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High throughput cellular screens to interrogate the human T and B cell repertoires

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There is growing consensus for an urgent need to develop efficient methods to analyze the human T and B cell response in order to understand its regulation, to unravel host-pathogen interactions and to design better vaccines. In this review we will consider different methods that have been developed to analyze human T and B cells and focus in particular on those based on high-throughput cell culture, such as memory B cell immortalization, plasma cell cultures and libraries of naïve or memory T cells. These culture-based methods can be used to interrogate with high efficiency the immune repertoires in order to characterize the frequency, function and specificity of naïve or memory T cells and to isolate potent and broadly neutralizing monoclonal antibodies.

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Introduction

In recent years we have witnessed the development of genome-wide approaches to study the human immune response and unravel mechanisms underlying infectious and autoimmune diseases. These approaches applied to complex cell populations in whole blood have led to the identification of distinct transcriptional profiles in systemic lupus erythematosus (SLE) [1], in latent *Mycobacterium tuberculosis* infection [2*] and in the response to vaccination [3], contributing to our understanding of the role of innate immunity and cytokine networks in immune responses. Current efforts are expected to provide novel insights into host–pathogen relationship by identifying mechanisms involved in both resistance and tolerance to infectious agents [4,5*]. Genome-wide approaches have been also applied to the study of antigen-specific lymphocyte populations isolated at different times after antigenic stimulation in both mice and humans and to characterize various

types of polarized T cells and memory subsets [6]. Transcriptional signatures that correlate with the development of memory, chronic stimulation, and cell exhaustion have been identified and revealed, among other principles, the importance of metabolic control of immune responses [7,8*,9–11,12*].

Clonal selection is at the basis of the adaptive immune system and high throughput methods are needed to analyze T and B cells at the single cell level. Deep sequencing of IgVH and TCR β genes has been used to explore the diversity of the T and B cell repertoires and the representation of individual clones among naïve and memory subsets in the same individual and between individuals [13–16]. These sequencing approaches are not problem free [17] and, although providing an in-depth analysis on distribution and representation of individual clonotypes, are limited to the analysis of only one of the two chains of the antigen receptor and therefore do not provide information on the receptor structure and antigenic specificity.

There are several reasons to develop high throughput cellular screens to analyze the human immune response. First, there is a need to understand its metrics, that is, specificity, frequency and class of memory cells required to mediate protection, as well as the individual variability in the magnitude and quality of the response. Second, there is a need to dissect protective from useless or harming responses. The availability of this information is at the basis of rational vaccine design that aims to maximize the response to the most favorable antigens and of the most appropriate class [18]. The basis for this approach, that we refer to as analytic vaccinology, is the production of recombinant antigens that present conserved epitopes in an immunodominant fashion combined with the use of adjuvants that elicit appropriate polarized effector T cells. At the forefront of this approach are current attempts to engineer epitopes of HIV-1 or influenza A virus and to use synergistic combinations of TLR agonists [8*,19,20]. An additional benefit of high throughput cellular screens is the possibility to take advantage of the extraordinary interindividual and intraindividual diversity of the human immune response to isolate potent monoclonal antibodies and T cell clones that can be used therapeutically.

Single cell analysis to dissect the adaptive immune response

Researchers working in the human system have extensively relied on cell culture methods to measure *in vitro*

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antigen driven responses and to isolate antigen-specific T and B cell clones. These methods have been continuously refined, for example with the introduction of fluorescent dyes to track cell division [21] or of an improved B cell immortalization method [22]. A breakthrough in the field came with the advent of flow based technologies to identify antigen-specific T and B cells directly *ex vivo* by staining with fluorochrome-tagged peptide-MHC (pMHC) tetramers [23] or protein antigen [24]. Both cell culture based and antigen-binding approaches can be adapted to high throughput analysis and have distinct characteristics that are particularly useful to address different types of questions. Below we will discuss recent applications of single cell analysis to the study of the adaptive immune response with particular emphasis on the human system (Table 1).

Naïve T cells

The human naïve repertoire is extraordinarily diverse because it contains an enormous number of distinct T cell clones, each represented by only a few cells. The analysis of the naïve T cell repertoire is of both fundamental and practical relevance to determine the extent of self-tolerance and to predict the antigenicity of vaccines and therapeutic proteins. However, this analysis has been hampered by the very low frequency of antigen-specific naïve cells and by their poor responsiveness to antigenic stimulation. Recently a method based on fluorochrome-labeled pMHC tetramers staining followed by magnetic bead enrichment has been used to enumerate antigen-specific naïve T cells in mice and humans [25,26]. Using this method it has been shown that the frequency of CD4⁺ naïve T cells specific for a given pMHC class II complex ranges from 1 in 200,000 to 1 in 2,000,000 [27].

In spite of recent improvements in the production of pMHC tetramers [28] and the development of multiplexed assays [29], this approach is not easily applicable to the study of the T cell repertoire specific for whole pathogens, considering that processing of even a single protein such as tetanus toxoid can give rise to several peptides that bind to the different MHC molecules characteristic of each individual. This limitation can be overcome by the use of a high throughput cellular screen method, which relies on cell cultures and antigen processing and presentation by professional APCs. The method is based on the polyclonal stimulation and expansion of naïve T cell pools in multiple replicate cultures to generate libraries that can be repeatedly screened for the presence of antigen-specific T cells using any antigen presented by autologous monocytes or EBV-immortalized B cells [30]. Using this method naïve CD4⁺ T cells specific for different protein antigens were estimated to range from 1 in 20,000 to 1 in 100,000 while those specific for bacteria ranged from 1 in 3000 to 1 in 10,000. This method has been used to identify naturally processed epitopes, to determine functional avidities among naïve

T cells and to isolate specific T cell clones [30]. A potential advantage of this approach is exemplified by a recent study showing that CD8⁺ T cell clones generated from naïve T cells have superior efficacy in adoptive immunotherapy [31]. In another approach, TCR isolated from naïve or memory T cell clones can be used to engineer antitumor T cells [32].

Effector and memory T cells

The memory T cell repertoire is much less diverse than the naïve repertoire since it is made up by T cell clones that have been expanded in response to antigenic stimulation *in vivo* and have acquired the capacity to persist in the memory pool [33]. The relatively high frequency of antigen-specific memory cells facilitates their characterization using a variety of approaches, ranging from antigenic stimulation *in vitro* to staining with pMHC tetramers. The limit of the tetramer technology has been pushed to the simultaneous detection of 15 pMHC specificities by combinatorial staining [29]. However, it is increasingly clear that antigen-specific T cells are heterogeneous as far as effector function, differentiation stage, and migratory capacity, which may differ even within a single clone [34]. The need to assess multiple parameters on the same cells has driven the development of sophisticated multiparametric analyses. Antigenic specificity, surface molecules, cytokine production, and phosphorylation of specific substrates can be assessed simultaneously using polychromatic flow cytometry for which validated protocols are becoming available [35–37]. Using this approach it has been possible to identify central memory cells and multifunctional T cells as correlates of protection [38,39–41].

The T cell library method described above has also been applied to the study of memory T cells [30]. This high throughput cellular screen allows the single cell analysis of large numbers of effector or memory T cells (typically 500,000 for each subset). The initial polyclonal expansion is instrumental for virtually unlimited screenings (different antigens assessed simultaneously). This approach revealed a distinct compartmentalization of pathogen-specific T cells in memory subsets defined by differential expression of chemokine receptors and cytokine production (Th1, Th2, Th17 or Th22) [42].

Memory B cells

Memory B cells are the progeny of antigen-specific naïve B cells that have been clonally expanded and selected through the germinal center reaction in the course of an immune response. In humans a large fraction of memory B cells carry switched isotypes and have undergone somatic mutations which increase the affinity of binding to the specific antigen. The memory B cell pool is remarkably stable over several years, even for a lifetime, and represents a repository of all antigenic specificities encountered by the individual [43]. The main impetus for the analysis of memory B cells lays in the monitoring of

vaccination and in the isolation of human monoclonal antibodies. Although the antibody response can be readily analyzed in the serum, the fine specificity and cross-reactivity of the antibodies produced can be better appreciated through the clonal analysis of memory B cells. This can be achieved either by isolating antigen-binding B cells and expressing their antibodies in an heterologous system or by immortalizing all memory B cells, analyzing the antibody produced in the culture supernatant, and isolating specific clones.

In the first approach antigen-specific memory B cells are stained with fluorochrome-conjugated antigen, isolated by cell sorting, and the VH/VL genes are amplified by RT-PCR and expressed in an appropriate system to produce recombinant antibodies [44]. This method has found a remarkable application in the systematic analysis of the repertoire of HIV-1 Env-specific antibodies produced by selected patients [45]. Interestingly when reverted to the germline configuration most of the antibodies were found to be polyreactive, indicating that polyreactivity might be required for the initial response to HIV-1 [46^{••}]. In another study a differential cell sorting strategy was used to retrieve, from a highly selected donor, rare memory B cells that bound to a resurfaced gp120 monomer with an intact CD4 binding site, but not to the same monomer carrying a point mutation in the CD4 binding site [47[•],48]. The antibodies retrieved from these memory B cells showed a very broad and potent neutralizing activity against divergent HIV-1 isolates. In summary, B cell sorting represents a powerful method for the isolation of specific monoclonal antibodies that is limited only by the requirement of intact antigens for memory B cell staining and cell sorting.

The second method to make human monoclonal antibodies is to immortalize human memory B cells and screen the antibody released in the culture supernatant in order to isolate a B cell clone producing the antibody of the desired specificity. The efficiency of memory B cell immortalization that can be achieved by EBV and CpG oligonucleotides [22] combined with the use of automated liquid handling devices allows high throughput screening of human monoclonal antibodies. Since the B cell clones release large amounts of antibody in the culture supernatant, the screening can be easily performed using functional assays such as virus neutralization. A relevant example is the isolation of antibodies that potently neutralize human cytomegalovirus infection without prior knowledge of their molecular target [49], an approach that can be generally applied to virtually any complex pathogen. Furthermore, the large number of specific B cell clones isolated using this method is instrumental to identify rare antibodies that show broad reactivity against different serotypes of a given pathogen. This is exemplified by the isolation of potent and broadly neutralizing antibodies to H5N1 influenza virus [50],

dengue virus [51], Group 1 influenza viruses, that is, H1, H2, H5, H9 [52[•]], and respiratory syncytial virus [53]. In the same vein, using an undisclosed method based on memory B cell stimulation combined with a sensitive neutralization screening it has been possible to isolate, from a highly selected patient, very potent and broadly neutralizing HIV-1-neutralizing antibodies that bind to a conformational epitope of trimeric gp160 that is not available on recombinant molecules [54].

There is also considerable interest in developing simple methods to analyze the composition of the human memory B cell repertoire. This approach would considerably extend the classical serological analysis by addressing function, fine specificity and crossreactivity of the antibody response at the clonal level. Immortalization of memory B cells with EBV and CpG or methods based on polyclonal activation using TLR agonists or T cell help has been also used to assess the frequency and fine specificity of memory B cells in limiting dilution assays [55–57].

Plasma cells

Plasma cells are the terminally differentiated nondividing products of the B cell lineage. They constitutively secrete antibodies at high rate and persist in survival niches of the bone marrow thus maintaining serum antibody levels constant [58]. At the peak of a secondary response (day 7 after boosting) antigen-specific plasma cells are also found in large numbers in peripheral blood and therefore represent an accessible source for the isolation of human monoclonal antibodies. Single cell RT-PCR can be used in a high throughput fashion to retrieve paired VH/VL genes from single plasma cells which are then expressed as single chain Fv fragments or intact IgG [59,60]. Using this method it has been recently shown that in immune donors the early plasma cell response to the new pandemic influenza virus is dominated by heterosubtypic antibodies [61[•]], as predicted by the original antigenic sin [62] and consistent with previous observations on heterosubtypic memory B cells [52[•]]. While the automation of this process allows screening of large numbers of plasma cell derived antibodies the method remains labor intensive since the screening to identify the most suitable antibodies can occur only after the antibodies have been expressed. To facilitate the screening process a method has been developed where single plasma cells are maintained alive in microcultures for several days (Corti *et al.*, unpublished data). In these conditions the antibody secreted at a rate of ~100 pg/cell/day accumulates in the culture supernatant at concentrations suitable for performing several parallel screening assays necessary to identify rare specificities.

Future perspectives

As our tools to analyze human T and B cells improve, two main themes emerge. The first is the functional heterogeneity of memory T cell subsets and the

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Table 1

Methods to analyze human T and B cells

Cell type	Methods	Refs	Application
Naive T cells	Fluorescent pMHC tetramers Naive T cell libraries	[25–27] [30*]	Antigenicity studies Naive repertoire analysis
Effector and memory T cells	Fluorescent pMHC tetramers Polychromatic flow cytometry Memory T cell libraries	[29*] [35–37,38*,39–41] [30*]	Correlates of protection Monitoring vaccination Distribution of antigen-specific T cells within memory subsets Identification of target epitopes
Memory B cells	Sorting of antigen-binding B cells, RT-PCR EBV immortalization and screening of supernatants Polyclonal stimulation, screening, and optionally RT-PCR	[44,45,46**,47*,48] [22,49–51,52*,53] [54–57]	Correlates of protection Monitoring vaccination Isolation of broadly neutralizing monoclonal antibodies
Plasma cells	Single cell RT-PCR Single cell culture, screening, and RT-PCR	[59,60,61*] –	Identification of target epitopes

compartmentalization of pathogen-specific memory cells within these subsets. The high frequency of naïve T cells specific for a given pathogen and the presence of characteristic microbial patterns will be instrumental to the development of a pathogen-driven primary T cell responses *in vitro*. It will be important to establish to which extent the *in vitro* primed cells resemble memory cells primed *in vivo* by the same pathogen to validate this approach as a surrogate of a primary immune response. Once validated this method may be used to predict antigenic epitopes and to dissect the innate signal that controls T cell polarization.

The second theme is the extraordinary diversity of the individual B cell response to pathogens. The approach of isolating from selected donors rare B cell clones producing potent and broadly neutralizing antibodies has given a new impulse to passive vaccination and serotherapy and provides important clues for vaccine design. While there is no doubt that serotype-specific antibody responses is driven by immunodominant epitopes that can vary extensively without loss of viral fitness, it is increasingly clear that there are also conserved although subdominant epitopes in viral glycoproteins that elicit neutralizing antibodies and are less prone to mutate. These findings call into question the conventional view of serotypes as absolute barriers for immunization and provide a rationale for attempts to develop vaccines capable of displaying these epitopes in an immunodominant form.

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