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T cell immunity is key to the pandemic endgame: How to measure and monitor it

Megan Schwarz [a,b], Slim Mzoughi [a,b], Daniel Lozano-Ojalvo [c], Anthony T. Tan [d], Antonio Bertoletti [d,**], Ernesto Guccione [a,b,e,*]

[a] Department of Oncological Sciences, Tisch Cancer Institute, Icahn School of Medicine at Mount Sinai, NY, USA
[b] Center for Therapeutics Discovery, Department of Oncological Sciences and Pharmacological Sciences, Tisch Cancer Institute, Icahn School of Medicine at Mount Sinai, New York, USA
[c] Precision Immunology Institute, Icahn School of Medicine at Mount Sinai, NY, USA
[d] Programme in Emerging Infectious Diseases, Duke-NUS Medical School, Singapore
[e] Bioinformatics for Next Generation Sequencing (BiNGS) Shared Resource Facility, Icahn School of Medicine at Mount Sinai, New York, NY, USA

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ABSTRACT

As vaccine deployment improves the healthcare emergency status caused by the SARS-CoV-2 pandemic, we need reliable tools to evaluate the duration of protective immunity at a global scale. Seminal studies have demonstrated that while neutralizing antibodies can protect us from viral infection, T cell-mediated cellular immunity provides long-term protection from severe COVID-19, even in the case of emerging new variants of concern (VOC). Indeed, the emergence of VOCs, able to substantially escape antibodies generated by current vaccines, has made the analysis of correlates of humoral protection against infection obsolete. The focus should now shift towards immunological correlates of protection from disease based on quantification of cellular immunity.

Despite this evidence, an assessment of T cell responses is still overlooked. This is largely due to technical challenges and lack of validated diagnostic tests. Here, we review the current state of the art of available tests to distinguish between SARS-CoV-2 antigen-specific Tcells and non-antigen specific T-cells. These assays range from the analysis of the T cell-receptor (TCR) diversity (i.e. Immunoseq and MHC tetramer staining) to the detection of functional T cell activation (i.e. ICS, AIM, Elispot, ELLA, dqTACT, etc.) either from purified Peripheral Blood Mononuclear Cells (PBMCs) or whole blood.

We discuss advantages and disadvantages of each assay, proposing their ideal use for different scopes. Finally, we argue how it is paramount to deploy cheap, standardized, and scalable assays to measure T cell functionality to fill this critical diagnostic gap and manage these next years of the pandemic.

1. Introduction

Since early 2020, the world has been devastated by a pandemic caused by SARS-CoV-2, the etiological agent of COVID-19. As of June 2022, there have been over 530 million cases and 6 million deaths worldwide (https://coronavirus.jhu.edu/map.html). After two years, the world is still facing the pandemic's far-reaching health, economic, and societal implications. Massive resources have been allocated to speed up the implementation and deployment of diagnostics and vaccinations strategies, ranging from rapid qPCR-based assays to detect viral RNA, to serology tests and innovative mRNA-based vaccines.

Since August 2020, the Center for Disease Control and Prevention (CDC) has used seroprevalence to first estimate the percentage of infected or convalescent individuals and once vaccine rollout began, to track population immunity.

Unlike streamlined antibody assays, there are no standardized methods to track cellular immunity, which is a critical aspect of population surveillance. There are, nonetheless, a plethora of suitable assays that could be used. The fact that these assays are not being used for population level analyses is due to their higher technical difficulty and to the initial underappreciation by the research community and public institutions of the essential role T cell immunity will play in providing long lasting protection from severe COVID-19.

* Corresponding author. Department of Oncological Sciences, Tisch Cancer Institute, Icahn School of Medicine at Mount Sinai, NY, USA.
** Corresponding author.
E-mail addresses: antonio@duke-nus.edu.sg (A. Bertoletti), ernesto.guccione@mssm.edu (E. Guccione).

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Abbreviations

VOC	Variants of Concern
TCR	T cell-receptor
ICS	Intracellular Cytokine Staining
AIM	Activation-Induced Markers
ELISpot	Enzyme-Linked Immune Absorbent Spot assay
ELISA	Enzyme-Linked Immunosorbent assay
qTACT	Quantitative PCR T cell activation assay
dqTACT	Direct Quantitative PCR T cell activation assay
TACTseq	T cell activation-sequencing assay
MHC	Major Histocompatibility Complex
PBMCs	Peripheral Blood Mononuclear Cells
CDC	Center for Disease Control and Prevention
APCs	antigen presenting cells
O/N	Overnight

2. The two arms of the adaptive immune system

The adaptive immune response consists of two complementary branches: humoral (antibody mediated) and cellular (T cell mediated) immunity. Antibodies are generated by B cells that recognize pathogenic agents in the blood and respond via: neutralization, antibody-mediated phagocytosis, antibody-mediated cellular cytotoxicity, and opsonization. While B cells are largely restricted to recognizing surface antigens (e.g. viral spike proteins), once inside cells, pathogens are no longer accessible to antibodies. T cells, through their diverse set of receptors (TCR), have the advantage of recognizing a wider range of epitopes displayed by infected or antigen presenting cells (APCs) onto either an MHC class I or II surface protein.

In the context of SARS-CoV-2 infection, while antibodies can protect us from infection, T cells can prevent severe disease by eliminating infected cells (Fig. 1).

In the general, healthy population, there is a correlation between cellular and humoral immunity (Ni et al., 2020), likely attributable to a robust $CD4^+$ T cell response, which promotes antibody production (Goel et al., 2021). However, several studies have reported a rapid (1–3 months post infection or vaccination) decrease in antibody titers and neutralizing capacity, particularly against variants of concern (VOCs), while T cells remain largely functional in recognizing viral-infected cells for longer (>9 months) (Bonifacius et al., 2021; Le Bert et al., 2021).

Of clinical relevance, individuals receiving B cell-depleting therapy, or who suffer from hematologic malignancies, fail to mount a humoral immune response following vaccination, and rely solely on T cell immunity for protection (Aleman et al., 2021; Bange et al., 2021; Oh et al., 2022).

In a recent review we have discussed the relevance of SARS-CoV-2 T cells' early temporal appearance, multi-specificity, and functionality for accelerated viral clearance and their correlation with protection from severe disease (Bertoletti et al., 2021). As we navigate these later stages of the pandemic, it will be crucial to monitor cellular immunity at a population level. As such, we need reliable and scalable methods to distinguish between antigen-specific and non-antigen specific T cells, based on: i) detection of SARS-CoV-2 specific T cells through analysis of the TCR repertoire (i.e. TCR sequencing and use of MHC-tetramers) (Fig. 2A); and ii) detection of functional T cell activation (i.e. based on *ex vivo* stimulation with viral peptides and downstream analysis)(Fig. 2B). Unlike humoral immunity, which has a simple and clear means of measurement (antibody quantification), the complex nature of cellular immunity does not lend itself to a single test for its quantification. Historically, a handful of assays have been used in clinical trials and vaccine development, including the enzyme-linked immune absorbent spot (ELISpot) assay, the enzyme-linked immunosorbent (ELISA) assay, and flow cytometry. Over the years, these protocols have been enhanced, automated, and multiplexed to answer more nuanced immunophenotyping questions. The starting material and quantification methods of these assays also vary, such that each comes with advantages and limitations, which will be described and compared in this review.

3. Assays to evaluate TCR diversity

These assays rely on the detection of SARS-2 specific T cells by characterizing the TCR diversity of each individual, without assessing their functionality.

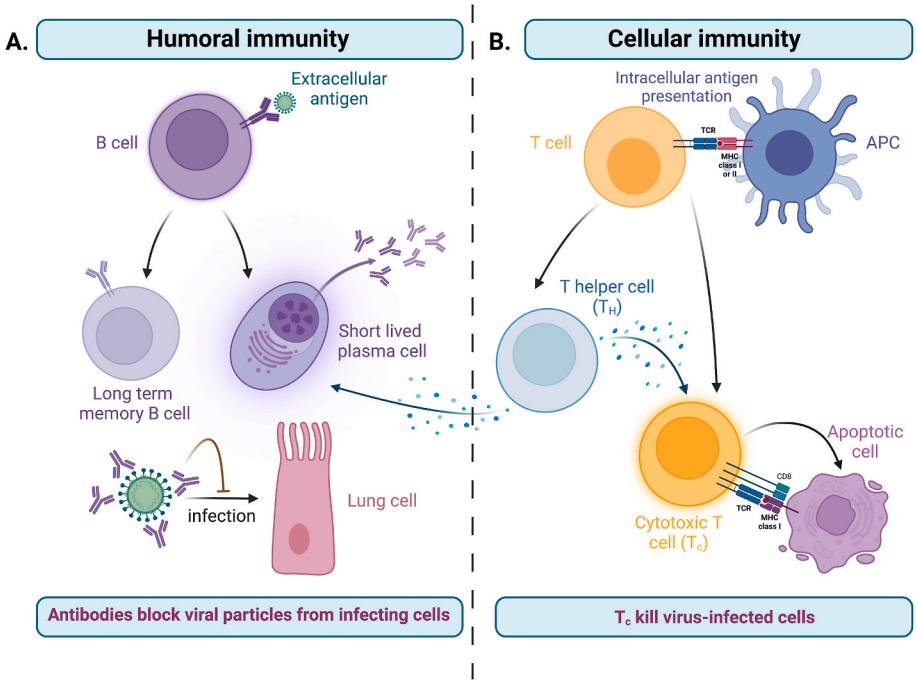

Fig. 1. The two arms of the adaptive immune response. A. The humoral response is initiated by the interaction of B cells with external antigens (e.g. viral particles). Antigen-specific B cells expand and differentiate into short-lived plasma cells that secrete antibodies, or long-term memory B cells that remain dormant until a secondary challenge event (i.e. re-infection). Antibodies coat viral particles and prevent their entry into cells, thus decreasing the risk of infection. **B.** The cellular immune is initiated when antigen-specific T cells interact with antigen that has been internalized (either by engulfment or infection) by APCs and presented on surface receptors. Once activated, T cells can differentiate into cytotoxic CD8 T cells that kill infected cells, or CD4 T helper cells that support and augment both the humoral and cellular immune responses (typically via secretion of cytokines).

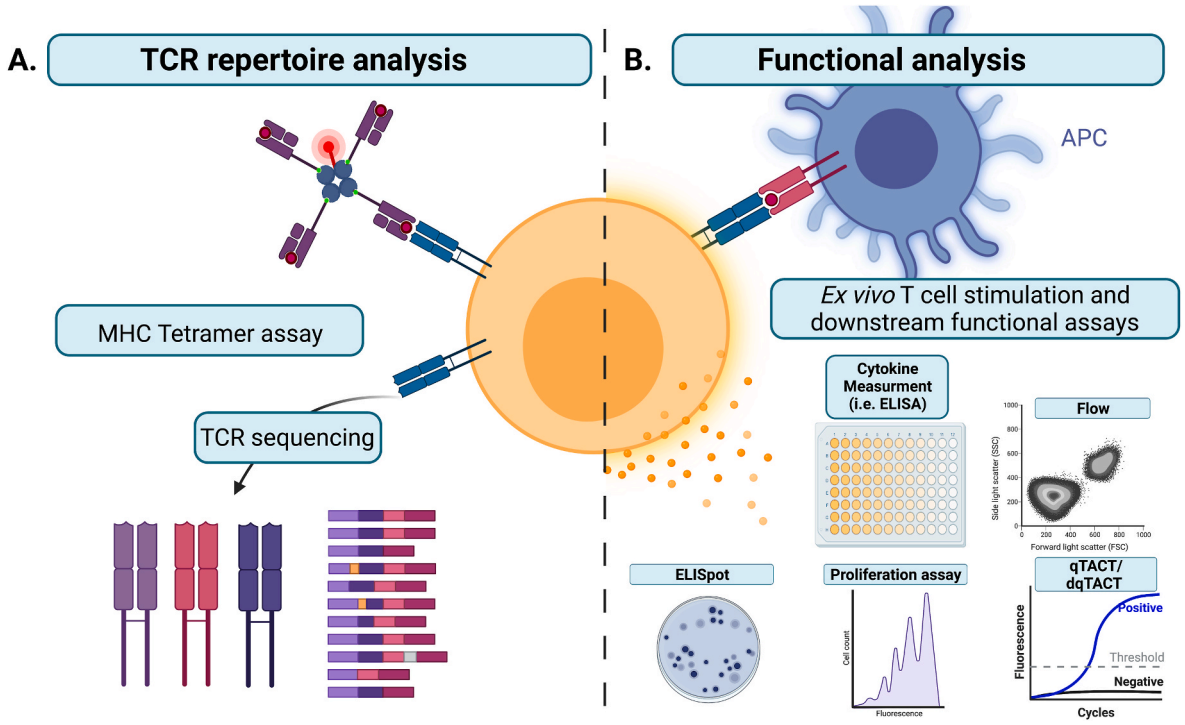

Fig. 2. Two approaches to quantifying cellular immunity. A. TCR repertoire analysis involves quantification of the number of T cells that recognize specific epitopes. MHC tetramer assays use fluorescently labeled, synthesized MHC-peptide complexes to bind antigen-specific TCRs. The bound TCRs can then be quantified and characterized by flow cytometry due to the fluorescent label. TCR sequencing allows for the profiling of an individual's entire T cell repertoire. **B.** Functional analyses require the activation of antigen-specific T cells (via peptides or proteins) and downstream quantification (either of activated T cells or byproducts of activation (e.g. cytokines)).

(i) Peptide-MHC tetramers

This technology is based on the ability of fluorescently labeled MHC-peptide (T cell epitope) tetrameric complexes (MHC tetramers) to bind TCRs located on the surface of antigen-specific T cells, which allows their direct visualization, quantification, and characterization by flow cytometry (Fig. 2A). At the beginning of the pandemic, the identification of SARS-CoV-2 epitopes for T cells was limited to the use of overlapping peptide pools predicted to bind common HLA-I and HLA-II. However, the early development of peptide-MHC multimers to detect SARS-CoV-2-specific $CD8^+$ and $CD4^+$ T cells during the pandemic provided an essential tool for the *ex vivo* study of TCR diversity not only during natural infection but also after vaccination. $CD8^+$ and $CD4^+$ T cell epitopes restricted by common human HLA class I (e.g., A1/ORF1a1637, A2/S269, A3/N361, A24/S1208, B7/N105, and B40/N322) and class II (e.g., DRB1*15:01S870-878 and DPB1*04/S167-180), respectively, led to insights into T cell immunity kinetics, magnitude, and immunodominance (Kedzierska and Thomas, 2022). The main limitations of this technology lie in the fact that MHC tetramers depend on the individual HLA genotype and are designed for specific peptide sequences (identified epitopes), which restricts the discovery of unknown epitopes.

(ii) Next generation sequencing (NGS) has been adapted to cellular immunity research with the arrival of the COVID-19 pandemic, which led to the development of the T-Detect COVID Test by Adaptive Technologies and Microsoft (McCarthy, 2021). Researchers sequenced the *TCRbeta* gene from healthy and infected subjects to derive a list of SARS-CoV-2 specific TCR sequences that are enriched in seropositive specimens (Fig. 2A). The assay is being used for two clinical trials (NCT04583982; NCT04494893). This NGS-based test provides information on the SARS-CoV-2 TCR repertoire without assessing function. From a technical standpoint, the protocol is lengthy and requires specific expertise and equipment. After blood is collected, samples must be shipped to a centralized lab, either fresh or frozen samples. Processing is arduous (Fig. 3A) and involves genomic DNA extraction and library preparation for NGS despite a simple sample collection process.

4. Assays to evaluate functional antigen-specific T cells

These assays rely on the characterization of the functional activation of antigen-specific T-cells (Fig. 2B). At the core of all these assays is the secretion of specific cytokines (e.g. IFN-γ), by *ex vivo* activated T cells. More specifically, 8-15mer peptides from various SARS-CoV-2 proteins can be incubated either with purified PBMCs (Fig. 4) or directly with blood (Fig. 5), to induce the downstream activation of antigen-specific T- cells that can be quantified and characterized to different degrees.

(i) The enzyme-linked immune absorbent spot (ELISpot) assay is the "gold standard" in the field of functional quantification of antigen-specific T cells. The ELISpot is designed to quantify antigen-specific T cells at single cell resolution(Cox et al., 2006; Czerkinsky et al., 1983). Stimulated cells are incubated on a plate coated with immobilized anti-cytokine antibodies (e.g. anti–IFN–γ). Cells that specifically recognize the antigen secrete cytokines that are locally captured by coating antibodies and can then be detected by incubation with a secondary, biotinylated antibody. Individually bound spots, each corresponding to a single antigen-specific T cell, are visualized by a colorimetric assay. A variation on the assay, Fluorospot, (Gazagne et al., 2003), leverages the use of differentially labeled fluorescent antibodies to detect more specific subsets of T cells that express multiple cytokines (Janetzki et al., 2014) (Fig. 4).

ELISpot advantages include high sensitivity and robust, highly reproducible data, which has been used in clinical trials (Lehmann and Zhang, 2012). Oxford Immunotec has commercialized an FDA-approved ELISpot assay to detect SARS-CoV-2 cellular immunity for diagnostic purposes (T.SPOT COVID)(Kruse et al., 2021). The main advantages of the ELISpot include its single cell resolution/accuracy, fast turnaround time (24 h), ability to be performed in a centralized location (PBMCs can

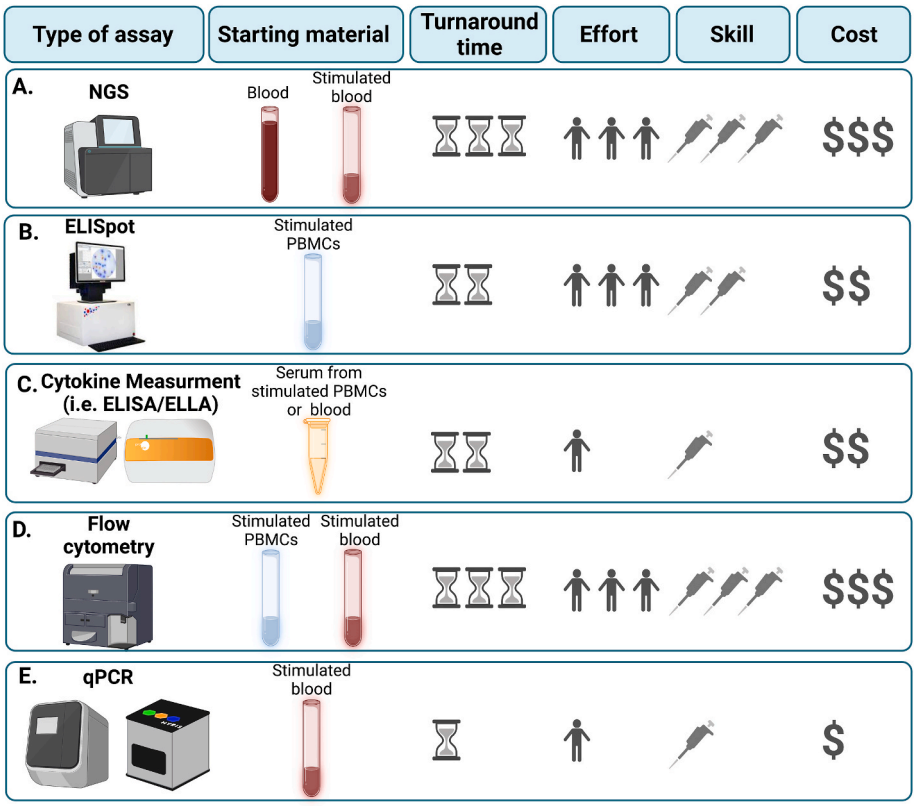

Fig. 3. Comparison of various cellular immunity assays. Each assay is compared in terms of their required starting material, turnaround time, effort needed in terms time and skill-level or personnel involved, and finally cost. **A.** Next Generation Sequencing; **B.** ELISpot; **C.** ELISA/ELLA; **D.** Flow Cytometry; **E.** qPCR-based assays (i.e. qTACT/dqTACT).

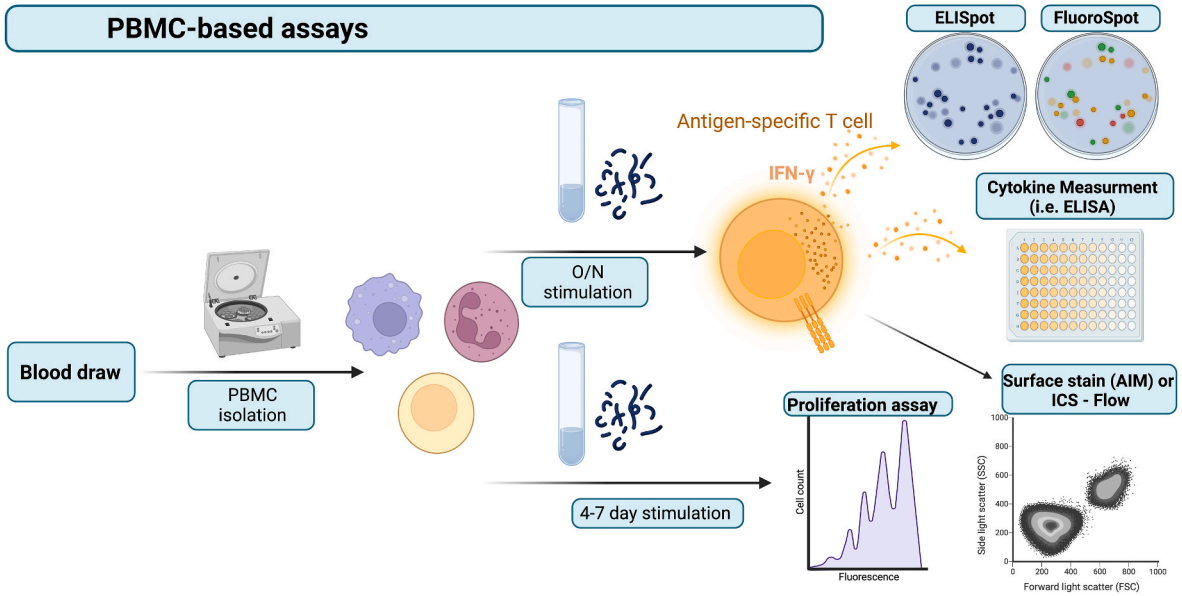

Fig. 4. Overview of functional analyses starting with PBMCs After blood is drawn, PBMCs are isolated. Once isolated, PBMCs are stimulated with a peptide pool overnight. Activated T cells can be directly quantified by ELISpot/Fluorospot and ELISA/ELLA assays, ICS, and surface staining (e.g. AIM). If cells are stimulated for longer periods of time (4–7) days and stained with a cell trace dye, a proliferation assay can be performed.

be cryopreserved and shipped), and cost (i.e. no expensive equipment/reagents required). The main disadvantages include the need for PBMC isolation, which introduces a technically challenging isolation step, and the risk of inefficient and unstandardized freeze/thawing procedures (Fig. 3B). Indeed, PBMCs are susceptible to changes in viability and functional responsiveness after cryopreservation (Mallone et al., 2011).

(ii) The Enzyme-Linked ImmunoSorbent Assay (ELISA) relies on plates coated with "capture" antibodies that bind to specific cytokines and quantify their concentration in sera of infected patients or in

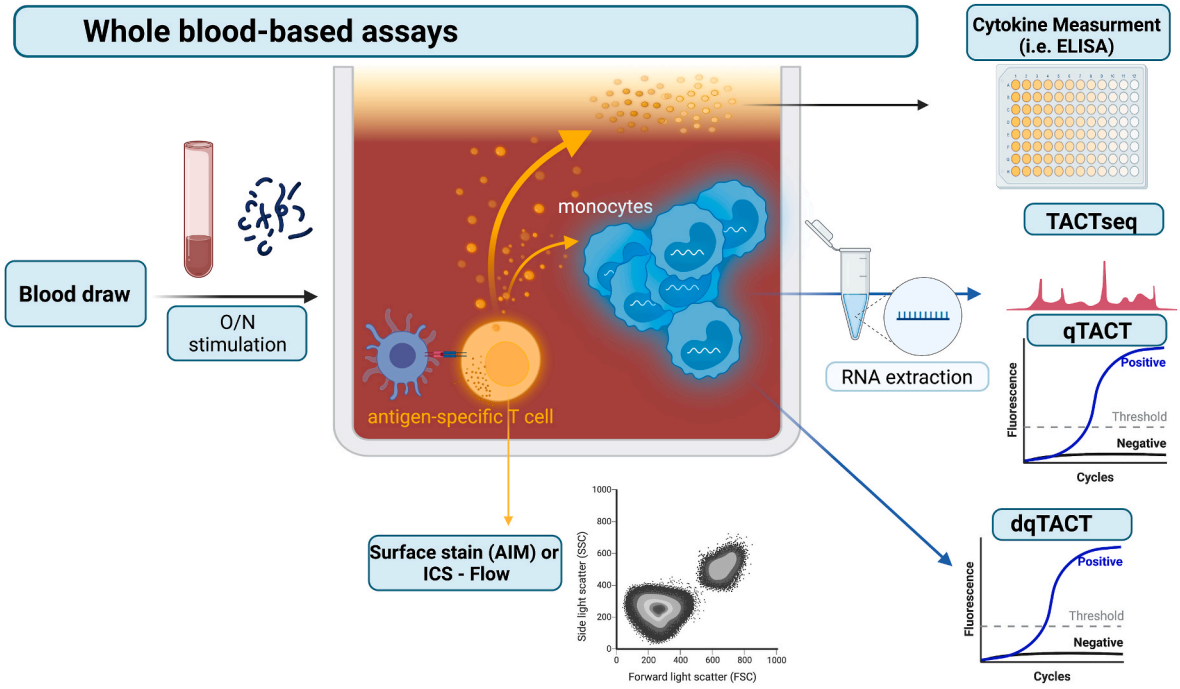

Fig. 5. Overview of functional analyses starting with whole blood. Freshly drawn blood is stimulated with a peptide pool overnight. During this time, peptides are processed and presented to T cells by APCs. Antigen-specific T cells are activated and produce cytokines/chemokines (e.g. IFN-γ), which, in turn, stimulates other cells in the blood (i.e. monocytes) that upregulate *CXCL10* mRNA expression. ELISA/ELLA assays quantify extracellular cytokines in the supernatant of the blood. Flow cytometry can be used to quantify upregulated T cell surface receptors (e.g. AIM) as well as intracellular cytokines (ICS). Whole blood total RNA can be extracted and used for TACTseq or qTACT. Alternatively, RNA extraction can be avoided and the whole blood used directly in the dqTACT assay.

supernatants from blood/PBMCs stimulated with SARS-CoV-2 peptides (Figs. 4–5). ELISAs are simple to implement and are available as commercial kits with clear instructions and reagents. Qiagen released an ELISA specifically geared to COVID-19 research (Quantiferon SARS-CoV-2), thus streamlining the use of this assay. ELISAs require minimal equipment - i.e. a plate reader to interpret their colorimetric output, which is converted to cytokine concentration by means of a serially diluted standard curve of known concentrations. An additional advantage of this assay is that, after whole blood stimulation, serum samples can be frozen and stored for later use, or kept for retrospective studies. Disadvantages include a relatively low sensitivity and specificity compared to other techniques (see below), and the inability to reliably cross compare data among different detection platforms.

The advent of multiplexed ELISAs allowed for the detection of multiple cytokines from a single sample, providing a more comprehensive view of T cell responses to antigen stimulation. ProteinSimple commercialized an upgraded version of the protocol, called ELLA, which uses pre-loaded microfluidics cartridges to which samples are added and then loaded onto an automated machine(Aldo et al., 2016). Unlike an ELISA, which requires manual plate loading, washing, and long (i.e. overnight) antibody conjugation, the ELLA requires only 10–15 min of sample preparation followed by a 90-min automated run time. Though ELLAs are a less labor and time intensive assay, and allow for reliable cross-comparison of data, they are significantly more expensive and require a specific machine to run, thus making them less widespread (Fig. 3C).

(iii) Flow cytometry and its spinoffs.

The ELISA and ELISpot assays are simple to perform, but also yield only basic results. In contrast to this is flow cytometry, which is used for extensive immunophenotyping. Despite the many variations on this assay, at its core, flow cytometry characterizes individual cells based on proliferation and surface or intracellular markers that are stained by antibodies. These experiments can be performed on PBMCs or whole blood (Fig. 4–5). The most common iterations are surface marker and intracellular cytokine staining (ICS), which characterize antigen-specific T cells based on extra- and intracellular proteins, respectively. Unlike ELISAs, where total cytokine concentration is quantified, flow cytometry can pinpoint the exact type of cell(s) secreting certain molecules, offering exceptional resolution. When differential cytokine expression is minimal, the activation-induced markers (AIM) assay offers a reliable means of quantifying antigen-specific cells, based on experimentally determined proteins that are upregulated in response to antigen recognition (Dan et al., 2016).

Flow cytometry analysis has been used extensively in COVID-19 research to understand what subsets of T cells (and other immune cell types) are up or downregulated and to perform functional analyses (e.g. T cell exhaustion). AIM, in addition, does not rely on prior knowledge of the epitope, HLA type, or cytokines to be analyzed. It is evident that a huge breadth of knowledge can be gained from this technique, however, with such complexity comes limitations. Flow cytometry requires high levels of skill/expertise, planning, effort, and cost (both from reagents and equipment) (Fig. 3D). As a result, a limited number of samples can be studied, preventing its use as an effective population surveillance method. Thus, flow cytometry is ideal for low throughput, research-oriented studies with the goal of asking specific biological questions that can eventually inform diagnostics and patient care.

(iv) NGS-based assays

We have recently developed an NGS-based assay to profile functional T cell activation (Schwarz et al., 2022). For this assay, named T cell activation-sequencing (TACTseq), we incubated peptides from SARS-CoV-2 spike or control DMSO in whole blood, from which RNA was extracted. The RNA was used for library preparation and sequencing. Our group used a cohort consisting of naïve (unvaccinated and uninfected) controls and individuals who had either been infected

with SARS-CoV-2 or had received two doses of an mRNA vaccine, to profile differentially expressed genes (Fig. 5). The advantage of this approach, which could be further implemented by a targeted amplification panel of 15–20 genes, is the possibility of capturing the variability of the response and measure cytokines produced by both T-cells and other myeloid cells in the blood. The cons are a longer turnaround time, a higher cost, and the need for skilled technical personnel.

(v) PCR-based assays

Given that TACTseq could not realistically be used for high throughput analyses, we focused on developing a probe-based qPCR assay (dqTACT, or direct and quantitative TACT) based on our RNA sequencing results (Schwarz et al., 2022). We identified *CXCL10* as the most robustly differentially upregulated gene in individuals who had been infected or vaccinated and developed a qPCR-based assay to detect it as a proxy for cellular immunity. Our focus was to design an assay that required limited benchtop experience, materials, and downstream analysis to reduce human error and increase accessibility. Whole blood is stimulated *ex vivo* with a negative control or a pool of SARS-CoV-2 peptides overnight. The next day, the blood is diluted and either frozen or used as input for a *CXCL10* probe-based qPCR reaction that includes a reverse transcription step. The relative *CXCL10* level, which is calculated by subtracting the background, is compared to an empirically calculated threshold value to determine whether T-cells responded (Schwarz et al., 2022) (Fig. 5).

When we set out to develop a novel T cell test, our goal was to address the main concerns with traditionally used assays, such as ELISpot and ELISA. For one thing, we eliminated the need for downstream blood processing, such as PBMC isolation, and adapted to using whole blood directly, which yields more reproducible results(Duffy et al., 2017). Originally, our protocol involved RNA isolation (qTACT, quantitative TACT)(Fig. 5), however, we were determined to eliminate this step (Schwarz et al., 2022). Overall, the protocol does not require much labor, unlike ELISAs and, at times, ELISpots. Furthermore, the only specialized equipment required is a qPCR machine, which are commonly used. Like an ELISA, no subjective interpretation of the data is necessary. Another benefit to this test is that it only requires a small volume of blood (<1 mL), enabling its use in subjects unable to have extensive volumes drawn (e.g. infants and children). Contrarily, PBMC isolation optimally requires 10–30 mL of blood.

In terms of scalability, our group has demonstrated the feasibility of conducting this assay at multiple sites on hundreds of individuals. In doing so, we are also gathering data on its reproducibility. Unlike any assay discussed thus far, dqTACT has an internal control (amplification of the *ACTIN* housekeeper), for internal normalization and quality check of the run and the sample (i.e. if cells are dead or degraded even *ACTIN* will be poorly amplified) (Schwarz et al., 2022).

As with all other tests, dqTACT does come with limitations. Primarily, it does not directly quantify the number of antigen-specific T cells, but rather their presence or absence. Further research is required to deconvolute the relationship between the magnitude of *CXCL10* expression and T cell number. Next, though the test offers a rapid turnaround time (<24 h), whole blood must be processed within 10–12 h after being drawn to avoid unspecific background and it cannot be frozen in the meantime. Once the overnight stimulation is complete, the samples can be frozen and do tolerate 3–5 freeze/thaw cycles without altering the results. Finally, the dqTACT test lacks the granularity of flow cytometry, offering no information on the characteristics of the antigen-specific T-cells (Schwarz et al., 2022). Overall, we think our assay is a promising alternative to ELISAs and ELISpots with the added advantages of additional simplicity, reduced need for skills or knowledge, and being internally controlled (Fig. 3E). One can imagine using this test for population surveillance, similar to the CDC's seroprevalence program.

5. Conclusions & future perspectives

The purpose of this review was to evaluate the assays being used to investigate cellular immunity during the COVID-19 pandemic. Unlike serological testing, the complexity of cellular immunity does not lend itself to a "one size fits all" test. The best assay is the one that addresses the questions being posed. The simplicity of tests such as ELISpot, ELISA, and ELLA assays allows for rapid, scalable, and minimally labor-intensive answers with limited phenotypic characterization. Flow cytometry is the ideal choice for complex questions about the type of quantity of T cell subsets, however, its high barriers to entry restrict its scalability. NGS-based assays, such as T-Detect and TACTseq are limited by the need for skilled labor and specialized equipment. Our recently developed qPCR-based assays (qTACT and dqTACT) boast the clear advantage of accessibility, which is similar to ELISA/ELLA assays, with the added bonus of having an internal control and unbiased data analysis.

The pandemic has given us the opportunity to reflect on how cellular immunity is quantified at a population level and realize the need for harmonization and reproducibility among labs. This is not a new concept; in 2009, an initiative called minimal information about T cell assays (MIATA) was launched to address the need for transparency and reproducibility for such assays(Britten et al., 2011; Janetzki et al., 2009). MIATA provides guidelines on the minimal amount of information needed to properly report T cell assays such that they can be performed by other labs. Such an initiative is especially crucial in times like the pandemic, where there is an incentive for the rapid development of novel tools to quantify cellular immunity. For example, a recently described nanowire-based assay that promises rapid and accurate T cell quantification(Nami et al., 2022) or a skin test (similar to the tuberculin skin test) that has just received the greenlight to proceed with in-human trials (NCT05216510). This pandemic has also shown a lack of consensus among scientists and public health officers as to the best method of surveilling cellular immunity, unlike seroprevalence that was immediately monitored by the CDC. Going forward, it is paramount that such a surveillance program is implemented should we hope to fully understand the extent of immunity among the population and have the ability to identify vulnerable groups.

Artwork created with BioRender.com.

CRediT authorship contribution statement

Megan Schwarz: Conceptualization, Writing – original draft, Visualization. **Slim Mzoughi:** Conceptualization, Writing – review & editing, Supervision. **Anthony T. Tan:** Writing – review & editing, Supervision. **Antonio Bertoletti:** Conceptualization, Writing – review & editing, Supervision, Funding acquisition. **Ernesto Guccione:** Conceptualization, Writing, Visualization, Writing – review & editing, Supervision, Funding acquisition.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Ernesto Guccione has a patent pending to Hyris. Megan Schwarz has a patent pending to Hyris. Antonio Bertoletti has a patent pending to Hyris. Anthony Tan has a patent pending to Hyris. Daniel Lozano-Ojalvo a has patent pending to Hyris.

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