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Understanding and making use of human memory B cells

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Summary: The work of our laboratory has focused on the study of human memory B cells. Using an *in vitro* approach we dissected the triggering requirements of B cells and unveiled a distinct role for TLRs in the activation of naive versus memory B cells. Using an *ex vivo* approach we analyzed the dynamics of memory B cells and ASCs and the kinetics of serum antibodies during secondary immune responses and in steady state conditions and used these quantitative data to build up a model of serological memory. According to this model memory B cells behave as 'stem cells' capable of generating plasma cells and antibodies in an antigen-dependent as well as in an antigen-independent fashion. Finally we developed an efficient method to interrogate human memory B cells and to isolate human monoclonal antibodies. This method can be exploited for the production of neutralizing antibodies for serotherapy and for "analytic vaccinology".

Introduction

T-cell-dependent antibody responses are initiated when B cells receive stimulatory signals from the antigen-triggered B-cell receptor (BCR) and CD40, which is triggered by CD40L upregulated on T-helper cells upon cognate interaction with B cells. Productive signaling events lead to B-cell expansion, to the generation of short-lived antibody-secreting cells (ASCs), and, following the germinal center reaction, to high-affinity memory B cells and long-lived plasma cells (1–4). Soon after antigenic stimulation, serum antibodies reach a peak level and are subsequently maintained at lower but constant levels for a lifetime. The maintenance of serum antibody level after immunization, also defined as 'serological memory', is the desirable output of vaccination, because it provides immediate protection against pathogens or toxins. The cellular bases of this phenomenon, however, remain to be defined. Below we review data from our laboratory regarding the activation requirements of naive and memory B cells and the *in vivo* dynamics of memory B cells, ASCs, and serum antibodies during an immune response and in the steady state. We also

discuss a model of serological memory based on memory B cells and their capacity to proliferate and differentiate to ASCs in response to antigen-independent environmental signals. We finally illustrate an example of how memory B cells can be interrogated and exploited to make human monoclonal antibodies.

Differential requirements for the activation of memory and naive B cells

Human memory and naive B cells can be identified using a combination of markers that include immunoglobulin (Ig) isotypes, CD27, which is expressed on most memory B cells (5), and ABCB1, a multidrug transporter molecule that precisely discriminates naive from memory and transitional B cells (6).

More than 20 years ago, we demonstrated that human memory B cells proliferate and differentiate to ASCs in response to bystander T-cell help, that is to $CD4^+$ T-cell clones activated by a third-party antigen, which stimulate B cells in a non-cognate fashion via CD40L and cytokine production (7, 8). The results of these early studies have been subsequently confirmed and extended by other laboratories (9, 10). More recently, we found that memory B cells constitutively express Toll-like receptor 2 (TLR2), TLR6, TLR7, TLR9, and TLR10 and, when triggered by the corresponding TLR agonists, such as CpG or R848, proliferate and differentiate to ASCs (11, 12). Differences among memory B-cell subsets were observed. For instance, IgM memory B cells were able to respond to CpG in the absence of cytokines, while switch memory B cells did so only in the presence of interleukin-2 (IL-2) or IL-15. In contrast, naive B cells did not express TLRs constitutively and did not respond directly to microbial products. However, they rapidly upregulated TLRs upon BCR stimulation (12). These data suggested that microbial products play a role in memory B-cell homeostasis.

Using the ABCB1 marker to isolate highly pure populations of human naive B cells (6), we recently addressed the requirements for naive B-cell activation (13). We found that BCR triggering (by anti-Ig) and cognate T-cell help (provided by $CD4^+$ T cells recognizing a superantigen on B cells) were not sufficient to induce full cell expansion and differentiation. The accumulation of proliferating B cells, isotypic switch to IgG and IgA, and differentiation to ASCs required a third signal that was efficiently delivered by agonists that triggered any of the TLRs that are upregulated in naive B cells upon BCR triggering. TLR agonists acted directly on B cells and were required irrespective of the nature of T-helper cells present. Supernatants of dendritic cells (DCs) stimulated by DC-specific TLR agonists were also capable of enhancing B-cell responses, although to a much lower and variable extent. The requirement for TLR engagement for human naive B-cell activation is consistent with a recent report in the mouse system (14). However, it is worth noting that there are important differences both in the pattern and in the regulation of TLR expression between humans and mice. For instance, mouse naive B cells constitutively express TLRs and proliferate in response to lipopolysaccharide (LPS) (15) or CpG (16) in the absence of antigenic stimuli. The coupling of BCR stimulation to TLR expression endows the human immune system with a high degree of specificity, because it allows focusing of innate signals only on antigen-stimulated naive B cells.

In conclusion, while human naive B cells require three signals to be activated, two of which are antigen specific, memory B cells can be activated to proliferate and differentiate in an antigen-independent fashion by microbial products, cytokines, bystander T-cell help, and, possibly, other stimuli yet to be defined (*Fig. 1*). The fact that all human memory B cells can be polyclonally activated by a variety of environmental stimuli may be related to the high spontaneous *in vivo* turnover of these cells, as discussed below.

Mechanisms that maintain serum antibody levels

Serum antibodies represent a particularly informative and easy-to-measure parameter to evaluate the immune status of

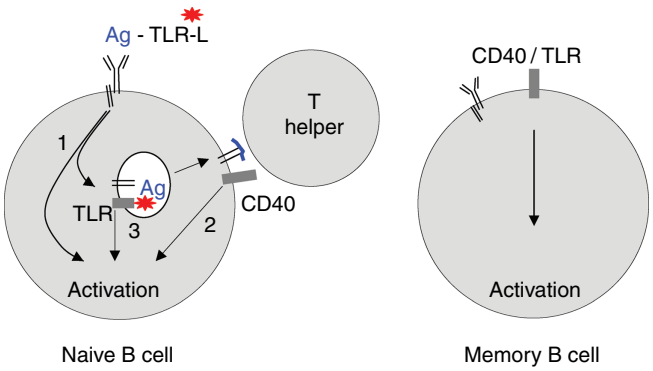

Fig. 1. Differential requirements for the activation of human naive and memory B cells. The activation of naive B cells (left) requires the integration of three signals: signal 1 delivered by antigen through B-cell receptor (BCR), signal 2 delivered by antigen-specific T-helper cells via CD40 following cognate interaction, and signal 3 delivered by microbial products acting on Toll-like receptors (TLRs). Note that naive B cells do not express TLRs, which are induced by signal 1. The activation of memory B cells (right) can be effectively triggered in the absence of BCR stimulation via TLRs or bystander T-cell help.

an individual, to reveal previous antigenic exposure, and to estimate the degree of protection. In spite of the wide use for diagnostic purposes and to monitor the efficacy of vaccination, little is known on how serum antibody level is regulated at different times after immunization and how it can be maintained constant in the absence of antigenic stimulation over a long period of time.

Mouse studies have identified two distinct mechanisms that can contribute to sustain serum antibody level. The first is the longevity of plasma cells (4, 17). One study demonstrated that antigen-specific plasma cells can survive in appropriate niches of the bone marrow for several months without dividing (18). Another study showed that the serum antibody level that is reached following immunization is maintained constant for up to one year but declines about 10-fold if the mice are irradiated, indicating that radiation-resistant plasma cells can survive with a half-life of approximately 130 days (19). The latter result suggests that dividing cells contribute to the maintenance of serum antibodies, a mechanism that can be particularly significant if the time of observation exceeds one year.

Another mechanism that sustains antibody levels is continuous antigenic stimulation by persisting antigen (20). Mice immunized with a protein antigen show a very transient antibody response in the serum, while the same antigen expressed by a live virus induces sustained antibody levels (21). Antigen persistence on follicular DCs (FDCs) may be relevant to this phenomenon, but the role of these cells in maintenance of serum antibody level remains to be addressed. Although mouse studies have been very informative, one should consider the great differences in lifespan and degree of environmental antigenic exposure between humans and experimental mice raised in specific pathogen-free environments.

Human studies have shown that the peak level of serum antibodies that is reached following acute infection or immunization declines initially over a period of a few months, but serum antibodies are then maintained at constant level for decades and eventually for a lifetime in the absence of additional antigenic stimulation. A striking example is the fact that serum antibodies to vaccinia virus (22), as well as vaccinia virus-specific memory B cells (23), are still detected more than 50 years after vaccination, in spite of the fact that this virus is highly cytopathic, is non-persistent, and does not circulate in the population. Several considerations suggest that the longevity of plasma cells and/or persistence of antigen are not sufficient to account for the extraordinary longevity of human serological memory. For instance, if human plasma cells were to survive for a lifetime, then their mass (and consequently total serum Ig levels) should increase steadily with the age of the individual, a phenomenon that is not observed. Furthermore, it has been recently shown that upon booster immunization, newly formed ASCs displace old plasma cells from the bone marrow (24). This finding is consistent with a substantial turnover of plasma cells in the bone marrow dependent on antigenic stimulation and to competition for survival niches between resident plasma cells and new plasma cells migrating to the bone marrow from the periphery.

If long-lived plasma cells in the bone marrow continually turn over, then homeostatic mechanisms must exist to sustain serum antibody level. By combining two quantitative assays to measure the amount of serum IgG and the frequency of IgG-producing memory B cells in the blood, we found that under steady state-conditions (i.e. at least one year after antigenic boost), there was a striking correlation between the frequency of antigen-specific B cells in the memory pool and the antigen-specific IgG levels in serum (11). This correlation was found for different antigens and in individuals that have wide distribution of serum antibodies and memory B cells following a standard vaccination. This observation suggested that under steady-state conditions, memory B cells, plasma cells, and antibodies are part of a homeostatic system and maintain their pool size by continual division and differentiation in the absence of antigen. Below we review evidence for this homeostatic maintenance of serological memory.

Memory B-cell dynamics in the absence of antigen

A homeostatic maintenance of serological memory requires that the entire memory B-cell pool continuously divides and differentiates in response to antigen non-specific stimuli, a fact that is consistent with the previously described activation properties of memory B cells. Recent studies using *in vivo* labeling with heavy glucose (25) or *ex vivo* Ki67 expression (6) indicated that approximately 2% of human memory B cells divide every day. A subset of mouse memory B cells, called preplasma cells, was also shown to proliferate and differentiate and did so in the absence of antigen (26, 27).

We found that under steady-state conditions, there is a continuous low rate production of ASCs that can be detected in peripheral blood (approximately 200 per million mononuclear cells) (11) (*Fig.* 2). These ASCs are $Ki67^+$ and secrete antibodies of memory specificities, all proportionally to the frequency of specific memory B cells (11). We also found that upon tetanus toxoid (TT) boost, the

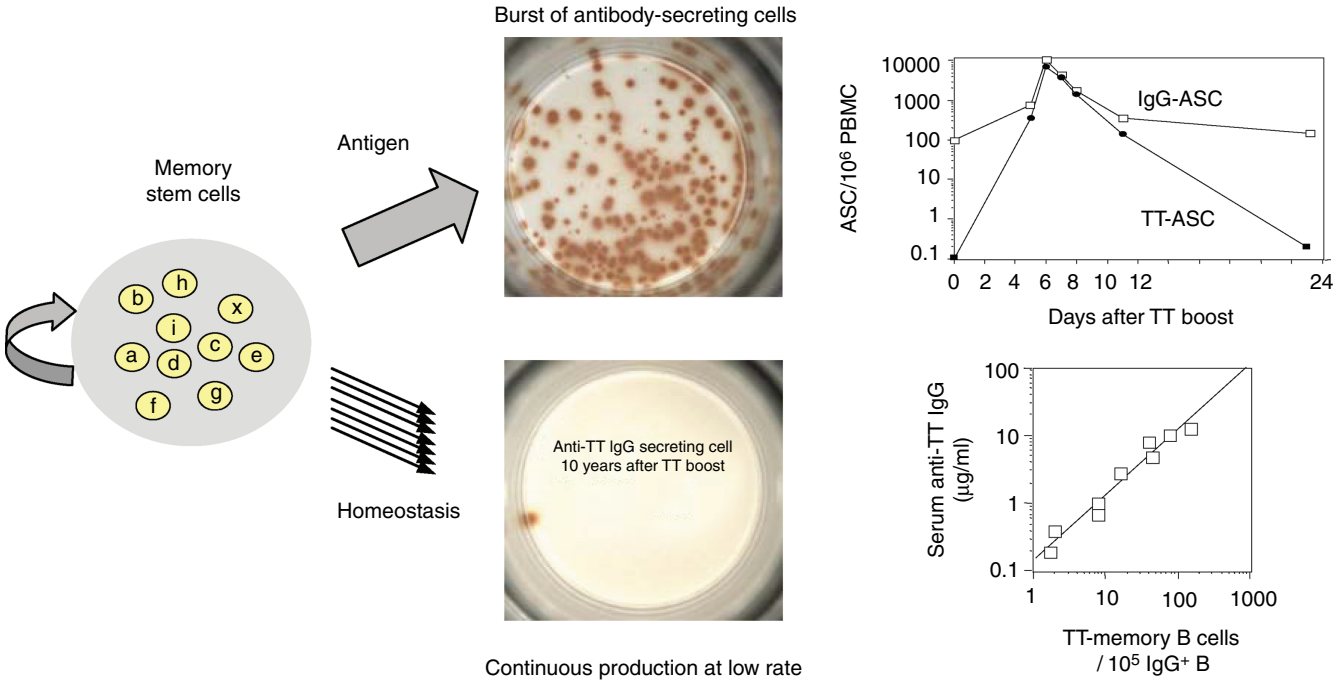

***Fig. 2.* Dynamics of memory B cells and antibody-secreting cells (ASCs) in the presence or absence of antigen.** Upon antigenic boosting (upper panels), specific memory B cells selectively proliferate and differentiate generating a burst of ASCs (measured by enzyme-linked immunospot assay analysis) that peaks on day 6 and day 7. In the absence of antigen (lower panels), all memory B cells turn over at low rate and continually spill out ASCs of all memory specificities. In this condition, there is a good correlation between the frequency of memory B cells and serum antibody levels. Shown are tetanus toxoid (TT)-specific ASCs 6 days and 10 years after a booster immunization.

number of circulating ASCs producing antibodies of other unrelated memory specificities increased. This finding is consistent with an increased polyclonal activation of all memory B cells, possibly due to increased bystander T-cell help. It is of note that the transient and polyclonal nature of this bystander response is not expected to lead to a significant increase in serum antibody levels.

Taken together, the above findings delineate an antigen-independent mechanism for continuous plasma cell generation and homeostasis that can contribute to maintain serum antibody levels. It is important at this point to understand the differential contribution of antigen-dependent and antigen-independent mechanisms to the serological response and memory.

Memory B cell, ASC, and antibody dynamics following booster immunization

To obtain quantitative data that would allow us to discriminate antigen-dependent and antigen-independent components in the serological response, we studied the dynamics of memory B cells and ASCs and the kinetics of serum antibody level following immunization (*Figs 2 and 3*). We found that upon boost with TT, large numbers of TT-specific ASCs (up to 10^4/ml) appeared in peripheral blood on day 6 and day 7 and returned to baseline levels by day 15. TT-specific serum IgG antibodies increased 50- to 100-fold from day 5 to day 10, reaching levels as high as 100 μg/ml. The antibodies remained at plateau levels for approximately one month and declined progressively over a period of six to eight months with a half-life of approximately 40 days to reach eventually a plateau that was stable for the rest of the observation time, in some cases up to one year. The plateau was 10- to 50-fold lower than the peak levels but was higher than the preboost level and correlated with an increased frequency of specific memory B cells.

A detailed analysis of the above experimental data allowed us to draw several conclusions. First, antigen boosting induces a rapid increase in serum antibodies that was sustained only for a few months. This is in contrast to the mouse system, where serum antibodies are maintained constant at peak levels for about a year. Second, most of the antigen-induced ASCs, which are responsible for the rapid increase in serum antibodies, are short lived and are eliminated, as no further increase is observed after day 10. If, however, all ASCs were short lived, serum antibody levels should start to decline on day 10 with a half-life of approximately 20 days, which is the half-life of human IgG1, the predominant isotype induced by

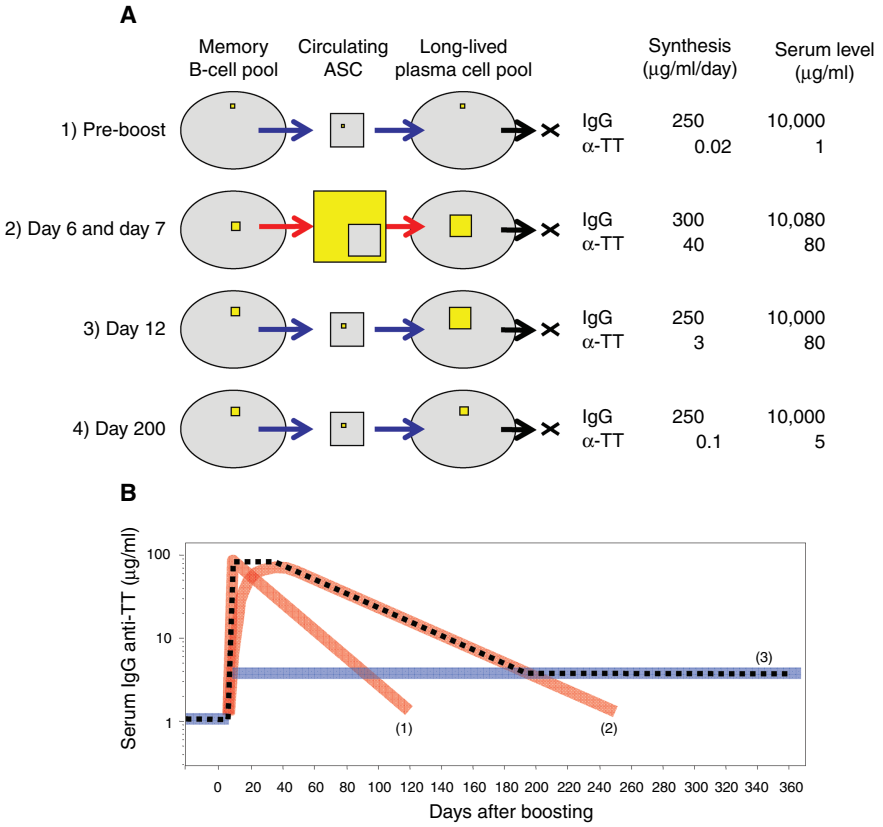

***Fig. 3.* Kinetics of memory B cells, antibody-secreting cells (ASCs) and serum antibodies following a booster immunization.** (A). In the absence of antigenic stimulation (1), memory B cells are in a dynamic equilibrium with plasma cells and antibodies. On day 6 and day 7 after booster immunization (2), large numbers of memory B cells are generated in an antigen-dependent fashion (red arrows). Some of these cells enter the bone marrow in part by normal turnover or by displacing old plasma cells, while most die by day 10. On day 12 (3), there is a large population of long-lived plasma cells that are rescued in the bone marrow. These cells slowly disappear until a new equilibrium is reached (4). The fraction of antigen-specific B cells is shown in yellow, while the total B-cell pool is in gray. Red and blue arrows indicate antigen-dependent and antigen-independent processes, respectively. Synthetic rate of IgG and serum antibody levels is indicated on the right side. (B). The experimental curve of antibody response following TT-boost is shown in a dotted black line. The contributions of antigen-induced short-lived (1) and long-lived plasma cells (2) are shown in red. The contribution of antigen-independent mechanisms (3) is shown in blue.

TT vaccination. The sustenance of serum antibody after day 10 indicates that some cells are rescued in the bone marrow as long-lived plasma cells.

A relevant question at this point is what is the half-life of human plasma cells in the bone marrow. If these cells live forever, the antibody they produce should accumulate in the serum for several days until a plateau is reached, where the rate of synthesis equals the rate of degradation. Alternatively, if they have a long yet limited lifespan, the antibody they produce should accumulate initially and then decline as the cells die. The experimental data on the kinetics of serum antibodies and ASCs allow us to estimate that the half-life of human plasma cells generated during an immune response (and by extrapolation of all long-lived plasma cells) is approximately 40 days.

Additional quantitative parameters could be estimated from a typical secondary response to TT. The total serum IgG1 concentration of 10 mg/ml and a half-life of 20 days allow us to estimate a synthetic rate of ~250 μg/ml/day. Thus, approximately 100 μg of anti-TT-specific IgG1 antibodies are produced in two days (from day 6 to day 7) by short-lived ASCs. Based on the assumption that the half-life of all bone marrow plasma cells is the same of that of TT-specific plasma cells, that is approximately 40 days, and therefore their turnover is 1% per day, it is estimated that on day 12 after boost, 1–2% of bone marrow plasma cells are TT specific and produce 2.5–5 μg/ml/day of anti-TT antibody. This rate of production is sufficient to sustain serum antibody levels in the plateau phase (when antibodies produced by short-lived ASCs are replaced by those synthesized by long-lived plasma cells) and is consistent with the subsequent decline phase due to the turnover of long-lived plasma cells.

Taken together, our studies delineate antigen-dependent and antigen-independent (polyclonal) components that together contribute to the serological response observed after immunization and to the development of memory. Accordingly, a 'short-term serological memory' and a 'long-term serological memory' could be defined. The first is mediated by the antigen-driven production of short-lived and long-lived plasma cells, whereas the second is mediated by antigen-independent homeostatic activation of all memory B cells leading to a sustained production of plasma cells of all memory specificities.

On the one hand, upon antigenic stimulation, specific memory B cells proliferate and give rise to large numbers of plasma cells and antibodies while maintaining and possibly increasing their pool. On the other hand, in the absence of

antigen, all memory B cells continually proliferate at a low rate, spilling out plasma cells that replace those that turnover, thus maintaining constant antibody levels. Therefore, we propose that memory B cells function as 'memory stem cells' capable of maintaining their own pool size and to continually generate and maintain a pool of differentiated cells with limited lifespan.

Vaccination, memory B cells, and antibodies

Successful vaccines rely on the generation of protective antibody level. Protection is relative to a particular pathogen and may be mediated by preformed antibodies, by antibodies that are rapidly produced by ASCs derived from proliferating memory B cells, or by a combination of both. In the case of toxins, there is often no time to mount a secondary proliferative response, and therefore protection relies entirely on pre-existing neutralizing antibodies. The serological response described above showed that a high level of antibodies can be induced within a week after antigenic boosting and is maintained for a few months. High numbers of memory B cells are not required for this secondary antibody response. Indeed, we found that the magnitude of the antibody response does not correlate with the frequency of existing antigen-specific memory B cells, but rather it is inversely correlated with the preboost antibody level (E. Traggiai, unpublished data). This finding is consistent with the negative feedback effect of existing antibodies on secondary responses (28). In contrast, high numbers of memory B cells are required to sustain serum antibodies over a period of several years. Therefore to induce long-lived protection, vaccines should generate memory B cells at frequencies that are high enough to maintain protective antibody levels. This frequency is achieved upon natural infection or immunization with live vaccines, such as vaccinia virus. In contrast, 'weak' immunogens, such as protein antigens, are very poor inducers of memory B cells, and repetitive immunization appears to be required to increase memory B-cell frequency up to an appropriate level.

Human monoclonal antibodies from memory B cells

Together with active vaccination, passive vaccination, also called serotherapy, acting through the administration of preformed specific antibodies, has been one of the great contributions of immunology to medical treatments. Monoclonal antibodies can be used to offer immediate protection against a variety of toxins and pathogens, including emerging ones, such as severe acute respiratory syndrome (SARS) or H5N1 influenza, for which polyclonal human Igs from hyperimmune sera are not available in sufficient amounts.

Monoclonal antibodies represent an ideal alternative to hyperimmune sera (29). They can be produced by immortalizing memory B cells with Epstein–Barr virus (EBV) or by fusing a B cell with an appropriate partner cell to produce hybridomas. These methods, which have been used (30–32), have a very low efficiency, and therefore alternative strategies have been developed. These alternatives include the humanization of murine monoclonal antibodies through protein engineering (33), the selection of antibodies from phage display libraries of human antibody fragments (34), and the immunization of transgenic mice carrying human Ig loci, followed by the production of monoclonal antibodies using hybridoma technology (35). Although these methods have led to the development of several therapeutic monoclonal antibodies against cytokines or surface molecules, their impact on infectious disease therapy has been less pronounced (36). Indeed, the number of therapeutic antibodies against infectious agents is still limited, and only one is currently in use to prevent respiratory syncytial virus (RSV) infection in newborns (37).

There is an obvious advantage to using human B cells to produce monoclonal antibodies. First, humans can mount powerful immune responses, which include antibodies with long complementarity-determining region 3 (CDR3) regions. Second, antibodies are fully human and have been selected in a human body, minimizing the risk of cross reactivity with self-antigens. Third, the human immune response is directed against the virulent pathogen and can target all the components necessary for infection and virulence, which are usually invisible to the immune system of a different host, for instance a mouse.

We recently described an improved method of EBV transformation of human B cells, based on the addition of a TLR agonist during EBV transformation and cloning (38). We are using this method to isolate neutralizing and non-neutralizing monoclonal antibodies against a variety of targets including viruses, such as SARS coronavirus, toxins, and parasites. As an example, we have been able to isolate 35 independent monoclonal antibodies. These antibodies from one individual recovered from SARS infection neutralize with high potency virus infection *in vitro*, that is at low antibody concentration (38, 39). Using a panel of antibodies with different neutralizing titers, we found that the *in vitro* potency precisely correlates with the *in vivo* efficacy, measured as the reduction of viral titers in the lungs (K. Subbarao and A. Lanzavecchia, unpublished data).

We envision that this method may be used not only to isolate therapeutic antibodies for passive vaccination but also to analyze the antibody repertoire in immune or vaccinated

individuals to identify neutralizing, enhancing, or irrelevant epitopes, thus guiding the formulation of candidate vaccines (40). This 'analytic vaccinology' will be particularly useful in the case of highly variable viruses, such as hepatitis C virus or human immunodeficiency virus, or highly complex pathogens such as *Plasmodium*.

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