ABSTRACT In this article, we highlight the advantages of isolating human monoclonal antibodies from the human memory B cells and plasma cell repertoires by using high-throughput cellular screens. Memory B cells are immortalized with high efficiency using Epstein-Barr virus (EBV) in the presence of a toll-like receptor (TLR) agonist, while plasma cells are maintained in single-cell cultures by using interleukin 6 (IL-6) or stromal cells. In both cases, multiple parallel assays, including functional assays, can be used to identify rare cells that produce antibodies with unique properties. Using these methods, we have isolated potent and broadly neutralizing antibodies against a variety of viruses, in particular, a pan-influenza-A-neutralizing antibody and an antibody that neutralizes four different paramyxoviruses. Given the high throughput and the possibility of directly screening for function (rather than just binding), these methods are instrumental to implement a target-agnostic approach to identify the most effective antibodies and, consequently, the most promising targets for vaccine design. This approach is exemplified by the identification of unusually potent cytomegalovirus-neutralizing antibodies that led to the identification of the target, a pentameric complex that we are developing as a candidate vaccine.

MANY WAYS TO MAKE HUMAN MONOCLONAL ANTIBODIES

Today, several methods are available to isolate human monoclonal antibodies. The first efficient method described was the panning of phage display libraries constructed from the Ig variable genes of immunized or infected individuals (1) or from random synthetic libraries (2). While this method has led to the isolation of several neutralizing antibodies against multiple pathogens, the resulting antibodies do not represent necessarily the natural antibody repertoire, since the antibody fragments are generated from the random pairing of immunoglobulin VH and VL variable regions. Thus, in the case of phage libraries, it is unlikely that a given VH/VL pair went through a selection process, including the negative selection for self-reactivity. Another significant drawback is that target antigens must be known a priori, since the selection is based on binding to a purified antigen, rather than for instance neutralization. Consequently, this system is not suitable to identify new neutralizing targets within complex pathogens. In addition, selection for high-affinity binding does not necessarily translate into higher protection if the epitope recognized is not readily available on the viral spikes. An additional problem of this approach that was frequently encountered is that the antibodies isolated in Escherichia
coli or yeasts may be expressed suboptimally in mammalian cells.

Mice that carry human immunoglobulin loci produce fully human antibodies in response to immunization with the significant advantage that, being mice, they can be immunized and make antibodies to human antigens (3). This system has become the method of choice for the isolation of antibodies specific for human antigens such as cytokines or cell surface molecule. However, it is less suitable to make antibodies against human pathogens such as HIV, hepatitis C virus (HCV), hepatitis B virus (HBV), cytomegalovirus (CMV), that do not infect mice. In addition, the immune response of mice is often suboptimal, possibly owing to the mismatch between human Ig and mouse Fc receptors. Thus, the isolation of antibodies from immune donors offers the advantage of fully exploiting the strength of the human antibody response to a human pathogen.

An important advance in this field was represented by the use of single-cell reverse transcription (RT)-PCR to isolate the immunoglobulin heavy and light chain variable gene pairs from single B cells, followed by expression of the full antibody by transfection of H and L chain genes in 293 cells (4). The single-cell RT-PCR and expression approach has been also used to isolate antibodies from plasma cells isolated soon after a booster vaccination (5), and from memory B cells, which can be isolated according to their capacity to bind fluorescently labeled antigens (6). The latter approach has been particularly successful in the isolation of broadly neutralizing antibodies against the CD4 binding site of gp120. However, staining and sorting B cells with labeled antigens is not generally feasible whenever the target antigen is not available or unknown. In conclusion, the single-cell RT-PCR and expression approach has been very useful, especially when antigen binding can be used to enrich for specific cells or when specific cells are highly represented in the population, as is the case for plasma cells collected after antigenic boost. A significant limitation of this approach is that the cloning and expression of individual antibodies is very labor intensive, and, consequently, the throughput has been generally limited to a few hundred clones. However, recent technological advances using nanofluidic devices have considerably increased the throughput of this approach (7).

A simple and effective alternative to the single-cell RT-PCR and expression method is the direct screening of the antibodies produced by plasma cells or by activated or immortalized memory B cells. This can be achieved by using methods based on cell cultures, such as those described in this article, that allow instead the interrogation of the cell-derived monoclonal antibodies in their natural secreted form (Fig. 1). In 2004, we reported an efficient method of memory B-cell immortalization using EBV and CpG (8), and, in 2011, we demonstrated the feasibility of culturing single plasma cells and identifying those producing antibodies with special properties (9). These methods will be described in detail below. In 2008, Spits and coworkers developed an alternative method to immortalize human memory B cells by using retroviruses encoding the antiapoptotic factors Bcl-6 and Bcl-xL (10). Memory B cells are transduced under polyclonal conditions (typically 100 cells per well) in the presence of IL-21, and CD40L-expressing cells differentiate into long-lived antibody-secreting cells that still expressed their B-cell receptor on the cell surface. These cells also maintained a low-level activation-induced cytidine deaminase-mediated mutation activity that represents a possible source of antibody clone instability, but which can also be used to increase antibody affinity through an in vitro iterative selection process. In 2009, Walker et al. described the isolation of an HIV-1 broadly neutralizing antibody able to recognize the Env protein only in the trimeric native form by using another approach that relied on the in vitro activation and expansion of human memory B cells for approximately 10 days, followed by the screening of culture supernatant and lysis of all expanded cells (11). The details of this method have not been disclosed, but Connors and colleagues have recently described and published in detail a similar approach (12). The antibody-containing supernatants are used in functional sensitive assays to identify the wells from which the antibody genes can be retrieved for cloning and expression in 293 cells. A significant drawback of the latter two methods is related to the low levels of antibodies produced in the culture supernatants. In the Bcl-6/Bcl-xL transduction and the polyclonal stimulation technologies, the average productivities are claimed to be ~0.1 to 1 μg/ml and 0.01 to 0.1 μg/ml, respectively. The low antibody levels might limit the testing and identification of antibodies in several types of functional assays that could require higher levels of antibody.

EFFICIENT IMMORTALIZATION OF MEMORY B CELLS BY USING EBV AND CpG

Memory B cells are readily accessible in peripheral blood and persist for the lifetime of an individual and are therefore an excellent source to isolate monoclonal antibodies. Work in the late 1970s showed that human memory B cells could be immortalized with EBV and
that this method could be used to isolate human monoclonal antibodies (13). However, the efficiency of B-cell immortalization was low, typically in the order of 0.1%, and therefore did not allow an efficient interrogation of the human memory repertoire. In 2004, we reported that the efficiency of EBV immortalization could be dramatically increased by the addition of a toll-like receptor (TLR) agonist, in particular, CpG or R848 that trigger TLR9 and TLR7, which are expressed at high levels on human B cells (8, 14).

The basis for the powerful synergy between EBV and TLR stimulation was clarified in a subsequent study where we examined the requirements for the activation of human B cells (15). In this study, we reported that maximal stimulation of B cells requires three distinct signals: signal 1 delivered by B-cell receptor (BCR) stimulation, signal 2 delivered by T-helper cells primarily via CD40L/CD40 interaction, and signal 3 delivered by a TLR agonist (Fig. 2A). In particular, while BCR cross-linking and cognate interaction with activated T cells provided a suboptimal stimulus, addition of CpG or R848 potently boosted B-cell proliferation and differentiation to antibody-producing cells. Interestingly, EBV is known to activate B cells by expressing two proteins, LMP2A and LMP1, that mimic activated BCR and CD40, respectively, but there is no evidence that EBV may activate TLR as well (16). Thus, the three-signal model of B-cell activation is consistent with the potent synergy that TLR agonists have on B-cell immortalization (Fig. 2B).
The method has been adapted to high-throughput cultures performed in 384 plates. In a typical experiment, memory B cells are isolated by positive or negative selection according to the isotype expressed (IgG or IgA), incubated with EBV and CpG, and seeded in multiple 384 plates in the presence of irradiated allogeneic peripheral blood mononuclear cells (PBMCs) as feeder cells. The efficiency of immortalization, as demonstrated

**FIGURE 2** Efficient immortalization of human memory B cells by combination of EBV and CpG and isolation of rare neutralizing antibodies. (A) BCR stimulation mediated by antigen, T-cell help mediated by CD40L/CD40 interaction, and TLR stimulation provide three synergistic stimuli for activation of human B cells (15). (B) EBV encodes LMP2a and LMP1 that mimic constitutively activated BCR and CD40, thus providing signal 1 and signal 2. Addition of TLR agonists potently synergizes with the viral genes leading to efficient activation and immortalization (8). (C) Immortalization efficiency of IgG+ memory B cells plated at 1 cell/well in 384 culture plates containing irradiated allogeneic PBMC. Shown is the concentration of IgG in culture supernatants on day 10. Positive values above 2.5 OD correspond to IgG concentrations >1 μg/ml. The efficiency was calculated according to the Poisson distribution. (D) Example of a primary screening for MPV-neutralizing antibodies. In preliminary experiments, culture conditions were defined to achieve cytopathic effect by using primary MPV isolates. Culture supernatants were incubated with MPV followed by addition of LLC-MK2 cells. Living cells were measured by using a colorimetric assay on day 8. Several antibodies were isolated, including one (MPE8) that neutralizes four different paramyxoviruses. Shown is the total numbers of cultures screened. (E) Blood was collected from an immune donor 2 weeks after vaccination with a seasonal vaccine, and IgG memory cells were immortalized and plated at 3 cells/well in 384-well plates. The supernatants were screened for the presence of antibodies that bind to either H1 HA (CA09) present in the vaccine or to H5 HA (VN04) that represents a heterologous group 1 HA. doi:10.1128/microbiolspec.AID-0018-2014.f2
by the production of high levels of IgG is close to 30% (Fig. 2C). Given an efficiency immortalization of 20 to 30%, we typically seed 3 cells/well in order to have clonal growth. This procedure allows the avoidance or minimization of the need to subclone positive cultures and the easy rescue of the few clones that may show poor growth in vitro. Using this method and taking advantage of an automated liquid-handling system and fully integrated immunoassays, we can typically screen repertoires of >10^4 IgG memory B cells. An even higher number of cells can be screened by plating higher numbers of cells per culture (up to 30 to 100). In the latter case, subcloning of positive cultures will be required in order to isolate a clone making the desired antibody. Importantly, in all cases, the antibody is present at high concentrations in the culture supernatant so that multiple assays can be performed in parallel, including functional assays such as virus neutralization or bacterial opsonophagocytosis killing assays. An example of a screening by neutralization is provided in Fig. 2D. Of almost 50,000 culture supernatants of immortalized B cells from three donors, several were found to protect cells from the cytopathic effect of metapneumovirus (MPV), and one clone, named MPE8, was found in a parallel assay to neutralize respiratory syncytial virus (RSV) as well. Another example (Fig. 2E) shows how it is possible to rapidly identify in B cells obtained from an individual immunized with the pandemic H1N1 strain so-called heterosubtypic antibodies that bind to H1 and cross-react with the heterologous H5 hemagglutinin.

A significant advantage of the EBV-based method is that the immortalized B cells secrete high amounts of antibodies but also maintain expression of BCR, although at a low and variable level. Consequently, antigen-specific cells can be selected from a polyclonal population by panning or staining with isotype-specific antibodies or with the specific antigen. In addition, we never found evidence of ongoing somatic mutations in EBV-immortalized clones. EBV-immortalized B-cell clones maintain constant productivity with antibodies typically recovered in the culture supernatants at concentrations ranging from 5 to 50 μg/ml in static cultures. Thus, antibodies can be easily prepared from the culture supernatant in milligram amounts. In the past 10 years, the EBV+CpG immortalization method has been used in our laboratory to interrogate memory B and isolate monoclonal antibodies specific for a variety of human pathogens, toxins, and self-antigens (Table 1).

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SARS, severe acute respiratory syndrome.

PVM, pneumonia virus of mice; RSV, lovivne-RSV.

GM-CSF, granulocyte-macrophage colony-stimulating factor.

MERS, Middle East respiratory syndrome.
LONG-TERM CULTURE OF SINGLE PLASMA CELLS AND THEIR INTERROGATION USING MULTIPLE ASSAYS

We originally reported that antigen-specific plasma cells appear in high numbers in peripheral blood 1 week after booster immunization (17) when they may account for up to 40 to 90% of total Ig-secreting cells (17). While other laboratories have used this source of specific plasma cells to clone VH/VL pairs by RT-PCR and produce the antibodies recombinantly (5), we were interested to develop a method that would allow us to rapidly focus on rare antibodies, for instance, capable of binding two different viruses. We reasoned that if single plasma cells could be maintained in culture for long periods of time in a small volume, the antibody released in the supernatant could reach concentrations sufficient to perform several parallel assays. We found that single plasma cells isolated from blood or bone marrow could be maintained in culture for several weeks when cultured on a monolayer of immortalized stromal cells. In these conditions, single plasma cells secreted Ig at a constant rate of approximately 100 pg/cell/day (Fig. 3A). We also found that, in the absence of stromal cells, recombinant IL-6 was sufficient to maintain plasma cell viability at least for 3 to 4 days (9).

The plating efficiency in IL-6-dependent cultures is high, ranging from 50 to 100% (Fig. 3B). In a typical experiment, we isolate CD138+ CD27hi cells by cell sorting and seed them in 384 well plates at 1 cell/well in 50 μl of complete medium supplemented with IL-6. After 3 to 4 days, the supernatants are removed and tested in multiple parallel assays by using an automated liquid-handling system. The throughput and efficiency of the method is illustrated by the finding that the FI6 pan-influenza-neutralizing antibody was isolated every year for 5 consecutive years from the same donor from plasma cells collected after infection or vaccination (D. Corti, unpublished data). An example of the high throughput of this method is shown in Fig. 3C and D, where 13,000 plasma cell cultures were screened to determine the fraction of hemagglutinin (HA)-specific antibodies that cross-react with the heterologous H5 HA. This same method was used to clone rare IgE antibodies from an allergic individual.

Once identified, the positive cultures are subjected to single-cell RT-PCR and the specific VH/VL pairs are isolated, and the recombinant antibodies are expressed by using appropriate vectors. This high frequency of somatic mutations might represent a potential problem to antibody sequencing, because the mutated sequences may no longer be complementary to the oligos used for VH/VL gene amplification. To overcome this potential problem, we use, in a stepwise fashion, multiple sets of primers spanning different regions of the variable region leader sequence that allow us to obtain, with almost 100% efficiency, VH and VL sequences from all isolated plasma cell clones.

A TARGET-AGNOSTIC APPROACH FOR ANTIGEN DISCOVERY FOR VACCINE DESIGN

The high throughput of the cell-based screens and the possibility of directly assessing the function of the antibody (rather than just binding) are essential elements to implement a target-agnostic approach that aims at the identification of the most effective antibodies and, through the analysis of their specificity, of the most promising targets for vaccine design. This approach was originally proposed by Dennis Burton as a way to identify conserved epitopes in the highly variable viral glycoproteins, such as the Env of HIV-1 (18), but in a broader sense can be also used to identify in complex pathogens the molecules that induce the most potent neutralizing response, without prior knowledge of their nature.

A relevant example is provided by human cytomegalovirus (HCMV), a herpes virus that uses several glycoproteins to infect human cells. Previous studies focused on gB or gHgL, which are abundant proteins that were known to be targeted by neutralizing antibodies. Using a target-agnostic approach, we isolated from immune donors a large panel of antibodies based on their capacity to neutralize infection of fibroblasts or epithelial cells by a primary HCMV isolate and identified a group of unusually potent antibodies that selectively neutralized infection of epithelial, endothelial, and myeloid cells at concentrations 1,000-fold lower than antibodies specific for gB or gHgL (19). We found that these antibodies recognize multiple antigenic sites on a pentameric complex of gH/gL/pUL128/pUL130/pUL131A, which was previously described to be required for infection of endothelial epithelial and myeloid cells but was not known to be the target of neutralizing antibodies. We then produced a soluble pentameric complex that correctly displays all the neutralizing epitopes and elicits in mice very high titers of neutralizing antibodies. In addition, the soluble recombinant pentamer produced is used to identify the cellular receptors and dissect the mechanisms of virus entry in different cell types. We refer to this process as analytic vaccinology, since it is essentially based on the analysis of the antibody response to the pathogen (Fig. 4).
Another example of analytic vaccinology is provided by the analysis of the neutralizing antibody response to the F protein of RSV (20). Previous studies suggested that the postfusion F protein could be developed as a vaccine, since it was recognized by palivizumab, a neutralizing monoclonal antibody that is used to prevent RSV infection in newborns (21). In contrast, we found that, of 30 neutralizing antibodies tested, only 4 bound to the postfusion protein while the remaining, including the MPE8 antibody that cross-neutralizes four paramyxoviruses, were specific for the prefusion F conformation. These findings suggest that the prefusion protein should be considered as the most effective vaccine capable of inducing a broad spectrum of antibodies that neutralize but fail to react with the abundant postfusion F protein. Efforts to produce a stabilized prefusion form will be facilitated by the use of the antibodies that can be used as probes to test the conformation and stability of different constructs.

**FIGURE 3** Cultures of single plasma cells are instrumental for the rapid identification of rare antibodies. (A) Survival of single CD138+ plasma cells isolated from peripheral blood (open circles) or bone marrow (filled circles) in the presence of IL-6 and stromal cell monolayers. Shown is the cumulative production of IgG in cultures containing single plasma cells. (B) Plasma cells were isolated from peripheral blood as CD38+ CD138+ and plated at 0.5 cell/well in the presence of IL-6. IgG, IgA, IgM, and IgE levels were measured in each culture supernatant. The efficiency of cloning as estimated from the frequency of Ig-containing cultures was estimated to be approximately 70%. (C), (D) HA-specific antibodies produced by plasma cells isolated in 2009 following infection with H1N1 CA09 cross-react extensively with H5-HA (VN04). (C) In contrast, plasma cells isolated from the same donor in 2010 following vaccination with the seasonal trivalent vaccine are largely vaccine specific (D). doi:10.1128/microbiolspec.AID-0018-2014.f3
FIGURE 4 A target-agnostic approach to antibody discovery and vaccine design. In analytic vaccinology, donors that have developed a protective response are identified and memory B cells/plasma cells are interrogated to identify the most effective neutralizing antibodies in terms of potency and breadth. The antibodies are then used to identify the target antigen and to probe its correct conformation when the latter is produced as a recombinant vaccine. The vaccine is expected to elicit antibodies of the same quality as those originally isolated. In addition, the recombinant molecules can be used to identify cellular receptors. doi:10.1128/microbiolspec.AID-0018-2014.f4

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Isolation of Human Monoclonal Antibodies from Memory B Cells and Plasma Cells