DEVELOPMENT OF AN IMMUNE TUBERCULOSIS ANTIBODY LIBRARY: MONOCLONAL ANTIBODY GENERATION FOR α-CRYSTALLINE ANTIGEN

NURUL HAMIZAH BINTI HAMIDON

UNIVERSITI SAINS MALAYSIA
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by

NURUL HAMIZAH BINTI HAMIDON

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LIST OF ABBREVIATIONS

Abs	Absorbance
ABTS	2,2'-azimo-bis(3-ethylbenzenethiazoline-6-sulphonic
bp	Base pair
βME	β-mercaptoethanol
cDNA	Complementary deoxyribonucleic acid
CDR	Complementary determining region
CH1	Constant heavy chain 1
CH2	Constant heavy chain 2
CH3	Constant heavy chain 3
CH4	Constant heavy chain 4
DNA	Deoxyribonucleic acid
H_2O	Distilled water
dNTP	Deoxynucleotide triphosphate
dsDNA	Double stranded DNA
E.coli	*Escherichia coli*
eGFP	Enhanced green fluorescence protein
ELISA	Enzyme-linked immune assay
EtBr	Ethidium bromide
EtOH	Ethanol
Fv	Fragment variable
HC	Heavy Chain
His	Histidine
Hour	hr
HRP	Horseradish peroxide

IPTG	Isopropyl-beta-D-thiogalactopyranoside
Ig	Imunoglobulin
Ig	Imunoglobulin A
Ig	Imunoglobulin D
Ig	Imunoglobulin E
Ig	Imunoglobulin M
J	Junction
LC	Light chain
Min	Minute
Mtb	*Mycobacterium tuberculosis*
Kan	Kanamycin
kDa	Kilodalton
OD	Optical density
PBS	Phosphate buffer saline
PBST	Phosphate buffer saline containing Tween 20
PCR	Polymerase chain reaction
pI-pXI	Phage coat protein 1,2,3,4,5,6,7,8,9,10 and 11
PTM	Milk powder in PBST
RE	Restriction site
RNA	Ribonucleic acid
RIN	RNA integrity number
s	Second
scFv	Single-chain fragment variable
ssDNA	Single stranded DNA
SDS	Sodium dodecyl sulphate

SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SHM	Somatic Hypermutation
Tb	Tuberculosis
U	Unit
V	Variable/Voltan
V-gene	Variable gene

LIST OF SYMBOLS

°C	Degree Celsius
g	Gram
g	Gravity
L	liter
µg	Microgram
µL	Microliter
mg	Miligram
mL	Mililiter
ng	nanogram
nm	nanometer
rpm	Revolution per minute
U	Unit of enzyme
v/v	Volume/volume
w/v	Weight/volume

PEMBANGUNAN PERPUSTAKAAN ANTIBODI IMUN BAGI TUBERKULOSIS: PENJANAAN ANTIBODI MONOKLONAL UNTUK ANTIGEN α-KRYSTALIN

ABSTRAK

Antibodi adalah amat penting untuk aplikasi terapeutik dan diagnostik untuk penyakit berjangkit. Perpustakaan imunisasi adalah perpustakaan yang berguna untuk penyakit berjangkit kerana ia akan membolehkan pengkayaan selektif antibodi terhadap antigen. Generasi perpustakaan antibodi adalah perlu untuk menghasilkan monoclonal antibodi. Perpustakaan antibodi ScFv imunisasi dihasilkan daripada enam orang penderma (pesakit batuk kering). Semua antibodi V gene yang mungkin diperkayakan dengan PCR konvensional kemudiannya diklonkan dalam pLABEL, vektor fajmid. Pengklonan dua langkah telah digunakan untuk menjana perpustakaan antibodi. Anggaran saiz perpustakaan mini HC dan perpustakaan scFv yang dijana adalah 10^7 dan 10^9. Kadar penyelitan untuk teknik pengklonan dua langkah hanya mencapai 80% sahaja dan α-kristal antigen digunakan untuk verifikasi perpustakaan antibodi dan pemencilan monoklonal. Pemilihan monoklon antibodi dijalankan dengan mengunakan konvensional *panning*. Tiga monoklon scFv antibodi telah berjaya diperoleh untuk α-kristal antigen (Rv2331c) M.tuberculosis jujukan antibodi ini dianalisis menggunakan perisian IMGT. Klon tersebut adalah IGKV3 dan IGHV2 untuk klon 1H, IGKV4 dan IGHV2 untuk klon 1B and IGLV1 dan LGHV1 untuk klon 10G. Antibodi diperkaya juga dinilai dalam bentuk larut untuk aktiviti mengikat. Kesimpulannya, perpustakaan scFv imun yang pelbagai ini telah dihasilkan dan mampu menjana antibodi monoklonal scFv yang larut air terhadap α-kristal.

DEVELOPMENT OF AN IMMUNE TUBERCULOSIS ANTIBODY LIBRARY: MONOCLONAL ANTIBODY GENERATION FOR α-CRYSTALLINE ANTIGEN

ABSTRACT

Antibodies are very important for therapeutic and diagnostic applications for infectious diseases. Immune libraries are useful for infectious disease applications because it would allow for better and selective enrichment of antibodies against disease antigens. The generations of antibody libraries are necessary to produce the monoclonal antibodies. Immune scFv antibody library was generated from six donors (tuberculosis patients). Conventional PCR method was used to amplify all possible antibody genes, which were then cloned into the pLABEL, phagemid vector. A two-step cloning procedure was done to generate the antibody library. The library size of the mini HC library and the scFv library constructed was approximately around 10^7 and 10^9 individual clones respectively. The insert rate for two-step cloning was only 63% and α-crystalline antigen was used for verification of the antibody library and monoclonal isolation. Conventional plate panning technique was used for selection of monoclonal antibodies. Three monoclonal scFv antibodies were successfully obtained for the *Mycobacterium tuberculosis,* α-crystalline (Rv2331c) antigen and the antibody sequence was analyzed using the software, VBASE2. The clones were IGKV3 and IGHV2 for clone 1H, IGKV4 and IGHV2 for clone 1B and IGLV1 and LGHV1 for clone 10G. The antibodies enriched was also evaluated in soluble form for binding activities. In conclusion, a diverse immune scFv library was constructed from which soluble monoclonal scFv antibodies against α-crystalline antigen was obtained.

CHAPTER 1

INTRODUCTION

1.1 Tuberculosis

Tuberculosis (Tb) is important and highly infectious disease This disease is caused by the *Mycobacterium tuberculosis* (*Mtb*) bacterium and is considered a health threat to the world population. Tb is an airborne disease that can cause severe lung disability and high mortality. It is predominant in regions such as Southeast Asia and Africa (Demissie et al., 2006). It has become endemic because the human population density has increased from the 17^{th} to 19^{th} centuries (Acosta et al., 2011) with an estimated total of 480,000 people infected with multi drug resistant Tb (MDR Tb) (Prasad et al., 2014). *Mtb* is an acid-fast bacillus bacterium that grows very slow but can infect most mammals in the world (Collins, 1989). However, this disease only can be transmitted from human to human. Most infected individuals never realise they are infected because 90% of them never develop clinical symptoms in their lifetime and only about 2% of immunocompromised people become ill with primary tuberculosis (Garay, 1996).

Bacille Calmette Guerin (BCG) is the only licensed vaccine against Tb (Goonetilleke et al., 2003). Most of the world's population is vaccinated with BCG during birth and later again during childhood because BCG can be effective in protecting young children against severe forms of Tb effectively, especially for meningeal (Fine, 1995). However, BCG is relatively not successful in preventing Tb infection in adults because BCG-immunized individuals can still be infected with

Mtb and develope active tuberculosis. The efficacy of BCG vaccine in human trials has varied from 0% to 80% (Flynn et al., 2004).

The protection of BCG vaccine is debatable because a report on the efficacy to prevent adults from pulmonary Tb is contradictory (Jacob et al., 2009). BCG implementation becomes complicated because WHO has reported that there is no vaccine to children born from mother infected with positive HIV (Hesseling AC et al., 2009). However to prevent Tb, BCG remains as a standard vaccine in most countries across the world because it is the only cheap and efficient vaccine available in preventing the life threatening Tb from young children and infants.

1.1.1 α-crystalline antigen

The 16 kDa antigen of *Mtb* was previously referred to as the 14 kDa antigen (Jackett *et al.*, 1988; Engers *et al.*, 1986). It is also known as HSP16.3 that is correlated to low molecular weight heat-shock proteins (Beck et al., 2005). This *Mtb* is strictly aerobic but it can survive in microaerophilic environments as long as the changes is not too extreme (Wayne and Hayes, 1996). The α-crystalline antigen major protein located in the membrane layer of *Mtb* (Lee et al., 1992).

This 16 kDa antigen plays a role in the survival of *Mtb* against macrophages activity(Kennaway et al., 2005). The physiology of this antigen is unique because it cannot be detected outside of *Mtb bacillus Calmette-Guerin* (BCG) (Yuan et al., 1996; De Smet et al., 1996). The α-crystalline antigen has been targeted as one of the component antigens in vaccine strategies. This antigen is also targeted for protective immune responses against primary and reactivation of latent Tb infections (Taylor et al., 2012). This antigen contains a specific B-cell epitope in the *Mtb* complex (Demkow et al., 2002).

There is an association between *Mtb* cell wall thickening and 16 kDa antigen (Lee and Horwitz, 1999; Cunningham and Spreadbury, 1998). This antigen is not expressed during the exponential but rather during the latency phase (Yuan et al., 1996). These properties make the 16 kDa antigen usefuk as a potential vaccine candidate to protect against of *Mtb* infection.

16 kDa antigen or HSP16.3 is an important agent for infection that will trigger cellular and humoral immune responses. Therefore, the study of this antigen in its role in pathogenicity and immunity of Tb is important (Siddiqui et al., 2014). The antigen specificity has made it a great candidate for detecting the disease at an early stage of infection (Rabahi et al., 2007; Verbon et al., 1992). This antigen is suitable for evaluation of a immune Tb library because it carries epitopes specific to Tb and is exposed to B-cell recognition (Coates et al., 1981).

1.2 Immune system

The human immune system is able to develop various defense mechanisms during infection to protect the body. The defense mechanisms act against virulence factors and destroy the invading pathogens in the body (Boyden, 1996). Antibodies have been proven over the years to be a desirable tool in both diagnostic and therapeutics management (Behring, 1890). There are two different types of protection for human, innate and adaptive immunities.

The function of innate immunity is to fight off antigens when it appears in the body (Kindt et al., 2007). Two main functions of innate immunity are to determine the adaptive immune response and to kill the pathogen (Medzhitov and Janeway, 1997; Fearon and Locksley, 1996). The physical barriers such as chemicals, skin and immune cells can attack foreign particles and ensure they never gain access into the

body. However, there are several strategies for pathogens to escape from being eliminated from the immune system. This includes the possibility of mimicking the host cell surface and continual variation of antigen surfaces.

Second line of defense for human is adaptive immune response. The function is to block the infiltration of foreign pathogens. It is more complex than the innate immune response because it has the capability to differentiate self from non-self antigens and kill foreign microorganisms. It provides defense to eliminate and generate a memory towards it when the body is re-infected by the same foreign pathogen (Alberts et al., 2002).

Adaptive immune system can be divided into two, cell-mediated immune system (T-lymphocytes) and humoral immune system (B-lymphocytes) (McCullough and Summerfield, 2005). B-lymphocytes in the humoral immune system are responsible for the production of antibodies. Plasma cell secreting antibodies are derived from B-lymphocytes. It can mature and develop into plasma cells when antigen bind to the B-cell receptor (Yancopoulos and Alt, 1986).

1.2.1 Antibody structure and class

Antibodies are glycoproteins, also know as immunoglobulins (Ig). Antibodies are present in the tissue and serum of all vertebrates and mammals. It has a unique structure affinity to the target antigen (Padlan, 1994). B-cells are produced and mature in the bone marrow (Yancopoulos and Alt, 1896) and are responsible for humoral immune response to recognize the target antigen. Antibody is a heavy globular plasma protein with a size approximately 150 kDa.

The basic Y shaped structure of all antibodies is similar (Figure 1.1). Antibodies have two types of polypeptide chains, heavy chains (HC) and light chains (LC) (Janeway et al., 2001). The HC fragment is larger and longer than LC with a size approximately 50 kDa or more. HC fragment can be further divided into four constant domains (C_H1-C_H4) (Normansell, 1987) while LC only have two different fragments with sizes around 22 kDa. However, the LC that is present in the antibody can only be in one form at a given time, either lambda or kappa fragment. LC fragments are linked to each other and to the HC fragment by disulphide bonds (Schroeder and Cavacini, 2010).

Human antibodies can be divided into five different classes, namely IgG, IgA, IgD, IgE, and IgM (Davies and Metzger, 1983) classified based on their physiochemical, structural and immunological properties. IgM is predominant in the primary immune response (Cruse and Lewis, 2010) and structurally has an additional constant domain but does not contain the hinge region (Collins et al., 2002). IgA is predominant in mucus secretions and can exist as a monomer or dimer. IgE antibody plays a role against parasites and is normally found on basophils and mast cells of an individual (Cruse and Lewis, 2010).

IgD has a same basic structure as IgG but with an extended hinge region with an unknown function. However, IgG is the most widely used antibody isotype for therapeutics compared to other classes of antibodies (Carter, 2006). IgG is the main class antibodies to protect the body against bacterial and viral infections. It functions in humoral immune responses can be further divided to four different subclasses; IgG1, IgG2, IgG3, and IgG4. Each subclass of IgG antibody has different flexibility of the hinge region and number of disulfide bonds (Schroeder and Cavacini, 2010).

The paratope is an antigen-binding site on antibodies while the epitope is a part of the antigen that is specifically recognized by antibodies (Hoogenboom and Chames, 2000). Both HC and LC antibody fragments are important for antigen binding and each one of them have different complementary determining regions (CDR).

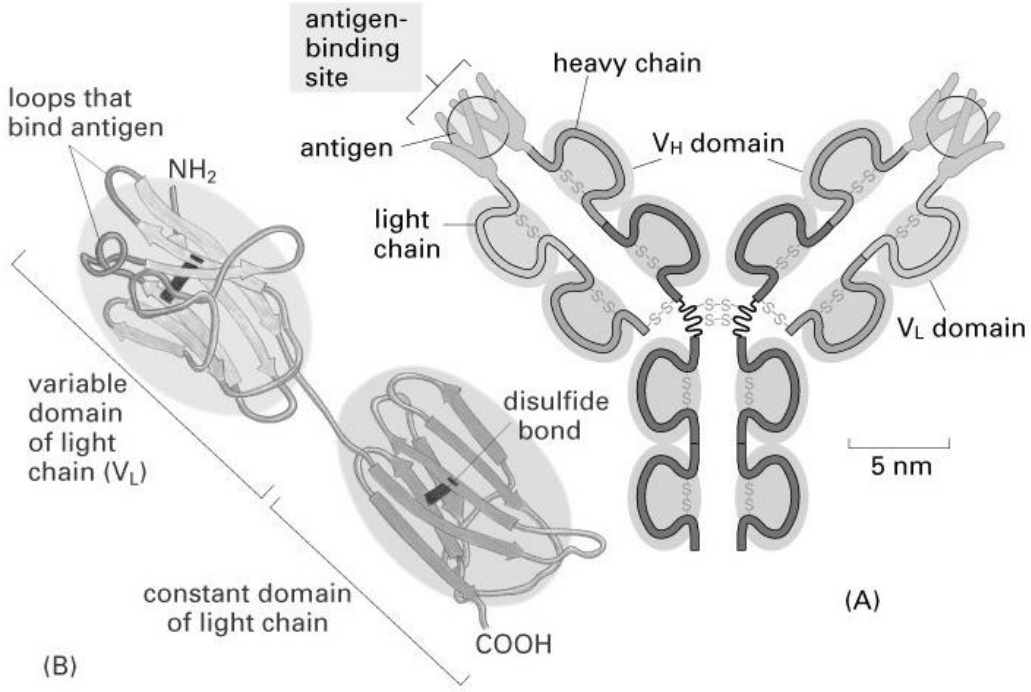

Figure 1.1 Typical structure of antibody. (a) Antibody consists of two HC and two LC, which are linked together by disulphide bonds. One bond are linked together LC and HC, two bonds linked two HC and several bonds linking antibody subdomains. (b) Finger-like loops of a single LC whereby binding of antigen occur (Alberts et al., 2013).

1.2.2 Antibody diversity

Generally, before process maturation the diversity of antibodies is created at the germline level. During B cell development, the variable (V) and junction (J) segments for LC and V, D, and J segments for HC are joined together to form a functional of HC and LC repertoire by VDJ recombination (Marrack and Kappler, 1987). VDJ segments rearrangement will create a unique HC and LC repertoire with large diversities. This creates the initial repertoire of antibody sequences that contributes to the primary diversity.

Mutations of the rearranged variable region gene called somatic hypermutation (SHM) could also create antibody diversity (Di Noia and Neuberger, 2007). SHM allows for the secondary diversity to be generated resulting in higher affinity antigen binding sites (MacLennan, 1994). SHM mutations are mainly substitutions and alter the specificity of the encoded antibodies between ~150 to 200 bp downstream from promoter (Rada and Milstein, 2001).

Random pairing of HC and LC repertoire also contributes to antibody diversity. The random combination of the associated HC and LC repertoire will increase the diversity of antibodies. Not only that, the diversity of antibodies also can be increase by changes of amino acid at CDR3 where the nucleotides can either be lost or added during VDJ rearrangement (Parslow et al., 2001).

1.2.3 Recombinant antibodies

Advances in molecular biology have led to the ability to produce antibodies *in vitro* without depending on hybridoma technology and animals. Recombinant antibodies can be used in all applications without triggering the intense immunogenic reactions in patients because the antibodies are derived from synthetic or natural human genes

(Weiner, 2006). The application of recombinant antibodies has increased over the last few years with the introduction of phage display technology (Frenzel et al., 2013). It has highly specific diagnostic and therapeutic applications. This method also has an advantage in that it needs less purified antigen to produce antibodies (Baldi et al., 2007). Antibody genes are isolated and then incorporated into plasmid DNA vectors, which are then transferred into new expression hosts. Antibodies fragments are typically displayed on bacteriophage surfaces, a virus that infects bacteria by introducing the antibody DNA to the phage genome (McCafferty et al., 1990).

However, the *Escherichia coli* (*E.coli)* folding machinery has limitations. It only allows small fragments to be display (Dubel et al., 2010; Hoet et al., 2005). Therefore, only variable region genes are used instead of whole antibody molecules because antibodies fragments are more easily assembled in microorganism than whole antibody molecules. This makes presentation on phage surfaces suitable for smaller antibodies fragment.

There are many antibody formats expressed as soluble proteins in prokaryotic and eukaryotic cells and these include disulfide-bond stabilized scFv (single chain fragment) (Schmiedl et al., 2000), single chain Fab fragments (scFab) combining scFv and Fab properties (Hust et al., 2007) as well as multi- and dimeric antibody formats (Hudson and Kortt, 1999; Hu et al., 1996) or minibodies (miniAbs) (Hu et al., 1996). Single domain antibody (sdAb) or nano body is the smallest antibody that contains of 11 only a single variable domain, either LC or HC fragment (Muyldermans, 2013) (Figure 1.2).

Fab and scFv are the most widely used antibody fragments for phage display. Fab is a larger format that consists of the constant and variable regions of the

antibody. The disulphide bond is formed between the LC fragments to the CH1 domain of the HC fragment (Williams and Barclay, 1998). Fab format requires the expression, transportation, and assembly of both HC and LC in the periplasmic space (Foudeh et al., 2012). The scFv format only consists of variable regions HC and LC fragments with a short repetitive linker sequence (glycine and serine) to hold both fragments together. However, scFv molecule can form a dimer, trimer or tetramer depending on the length of the linker sequence (Hudson and Kortt, 1999).

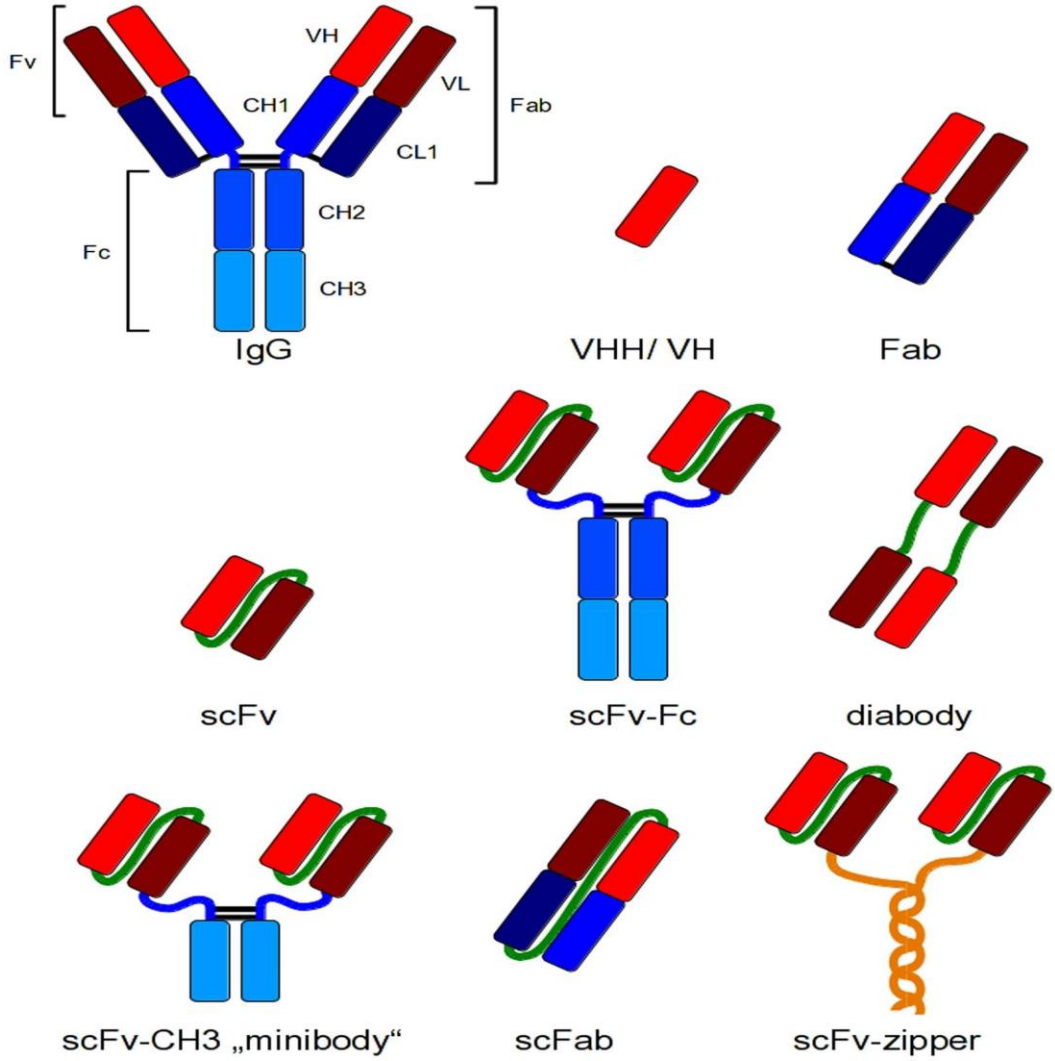

Figure 1.2 Recombinant antibody formats for different applications compared to IgG. Red and dark red: Variable regions; Blue: Constant regions; Green: Peptide linkers; Yellow: Amphiphatic helices used for dimerization of scFv fragments (Frenzel et al., 2013).

1.2.4 Antibody libraries

The collection of different antibody molecules cloned in phage vectors is called antibody phage library (Figure 1.3). Immune and naive libraries are constructed from natural human antibody gene diversity while synthetic and semi-synthetic libraries are constructed using chemically synthesized oligonucleotides based on designated gene sequences to exhibit similar diversities found in natural human antibody.

A naïve or non-immune library is generated using the IgM repertoire from human B cells. It can be generated from spleen, bone marrow or from animal sources (Mark et al., 1991). Donors for this library must be healthy and have not been infected with any diseases at the time of collection. To generate a naïve repertoire, IgM antibody is a suitable immature repertoire that provides unbiased characteristics because it is produced at the early stage of development (Dobson et al., 2015). The large repertoires of this naïve library are generated from a pool collection of healthy donor and rearranged *in vitro* to increase the diversity (Marks, 2004). Advantages of this library are (i) a single library can be used for different types of antigens (ii) isolation of high affinity antibodies when large library repertoires are used. However, the construction of this library is very challenging and is time consuming because the size and diversity of the library required is larger compared to the immune library. The quality of the library generated is also influenced by the diverse expression of the V-gene repertoire due to the variation in immunity and genetic history of the donors.

An immune library is normally derived from IgG mRNA of B-cells from animals or humans that have been exposed and infected by a certain type of disease (Azzazy and Highsmith, 2002; de Carvalho Nicacio et al., 2002). It is a typical library used in medical research because it is able to produce high quality and

affinity antibodies against the target antigen (Bazan et al., 2012). The size of an immune library can be smaller compared to other libraries because the repertoire consists of antibody with higher affinity against certain types of diseases. It is because of the polarization of the V-gene repertoire for antigen specificity in the B cells (Rahumatullah et al., 2015). Some of the antibodies have undergone affinity maturation through the immune system resulting in enrichment of antigen specific antibodies (Clackson et al., 1991; Skerra and Pluckthun, 1988). Some of the disadvantages of an immune library are (i) lack of immune response to self or toxic antigens, (ii) long time required for animal immunization, (iii) new antibody library must be constructed for different types of diseases and (iv) unpredictable immune responses to the target antigen (Cai and Garen, 1995).

The synthetic library is constructed *in vitro* by chemically synthesized oligonucleotides that are assembled *in vitro* resulting in a fully synthetic construction of the antibody gene (Knappik et al., 2000). The repertoire can be made by randomizing sequences using PCR technique or direct mutagenesis in the CDR3 region. There are several strategies to generate the synthetic repertoire including diversifying all three CDR loops in V-gene segment (Garrard and Henner, 1993) and randomizing the LC and HC repertoires (Akamatsu et al., 1993). This library is made up of a fixed framework and the diversity can be generated by synthetic oligonucleotides (Sidhu and Fellouse, 2006). Synthetic antibody repertoires can be easily generated using bioinformatics technology. Using this technology, stable and suitable structure of antibody can be identify (Ponomarenko et al., 2008). The advantage of such a library is that the repertoire has the potential to be controlled and the diversity can be clearly defined.

For a semi-synthetic library, the genetic information can be obtained from donors and randomized framework can be synthesized. This library is generated by fusion of the synthetic and naïve libraries (De Kruif et al., 1995). Naïve repertoire also can be presented in a semi-synthetic library. Therefore, these libraries can be used to produce antibody against any target antigens.

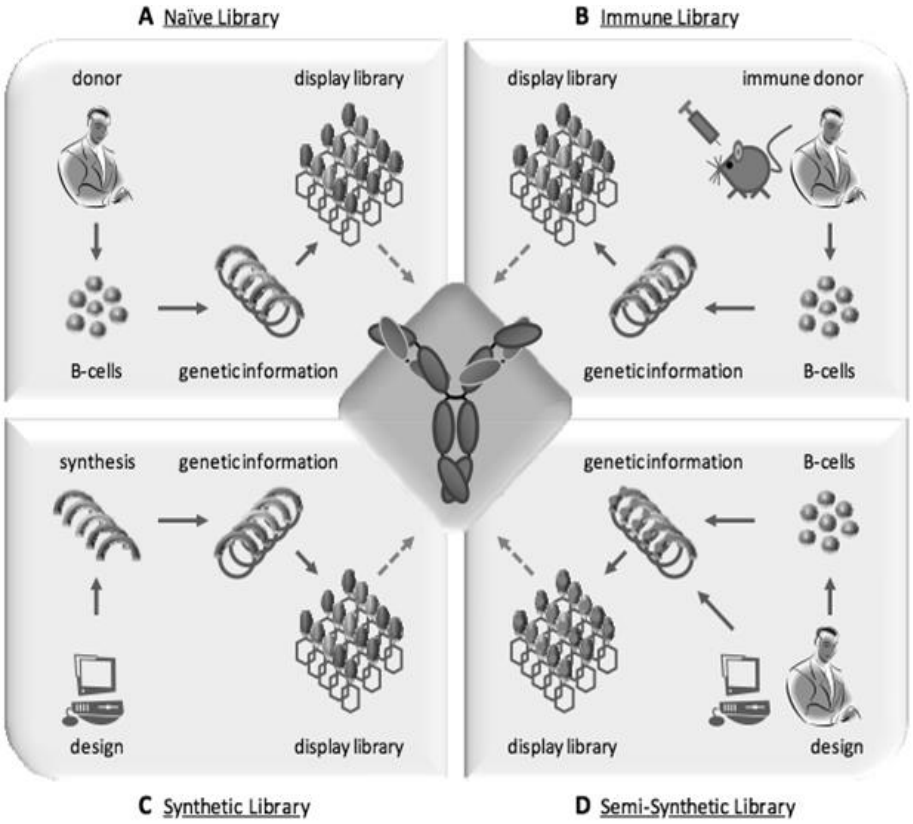

Figure 1.3 Different types of antibody libraries. (a) Generation of naïve libraries is from the B-cells isolation of non-immunized donor. (b) Generation of immunized libraries is from the B-cells isolation of immunized donor. (c) Generation of synthetic libraries are fully based on the computational design of the gene sequence (d) Generation of semi-synthetic libraries are combination of pre-designed segments and natural sources of gene sequence (Ponsel et al., 2011).

1.3 Phage display technology

Phage display is one of the techniques used for antibodies identification by affinity isolation against target antigen. Smith and colleagues first introduced this method in 1985 as a means to present foreign sequences on phage surfaces (Smith, 1985) and which has become an important tool for molecular evolution studies. This powerful technique allows for billions of different proteins or peptide, which are functionality intact (Paschke, 2006) to be displayed. It involves the expression of proteins, antibodies and peptides on the filamentous phage (Azzazy and Highsmith, 2002).

Phage display allows the presentation of large peptide and protein libraries on the surface of filamentous phage, which leads to the selection of peptides and proteins, including antibodies, with high affinity and specificity to almost any target. The bacteriophage (or simply phage) mostly used in phage display technology, are single-stranded DNA viruses that infect a number of gram-negative bacteria.

The presented antibodies can be used in a way similar to monoclonal antibodies that are important for cancer therapy and elucidating the specificity of autoimmune antibodies (Azzazy and Highsmith, 2002). It allows for rapid generation of monoclonal antibodies (Willats, 2002) that can be used for diagnostics and therapy. This technique is fast becoming a popular approach for *in vitro* selection and includes applications such as identification of interacting partners, evolution of enzymes and generation of antibodies (Li and Caberoy, 2010).

1.3.1 Structure of bacteriophages

M13 helper phage is the most common filamentous phage used for phage display (Barbas and Barbas, 1994). In a phagemid system, the non-lytic M13 helper phage will co-infect *E.coli* cell containing F' pilus, for example XL1 blue, ER 2738 and TG1 bacteria strains for phage packaging. Bacteriophage is a single stranded virus DNA that only infects gram-negative bacteria using the F' pilus as a receptor (Russel, 1991). The size of a bacteriophage is around 6-10 nm diameter, 2000 nm in length and consists of 11 different genes (Zimmermann et al., 1986). The functions of these 11 genes can be divided into 4 types, i) coat protein (III, VI-IX), ii) phage assembly (I, IV and XI), iii) DNA replication (II and X) and iv) binding (ssDNA) (Figure 1.4). The bacteriophage mainly consists of 2700 copies of pVIII protein and 3-5 copies each of the pVII and pIX proteins that form the phage head protein. PIII and pVI makes up the phage tail protein with each having 5 copies of protein for phage infection and stability (Russel et al., 1997). PIII and pVIII can be used for displaying peptide or protein in applications of phage display (Rodi and Makowski, 1999).

Life cycle of M13 phage can be divided into three stages, attachment, DNA replication and exportation. Infection starts when p3 of M13 phage attach to F pilus of *E.coli*. Single-stranded DNA of M13 enters the bacteria and convert into replicative form. Using replicative form as a template, DNA is replicative by rolling circle replication mechanism. Phage particles are assembled and extruded out bacterial membrane without cell lysis (Marvin, 1998).

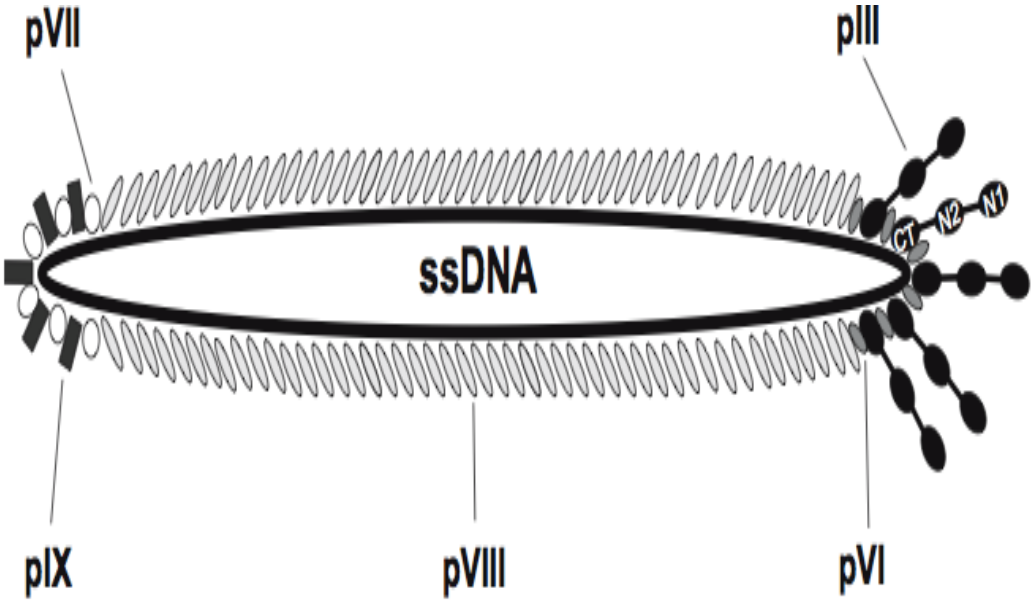

Figure 1.4 Structure of filamentous phage particle. The coat proteins of the filamentous phage such as pIII, pVI, pVII, pVIII and pIX. During the phage assembly process, this end emerges first from the bacterium. The incorporation of the minor coat proteins pIII and pVI on the other end of the particle terminates the assembly process. pIII consists of three domains (N1, N2, and CT), which are connected by flexible linker regions. The binding of the N2 domain to the F pilus initiates the infection (Paschke, 2006).

1.3.2 Display formats

The coat proteins that are commonly used for phage display are pVIII and pIII (Smith and Petrenko, 1997). They are normally involved in the cloning of recombinant phage antibodies and peptides (Dente et al., 1994). Foreign sequence either protein or peptide are inserted at the C-terminus of the pIII and pVIII on the vector. While, linker sequence of the HC and LC repertoire are normally inserted at the N-terminus. There are thousands of copies of pVIII coat protein that form the long cylindrical phage particle. However, there are only 3-5 functional domains for pIII coat protein (Webster. 1996). The pVIII coat protein only allows for short peptides or protein to be displayed on it because it could interrupt the interaction during assembly. On the other hand, pIII coat protein can tolerate large foreign molecules while maintaining the infectivity. Therefore, pIII coat protein is a suitable target protein for phage display. The recombinant antibodies are normally expressed as pIII fusion proteins. The antibodies will bind to the target antigen and the bound phage is detected with HRP labeled antibody (Dente et al., 1994).

1.3.3 Antibody phage display

Phage display has become a powerful application since 1985 that allows *in vitro* mimicking of the natural human antibody responses. This technique is a robust scientific research and diagnostic tool because it can isolate recombinant antibody repertoires with unique specificity (Winter et al., 1994; Griffiths, 1993). Antibody genes are fused to gene pIII of phage particle to generate an antibody phage library and create an association between antibody genotype and phenotype (Barbas et al., 1991). The single antibody will be display by phage particle in accordance to the antibody genotype encoded by phage particle (Winter et al., 1994).

ScFv (~35 kDa) and Fab (~60 kDa) antibody formats are favored in constructing phage antibody libraries due to difficulties displaying and assembling large antibody formats on the phage surface. Fab fragments are more stable than scFv because it consists of disulphide bonds and constant domains that hold the molecule together. Fab is normally chosen over the scFv format when a complete antibody format is needed as the final product (Feldhaus et al., 2003). The removal of the scFv linker might result in the changing of antigen binding. However, scFv with a smaller size has advantages compared to Fab because it has a better production yield as a fusion protein and easier to construct. However, to construct antibody libraries of either Fab or scFv it depends on the purpose of the antibodies for use in downstream applications (Carmen and Jermutus, 2002).

1.3.4 Biopanning

Biopanning is a process to identify unique phage clones that binds with the target antigen (Willats, 2002). This process is carried out to determine the antigen specific clones (Nissim et al., 1994; Clackson et al., 1991). The physical interaction between the phage particle and specific antibody allows for *in vitro* selection (Romanov, 2003) (Figure 1.5). The phage library or phage clones is produced with standard molecular biology techniques involving the exploitation of DNA vectors to accommodate V-gene inserts and accommodate packaging to mature phage particles by making use of the *E.coli* machinery (Galanis et al., 1997).

This biopanning technique is based on collection of repetitive cycles of amplification, incubation, washing and reselection of phage particles (Bazan et al., 2012). After several rounds of affinity selections of the libraries, an enriched pool of antigen binding phage particles can be isolated. The target antigen can be

immobilized onto microtiter plate wells, plastic tubes and columns with target-activated matrix (Lomonosova et al., 2011) PVDF membrane (Liu et al., 2002), column matrix (Garet et al., 2010) and magnetic beads (Noronha et al., 2002). Target phage is captured during incubation and after that the washing step is carried out. This step was introduced to remove unbound phages and contaminants (Goodchild et al., 2005). Washing step is very important to wash off non-specifically bound phage from the microtiter plate. To obtain a strong binder of antibodies, more stringent washing conditions can be introduced. The antibody that is bound to the target antigen can be eluted either using base or acid solutions (Hoogenboom et al., 1998). For the next round of biopanning, the rescued phage clones are infected again with TG1 to be amplified as input phage. This continuous process will provide a concentrated pool of a selected population of antibodies after several rounds.

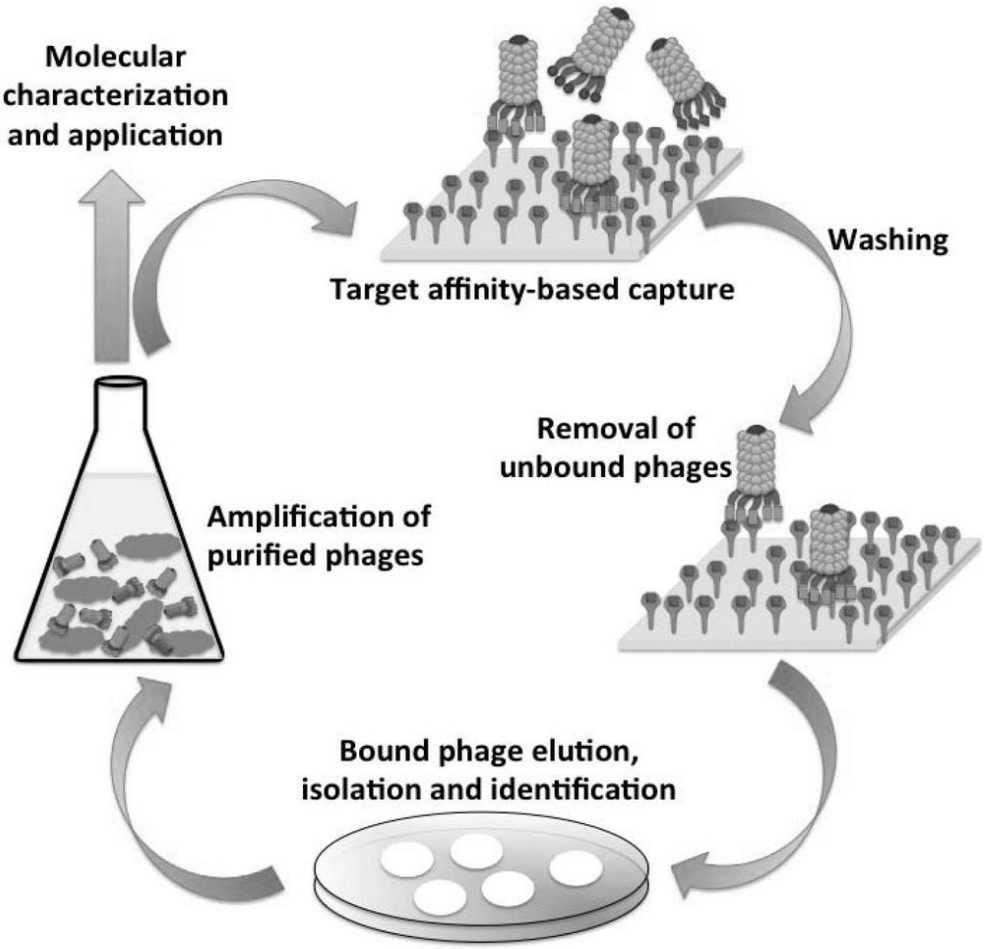

Figure 1.5 Biopanning process of phage display libraries. The antibodies are presented on the phage particle and add into immobilized target antigens for binders. The unbound phages are removed by several washing steps, elution and propagation of the bounded phages (Singh et al., 2013).

1.4 Applications of phage display

Phage display has provided a valuable alternative for the isolation of numerous peptides and proteins. It has been proven successful in generating antibodies against target antigens that are useful in a number of applications (Bratkovic, 2010). The advantages of phage display technology are mainly the speed to obtain monoclonal antibodies and an animal-free process. V-gene pool of synthetic libraries and healthy donors can be made artificially by generating diversity synthetically genes in the CDR.

Phage display technology can also be used for direct isolation of antibodies against intracellular target molecules. Intrabodies are very good in modifying and visualizing the function of intracellular targets. Highly diverse scFv has been screened to select intrabodies due to the stability of scFv in reduced environments (Cardinale and Biocca, 2008). Phage display also has been widely used in anti-tumor activity. Antibody with tumor specificity can be used in cell toxic conjugates and target directed delivery of imaging agents (Zhou and Marks, 2009). Not only that, it also can reproduce antigenic epitopes by selecting anti-idiotypic antibodies (Zhikui et al., 2010). The potential application of phage derived human monoclonal antibodies for therapy and diagnostics is vast.

1.5 Problem statement and rationale of study

Tuberculosis (Tb) is an infectious disease associated with high mortality and causes 2-3 million deaths every year. The number of Tb infections has constantly increased over the past few years and the emergence of drug resistant strains for Tb (XDR, TDR) increases the urgency for alternative treatments. The concept of antibody-based therapy for infectious diseases is increasing in recent years because it provides a potentially suitable alternative treatment especially for those infected with *Mtb* resistant strains (Saylor et al., 2009). However, a specific antibody therapy for Tb infection is not available. The challenge in antibody-based therapy is the discovery of effective monoclonal antibodies. Human antibodies have fewer side effects compared to conventional murine monoclonal antibodies. Therefore, phage display is the suitable method for generation of human monoclonal antibody.

In order to obtain good quality antibodies against a certain disease, the use of immune antibody libraries are preferred due to its skewed repertoire. This makes the generation of an immune Tb library very useful in terms of research and also lead to candidate isolation. As there is still no Tb immune antibody library reported to date, the generation of a Tb immune scFv antibody library is important to help with the growing concerns of Tb infection in the world. The focus on antibody-based immunity allows for a new perspective to be looked upon for Tb immunity. This is because traditional concepts of Tb immunity only involve the T-cell arm in the immune system with little focus given to the B-cell arm. However, this is no longer the case as several studies in recent years have highlighted the importance and role of B-cell immunology in Tb immunity (Cooper, 2009). This makes the role of an immune Tb library even more important in terms of the development of potential strategies for Tb therapy in the future.