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Genetic manipulation of B cells for the isolation of rare therapeutic antibodies from the human repertoire

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ABSTRACT

Antibody based therapies are increasingly applied to prevent and treat human disease. While the majority of antibodies currently on the market are chimeric or humanized antibodies from rodents, the focus has now shifted to the isolation and development of fully human antibodies. By retroviral transduction of B cell lymphoma-6 (BCL-6), which prevents terminal differentiation of B cells and, the anti-apoptotic gene B-cell lymphoma-extra large (Bcl-xL) into primary human B cells we efficiently immortalize antibody-producing B cells allowing the isolation of therapeutic antibodies. Selection of antigen-specific B cell clones was greatly facilitated because the transduced B cells retain surface immunoglobulin expression and secrete immunoglobulin into the culture supernatant. Surface immunoglobulin expression can be utilized to stain and isolate antigen specific B cell clones with labeled antigen. Immunoglobulins secreted in culture supernatant can directly be tested in functional assays to identify unique B cell clones. Here we describe the key features of our Bcl-6/Bcl-xL culture platform (AIMSelect).

1. Introduction

Human monoclonal antibodies represent an important and growing class of prophylactic and therapeutic drugs for a wide range of indications including infectious diseases, cancer, autoimmune- and inflammatory diseases [1–4]. Here we describe a novel and powerful method to select and culture human antibody-producing B cell clones and use these cells to isolate and improve monoclonal antibodies. We compare these technologies with traditional antibody discovery methods. Many of the antibody therapeutics currently used to treat disease are mouse-derived monoclonal antibodies generated by using hybridoma technology [5]. Humanization of these mouse antibodies is necessary to prevent the host immune system from generating anti-mouse or anti-idiotypic antibodies against the therapeutic antibodies that could cause an anaphylactic (allergic) reaction and diminish the function of the therapeutic antibody [6,7]. Although these engineered mouse antibodies resemble human proteins, anti-idiotypic antibodies still regularly appear [8–10]. As an alternative for humanization of rodent antibodies, several groups have used transgenic mouse models that contain human immunoglobulin gene loci for the development of therapeutic antibodies [11,12].

In contrast to mouse, the discovery of monoclonal antibodies derived from human B cells using long-term culture methods has long been hampered by technical difficulties. Early work showed that human monoclonal antibodies can be isolated by immortalizing B cells with Epstein-Barr virus (EBV) [13] or by fusing B cells with an appropriate partner to produce hybridomas [14,15]. However, these methods are inefficient and suffer from clonal instability and variation in immunoglobulin production. Therefore alternative strategies have been developed, several will be described below. More recent the EBV transformation efficiency has been greatly enhanced with the use of Toll Like Receptor agonists and this has led to the discovery of several unique antibodies [16,17]. Nevertheless, EBV-transformed lymphoblastoid cells frequently suffer from genomic instability and loss of antibody production that could hamper antibody discovery [18–21]. An alternative method to culture B cells was presented by Banchereau and collaborators in 1991 [22]. In this method B cells stimulated via CD40 proliferate in the presence of cytokines such as IL2, IL-4 or IL-21. However, primary human B cells have a limited proliferative life span *in vitro* when isolated cells are stimulated with CD40L and cytokines, which prevents the establishment of long-term cultures of B cell clones.

Abbreviations: AID, activation-induced cytidine deaminase; BCL-6, B cell lymphoma-6; Bcl-xL, B-cell lymphoma-extra large; BCR, B-cell receptor; EBV, Epstein-Barr virus; GALV, gibbon ape leukemia virus; IRES, internal ribosomal entry sequence; MMLV, moloney murine leukemia virus; RSV, respiratory syncytial virus; SHM, somatic hyper mutation.

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Single cell PCR methods circumvent the need for prolonged B cell culture and cell division [23,24]. This method relies on the targeted selection of antigen specific cells, as characterization of the cognate VH/VL sequences and production and characterization of the antibody is laborious. Wrammert et al. [25] isolated peripheral blood plasmablasts shortly after immunization and found most of these sequences to be antigen specific but not very heterogeneous. Alternatively, cell surface B cell receptor (BCR) expression can be utilized to select antigen specific cells. This is realized by incubation of a pool of B cells with fluorescent-labeled antigen followed by single cell sorting of positive cells and VH and VL single cell PCR. Subsequently recombinant antibody is produced to confirm specificity and functionality. In line with this method Jin et al. [26] published a method in which B cells are placed on a microwell array chip which are then checked for antigen specificity [26,27]. Using this 'immunospot array assay on a chip' thousands of B cells can simultaneously be screened for antigen specificity.

Several other human antibody isolation methods have been developed that completely circumvent (prolonged) B cell culture. The most widely applied techniques use antibody display libraries [28]. These consist of randomly combined heavy and light chain regions obtained through RT-PCR amplification from mRNA of B-cell pools. Large libraries need to be build to be able to isolate panels of high affinity clones [29,30]. As the pairing of heavy and light chain sequences is random most of the VH/VL combinations will not have been present in the original repertoire. In addition, antibodies isolated from display libraries can often only be tested in protein binding assays like ELISA, but not for functional activity like virus neutralization. As plate bound antigens regularly do not mimic the original conformational structure of a protein, ELISA based screenings may result in many false positive (and negative) results.

Technological innovation has also let to the discovery of antibodies directly from serum by liquid chromatography tandem mass spectrometry. Here antibodies are enriched after binding to purified antigens before the amino acid sequences are retrieved [31]. This method allows for interrogation of the circulating antibody population. Nevertheless, detailed functional analysis can only be performed after recombinant antibody production and serum titers for specific antibodies are generally only high enough during acute infection and the weeks thereafter.

In the past couple of years AIMM Therapeutics has build on the B cell culture method pioneered by Banchereau and colleagues [22] to develop a technology that establishes instant and unlimited proliferation of human B cells that express the BCR and in addition secrete antibodies [32]. As mentioned above B cells stimulated with CD40L and cytokines initially proliferate vigorously but eventually undergo terminal differentiation to plasma cells and subsequently die (Fig. 1A). We found that ectopic expression of BCL-6 into activated B cells inhibited terminal differentiation while allowing continued responsiveness to cytokines [33] (Fig. 1B). BCL-6 is a transcriptional repressor expressed in Germinal Center B cells [34] that plays a role in the prevention of plasma cell differentiation (Fig. 1) [35]. The Bcl-6-expressing B cells, however, showed a high degree of apoptosis which could be overcome by co-expression of the anti apoptotic molecule BCL-xL [36]. BCL-6 and Bcl-xL introduction is achieved by retroviral transduction of the coding regions for these proteins. The transduced B cells can be expanded (Fig. 1 C) and cloned in the presence of the ligand for CD40 and the cytokine IL-21. Below we will elaborate on the characteristics of this culture system.

2. Genetic reprogramming of B cells

Gene transfer into lymphocytes by traditional methods like calcium phosphate precipitation, liposome formation or electroporation is inefficient but more importantly stable gene integration is generally absent. Viral transduction however leads directly to stable gene integration into the genome of the target cell and can be very efficient if the proper virus envelope is chosen. Both retroviral and lentiviral transductions are suitable for efficient gene transfer [37]. While retroviral integration is dependent on cell division, lentiviral transduction can also be applied to non-dividing cells like plasma B cells. Large-scale preparation of recombinant retrovirus can easily be achieved by using stable producer cell lines such as the Phoenix expression platform [38]. Production of high titer lentivirus tends to be more cumbersome mainly because of the toxicity of the expressed virus proteins and envelopes. As such, we routinely use a Moloney Murine Leukemia Virus (MMLV) based platform using Gibbon Ape Leukemia Virus (GALV) envelope expressing producer cells [39,40]. The transfer vector is set-up such that Bcl-6, Bcl-xL and the GFP marker proteins are simultaneously translated from the same viral RNA (Fig. 2). This multicistronic approach is achieved by placing a 'self-cleaving' 2A peptide sequence [41] between the BCL-6 and BCL-xL coding regions and an Internal Ribosomal Entry Sequence (IRES) upstream of the GFP reporter gene. Viral transduction efficiencies are high and unbiased. We have been able to transduce and grow IgA, IgM and IgG1 to IgG4 expressing cells isolated from peripheral blood and in addition also B cells from tonsil, gut or inflamed tissues.

3. Isolation of B cells

Successful isolation and identification of therapeutic antibodies depends on the presence of antigen specific memory B cells. Therefore the selection of B cell donors is of critical importance. If possible B cells are isolated from donors that have had recent exposure to the antigen of interest, either by vaccination or natural encounter. Studies with Influenza vaccine have show that the highest frequency of antigen specific memory cells in the blood are reached on day 14–21 post booster vaccination and can make up 1% of the B cell population [25]. Recent efforts on the isolation of very rare broadly neutralizing antibodies from HIV infected individuals have focused on the memory B cells from so-called elite-neutralizers [42]. Plasma antibodies from these patients (representing 1% of total HIV patients) showed potent neutralizing activity against a broad panel of HIV isolates. Subsequent screening of the memory B cell pool of these patients resulted in a panel of 17 broadly neutralizing HIV antibodies.

Memory B cells can be isolated from blood or frozen PBMC with a flow cytometer using the cell surface markers CD19 and CD27, combined with an IgA, IgM or IgG Immunoglobulin stain if necessary [43]. Subsequently the isolated B cells are stimulated on irradiated CD40-L expressing L-cells in the presence of IL-21 and transduced with retrovirus. Transductions efficiencies of 60–80% are routinely achieved as can be determined by expression of GFP in those cells. Sequence analysis of a large panel (>100) of antigen specific B cell clones showed that the distribution of the different VH segments was similar to that reported for non-manipulated memory B cells (Fig 3.) (www.imgt.org) [44].

Based on the expected frequency of the antigen specific B cells, cells can be grown in small pools (5–200 cells per well) or subjected to labeled antigen sorting, as discussed in sections 5 and 6.

4. Characteristics of transduced B cells

Culture of transduced B cells is performed in the presence of irradiated mouse fibroblast L cells expressing CD40L (CD40L-L cells) and IL-21. Transduced cells have a stable doubling time of 25–36 h and can be cultured for at least six months which allows for ample time to perform all necessary screening assays. The cells