractive compensation-dependent systems. Mass cytometry, where cellular markers are identified through the detection of metal-ion-tagged antibodies, rather than fluorophore-tagged, have bypassed many of these limitations and introduced the ability to measure in excess of 50 simultaneous markers[7]. This system is highly sensitive and resistant to the compensation-based errors that can result in erroneous cellular classification, but is lower throughput than its fluorescent-based counterparts and requires intensive reagent quality control. However, due its ability to reliably produce extraordinarily high-dimensional datasets, this approach continues to find increased applications in immunology, including COVID-19 studies[8].

A notable new generation of cytometers — spectral flow cytometers — have replaced traditional compensation methods in favor of spectral unmixing[9]. Widespread adoption of this technology has made access to antibody panels discerning 30 or more cellular markers relatively commonplace within large research institutions. Rapid application of spectral flow panels resulted in the broad characterization of lymphocyte responses across COVID-19 disease severity, including the identification of broad patient 'immunotypes' with potential therapeutic implications, within months of the start of the pandemic[10]. Simultaneously, deeper immune profiling of more restricted populations, such as our own study of B lymphocytes, provided fine characterizations of these populations and their relation to responses identified in other critical areas of human disease[11]. Regardless of populations of interest, the high-dimensional nature of these data and the development of approachable analysis tools have made the application of unsupervised analysis methods standard — allowing for deeper probing of cytometric data to uncover unexpected relationships between cellular markers.

Another critical use of flow cytometry is the identification of antigen-specific B cells using antigens labeled with different fluorochromes. When integrated within multi-marker panels, this approach is central for the identification of the specific source of early and late anti-COVID B-cell responses, including memory compartments generated in response to previous infection or vaccination. The enumeration of such cells in different memory compartments with different requirements for induction, maintenance and re-activation will be critical for our ability to predict, measure and manipulate the abundance of the more

desirable memory responses. A frequently ignored implication of memory studies is the possibility that different vaccines in individual subjects might induce protective B-cell responses that are not reflected in serum antibody titers due to uncoupling of the generation of memory B cells and antibody-producing plasma cells, as well as differences in the longevity of the two compartments. This scenario would advocate for the measurement of antigen-specific memory responses in addition to antibody titers to monitor vaccine efficacy and lasting immunity.

Single-cell multi-omics and the B-cell repertoire

The capabilities and value of single-cell sequencing are now well established across disciplines, with the technology used to great effect in the earliest characterizations of the inflammatory profiles that define severe COVID-19 (ref. [12]). In a rapidly developing field, current iterations of single-cell technologies enable multi-omics assessment that extends well beyond the transcriptome — making available the simultaneous evaluation of cell-surface phenotypes[13], antigen specificity[14], chromatin accessibility[15] and the adaptive immune repertoire[16]. The vast depth of information available through these combined approaches has propelled single-cell sequencing to the forefront of immunologic investigation into COVID-19 (refs. [16,17]).

However, while single-cell sequencing technology has proven its worth in a variety of applications, the unique biological properties of the B-cell receptor (BCR) make it particularly appealing for understanding the developmental pressures associated with the rapidly developing humoral immune response. As in T-cell repertoire analysis, by sequencing recombined VDJ (variable, diversity, joining) sequences at a single-cell level, clonotypes can be readily identified with repeat identification of sequences that directly indicate a lineage expansion event[18]. Using these expanded clonotypes as indicators of selection, *v*- or *j*-gene biases can be identified and characterized in the context of acute infection[18]. In addition to these simple assessments, the somatic alterations across the BCR as a result of B-cell selection, namely somatic hypermutation and class-switch recombination, provide a wealth of information beyond gene-selection asymmetries that are unavailable in other cell types. These additional parameters, when appropriately used, contribute detailed information about the developmental trajectories of individual clonotypes, positive and negative selective pressures acting on the compartment, and even the likely cytokine milieu of the microenvironment. These types of analyses, that have been used to great effect in the identification of disrupted mechanisms of tolerance in human autoimmunity[19], have now been wielded similarly in COVID-19 to begin to answer long-standing questions about the emergence of autoreactive antibody responses in primary viral infection[20].

The combination of the single-cell repertoire alongside traditional transcriptomics and antibody-derived tag (ADT)-based sequencing allows for new compiled analysis pipelines[21] — providing the potential for extraordinarily detailed developmental trajectories in primary human immunity. Although much of this work has focused on blood, the sampling of tissue outside of the blood, including fine-needle aspirates from human secondary lymphoid tissue, has increasingly allowed probing of developmental B-cell responses directly at the differentiation site[22]. These studies are exciting and feasible, and promise to close many of the gaps in current understanding of both extrafollicular and germinal center B-cell origins.

ASC functional assessment and clonotype specificity testing

A commonly identified feature of B-cell responses in severe COVID-19 has been the rapid expansion of antibody-secreting cells (ASCs — that is, plasma cells and plasmablasts) in the blood during the acute phase of infection. Despite the presumed contribution of ASCs to the early antiviral antibody response, the specificities of these cells are difficult to assess due to their downregulation of cell-surface B-cell receptors rendering traditional cytometry-based antigen specificity assays ineffective. Intracellular flow cytometry (ICS) methods of antigen-specific immunoglobulin staining have been pursued, but the need for cell fixation makes the technique incompatible with downstream antibody discovery. Historically, ELISpot (enzyme-linked immunosorbent spot) assays have been performed on these cells to identify antigen-specific antibody secretion, although these assays are low throughput, targeted at a single antigen and require significant numbers of fresh cells for each individual assay. However, a new approach has emerged to help parse the contribution of circulating ASCs to the overall antiviral response. Through short-term ASC culture and collection of media enriched with newly synthesized antibodies (MENSA), the bulk specificities of circulating ASCs enriched following infection can be readily assessed[23,24]. MENSA collection and testing offers the ability to interrogate multiple antigens simultaneously, and to assess neutralizing function to provide a snapshot of the antibodies from circulating early minted ASCs in contrast to the serum or plasma, which offers the entire immunological or microbial history. Hence, MENSA provides a unique immune snapshot of antibody specificities from the new active infection, compared to historical serum antibody measurements.

Although short-term ASC culture is informative, longer-term ex vivo study may be required for functional assessment. Despite the amenability of some lymphocyte populations to long-term culture (including memory B cells), circulating ASCs have lagged significantly due to the predisposition of these terminally differentiated B cells to die early in culture[25]. In vivo, long-lived plasma cells require specialized microniches for maturation and survival. By harnessing factors secreted by bone marrow mesenchymal stromal cells, and providing survival signals and a hypoxic environment that mimics the natural bone marrow microniche[25], we have developed a cell-free plasma cell survival system (PCSS) that allows for the survival and maturation of these temperamental cells for months[25] (Fig. 2). This system enables interrogation of intrinsic survival programs that drive ASC maturity and longevity[26,27], and may provide a useful platform for directed genetic editing of ASCs ex vivo for therapeutic use[28].

Therapeutic mAb identification

Studies of circulating ASCs generally, alongside antigen-specific memory, have been critical for discovery of functional mAbs against COVID-19 for use as therapeutics or prophylaxis. Classic approaches to single-cell screening and hybridoma creation can require years, and even high-throughput techniques such as emulsion-based single-cell methods can be challenging for differentiation of antigen binding from neutralization. Fortunately, a recent technology developed by Berkeley Lights, Inc. (BLI) offers the ability to prescreen ASCs for neutralizing potential on a single-cell basis to then capture mRNA from these cells. The BLI technology uses OptoElectroPositioning, which uses light-induced electrokinetics to position and manipulate individual cells in sub-nanoliter chambers[29] (Fig. 2). Using the PCSS to stabilize these cells, this optofluidic platform accelerated discovery of mAbs for therapeutic potential during the COVID-19 pandemic[5,30] and resulted in the highly efficacious and widely distributed

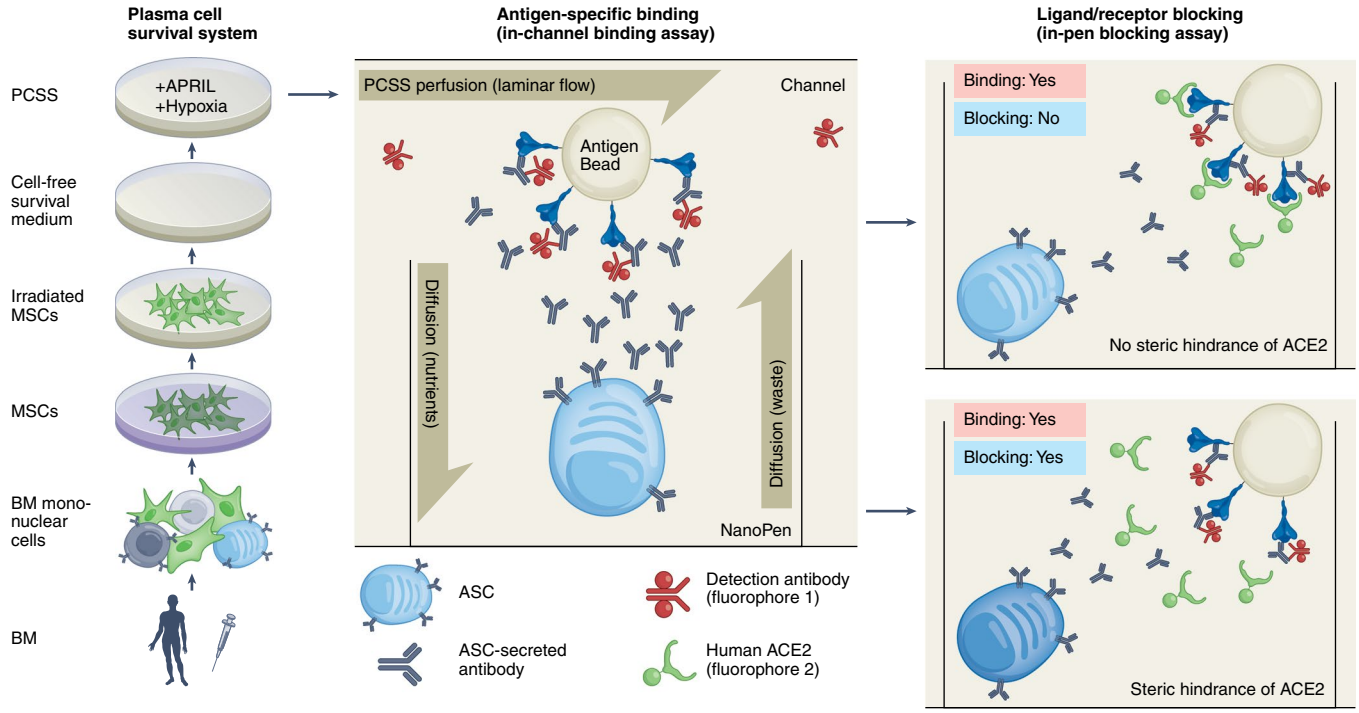

Fig. 2 | Function-based pre-selection of antigen-specific ASCs. Left: development of the PCSS. Primary mesenchymal stromal cells (MSCs) derived from human bone marrow (BM) are expanded and irradiated. The resulting culture medium is then collected and supplemented with APRIL (A Proliferation-Inducing Ligand) and hypoxic conditions to promote ASC survival. Middle: single-cell detection of antigen-specific ASCs using an in-channel binding assay. A single-assay mixture of antigen-coated beads and fluorescently labeled detection antibodies is imported into the channel above the NanoPen chambers. The PCSS is perfused through the chip to maintain survival of penned ASCs, the secreted antibodies of which diffuse into the channel and bind the beads. Accumulation of fluorescence on the beads leads to the development of fluorescent halos (blooms) in the channel adjacent to the pens that contain antigen-specific ASCs. Right: single-cell functional screening for antigen-specific ASCs using an in-pen receptor-blocking assay. After positioning antigen-coated beads into pens and saturating the antigens, an assay mixture of fluorescent receptor (angiotensin-converting enzyme 2 (ACE2)) and fluorescently labeled detection antibodies targeting antigen-specific receptor-binding domain (RBD)-binding antibodies are imported into the channel and allowed to diffuse into pens. The PCSS is perfused through the chip to maintain survival of penned ASCs. The upper panel shows that RBD-binding ACE2-non-blocking antibodies result in accumulation and detection of both fluorophores; the lower panel shows that ACE2-blocking antibodies exhibit signals for RBD-binding antibodies only. ASCs secreting blocking antibodies are then exported from specific pens for downstream interrogation.

mAb therapy developed for pre-exposure prophylaxis[5,31].

A unique advantage of the BLI system is the feasibility and implementation of ASC functional readouts — that is, neutralization potency and receptor blockade[5] (Fig. 2) — which offers increased efficiency and rapid scalability. Emulsion-based single-cell methods do not allow for direct interrogation of cells or a secreted product in supernatants, and while other target-based screening microtools allow for direct single-cell manipulation and recovery of living cells (such as microcapillary arrays[32], microfluidic chambers[33] and micro-engraved/microwell-array systems[34]), they have not been shown to accommodate a neutralization assay. Beyond the identification of therapeutics, these novel approaches will allow for continued probing of ASC biology, including the potential for proliferation and secretory dynamics. This platform has already yielded results for COVID-19, allowing for the generation of monoclonal and bi-specific antibodies that are effective against spike mutations associated with emerging viral variants[5,30,31].

Whether ASCs are identified and collected through function-based assays or more generic single-cell VDJ sequencing, the paired identification of intact heavy- and light-chain sequences from quality-controlled samples provides an opportunity to directly evaluate antigen specificities of identified clonotypes of interest. Combined with increasingly mechanized gene synthesis and custom antibody production[5], the field inches closer to the long-pursued possibility of deeply screening a donors' circulating BCR for specificity within the context of identified cell-surface and transcriptomic features. Although still costly, this single-cell-to-antibody pipeline is increasingly used to identify functional antigen specificities within unique B-cell populations that are emerging in COVID-19 and characterized directly through single-cell phenotyping[20].

Antibody screening

Taking advantage of these single-cell approaches, and the more generalized need for antigen specificity screening at the serological level, COVID-19 has brought focus on a myriad of high-throughput technologies that aim to provide a detailed understanding of broad antibody targeting. Due to its high-throughput and easily customizable antigen sets, Luminex-based fluorescent and Meso Scale platforms have become invaluable tools for antiviral specificity assessment across comprehensive sets of SARS-CoV-2-based antigens[11,35–37]. Similarly, targeted systems have been developed through both chip-based and flow-based technologies, with multiplex identification of COVID-19 antigen specificity emerging as a cornerstone

approach to serological assessment throughout the pandemic.

In addition to antiviral reactivity, a surprising connection between the B-cell responses present in severe COVID-19 and those identified in patients with active autoimmune disorders has driven a need for comprehensive assessments of both antiviral and self-targeted antibodies[11]. With no clear standard approach for these screening strategies, groups have relied on commercially available protein arrays that reflect the entire human proteome to identify self-targeted antibody responses in COVID-19 (ref. [31]), phage-display-based screening of antiviral targeting[32], or complex combinations of in-house-validated bead-based platforms to synthesize antiviral and anti-self-targeting into a comprehensive strategy[33]. A notable emerging technology, the REAP system, offers the potential to screen an expansive selection of the exoproteome for autoreactivity, and has been used to great effect in the identification of autoreactivity in COVID-19 (ref. [38]). Commercial avenues for large-scale autoreactivity testing have also emerged as widely used technologies, despite high expense and the need for a fair amount of individual target validation[39]. Importantly, although these tools have application in identifying broad autoreactivity against a variety of targets, existing US Food and Drug Administration (FDA)-approved autoantibody testing pipelines that are routinely employed in healthcare settings should be considered as established methods for identification of clinically relevant autoreactive features of severe infectious disease[20].

Conclusions

The questions informed by these various technologies have played a critical role in the extraordinarily public investigation of COVID-19. High-dimensional cytometry, paired with single-cell multi-omics and antibody repertoire analysis, has helped reveal the intensity, quality and even pathological potential of the B-cell compartment in severe COVID-19. Multiplex antibody screening has yielded critical information about the duration of responses resulting from both infection and vaccination, and their durability in the face of emerging SARS-CoV-2 variants. Single-cell functional studies have allowed for the rapid identification of therapeutic mAbs, which have been deployed to great effect throughout the pandemic, and may reveal therapeutic targets to intervene in misdirected humoral immune targeting. Together, these technologies have allowed the B-cell field to reliably address some of the most rapidly evolving and important questions emerging throughout the pandemic — informing not only scientific understanding, but also human health and public policy.

However, in addition to technological advance, the rapid pace of discovery since November 2019 is a direct reflection of the willingness of the immunological community to alter research trajectories toward unfamiliar and often dangerous work in support of the common good. It is also a testament to the value of continuing technological development aimed at basic science discovery in human immunology. Through targeted funding opportunities and the creation of critical national research consortia (SeroNet, PRISM and RECOVER, among others), laboratories at the forefront of the investigation of B-cell biology were able to deploy new technology on an emerging global threat at an unprecedented pace. Now firmly embedded in the COVID-19 literature, these technologies will serve as a benchmark for future investigations and applications — driving critical understanding of developing humoral immunity and B-cell effector responses even beyond the realm of infectious disease.

Despite the unprecedented pace of developments enabled by the confluence of technological and scientific prowess, there remains a need for additional advances in our ability to interrogate the B-cell and antibody immune response to SARS-CoV-2 infection and vaccination. Areas of particular need include extensive antigen platforms for the analysis of autoreactivity of serum and mAbs. Although such platforms currently exist, there is a need for robust and affordable screening systems that, in turn, should be subjected to cross-validation. Similarly, it will be important to continue to develop methods for high-throughput identification of antiviral and autoreactive ASCs. These capabilities would enable investigators to readily determine the protective and pathogenic potential of serum antibodies that are generated in response to vaccination, and of mAbs considered as candidates for passive prevention and treatment of COVID-19 infection. In all, a final goal should be the generation of B-cell and antibody signatures that are associated with favorable responses to infection and vaccination and, conversely, capable of predicting acute and long-term autoimmune complications. ❐

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Competing interests

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