IDENTIFICATION OF ANTIGENS RECOGNIZED BY THE MONOCLONAL ANTIBODY, IBMR3 ON LYMPHOCYTES AND ESTABLISHED CELL LINES

FARIZAN BINTI ARIS

UNIVERSITI SAINS MALAYSIA
2016

IDENTIFICATION OF ANTIGENS RECOGNIZED BY THE MONOCLONAL ANTIBODY, IBMR3 ON LYMPHOCYTES AND ESTABLISHED CELL LINES

by

FARIZAN BINTI ARIS

Thesis submitted in fulfillment of the requirements
for the degree of
Doctor of Philosophy

September 2016

ACKNOWLEDGMENT

How to say thank you, when you can't thank enough. First and above all, I praise Allah, The Almighty for providing me this opportunity and granting me the capability, the strength and the patience to proceed successfully. This project could have not been completed without the help of some very important and special people.I wish to convey my sincere gratitude to many people who have accompanied and supported me all the way. To Prof Ishak Mat, my supervisor who deserves special recognition for being a great supervisor in providing inspiring scientific discussions and study facilities. I am greatly appreciate my co-supervisor Dr. Mohd Nazri Ismail who monitored my work and took efforts in reading and providing me with valuable comments on the thesis writing.

Many thanks to my close friend as well as project partners, Ekhlas, who played such important roles along the journey, as we mutually engaged in making sense of the various challenges we faced and in providing encouragement to each other during times when it seemed impossible to continue. I would never forget the company I had from my fellow research scholars, Rabiatul, Hazwani and Madziatul. I enjoyed my work with their encouragement, cooperation and moral support. I would also like to express my greatest appreciation to my best friends, Indah and Zulinda who were always available to support, encourage and motivate me during difficulties. Thank you to Cik Mah and family who helped taking care of my children while I was working in the lab. I would like to thank the Oncological and Radiological Department of IPPT especially Rafedah, Rohanizah and Ira Maya for their valuable help.

My parents taught me the value of hard work by their own examples. I want to thank my mother for her silent prayers. Unfortunately, my father is no longer with us to celebrate the completion of my thesis. However, the encouragement and motivation that was given to me by my father is always remembered. I warmly thank and appreciate my mother and father-in-law for their material and spiritual supports. I also would like to thank my brothers, sister, and brothers and sisters-in-law, who have provided assistance in numerous ways. . Finally, without the support and sacrifice of my lovely husband, Norazman and my children Aufa and Ilham, I would never have succeeded. I would also like to share this moment of happiness to my newly born twin, Layla and Sarah who have being one of my inspiration to complete my study . I can just say thanks for everything and may Allah give you all the best in return.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

°C	Degrees Celsius
ADC	Antibody drug conjugate
ADCC	Antibody dependent cell cytotoxicity
Ag	Antigen
ALL	Acute Lymphoid Leukemia
AML	Acute Myeloid Leukemia
APS	Ammonium persulfate
Bis	N, N‟-methylene bisacrylamide
BSA	Bovine serum albumin
CCS	Cell culture supernatant
CD	Cell differentiation
CDC	Cell dependent cytotoxicity
cDNA	Complementary DNA
CEA	Carcinoembryonic antigen
DAB	3,3'-Diaminobenzidine 2
dH2O	Double-distilled water
DMEM	Dulbecco's Modified Eagle's Medium
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic Acid
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ELISA	Enzyme-Linked Immunosorbent Assay
ER	Estrogen receptor

et al.	and others
FAB	French-American-British
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
g	Gram
HAMA	Human anti-mouse antibody
HLA-DR	Human leukocyte antigen
HPLC	High performance liquid chromatography
HRP	Horseradish peroxidase
Ig	Immunoglobulin
IL	Interleukin
IL-R	Interleukin receptor
KDa	Kilodalton
MAb	Monoclonal antibody
Min(s)	Minute(s)
mL	Milliliter
mM	Millimolar
MTS	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NaCl	Sodium chloride
ng	Nanogram
nM	Nanomolar
NRS	Normal rabbit serum
PAGE	Polyacrylamide gel electrophoresis
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline

PHA	Phytohaematoglutinin
PSA	Prostate serum antigen
PWM	Pokeweed mitogen
rpm	Rotations per minute
RPMI	Roswell Park Memorial Institute
RT	Room temperature
RT-PCR	Reverse transcription-PCR
Sec(s)	Second(s)
TAA	Tumor associated antigen
TBS-T	Tris Buffered Saline with Tween 20
TEMED	N,N,N‘‘,N‘‘-Tetramethylethylenediamine
Tris	Tris-(Hydroxymethyl)-Aminomethane
v/v	Volume per volume
VEGF	Vesicular Endothelial Growth Factor
WHO	World Health Organization
µg	Microgram
µL	Micro liter
µM	Micro molar

PENGESANAN ANTIGEN YANG DIKENALPASTI OLEH ANTIBODI MONOKLON IBMR3 PADA SEL LIMFOSIT DAN TITISAN SEL

ABSTRAK

Antibodi monoklon menunjukkan kegunaan yang tinggi dalam penyelidikan dan klinikal. Antibodi monoklon IBMR3 (IBMR3 MAb) telah menunjukkan keupayaan mengcam molekul yang mungkin berhubungkait dengan kompleks reseptor interleukin-4 (IL-4R). Selama ini, IL-4R sering dikaitkan dengan kanser. Tujuan penyelidikan ini adalah untuk mengesan pengekspresan antigen IBMR3 (IBMR3 Ag) pada sel mononuklear darah periferal (PBMC) dan panel titisan sel. Kaedah pewarnaan imuno menunjukkan IBMR3 MAb boleh mengcam antigen pada beberapa jenis sel termasuk sel epithelial, fibroblas, haematopoitik, kanser dan normal. Yang menariknya, IBMR3 Ag diekpres dengan signifikan dalam ruang sitoplasma tetapi tidak diekspres pada permukaan sel kecuali pada sel limfosit normal. Analisis pengekspresan IBMR3 Ag menunjukkan pengekspresannya adalah tinggi pada limfosit teraktif berbanding limfosit tidak teraktif tanpa mengambilkira jenis ejen mitogenik yang digunakan. Limfosit yang diaktifkan oleh CD3 MAb menunjukkan pengekspresan tertinggi (60.00 ± 1.94%) diikuti oleh limfosit yang diaktifkan oleh rangsangan PHA (41.23 ± 0.63%). Namun begitu, kombinasi rangsangan PHA dan IL-4 (38.78 ± 4.82%) menunjukkan pengekspresan yang hampir sama dengan pengekspren akibat rangsangan PHA sahaja (41.23 ± 0.63%). Rangsangan oleh IL-4 sahaja (22.51 ± 1.00%) dan PWM (22.00 ± 1.00%) sahaja menunjukkan pengekspresan pada kadar yang hampir sama dengan kawalan tanpa rangsangan (20.61 ± 0.88%). Asai MTS telah digunakan untuk menyiasat kesan inkubasi IBMR3 MAb dengan kultur PBMC dan sel Jurkat. Berdasarkan kepada

keputusan, proliferasi PBMC telah terencat sebanyak 10.00 ± 5.30% dan 6.60 ± 5.10% pada tempoh inkubasi masing-masing selama 48 dan 72 jam. Sebaliknya, apabila IBMR3 MAb diinkubasi dengan sel Jurkat rangsangan proliferasi yang ketara diperoleh sehingga 57.90 ± 14.40% selepas 48 jam inkubasi. Namun, inkubasi lebih lama pada 72 jam menunjukkan penurunan proliferasi sebanyak 16.42 ± 12.70% berbanding kawalan. Analisis spektrometri jisim pada sel Jurkat menemukan lapan calon protin bagi molekul IBMR3, yang mana tujuh daripadanya adalah molekul yang selalu dihubungkaitkan dengan kanser. Salah satu protein dengan skor signifikan yang tinggi adalah YWHAZ. Pengekspresan melampau YWHAZ sering dikaitkan dengan kesan malignan pada banyak jenis kanser. Tambahan pula, subsrat bagi YWHAZ adalah protin yang diketahui terlibat dalam tapakjalan isyarat proliferasi IL-4. Oleh itu, IBMR3 Ag adalah berkemungkinan molekul yang mengambil bahagian dalam proses pembentukan tumor melalui penglibatannya dalam proliferasi sel.

IDENTIFICATION OF ANTIGENS RECOGNIZED BY THE MONOCLONAL ANTIBODY, IBMR3 ON LYMPHOCYTES AND ESTABLISHED CELL LINES

ABSTRACT

Monoclonal antibodies have demonstrated significant utilities in both research and clinical applications. IBMR3 monoclonal antibody (IBMR3 MAb) has been shown to recognize a molecule that may be associated with the human interleukin-4 receptor (IL-4R) complex. IL-4 has been shown to be extensively associated with cancer. The aim of this study is to identify the IBMR3 antigen (IBMR3 Ag) expressed in normal peripheral blood mononuclear cells (PBMC) and in a panel of established cell lines. Immunostaining revealed that IBMR3 MAb can recognize antigen in a variety of cell types, including epithelial, fibroblast, haemopoietic, cancer, and normal cells. Interestingly, IBMR3 Ag was found to be expressed in the cytoplasm but was absent on the cell surface except for normal lymphocytes. The analysis of IBMR3 Ag expression on lymphocytes showed that IBMR3 Ag expression was higher on activated lymphocytes compared to resting lymphocytes regardless of the type of mitogenic agents used. The lymphocytes activated by CD3 MAb demonstrated the highest expression (60.00 ± 1.94%) followed by those activated only by phytohaematoglutinin (PHA) stimulation (41.23 ± 0.63%). However, the combination of PHA with interleukin 4 (IL-4) stimulation (38.78 ± 4.82%) exhibited almost the same expression as PHA alone (41.23 ± 0.63%). Stimulation by IL-4 alone (22.51 ± 1.00%) and pokeweed (PWM) alone (22.00 ± 1.00%) produced almost the same level of expression as unstimulated control (20.61 ± 0.88%). MTS assay was used to investigate the effect of IBMR3

MAb incubation in PBMC and Jurkat cultures. According to the results, the PBMC proliferation was slightly inhibited by 10.00 ± 5.30% and 6.60 ± 5.10% at 48 and 72 hours of incubation time, respectively. Conversely, when IBMR3 MAb was incubated with Jurkat cells, significant proliferation stimulation was observed and reached up to 57.90 ± 14.40% after 48 hours of incubation. However, longer culture incubation at 72 hours decreased culture proliferation by 16.42 ± 12.70% compared with control. Mass spectrometry on Jurkat cells revealed eight candidate proteins for IBMR3 Ag , seven of which are common molecules associated with cancer. One of the proteins with high significance score was YWHAZ. Overexpressed YWHAZ has been correlated with malignant outcomes of several cancers. Moreover, the substrate for YWHAZ binding is known to be a protein involved in the IL-4 proliferation signaling pathway. Therefore, IBMR3 Ag could be a molecule that participates in tumorigenesis through involvement in cell proliferation.

CHAPTER 1

INTRODUCTION

1.1 Background of the study

Monoclonal antibody (MAb) is a biomolecule that is widely used in research and clinical applications. Antibody–antigen binding, which was envisioned as the "magic bullet" concept by Paul Ehrlich over 100 years ago, is a potentially important basis in developing monoclonal antibodies as potential alternative for unspecific chemical anticancer agents. Treatment with antibodies, which are naturally occurring in the human body, is expected to produce fewer side effects compared to chemical or radioactive agents (Hansel et al., 2010). However, the method to achieve optimal delivery to the target tissue remains unknown (Coulson et al., 2014). Through hybridoma technology, which was first developed by Kohler and Milstein in 1975, a monoclonal antibody can be generated against anything that initiates antibody production (Kohler and Milstein, 1975). Thus, any type of antibody can be potentially created. Antibodies are common and essential tools for many research applications, including Western blot analysis, immunohistochemistry, immunocytochemistry, enzyme-linked immunosorbant assay (ELISA), immunoprecipitation, and flow cytometric analysis. In clinical applications, antibodies are used in managing many diseases, including cancer. At present, monoclonal antibodies are progressively being developed for a larger spectrum of therapeutic applications as well as for cancer diagnosis, prognosis, therapy, surveillance, and monitoring devices.

Cancer is a disease caused by the abnormal proliferation of cells. Pathologically, cancer is categorized as benign or malignant. A benign tumor remains locally in its original location, whereas a malignant tumor can invade surrounding tissues and spread

throughout the body of a host. The ability to invade and spread, which is referred as metastasis, makes cancer a fatal disease (Hanahan and Weinberg, 2000). Cancers are classified into several major groups, including carcinoma, sarcoma, and leukemia or lymphoma. Approximately 90% of human cancers are malignancies of epithelial cells known as carcinoma. Sarcomas, which are usually rare, are tumors of connective tissues, bones, cartilages, and fibrous tissues. Roughly 8% of human malignancies are identified as leukemia and lymphoma, which result from blood-forming cells and cells of the immune system, respectively (Cooper and Hausman, 2000).

Interleukin 4 (IL-4) cytokine is one of the essential lymphocyte growth factors for fundamental cell functions (Luzina et al., 2012). The expression of IL-4 cytokine and its receptor in a number of cancer types are well-documented (Suzuki et al., 2015, Venmar and Fingleton, 2014). However, the role and involvement of cytokine and cytokine receptors in cancer remain controversial (Li et al., 2009a). Four synthetic peptides were used in a previous study to generate monoclonal antibodies against human IL-4 receptor (IL-4R) to characterize the role of the receptor in cancer progression or tumorigenesis (Mat, 1992b). These peptides correspond to selected sequences of human IL-4R amino acids from the cDNA sequence published in 1990 (Galizzi et al., 1990). These peptides that represent the extracellular and intracellular domains of the IL-4R complex were chosen because of their potential immunogenicity. The amino acid sequences of the peptides are shown in Figure 1.1. The resulting MAb raised against commercially prepared synthetic peptides were expected to cross-react with the native human IL-4R protein. However among the generated MAbs, only IBMR3 was reactive against native and denatured antigens. In a preceding functional study, IBMR3 MAb did not significantly inhibit the IL-4 induced proliferation of peripheral blood lymphocytes. In the assay, IBMR3 was added simultaneously with

recombinant IL-4 to a lymphocyte culture (Mat, 1992a). However, a previous work determined that the preincubation of the with IBMR3 MAb before adding IL-4 to the culture system inhibited the subsequent proliferative response of MO7 (IL-3 and IL-4 dependent monocyte cell line) cells. These preliminary data demonstrated the possibility that IBMR3 MAb may recognize a part of the human IL-4 receptor complex at low affinity (Mat, 1992a).

1MGWLCSGLLFPVSCLVLLRVASSGN**MKVLQEPTCVSDY**MSISTCEWKMNGP
TNCSTELRLLYQLVFLLSEAHTCVPENNGGAGCVCHLLMDDVVSADNYTLDL
WAGQQLLWKGSFKPSEHVKPRAPGNLTVHTNVSDTLLLTWSNPYPPDNYLYN
HLTYAVNIW**SENDPADFRI**YNVTYLEPSLRIAASTLKSGISYRARVRWAQCYNT
T**WSEWS**PSTKWHNSYREPFEQHLLLGVSVSCIVILAVCLLCYVSITKIKKKEWW
DQIPNPARSRLVAIIIQDAQGSQWEKRSRGQEPAKCPHWKNCLTKLLPCFLEHN
MKRDEDPHKAAKEMPFQGSGKSAWCPVEISKTVLWPESISVVRCVELFEAPVE
CEEEEEVEEEKGSFCASPESSRDDFQEGREGIVARLTESLFLDLLGEENGGFCQQ
DMGESCLLPPSGSTSAHMPWDEFPSAGPKEAPPWGKEQPLHLEPSPPASPTQSP
DNLTCTETPLVIAGNPAYRSFSNSLSQSPCPRELGPDPLLARHLEEVEPEMPCVP
QLSEPTTVPQPEPETWEQILRRNVLQHGAAAAPVSAPTSGYQEFVHAVEQGGT
QASAVVGLGPPGEAGYKAFSSLLASSAVSPEKCGFGASSGEEGYKPFQDLIPGC
PGDPAPVPVPLFTFGLDREPPRSPQSSHLPSSSPEHLGLEPGEKVEDMPKPPLPQE
QATDPLVDSLGSGIVYSALTCHLCGHLKQCHGQEDGGQTPVMASPCCGCCCG
DRSSPPTTPLRAPDPSPGGVPLEASLCPASLAPSGISEKSKSSSSFHPAPGNAQSSS
QTPKIVN**FVSVGPTYMRVS** 825

Figure 1.1 Amino acid sequence of the human IL-4 receptor cDNA. The sequences that are used to synthesize peptides to raise IBMR3 are indicated in bold letters (**MKVLQEPTCVSDY** is peptide 5; **SENDPADFRI** is peptide 2; **WSEWS** is peptide 3; and **FVSVGPTYMRVS** is peptide 4) Adopted from (Mat, 1992a).

1.2 Problem Statement

A considerable number of publications reported that IL-4R was overexpressed in many cancer types, such as melanoma, breast carcinoma, and ovarian carcinoma. The level of IL-4 cytokine was correlated with malignant transformation (Li et al., 2005, Kawakami

et al., 2004, Kaklamanis et al., 1996). Furthermore, the upregulated expression of IL4-R was observed in glioma, breast, lung, colon, and many other types of malignancies (Joshi et al., 2014, Todaro et al., 2008, Prokopchuk et al., 2005, Murata et al., 1998). However, the role of IL-4R in cancer remains debatable. *In vivo* and *in vitro* studies indicated that IL-4 is a tumor growth-supporting cytokine in murine myeloma and colon cancer cell line (Li et al., 2009a, Li et al., 2008a). In contrast, IL-4 also inhibited the proliferation of human renal, breast, and colon cancer cells (Gooch et al., 2002, Okada et al., 2001). At present, continuous efforts are carried out to understand the function of IL-4R in cancer although IL-4 is considered as the most understood cytokine (Banderas et al, 2012; Koller et al., 2010).

IBMR3 MAb was previously raised against the IL-4R peptide (Mat, 1992). The result of ELISA indicated that IBMR3 MAb binds to the peptide that it was raised against (Mat, 1992).). However, IBMR3 MAb failed to inhibit the proliferation of cell culture when it was added with IL-4 cytokine . (Mat, 1992).. Thus IBMR3 MAb may bind to IL-4R associated molecule yet it was not clear what IBMR3 Ag is. Previous results showed that IBMR3 MAb recognized protein with different molecular weights between Jurkat and normal PBMC lysates (Abas et al., 2008). We hypothesized that IBMR3 Ag may play a role during transformation from normal to cancer cells. Hence, characterization and identification of IBMR3 Ag may help to understand the importance and involvement of the molecule in cancer , thus provide basis for possible application of IBMR3 MAb in cancer management.

1.3 Objectives of the Study

1. To analyze IBMR3 antigen expression and localization on lymphocytes as well as on Jurkat, HT29, Hep2 and 3T3 cell lines.
2. To examine the modulation of the IBMR3 antigen expression on mitogen-activated versus resting normal lymphocytes and Jurkat cells.
3. To determine IBMR3 MAb binding effect on normal PBMC and Jurkat proliferation.
4. To identify the IBMR3 antigen in Jurkat cells.

1.4 Literature review

1.4.1 Antibody and its general characteristics

Antibody is a group of glycoproteins also commonly known as immunoglobulins. Globulin is among the three types of human serum protein besides albumin and fibrinogen (Schroeder and Cavacini, 2010). The term globulin encompasses a heterogeneous group of proteins that typically have high molecular weight, and both its solubility and electrophoretic migration rates are lower than those of albumin. Among these proteins, gamma globulins were found to be immunologically active and were therefore later called immunoglobulins (Louise and Louise, 2009).

In nature, antibodies can be found in body fluids, including blood plasma, tears, saliva, and colostrum. Immunoglobulin molecules have five distinct isotypes recognized in higher mammals: IgM, IgG, IgA, IgD, and IgE. These immunoglobulin types differ in characteristics, such as molecular weight, serum concentration, and carbohydrate component (Louise and Louise, 2009). The primary function of antibodies is to neutralize bacterial toxins or certain viruses following their binding with antigen. A

secondary interaction of antibodies with another effector agents is usually required to dispose larger antigens such as bacteria (Späth, 1999).

1.4.2 Structure of a typical antibody molecule

Most antibodies used in cancer diagnosis and therapy are derived from the IgG isotype. The IgG isotype antibody consists of two antigen-binding fragments (Fabs) connected through a flexible region (hinge) to a constant (Fc) region (Figure 1.2). This structure has two pairs of polypeptide chains, with each pair containing a heavy and a light chain of dissimilar sizes. Both heavy and light chains are folded into immunoglobulin domains. The variable domains in the amino-terminal part of the molecule are the domains that identify and bind antigens. The rest of the molecule is composed of constant domains that vary among immunoglobulin isotypes that give them unique properties and functions. The Fc portion of the immunoglobulin serves to bind a variety of effector molecules of the immune system aside from being associated with the serum half-life of antibodies (Schroeder and Cavacini, 2010, Trail et al., 2003).

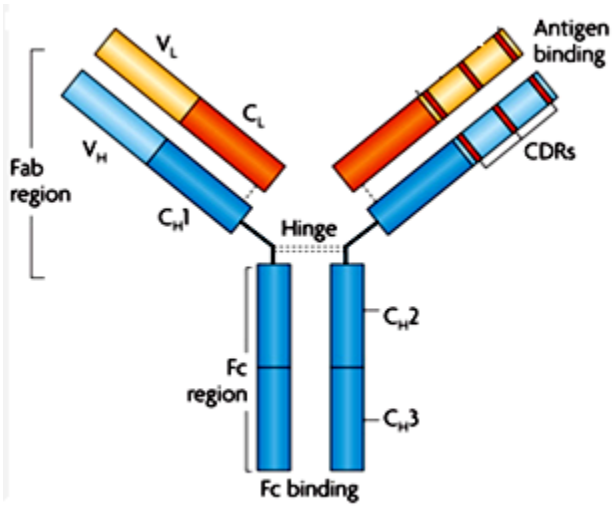

Figure 1.2 Schematic structure of an immunoglobulin G (IgG). Adopted from (Hansel et al., 2010).

1.4.3 Monoclonal versus polyclonal antibodies

The decision of whether to use a polyclonal antibody (PAb) or monoclonal antibody (MAb) depends on a number of factors. The most important factor is its intended use. The production of PAb is more rapid at several months, inexpensive, and requires less technical skill than that of MAb (Ritter, 2000). PAb consists of heterogeneous antibodies that bind to multiple epitopes. Thus, PAb is less sensitive than MAb to small changes in epitope structure due to genetic polymorphism, denaturation, or glycosylation. These antibodies are stable over a wide range of pH and salt concentrations (Lipman et al., 2005). PAb is particularly useful in applications that recognize more than one epitope (antibody binding site) on a target molecule or where the molecule of interest is highly conserved. The most common application of PAb is for the detection of multiple epitopes as secondary, conjugated reagents for indirect immunoassays. In the application, polyclonal binding to the primary layer antibody leads to considerable amplification of the signal detection (Pohanka, 2009). A major problem of PAb use is on its limited amount of production. A PAb can never be exactly reproduced as different animals cannot produce identical responses toward the same immunogen. For this reason, different batches of PAbs will have different ranges of specificities and affinities (Lipman et al., 2005).

By contrast, the generation of MAb is time consuming, costly, and requires highly trained technical skill. The generation of MAb-producing cells, the hybridomas, can take up to a year or longer (Ritter, 2000). The main advantages of MAbs are their homogeneity and consistency. Once characterized, the behaviors of MAbs are predictable. Identical MAbs can be produced infinitely. The greatest effect of MAbs is their use for discovering and characterizing the structure and function of novel molecules. Nearly all molecules that are known to be important in immune response

were previously identified through the generation of specific MAbs. The homogeneity of MAb provides excellent specificity towards a single epitope (Lipman et al., 2005, Pohanka, 2009). However the same monospecificity also limits their utility as MAb functions are susceptible to slight changes in epitope structure. To date, MAbs have demonstrated success in broad applications, and humanized MAbs are now the fastest-growing group of biotechnology-derived molecules in clinical trials (Nelson et al., 2010).

1.4.4 MAbs and their clinical applications

MAbs are monospecific antibodies made by identical clones of plasma cells in contrast to PAbs, which are made from several different cell clones. Since the introduction of the revolutionary MAb technology by Kohler and Milstein in 1976, the continuous production of large amounts of single-type antibodies known as MAbs have been made possible (Köhler and Milstein, 1976). Many efforts to improve MAb treatment efficacy while decreasing toxicities have led to the development of a large variety of engineered MAbs for research, diagnosis, and therapy tools. In various categories, MAbs are used in their naked, conjugated, chemically-modified, fragmented, or genetically recombinant modified forms (Sapra and Shor, 2013).

1.4.5 MAb production

The production of MAbs is achieved using a couple of different techniques, one of which involves the formation of hybridoma cells. Hybridomas are generated by the fusion of immune spleenocytes with myeloma. The spleen cells are extracted from a mouse previously exposed to the antigen of interest. The resulting hybridoma when grown produces certain antibodies constitutively as an outcome of the antibody-

producing spleen cells and rapidly growing immortal myeloma cells (McCullough and Spier, 1990, Kohler and Milstein, 1975). However, MAbs isolated from animal hosts, such as murine species, induce human anti-mouse antibody (HAMA) response in humans (Tjandra et al., 1990).

To decrease immunogenicity, MAbs are commonly modified into chimeric or humanized constructs. Genetic engineering is used to fuse the antigen binding region (variable domains of the heavy and light chains, VH and VL) from murine-derived MAbs with the constant region from human sources (Figure 1.3). The chimeric MAbs successfully improved the efficacy and reduced the HAMA response of some therapeutic MAbs (Hwang and Foote, 2005). However, the perception of murine-associated risk drove the MAb technology to the production of humanized MAbs (Reichert et al., 2005). These antibodies are constructed with only antigen-binding regions derived from a mouse and the remainder regions from a human source. Later, to circumvent the immunogenicity issue of murine-derived antibodies, fully human antibodies were generated in transgenic mice. With the advent of DNA recombinant technology, this production was achieved by replacing antibody genes in mice with human genes. Thus, fully human antibodies are produced by transgenic mice in response to immunization with a certain antigen (Hwang and Foote, 2005). Another method to produce these types of antibodies is by fusing antibody-encoding genes with a capsid protein of a bacteriophage. In addition to phage display antibodies, antibody fragments or full molecules can also be presented in yeast, bacteria, and mammalian cells (Deantonio et al., 2014, An, 2008). In the early stage of DNA recombinant technology in the 1980s, the generation of human hybridomas was derived from human lymphocytes and myeloma cell lines. Nonetheless, the approach was proved to be unreliable, produced insufficient antibody quantity, and was vulnerable to

contamination. Another limitation is that, "immunizing" patients in a controlled manner and then collecting the resultant lymphocytes was not allowed for ethical reasons (Nissim and Chernajovsky, 2008).

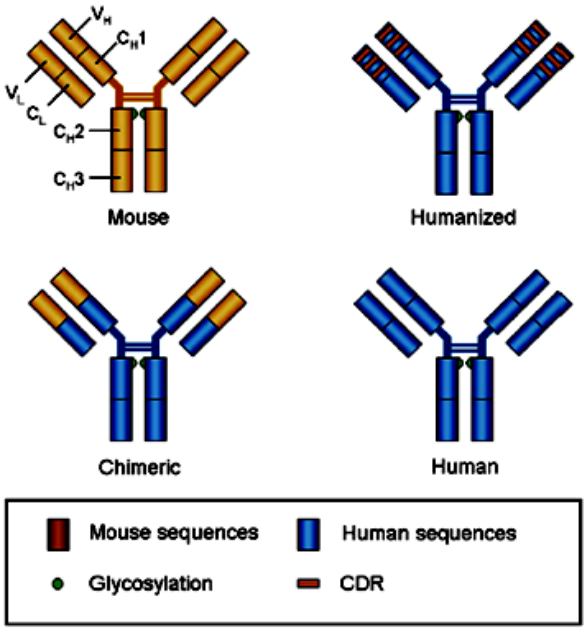

Figure 1.3 Modifications of MAbs. Adopted from (Kim et al., 2005).

1.4.6 Categories of MAbs for therapeutic use

Human antibody production by conventional hybridoma technology is difficult because the *in vivo* immunization of humans is not feasible for many antigens (Smith et al., 2013). In addition, the conventional hybridoma technology does not produce stable levels of antibodies. About 75% of the currently available therapeutic antibodies are full-length IgG molecules (Reichert, 2012). One reason for this situation is that IgGs are structurally stable, have long *in vivo* half-life, and confer Fc-mediated biological properties. Nonetheless, being whole molecules with a molecular weight of about 150 kDa, IgGs penetrate poorly from the vascular bed into a tumor tissue mass and clear slowly from the body (Chang et al., 2002). Aside from whole molecule antibodies (Zider and Drakeman, 2010), antibody fragments can also be generated into Fabs,

single-chain fragments, diabodies, minibodies, and others (Figure 1.4). Relative to whole molecules, these antibodies with smaller-sized fragments have better penetration and improved clearance characteristics (Chang et al., 2002). These characteristics make antibody fragments more suitable for imaging and radiotherapy. The lack of Fc region eliminates immunogenicity against Fc, and thus the therapeutic side effects may be reduced. Another advantage is that this construct can be produced at low costs. Even though these fragments have shorter plasma half-lives than whole antibodies, this setback can be compensated by PEGylation modification (Chapman, 2002).

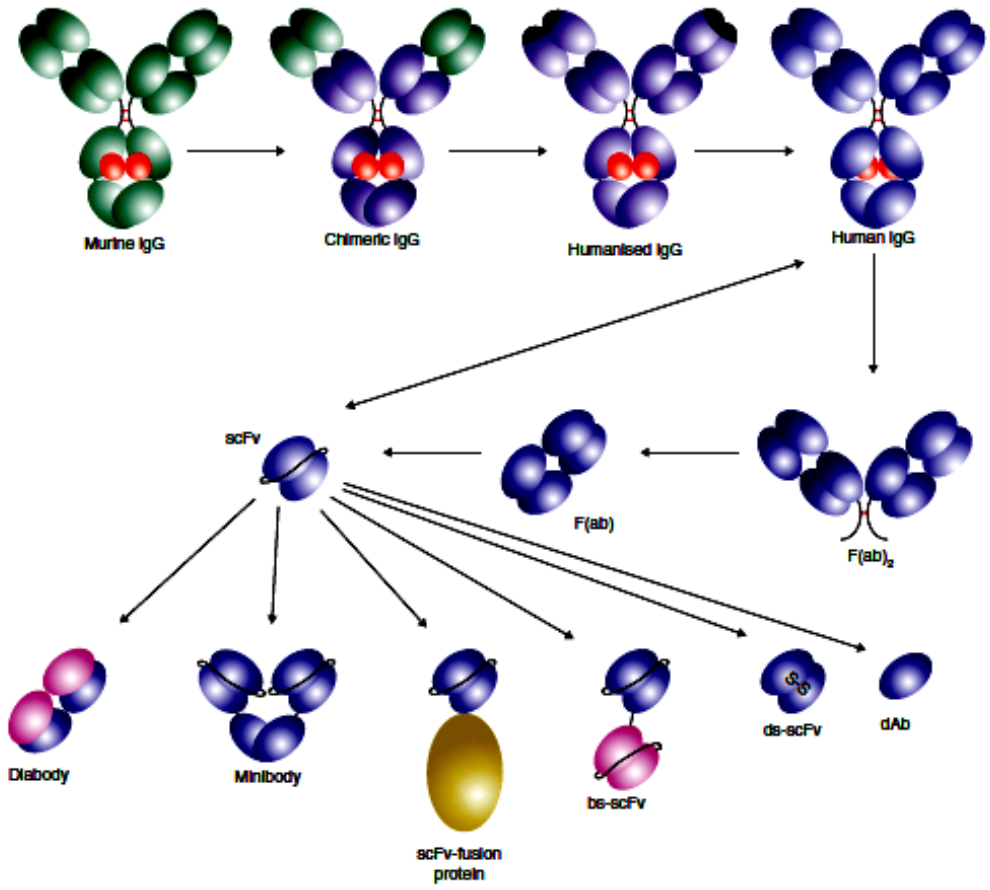

Figure 1.4 Recombinant antibodies for cancer therapy. Schematic diagram of the evolution of antibodies from murine (green shading) to humanized (blue shading) and various fusion proteins. Red spheres indicate glycosylation in the CH2 domain, pink shading indicate the VH/VL domains from one scFv, and yellow shading represents any fusion protein partner. bs: Bispecific; dAb: Single-domain antibody; ds: Disulphide; scFv: Single-chain antibody fragment. Adopted from (Zider and Drakeman, 2010).

1.4.7 Clinical applications

Various anti-hemagglutinin MAb approaches have become major targets for influenza research to neutralize the influenza virus (He et al., 2013b). Besides, MAbs against cluster of differentiation (CD) markers are extensively used to study many diseases, especially hematological malignancies. Examples include anti-CD34 and anti-CD44 MAb, which are employed to study mesenchymal damage caused by chemotherapy for leukemia, myeloma, myelodysplasia, and lymphoma to identify treatment side effects (Kemp et al., 2010).

Recently, ELISA based on an MPT64 antibody has been found useful for the diagnosis of pulmonary tuberculosis in both sputum-smear-positive and -negative patients (Zhu et al., 2012). Serological markers, such as anti-*Saccharomyces cerevisiae* antibodies and perinuclear antineutrophil cytoplasmic antibodies, and the recently reported anti-glycan antibodies, anti-laminaribioside, anti-chitobioside, and anti-mannobioside carbohydrate antibodies are widely utilized for the serological diagnosis and stratification of inflammatory bowel disease patients (Werner et al., 2013). MAbs are also used as diagnostic tools in supporting the diagnosis of specific forms of cancer, such as calretinin detection in mesothelioma (Chhieng et al., 2000).

For therapeutic applications, MAbs have rapidly become a clinically important class of drug. In 2012, more than 26 antibodies were marketed in the United States and Europe, and more than 350 were under clinical evaluation worldwide. These antibodies are used for the treatment of a wide range of diseases, including organ transplantation, autoimmunity and inflammation, cancer, cardiovascular diseases, infectious diseases, and ophthalmological diseases (Reichert, 2012, Chan and Carter, 2010) .

MAbs are also used as prognostic tools in treatment selection, e.g., anti-HER2 in

breast cancer and as the basis for targeted imaging studies, including anti-CEA- and anti-CAIX-based imaging (Pillay et al., 2011). In most cases, more than single antibodies are necessary to demonstrate sufficient sensitivity or specificity to be used for any indications mentioned above.

1.4.8 MAbs for cancer treatment

Conventional anticancer therapeutics remains important and widely used as treatment options for many types of cancers. However, the broad specificity results in toxicities, which usually limit the optimal dosing of anticancer drugs. Thus, suboptimal dosing eventually leads to relapse, drug resistance, and limited efficacy among cancer patients (Hackbarth et al., 2008, Gonzalez-Angulo et al., 2007, Kayl and Meyers, 2006). The challenge to find specific agents is selecting tumor cells over normal cells despite the fact that they share many common features. One possible strategy to overcome this is to harness the property of precision-guided MAbs to deliver the drugs to the tumor cells. Antibodies can exert an anti-tumor effect through four mechanisms, namely, perturbation of tumor cell signaling, activation of complement-dependent cytotoxicity (CDC), antibody-dependent cellular cytotoxicity (ADCC), and induction of adaptive immunity (Scott et al., 2012a), as described below.

1.4.9 Mechanism of action of therapeutic antibodies for cancer treatment

IgG2 and IgG4 are designed primarily for signaling perturbation, and they do not activate CDC (Shuptrine et al., 2012). Increased expressions of growth factor receptors during tumorigenesis make them an interesting target for therapeutic antibodies (Di Lorenzo et al., 2002). MAbs can exert antitumor effects by blocking ligand binding to growth factor receptors, thereby inhibiting proliferation, inducing apoptosis, or

sensitizing tumor to chemotherapeutic agent (Mendelsohn and Baselga, 2000). Cetuzimab and Panitumumab, which are currently approved for the treatment of cancers of the head and neck and colon, are reported to function by binding to the epidermal growth factor receptor (EGR) (Li et al., 2005). They prevent EGF binding, which activates tumor proliferation and neoangiogenesis, thus reducing cell proliferation and tumor angiogenesis.

Both ADCC and CDC are mediated through the Fc region of antibodies, with the MAbs of the IgG1 isotype being the most effective at inducing ADCC and CDC (Natsume et al., 2009). CDC causes cell destruction through complement system activation. In CDC, the Fc portion activates the classical complement system following its interaction with C1q (component of the complement system), thus leading ultimately to the formation of a membrane-attack complex. Both IgG3 and IgG1 have been found to be essential for the interaction between IgG and C1q (Shuptrine et al., 2012). CDC was reported to contribute to the antitumor activity of the anti-CD20 rituxiMAb, a MAb used in the treatment of non-Hodgkin's lymphoma (Di Gaetano et al., 2003). Recently, Moore and co-workers (2010) described the generation of a series of Fc variants with improved ability to recruit complement. The variants generated enhanced the cytotoxic potency of the anti-CD20 monoclonal IgG1 antibody, OcrelizuMAb up to 23-fold against tumor cells in CDC assays, and they demonstrated a correlated increase in C1q binding affinity, thus improving the CDC activity (Moore et al., 2010).

The Fc domain of antibodies can activate ADCC by interacting with the Fc receptors (FcγRs) in the effector immune cells. Natural killer (NK) cells that predominantly express FcγRIIIA are the main effectors of ADCC apart from macrophages and granulocytes, which have been shown to mediate ADCC to a lesser

extent (Stewart et al., 2014). Through the interaction between the FcγRs in the effector cells and the MAb Fc domain, NK cells release cytokines, such as interferon-γ (IFN-γ), and cytotoxic granules containing perforin and granzymes, which enter the target cell and promote cell death by triggering apoptosis (Nimmerjahn and Ravetch, 2007). The balance in the expression between activating and inhibitory FcγRs seems to be an important determinant of clinical efficacy because *in vivo* studies indicated that Trastuzumab and Rituxumab anti-tumor effects require functional activating FcγRs. Mice lacking the expression of FcγRIIIB exhibit a greater anti-tumor response after antibody treatment compared with mice with a deficient expression of activating FcγRIIIB (Clynes et al., 2000).

Tumor antigens are processed by dendritic cells (DCs) through endocytic phagocytosis and are presented in the major histocompatibility complex class II (MHC II). The main function of the MHC II molecule is to present processed antigens, which are derived primarily from exogenous sources, to activate CD4+ T-lymphocytes. Likewise, DCs are capable of cross-presenting the phagocytozed tumor antigen in the MHCI molecule to generate tumor-specific CD8+ cytotoxic T cells (CTLs). Upon activation, CTLs kill tumor antigen-loaded on MHCI cells or further differentiate into specific memory cells. (Holling et al., 2004). This mechanism was suggested to contribute to the clinical efficacy of some antibodies. CTLs had been shown to be capable of killing primary ovarian and melanoma cells after being activated by DCs loaded with antibody-coated ovarian and melanoma cells (Cioca et al., 2006). In an *in vitro* study investigating cetuximab immune-mediated effects that contribute to the antibody anti-tumor effectiveness for colon cancer, DCs loaded with drug-treated cetuximab-coated colon cancer cells were used to generate CTLs. The consequent DC-mediated cross-priming of antigens elicited a robust CTL anti-tumor response (Correale

et al., 2012).

1.4.10 Types of MAb conjugates

In addition to the development of naked or unconjugated MAb based on effector function and signaling inhibition, MAbs that attach to toxic agents are also being developed. The conjugation of MAb with radioisotopes, toxin, cytotoxic drugs, or liposomes can increase the specificity of the toxic agents, improve treatment efficacy, or reduce systemic toxicity (Trail, 2013).

1.4.10(a) Immunotoxins

Antibody–toxin conjugates, which are also called immunotoxins, are combinations of toxin molecules derived from plants, fungi, or bacteria that are chemically conjugated or genetically fused to an antibody or an antibody fragment specific to the antigen on the tumor surface (Trail, 2013). Natural whole toxin molecules usually possess cell-binding properties responsible for non-specific toxicity toward normal tissue (Romano et al., 2007). Thus, first-generation immunotoxins have major limitations, including serious side effects and lack of specificity and immunogenicity. Redesigned versions of toxin units through protein truncation, mutation, and domain elimination were generated eventually (Becker and Benhar, 2012). For example, truncated versions of *Pseudomonas aeruginosa* exotoxin A (PE), including PE35, PE38, and P40, have been used for conjugation to antibodies. Similarly, truncated or mutated forms of diphtheria toxin (DT) were also generated. Both DT and PE exert their toxicity upon internalization inside the cells. Soon after the immunotoxin binds to the target molecule, PE-based immunotoxin undergoes receptor-mediated endocytosis (Słomińska-Wojewódzka and Sandvig, 2013). In the endosome, the protease cleaves the

immunotoxin into two subunits. One of the subunits is further transported to the endoplasmic reticulum (ER) through the trans-Golgi network before reaching the cytosol. Finally, the catalytic ADP ribosylation of elongation factor II occurs, leading to apoptotic cell death (Słomińska-Wojewódzka and Sandvig, 2013).

LMB-2 immunotoxin is composed of the murine anti-CD25 MAb linked to a scFv of PE38 (PE at 38 kDa size). In the interleukin-2 receptor (IL-2R) molecule, CD25 is only expressed after T-cell activation but is not expressed in the resting lymphocytes (Chao et al., 2002). IL-2Rs have been greatly expressed in autoimmune diseases and various hematopoietic malignancies (Wilde et al., 2014, Houghton and Arceci, 2010). LMB-2 was the first recombinant immunotoxin that induced a major response in cancer. In one of the clinical trials, one patient with hairy cell leukemia achieved complete remission, whereas the remaining three patients achieved partial remission. Partial remissions were also observed in 1 patient with cutaneous T-cell lymphoma, 1 patient with chronic lymphocytic leukemia, and 9 patients with adult T-cell leukemia Hodgkin's disease. Responding patients had 2–5 log reduction of circulating malignant cells, improvement in skin lesions, and regression of lymphomatous masses and splenomegaly (Kreitman et al., 2000). LMB-2 was evaluated in patients with metastatic melanoma as a combinatorial approach (with MART-1 and gp100-specific peptide vaccination), and the results showed a 79% reduction of circulating CD25+CD4+ T cells and 51.4% reduction of FOXP3+CD4+ T regulatory cells (Tregs) in patients' peripheral blood (Powell et al., 2007). Tregs possess the ability to suppress T-cell responses and regulate tolerance to self-protein (Corthay, 2009).

Although they showed promising results during pre-clinical evaluations, some PE-based immunotoxins, including OVB3-PE and ERB-38, failed to induce a favorable

response in patients. Indeed, hepatoxicity was observed in all five breast cancer patients and one esophageal cancer patient (Pai-Scherf et al., 1999, Pai et al., 1991).

The early generation of a plant toxin, ricin A chain (RTA), demonstrated impressive pre-clinical results. The elimination of a cell-binding domain successfully avoided interference in the macrophages and in the endothelial system uptake. Moreover, deglycosylation of the RTA reduced the binding to the mannose receptor, thus resulting in a longer half-life in mice and a better therapeutic index (Ponder and Waring, 2012). The RTA-based immunotoxin enters the cell by endocytosis upon binding to the target molecule. The endosome unites with the Golgi system and then with the ER to deliver the immunotoxin. In the ER, the RTA is reduced through a disulfide bond cleavage into A and B chains (Becker and Benhar, 2012). From the ER, the A chain is translocated to the cytosol, where the RTA inactivates the ribosomes, eventually stops protein production in the cell, and causes cell death. The RTA-conjugated antibodies against CD25 and CD30-positive Hodgkin's lymphoma have also been exploited in clinical studies because of the high concentrations of lymphocyte activation markers, such as CD25 and CD30, being expressed in Hodgkin cells. The results demonstrated the partial remission of 2 out of 15 patients and 1 out of 17 patients for CD25 and CD30, respectively (Engert et al., 1997). The following clinical trial also showed a few patients responding probably because of the low number of CD30+ peripheral blood mononuclear cells and the binding of antiCD30 to soluble CD30 antigen. However, RTA-based immunotoxin applications were usually compromised because of dose-limiting toxicities, including vascular leak syndrome, myalgia, hepatic damage, and immunogenicity (Słomińska-Wojewódzka and Sandvig, 2013).

In fact, toxins of human origins are currently being developed to diminish the

immunogenicity or toxicity concerns of plants or bacterial immunotoxins. The toxins include pro-apoptotic proteins, RNAses, or kinases (Lorberboum-Galski, 2011). Human RNAses can induce the apoptosis of cancer cells. The internalization and effective entry into the cytoplasm of RNases degrade RNAs, thus causing p53-independent apoptosis (Lorenzo and D'Alessio, 2008).

1.4.10(b) Antibody drug conjugates

An antibody drug conjugate (ADC) consists of a MAb chemically coupled to a linker and a cytotoxic drug. Initially, ADC constitutes internalizing and non-internalizing MAb, following binding to target antigens. Non-internalizing ADC utilizes linkers designed to be cleaved or hydrolyzed by enzymes, such as cathepsin, matrix metalloproteinases, or acidic pH, present at the tumor site. Unlike non-internalizing ADC, the internalizing ADC delivers cytotoxic drugs more effectively when the metabolic potential of the endosomes and lysosomes can be utilized for drug release. Linkers used to conjugate drug should be stable in circulation to support the successful delivery of ADC (Lu et al., 2016, Dubowchik and Walker, 1999).

Previously, ADC incorporated drugs that display clinical activity as free drugs, such as methotrexate, vinblastine, and doxorubicin. Generally, these ADCs require improvement as high dosage levels are requisite to achieve significant antitumor activity. To increase doxorubicin conjugate potency, for example, increasing the drug:MAb ratio over a range of 1–25 molecules of drug per one molecule of MAb was achieved apart from the use of a branched linker and polymeric carriers (Trail, 2013). Currently, most ADC programs undergoing clinical testing incorporate cytotoxic agents that are 100–1000-fold more potent. These agents, which previously failed as free drugs because of high toxicity and lack of therapeutic index, include calicheamicin,

maytansinoids, auristatin, and duocarmycins. Cytotoxic drugs currently being exploited to construct ADC fall into two categories that target cell microtubules and DNA molecules (Trail, 2013).

Probably the most advanced drug conjugate (auristatin analogs) is the vc-MMAE anti-CD30, also called SGN-35. Upon antigen-specific binding and cell internalization, the conjugate linker is cleavable by lysosomal enzymes and releases active MMAE. MMAE binds to the vinca binding site of tubulin and inhibits tubulin polymerization resulting in the GM2/M phase arrest in the cell cycle and ultimately in cell death. The drug can kill cells in close proximity even if the cells lack target antigen expression because of the membrane permeable properties (Okeley et al., 2010). Among CD30+ patients with relapse lymphoma, almost 50% had a reduction in tumor burden in the SGN-35 phase 1 trial including 11 complete remissions. The same unconjugated anti-CD30 MAb evaluated in a similar population of patients showed minimal efficacy (Younes et al., 2010). These data indicate the importance of drug delivery through MAb targeting, and incorporating an additional effector mechanism of MAb-mediated drug

delivery can improve unconjugated MAb activity. Unlike MMAE, the MMAF ADC analog is highly impermeable and therefore does not exhibit a significant bystander killing activity. However, MMAF may theoretically provide an important advantage because of its reduced off-target toxicity.

Other examples of ADC with microtubule-targeted drugs are DM1 and DM4. Generally, the anti-tumor activity of these maytansinoid conjugates correlate with their disulfide linker stability. HuC242-SPP-DM1 seems less stable with a shorter half-life than hu242-SPDB-DM4, which shows better results in pre-clinical models. The bystander killing effects were considered to contribute to the explanation for the

improved anti-tumor activity (Kozak and Raab, 2013).

Chemical compounds that target DNA are also used as payloads for ADCs. Calicheamicin from *Micromonospora calichensis* is one of the most potent DNA cleaving agents (Lee et al., 1992). An anti-CD22 calicheamicin conjugate is considered the most advanced calicheamicin-based ADC under clinical evaluation for the treatment of aggressive and indolent non-Hodgkin lymphoma (Kantarjian et al., 2012).

1.4.11 Peripheral blood and surface marker proteins

1.4.11(a) Types of cells present in human peripheral blood mononuclear cells

The peripheral whole blood contains a mixture of different types of cells largely divided into red blood cells, white blood cells, and platelets. To study cells that are involved in the immune system, drawn peripheral whole blood is usually processed to isolate white blood cells by density gradient centrifugation. This method commonly uses a high-density polysucrose solution (Fuss et al., 2001). The sucrose polymer separates blood cells based on density of below and above 1.007 g/ml. During centrifugation, red blood cells are aggregated by polysucrose and rapidly sedimented. Granulocytes become slightly hypertonic, thus increasing their sedimentation rate, and pelleting occurs at the bottom of the centrifugation tube. Polymorphonuclear cells (PMNs), which include neutrophils and eosinophils, are sedimented, and they become denser than 1.007 g/ml. Three major fractions are obtained after centrifugation (Fuss et al., 2009). The top fraction consists of blood plasma, which has the lowest density; the second fraction is the polysucrose solution; and the bottom fraction comprises red blood cells, PMNs, and granulocyte sediments. Lymphocytes and other mononuclear cells called PBMC remain as a thin layer between the plasma and the polysucrose interface. This layer, which is

usually referred to as a buffy coat, is composed of the cells collected when this method is used for PBMC isolation (Figure 1.5). These cells comprise of mainly lymphocytes, monocytes as well their subtypes including B cell, T cells, NK and more (de Almeida et al., 2000).

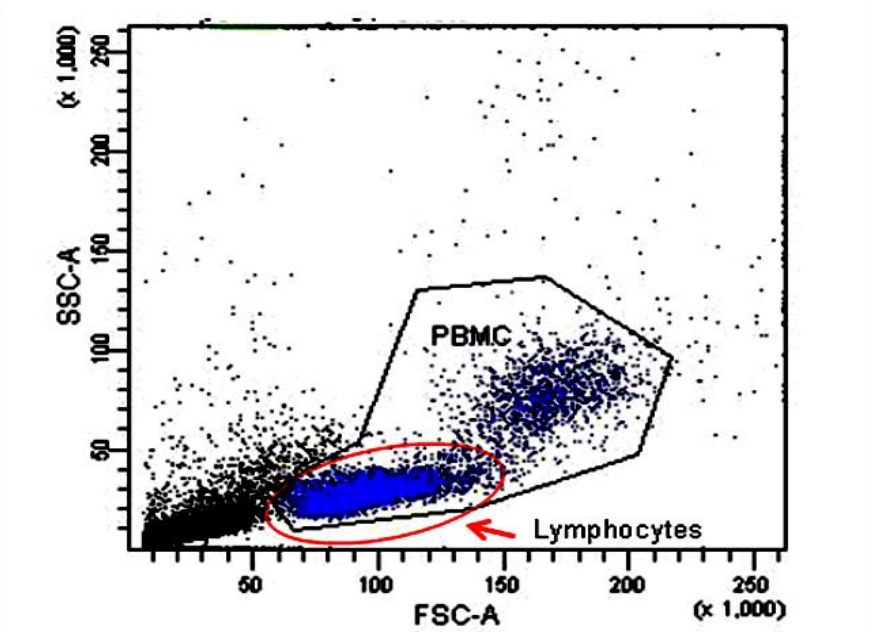

Figure 1.5 Flow cytometric analysis of cell population after PBMC isolation using density gradient centrifugation.

1.4.11(b) Peripheral blood CD markers

Peripheral blood cells express various protein molecules on their cell surface. For each cell subtype, unique molecules or proteins are expressed and dedicated specifically to a particular subtype (Maecker et al., 2012). During the cell lifetime, different protein molecules can be expressed by cells that are associated with the stages of cell development. Therefore, these proteins have become signature proteins or markers to a specific peripheral blood subtype. Almost 400 protein markers (Zola, 2007) known as CD markers can be found on the surface of cells in the human body. All cells have at least one CD marker, and these markers are mostly useful in classifying leucocytes.

1.4.12 Key lymphocyte CDs

1.4.12(a) CD3

In the early development of T cells, CD3 protein is expressed in the cell cytoplasm. At a later development stage, the CD3 migrates to the cell membrane. The CD3 is found in all mature T cells. Virtually no other cell type expresses CD3 except a small number of Purkinje cells (DeJarnette et al., 1998). The consistent expression of CD3 on T cells is utilized to distinguish T-cell lymphomas and leukemias from superficially similar B-cell and myeloid neoplasm (Dave, 2009).

1.4.12(b) CD4

CD4 is a glycoprotein found on the surface of immune cells such as T helper cells, monocytes, macrophages, and dendritic cells (Biswas et al., 2003). CD4 is usually referred to as a T helper cell because one of its main roles is to send signals to other types of immune cells to activate them (Germain, 2002). Initially, two subsets of CD4 T cell were identified to comprise Th1 and Th2. To date, at least seven distinct CD4 T-cell subsets have been shown to exist, namely, Th1, Th2, Th17, Th9, Th22, Tfh, and iTreg cells (Ivanova and Orekhov, 2015). CD4 T cells have the capacity to help B cells make antibodies, induce macrophages to develop enhanced microbicidal activity, and recruit neutrophils, eosinophils, and basophils to sites of infection and inflammation (Alberts et al., 2002). CD4 is the main receptor of the HIV virus binding on lymphocytes, and this binding results in a series of conformational changes and eventually the entry of the virus into the host cell (Landi et al., 2011).

1.4.12(c) CD8

CD8 is a transmembrane glycoprotein that serves as a co-receptor for the T-cell

receptor. CD8 is predominantly expressed on the surface of CTL, but it can also be found in NK cells, cortical thymocytes, and dendritic cells (Xiao et al., 2007). CTLs are activated when they recognize MHC class I proteins altered by infiltrated cells. Activated CTLs secrete the essential cytolytic mediators (perforin and granzyme) and induce apoptosis in target cells, such as tumor cells or viral infected cells (Ito and Seishima, 2010). CD8 expression usually remains in T-cell lymphoblastic lymphoma and hypo-pigmented mycosis fungoides infection but is frequently lost in other T-cell neoplasms (Carulli et al., 2009).

1.4.12(d) CD19

The B-lymphocyte marker, CD19, is specifically expressed on the surface of B cells as well as follicular dendritic cells. The surface density of CD19 is highly regulated throughout B-cell development (Sato et al., 1997). However B cells lose their protein expression during terminal plasma cell differentiation. CD19 expression is highly conserved in most B-cell tumors although the level of expression is variable according to different subtypes. CD19 levels can potentially be useful as a diagnostic tool in distinguishing certain lymphoma subtypes (Wang et al., 2012).

1.4.12(e) CD56/CD16

In human peripheral blood, five NK subpopulations can be defined on the basis of the relative expressions of the CD16 or FcγRIIIA and CD56 markers (Poli et al., 2009). Some NKs may express a comparatively high number of one CD marker than the other, and some express may very low and even no expressions at all of one CD marker. These expression levels are identified as bright, dim, positive, and negative expressions. For instance, the two major subsets are $CD56^{bright}$ $CD16^{dim/-}$ and $CD56^{dim}$ $CD16^{+}$,