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The MegaPool approach to characterize adaptive CD4+ and CD8+ T cell responses

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Abstract

Epitopes recognized by T Cells are a collection of short peptide fragments derived from specific antigens or proteins. Immunological research to study T cell responses is hindered by the extreme degree of heterogeneity of epitope targets, which are usually derived from multiple antigens; within a given antigen, hundreds of different T cell epitopes can be recognized, differing from one individual to the next because T cell epitope recognition is restricted by their ability to bind to major histocompatibility complex (MHC) molecules, which are extremely polymorphic in different individuals. Testing large pools encompassing hundreds of peptides is technically challenging because of logistic consideration ad solvent-induced toxicity. To address this issue, we developed the MegaPool (MP) approach based on sequential lyophilization of large numbers of peptides that can be used in a variety of assays to measure T cell responses, including ELISPOT, Intracellular Cytokine Staining (ICS), and Activation Induced Marker (AIM) assays and have been validated in the study of infectious diseases, allergies, and autoimmunity. Here we describe the procedures for generating and testing “MegaPools”, starting with peptide synthesis and lyophilization, as well as guidelines and recommendations for their handling and experimental usage. Overall, the MP approach is a powerful strategy for studying T cell responses and understanding the immune system’s role in health and disease.

Basic Protocol 1: A method for the generation of peptide pools (“MegaPools”)

Basic Protocol 2: MegaPool testing and quantitation of antigen-specific T Cell responses

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AUTHORS CONTRIBUTIONS

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CONFLICT OF INTEREST STATEMENT

A. Sette is a consultant for Gritstone Bio, Flow Pharma, Arcturus Therapeutics, ImmunoScape, CellCarta, Avalia, Moderna, Fortress, and Repertoire. S.C. is a consultant for Avalia. La Jolla Institute for Immunology (LJI) has filed for patent protection for various aspects of MPs design. All other authors declare no conflict of interest.

Keywords

AIM; Epitopes; Infectious diseases; Lyophilization; T cell; SARS-CoV-2

Introduction

T cell responses are key elements of the body's reaction to vaccination and infection and have likely evolved to provide a synergistic line of defense alongside antibody responses. T cells recognize epitopes, which are peptide fragments derived from the cellular processing of protein antigens, through their T cell receptors (TCRs), which are highly specialized proteins on the surface of T cells (Punt, Stranford, Jones, & Owen 2018). T cells can be grouped into a series of subsets based on their function or protein/gene expression. There are several types of T cells, with the most prevalent being CD4+ T cells, also known as helper T cells, and CD8+ T cells, which are referred to as cytotoxic T cells or killer T cells.

Because of their key role in adaptive immunity, measuring T cell responses is an important component of vaccine and diagnostic evaluations, in immunotherapy applications and in the general study on immunopathology. The most common and well-known methodologies to measure antigen-specific T cell responses are cytokine-based assays (e.g. IGRA, ELISPOT and Intracellular Cytokine Staining (ICS)) (Tian et al., 2018), Tetramer/multimer staining assays (Klenerman, Cerundolo, & Dunbar, 2002), and Activation Induced Marker (AIM) assays, which are agnostic towards particular cytokine functionality (Dan et al., 2016; Poloni et al., 2023) (see: Basic Protocol 2 section). The different approaches represent the fundamental tensions that exist between ease of performance, robustness, cost and throughput, on the one hand, and physiological relevance,, complexity, depth of analysis and granularity of information on the other. A comprehensive discussion of the available techniques is beyond the scope of the present protocol and some recent reviews have discussed the topic (Gondre-Lewis et al., 2023; Peters, Nielsen, & Sette, 2020; Sidney, Peters, & Sette, 2020).

In the following protocol, we will describe the generation and use of specialized reagents for probing and characterizing both CD4+ and CD8+ T cell responses. These reagents allow for the efficient elucidation of adaptive T cell immunity against any target of known sequence, and in the context of infectious disease, cancer, allergy and autoimmunity. With the capacity to probe large sequences, the regents described below are of value for use in epitope discovery, crucial for vaccine design, and diagnostic purposes.

Human T cell responses generally recognize multiple epitopes restricted by a diverse set of HLA molecules

Measurement of human T cell responses has to contend with the large diversity of epitope targets recognized (Livingstone & Fathman, 1987; Peters et al., 2020). This diversity originates from two main interdependent sources: the diversity of the antigens recognized, and the diversity of HLA molecules, which bind and present epitopes recognized by T cells. Importantly, this diversity is consequential because responses to different antigens and

epitopes can be directly related to the preservation of T cell responses, the counteracting of immune escape, or associated with differential disease outcomes (Dillon et al., 2015; Schulten, Westernberg, et al., 2018) (Grifoni & Sette, 2022).

The genes encoding HLA molecules are polygenic and highly polymorphic. In humans, there are three main class I loci (A, B and C) and several different class II loci (DRA, DRB1, DRB3/4/5, DPA, DPB and DQA and DQB), resulting in the expression of four different types of HLA class II heterodimers. Because of heterozygosity and the high degree of HLA polymorphism, each individual can express up to 14 different HLA molecules (8 HLA class II and 6 HLA class I). Given that each HLA molecule binds a different set of peptide specificities, HLA polygeny and polymorphism is a powerful force, amplifying the diversity of epitope repertoires recognized in humans (for review, see e.g., (Little & Parham, 1999; Madden, 1995; Parham, 1988; Sidney, Peters, Frahm, Brander, & Sette, 2008; Stevanovic, 2002).

At the same time, this large diversity poses unique challenges for approaches to measure T cell responses (McKinney et al., 2013; Nilsson, Grifoni, Tarke, Sette, & Nielsen, 2021; Sidney et al., 2020). Utilizing just a handful of epitopes and HLA molecules will most likely result in biased and incomplete coverage of the T cell responses, and provide a skewed and incomplete assessment of responses. This situation is made more complex by the fact that different HLA variants are expressed at different frequencies in different ethnic groups and geographic regions (Gonzalez-Galarza et al., 2020). Perhaps unsurprisingly, epitopes presented by HLA molecules that are the most frequent in Caucasians populations, are the ones that have been most highly characterized to date in the literature. This ethnic bias in coverage, a result of approaches utilizing few epitope specificities, has been repeatedly noted, and is a serious limitation that needs to be addressed in developing approaches to study T cell responses in human populations (Sette, Chesnut, Livingston, Wilson, & Newman, 2000; Sette et al., 2001; Sidney et al., 2020).

The MegaPool (MP) approach

To address several of the concerns discussed above, including the need to probe for responses to a wide breadth of epitopes, and in the context of diverse populations, our group developed the MegaPool (MP) approach (Carrasco Pro et al., 2015; Sidney et al., 2020). This approach, based on testing large pools of peptides and/or epitopes, provides an efficient means for comprehensive analysis of T cell responses in virtually any donor and has many advantages compared to other competing technologies (Table 1). To provide a comprehensive evaluation of diverse targets in a diverse population, it may be necessary to test peptides pools that can encompass several hundred different peptides. However, pooling such large numbers of peptides can result in a stock solution that is relatively diluted when each separate peptide is considered. Also, requiring the addition of a relatively large amount of solvent (such as DMSO) into test cell cultures will contribute to cell toxicity, if a sufficiently high enough amount of each peptide is required (de Abreu Costa et al., 2017; Verheijen et al., 2019). To overcome this limitation, we developed a sequential lyophilization approach that achieves much higher concentrations of each peptide in viable amounts of

solvent, thus eliminating or reducing DMSO (or other solvent) toxicity (see: Basic Protocol 1 section).

There are three main approaches to generate the MPs that we routinely utilize in our research. The first corresponds to the use of sets of overlapping peptides spanning the entire sequence of an antigen(s) of interest (Maecker et al., 2001). The second is based on assembling sets of predicted HLA binding epitopes (Peters et al., 2020). Finally, sets can be assembled to consider previously identified and experimentally defined epitopes (Dhanda et al., 2019). Each of these approaches is associated with distinctive advantages and disadvantages.

The use of overlapping peptides is the most comprehensive and unbiased, as it does not rely on any predictive algorithm, or *a priori* knowledge of which epitopes are recognized in a given human population and is irrespective of known HLA phenotype. By selecting a 15-mers size overlapping by 10 amino acids, it further allows to simultaneously assess CD4+ and CD8+ T cell responses in flow cytometry approaches by setting appropriate gating on the responding T cell populations. By definition, however, this approach requires the highest number of peptides, flow-cytometry based techniques, which are less high-throughput and cost-effective, and consequently, the highest number of cells required for testing if individual epitope identification is further desired.

MPs based on predictive approaches allow to reduce the number of peptides tested. However, this approach requires careful consideration of HLA polymorphism to ensure good and appropriate population coverage. Furthermore, this approach requires separate pools for CD4+ and CD8+ T cell detection, and will also detect a fraction of the total response, depending on the comprehensiveness of the HLA predictions and allele coverage. Additionally, there is the intrinsic limitation that predictive algorithms, while highly effective and accurate in most cases, are not 100% sensitive and specific, meaning that some epitopes may be missed.

Finally, MPs based on curated epitopes are effective, since they include epitopes experimentally shown to be recognized by T cells. Epitope sequences can be retrieved by using web databases such as IEDB (https://www.iedb.org). The main limitation of this approach is that, by definition, it relies on the assumption that the epitope repertoire associated with a given indication has already been thoroughly defined in multiple HLA alleles and accounting for diverse ethnic backgrounds representative of the general worldwide population.

In general, MPs are envisioned for determining antigen-specific T cell responses for any particular indication, such as but not limited to allergies, autoimmunity, and bacterial or viral infectious diseases. However, MPs designed with overlapping peptides spanning an individual antigen or a combination of several could be initially used to screen for donor responsiveness, and further introduced into an epitope screening pipeline, and sequentially deconvoluted to map individual peptide CD4+ T cell reactivity. This multi-step approach has been recently employed for genome-wide screening and epitope identification of SARS-

CoV-2, common cold coronaviruses, and Bordetella pertussis (da Silva Antunes et al., 2023; Tarke, Sidney, Kidd, et al., 2021; Tarke et al., 2023).

In the following sections, we will give a brief account of the main MPs we have defined to probing and characterizing both CD4+ and CD8+ T cell responses for several different indications (Table 2). These pools exemplify each of the considerations above, and demonstrate how they can be efficient tools for characterizing immune responses in a wide range of different immunological contexts.

Allergens

MPs have been used extensively to characterize allergen specific T cell responses. The first use of MPs was associated with the characterization of timothy grass responses, addressing both known and novel allergens (Hinz et al., 2016; Oseroff et al., 2010; Schulten et al., 2013), and was later expanded to a broad collection of allergens (Oseroff, Sidney, Vita, et al., 2012). Further studies investigated Japanese Cedar (Oseroff et al., 2016), Cockroach (Birrueta et al., 2019; da Silva Antunes, Sutherland, et al., 2021; Dillon et al., 2015; Oseroff, Sidney, Tripple, et al., 2012), Dust Mite (Hinz et al., 2015; Seumois et al., 2020), murine (da Silva Antunes, Pham, et al., 2018; Grifoni, da Silva Antunes, et al., 2019; Schulten, Westernberg, et al., 2018) and cow milk (Lewis et al., 2023) allergens.

Some of the salient and original applications of MP include the characterization of T cell responses to antigens identified by immunoproteomic approaches (Oseroff et al., 2017; Schulten et al., 2013), epitopes differentially recognized in different clinical manifestations (asthma versus rhinitis) (Dillon et al., 2015) or different levels of sensitization (Schulten et al., 2019), and detection of T cell responses in non-allergic subjects. Interestingly, by probing these reagents allergic and non-allergic subjects were both found to mount T cell responses, but were associated with different phenotypes (da Silva Antunes, Pham, et al., 2018; Grifoni, da Silva Antunes, et al., 2019; Hinz et al., 2016; Schulten, Westernberg, et al., 2018; Yu, Westernberg, et al., 2021). Moreover, specific mouse-allergen derived epitopes were identified by coupling mass-spectrometry and bioinformatic approaches (da Silva Antunes, Pham, et al., 2018). Dust mite peptide pools were used to define the transcriptional profiles associated with allergic asthma (Seumois et al., 2020), and timothy grass and cockroach specific MPs were used to follow T cell reactivity in the context of allergen specific immunotherapy (Rudman Spergel et al., 2021; Schulten et al., 2016; Schulten, Tripple, et al., 2018; Schulten et al., 2014). Lastly, timothy grass and mouse allergen MPs were used to follow the modulation of T cell responses associated with allergen exposure in non-allergic subjects (Hinz et al., 2016; Yu, Westernberg, et al., 2021) and most recently to reveal recognition of allergen-specific responses by Gamma Delta T cells (Yu, Wang, Garrigan, Sutherland, et al., 2022).

Autoimmunity

MPs have also been utilized in the study of autoimmunity, specifically in the study of neurodegenerative and cardiovascular diseases. In the case of Parkinson’s Disease (PD), MPs derived from alpha-synuclein (Lindestam Arlehamn et al., 2020; Singhania, Pham, et

al., 2021; Sulzer et al., 2017), tau (Lindestam Arlehamn et al., 2019), and other neuronal-associated antigens (Dhanwani et al., 2020) have been studied. Similar studies have been conducted in the context of Alzheimer's Disease (AD). The results thus far indicate an autoimmune component in PD, possibly associated with early disease stages (Lindestam Arlehamn et al., 2020), with enhanced reactivity against alpha-synuclein (Sulzer et al., 2017), and possibly other antigens. No increased reactivity against neuronal antigens has thus far been associated with AD (Dhanwani et al., 2020).

In the case of cardiovascular disease, pools of predicted class II binding peptides derived from apolipoprotein B (APOB) were utilized to identify dominant epitopes, and responses to these APOB epitopes correlated with coronary artery disease severity (Roy et al., 2022). In both neurodegenerative and cardiovascular disease applications, an initial restimulation step was necessary to detect antigen-specific responses, potentially reflective of lower frequency of autoimmune T cells compared to other indications.

Bacterial antigens

Several studies have described MPs encompassing different bacterial targets. The first target was MTB, where a genome-wide screen revealed an unprecedented breadth of responses targeting many known and novel antigens (Lindestam Arlehamn et al., 2013). This resulted in the development of the MTB300 MP (Lindestam Arlehamn et al., 2016), which was utilized in over 14 different studies since 2020 (Chihab et al., 2023; Day et al., 2021; Du Bruyn et al., 2022; Du Bruyn et al., 2021; Foreman et al., 2022; Hoft et al., 2023; Kauffman et al., 2021; Ogongo et al., 2021; Patankar et al., 2020; Pomaznoy et al., 2020; Riou et al., 2020; Riou et al., 2021; Robison et al., 2021; Sakai et al., 2021; White et al., 2021; Wood et al., 2020; Woodworth et al., 2021). In addition to being used primarily for the characterization of human CD4+ T cell reactivity, this pool has also been used, based on the similarity of peptide binding repertoires, to probe reactivity in non-human primates (Foreman et al., 2022; Kauffman et al., 2021; Mothe et al., 2015; Sakai et al., 2021; White et al., 2021; Wood et al., 2020), and mice (Patankar et al., 2020). Additional MPs were designed to explore the relationship with conservation in other Mycobacteria (Lindestam Arlehamn et al., 2022), to perform a quantitative analysis of TCR and epitope repertoire composition (Glanville et al., 2017; Scriba et al., 2017), to study specific gene deficiencies in humans (Kong et al., 2018; Martinez-Barricarte et al., 2018), to study the role of CD8+ T cell responses (Pomaznoy et al., 2020), and to derive immune signatures associated with transcriptional profiles and different disease outcomes (Singhania, Dubelko, et al., 2021).

Bordetella pertussis is another target where MPs have been described and validated (da Silva Antunes, Babor, et al., 2018; da Silva Antunes et al., 2023; da Silva Antunes, Quiambao, et al., 2021; da Silva Antunes et al., 2020). Briefly, a series of studies defined epitopes encoded in the antigens included in the acellular Pertussis (aP) vaccine currently in use (Bancroft et al., 2016). These epitopes were used to generate MPs that revealed a long-lasting polarization of responses as a function of the original priming, and unveiled transcriptomic profiles associated with human CD4+ T cell responses to vaccine antigens (da Silva Antunes, Babor, et al., 2018), in addition to clonality assessment of TCR repertoires (Singhania, Pham, et al., 2021). Recently, a whole-genome screening of *Bordetella pertussis*

revealed a highly diverse T cell repertoire and identified epitopes derived from antigens not included in the aP vaccine (da Silva Antunes et al., 2023), which resulted in the development of several MPs that aid the characterization of CD4+ T cell responses to these antigens. Additional studies described and validated CD4+ T cell human epitopes from the Tetanus toxoid protein, which is also in the multivalent Tetanus Diphtheria acellular Pertussis (TDaP) vaccine (da Silva Antunes et al., 2017).

MPs for Flaviviruses

In terms of application of the MP approach to viral targets, early efforts focused on flaviviruses, and in particular on dengue virus (DENV). Initial studies using predicted and experimentally confirmed epitopes spanning the entire proteome of the four DENV serotypes defined a wealth of CD4+ and CD8+ epitopes recognized by T cells derived from individuals previously infected with DENV from different endemic areas, such as Sri Lanka and Nicaragua (Grifoni, Angelo, Lopez, et al., 2017; Grifoni, Moore, et al., 2019; Tian, Grifoni, Sette, & Weiskopf, 2019; Weiskopf et al., 2015; Weiskopf et al., 2016; Weiskopf et al., 2014) and participants in vaccine studies utilizing a live attenuated vaccine (Angelo et al., 2017), a challenge model (Grifoni, Angelo, Sidney, et al., 2017), as well as HLA transgenic mice (Weiskopf et al., 2011). This led to the generation of MPs of experimentally defined epitopes covering both CD4+ and CD8+ T cell responses, which were validated in endemic different populations such as Sri Lanka, Nicaragua, India and Brazil (Grifoni, Angelo, Lopez, et al., 2017; Weiskopf et al., 2015).

The analysis of T cell responses associated with infection and vaccination with different flaviviruses was further expanded to other members of this viral family, such as Yellow Fever (YF), Japanese encephalomyelitis (JEV), West Nile virus (WNV) and Zika virus (ZIKV) (Grifoni, Pham, et al., 2017; Grifoni, Voic, et al., 2020; Mateus, Grifoni, Voic, et al., 2020). These pools were utilized to broadly describe patterns of reactivity and cross-reactivity (Grifoni, Pham, et al., 2017; Schouest et al., 2021). Overall, the results illustrated how the MP approach is broadly applicable to viral infection and vaccination targets.

SARS-CoV-2 and coronaviruses

MPs have played a key role in the study and elucidation of T cell responses to SARS-CoV-2 in the context of infection and vaccination. The first description of CD4+ and CD8+ T cell responses in the context of convalescent individuals defined the key characteristics of a successful immune response utilizing MPs of overlapping peptides spanning the entire proteome, but also MPs based on the use of bioinformatically predicted epitopes (Grifoni, Sidney, et al., 2020; Grifoni, Weiskopf, et al., 2020). These studies highlighted, as previously described for other systems, that MPs based on predicted HLA binders are remarkably effective, and recapitulate at least 50% of the total response, while requiring substantially less peptides and cells for the analysis (Grifoni, Weiskopf, et al., 2020).

These SARS-CoV-2 pools were made broadly available to the scientific community to over 110 laboratories in four different continents, and were utilized in a large number of studies, resulting thus far in over 80 publications (Ansari et al., 2021; Ansari et al., 2022; Apostolidis

et al., 2021; Arunachalam et al., 2022; Banki et al., 2022; Bhuiyan et al., 2022; Blixt et al., 2022; Boland et al., 2022; Bosteels et al., 2022; Bowen, Addetia, et al., 2022; Bowen, Park, et al., 2022; Brasu et al., 2022; Bueno et al., 2022; Cheon et al., 2021; Chiuppesi et al., 2022; Costa et al., 2022; da Silva Antunes, Pallikkuth, et al., 2021; Dan et al., 2021; Dentone et al., 2022; Galvez et al., 2022; Gao, Cai, Grifoni, et al., 2022; Gao, Cai, Wullimann, et al., 2022; Garcia-Valtanen et al., 2022; Gazzinelli-Guimaraes et al., 2022; Geers et al., 2023; GeurtsvanKessel et al., 2022; Goel et al., 2021; Grifoni et al., 2021; Grifoni, Sidney, et al., 2020; Grifoni, Weiskopf, et al., 2020; Hassan et al., 2020; He et al., 2022; Hsieh et al., 2022; Jin et al., 2021; Keeton et al., 2021; Keeton et al., 2022; Keeton et al., 2023; Lederer et al., 2022; Madelon, Heikkila, et al., 2022; Madelon, Lauper, et al., 2022; Mateus et al., 2021; Mateus, Grifoni, Tarke, et al., 2020; Meckiff et al., 2020; Mele et al., 2021; Melo-Gonzalez et al., 2021; Murugesan et al., 2021; Nelson et al., 2022; Ogbe et al., 2021; Painter et al., 2021; Paul et al., 2022; Peluso et al., 2022; Perez-Gomez et al., 2022; Petrone et al., 2021; Petrone, Picchianti-Diamanti, et al., 2022; Petrone, Tortorella, et al., 2022; Pino et al., 2021; Poon, Byington, et al., 2021; Poon, Rybkina, et al., 2021; Premkumar et al., 2020; Ramirez et al., 2022; Rydyznski Moderbacher et al., 2022; Rydyznski Moderbacher et al., 2020; Schultz et al., 2022; Shaan Lakshmanappa et al., 2021; Singh et al., 2022; Soto et al., 2022; Sun et al., 2022; Tarke, Coelho, et al., 2022; Tarke, Potesta, et al., 2022; Tarke, Sidney, Kidd, et al., 2021; Tarke, Sidney, Methot, et al., 2021; Ukey et al., 2022; Valencia et al., 2022; Van Damme et al., 2020; Vikkurthi et al., 2022; Weiskopf et al., 2020; Williams et al., 2023; Yu, Narowski, et al., 2022; Yu, Wang, Garrigan, Goodwin, et al., 2022; Zhang et al., 2022). While an in-depth review is beyond the scope of this report, the MPs helped clarify a diverse set of topics and concerns related to SARS-CoV-2, including elucidation of responses in the acute phase of infection, responses to vaccination, breakthrough infections, kinetics and features of responses in the memory phase, responses in vulnerable and immunocompromised individuals, health care workers, and responses in children.

Several additional insights of SARS-CoV-2 infection were also addressed with specific MPs. These include the demonstration of cross-reactive pre-existing memory T cell responses (Mateus, Grifoni, Tarke, et al., 2020), and the demonstration that both CD4+ and CD8+ T cell responses are remarkably preserved in the context of the different variants that originate throughout the pandemic (Tarke, Coelho, et al., 2022; Tarke, Sidney, Methot, et al., 2021). Additional MPs were subsequently generated, based on experimentally defined epitopes, and used to derived immunodiagnostic strategies to address vaccination and infection history (Grifoni et al., 2021; Tarke, Sidney, Kidd, et al., 2021; Yu, Wang, Garrigan, Goodwin, et al., 2022). In parallel, specific MPs were also derived to follow responses to other coronavirus species, such as the main common cold coronaviruses (da Silva Antunes, Pallikkuth, et al., 2021; Tarke et al., 2023; Yu, Narowski, et al., 2022).

Broad application to study responses to human viral pathogens

While perhaps flaviviruses and SARS-CoV-2 represent “poster child” applications of MPs in the study of viral disease, several additional viral indications have been addressed by the MP approach. These include influenza, where pools addressing different subtypes and antigens have been validated (Meckiff et al., 2020; Poon, Byington, et al., 2021; Yu, Grifoni,

et al., 2021), and other common respiratory viruses such as Rhinovirus (Grifoni, Mahajan, et al., 2019), Metapneumovirus (HMPV) (Meckiff et al., 2020), Parainfluenza virus (HPIV) (Meckiff et al., 2020), and Respiratory syncytial virus (RSV) (Yu, Narowski, et al., 2022), as well as herpesviruses such as Epstein-Barr Virus (EBV) (Carrasco Pro et al., 2015; Dan et al., 2016), Cytomegalovirus (CMV) (Carrasco Pro et al., 2015; Dhanwani et al., 2021), and Varicella Zoster Virus (VZV) (Voic et al., 2020). Recently, MPs were developed and validated for detection of HIV-specific and CD4+ and CD8+ T cells (Al-Kolla et al., 2022). Murine poxvirus epitopes were first described in the mid 2000s providing rational for the characterization of smallpox vaccines (Pasquetto et al., 2005; Tscharke et al., 2005), followed more recently by the design and validation of specific MPs broadly encompassing orthopox and MPOX-derived epitopes for monitoring of infection and vaccination (Grifoni et al., 2022).

BASIC PROTOCOL 1: A method for the generation of peptide pools (“MegaPools”)

This section describes guidelines on how to produce MPs. The initial step encompasses synthesis of peptide sets that are produced and lyophilized, as common practice in the field. They can be purchased from any supplier. However, all MPs depicted in this manuscript were generated from peptides synthesized by TC peptide lab or Mimotopes. Each peptide is then solubilized, and a pool generated by combining equivalent amounts of each solubilized peptide. The resulting pool is then re-lyophilized. If necessary, the procedure is repeated until a white, reasonably fluffy, powder (lyocake) is obtained. Any synthesized peptides can be pooled for use into a MP. Generally, given the high cost of large sets of peptides, purified preparations are not necessary and the of use crude peptides is acceptable. In the vast majority of cases these will achieve >70% purity. Further, large scale synthesis of crude peptide can usually be obtained in microtube racks, which allow efficient dilution of peptide stocks using, for example, multi-channel pipettors. Purified peptides are excellent if resources are available, or use necessitates. The final MP amount required should be calculated based on the number of samples or assays that need to be tested, but we recommend starting this protocol with 1–2mg per individual peptide.

Materials

Peptides (TC peptide lab (San Diego, CA) or Mimotopes (Victoria, Australia).

Falcon Conical Tubes, 15 and 50mL

Fast - Freeze™ Flask Adaptors ¾” to ¾” (Labconco® Cat# 7457200)

Fast - Freeze™ Flask 900 mL (Labconco® Cat# 7540901)

Fast - Freeze™ Tube Holder 50mL (Labconco® Cat# 7379500)

Fast - Freeze™ Filter Paper 50mL (Labconco®A-75448)

FreeZone® Benchtop Freeze Dryer, 4.5L Capacity (Labconco® Cat#720401000)

Peptide preparation and pooling

1. Resuspend peptides intended for use in MPs at a high concentration (≥20 mgs/ml) in 100% DMSO.

   For some peptides, it may be necessary to reduce concentration and/or use water. Similarly, in a few cases, precipitates might from in the solubilized pools. This can be addressed by further diluting the pools in water.

2. Pool a uniform and desirable amount of each peptide in a suitable vessel, such as a 15 or 50 ml conical tube.

   To facilitate efficient lyophilization, the final total volume of pooled peptides should not exceed 20% of the capacity of the vessel.

3. Dilute pooled sample 2:1 with H_2O (i.e., max 15 ml total volume in a 50 ml conical tube).

4. Place diluted sample in a −80°C freezer for 24 hours.

   Ideally, samples should be frozen at an angle (~45 degrees) to maximize surface area of the liquid sample in the tube. The rack used to hold the pools in the lyophilized will ideally be frozen at the same time, to facilitate maintenance of sample temperature (and prevent thawing) when subsequently moving to the lyophilizer.

Lyophilization

5. Operate the lyophilization unit as per the manufacturer's specifications. If using the 4.5L Capacity Benchtop Freeze Dryer unit, set conditions at −105°C at 0.1–0.01 mBar. Verify that all parameters have been set as prescribed by the manufacturer.

6. Remove the accessory drying chamber (manifold) from the collector chamber lid, and using a soft, lint-free cloth or paper towel, wipe the port gasket(s) and sealing surfaces of the drying chamber/manifold and collector chamber lid to remove any dirt or contaminants that could cause a vacuum leak or contaminate your sample.

7. Check that each sample valve is closed or in the "vent" position, then start the instrument collector and allow the refrigeration system to reach its specified operating temperature (−105°C for our model). Start the vacuum when collector temperature is at −40°C or colder.

   Once the system pressure is at 0.1–0.01 mBar, and the temperature is −105°C, the system is ready for use.

8. Moving quickly to avoid thawing, retrieve samples from the freezer, and using a 50ml tube holder, place the samples into a pre – chilled Fast - Freeze™ Flask.

   Do not overload the beaker; utilize additional flasks if necessary.

9. Slowly open the port valves to commence lyophilization.

   Abrupt opening may result in burping the sample against jar filters, or into the instrument, potentially causing cross contamination. Vacuum pressure will temporarily increase, but if the vessel is properly sealed and mounted it will go back down to operating range (e.g., 0.1–0.01 mBar).

10. Check sample and system periodically to ensure proper temperature and pressure is maintained, and that the sample has not thawed.

11. When the sample has completely dried, close the pressure valve and remove the beaker from the lyophilizer. It may take up to 24 hours to dry a sample, depending on the volume and sample mixture, but most samples are typically dried between 16–18 hours.

12. Resuspend the sample in water using the same initial volume of water as was used to dilute the original sample (i.e. 2 -parts water to 1-part sample in DMSO), and then refreeze the sample.

    This will facilitate further removal of any left over DMSO.

13. After the sample is frozen, return it to the freeze dryer, repeating steps 1–8 above, as necessary. When the sample is completely dried, close the valve and carefully remove the flask from the machine.

    A successfully dried product will have a consistent white almost fluffy powder appearance.

MPs resuspension and storage

14. Resuspend the lyophilized MP in 100% DMSO (SIGMA D2650) down to 1mg/mL/peptide.

    The final concentration of each individual peptide in the MP is uniform as long as the same amount of peptide is pooled, irrespective of the number of peptides contained in each MP. In order to use the MP in cell stimulation assays, the peptides should be resuspended in enough volume to go into solution, but not too concentrated (viscous) for accurate pipetting

15. Perform additional dilutions with H_2O, if concentrations lower than 1mg/mL are necessary.

    Avoid dilution with DMSO to minimize cytotoxicity in cell culture. In this case, it is advised to maintain a 1 mg/ml DMSO mother stock, but dilute smaller aliquots.

16. Aliquot diluted pools in small aliquots (default volume aliquots of 25 or 50μL are optimal) to prevent freeze-thaw cycles.

17. Store aliquoted MPs at −20°C and inspect overtime for appearance. If routinely precipitation is noted, re- lyophilize the MPs.

BASIC PROTOCOL 2: MegaPool testing and quantitation of antigen-specific T Cell responses

MPs are a complex mixture of multiple peptides and therefore unfeasible to perform mass spectrometry analysis for quality purposes. The assessment of quality of MPs is generally assessed by testing the bioactivity on a T cell assay. This section describes guidelines on how to to assess the quality of MPs and their use in an immunological assay to quantify antigen-specific responses. Specifically, the immunological assay described herein is an Activation Induced Marker (AIM) assay using PBMCs as biological source material. This protocol also describes the steps of thawing and washing cryopreserved PBMCs prior to cell culture and stimulation, and how to perform a flow cytometry staining for membrane markers. This will allow identifying co-stimulatory receptors that are expressed on activated T cells after antigen-specific stimulation with MPs. Although this protocol is focused on an AIM assay, MPs can be used in other assay modalities to assess antigen-specific responses and/or validate MPs quality such as ICS or ELISPOT assays, and by using whole blood instead of PBMCs.

Materials

Peripheral blood mononuclear cells (PBMCs)

Benzonase (Sigma, E8263)

Spike MP, CD4RE MP, CD8RE MP and EBV MP

Phosphate-buffered saline (PBS, GIBCO BRL 10010–023)

HR5 media (see recipe)

MACS Buffer (see recipe)

CD40 antibody (Miltenyi Biotech, 130–094-133)

Conjugated antibodies (see Table 3)

Laminar flow hood (Labconco Purifier BSC Class II, or equivalent)

Tissue culture microscope (Nikon or equivalent)

Falcon Polypropylene conical tubes and cell culture flasks

96-well round bottom cell culture–treated plate (Grenier Bio-One, cat. no. 655180)

Multichannel pipettes (1000, 200, 20 and 0.5 μl)

ZE5 Flow cytometer (Biorad)

Thawing of PBMCs

1. Submerge cryovials with frozen PBMCs into a 37°C water bath. Hold the vials in the bath until all but a tiny bit of ice remains in the vial. Do not allow the cells to warm completely to 37°C.
2. Transfer the cells into the cold conical propylene tubes with cold culture medium (10 mL HR5+20 μL benzonase per vial used) and centrifuge the cells at 1200 rpm at 4°C for 7 min.
3. Count the cells and determine cell viability.
4. Add HR5 media to adjust the cell concentration to 10×10^6 cells/ml.

   If using fresh PBMCs advance to step 5.

Cell Stimulation

5. Prepare MP stimulation solution at the desirable concentrations. Adjust the volume depending on the number of samples used per each experiment.

   When a new MP is used for the first time, it is advisable to perform a titration with cells known to respond to the stimulus. If it's not feasible to do a titration, the default recommended final concentrations (for each peptide) are 1 μg/mL for $CD4^+$ and $CD8^+$ MPs, unless indicated differently for each individual MP.
6. Plate in 96 well U-bottom, 100μL of stimuli at a 2X concentration.
7. Plate 100μL of cells at a concentration of 10×10^6 cells/ml to plate 1×10^6 cells per each well.

   The assay can be performed by plating a range of $0.5–2\times10^6$ cells/well. If lower cells are available, consider removing one of the conditions from the assay. If more than 2×10^6 cells per condition are available, consider to carry out the experiment in duplicate or triplicate.
8. *Optional:* If the membrane staining will include the activation marker CD40L, then add CD40 antibody in the solution per each donor before plating for stimulation.
9. Incubate plate for a total of 24 hours at 37°C/5% CO_2.

Flow Cytometry antibody surface staining

10. To perform the membrane staining after 24 hours stimulation, prepare a cocktail of antibodies as shown in the Table 3.

    Volumes and fluorochromes combinations are optimized for the Biorad ZE5, please change the fluorochrome combinations and perform an additional antibody titration if using a different instrument.
11. After stimulation, spin 96 U-bottom plate at 1400 rpm at 4°C for 2 min.

12. Wash plate using 200μL PBS at 1400 rpm at 4°C for 2 min.
13. Add in each well 100μL of antibody mix.
14. Incubate for 30 min at 4°C. Protect from light.
15. After incubation, add 100μL MACS buffer and spin plate at 1400 rpm at 4°C for 2 min.
16. Wash 2X plate using 100μL MACs buffer at 1400 rpm at 4°C for 2 min.
17. Resuspend in 100μL of PBS per 1×10^6 cells plated (if you plate 0.5×10^6 cells resuspend in 50μL; if you plate 2×10^6 cells resuspend in 200μL).
18. Acquire samples in a Flow Cytometry machine.

REAGENTS AND SOLUTIONS

HR5 media

1000mL RPMI (Omega RP-21)

50mL Heat Inactivated Human Serum AB (GeminiBio 100–512)

10mL Pen-Strep (GeminiBio 400–109)

10mL L-Glutamine (Glutamax) (Gibco 35050061)

MACS Buffer

500ml PBS pH 7.4 In Vitrogen (#10010–0230)

2.5g BSA Sigma (A-3294) stored at 4°C (Deli cabinet)

2ml 0.5 M EDTA

Filter through .2μ filter and degas for 30 minutes

Store at 4°C

COMMENTARY

Background information

To evaluate the targets of T cell responses to diverse pathogens or allergens, testing peptide pools with hundreds of peptides may be necessary. However, challenges associated with pooling and testing a large number of peptides can hinder the identification and quantification of T cell responses. Here, we developed a sequential lyophilization method to increase peptide concentrations and reduce solvent toxicity for further use in biological assays and assessment of antigen-specific responses such as, but not restricted to, AIM assays.

AIM assays are based on TCR-dependent upregulation of co-stimulatory immune molecules, independent of cytokine response, and have been successfully used in various studies to

detect T cells specific to viruses, bacteria or allergens. In this particular protocol, we are using as example SARS-CoV-2 MPs (see section below: Understanding results), which have been applied in more than 80 studies thus far, and OX40, 4–1BB and CD69 as activation markers. In this assay, CD4+ T cell responses are measured utilizing OX40+CD137+ dual marker combination and CD8+ T cell responses measured utilizing 4–1BB+CD137+ dual marker combination. The use of other activation markers and/or marker combination can be used as discussed previously (da Silva Antunes et al., 2017; Dan et al., 2016; Reiss et al., 2017).

Critical Parameters

To avoid contamination or vacuum leaks prior to start up the MP lyophilization, remove any baffling (if equipped) and wipe with a soft cloth or paper towel to remove any remaining moisture within the drying chamber, and ensure that the collector chamber and drain line are free of any residual moisture. It is recommended that all the samples be covered using a filter paper, parafilm or Kim wipe as an extra precaution to avoid contamination of samples during the freeze dry process.

Because of the intrinsic solubility and numbers of epitopes, there can be great variability in solubility and viscosity between different MPs, and even different batches of the same MP. Additionally, storage conditions and multiple freeze-thaw cycles can affect the stability of the peptides in the MP. To maximize cellular responses and lengthen the shelf life of the pool, once the MP is removed from the freezer, it can be stored at 4°C for 2 weeks or refrozen. Avoid more than 5 freezing-thawing cycles.

When assessing MP reactivity, the most important and mandatory control in the AIM assay is a control of specificity or negative control. For that purpose, all assays should be performed with a DMSO stimulation control in parallel, at a concentration matching the same exact concentration of MPs used. It is highly recommended to perform this control in triplicate if enough PBMCs are available. The DMSO signal should then be subtracted to the MP-specific signal.

The second most important control in this assay is a positive control. These control is used to ensure that a detection of signal is observed by validating the expression and/or upregulation of the different activation markers. Typical positive controls used in this assay are polyclonal stimulation agents such as PHA, PMA/Ion, α-CD3/CD28 or SEB.

Another control, but optional, is the use of a MP that is not associated with the biological target that the user is interested, typically named as irrelevant MP control. This allows for an internal assay control of the donor response or sample being interrogated. Typical MPs used as irrelevant controls are MPs associated with generalized responses across a human population like those elicited after vaccination (e.g. Pertussis, Tetanus or SARS-CoV-2) or after infection with ubiquitous pathogens (e.g. CMV or EBV).

Another optional control is the inclusion of PBMC samples from donors that are not expected to respond to a specific MP (negative donors), such as for example using HIV negative donors to test reactivity to an HIV MP. This control will identify if any

undesired reactivity of a MP or issues with the MP synthesis occurred (e.g. lyophilization contamination). Alternatively, PBMC samples from donors that are expected to respond to a specific MP can be included (positive or control donors), such as donors with a particular indication, donors that have experienced a previous clinical diagnosed disease or undergone a particular vaccination schedule (e.g. Pertussis, Tetanus or SARS-CoV-2). This control can be particularly valuable in the monitoring and assessment of longitudinal response stability (da Silva Antunes, Sutherland, et al., 2021).

Troubleshooting

Fluctuations in the vacuum readings as well as a loud suctioning grunt during the machine start up is often an indicator of a vacuum leak. This can be resolved by making sure all the knobs on the valves are in the “Vented” position which closes the valves, allowing the drying chamber pressure to drop to working condition. It is important to also inspect the lid as well as the gaskets on the chamber/manifold for proper placement and/or any damage or degradation that may cause any unwanted vacuum leaks.

If samples begin to thaw before being placed into the freeze dry unit, it is necessary to have them refrozen to facilitate freeze drying efficiency and avoid sample loss. Liquid Nitrogen can be employed to help with this after a short period in the −80°C freezer. Flasks can be further insulated using a neoprene cozy and, or aluminum foil, to minimize sample thawing. The cozy can also function to protect the vessel from breakage in case it falls.

While the Labconco® Benchtop Freeze Dryer is our Lyophilizer of choice, solvent removal from peptide pools could be performed using a standard Speed Vacuum Concentrator, which will remove a large amount of solvent relatively quickly. However, this type of device does not do as complete a job as a Freeze Dryer, and thus may pose some issues with DMSO toxicity in the final sample.

Regarding the AIM assay If using frozen PBMCs that are not thawed properly, viability and cell recovery can be compromised, and the cells may not perform optimally in the AIM assay. In general, cells should be thawed quickly and kept cold while still in the presence of DMSO. Cells with DMSO intercalated into their membranes are very fragile, and must be pelleted and handled gently.

When performing the surface staining steps, it is important to minimize the exposure to light as the antibodies used for this assay are conjugated with fluorochromes that are sensitive to light. The volume for each antibody depends on the Flow cytometer used to acquire the sample and on the manufacturers’ recommendation. As such it is advisable to perform an antibody titration to assess the optimal configuration based on the instrument. Similarly, fluorochromes combinations are machine-dependent and should be chosen according to the Flow cytometer used for the specific experiment.

When interpreting results some MPs pools can be used to simultaneously detect CD4+ and CD8+ T cell responses, while others are optimized to detect exclusively CD4+ or CD8+ T cell responses. If unsure, please refer to the details about the specificity of the MP you are using.

The number of peptides that can be pooled into a single MP depends on several factors that affect solubility, such as the number of peptides with a high proportion of hydrophobic amino acids, the size of peptides, or the presence of salts in the peptide preparations, to name a few. As a general rule MPs should not exceed 300–400 peptides. However, a strategy to test higher number of peptides could be to use multiple pools for simultaneously stimulation directly in the culture assay (Yu et al., 2022).

Understanding results

Within this segment, we describe and visually outline the detection and characterization of antigen-specific responses to SARS-CoV-2 after employing the AIM assay, as mentioned above. To detect SARS-CoV-2 T cell reactivity, a MP of overlapping peptides spanning the entire Spike sequence and two MPs optimized for detection of non-Spike (remainder of SARS-CoV-2 proteome) reactivity in CD4+ and CD8+ T cells were employed (CD4RE and CD8RE, respectively). The combination of these pools allowed to discriminate 4 groups of subjects with different SARS-CoV-2 infection and COVID-19 vaccine status as described below. An EBV MP was used as control (Figure 1). CD4+ and CD8+ T cells responses were calculated as percent of total CD4+ (OX40+CD137+) or CD8+ (CD69+CD137+) T cells. The background was removed from each stimulation by subtracting the signal from wells stimulated with DMSO. The gating strategy utilized is shown in Figure 2, as well as an example of reactive CD4+ and CD8+ T cell responses to SARS-CoV-2 Spike, CD4RE and CD8RE MPs and EBV MP from a representative donor.

As expected Spike SARS-CoV-2 specific-T cells can be detected in individuals that have been vaccinated with COVID-19 vaccines (non-infected and vaccinated; I−V+) and infected with SARS-CoV-2 (infected and non-vaccinated; I+V−) or both (Infected and then vaccinated; I+V+). Conversely no responses are observed in individuals neither infected nor vaccinated (non-infected, non-vaccinated; I−V−). Importantly, CD4RE and CD8RE responses are only detected in convalescent subjects (I+V− or I+V+) and not in unexposed (I−V−) or vaccinated (I−V+) subjects, as expected since all vaccinated donors included in this study received exclusively Spike-based mRNA vaccines. Also, EBV reactivity was detected and observed at equal levels across all the groups. Overall this data demonstrates the attributes of the MP approach and versatility of MP design to detect antigen-specific responses to SARS-CoV-2 and EBV, complex pathogens composed of multiple antigens. Importantly, due to the intrinsic nature and diversity of the epitopes selected, the MPs employed in this section allowed to discriminate responses specific to COVID-19 vaccination or SARS-CoV-2 infection, highlighting the potential use of MPs as immunodiagnostic tools. For complete details on the use and execution of this protocol for this particular experimental setting and/or interpretation of the data, please refer to (Yu, Wang, Garrigan, Goodwin, et al., 2022).

Time Considerations

On average, it takes about 16–18 hours per run to completely lyophilize a sample, and two runs in total with an additional dilution step to extract all the DMSO from a peptide sample. For the lyophilization of any MP, if time is of the essence, the drying process can be accelerated first with the speedvac, then the lyophilizer utilized to further remove residual

DMSO, though this does incur the potential risks involved with additional sample handling. Use of a speedvac must also proceed with caution, as doing the process too quickly risks loss of sample along with the solvent.

The AIM assay requires 2 days to be completed. The thawing of PBMCs should take less then 1 hour, although more time may be needed if more than 10 samples are used at the same time. If using fresh PBMCs plan ahead the time allocated for blood separation before PBMCs are processed. The T cell culturing period with MPs should be exactly 24h. The surface staining should be performed in the following day and immediately after stimulation. If not possible the plate should be removed from the incubator and placed at 4°C until the surface staining is performed. Samples should be then immediately acquired in a Flow Cytometer machine. However, fixation of the PBMCs samples can be performed after the last step of the protocol to extend the period before sample acquisition.

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DATA AVAILABILITY STATEMENT

Materials availability: MPs described in this protocol will be made available to the scientific community upon request, depending on material availability, and following execution of a material transfer agreement (MTA), by contacting A.S. (alex@lji.org).

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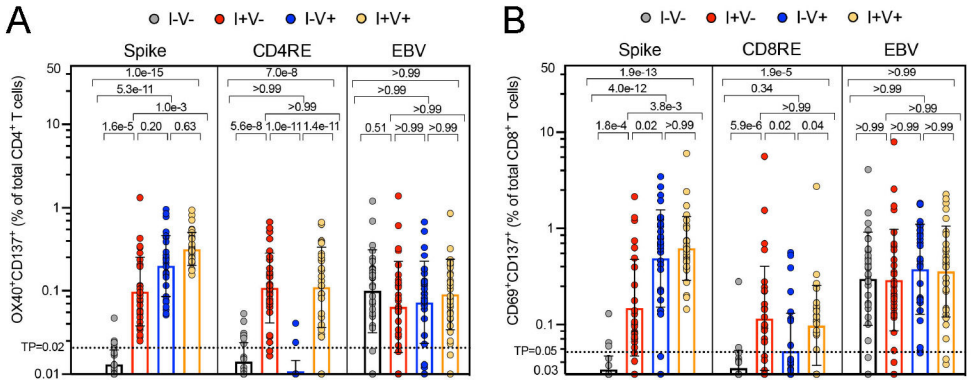

Figure 1. SARS-CoV-2-specific CD4+ and CD8+ T cell responses in 4 study groups of subjects with different SARS-CoV-2 infection and COVID-19 vaccine status.

A T cell-based classification scheme has been previously developed with the aid of MPs that can discriminate responses exclusively from COVID-19 vaccination (i.e. using a MP targeting Spike) and from SARS-CoV-2 infections (i.e. using MPs targeting the remainder of SARS-CoV-2 proteome). T cell reactivity was assessed by AIM assay and SARS-CoV-2-and EBV specific CD4+ and CD8+ T cell responses were measured as percentages of AIM+ (OX40+CD137+) CD4+ T cells (A) or AIM+ (CD69+CD137+) CD8+ T cells (B) after stimulation of PBMCs with peptides pools encompassing spike-only (Spike) MPs or the experimentally defined CD4RE and CD8RE MPs representing all the proteome without spike. EVB MP was used as a control. Graphs show individual response to each MP plotted as background subtracted against DMSO negative control. Geometric mean with standard deviation (SD) for the 4 different groups is shown. Kruskal-Wallis test adjusted with Dunn's test for multiple comparisons was performed, and p values < 0.05 considered statistically significant. I−V−, unexposed and unvaccinated (n = 30); I+V−, infected and non-vaccinated (n = 30); I+V+, infected and then vaccinated (n = 30); I−V+, non-infected and vaccinated (n = 30). Threshold of positivity (TP) is indicated. Median response each group is shown. This figure was adapted from (Yu, Wang, Garrigan, Goodwin, et al., 2022).

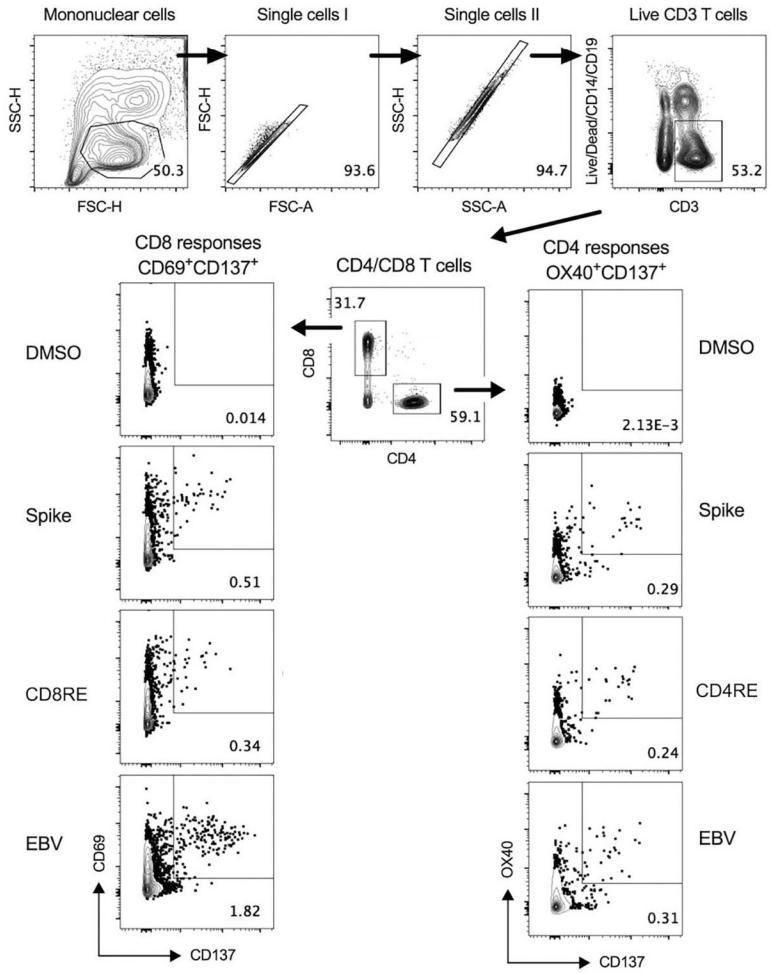

Figure 2. Gating strategy and representative CD4+ and CD8+ T cell responses plots of the activation induced marker (AIM) assay

Representative gating of live CD3+ T cells, CD4+ T cells, CD8+ T cells and reactive OX40+CD137+ CD4 or CD69+CD137+ CD8 T cells from donor PBMCs is shown. Briefly, mononuclear cells were gated out of all events followed by subsequent singlet gating. Live CD3+ cells were gated as Live/Dead-CD14-CD19-CD3+. Cells were then gated as CD4+CD8− or CD4−CD8+ T cells, and reactive OX40+CD137+ CD4+ or CD69+CD137+ CD8+ T cells were gated and calculated as percent of total CD4+ or CD8+ T cells. Representative CD4+ and CD8+ antigen-specific responses plots after stimulating with DMSO (negative control) and SARS-CoV-2 or EBV specific MPs are shown. This figure was adapted from (Yu, Wang, Garrigan, Goodwin, et al., 2022)

Table 1.

MegaPool approach comparison with competing technologies

Other technologies	Advantages	Limitations
MegaPool approach	• Exhaustive HLA coverage • Comprehensive coverage of diverse and broad epitope repertoire • Enhanced specificity • Ease and speed of production • Allows ex vivo assessment bypassing proteolysis • Broad applicability (i.e. AIM, ICS, ELISPOT assays using either PBMCs or whole blood)	• Epitope Masking • Limited Information on fine specificity • Requires specialized equipment
Multimers/tetramers	• Allows ex vivo assessment bypassing proteolysis • Enhanced specificity • Enhanced sensitivity	• Need for predefinition of exact epitope and HLA restriction • Increased complexity/need for specialized reagents • Higher cost • Limited applicability, throughput and repertoire
Use of whole organism (Live pathogens, cell lysates, supernatant or extracts)	• Broad range of antigens • Native conformation of antigens • Allow measuring post-translationally modified epitopes	• Potential toxicity/biosafety issues • Cell lysates do not contain non-structural proteins or secreted antigens • Requires longer time for antigen-processing • Complexity and heterogeneity • Limited applicability
Antigens (Recombinant or purified)	• Allow measuring post-translationally modified epitopes • Reduced complexity and heterogeneity	• Potential toxicity/biosafety issues if protein is a toxin or has immunomodulatory effects • Requires longer time for antigen-processing • Limited applicability

Table 2.

List of MPs developed and targets of responses

Indication	Pathogen/ Organism	Pool Name	Source	Peptide/Epitope type	Number of peptides	Reference
Infectious diseases (Viruses)	Cytomegalovirus	CD4 CMV	Whole proteome	Experimentally defined	313	Carrasco Pro et al., 2015 (Carrasco Pro et al., 2015)
	Cytomegalovirus	CD4 CMV235	Whole proteome	Experimentally defined	235	Dhanwani et al., 2021 (Dhanwani et al., 2021)
	Cytomegalovirus	CD4 CMV	Whole proteome	Experimentally defined	198	Carrasco Pro et al., 2015 (Carrasco Pro et al., 2015)
	Dengue Virus	DENV_CD8	Whole proteome	Predicted and experimentally defined	268	Grifoni et al., 2017 (Grifoni, Angelo, Lopez, et al., 2017)
	Dengue virus	DENV_CD4	Whole proteome	Experimentally defined	180	Grifoni et al., 2017 (Grifoni, Angelo, Lopez, et al., 2017)
	Dengue/Zika virus cross reactive	DENV/ ZIKV_CR	Whole proteome	Experimentally defined	94	Schoeust et al, 2021 (Schouest et al., 2021)
	Ectromelia, Vaccinia and Variola virus	OP-CD4-E MP	Whole proteome	Experimentally defined	300	Grifoni et al., 2022 (Grifoni et al., 2022)
	Epstein-Barr virus	CD8 EBV	Whole proteome	Experimentally defined	218	Carrasco Pro et al., 2015 (Carrasco Pro et al., 2015)
	Epstein-Barr virus	CD4 EBV	Whole proteome	Experimentally defined	83	Dan et al., 2016 (Dan et al., 2016)
	HCoV-229E	229E	Whole proteome	Spike (S) Overlapping/ Rest of proteome (R) Predicted	225	da Silva Antunes et al., 2021 (da Silva Antunes, Pallikkuth, et al., 2021)
	HCoV-HKU1	HKU1	Whole proteome	Spike (S) Overlapping/ Rest of proteome (R) Predicted	320	da Silva Antunes et al., 2021 (da Silva Antunes, Pallikkuth, et al., 2021)
	HCoV-NL63	NL63	Whole proteome	Spike (S) Overlapping/ Rest of proteome (R) Predicted	280	da Silva Antunes et al., 2021 (da Silva Antunes, Pallikkuth, et al., 2021)
	HCoV-OC43	OC43	Whole proteome	Spike (S) Overlapping/ Rest of proteome (R) Predicted	294	da Silva Antunes et al., 2021 (da Silva Antunes, Pallikkuth, et al., 2021)
	Human immunodeficiency virus	HIV CD4	Whole proteome	Experimentally defined	164	Al-Kolla et al., 2022 (Al-Kolla et al., 2022)
	Human immunodeficiency virus	HIV CD8	Whole proteome	Experimentally defined	187	Al-Kolla et al., 2022 (Al-Kolla et al., 2022)
	Influenza A	HA-Influenza MP	Hemagglutinin (HA)	Experimentally defined/Predicted	161	Meckiff et al., 2020 (Meckiff et al., 2020)
	Influenza A	Flu-Other	Non-Hemagglutinin proteome	Experimentally defined	169	Yu et al. 2021 (Yu, Grifoni, et al., 2021)

Indication	Pathogen/ Organism	Pool Name	Source	Peptide/Epitope type	Number of peptides	Reference
	Influenza A	flu-CD8	Whole proteome	Experimentally defined	400	Poon et al., 2021 (Poon, Byington, et al., 2021)
	Japanese encephalitis virus	JEV CD4	Whole proteome	Predicted	239	Grifoni et al., 2020 (Grifoni, Voic, et al., 2020)
	Japanese encephalitis virus	JEV CD8	Whole proteome	Predicted	310	Grifoni et al., 2020 (Grifoni, Voic, et al., 2020)
	Metapneumovirus	HMPV	Whole proteome	Predicted	107	Meckiff et al., 2020 (Meckiff et al., 2020)
	Monkeypox virus	MPx-CD4-PMP	Whole proteome	Predicted	276	Grifoni et al., 2022 (Grifoni et al., 2022)
	Monkeypox virus	MPx-CD8-PP1-P5 MP	Whole proteome	Predicted	1647	Grifoni et al., 2022 (Grifoni et al., 2022)
	Parainfluenza virus	HPIV	Whole proteome	Predicted	256	Meckiff et al., 2020 (Meckiff et al., 2020)
	Respiratory syncytial virus	RSV	Whole proteome	Experimentally defined	216	Yu et al. 2022 (Yu, Narowski, et al., 2022)
	Rhinovirus	Rhinovirus	Whole proteome	Experimentally defined	136	Grifoni et al., 2019 (Grifoni, Mahajan, et al., 2019)
	SARS-CoV-2	CD4RE	Non-spike proteome	Experimentally defined	284	Grifoni et al., 2021 (Grifoni et al., 2021)
	SARS-CoV-2	CD8RE	Non-spike proteome	Experimentally defined	621	Grifoni et al., 2021 (Grifoni et al., 2021)
	SARS-CoV-2	CD8A	Whole proteome	Predicted	314	Grifoni et al., 2021 (Grifoni et al., 2021)
	SARS-CoV-2	CD8B	Whole proteome	Predicted	314	Grifoni et al., 2021 (Grifoni et al., 2021)
	SARS-CoV-2	CD4R	Non-spike proteome	Predicted	221	Grifoni et al., 2020 (Grifoni, Sidney, et al., 2020)
	SARS-CoV-2	SARS2	Whole proteome	Spike (S) Overlapping/ Rest of proteome (R) Predicted	474	da Silva Antunes et al., 2021 (da Silva Antunes, Pallikkuth, et al., 2021)
	SARS-CoV-2	CD4E	Whole proteome	Experimentally defined	280	Tarke et al., 2021 (Tarke, Sidney, Kidd, et al., 2021)
	SARS-CoV-2	CD8E	Whole proteome	Experimentally defined	454	Tarke et al., 2021 (Tarke, Sidney, Kidd, et al., 2021)
	Vaccinia and Variola virus	OP-CD8-EMP	Whole proteome	Experimentally defined	238	Grifoni et al., 2022 (Grifoni et al., 2022)
	Varicella zoster virus	VZV	Whole proteome	Glycoprotein E (gE) Experimemntaly definied / Rest of proteome (R) Predicted	335	Voic et al., 2020 (Voic et al., 2020)
	West Nile	WNV CD4	Whole proteome	Predicted	244	Grifoni et al., 2020 (Grifoni, Voic, et al., 2020)

Indication	Pathogen/ Organism	Pool Name	Source	Peptide/Epitope type	Number of peptides	Reference
	West Nile	WNV CD8	Whole proteome	Predicted	324	Grifoni et al., 2020 (Grifoni, Voic, et al., 2020)
	Yellow Fever	CD4_YF; CD4_YF_rev	Whole proteome	Predicted and experimentally defined	215;275	Mateus et al, 2020; Grifoni et al., 2020 (Grifoni, Voic, et al., 2020; Mateus, Grifoni, Voic, et al., 2020)
	Yellow Fever	YF_CD8	Whole proteome	Predicted	368	Grifoni et al., 2020 (Grifoni, Voic, et al., 2020)
	Zika virus	ZIKV CD4	Whole proteome	Predicted	209	Grifoni et al., 2017; Grifoni et al., 2020 (Grifoni, Pham, et al., 2017; Grifoni, Voic, et al., 2020)
	Zika virus	ZIKV CD8	whole proteome	Predicted	309	Grifoni et al., 2017; Grifoni et al., 2020 (Grifoni, Pham, et al., 2017; Grifoni, Voic, et al., 2020)
Infectious diseases (Bacteria)	Bordetella pertussis	BP(E)VAC	Acelullar vaccine antigens (FHA, Fim2/3, PRN and PtTox)	Experimentally defined	132	Dan et al., 2016 (Dan et al., 2016)
	Bordetella pertussis	BP(E)R	Non-vaccine antigens proteome	Experimentally defined	170	da Silva Antunes et al., 2023 (da Silva Antunes et al., 2023)
	Clostridium tetani	TT	Tetanus toxoid	Experimentally defined	125	da Silva Antunes et al., 2017 (da Silva Antunes et al., 2017)
	Mycobacterium avium	MAC-specific pool 1–3	Whole proteome	Predicted	628	Lindestam Arlehamn et al., 2022 (Lindestam Arlehamn et al., 2022)
	Mycobacterium avium and Mycobacterium tuberculosis	MAC/Mtb-specific pool 1–3	Whole proteome	Predicted	440	Lindestam Arlehamn et al., 2022 (Lindestam Arlehamn et al., 2022)
	Mycobacterium tuberculosis	MTB300	Whole proteome	Experimentally defined	300	Lindestam Arlehamn et al., 2016 (Lindestam Arlehamn et al., 2016)
	Mycobacterium tuberculosis	Type 1	Whole proteome	Experimentally defined	113	Scriba et al., 2017 (Scriba et al., 2017)
	Mycobacterium tuberculosis	Type 2	Whole proteome	Experimentally defined	122	Scriba et al., 2017 (Scriba et al., 2017)
	Mycobacterium tuberculosis	MTBCD8	Whole proteome	Experimentally defined	113	Pomaznoy et al., 2020 (Pomaznoy et al., 2020)
	Mycobacterium tuberculosis	ATB116	Whole proteome	Experimentally defined	116	Panda et al., 2023 (pre-print)
	Non-tuberculous mycobacteria	NTM-specific pool 1–3	Whole proteome	Predicted	516	Lindestam Arlehamn et al., 2022 (Lindestam Arlehamn et al., 2022)
Allergy	Cockroach	CR	Major cockroach allergens	Experimentally defined	228	Schulten et al., 2019 (Schulten et al., 2019)
	House Dust Mite	HDM	Major house dust mite allergens	Experimentally defined	75	Hinz et al., 2015 (Hinz et al., 2015)

Indication	Pathogen/ Organism	Pool Name	Source	Peptide/Epitope type	Number of peptides	Reference
	Mouse	LoMo	Mouse urinary oligopeptides (Low molecular weight fraction)	Experimentally defined	225	da Silva Antunes et al., 2018 (da Silva Antunes, Pham, et al., 2018)
	Mouse	HiMo	High molecular weight fraction of mouse allergen extracts	Experimentally defined	106	Schulten et al., 2018 (Schulten, Westernberg, et al., 2018)
	Timothy Grass	TG P20	Major TG allergens	Experimentally defined	20	Schulten et al., 2013 (Schulten et al., 2013)
	Timothy Grass	PUTGA P19	Non-dominant TG allergens	Experimentally defined	19	Schulten et al., 2013 (Schulten et al., 2013)
	Cow Milk	MT111	Cow milk extract	Experimentally defined	111	Lewis et al., 2023 (Lewis et al., 2023)
Autoimmuniy	Self	*α*-syn	Alpha-Synuclein	Experimentally defined	13	Lindestam Arlehamn et al., 2020 (Lindestam Arlehamn et al., 2020)
	Self	APOB	Apolipoprotein B	Experimentally defined	20	Roy et al., 2022 (Roy et al., 2022)

Table 3 –

List of antibodies used in Basic Protocol 2

Membrane Antibody	Fluorochrome	Clone/Source/catalog
CD8	BUV496	RPA-T8/BD/612942
CD3	AF532	UCHT1/Life Tech/58-0038-42
LIVE/DEAD	aqua	eBioscience/65-0866-18
CD14	V500	M5E2/BD/561391
CD19	V500	HIB19/BD/561121
CD4	BV605	RPA-T4/BD/562658
CD40L	PerCP-ef710	24-31/eBioscience/46-1548-42
CD69	PE	FN50/BD/555531
OX40	PE-Cy7	Ber-ACT35/Biolegend/350012
CD137	APC	4B4-1/Biolegend/309810