Global Vaccines and Immunology

Review Article

Microsystem enabled single cell monoclonal antibody cloning for antibody immunotherapy of HIV infection (features)

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Monoclonal antibodies (mAbs) have well established their valuable roles as therapeutic proteins. Second generation of mAbs as therapeutical products for immunotherapy of HIV infection has made significant progress in recent studies. A recent small phase 1 clinical trial has demonstrated that monoclonal antibodies could be safe and effective in reducing HIV-1 viraemia in human. This study also suggested that passive transfer of mAbs will provide a potential alternative strategy for HIV-1 prevention, therapy, and cure. Single cell mAb cloning using either hydridoma technique or fluorescent activated cell sorting (FACS) is less efficient. Microsystem will enable a more efficient approach for single cell MAb cloning of bNAbs for HIV infection.

Production of monoclonal antibodies (mAbs)

Monoclonal antibodies (mAbs) are highly desirable as therapeutic proteins, and also widely defined as the most significant class of biologics for use as pharmaceuticals and diagnostics due to their specificity of binding, homogeneity, ability to be produced in unlimited quantities and predictable safety [1]. Since the first therapeutic mAb was commercialized in 1986, product approvals and sales of mAbs have grown significantly. Forty seven mAbs have been approved and marketed in the United States and Europe as of November 10, 2014 [2-5]. Advancement of understanding of diseases at a molecular level drives the rapid antibody product development. Techniques including genomics, proteomics and systems biology continue to provide important new targets for modulating disease. MAbs often provide the most rapid route to a clinical proof of concept for activating, inhibiting, or blocking these new targets, and therefore are often the first product candidates advancing to clinical trials. Increasing and aging worldwide population and the increasing standard of living in emerging markets also fuel the growth in mAbs sales and global market expansion of the pharmaceutical market [6]. More than 200 mAbs are in clinical trial and over 300 mAb candidates are currently in development. In 2015, the sales revenue of therapeutic antibodies is $80 billion and is predicated to reach $150 billion in 2020.

The classic method of mAb production is the hybridoma technique [7-10] (Figure 1). Antibody-secreting B lymphocytes (B cells) are isolated from animals immunized with an antigen. Isolated B cells are immortalized by chemically-induced fusing with a tumor cell line (a myeloma). The fused cells are called hybridomas and can be maintained in vitro. Standard techniques could generate 10^3-10^4 clones in each experiment. A substantial amount of work is needed to identify those cells that produce antibodies with a defined specificity. Traditional methods to screen for specific clones within these populations have relied on depositing cells into 96- or 384-well microtiter plates at densities of approximately one cell per well. After 7-14 days, the supernatants from these cultures are then assessed for the antibody secretions. Positive wells are selected for single cell cloning expansion. Limiting-dilution method for single-cell selection needs the hundreds to thousands of ELISA tests. Overall efficiency of this method is very low both practically and economically. All of the antibodies produced by descendants of one hybridoma cell are identical. Antibodies that are produced by hybridomas are known as monoclonal antibodies.

Advancement of technologies for speeding up the process

There have been several attempts to speed up the production of mAbs. ClonePixFL technology from Genetix Ltd plates out cells in

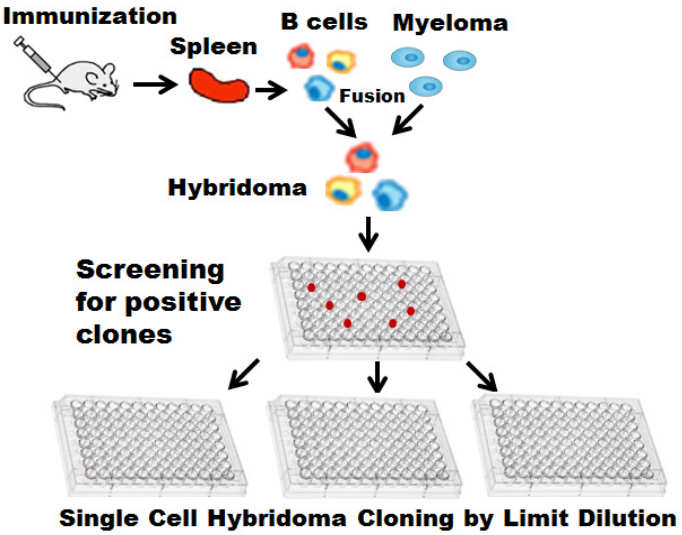

Figure 1. Hybridoma technique. Isolated parimary B cells fused with myeloma to form hybridoma. Positive antibody secreting hybridoma will be screening extensively by limit dilution.

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Received: January 10, 2016; **Accepted:** February 12, 2016; **Published:** February 16, 2016

semisolid medium, allow cells to grow 5-14 days into colonies. The secreted protein of interest is trapped in the vicinity of the colony and illuminated by a fluorescently-labeled antibody present in excess in the medium that diffuses freely through the medium until it recognizes and complexes with the secreted protein. The amount of fluorescence accumulating around a colony is proportional to the amount of target protein secreted by the colony. The positive clones can be selected and picked up based on quantitative protein secretion or specific protein production [11,12]. Integration of novel clone selection algorithms developed by Invitrogen and ClonePix technique allows for rapid screening through thousands of clones in a few hours.

Fluorescent activated cell sorting (FACS) technique sorts single cells based on the expression patterns of specific cell surface markers [13,14]. This approach has been applied extensively to select antigen-specific B cells using antigen coated magnetic beads [15], fluorochrome-labeled antigens via multi-parameter FACS [16-19]. The major advantage of FACS technology in this application is that cells to be sorted can be clearly distinguished in terms of their stage of development and differentiation based upon the expression patterns of specific cell surface markers. In general, B cells at any stage can be sorted, but class-switched memory B cells and antibody-secreting cells (ASCs, i.e. plasmablasts and plasma cells) are of special interest to obtain relevant mAbs as they bear somatically mutated B cell antigen receptors (BCRs) with high affinities. Whereas for FACS, the correlation is not straightforward between cells that stain positive and those that actually secrete the antibodies. Both methods have improved the efficiency of screening by serial dilution.

Antibody (Ab) immunotherapy of HIV infection

MAbs have been successfully used as immunotherapy products for cancer [20] and autoimmune disease [21,22]. Passive transfer of first generation broad neutralization antibodies (bNAbs) including b12, 2G12, 2F5 or 4E10 has been proved to protect against simian immunodeficiency viruses (SIVs) that express the HIV-1 envelope glycoproteins infection in macaques [23-29]. Clinical studies have discovered many human antibodies that can neutralize multiple strains of HIV [30-35]. Antibodies have been proved to be effective in suppressing HIV-1 infection in humanized mice [36], rhesus monkeys [37], and macaques [38]. A recent small phase 1 clinical trial [39] included 29 volunteers (17 HIV-infected and 12 uninfected) demonstrated that a single infusion of an experimental antibody 3BNC17 significantly reduced HIV levels in infected people for as long as 28 days. For the first time, this study established that passive infusion of single bNAbs can have profound effects on HIV-1 viraemia in humans and can be used as immunotherapy products.

Significance of single cell monoclonal antibody (mab) cloning

Elicitation of potent broadly neutralizing antibodies (bnAbs) against HIV viruses is one of the key attributes of humoral immune response to HIV vaccines. Antibody responses to vaccination have been mainly measured as overall serum binding or functional titer such as neutralization to the HIV viruses. While serum antibody titers provide a general humoral response to potential HIV vaccines, polyclonal antibodies are of little value for defining the critical components of the host humoral response to HIV vaccines. Profiling monoclonal antibodies (mAbs) generated from vaccinated animal models or clinical samples will reveal crucial aspects of the immunological response to potential HIV vaccines. Generated mAbs can be further characterized for their binding affinity, neutralization, and cross-reactivity. These details will provide valuable information for HIV vaccine designs. These mAbs can also be used as Ab immunotherapy for HIV infection. Hybridoma and EBV immortalized B cell lines have been used to generate human monoclonal antibodies. But their overall transformation efficiencies are extremely low (about 1-3%) [40-45]. Development of single cell mAb cloning techniques will be crucial for generating potential Ab immunotherapy for HIV infection.

Single cell monoclonal antibody (mab) cloning using FACS

Single cell Ab cloning techniques were originally developed to study mechanisms that control tolerance in the B cell compartment [46]. Wardemann and coworkers have found that a portion of newly generated B cells in the human bone marrow expressed self- and polyreactive antibodies and their development was regulated at two independent self-tolerance checkpoints [46]. The techniques were modified to identify B cells which secrete broad neutralization Abs [47-50] or screen single B cells expressing Abs that bind to the HIV-1 envelope spike [51,52]. The single cell Ab cloning techniques successfully cloned many naturally arising, broad and potent HIV-1-neutralizing antibodies (bNAbs) that were up to 2 to 3 orders of magnitude more potent than those previously discovered [47-67]. The single cell Ab cloning techniques have also been used to isolate mAbs from other animal models such as rhesus macaque [69,70].

As illustrated in Figure 2, single memory B cells were isolated using fluorescence activated cell sorting (FACS) based on surface expressed markers such as gp140 [52], or a modified gp120 core [53,54], gp41 [68], gp160Δc^{BaL} [55] or a combination of a serial markers including CD3, CD27 and CD19 etc [54]. Some studies also used engineered protein probes to identify and sort epitope-specific B cells [66,67]. Immunoglobulin (Ig) genes of isolated single B cells were amplified using single cell reverse transcription and polymerize chain reaction (RT-PCR). Amplified heavy- and light-chain antibody regions were cloned into eukaryotic expression vectors and transfected into human embryonic kidney (HEK) 293 or 293 T cells to produce monoclonal human antibodies of the same specificity in vitro. Recombinant antibodies were purified from supernatants and tested for antigen reactivity at the end using ELISA.

Single cells sorted using FACS were based on surface markers which can distinguish their development stages and differentiation status. Ig-cloning step is time-consuming and prevents high-throughputs analysis of the B cell repertoires [68]. The single cell sorting using FACS is based on the surface markers not antigen specificity. Antigenic specificity was tested at the end of procedure. Single cell isolation was

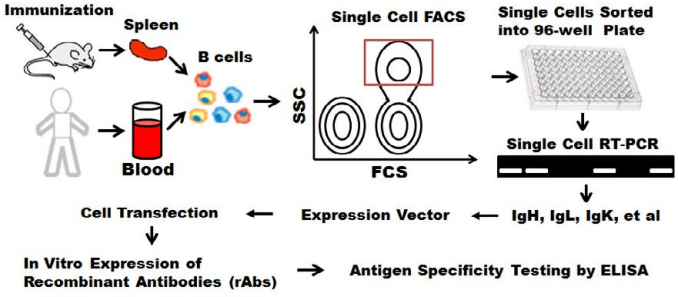

Figure 2. Single cell mAb cloning using FACS. Single cell sorted using FACS.IgG genes are amplified using single cell RT-PCR, transfected into 293 T cells with appropriate expression vector for recombinant mAbs.

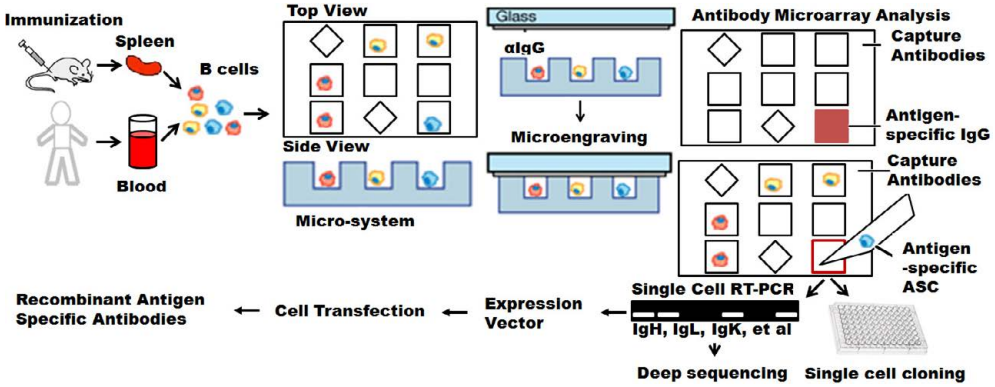

Figure 3. Microsystem enabled single cell monoclonal antibody cloning. Antigens-specific antibody secreting cells (ASC) are identified based on antigen-specific IgG secretion using either microengraving or on-ship immunoassay. Identified single cells can be directly used for single cell cloning. IgG genes isolated from these single cells can be amplified with single cell RT-PCR for either deep sequencing or in in vitro recombinant mAb production.

performed in a random way, therefore this approach is far less efficient than screening method based on antigen-specific secretions.

Microsystem enabled single cell monoclonal antibody (MAb) cloning for Ab immunotherapy of HIV infection

The time required per assay of antibody secretion using ELISA, as well as the small numbers of cells screened per 96 or 384-plate, limit the overall efficiency of the process, both practically and economically. Microsystem provides dense arrays of microwells with subnanoliter volumes to compartmentalize 10^4-10^6 cells for single cell assay [71,72]. Therefore, cell-based microarray [73] and microengraving techniques [74,75] can overcome above limitations to isolate, rapid screen and detect single cells secreting antibodies with the desired reactivity from a large numbers of individual cells. These techniques allow both the high throughput processing, retrieval and cloning of single cells with specificities of interest.

A microarray enabled assay that are defined as immunospot array assay on a chip (ISAAC) enable rapid and efficient manipulation of live single cells to isolate antigen-specific Ab-secreting cells from human peripheral blood. As shown in Figure 3, the chip surface is coated with capture Abs, and the Abs secreted by the Ab-secreting cells are then trapped on the surface around the cells. The binding between capture Abs and targets is detected using fluorescently labeled secondary detection Abs. As a result, the formation of distinct fluorescent spots can be easily distinguished from nonspecific signals [73]. The mechanism of ISAAC is based on ELISPOT.

Strategies that use microengraving technique [74-81] are based on the same principles as ELISA. Microengraving technique uses an engineered micro-tool that is fabricated by a soft lithographic method which modify the biocompatible polymer polydimethylsiloxane (PDMS). Microengraving technique uses the microfabricated array of subnanoliter wells to isolate individual cells spatially and print protein microarrays, where each spot on the array contains the proteins secreted by a single cell [74]. Unique IDs for each micro-well make it possible to position and track individual cells. The array is typically composed of 84,672 microwells, each with dimensions of 50μm length, width, and depth arranged over an area of 1"× 3" on a polymeric stamp. Each well holds a volume of approximately 125pL.

The microengraving technique was originally developed to generate microarrays comprising the secreted products of single cells. This method enabled a rapid and high-throughput system for screening of hybridoma cells producing antigen-specific Abs, and subsequent recovery and clonal expansion of single hybridoma cells of interest [75]. Microengraving technique was applied to collect multiparametric datasets that describe the specificity, isotype and apparent affinity of the Abs secreted from many individual primary B cells [76]. Microengraving technique was used to reveal isotype-specific autoreative B cells in Sjogren's syndrome [77]. This technique was applied to detect and isolate auto-reactive human Abs from primary B cells and obtain comprehensive Ab immune profiles of B cells isolated from HIV patients [78].

Ig gene cloning is a time consuming process. Both high-throughput methods of ISSAC and microengraving provide compelling advantages for the early and rapid identification of cells secreting antigen-specific antibodies with high affinities and also enable the screening of multiple different clones with distinct specificities in parallel. The advantages provided with microsystem will allow us to single cell cloning HIV vaccine antigen-specific mAb for Ab therapy of HIV infection [79]. Isolated HIV vaccine antigen-specific B cells will also provide nature V_H and V_L pairs for subsequent deep sequencing [80-89]. These studies, illustrated in Figure 3 will allow us to functionally characterize HIV vaccine antigen-specific Abs, and to understand the evolution of broad neutralization Abs during the vaccination and maturation process. These methods can also be applied to characterize and sequence HIV vaccine antigen-specific B cells at different anatomical sites, such as blood, bone marrow, mucosal, etc. to reveal the insights of adaptive immune responses in response to HIV infection or vaccines. Meanwhile, the mutiplxed methods can also integrated into the single-cell analytical system to reveal the cell-cell heterogeneities [90-93].

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