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Monoclonal Antibody-Based Therapeutics for Melioidosis and Glanders

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Abstract: Problem statement: *Burkholderia pseudomallei* (BP) and *B. mallei* (BM) were two closely related pathogenic gram-negative bacteria. They were the causative agents of melioidosis and glanders, respectively and are recognized by CDC as category B select agents. Significant efforts had been devoted to developing the diagnostic and therapeutic measures against these two pathogens. Monoclonal antibody-based therapeutic was a promising targeted therapy to fight against melioidosis and glanders. Valuable findings have been reported by different groups in their attempt to identify vaccine targets against these two pathogens. **Approach:** Our group has generated neutralizing Monoclonal Antibodies (MAbs) against BP and BM and characterized them by both *in vitro* and *in vivo* experiments. We present an overview of the MAb-based therapeutic approaches against BP and BM and demonstrate some of our efforts for developing chimeric and fully human MAbs using antibody engineering. **Results:** Throughout conventional mouse hybridoma technique and antibody engineering (chimerization and *in vitro* antibody library techniques), we generated 10 chimeric MAbs (3 stable MAbs and 7 transient MAbs) and one fully human MAb against BP and BM. In addition, we present the reactive antigen profiles of these MAbs. Our approaches had potentials to accelerate the development of therapeutics for melioidosis and glanders in humans. **Conclusion:** Our experience and findings presented here will be valuable for choosing the best antigenic targets and ultimately for the production of effective vaccines for these two pathogens.

Key words: Melioidosis, glanders, *Burkholderia pseudomallei*, *B. mallei*, monoclonal antibody engineering

INTRODUCTION

Melioidosis and glanders are caused by the closely related species *Burkholderia pseudomallei* (BP) and *Burkholderia mallei* (BM), respectively. BP is a gram-negative, facultative anaerobic, motile bacillus commonly found in the soil and stagnant waters (Leelarasamee, 2004). BP is endemic to Southeast Asia and northern Australia, where melioidosis is an ongoing public health problem (Cheng and Currie, 2005; Mukhopadhya *et al*., 2007). BP infection is often due to either direct inoculation into wounds and skin abrasions or inhalation of contaminated materials (Chaowagul *et al*., 1989; Leelarasamee and Bovornkitti, 1989). The clinical manifestation ranges from subclinical to acute and chronic forms (Cheng and Currie, 2005). Symptoms include pain (chest, bones and joints), cough, skin infections, lung nodules and pneumonia. Depending on whether the disease is acute or chronic, melioidosis can mimic other infections, including glanders, typhoid fever, malaria, tuberculosis and bacterial sepsis (Chaowagul *et al*., 1993; Koponen *et al*., 1991). The overall mortality rate in individuals infected with BP is 50% in northeast Thailand (35% in children) and 19% in Australia (Wiersinga *et al*., 2006). Although melioidosis is primarily found in Southeast Asia and Australia, worldwide spreading evidence has been reported (Currie *et al*., 2008). Moreover,

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numerous studies revealed that BP could be intrinsically resistant to many antibiotics. Despite therapeutic regimens with certain antibiotics, the mortality rate of melioidosis remains very high (White, 2003). BP infection appears to be consistent with the reported cases of melioidosis occurring in previously healthy US helicopter crews during the Vietnam War, possibly as a consequence of the inhalation of contaminated dusts or mist (Howe *et al*., 1971). Healthy individuals can develop melioidosis but the majority cases have some underlying immunosuppressive condition, particularly diabetes but also chronic renal disease, thalassemia or alcoholism (Ip *et al*., 1995). In naturally occurring melioidosis, the mortality rate in acute cases can exceed 50% and prolonged treatment with antibiotics may result in only temporary control of the infection, with 10-15% of patients relapsing when antibiotic therapy is withdrawn. The treatment of melioidosis is often problematic because the bacteria are inherently resistant to many of the commercially available antibiotics and successful therapy often requires extended treatment regimens.

Glanders is generally confined to equines in parts of the Middle East, Asia and South America (Reckseidler *et al*., 2001). BM is a host-adapted pathogen that does not normally persist in nature. In humans, it is primarily an occupational disease, affecting individuals in close contact with infected animals such as veterinarians, grooms and farmers. However, a number of laboratory-acquired cases of glanders have also been reported. BM infection primarily results from the contamination of wounds, abrasions or breaks in mucous membranes. Some studies indicated that BM is highly infectious in humans by aerosol route (Howe *et al*., 1971). In horses and humans, BM infection can present as either nasal-pulmonary infections or cutaneous infections and the disease may develop either acutely or chronically (Whitlock *et al*., 2007).

There are considerable potentials for these 2 pathogens to be used as Biological Warfare Agents (BWA) (Wiersinga *et al*., 2006). It has reported that BM was used to attack military horses during World War I and there is direct evidence that BM was also used in World War II and during the Soviet invasion and occupation of Afghanistan (Fong and Alibek, 2005). No effective methods of prevention of either melioidosis or glanders caused by BP and BM currently exist. Studies revealed that BP could be intrinsically resistant to many antibiotics. Development of specific MAbs against these 2 pathogens could provide an important countermeasure in prevention and treatment of the endemic infectious melioidosis and glanders. Unlike vaccines, antibodies can confer passive protection regardless of the immune status of the infected host. In comparison with antimicrobial therapy, MAb therapy against BP and BM infection is significantly promising due to high specific function and low toxicity (Casadevall, 2002). Currently, specific antibodies that protect against infections of highly pathogenic BP and BM that military or civilian populations may encounter in biological warfares have not been developed. There has been a rising interest in these 2 pathogens, mainly not only focusing on their characterization but also identifying vaccine targets (Bondi and Goldberg, 2008; Estes *et al*., 2010).

Progress of genome sequencing of BP and BM has provided new insights into a better understanding of the pathogenicity, their survival mechanism(s) in the host cell's environment, their potential virulence factors and vaccine candidates (Felgner *et al*., 2009; Hara *et al*., 2009; Jones *et al*., 1996). The survival within host cells and the results of the various vaccine regimens against BP and/or BM (Bondi and Goldberg, 2008; Whitlock *et al*., 2007) suggests that a cell-mediated immune response, in addition to a humoral response, will be necessary for complete protection from BP and BM infection. The similarity of genomes (DNA-DNA homology and base sequence of the 16S rRNA) and phenotypic characteristics of these 2 pathogens (Anuntagool and Sirisinha, 2002; Rogul *et al*., 1970) suggests that vaccines developed against one of these bacteria may be suitable for clearance of the other (Groot and Rappuoli, 2004).

In response to national need for developing countermeasures against biological warfare agents, we have initiated studies on rapid diagnosis, therapeutic MAb development and multi-agent vaccines against BP and BM. To develop high-performance MAbs specific to BP and BM as potential therapeutics we used 2 different approaches based on principles using mouse hybridoma production technique and phage-displayed single chain antibody (scFv) technique. The lead MAbs with good neutralizing activity against BP and BM were converted to chimeric and humanized format which can be used to prevent and treat diseases of melioidosis and glanders in humans without having severe side effects. Due to close genetic and antigenic homology among many BP and BM species have made isolation of highly species-specific MAbs very difficult (Anuntagool and Sirisinha, 2002; Cravitz and Miller, 1950). However, good neutralizing MAbs that cross-react to both BP and BM could be extremely effective for the therapeutic purpose. Using many different isolates or strains of BP and BM from different geographic regions in the world, generation of MAbs is highly challenging. Since MAb therapy can provide immediate immunity against BP and BM, an overview

of current technologies for fast-performance production of neutralizing MAbs would be useful for successful development of MAb-based therapies for melioidosis and glanders.

In this review article we discuss our progress during the past over 5 years for therapeutic MAb technology that has proved to be one of modern biology's most sophisticated tools for both basic research and clinical therapies against a wide spectrum of human diseases. There are advantage and disadvantage in development of ideal therapeutic MAbs. Human or humanized MAbs have long half-life, low toxicities and high specificity-much less likely to select for resistance of non-targeted organisms. Since human MAbs are important immune modulators, actions of human antibody may or may not rely on other host mediators. Therefore, it could reduce the damage that results from host inflammatory responses and provide synergistic effects in treatment efficacy against infections when combined with conventional antimicrobial therapy, especially in immune compromised patients. Treatments require knowledge of the causative microbial agents which in turn requires rapid microbiological diagnosis. The objectives of this review are to better understand the passive immunity and MAb-based therapy against *Burkholderia* infections and to be familiar with the history of MAb development and use in treatment against melioidosis and glanders in human. In addition, we are to be familiar with the pathogenesis associated with 2 pathogenic *Burkholderia* infections and the development of high-performance MAbs against 2 pathogens using antibody engineering. However, manufacturing and economic hurdles may continue to limit the development of therapeutic MAbs against BP and BM. Ultimately, protective antibodies can provide immediate immunity against BP and BM and an important stimulus for the development of MAb-based therapy for melioidosis and glanders.

Technological progresses in MAb production systems: Kohler and Milstein (1975) discovered a method to produce hybridomas and MAbs by immortalizing antibody-producing B cells in culture. For the first time, it was possible to produce *in vitro* very large quantities of an immunoglobulin of a defined specificity and a single isotype. Since MAb is produced by a single clone of cells having a single binding specificity for an antigenic determinant, the end results after long and laborious processes constitute biological research tools, molecular biology and immunology for the diagnostic testing, purifying substances, targeting specific cell types and many other uses which can be discovered in the future. Hybridoma technology has been rapidly exploited and developed for making humanized MAbs for (pre)clinical use (especially in the fields that effective therapies are urgently needed) (Vaswani and Hamilton, 1998; Weiner *et al*., 2010). Many new detail methods (Fig. 1) for therapeutic use have been developed for making human hybridomas (Fig. 2) (Li *et al*., 2006; Karpas *et al*., 2001; Traggiai *et al*., 2004; Vaisbourd *et al*., 2001), human MAbs via transgenic mice (Fishwild *et al*., 1996; Jakobovits, 1995; Kuroiwa *et al*., 2009; Lonberg, 2005; 2008; Lonberg and Huszar 1995) and recombinant human MAbs from phage-displayed fragments (Gao *et al*., 2002; Huls *et al*., 1999; Irving *et al*., 1996; Low *et al*., 1996; Thie *et al*., 2008; 2011; Zou *et al*., 2007). Phage displayed MAb has proved much less successful because the phages are sticky and because a small number of specificities quickly come to dominate in the phage population. Therefore, humanization of mouse MAbs via antibody engineering, are still a good approach to analyze complex surface antigens of target infectious agents. Current marketing status of therapeutic MAbs approved by U. S. Food and Drug Administration is summarized (Table 1).

A platform technology for ideal therapeutic MAbs should constitute the most complete technology solution available in the marketplace for generating fully human antibodies and enables us to produce antibodies that meet the industry standard in that they are (1) 100% human, (2) of a very high affinity and (3) can be produced and manufactured relatively quickly and efficiently (Dűbel, 2004; Reichert, 2002). Current human antibody technologies offer the following advantages over other conventional antibody technologies. Fully human antibody development system, unlike humanization techniques, can be generated antibodies with 100% human protein sequences, which permit the development of end products with a favorable safety profile. Additionally, fully human MAbs are likely to be eliminated less rapidly from the human body, potentially reducing the frequency and amount of dosing. Human antibodies having high affinity to target antigen take advantage of the human body's natural affinity maturation process (whereby antibodies evolve over time to have higher affinity to targets) (Vaughan *et al*., 1998). Since high affinity antibodies have been generated against a wide range of target antigens, these MAbs are produced without the need for any subsequent engineering to make them more human-a process that at times has proven to be challenging and time consuming. Rapid development capabilities of fully human antibodies can accelerate the progress from immunization to the clinic.

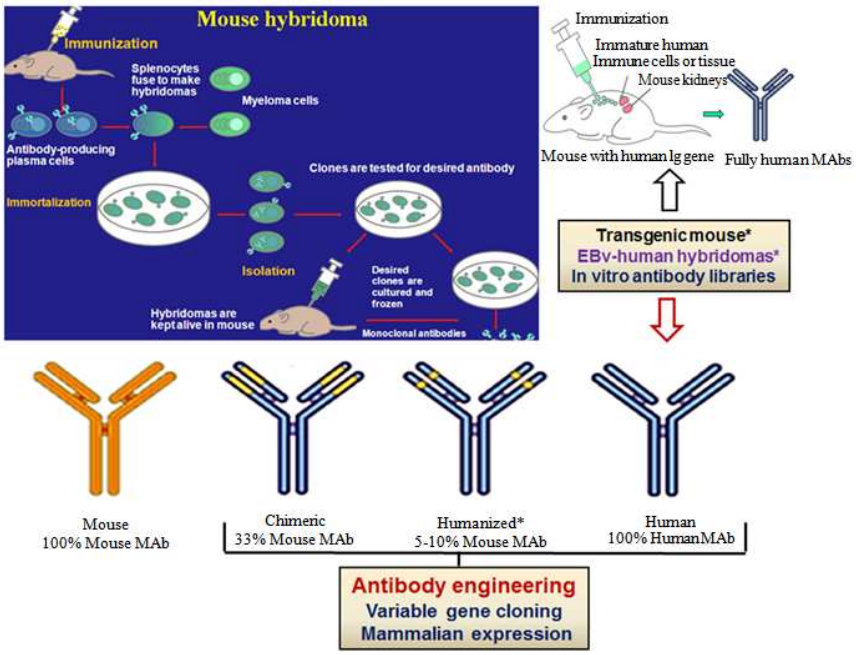

Fig. 1: Progress of current technologies for production of monoclonal antibody. Throughout the mouse hybridoma technique and antibody engineering (chimerization and *in vitro* antibody library techniques), we generated 10 chimeric MAbs (3 stable MAbs and one fully human MAb against BP and BM. *Other techniques [transgenic mouse, Epstein-Barr virus (EBv)-hybridoma and humanization via CDR-grafts] are also promising to make efficient human (ized) MAbs

Development of fully human MAbs against targeted agents

Problem statement:

1. Lack of a suitable fusion partner
2. Instability of hybrids
3. Limitation of source of human B cells

To solve potential problems:

1. Activation of human B cells by IL-4 and CD40
2. Epstein-Barr virus (EBv) - hybridoma techniques
3. Transgenic mice

3 major approaches for human MAb production

	Hybridoma techniques	EBv immortalization technique	EBv- hybridoma technique
Advantages	Fusion efficiency is high	Epstein-Barr virus is the only virus known to immortalize human B lymphocytes.	PEG-resistant/ HAT sensitive human myeloma cell line, Karpas 707H is available.
Disadvantages	1. Lost human chromosome 2. Lost Ab production	1. Since immortalization ratio is very low, adequate enrichment of antigen-specific cells to be fused is required. 2. Time consideration is required for the preparation of the EBv-containing culture supernatants, and preparation of the memory B cells to be immortalized since long-term stabilization of cell line takes 3 to 5 weeks.	Ethical consideration due to Ab production by human cancer cell line
Maximum Secretion (μg/ml)	~3-11	~0.1-16	~200
Current Status	Successful	Successful	Promising

Fig. 2: Human hybridoma technology. Three major approaches have been developed to produce fully human MAbs. For a small-scale production, the conventional human hybridoma technique can be used by direct fusion of memory B cells with human myeloma cells. The EBv-transfected hybridoma technology via human Karpas 707H myeloma cells as a fusion partner is also promising for successful development of fully human MAbs, in terms of production levels, purification and application in the downstream

Table 1: Current marketing status of therapeutic MAbs approved by the U.S. Food and Drug Administration

Therapeutic MAbs Type	Product	Target	Therapeutics	Marketer	Current, 2011 Approved
Murine	OKT3	CD3	Allograph rejection	Johnson and Johnson	June, 1986
Murine-Radiolabeled	Zevalin	CD20	Non-Hodgkin's lymphoma	IDEC Pharmaceuticals and Schering AG	March, 2002
Murine-Radiolabeled	Bexxar	CD20	CD20+ Follicular, Non-Hodgkin's lymphoma	Corixa Corp. and GlaxoSmithKline	June, 2003
Chimeric	ReoPro	GPIIb, IIIa	PTCA adjunct	Centocor (Johnson and Johnson) and Eli Lilly	December, 1994
Chimeric	Rituxan	CD20	Non-Hodgkin's lymphoma	Biogen-Idec and Genenech	November, 1997
Chimeric	Simulect	CD25	Organ rejection prophylaxis	Novartis	May, 1998
Chimeric	Remicade	TNF-α	Rheumatoid arthritis, Crohn's disease	Centocor (Johnson and Johnson)	August, 1998
Chimeric	Erbitux	EGFR	Colorectal cancer	ImClone Systems and Bristol Myers Squibb	February, 2004
Humanized	Zenapax	CD25	Organ rejection prophylaxis	Roche and Protein Design Labs	December, 1997
Humanized*	Synagis	RSV-F protein	Respiratory syncytial virus (RSV) disease	Medimmune	June, 1998
Humanized	Herceptin	HER-2	Metastatic breast cancer	Genentech	September, 1998
Humanized	Campath	CD52	Chronic lymphocytic leukemia	Millennium Pharma ceuticals and Berlex Lab	July, 2001
Humanized	Xolair	IgE	Moderate to severe persistent asthma	Genentech, Tanox, and Novartis	June, 2003
Humanized	Avastin	VEGF	Metastatic colorectal / breast cancers	Genentech	February, 2004
Humanized	Tysabri	α4 subunit	Multiple sclerosis and Chron's disease	Biogen-Idec and Elan Corp.	November, 2004
Humanized	Lucentis	VEGF-A	Wet age-related macular degeneration	Genentech	June, 2006
Humanized	Soliris	Complement C5	Paroxysmal nocturnal hemoglobinuria	Alexion Pharmaceuticals, Inc	March, 2007
Humanized	Cimzia	TNF-α	Rheumatoid arthritis, Crohn's disease	UCB	April, 2008
Humanized	Actemra	Anti-IL6R	Rheumatoid arthritis	Roche	January, 2010
Phage-displayed	Humira	TNF-α	Rheumatoid arthritis	Abbott Labs / Cambridge Antibody Technology	December, 2002
Transgenic	Vectibix	EGFR	Metastatic colorectal cancer	Amgen/Abgenix	September, 2006
Transgenic	Simponi	TNF-α	Rheumatoid and psoriatic arthritis	Centocor (Johnson and Johnson)	April, 2009
Fully human	Ilaris	IL-1β	Cryopyrin-associated periodic syndromes	Novartis	June, 2009
Transgenic/ hybridoma	Arzerra	CD20	Chronic lymphocytic leukemia	Genmab / GlaxoSmithKline	October, 2009
Fully human	Stelara	IL-12/IL-23	Plaque psoriasis	Centocor	November, 2009
Fully human	Prolia	RANKL	Postmenopausal osteoporosis	Amgen	June, 2010
Transgenic	Vervoy	CTLA-4	Metastatic melanoma	Bristol Myers Squibb	March, 2011

*Among 27 MAbs (3 murine, 5 chimeric, 11 humanized, 8 fully human) for therapeutic use, only one MAb is licensed for use against the RSV infection

In order to diverse selection of antibodies responding to many target antigens, the technology should have a potential to generate high affinity human antibodies of all isotypes and subclasses that recognize more antigen structures. In addition, the technology should have been able to create large panels of MAbs against many potentially relevant antigens.

For a given antigen target, the ability to select a product candidate from a pool of multiple therapeutic MAbs could be imperative in selecting the optimal antibody product for development. Here we present our extensive efforts (mouse MAbs to human MAbs) to generate high-performance therapeutic MAbs against BP and BM. These MAbs can be useful to study Burkholderia pathogenicity and to prevent melioidosis and glanders diseases of humans and animals.

Production and characterization of mouse MAbs against BP and BM: To generate hybridomas and MAbs against BP and BM, in our initial attempt we hyper-immunized BLAB/c mice with whole cell antigens and Lipid-Associated Membrane Proteins (LAMPs) with Lipopolysaccharides (LPS) from heat-killed BP ATCC 23343 and BM ATCC 23344. Thereafter, to make diverse MAb pools in other immunization attempts, the antigen was prepared as several types such as whole cell antigens of heat-inactivated (65°C for 90 min) BP and BM, capsular

polysaccharides (>200-kDa exo-polysaccharides) on surface of all BP strains and about 50% of the BM strains and LAMPs/LPS, known to have different epitopes between BP and BM. Throughout three I-steps (immunization, immortalization and isolation) (Fig. 1), the antisera of immunized mice had a titer of approximately $1{:}10^6$ against the whole bacteria by ELISA and a titer of approximately $1{:}10^5$ against the capsular and LPS antigen. As expected, polyclonal antisera from mice immunized by whole cell antigens of BP (or BM) cross-reacted strongly with the whole cell antigens of BP, BM and *B. thailandensis* (BT) by either ELISA or Western Blotting (WB) (Feng *et al*., 2006) We identified hybridomas producing BP- and BM-specific MAbs that do not cross-react with other 8 non-pathogenic *Burkholderia* species [*B. cepacia* (ATCC 700070), *B. vietnamiensis* (ATCC BAA-248), *B. stabilis* (ATCC BAA-67), *B. ambifaria* (ATCC BAA-244), *B. caledonica* (ATCC BAA-462), *B. kurureinsis* (ATCC 700977), *B. multivorans* (ATCC BAA-247) and *B. fungorum* (ATCC BAA-463)]. All BP and BM-specific MAbs obtained were characterized for their specificity and the nature of bacterial antigens they reacted to [glycoproteins, capsule polysaccharides and LPS] (Zou *et al*., 2008). We continued our attempt of developing more hybridomas for specific MAbs against BM by immunizing 2 clinical bacterial isolates (BM005 and BM007) other than the prototype BM ATCC 23344 strain. Similar attempt to MAbs against BP was conducted by immunizing mice with 3 different clinical isolates (BP8324, BP430 and BP428) other than the BP ATCC 23343. All together we generated and characterized 108 individual hybridomas producing MAbs that reacted strongly with BP and/or BM by ELISA (Feng *et al*., 2006; Zou *et al*., 2008). Immunoglobulin isotype of each MAbs was also determined. Most MAbs belong to type IgG or IgM with a few belonging to type IgA. In addition, we examined binding properties and cross-reactivity of the MAbs to non-Burkholderia species of bacteria or to a closely related species of *Pseudomonas aeruginosa* using ELISA and WB. MAb affinity ranking to target antigens has been determined by using Biacore's Surface Plasmon Resonance (SPR) biosensor technology (Canziani *et al*., 2004; Wohlhueter *et al*., 1994). To ensure the selected MAbs were able to recognize as many different strains or clinical isolates of pathogenic BP and BM, we examined each MAbs for their reactivity to 16 different strains or clinical isolates of BM and 13 different strains or clinical isolates of BP. Anti-BP LPS MAbs often cross-reacted with the BT LPS. We also examined the bactericidal activities of 47 candidate MAbs by in vitro studies. The opsonic assay using dimethyl sulfoxide-treated human HL-60 cells as phagocytes revealed that 19 out of 47 tested MAbs (40%) have prominent bactericidal activities against BP and/or BM. Then, the *in vivo* protective efficacy of selected MAbs was evaluated using female 8-10 week-old BALB/c mice challenged intranasally with predetermined dose (20×LD50) of the bacteria. Groups of six mice under anaesthetization were intravenously injected with 100 µg of purified MAb in 0.4 mL^{-1} of PBS or control serum. Mice were allowed to rest for 2 h prior to challenge intranasally with BP or BM. The mice after challenge were observed daily and mortality rate was recorded over a period of 3 weeks for BP and 2 weeks for BM (Zhang *et al*., 2011). Mouse protection assay by lead MAbs from our group and other investigators showed that these MAbs reactive to either the capsular polysaccharides or LPS were effective in protecting mice against lethal bacterial challenge challenge (Jones *et al*., 2002; Trevino *et al*., 2006; Zhang *et al*., 2011).

Affinity improvement of neutralizing MAbs: Our lab has successfully developed the high-performance MAbs that can ultimately be used into therapeutic for the prophylaxis and treatment of melioidosis and glanders. The rationale that underlies past 3 year study was that we would improve specific MAbs against BP and BM using approaches based on 2 completely different principles-affinity improvement by single chain antibody (scFv) phage display technique (Irving *et al*., 1996; Low *et al*., 1996; Kim *et al*., 2011a; Thie *et al*., 2008; 2011) and chimerization of lead MAbs via antibody engineering (Kim *et al*., 2011b). The traditional mouse hybridomas technique would most likely produce MAbs that recognize bacterial antigens processed by professional antigen-presenting cells in mice. The functional full human IgG format of scFv MAbs might have the unparalleled advantage in the applicability of antibody therapies to diseases of melioidosis and glanders in humans. Overall, we generated and characterized 108 different mouse MAbs against BP and BM (Feng *et al*., 2006; Zou *et al*., 2008). We also studied more than 40 human scFv MAbs obtained by panning of human scFv phage library against BP and BM and eventually selected 9 scFv MAbs (Zou *et al*., 2007). The specificity (cross-reacting to other *Burkholderia* species or non-*Burkholderia* species bacteria) and sensitivity (reacting to other clinical strains or isolates of BP and BM) as well as the nature of bacterial antigens recognized by these MAbs were all carefully studied. We completed the testing of the representative mouse MAbs from each of the 8 different groups of MAbs (Zou *et al*., 2008) for

in vitro antibody-mediated bacterial killing assays and bacteria opsonic assays against BP and BM. Furthermore, we have identified 2-3 most effective mouse MAbs of protecting animals against lethal infections of BP and BM, respectively (Zhang *et al*., 2011).

The neutralizing activity of MAbs is closely related to their antigen binding affinity. Many approaches have been developed to improve antibodies, including random Complementarity-Determining Regions (CDRs) mutagenesis, chain shuffling and error prone PCR (Wark and Hudson, 2006). Among these approaches, random mutagenesis on hotspots in CDRs (Yau *et al*., 2005), mainly CDR3 is most commonly used and successful (Irving *et al*., 1996). Therefore, we have first focused on site-directed mutational approach at CDR3. Phage-displayed scFv antibody technology with scFvs displayed on M13 phage has been well established (Thie *et al*., 2011; Yau *et al*., 2005; Zou *et al*., 2007). We used this system to build mutation scFv libraries for the selected MAbs. An engineered antibody for improving their antigen binding affinity is actually one of the most extensively studied areas of antibody engineering. There are basically two approaches to improve antibody affinity. The first approach was to create very large libraries of randomly mutated CDRs or the entire variable domains and then select for higher affinity variants from this large collection of mutants (Kim *et al*., 2011a; Wark and Hudson, 2006). The second approach is to make small libraries by focused CDR3 mutagenesis that may increase the likelihood of identifying higher affinity variants. Combinations of different mutations that lead to small increases in affinity often have addictive or synergistic effect and lead to a greater improvement in affinity (Chowdhury and Wu, 2005).

Construction and panning of phage-displayed scFv libraries containing mutations in CDRs for affinity improvement: To improve antibody binding affinity to target antigens, we used a process targeting random mutations to the hotspots in the variable regions of MAbs (Yau *et al*., 2005). During somatic hypermutation mutations in genes encoding the antibody variable domains are preferentially focused in certain regions termed “hotspots”. The essence of the approach is to mimic in vitro the natural somatic hypermutation process that underlies affinity maturation of antibodies *in vivo* (Neuberger and Milstein, 1995). The consensus sequence of hotspots is the tetranucleotide, RGYW (in which R can be A or G, Y can be C or T and W can be A or T). The AGY serine codons may also constitute hotspots (Wark and Hudson, 2006; Yau *et al*., 2005). The hotspots with value for further mutations generally locate at sites in contact with the antigens. To improve anti-Burkholderia MAb binding affinity, 6 mouse scFvs constructed previously were used to construct large highly-diversified phage-displayed mouse scFv libraries (Kim *et al*., 2011a).

Humanization of neutralizing MAbs: Treatments of various infectious diseases normally would require relatively short-term regimens compared with treatments of chronic forms of illnesses such as malignancies or autoimmune diseases (Adams and Weiner, 2005). However, potential human anti-murine immune response as well as subtle adverse effects produced by the foreign mouse antibodies in human is still major concerns in using mouse MAbs as therapeutics against infectious agents. At present, most therapeutic MAbs used in the clinic are either humanized MAbs or fully human MAbs (Table 1). A full format antibody molecule of human, mouse and most other mammalian species consists of a light chain and heavy chain linked together by S-S bonds. Both light and heavy chains contain highly variable N-terminal regions (VL and VH) and C-terminal constant regions. The VL and VH are critical for antigen specificity and binding affinity. Chimeric mouse-human MAbs generally are composed of VL and VH from the original mouse MAbs fused with the respective human light and heavy chain constant regions. Through molecular manipulation approaches, the high-performance MAbs can ultimately be used to effectively protect human victims against BP and BM infections.

Production and characterization of chimeric mouse-human MAbs against BP and BM: BP and BM as well as many other species in the *Burkholderia* family show a high homology at the nucleotide level (Rogul *et al*., 1970) by Basic Local Alignment Search Tool (BLAST) comparisons (Altschul *et al*., 1997). Serological studies also revealed BP and BM are antigenically closely related (Cravitz and Miller, 1950). Development of ideal therapeutic MAbs that can differentiate between all strains of BP and BM from other non-pathogenic *Burkholderia* species has been challenging due to the close homology. However, if the MAbs developed were to be used for therapeutic and not diagnostic purposes, MAbs that react strongly to both BP and BM are highly desirable. Furthermore, to design therapeutic MAbs for human diseases, it is important that the selected MAbs react not only to the particular strain of bacteria used as the immunogen, but to as many different strains and clinical isolates of these two closely related species of bacterial pathogens as possible (Brekke and Sandlie, 2003).

Table 2: Engineered therapeutic MAbs against BP and BM

Mouse hybridoma			Reactive antigen and specificity		Conversion and expression	
MAb ID	Immunization	Isotype	By WB[1]	By ELISA[2]	Chimeric MAb	Human MAb
BP7 10B11	BP430	IgG1/κ	Glycoproteins	BP, BM: +; BT: −	Stable	
BM-1	BM005	IgG1/κ	(Glyco)proteins	BP, BM: +		Transient
BP7 2F4	BP430	IgG2b/κ	Capsular polysaccharides	BP, BM: + ; BM005, BT: −	Transient	
BP2 I67	BP8324	IgG1/κ	Capsular polysaccharides	BP, BM: + ; BM005, BT: −	Transient	
BP7 2C6	BP430	IgG2a/κ	Capsular polysaccharides	BP, BM: + ; BM005, BT: −	Stable	
BP1 2E7	BP8324	IgG3/κ	Capsular polysaccharides	BP, BM: + ; BM005, BT: −	Transient	
BP1 7F7	BP8324	IgG3/κ	LPS	BP, BT: +	Stable	
BP A2	BP ATCC	IgG1/κ	LPS	BP, BT: +	Transient	
BP L30	BP ATCC	IgG2a/κ	LPS	BP, BT: +	Transient	
BML 5D11	BM ATCC	IgG2b/λ	LPS	BM: +; BP: weakly +	Transient	
BML 18F8	BM ATCC	IgG1/λ	LPS	BM: +; BP: weakly +	Transient	

[1]The nature of antigenic epitopes recognized by each MAb was characterized by Western blot analysis against heat-killed whole bacterial cell lysates with or without proteinase K-treatment (against protein epitopes), with or without sodium periodate-treatment (against carbohydrate epitopes); [2] ELISA was done against heat-killed whole bacteria of specified Burkholderia species [*B. pseudomallei* (BP) ATCC 23344 and *B. mallei* (BM) ATCC 23343] and clinical isolates (13 BPs and 16 BMs). BT indicates *B. thailandensis* ATCC 70038

In our previous studies, more than 100 mouse MAbs against BP and/or BM have been generated and characterized (Feng *et al*., 2006; Zou *et al*., 2008). Most importantly, many of these MAbs showed good protection efficacy against both pathogenic *Burkholderia* bacteria by an *in vitro* opsonic assay using HL-60 cells and mouse protection assay via intranasal challenge with a sub-lethal dose of the bacteria (Zhang *et al*., 2011). However, a recent mouse protection study against BP and/or BM infection has shown that MAbs against capsular polysaccharide, LPS and glycoproteins of BP and/or BM significantly reduce lethality of infections in mice. These MAbs fail to achieve full protection, especially with high dose challenges (Jones *et al*., 2002; Trevino *et al*., 2006; Zhang *et al*., 2011). Development of high-performance MAbs which could be used as therapeutics in exposed individuals is urgently needed to fill the current gap in defense against BWAs. Three major surface antigen [(glyco)proteins, capsular polysaccharides and LPS]-reactive MAbs against BP and/or BM were selected to further develop chimeric MAbs (Kim *et al*., 2011b). The ultimate goal was to develop superactive MAbs that can be used as therapeutics against BM and BP infections.

Recent advancements in entire genomic sequence information of BP and BM allowed us to identify potential vaccine candidates for melioidosis and glanders (Felgner *et al*., 2009). Since facultative intracellular BP and BM are capable of residing in host cell compartments, which can be escaped from either antibiotic or vaccines (Jones *et al*., 1996), it is believed that intracellular survival compartments may be a key element for determination of virulence factors. Therefore, it is likely that a cell-mediated immune response, perhaps in addition to a humoral response, may be critical for protection, as has been shown for other intracellular bacterial pathogens. Recent studies of the role of Burkholderia outer surface antigens in mouse protection by using MAbs post-nasal or lethal challenges of pathogenic Burkholderia species indicate that the Burkholderia surface antigens [Outer Membrane Proteins (OMPs), LPS and capsular polysaccharides] are potential protective antigenic targets (Felgner *et al*., 2009; Hara *et al*., 2009; Nelson *et al*., 2004; Plesa *et al*., 2004). Our ELISA data and Western blot analysis by 3 chimeric MAbs, using the treated antigens (heat, sodium periodate and proteinase K) and OMPs of BP and BM, revealed each chimeric MAb's reactivity to bacterial cell components and immunodominant antigens with different molecular size from 21-65 kDa (Kim *et al*., 2011b). Briefly, due to different antigen reactivities (surface glycoproteins, capsular polysaccharide antigens, LPS and/or Lipoproteins (LP) by therapeutic MAbs (Table 2), these MAbs would be useful for neutralizing BP and/or BM infection and studying the role of the major surface antigens in Burkholderia infection. Furthermore, since LP is a major component of the outer membrane of gram-negative bacteria, LP in culture supernatants of growing Burkholderia cells may induce pathologic changes associated with infections (Zhang *et al*., 1998). Our previous studies of in vitro neutralization and original MAb ranking using Biacore's SPR technique suggest that the surface epitopes (with little cross-reactivity to other Burkholderia bacteria) recognized by these MAbs are highly specific to target *Burkholderia* species. Thus, these cross-reactive antigens in BP and BM may be useful for determining potential vaccine targets against Burkholderia infection.

Humanization of the target MAbs could minimize possible side effects when used as human therapeutics (Chan *et al*., 2004; Reff *et al*., 1994). Therefore, the chimerization of these three MAbs against BP and BM is an important step in the preclinical development. The

utilization of bicistronic retroviral expression vectors containing the gene of interest (Thomas *et al*., 2003) and an amplifiable marker gene has been shown as an effective method in obtaining stable cell lines that express high levels of the chimeric MAb of interest (Bianchi and McGrew, 2003; Kim *et al*., 1998; Lucas *et al*., 1996). However, experience with the use of such vectors in high level expression of chimeric MAbs is currently limited. To obtain sufficient quantities of chimeric MAbs for the preclinical therapeutics, we established a platform technology to develop cell lines producing high levels of neutralizing chimeric MAbs against BP and/or BM. This include (1) construction of four major different mammalian expression vector systems with a Dihydrofolate Reductase (DHFR) amplification marker, (2) optimization of transfection/selection conditions in Chinese Hamster Ovarian (CHO) cells and other common mammalian host cells with the single-gene (heavy or light chain) vectors and double-gene vector (both heavy and light chain in the vector) system, (3) stable chimeric MAb production by CHO-DG44 cells and (4) affinity purification (Kim *et al*., 2011b). These techniques would be useful for accelerating preclinical development of therapeutic chimeric MAbs against targeted BP and BM.

Development of human scFvs MAbs by screening phage-displayed naive human scFv libraries against BP and BM: Much effort to make fully human MAbs has been devoted to the development of mouse MAbs that react specifically with BP and BM for diagnostic and/or therapeutic purposes. Our lab have been focused on the screening of a phage-displayed human scFv antibody library against heat-killed BP and BM for the generation of human scFv antibodies specific to the two pathogenic species of bacteria. Three phage-displayed human naïve scFv libraries from different sources have been acquired and propagated from the *E. coli* stocks (XL1-blue, TG-1 and HB2151). Although we initially tested 3 different phage-displayed human naïve scFv libraries, we subsequently focused on one particular phage-displayed human naïve scFv library that had provided most consistent results, for more extensive screenings for scFvs against heat-killed whole cell bacteria as well as SDS lysates (coated on immune-sticks) of BP ATCC 23343 and BM ATCC 23344.

Using two different panning procedures against BP and BM, we obtained seven different human scFv phage antibodies that interacted with the heat-killed whole bacterial cells of BP and BM. Our results demonstrate that panning of a human scFv antibody library against heat-killed whole bacterial cells may provide a valuable strategy for developing human MAbs against the highly pathogenic BP and BM. We successfully obtained more than 40 clones of human scFv phage MAbs that reacted with BP and BM. All these selected clones of scFv phage MAbs were characterized by DNA sequencing and classified into different families. These human scFv phage libraries were useful for screening against whole cell antigens of heat-killed BP ATCC 23343 and BM ATCC 23344 of interest large stocks of helper phages (VCSM13 and M13 KO7).

To reconstruct of scFv into fully human IgG1, we constructed recombinant genes of light and heavy chains of full human format IgGs by fusing the VL and VH sequences from each of 4 scFvs (BM1, 3A2, 1F3 and 1D7) with the human light (kappa) and heavy chain (gamma) constant region sequences, respectively. The fusion genes were then cloned in the mammalian expression vector pcDNA3. Expression of one human format IgG1 (BM1) from transiently transfected CHO cells and 293T cells was proved to be successful. The specificity and relative binding activity of transiently expressed human anti-BM1 was tested and found to be the same as that of the original scFv MAb and mouse MAbs (Zou *et al*., 2007).

Stable MAb production: Cells used for the production of therapeutic MAbs must comply with various requirements to ensure approval for the MAb as a drug (Reichert, 2011). One of those requirements is to use a thoroughly defined clone. It must be guaranteed that the cells used for MAb production are derived from a single clone with a specifically-defined integration site. The generation of a stable clone often requires 6 months or more due to selection procedures and adaptation to serum-free conditions. A variety of systems for selecting transfected cells exists, including resistance to antibiotics such as neomycin, hygromycin and puromycin, Dihydrofolate Reductase (DHFR) (Kim *et al*., 1998; Lucas *et al*., 1996) and Glutamine Synthetase (GS) systems (Jun *et al*., 2006). The aim of selection is to identify high producing clones, but this is tedious and laborious processes. Although several methods for isolating clones are used, the most popular subcloning is used by limiting dilution using multi-well plates or cloning cylinders. Once a stable clone is selected, it must be adapted to serum-free suspension culture so that it can be used in efficient large-scale production This adaptation process is again time consuming and bears the risk that the clone may lose its desired high producing properties. By applying novel transfection technologies, mammalian cells can be transfected directly in a serum-free environment. This saves significant time and reduces the risk on the way to find the right clone.

In our attempt to establish stable cell lines that express the chimeric MAbs against BP and BM, we introduced the chimeric heavy and light chain genes

cloned in an expression vector that carries the DHFR or GS amplifiable selection markers, into the DHFR-deficient Chinese Hamster Ovarian cells (CHO-DG44) (Kim *et al*., 2011b). CHO-DG44 with the expression vector integrated into the chromosomes was selected and the copy numbers of the integrated chimerical heavy and light chain genes was further amplified by selective agents such as Methotrexate (MTX) or Methionine Sulfoximine (MSX). Stably transfected CHO-DG44 cells producing highest levels of the selected chimeric mouse-human MAbs with the best neutralizing activities against BP and BM have been established by using two bicistronic vectors with amplification markers. The humanized MAbs produced were successfully purified and re-analyzed for their binding affinity and neutralizing activity against BP and BM. Selection of MAbs for further improvement of antigen binding affinity was based on in vitro bacterial killing and *in vivo* animal protection assays. To construct antibody expression vector, we used the mammalian expression plasmid pIRES which contains two Multiple Cloning Sites (MCS-A and MCS-B) and allows expression of two genes at high level simultaneously. We modified pIRES by inserting the DHFR gene separately at the MCS-B (pIRES-DHFR). The genes of chimeric heavy or light chains was introduced into MCS-A on separate plasmid of the pIRES-DHFR vector. In brief, overlapping PCR technique was used to assemble full fragments of heavy or light chain genes containing Kozak sequences, synthetic leader heavy (light) sequences and human constant gamma 1 (kappa) sequences and restriction sites. These two constructed vectors (pIRES-H-DHFR and pIRES-L-DHFR) was cotransfected into CHO-DG44 cells by lipofectamine 2000 reagent and amplified in culture medium containing stepwise increments of MTX (Kim *et al*., 2011b). DHFR amplification is based on systematic increases of MTX in medium without added nucleosides. Cells are qualified for next amplification step after regaining their polygonal morphology (2-3 weeks). Since the concentration of MTX is expected to increase about 16,000× (from 0.005 μM to about 80 μM) the amplification process will take about 4 months taking into consideration that 4 times increase in MTX is applied in each amplification step (Kim *et al*., 1998). At certain time points of MTX amplification, single clones are separated and tested for expression level and binding affinity by ELISA.

We established a platform technology for fast-performance production of stable chimeric MAbs against BP and/or BM by CHO-DG44 cells using the effective expression vector systems (Kim *et al*., 2011b). Overall, we have generated 6 mammalian expression vectors, incorporating an Internal Ribosome Entry Site (IRES) and Foot and Mouse Disease Virus (FMDV)-derived 2A self-processing sequence that efficiently mediated the (co)expression of two transgenes in multiple mammalian cell types. These 6 vectors would be useful for various cMAb productions in the laboratory but also genetic immunotherapy strategies where more than one gene product is needed to mount an effective immune response. The utilization of either bicistronic pIRES-H(L)-DHFR or p2A-H(L)-DHFR vectors has been shown as the most effective method in obtaining stable cell lines that express high levels of the cMAbs (Kim *et al*., 2011b). Therefore, two systems utilizing single gene vectors might be more relevant than other systems for maintaining intact binding properties after chimerization and higher level production of chimeric MAbs by mammalian host cells.

Recent progresses in antibody engineering, cell transfection technology and expression systems containing selective markers allow highly efficient transfection of mammalian cells for the fast production of therapeutic MAbs (Chowdhury and Wu, 2005; Reichert, 2011; Wurm, 2004). The efficient transfection of cells not only facilitates stable cell clone generation, but also allows true preclinical development of cMAb production in transiently transfected cells for small-scale production. Using the DHFR double deletion mutant ($dhfr^-/dhfr^-$) CHO-DG44 cells, we were able to amplify the chimeric antibody gene associated with the amplifiable DHFR gene by systematic increases of MTX in medium without added nucleosides. For preclinical development of therapeutic chimeric MAbs, it is critical to obtain a thoroughly guaranteed stable clone derived from a single clone producing stable cMAbs. Using the bicistronic pIRES-H (L)-DHFR vector system, approximately six months was usually required due to selection/amplification and adaptation to serum-free conditions. By using cloning cylinders and applying a less toxic Lipofectamine (LF-LTX) reagent to the highly confluent CHO-DG44 cells (~90%), we significantly reduced the time and increased the probability of finding the positive clone. Although tedious time is still required for these procedures, the end-results obtained through the selection/amplification processes allow us to use chimeric MAbs resembling the original human MAbs for all kinds of clinical applications, potentially leading to better results in therapeutic development against Burkholderia infection.

Considering the production levels and binding properties of chimeric MAbs produced by transient transfection using 8 different mammalian host cells, the cell lines (CHO-K1, COS-7, 293T and BHK-21) were more productive than other mammalian cells tested (Kim *et al*., 2011b). However, for large-scale

production, the CHO-DG44 cells are only available in obtaining stable clones due to the property of deficient DHFR genes within host chromosomes. Thus, we can continuously amplify the target antibody gene up to certain MTX levels without causing any cell damage. When producing therapeutic MAb, the CHO-DG44 cell line makes an ideal cell factory since their therapeutic MAbs largely correspond to genuine human MAbs with similar patterns of post-translational modification and glycosylation (Wurm, 2004). The availability of commercial media of high quality for cell culture is an additional factor that would support large-scale production of cMAbs in this cell line.

Efficient expression of 3 lead chimeric MAbs against BP and BM: A new development of antibody engineering technology not only facilitates a simpler approach for the generation of stable cell clones, it also turns more humanized MAb production in mammalian cells (Adams and Weiner, 2005; Dubel, 2007; Thie *et al*., 2008). This allows us to use certain amounts of MAbs resembling the original human MAb for human applications, potentially leading to better results in clinical trial and therapeutic development. The demand for therapeutic MAbs is increasing. More and more MAbs are required for research applications and numerous new MAb-based therapeutics are in preclinical or clinical testing, constituting an increasing need for new clinical-grade expression systems and large-scale production capabilities (Thie *et al*., 2008; Weiner *et al*., 2010). The major reason for choosing mammalian expression system is due to the post-translational modifications of the therapeutic MAbs largely correspond to the genuine human patterns and that correspondence is indispensable when it comes to mediating immune effector functions. Since the engineered MAb production rates in mammalian cells are relatively low, many different efforts have been made to improve those rates. The efforts have been focused on improvements of expression vectors, antibody gene transfection, medium development, high-throughput screening methods and downstream process for purification of end product MAbs. However, although these improvements have given to a leading role to mammalian cells in the large scale production of therapeutic MAbs, little improvement has been made with respect to small-scale MAb production in mammalian cells.

The most frequently used cell lines tor MAb production are CHO cells, Baby Hamster Kidney (BHK-21), transformed African green monkey kidney Cells (COS-7), mouse myeloma (NSO), transformed human embryonic kidney with T antigen (293T). Commercial media of high quality are available for cell culture. When it comes to therapeutic MAb production, however, cell culture processes are executed in serum-free media to reduce cost of downstream processes. Modern media compositions support excellent cell culture performance in the absence of serum provided peptides, growth factors and an undetermined collection of proteins, lipids, carbohydrates and small molecules. Efficient gene transfer into mammalian cells relevant for MAb production purposes has been a major challenge. Viral vector systems usually have the advantage of high transfection efficiencies compared to non-viral methods. However, these methods suffer from several limitations such as the time-consuming and laborious construction of vectors, elevated laboratory costs due to the high level of safety requirements, limitation of insert size and possible immunogenic reaction in clinical trials (Thomas *et al*., 2003). Non-viral gene transfer methods include calcium phosphate, lipofectamine reagents, electroporation and ballistic gene transfer (Nildome and Huang, 2002). Whereas electroporation and ballistic techniques usually lead to high cell mortality, calcium phosphate and lipofectamine often result in low transfection efficiencies especially in suspension cells. The electroporation-based transfection technology is a valid alternative and has been proven to be efficient even in suspension cells relevant for MAb production.

CONCLUSION

BP and BM, the causative agents of melioidosis and glanders, respectively, are priority pathogens which have increased the interest of the scientific community as category B select agents. They are highly virulent pathogens that might have been developed as bioweapons. There are no effective vaccines available to either of these 2 pathogens. During the past 5 years we have successfully developed high-performance therapeutic MAbs against BP and BM [from mouse MAbs to chimeric MAbs or fully human MAbs] to prevent melioidosis and glanders diseases of humans and animals. Throughout the conventional hybridoma technique, total 108 MAbs against BP and BM were generated and characterized (Zou *et al*., 2008). Six lead MAbs were evaluated using BALB/c mice challenged intranasally with a lethal dose of the bacteria (Zhang *et al*., 2011). Some of MAbs have been developed into chimeric MAbs, or fully human MAbs through antibody engineering [variable gene cloning, mammalian expression and mouse (or human) scFv antibody library techniques]. Overall, we generated 10 chimeric MAbs (3 stable MAbs and 7 transient MAbs) and one fully human MAb against BP and BM (Table

2). Other techniques [transgenic mouse, Epstein-Barr virus (EBv)-hybridoma and CDR-graft] (Fig. 1 and 2) are also promising to make efficient human(ized) MAbs, although these 3 approaches were not tried in our laboratory. Furthermore, a platform technology for ideal therapeutic MAbs against BP and BM (construction of expression vector systems and optimization of transfection/selection for stable production) has been established (Kim *et al*., 2011b).

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