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Dissecting the human immunologic memory for pathogens

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Summary: Studies on immunologic memory in animal models and especially in the human system are instrumental to identify mechanisms and correlates of protection necessary for vaccine development. In this article, we provide an overview of the cellular basis of immunologic memory. We also describe experimental approaches based on high throughput cell cultures, which we have developed to interrogate human memory T cells, B cells, and plasma cells. We discuss how these approaches can provide new tools and information for vaccine design, in a process that we define as 'analytic vaccinology'.

Keywords: immunologic memory, T cells, B cells, plasma cells, analytic vaccinology

Introduction

Immunologic memory is an exclusive feature of the adaptive immune system and is based on clonal selection, expansion, differentiation, and persistence of antigen-specific T and B cells. It confers the ability to respond with greater speed and vigor to the re-encounter with the same pathogen and consequently protects the host from re-infection without causing overt disease. Immunologic memory is at the basis of vaccination, which represents the most effective method of preventing infectious diseases and definitely the most important contribution of immunology to human health.

From primary responses to memory cells

Memory T cells, memory B cells, and plasma cells are the progeny of antigen-specific naive cells that have been clonally expanded following antigenic stimulation and survive once antigen has been eliminated. In the course of the primary response, naive T cells are stimulated by antigen presented by activated dendritic cells (DCs). During T-DC interaction in secondary lymphoid organs, the integration of antigenic, costimulatory, and cytokine signals determines the quality of the ensuing immune response, i.e. the magnitude of clonal expansion, the proportion of effector and memory

cells, and their functional properties (1). Besides DCs that produce polarizing cytokines in different proportions and amounts in response to microbial and endogenous danger stimuli (2), other innate cells can participate in the initial phase of T-cell activation. Monocytes, natural killer (NK) cells, and basophils are transiently recruited to lymph nodes, where they can present antigens and produce T-cell polarizing cytokines (3–5).

Only a small fraction of the expanded cells found at the peak of the immune response survive as memory cells. Classical studies demonstrated that the precursors of memory T cells could already be identified at the peak of the primary response as IL-7R^{hi} cells, whereas the more abundant KLRG1^{hi} cells represent short-lived effector cells (6–8). The ratio between effectors and memory precursors is dependent on the strength of stimulation by antigen and cytokines, which in turn determine the differential expression of transcription factors such as T-bet and eomesodermin (9, 10). In addition, the immunosuppressive drug rapamycin administered during the induction phase of the response can selectively expand memory CD8⁺ T-cell precursors at the expense of effector T cells, suggesting the possibility of enhancing memory generation using a licensed drug (11).

Memory T cells are maintained for a lifetime in the absence of the specific antigen and continuously recirculate in secondary lymphoid organs and in peripheral non-lymphoid tissues as central memory (T_{CM}) and effector memory (T_{EM}) T cells, respectively. Their survival is dependent on exogenous cytokines that are present in distinct cellular niches. For CD4⁺ and CD8⁺ memory T cells, the survival cytokines are IL-7 and IL-15, which maintain these cells in a state of slow but continuous proliferation (12). The expression of IL-7 receptor on memory precursors is consistent with a role for this cytokine in rescuing memory cells in the effector to memory transition (13).

Recent studies in the mouse model revealed that some memory T cells may not recirculate but may instead persist in a resting state. After acute infection with herpes simplex virus, specific CD8⁺ T cells remained resident in the skin and in latently infected ganglia, where they efficiently and locally controlled new infection with this virus (14). It has been suggested that such tissue-resident memory T cells provide first line immunity at portals of pathogen entry, such as the skin or vaginal epithelium, providing a protective mechanism that would be of particular relevance for pathogens such as human immunodeficiency virus-1 (HIV-1) (15). Furthermore, in response to weak antigenic stimulation, memory CD4⁺ T cells survived in the bone marrow in contact with IL-7-producing

stromal cells (16). These sessile T cells were non-dividing and could be recalled following secondary challenge, suggesting that antigen or antigen-presenting cells must be carried to the bone marrow.

The B-cell response is initiated at the boundary between T and B-cell areas, where activated T cells, which have been primed by antigen-presenting DCs, encounter antigen-specific B cells that have captured and processed native antigens. The cognate T–B interaction leads to the expansion of antigen-specific B cells and to their differentiation into short-lived plasma cells, which produce unmutated antibodies, usually of the IgM isotype. This extrafollicular response is followed by the formation of the germinal center (GC) reaction. Follicular helper T cells (T_{FH}) drive proliferation, isotype switch, and affinity maturation of antigen-specific B cells, leading to the generation of memory B cells and long-lived plasma cells that produce high affinity somatically mutated antibodies of switched isotypes (17). The GC reaction is driven by persisting antigen on the surface of follicular dendritic cells (FDCs) and can be sustained for several weeks and even months (18). As a consequence, the antibodies with highest affinity may be produced at late time points in the immune response, when the infectious pathogen has already been cleared. This notion is also supported by the finding of a progressive increase in the potency of neutralizing monoclonal antibodies isolated over a period of 2 years following recovery from SARS-CoV infection (Davide Corti, unpublished data).

Plasma cells that emerge from GCs home to the bone marrow, where they survive in niches organized by stromal cells that provide, in association with accessory cells, the attracting chemokine CXCL12, and survival cytokines such as interleukin-6 (IL-6) and a proliferation-inducing ligand (APRIL) (19). Long-lived plasma cells continually secrete antibodies, thus maintaining serum antibody levels constant for the lifetime of the individual. In a study performed on serial samples, serum antibody titers were found to be maintained constant over several decades (20). Interestingly, during infections, new survival niches become transiently available in inflamed organs, providing extra capacity to sustain the specific antibody response (21). There are, however, at least two mechanisms that limit plasma cell survival. First, immune complexes can kill plasma cells by engaging the inhibitory FcγRIIB (22). Second, newly formed plasma cells can effectively compete for survival niches and displace old plasma cells from the bone marrow (19). Indeed, in the steady state, human peripheral blood plasma cells comprise both newly generated and old plasma cells, which are likely to be displaced from the bone

marrow, suggesting a slow turnover of the plasma cell pool (23).

Memory B cells recirculate in secondary lymphoid organs and appear to be slowly dividing (24). A specific survival cytokine, however, has not been defined yet. Human memory B cells proliferate and differentiate into plasma cells *in vitro* in the absence of antigen in response to polyclonal stimuli such as Toll-like receptor (TLR) agonists, CD40 ligation, and common γ -chain (γ c) cytokines, suggesting a possible mechanism for their homeostatic maintenance and for the sustained generation of plasma cells (25). Following antigenic boost, antigen-specific memory B cells rapidly proliferate and differentiate, giving rise to a burst of circulating plasma cells. Typically, these newly generated plasma cells reach peak levels in the blood on day 7, and antibody titers increase concomitantly to reach a plateau on day 10 (25). Intriguingly, we were able to detect rare plasma cells among the newly generated plasma cell pool, which produce antibodies against antigens to which the donor was immune but had not been exposed over the last several years (25). This finding, which is now corroborated by high throughput repertoire analyses (Dora Pinto, unpublished data), would be consistent with a role for polyclonal activation of memory B cells in replacing old plasma cells, which are displaced from the bone marrow (26). Admittedly, this turnover must occur at low rate, since serum antibody levels are not significantly decreased following depletion of B cells by anti-CD20 antibodies.

In humans, a large fraction of circulating B cells express membrane IgM and carry somatically mutated Ig genes. These cells may comprise both *bona fide* T-dependent memory B cells as well as a circulating equivalent of mouse marginal zone B cells that respond to blood-borne pathogens (27, 28). Recent studies in the mouse suggest that B-cell memory appears in IgM⁺ and IgG1⁺ subsets that are present both in GCs as well as outside of B-cell follicles. After secondary challenge, the IgG⁺ subset differentiated into plasma cells, whereas the IgM⁺ subset re-initiated a GC reaction (18). It will be important to translate these studies into the human system and to dissect the cellular basis for the rapid and transient burst of circulating plasma cell observed 1 week after booster immunization (25) and for the secondary response in GCs that develop over a period of several weeks.

Dissecting human memory cells

Studies in experimental animals have exploited the power of genetic approaches to express, delete or track single elements of the immune response and have thus been instrumental in

dissecting basic mechanisms that define the dynamics and anatomy of the immune response and memory generation. However, it is now of importance to translate the concepts learned from experimental models into the human system, taking into consideration the complexity of human genetics and also the sophistication of human pathogens. It is well recognized that there is a need to shift our efforts towards human immunology if we want to fully exploit the power of this system for human health (29).

The study of memory cells in individuals that have developed an adaptive immune response against a given pathogen can provide detailed information about the recognized target antigens and the class of the response. This information is relevant to define the quality of the response, to dissect the mechanisms of immunity versus immunopathology, and to design preventive and therapeutic vaccination strategies. In humans, we can easily assess the properties and functions of memory T and B cells, at least of those which circulate in the blood. Below, we review current approaches to dissect memory T and B-cell subsets and to analyze their antigenic repertoire.

Central memory and effector memory T cells

Cell surface markers have been particularly useful to dissect the functional heterogeneity of human memory T cells. The combinatorial expression of adhesion molecules and chemokine receptors allows for tissue specific homing of memory and effector leukocytes and thus a segregation of the immunologic memory in terms of tissue localization (30, 31). Initial studies in the human system led to the notion that two functionally distinct subsets of memory T cells can be identified based on the expression of lymph node homing receptors (32). T_{CM} cells express CCR7 and CD62L and, like naive T cells (T_N), patrol the T-cell areas of secondary lymphoid organs. T_{CM} have limited effector function but have a low activation threshold, retain high IL-2 production and proliferative capacity, and can rapidly differentiate to effector cells upon encountering the specific antigen. In contrast, T_{EM} cells lack CCR7 and CD62L and express receptors for homing to peripheral or inflamed tissues such as CCR6, CCR4, CXCR3, or CCR5. T_{EM} cells are heterogeneous in terms of homing receptor expression and effector function and comprise the classical T-helper cell subsets Th1, Th2, Th17, as well as cytotoxic T lymphocytes (CTLs). Surface molecules other than homing receptors can be used to further dissect memory subsets. The costimulatory molecules CD28 and CD27 are expressed by T_{CM} and by some T_{EM} cells and are lost on the most differentiated T_{EM} cells

(33, 34). The relative distribution of antigen-specific T cells within T_{CM} and T_{EM} subsets may represent a useful correlate of protection. For instance, an increased frequency of antigen-specific T_{CM} cells producing high levels of IL-2 is characteristic of individuals that control chronic infectious agents such as HIV-1, hepatitis C virus (HCV), and *M. tuberculosis* (35–38).

The expression of tissue-homing receptors also delineates subsets of circulating memory T cells that preferentially home to the skin or gut (30). These receptors are imprinted in developing T cells by DCs that process tissue-derived vitamins (39). Thus, skin-derived DCs convert locally produced vitamin D3 into active metabolites that induce the expression of CCR10, which enables migration towards skin-specific chemokine CCL27 that is secreted by keratinocytes (40). Accordingly, gut DCs convert food-derived vitamin A into retinoic acid that induces the expression of the gut-homing receptors $\alpha 4\beta 7$ and CCR9 (41).

Studies in the mouse system confirmed the differential distribution of T_{CM} and T_{EM} in lymphoid versus peripheral tissues in the steady state (42, 43). In this system, it was found that in particular circumstances, as in inflammatory conditions, T_{EM} cells can enter lymph nodes. Thus, $CD8^+$ T_{EM} cells, which are excluded from resting lymph nodes, can migrate in a CXCR3-dependent fashion to acutely inflamed lymph nodes and kill antigen-presenting DCs, thus curtailing the immune response (44). In addition, $CD4^+$ T_{EM} cells were shown to enter chronically stimulated lymph nodes where they licensed DCs by constitutively expressed CD40L, thus facilitating immune response to non-cognate antigens (45). Taken together, these findings suggest that T_{EM} cells can participate, together with T_{CM} , in secondary immune responses.

The lineage relationship between T_{CM} and T_{EM} has been the subject of intense investigation. The initial finding that antigenic stimulation leads to an irreversible differentiation from T_{CM} to T_{EM} led to the proposal of a linear differentiation model, suggesting that T_{CM} cells are differentiation intermediates that retain proliferative capacity and differentiation potential, while T_{EM} cells are more differentiated cells with limited proliferative potential and differentiation capacity (1). According to this model, T cells differentiate along a one-way linear pathway, the progression being determined by the cumulative strength of stimulation received by T cells. The stochastic interaction with antigen-presenting DCs and the different concentrations of cytokines, to which proliferating cells are exposed, would account for the generation of different fates, even within a single clone. This proposition has been corroborated by new methods that facilitate the analysis of the progeny of single T cells (46, 47).

In several experimental systems, it has been shown that T_{CM} cells confer long-term protection upon adoptive transfer, while T_{EM} cells have only limited reconstitution capacity (48). The self-renewing capacity of T_{CM} cells is consistent with the presence of a 'memory stem cell'. Several studies have addressed the existence of such cells with the aim of identifying the conditions that may promote their generation. For $CD8^+$ T cells, Wnt signaling was shown to arrest effector T-cell differentiation and to promote development of memory stem cells (9).

The response of T_{CM} and T_{EM} cells to cytokines has been initially characterized in the human system (49). Using this approach, it was shown that T_{EM} cells readily proliferate in vitro in response IL-7 and IL-15 but fail to expand substantially due to a high degree of spontaneous apoptosis. In contrast, T_{CM} proliferated and spontaneously differentiated to T_{EM} -like cells, even in the absence of polarizing cytokines (50, 51). These findings are consistent with the notion that the T_{CM} population contains uncommitted precursors with self-renewing capacity as well as cells that are committed to differentiate into Th1 or Th2 in an antigen-independent fashion (pre-Th1 and pre-Th2). The sustained antigen-independent generation of T_{EM} from T_{CM} cells provides a plausible mechanism for the maintenance of a polyclonal and functionally diverse repertoire of T_{CM} and T_{EM} cells, in spite of rapid attrition of the latter.

T-helper cell subsets defined by cytokine production profiles

Functionally, memory T-helper cells can be distinguished according to their cytokine producing capacity, the expression of fate determining transcription factors and homing receptors (52). Some of the properties of these functional modules of adaptive immunity are summarized in Table 1. The first two subsets were identified more than 20 years ago, both in mice and humans (53, 54). Th1 cells produce the signature cytokine IFN- γ , while Th2 cells produce IL-4, IL-5, and IL-13. Th1 and Th2 programs are epigenetically imprinted by T-bet and GATA-3, respectively, and the distinct cytokine profiles are stably maintained upon restimulation in the absence of polarizing or maintenance factors. Th1 and Th2 responses are tailored to the elimination of different microbial pathogens, namely intracellular pathogens and viruses in the case of Th1 and helminths in the case of Th2. In addition, both lineages differ in the expression of chemokine receptors and therefore in homing abilities. CCR5 and CXCR3 are expressed by Th1 cells and CCR4, CCR3 and CRTh2 by Th2 cells – a property that is coordinately acquired during T-cell differentiation (55).

Table 1. Phenotypical and functional heterogeneity of CD4⁺ T-helper cells

Subset	Tx factor	Effector molecules	Homing receptors	Target cells	Function
Th1	T-bet	IFN- γ	CXCR3, CCR5	Macrophages	Bacteria
Th2	GATA-3	IL-4, IL-5, IL-13	CCR4, CCR3, CRTh2	Eosinophils	Parasites
Th17	ROR γ t	IL-17, IL-22, GM-CSF	CCR6 ⁺ CCR4	Neutrophils	Bacteria and fungi
Th9	PU.1	IL-9	n.d.	Mast cells	Helminthes
Th22	AHR	IL-22	CCR4 ⁺ CCR10	Epithelia	Skin protection?
T _{FH}	Bcl-6	IL-21	CXCR5	B cells	Antibody responses
Treg	FoxP3	TGF- β	CCR7, CCR6, CCR4	DC/T cells	Regulation
Tr1	n.d.	IL-10	CX3CR1	T cells	Regulation

n.d., not determined.

Additional T-helper cell subsets have been identified over the last few years. Th17 cells produce IL-17 as the hallmark cytokine as well as IL-22 and granulocyte-macrophage colony-stimulating factor (GM-CSF) (56–58). They express the transcription factor ROR γ t (59), are required for the elimination of fungi and extracellular bacteria (60, 61), and have distinct homing abilities based on preferential expression of CCR6 (62). Recently, Th22 cells have emerged as another T-helper subset based on the expression of IL-22 and the transcription factor aryl hydrocarbon receptor in the absence of IL-17 and ROR γ t (63–65). These cells have been implicated to play an important role in the skin due to the coordinate expression of the skin-homing receptors CCR10 and CLA and the production of IL-22, a cytokine, which stimulates production of antimicrobial peptides by keratinocytes (66, 67). Interestingly, Th22 cells comprise CD1a-autoreactive cells (68), suggesting recognition of lipid antigens presented by Langerhans cells in the skin. Th9 cells, which are characterized by IL-9 production, are specialized in the elimination of helminths and driven by the master regulator Pu.1 (69).

IL-10 was originally reported to be produced by Th2 cells but later found to be produced by virtually all T cells, including Th1 and T cells with regulatory functions (T_{reg} and T_r1) (70). IL-10 production by regulatory T cells was shown to be required for inhibition of Th1-dependent colitis elicited by gut flora in immunodeficient mice reconstituted with naive T cells and to influence host–pathogen interaction (71). IL-10 favors the persistence of intracellular bacteria and the establishment of a pathogen carrier state, while deletion or blockade of IL-10 leads to clearance of parasites, such as *Leishmania major* in mice (72). Recent studies revealed that in the case of infection by Th1-inducing intracellular pathogens such as *Toxoplasma gondii* and *Leishmania major*, the main source of host-protective IL-10 are Th1 cells, a finding that points to an intrinsic capacity for self-regulation by effector T cells (73). IL-10 production in Th1 cells is elicited by IL-12 and high antigen dose and is dependent on sustained ERK1 and ERK2

phosphorylation (74). It remains to be established whether fully differentiated Th17 cells, which are highly inflammatory, have a similar built-in mechanism of IL-10-mediated self-regulation.

While the classification of T-helper cell subsets represents a useful tool to functionally dissect the T-cell response, one has to consider the frequent occurrence of memory cells with multiple overlapping cytokine patterns that do not fit the current T-helper classification. Human T cells that produce IL-4 and IFN- γ have been described. They can be generated *in vitro* upon restimulation of polarized Th1 or Th2 cell clones under opposite polarizing conditions. This is accompanied by remodeling of the target cytokine genes (75). These studies, which have recently been reproduced in the mouse (76), demonstrate that effector T cells maintain the memory of the cytokines initially imprinted but remain capable of acquiring further cytokine production capacity by expressing the relevant transcription factors and by remodeling the target cytokine genes. This model is further supported by the finding that a small subset of Th2 cells has lost the capacity to differentiate into Th1 cells due to loss of inducible T-bet expression (75). Another example of memory T cells with double identity is provided by cells, that co-produce IL-17 and IFN- γ and express both CCR6 and CXCR3. This population is prominent in the gut and contains cells that proliferate in response to the mycobacterial antigen purified protein deriviate (PPD) (62, 77).

Heterogeneity of CD8⁺ T cells

Memory CD8⁺ T cells produce a variety of cytokines and possess cytolytic activity but are typically not categorized in distinct effector lineages. Several studies, both in mice and humans, demonstrated that the assessment of multiple parameters of CD8⁺ T-cell function could predict the outcome of vaccination or infection. Multiparameter flow cytometry can be used to simultaneously assess the production of several cytokines (IFN- γ , IL-2, and TNF) and chemokines (CCL4) in

antigen-specific memory T cells and expression of surface molecules such as CD107 as a surrogate marker of cytotoxicity. An inverse correlation was observed between the frequency of multifunctional CD8⁺ T cells and persistence of antigen load in chronic viral infection (78).

Another functional state of memory cells has been observed in settings of chronic infections by pathogens that have evolved strategies to resist acute innate and adaptive immune attacks. As a result of pathogen persistence, specific CD8⁺ T cells exhaust their cytokine production and proliferative capacity (79–81). Sustained negative signaling by the inhibitory receptor PD-1 has been mechanistically implicated in T-cell exhaustion. The role of PD-1 is illustrated by the reversal of T-cell exhaustion and concomitant increase in proliferation, cytokine secretion and cytotoxicity, and pathogen clearance upon blockade of PD-1 signaling with anti-PD-L1 antibody (82). Besides PD-1, other negative regulators, including CTLA-4, 2B4, and LAG-3, are expressed in chronically activated T cells and have been implicated in T-cell exhaustion (83).

Interrogation of memory T cells

Memory T and B cells represent a repository of the antigenic experience of the individual. Defining the distribution of antigen-specific memory cells in different memory compartments can provide useful information about the *in vivo* response following infection or vaccination. We describe below some approaches that we have used to analyze the antigenic repertoire of human memory T-cell subsets.

Using a carboxyfluorescein diacetate succinamidyl ester (CFSE) dilution assay, we observed that pathogen specific memory T cells are found predominantly within distinct memory subsets. For instance, T cells specific for *Candida albicans* were enriched in a CCR6⁺ Th17 cell subset, while T cells specific for *M. tuberculosis* were exclusively present within a CCR6⁺ CXCR3⁺ compartment, which contains cells that produce IL-17 and IFN- γ (62).

More recently, we established a method for high throughput interrogation of memory T cells (84). T-helper cell subsets isolated according to the differential expression of surface markers are distributed in multiple cultures and polyclonally activated and expanded in order to obtain a library of amplified T-cell blasts (Fig. 1). Individual cultures of the library can then be repeatedly screened in parallel for their capacity to proliferate in response to a variety of antigens presented by autologous monocytes or Epstein–Barr virus-immortalized B cells. This method is suitable for the analysis

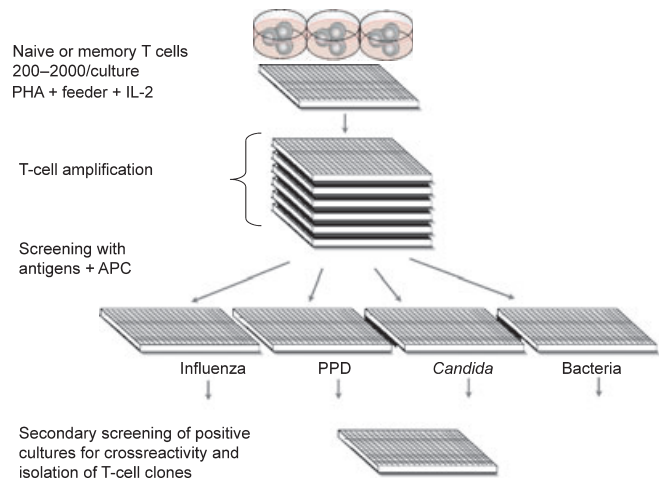


Fig. 1. Preparation and screening of T-cell libraries. Naive and memory T-cell subsets are isolated by cell sorting and clonally expanded in multiple replicate cultures, typically a few hundred. In a primary screening: each culture is tested for the capacity to proliferate in response to antigen in the presence of autologous antigen-presenting cell (either monocytes or Epstein–Barr virus-immortalized B cells). This process can be repeated as many times as necessary. Positive cultures are retrieved from the library and re-tested to determine crossreactivity and avidity, to identify the antigens and epitopes recognized and to isolate T-cell clones. In all cases, the frequency of specific T cells can be estimated.

of T-cell responses to complex antigens, even whole microbial organisms, presented by any HLA molecule.

The power of this approach is exemplified in the experiment shown in Fig. 2, in which libraries prepared from five memory CD4⁺ subsets from a healthy donor were interrogated with five different pathogens. For each pathogen, the responding cultures, as detected by thymidine incorporation, were found to segregate into one or more libraries. Memory CD4⁺ T cells specific for influenza virus were found in two distinct Th1 subsets, while cells specific for PPD were almost exclusively present in the CCR6⁺ Th1 subset. Memory T cells specific for *Candida albicans* were most prominent in the Th17 library, consistent with previous data (62), but positive cultures were also detected in CCR6⁺ Th1, Th2, and Th22 libraries. Memory T cells specific for bacteria were found in several libraries, although primarily those derived from Th17 and CCR6⁺ Th1. In different individuals, the pattern of distribution of pathogen-specific T cells within the memory compartments was fairly consistent, however, with some variability in the frequency, most likely reflecting the different antigenic experience (Federico Mele, unpublished data). This approach is particularly suitable for follow-up experiments that aim at further defining the specificity of responding T cells. For instance, cultures that score positive for a given pathogen can be reassessed for their reactivity against different strains or species of the pathogen to determine the extent of

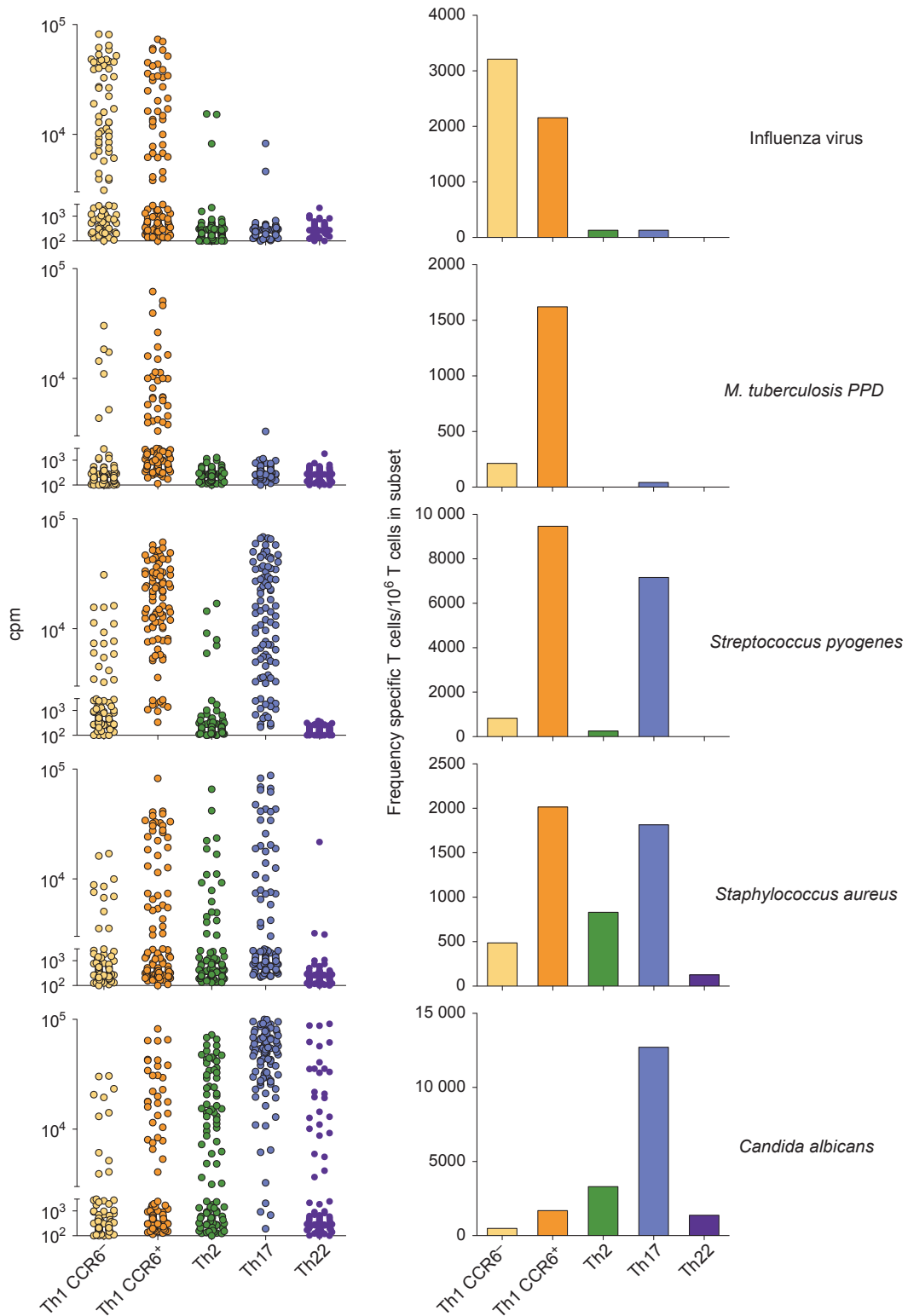


Fig. 2. Screening of memory T-cell libraries reveals pathogen-specific signatures. Memory Th1 (CCR6⁺ and CCR6⁻) Th2, Th17, and Th22 cells were isolated from a healthy donor according to the expression of surface markers. Libraries were prepared and screened for the proliferative response to five antigens in the presence of autologous monocytes. For each library, the proliferation of individual cultures is shown in the left panels and the estimated frequency of specific T cells on the right panel.

crossreactivity and to isolate crossreactive or strain-specific T-cell clones. The same cultures can also be tested with different antigenic fractions to determine the relationship between the class of the response (i.e. Th17 versus Th2) and the nature of the antigen (i.e. structural versus secreted proteins).

T-cell libraries were also prepared with naive T cells and were used to determine frequency and functional avidity of T cells specific for a variety of proteins and viruses as they occur in the naive repertoire (84). Thus, naive T-cell libraries can be used to predict antigenicity of complex antigens and to identify T-cell epitopes that are generated by the antigen-processing machinery and selected by HLA molecules.

Human monoclonal antibodies

For many years, the isolation of monoclonal antibodies represented the rate-limiting step in the analysis of the human antibody response. In recent years, however, several methods have become available to isolate with high efficiency human monoclonal antibodies. These methods follow two distinct approaches. The first is to isolate by cell sorting antigen-binding B cells or total plasma cells and to ‘rescue’ their antibody genes by single cell reverse transcriptase polymerase chain reaction (RT-PCR), which are then expressed in a heterologous system (85–87). This method is quite effective but is limited by the number of cells that can be processed.

We developed a different approach based on cells culture (Fig. 3). Human memory B cells are immortalized using Epstein–Barr virus (EBV) in the presence of a TLR agonist, such as CpG (88). In these conditions, approximately 30% of IgG⁺ memory B cells develop into an antibody-secreting clone that releases large amounts of antibodies into the culture supernatant. In another setting, plasma cells isolated from peripheral blood after a booster immunization are cultured in

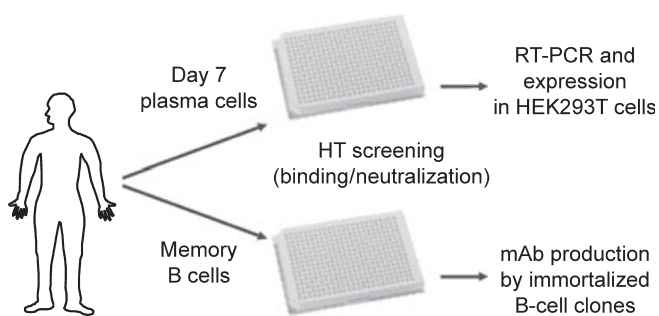


Fig. 3. High throughput cell culture methods can be used to isolate antibodies from memory B cells and plasma cells. Plasma cells are collected following antigenic boost, typically on day 7, and cultured for several days in conditions that preserve their viability. Memory B cells can be collected at any time following immunization and can be efficiently immortalized using Epstein–Barr virus in the presence of CpG.

conditions that maintain their viability and constitutive antibody production for several days, thus allowing convenient screening of the culture supernatant. The advantage of these culture-based methods is that large numbers of cells (typically $>10^4$) can be screened for antigen specificity using multiple assays, including functional assays. This is instrumental for the rapid identification of rare specificities, thus limiting the labor-intensive cloning and transfection to selected cases.

Using the B-cell immortalization method, we have isolated potent neutralizing antibodies to emerging pathogens such as SARS-CoV and H5N1 (88, 89) as well as broadly crossreactive antibodies against dengue and influenza A viruses (90, 91). Broadly neutralizing antibodies against influenza virus bound conserved epitopes in the stem region of the hemagglutinin (HA) and neutralized both *in vitro* and *in vivo* viruses belonging to different HA subtypes within group 1 viruses (91). Although less potent than classical antibodies directed against the globular head, these heterosubtypic antibodies did not select escape mutants, indicating that the virus cannot mutate this epitope without losing fitness. These findings suggest that human heterosubtypic antibodies might be suitable as single agents for passive serotherapy as well as probes to design HA molecules that display the conserved epitope in an immunodominant fashion. Interestingly, a headless hemagglutinin vaccine has been produced and has been shown to confer broad protection in an animal model (92).

Two studies further illustrate the potential of this approach for the identification of neutralizing targets within complex pathogens. By selecting monoclonal antibodies with the highest neutralizing activity against human cytomegalovirus (HCMV), we were able to identify several conformational epitopes on a pentameric complex, which represents a candidate subunit vaccine (93). Likewise, we were able to isolate from memory B cells of multiparous women a panel of monoclonal antibodies to VAR2CSA, a molecule expressed on the surface of *Plasmodium falciparum* infected erythrocytes that mediates adhesion to the placental endothelium (94).

Human monoclonal antibodies could be used therapeutically for passive or active vaccination. In a passive vaccination setting, neutralizing antibodies could be administered either as single agents or as a cocktail to minimize the selection of escape mutants. Monoclonal antibodies that broadly neutralize the four DENV serotypes were engineered to remove the Fcγ receptor-binding site. These antibodies retained virus neutralizing activity while losing infection-enhancing activity and were protective in a mouse model of lethal dengue infection (90). In an active vaccination setting, antibodies may be administered together with a vaccine to enhance the T-cell

and eventually the B-cell response. Indeed, antibodies are known to increase antigen uptake by targeting immune-complexes to Fc γ receptors expressed by antigen-presenting cells (95) and were shown to enhance B-cell responses to their target antigens by several hundredfold, depending on isotype and stoichiometry (96).

The current prevailing view is that protection against intracellular pathogens is exclusively mediated by CD4⁺ and CD8⁺ T cells, while antibodies do not play a significant role. However, it is worth reconsidering old studies showing that antibodies can promote fusion between lysosomes and phagocytic vesicles. In the case of *Toxoplasma gondii*, which resides in an intracellular vacuole that is unable to fuse with lysosomes, it has been shown that the fusion block can be overcome by IgG antibodies that engage Fc γ receptors and promote phagolysosomal fusion (97). Similarly, antibodies may be effective against other pathogens that possess a fusion escape mechanism such as *Mycobacterium tuberculosis* (98). Another mechanism by which antibodies can enhance the immune response to intracellular pathogens is by facilitating antigen uptake, processing and presentation to T cells (95). It is therefore foreseeable that human monoclonal antibodies may be used as adjuvant to enhance pathogen clearance and T-cell immunity.

From immunologic memory to vaccine design

Studies from several laboratories including our own suggest that it is possible to leverage on the analysis of human memory T and B cells to identify the antigens that are targeted by effector T cells and neutralizing antibodies and to identify the class of the respective immune response, thus providing a rational basis for vaccine design. This process of 'analytic vaccinology' takes advantage of the high throughput methods that we have described in this review.

Two pieces of information are crucial for the design of an effective T-cell-based vaccine: the immunodominant antigens and the class of T-cell polarization, which mediates protection. Predicting immunodominance has proved a difficult task, considering the constraints of antigen processing, the polymorphism of HLA molecules, as well as the multiplicity of proteins produced by a given pathogen in different amounts and in different compartments. Likewise, predicting the correct class of response has proven to be difficult, in view of the variety of pathogen-associated innate stimuli that provide polarizing cues for differentiation of effector T cells.

In this review, we outlined an experimental approach to achieve this relevant information. Using the T-cell library method, we can analyze memory T cells from immune donors

to identify the class and specificity of *in vivo* primed T cells. This approach can be implemented by cell culture experiments that aim at reconstructing *in vitro* the primary response from its key elements, namely naive T cells, antigen-presenting cells, and pathogens. By analyzing and perturbing these cultures, it is possible to gain insights into the nature of the innate signals and the polarizing cytokines involved in pathogen-specific responses (Christina Zielinski, unpublished data).

For antibody-based vaccines, it is essential to identify the antigens that elicit neutralizing antibodies and manufacture them in a way that preserves the correct conformation. A particular challenge is posed by variable pathogens such as influenza virus or HIV-1 that can easily escape the neutralizing antibody response (99). The new methods for high throughput analysis of the human antibody response offer, for the first time, the possibility of isolating broadly neutralizing antibodies that target conserved epitopes. Once such antibodies and the target epitopes are identified, it should be possible to produce antigens that display the target epitope in an immunodominant fashion (Fig. 4). This approach should therefore focus the immune response to the most conserved protective epitopes. Human monoclonal antibodies can be instrumental not only as discovery tools to identify the relevant epitopes but also as quality control tools to ensure that the recombinant vaccines produced retain the correct conformation. This is particularly important in attempts to reconstruct conformational epitopes using recombinant proteins expressed in heterologous systems or on molecular scaffolds (100).

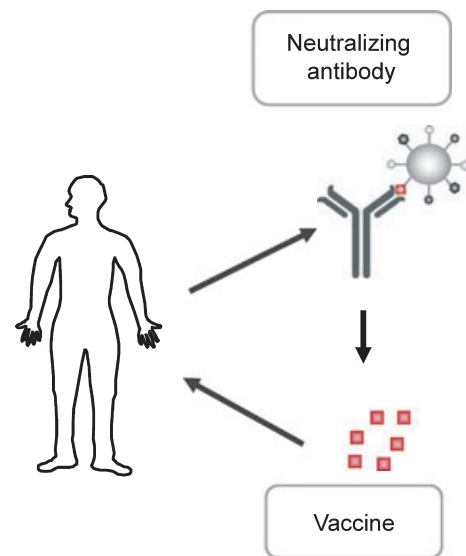


Fig. 4. Analytic vaccinology. Human monoclonal antibodies selected for properties such as potency, breadth, and failure to select escape mutants can be used not only as prophylactic or therapeutic drugs but also as tools for vaccine design to identify the target antigen and to optimize the expression of the relevant epitope.

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