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Immune Responses Against *Mycobacterium tuberculosis* and the Vaccine Strategies

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1. Introduction

Tuberculosis (TB) is an infectious disease caused by *Mycobacterium tuberculosis* (Mtb). Robert Koch identified Mtb as a causative agent of TB at 1882 (Sakula, 1983). Since then, TB has been one of the most important infectious diseases for human being. According to the WHO report regarding the global burden of TB in 2009, there were 9.4 million incident cases of TB with approximately one third of the world total population being infected (World Health Organization, 2010). Especially, coinfection of Mtb and HIV is a serious issue in sub-Sahara Africa area. Distribution of multi-drug-resistant TB or extensively drug-resistant TB has been another issue in TB.

The epidemiological studies revealed that only 10 to 30% of people who exposed Mtb are infected with Mtb and that 90% or more of the infected people do not develop TB. Only 5% shows the symptoms within 1 year and 95% of the infected individuals are considered to remain infected latently for the long time. Further, only 5% of individuals who have persistently infected Mtb show internal reactivation, i.e., show the overt symptoms the long time later (North and Jung, 2004). These facts indicate that immune responses that are necessary to contain Mtb are induced in the majority of Mtb-infected individuals. Induction of appropriate immune responses against protective Mtb antigens in appropriate stages is necessary for preventing TB. In this chapter, we review the aspect of immune responses and the vaccine strategies against Mtb.

2. Protective immunity against Mtb

2.1 Immune cell effectors against Mtb

Mtb is a facultative intracellular bacterium that survives in phagosomes of alveolar macrophages. In general, effective immune responses against intracellular pathogens are based on the cellular arm (T cells), not on the humoral arm (antibodies) of immune responses (reviewed in Stenger & Modlin, 1999; Kaufmann, 2003). The followings are effective cell subsets that have been considered to be important for protection against Mtb infection.

2.1.1 CD4$^+$ T cells

Intracellular bacteria in phagosomes including Mtb are processed via major histocompatibility complex (MHC) class II-mediated antigen processing pathway and antigens of the bacteria are presented to CD4$^+$ helper T cells (reviewed in Kaufmann, 2003; Flynn & Chan, 2001; Cooper, 2009). Therefore, CD4$^+$ T cells are considered to be the principal effectors against Mtb. Mice that have a deletion in MHC class II or CD4 gene have been shown to be succumbed against Mtb challenge infection (Ladel et al., 1995; Caruso et al., 1999). CD4$^+$ T cells are divided to mainly two subsets depending on the difference of cytokines produced, type 1 helper T cells (Th1) and type 2 helper T cells (Th2). Th1 produces interferon (IFN)-γ, tumor necrosis factor (TNF)- α, and/or interleukin (IL)-2, and contribute to macrophage activation or granuloma formation through effects of these cytokines. IFN-γ has been reported to be the most critical for the protective immunity through analyses of mice or humans which have a deletion in genes encoding IFN-γ or the receptor (Cooper et al., 1993; Flynn et al., 1993; Ottenhoff et al., 1998). Therefore, Th1 has been considered to play a pivotal role for protection against Mtb infection. IFN-γ amounts or the number of IFN-γ-producing T cells has been considered to be a relevant marker for induction of a protective immunity against Mtb (Ellner et al., 2000; Walzl et al., 2011), although it is not the unique marker (Agger & Anderesn, 2001). This cytokine is also able to induce inflammation in the lesion, possibly to help aggravating the disease. Th2 produce IL-4, IL-5, and/or IL-10 and contribute to antibody-mediated immune responses or type 1 allergy development. Therefore, Th2 are considered to have inhibitory effects for protective immunity against Mtb.

2.1.2 CD8$^+$ T cells

Theoretically, CD4$^+$ T cells are major effecter cells against phagosome-localized pathogens. However, CD8$^+$ T cells also have been shown to play an important role in protective immunity against Mtb by analysis of Mtb infection experiments to mice that have deficiency in β2-microglobulin gene critical for MHC class I expression (Flynn et al., 1992; Ladel et al., 1995). Mtb are reported to be cross-presented to CD8$^+$ T cells via MHC class I antigen presentation pathway. Kaufmann's group reported that Mtb-infected macrophages induce apoptotic process and then cross-presentation via apoptotic vesicles (Winau et al., 2006). The process may lead to induction of antigen-specific CD8$^+$ T cells. van Pinxteren and colleagues (2000) reported that CD8$^+$ T cells are particularly important in protective immunity at the latent phase of TB.

CD8$^+$ T cells contribute to protective immunity against Mtb in the following mechanisms (Smith & Duckrell, 2000; Kaufmann & Flynn, 2005). (1) Secretion of cytokines such as IFN-γ and TNF-α. IFN-γ production is essential for CD8$^+$ T-cell mediation of protective immunity against Mtb (Tascon et al., 1998). (2) Lysis of infected host cells through perforin and granzyme B secretion. (3) Direct killing of bacteria infected through granulysin secretion (Stenger et al., 1997). Stegelmann and colleagues (2005) showed that a subset of CD8$^+$ T cells coordinately expresses CC chemokine ligand 5 (CCL5, RANTES), perforin and granulysin, attracts Mtb-infected macrophages and kill the intracellular Mtb (Stegelmann et al., 2005).

2.1.3 Th17 cells

Th17 is a subtype of CD4$^+$ T cells and produce IL-17. Th17 promote migration of Th1, neutrophils, and monocytes to TB lesion in the presence of chemokines and contribute to protective immunity against Mtb (Khader et al., 2007).

2.1.4 CD1-restricted T cells

A subset of T cells is antigen-presented through CD1 molecules, not through conventional MHC class I (Ia) or II molecules. Genes encoding CD1 molecules are mapped outside of MHC and are less polymorphic than conventional MHC genes. CD1-restricted T cells have been reported to recognize glycolipids in mycobacterial cell wall such as mycolic acid (Beckman et al., 1994).

2.1.5. γδ T cells

Most CD4$^+$ and CD8$^+$ T cells express T-cell receptors of α and β protein chains. A minor subset of T cells expresses T-cell receptor of γ and δ protein chains (γδ T cells). γδ T cells tend to distribute in epithelial tissues and contribute to early protection against pathogens invading through epithelium. The phosphoantigen-specific γδ T cells (Vγ2Vδ2$^+$) displayed major expansion during BCG infection and a clear memory-type response after BCG reinfection in a macaque model (Shen et al., 2002)..

2.1.6 NK cells

NK cells have been associated with early resistance against Mtb (Junqueira-Kipnis et al., 2003). NK cells increased in the lung after aerosol infection of Mtb and produce IFN-γ and perforin. But in vivo NK cell depletion experiment had no influence on bacterial load within the lungs in mouse system, suggesting that NK cells do not play essential roles in early stage of Mtb infection.

2.1.7 Regulatory T cells

Regulatory T cells (Treg) are composed of a subset of T cells that express Foxp3 transcription factor and inhibit effector T-cell responses by both cell-to-cell direct contact and secretion of inhibitory cytokines such as IL-10 or transforming growth factor (TGF)-β. They also constitutively express CD25 molecule, α chain protein of the high affinity IL-2 receptor. So, this T-cell subset can be deleted in vivo with injection of anti-CD25 monoclonal antibody and the depletion experiments confirmed that it has been shown to be critical for prevention of autoimmunity and graft rejection. Treg have been reported to be induced in Mtb-infected mice and humans and this cell subset is considered to be important for the persistence of Mtb infection (Kursar et al., 2007).

2.1.8 Antibodies

In general, humoral immunity does not contribute to induction of the protective immunity against Mtb. However, antibodies specific for heparin-binding haemagglutinin (HBHA) and arabinomannan are reported to contribute to reduce the bacterial load in lung (Teitelbaum et al., 1998; Pethe et al., 2001). Antibodies specific for antigens presented on the surface of Mtb may have some effect to prevent invading Mtb into the host cells.

2.2 Protective antigens of Mtb

The analysis of genome of Mtb H37Rv strain reveals that 3,985 open reading frames exist in the genome (Cole et al., 1998). Since the report, DNA vaccines are possible to be constructed through the genome information. Information of mycobacterial genes and proteins is available from several comprehensive databases (e.g., The Pasteur Institute TubercuList [http://genolist.pasteur.fr/tuberculist/], The Institute for Genomic Research (TIGR) Comprehensive Microbial Resource (CMR) [http://cmr.tigr.org/tigr-scripts/CMR/CmrHomePage.cgi]). The most important issue in developing effective TB vaccines is to clarify which genes or gene products are important for the protective immunity against Mtb. The fact that viable Mtb, but not killed Mtb, can induce the protective immunity (Orme, 1988) leads to the speculation that mainly secretory proteins would be the protective antigens. In fact, the majority of the target antigens of the cellular arm of immunity has been reported to be secretory or cell-membrane proteins (Andersen, 1994). Representative protective antigens of Mtb have been clarified by analyses of T-cell responses against protein fractions separated with the electrophoresis analyses (Belisle et al., 2005). The followings are the representative protective antigens of Mtb reported.

2.2.1 Antigen 85 complex proteins

Antigen 85 (Ag85) complex proteins are mycobacterial major secreted proteins of 30 to 32 kDa. The proteins have mycolyl transferase function that is necessary for synthesis of lipid components of mycobacterial cell wall and fibronectin-binding function (Wiker & Harboe, 1992; Belisle et al., 1997). Ag85 complex proteins are composed of Ag85A (p32A), Ag85B (p30, MPT59, α antigen), and Ag85C protein. These proteins are well conserved in *Mycobacterium* genus. MPT51 protein has some homology to these proteins (Nagai et al., 1991; Ohara et al., 1995) and has been shown to be a protective antigen (Miki et al., 2004).

2.2.2 Low-molecular-mass secretory proteins

Low-molecular-mass proteins (less than 20 kDa) in Mtb culture fluid proteins (CFPs) have been reported to be major antigens that evoke T-cell responses (Boesen et al., 1995; Demissie et al., 1999). Among a variety of low-molecular-mass secreted proteins, early secreted antigenic target 6-kDa protein (ESAT6) and culture fluid protein 10 (CFP10) have been well studied (Berthet et al., 1998). Recently, ESAT6 and CFP10 proteins are widely used in whole blood IFN-γ release assays for TB diagnosis (Andersen et al., 2000) (QuantiFERON® TB Gold; Cellestis, Ltd., Carnegie, Victoria, Australia).

2.2.3 Heat shock proteins

A variety of heat shock proteins have been shown to be targets for antibodies and T cells in murine and human systems. Among them, heat shock protein 65 (Hsp65) is a major stress protein Mtb produces in macrophage cells. Mtb Hsp65 protein has more than 50% homology with *Escherichia coli* GroEL or human Hsp60 protein (Lee & Horwitz, 1995). Heat shock proteins have been shown to be expressed in Mtb in macrophages and induce protective immune responses.

2.2.4 Dormant phase proteins

Aforementioned proteins are expressed mainly in acute phage of TB. A different set of genes are expressed in late infection phase or chronic (dormant) phase of TB. DosR regulon is a unit of genes composed of 48 genes, the expression of which is regulated by DosR (Rv3133c). DosR regulon proteins are the major proteins expressed in chronic (dormant) phase of TB (Karalousis et al., 2004). HspX protein (Rv2031c) is one of the immunodominant antigens belong to DosR regulon proteins. It plays an important role in slowing the growth of Mtb as *hspX* gene-deleted Mtb mutants showed increased growth both in mice and in macrophages (Hu et al., 2006). T-cell responses specific for HspX were found in latent Mtb infection (Demissie et al., 2006). In addition, mycobacterial DNA-binding protein-1 (MDP1) has been shown to be expressed in the late phase of TB and induce humoral and cellular immune responses (Matsumoto et al., 2005; Suzuki et al., 2010). Analysis of dormant phase proteins is critical for development of therapeutic TB vaccines against persistent Mtb infection.

2.3 T-cell epitopes of Mtb proteins

T-cell epitopes are the peptides in antigenic proteins that bind to MHC molecules on antigen-presenting cells. In other words, T-cell epitopes are the peptides that stimulate T cells through the MHC-T-cell receptor interaction.

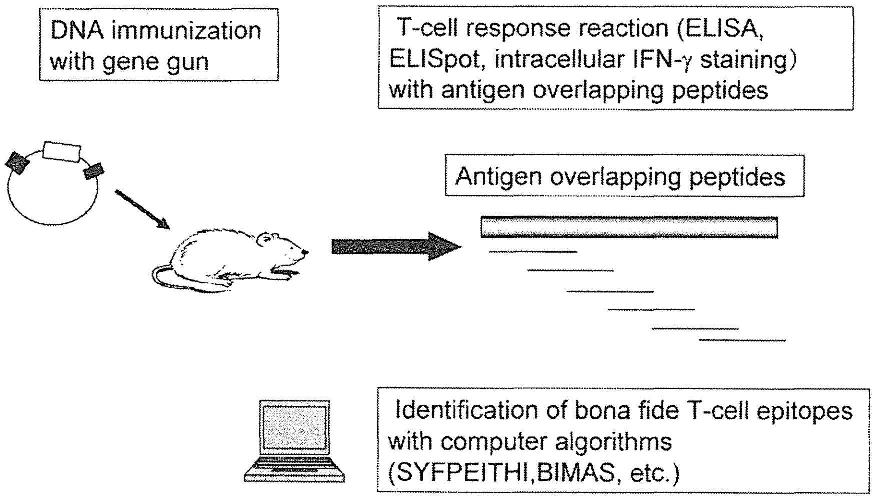
DNA immunization with gene gun
T-cell response reaction (ELISA, ELISpot, intracellular IFN-γ staining) with antigen overlapping peptides
Antigen overlapping peptides
Identification of bona fide T-cell epitopes with computer algorithms (SYFPEITHI,BIMAS, etc.)

Fig. 1. Shematic diagram for identification of T-cell with DNa immunization

Identification of T-cell epitopes in Mtb antigens is indispensable for accurate analysis of T-cell responses against Mtb antigens with specific MHC tetramers or intracellular cytokine staining. We realized that DNA immunization with gene gun bombardment is an excellent method for identification of Mtb T-cell epitopes as it is highly reproducible and efficiently

Antigen	Epitope peptide	MHC restriction	Reactive T cells	References
Ag85B	p30-38 (9-mer)	A*0201	CD8	Tang et al., 2011
	p183-192 (10-mer)	A*0201	CD8	Geluk et al., 2000
	p239-247 (9-mer)	A*0201	CD8	Geluk et al., 2000
	p10-27 (18-mer)	DR3, 52, 53	CD4	Mustafa et al., 2000b
	p19-36 (18-mer)	Promiscuous	CD4	Mustafa et al., 2000b
	p91-108 (18-mer)	Promiscuous	CD4	Mustafa et al., 2000b
MPT51	p51-70 (10-mer)	A*0201	CD8	Aoshi et al., 2008
	p191-202 (12-mer)	DR4 (Promiscuous)	CD4	Wang et al., 2009
Hsp65	p369-377 (19-mer)	A*0201	CD8	Charo et al., 2001
	p3-13 (11-mer)	DR3	CD4	Geluk et al., 1992
ESAT6	p72-95 (24-mer)	DR52, DQ2	CD4	Mustafa et al., 2000a
HspX	p21-29 (9-mer)	A*0201	CD8	Caccamo et al., 2002
(16kDa Protein)	p120-128 (9-mer)	A*0201	CD8	Caccamo et al., 2002
(DosR regulon)	p91-105 (15-mer)	A*0201	CD8	Geluk et al., 2007
	p31-50 (20-mer)	DR3	CD4	Geluk et al., 2007
Rv1733c (DosR regulon)	p161-169 (9-mer)	A*0201	CD8	Commandeur et al., 2011
Rv1733c	p181-189 (9-mer)	A*0201	CD8	Commandeur et al., 2011
Rv2029c (DosR regulon)	p161-169 (9-mer)	A*0201	CD8	Commandeur et al., 2011

Table 1. Human T-cell epitopes of Mtb antigens (examples)

induces T-cell responses (Yoshida et al., 2000). Therefore, we have used gene gun DNA immunization method for identification of CD8$^+$ and CD4$^+$ T-cell epitopes of Mtb antigens (Fig. 1). After immunization with plasmid DNA encoding Mtb antigens, immune spleen cells were examined for their IFN-γ responses to overlapping peptides covering full-length Mtb antigens by measuring IFN-γ levels by enzyme-linked immunosorbent assay (ELISA) or by counting the numbers of IFN-γ-secreting cells by enzyme-linked immunospot assay (ELISPOT). We combined these methods with computer algorithms to predict T-cell epitopes. T-cell epitope prediction algorithm programs we used are as follows. They are able to access through their websites. (1) SYFPEITHI Epitope program (http://www.syfpeithi.de/) (Rammensee et al., 1999), (2) the National Institutes of Health Bioinformatics and Molecular Analysis Section (BIMAS) HLA Peptide Binding Predictions (http://bimas.dcrt.nih.gov/cgi-bin/molbio/ken_parker_comboform) (Parker et al., 1994), RANKPEP MHC binding peptide prediction algorithm (http://immunax.dfci.harvard.edu/Tools/rankpep.html) (Reche et al., 2004), and (4) ProPred HLA-DR binding peptide prediction algorithm (http://www.imtech.res.in/raghava/propred/) (Singh & Raghava, 2001). These programs are helpful for narrowing down the amino acid regions of T-cell epitopes. However, the algorithms are still not perfect for exact identification of bona fide T-cell epitopes. A peptide that shows the highest score in these algorithms is not necessarily the best T-cell epitope. Experimental validation is definitely necessary to determine actual T-cell epitopes. A variety of

T cell epitopes of Mtb antigens have been reported. A comprehensive analysis of T-cell epitope data regarding *Mycobacterium* genus in the immune epitope database (IEDB; http://immuneepitope.org) was performed (Blythe et al., 2007). Huygen and colleagues have reported identification of murine T-cell epitopes of Ag 85 family proteins (Ag85A, Ag85B, and Ag85C) (Denis et al., 1998, D'Souza, et al., 2003) using intramuscular DNA immunization. We have used gene gun DNA immunization method for identification of murine CD8$^+$ and CD4$^+$ T-cell epitopes of Mtb antigens including MPT51 (Suzuki et al., 2004), MDP1 (Suzuki et al., 2010), and low-molecular-mass secretory antigens (CFP11, CFP17, and TB18.5) (Eweda et al., 2010).

In addition to murine T-cell epitopes, human T-cell epitopes have been reported (some examples are shown in Table 1). HLA-A02 is the most frequent HLA molecule in Caucasians and HLA-A*0201 represents the most frequent allele. HLA-A*0201-restricted CD8$^+$ T-cell epitopes have been identified in a variety of antigens including those derived from cancers, viruses, bacteria, and protozoan. Mtb-derived HLA-A*0201-restricted CD8$^+$ T-cell epitopes have been reported, including epitopes in Ag85A (Smith et al., 2000), Ag85B (Geluk et al., 2000), ESAT6 (Lalvani et al., 1998), and Hsp65 (Charo et al., 2001).

T-cell epitope (10-mer)	Position 1	2	3	4	5	6	7	8	9	10	
Anchor residues		L, M								L, V, I	
MPT51 p53-62	T	**L**	A	G	K	G	I	S	V	**V**	Aoshi et al., 2008
Ag85B p143-152	F	I	Y	A	G	S	L	S	A	**L**	Geluk et al., 2000
T-cell epitope (9 mer)	Position 1	2	3	4	5	6	7	8	9		
Anchor residues		L, M							L,V, I		
Ag85B p30-38	G	**L**	A	G	G	A	A	T	A		Tang et al., 2011
Ag85B p183-192	K	**L**	V	A	N	N	T	R	**L**		Geluk et al., 2000
Ag85B p239-247	G	**L**	A	G	G	A	A	T	A		Geluk et al., 2000
Hsp65 p369-337	K	**L**	A	G	G	V	A	V	**I**		Charo et al., 2001
HspX (DosR reg) p13-21	L	F	A	A	F	P	S	F	A		Caccamo et al., 2002
HspX (DosR reg) p120-128	G	I	L	T	V	S	V	A	**V**		Caccamo et al., 2002
Rv1733c (DosR reg) p161-169	I	A	D	A	A	L	A	A	**L**		Commandeur et al., 2011
Rv1733c (DosR reg) p181-189	A	**L**	L	A	L	T	R	A	**I**		Commandeur et al., 2011
Rv2029c (DosR reg) p314-322	E	**L**	A	A	E	P	T	E	**V**		Commandeur et al., 2011

Table 2. HLA-A*0201-restricted T-cells epitopes od Mtb antigens (examples)

Most HLA-A*0201-restricted T-cell epitopes were nonamer peptides (Falk et al., 1991, Parker, 1994), but some epitopes were decamer peptides. We reported an immunodominant HLA-A*0201-restricted T-cell epitope in MPT51 antigen (Aoshi et al., 2008; Tables 1, 2). Main anchor amino acid positions are, position 2 (Leu) and position 9 (Val), which were conserved in MPT51 p53-62 (TLAGKGISVV) (Table 2). MPT51 p53-62 decamer peptide was capable of binding to HLA-A*0201 and of stimulating $CD8^+$ T cells of HLA-A*0201-transgenic mice, but MPT51 p53-61 nonamer was not. The conformational and electrostatic differences between the nonamer and the decamer would affect their binding affinity to HLA-A*0201 molecule and following T-cell responses. Ruppert and colleagues (1993) studied in detail the role of different amino acid residues on each position of nonamer or decamer peptides for binding to HLA-A*0201 molecule. They suggested that nonamer or decamer peptide has different preference of amino acid residues for binding to HLA-A*0201 molecule. They showed that, for example, Tyr, Phe, Trp residues at positions 1, 3, and 5 in nonamer peptides, Gly residues at positions 4 and 6 in decamer peptides are preferable for binding to HLA-A*0201. According to their speculation, MPT51 p53-62 peptide seems to have better A*0201 binding features than MPT51 p53-61 peptide (Gly residues at positions 4 and 6 in MPT51 p53-62 peptide are suggested to be associated with good A*0201 binding). Interestingly, MPT51 p21-29 peptide (FLAGGPHAV) was not immunogenic in terms of IFN-γ production and cytolytic ability although the peptide showed high affinity to HLA-A*0201 as predicted by MHC binding algorithms (Aoshi et al., 2008). Previous reports showed a strong association between immunodominance and HLA binding affinity (Geluk et al., 1998). But, binding of peptides to the restricted MHC molecules is a prerequisite for T-cell epitopes, but all the peptides which show high affinity binding for MHC molecules are not necessarily immunodominant epitopes.

3. Vaccine strategies against Mtb

3.1 Recombinant BCG vaccines

Mycobacterium bovis bacillus Calmette-Guérin (BCG) is the only approved attenuated live vaccine to date against TB. Original BCG strain was reported approximately ninety years ago (1921) by Calmette and Guérin (Bloom & Fine, 1994). Despite the fact that BCG is among the most widely used vaccine throughout the world since then, TB still poses a serious global health threat. Whereas BCG is believed to protect newborns and young children against early manifestations of TB (Rodgrigues et al., 1993), its efficacy against pulmonary TB in adults is still a subject of debate (Bloom & Fine, 1994; Andersen & Doherty, 2005) and was reported to wane with time since vaccination (Sterne et al., 1998). Variable levels of the protective efficacy ranging from 0 to 80% have been reported in different studies (Fine, 1995). Moreover, the viable nature of BCG makes it partly unsafe in case of immunocompromised people such as HIV-infected individuals. This highlights the need to develop more effective, safe and reliable vaccines against TB (Kaufmann, 2010).

To improve the immunoreactivity of conventional BCG vaccines, a variety of recombinant BCG (rBCG) vaccines have been tried and evaluated for the protective efficacy (Stover et al., 1991). One of the problems of conventional BCG vaccines in induction of protective immune responses is that BCG is not effective for induction of $CD8^+$ T-cell responses compared with Mtb. BCG is less effective in MHC class I-mediated antigen presentation, which is prerequisite for $CD8^+$ T-cell induction (Mazzaccaro et al., 1996). To improve $CD8^+$ T-cell responses of BCG, Kaufmann and colleagues reported recombinant BCG (rBCG) introduced with listeriolysin O (LLO) gene derived from *Listeria monocytogenes* (Hess et al., 1998). *L. monocytogenes* escapes from the phagosomes into the cytoplasm shortly after infection into host macrophage cells. LLO, a thiol-activated cytolysin, has membrane-disrupting capability and plays a pivotal role in this process. The rBCG expressing LLO was reported to improve MHC class I-mediated antigen presentation of co-phagocytosed ovalbumin, suggesting that LLO endows BCG with an improved capacity to stimulate $CD8^+$ T cells. Further, the same group constructed urease C gene (*ureC*)-disrupted rBCG expressing LLO (Grode et al., 2005). Mycobacterial ureC increases pH value in the phagosome. The ureC deficiency induces low pH value in the phagosome, helping the LLO enzyme activity (to disrupt phagosome membranes), which leads enhancement of MHC class I-mediated $CD8^+$ T-cell responses.

Another promising rBCG is BCG overexpressing Ag85B protein (Horwitz et al., 2000). Ag85B is a major mycobacterial secreted protein and has been shown to be a protective antigen. Even though BCG does have endogenous Ag85B, overexpression of Ag85B in BCG was further enhanced the protective ability. In addition to these rBCG, attenuated auxotroph strains of Mtb have been examined (Guleria et al., 1996; Sambandamurthy et al., 2006; Larsen et al., 2009).

3.2 DNA Vaccination against TB

Many reports on DNA vaccination against Mtb have been published since 1996 (Huygen et al., 1996, Tascon et al., 1996). So far, a variety of Mtb antigen genes have been used for DNA vaccines, which include Hsp 65, Hsp 70, Ag85A, Ag85B, and ESAT6 (reviewed in Huygen, 2003 for early studies). DNA immunization with genes encoding dormancy regulon-encoded proteins has also been examined (Roupie et al., 2007). DNA immunization with naked DNA has been shown to efficiently induce cellular as well as humoral immune responses. DNA vaccines in most of these reports use needle injection through intramuscular or intradermal routes although some studies used gene gun (Sugawara et al., 2003). The DNA immunization with needle injection tends to raise predominant Th1 responses which are indispensable for induction of the protective immunity. On the other hand, gene gun DNA immunization is apt to produce "mixed type" (Th1 and Th2; producing IFN-γ and IL-4) T-cell responses which is not necessarily adequate for induction of the protective immunity (Tanghe et al., 2000). The difference is considered to be mainly due to the difference in the amount of antigen produced from the plasmids (high amounts in needle injection and low amounts in gene gun bombardment). Therefore, DNA vaccination with gene gun will need additional factors such as adjuvants for eliciting protective immunity against Mtb (D'Souza et al., 2002; Tollefsen et al., 2002; Li et al., 2006; Zhang et al., 2007).

Naked DNA vaccines have been evaluated as therapeutic TB vaccines as well as prophylactic TB vaccines. Lowrie and colleagues (1999) showed that intramuscular injection of mice with Hsp65 and MPT70 DNA vaccines reduced Mtb numbers in spleens and lungs after Mtb challenge by 1 to two $\log_{10}$ order compared with untreated mice. Further, they showed that three intramuscular injection of Hsp65 DNA vaccine eliminated residual Mtb after chemotherapy (isoniazid and pyrazinamid treatment) and immunosupprssive corticosteroid treatment. However, Orme's group reported that Ag85A DNA vaccine that was shown to induce protective immunity in mice when the vaccine was used as a prophylactic vaccine, could not give any therapeutic effect on the course of the infection in the lungs in mice earlier infected by Mtb aerosol (Turner et al., 2000). Moreover, they reported that vaccination with DNA encoding hsp60 of *Mycobacterium leprae* induced cellular necrosis throughout the lung granulomas when the DNA was given in a mouse immunotherapeutic model (Taylor et al., 2003). Repique and colleagues (2002) also reported that vaccination with a DNA vaccine cocktail containing ten Mtb antigen genes which had showed significant protective responses in mice could not prevent reactivation of disease in a murine latent TB model. These reports indicate that therapeutic TB vaccines still have room for further studies in terms of safety.

3.3 Improvement in immunization regimen: prime-boost immunization

Evaluation of vaccination has indicated that the repeated injection of the same vaccine has a limitation in terms of its overall immunological effects. Especially, DNA immunization has been reported to induce considerably strong immunological responses in the rodents, but not in the primates including human (Li et al., 1993). Instead of the repeated injection of the same vaccine, the heterologous prime-boost regimen including DNA vaccination, which is primed with naked DNA vaccination and boosted with recombinant viral vectors such as vaccinia virus and adenovirus, has been shown to evoke superior levels of immunity to DNA vaccine or recombinant virus alone (Ramshaw & Ramsay, 2000). The relatively low-level but persistent expression of immunogenic proteins in vivo by naked DNA vaccines has been suggested to be important for priming immunological responses and inducing enhanced cellular immunity (Ramshaw & Ramsay, 2000).

A variety of prime-boost regimens have been examined for Mtb infection (McShane & Hill, 2005). Many investigators examined the regimens in which priming with DNA vaccines and boosting with other immunization strategies. Feng and colleagues (2001) showed that priming with Ag85B DNA vaccine and boosting with BCG vaccine strengthened protective immunity against Mtb induced by BCG vaccine alone in mice. Skinner and colleagues (2003) also reported that priming with ESAT6 andAg85A DNA vaccines and boosting with BCG vaccine enhanced specific IFN-γ production from immune splenocytes compared with that by the DNA vaccine or BCG vaccine alone in mice. Ferraz and colleagues (2004) used DNA vaccines encoding mycobacterial Hsp70, Hsp65, and Apa antigens as priming vaccine and showed that the DNA vaccines enhanced BCG boosting effects. Romano and colleagues (2006) showed that immunization of BALB/c mice with Ag85A DNA vaccine first and boosting with BCG vaccine induced stronger protective immunity against Mtb challenge than that by Ag85A DNA vaccine alone. These results demonstrated that DNA vaccine priming and BCG vaccine boosting enhanced immune responses induced by BCG vaccine alone.

The regimens in which BCG vaccine was used as a priming vaccine also have been tried. As the BCG vaccine has been injected to people all over the world, this regimen seems to be reasonable. Derrick and colleagues (2004) showed that a polyvalent DNA vaccine encoding an ESAT6-Ag85B fusion protein protects mice against a primary Mtb infection and boosts BCG-induced protective immunity. Priming with BCG vaccine and intranasal boosting with MVA85A in mice enhanced Ag85A-specific $CD4^+$ and $CD8^+$ T-cell responses and strengthened protective immunity against aerosol Mtb challenge infection in mice (Goonetilleke et al., 2003). This regimen was reported in humans. McShane and colleagues

(2004) reported that in volunteers who had been vaccinated 0.5 to 38 years previously with BCG, vaccination with MVA85A induced substantially higher levels of antigen-specific IFN-γ-secreting T cells and that at 24 weeks after vaccination, these levels were 5 to 30 times greater than in vaccinees administered a single BCG vaccination.

3.4 Ongoing tuberculosis vaccine projects

A variety of TB vaccines have been evaluated (reviewed in Hoft, 2008; Kaufmann, 2010a, 2010b). WHO have showed a list of TB vaccine candidates (TB vaccine pipeline: http://www.stoptb.org/retooling/). These TB vaccine strategies are based on the prime-boost regimens and the vaccine candidates are categorized into three vaccine groups, namely, (1) priming vaccines, (2) boosting vaccines, and (3) therapeutic vaccines after Mtb infection. Some of vaccine candidates that have been ongoing are shown in Table 3.

Vaccine	Source	Explanation	References
Priming Vaccines			
rBCG30	UCLA (M. Horwitz)/NIAID	Ag85B recombinant BCG	Horwitz et al., 2000
rBCG Δ*ureC:Hly* (VPM1002)	Max Planck Inst. (S. Kaufmann) /VPM/TBVI	Listeriolysin O (LLO) recombinant BCG	Grode et al., 2005, Tchilian et al., 2009
mc^{2}6220, 6221, 6222, 6231	Albert Einstein College of Med.	ΔlysAΔpanCD (lysin/pantothetic acid-erquiring attenuated *M. tuberculosis*	Sambandamurthy et al., 2006
Booster Vaccines			
MVA85A/AERAAS-485	Oxford/Isis/Aeras/Ermergent	Ag85A recombinant Vaccinia Virus	McShane et al., 2001, Goonetilleke et al., 2003, McShcane et al., 2004, Scriba et al., 2010
AERAS-402/Crucell Ad35	Crucell/Aeras	Ag85A, Ag85B, TB10.4 recombinant Adenovirus type 35	Radošević et al., 2007 Abel et al., 2010
GSK M72	GSK/Aeras	PPE family protein Rv1196-Rv0125 fusion protein + AS01 adjuvant	Skeikey et al., 2004
SSI Hybrid I	Statens Serum Inst. (SSI)	Ag85B-ESAT6 fusion protein+IC31 adjuvant	Olsen et al., 2004 Aagaard et al., 2011
SSI HyVac 4/AERAS-404	SSI/Sanori Pasteur/ Intercell/Aeras	Ag85B-TB10.4 fision protein+IC31 adjuvant	Dietrich et al., 2005, Skeikey et al., 2010
rBCG30	UCLA (M. Horwitz)/NIAID	Ag85B recombinant BCG	Horwitz et al., 2000
HVJ-liposome/ Hsp65 DNA+IL-12 DNA	Kinki-chuo Chest Medical Center (Okada)	Hsp65 DNA+IL-12 DNA + HVJ-liposome	Yoshida et al., 2006
Therapeutic Vaccines			
MVA85A	Oxford	Ag85A recombinant Vaccinia Virus	McShane et al., 2001, Goonetilleke et al., 2003, McShcane et al., 2004
Hsp65 DNA vaccine	Cardiff Univ. (D. Lowrie)	hsp65 DNA Vaccine	Lowrie et al., 1999

Table 3. TB vaccine candidates (According to WHO TB Vaccine Pipeline)

BCG vaccines and rBCG vaccines are considered to be priming vaccines. For priming vaccines, BCG overexpressing Ag85B (rBCG30) and *ureC*-deleting BCG expressing lysteriolysin O (Δ*ureC hly*$^+$ BCG) have been evaluated. For booster vaccines, Ag85A recombinant vaccinia virus (MVA85A), Ag85B-ESAT6 fusion protein with adjuvant (SSI Hybrid I), and Ag85B-TB10.4 fusion protein with adjuvant (SSI HyVac4/AERAS-404) have been examined. MVA85A and Hsp65 DNA vaccine are candidate TB vaccine are candidate therapeutic TB vaccines. Human studies using the prime-boost regimens by these TB vaccine candidates have been publishing. Tchilian and colleagues (2009) reported that priming with Δ*ureC hly*$^+$ BCG and boosting with MVA85A induced protective immunity against Mtb infection in mice. The protective effects were much higher in Δ*ureC hly*$^+$ BCG vaccination than that in parental BCG vaccination. MVA85A boost immunization enhanced

Ag85A-specific T-cell responses, but did not affect bacterial numbers in the lung after Mtb aerosol infection. Scriba and colleagues (2010) reported that vaccination with MVA85A in healthy adolescents and children from a TB endemic region, who received BCG at birth, is safe and induces polyfunctional CD4$^+$ T cells co-expressing IFN-γ, TNF-α, and IL-2. Further, Abel and colleagues (2010) reported that vaccination with AERAS-402 (Adenovirus type 35 expressing a fusion protein created from the sequences of Ag85A, Ag85B, and TB10.4) is safe and immunogenic in healthy South African BCG-vaccinated adults.

4. Conclusion

Mtb, a causative agent of TB, is a unique facultative intracellular bacterium. The cellular immunity is essential for protection against Mtb. The main effectors are type 1 CD4$^+$ T cells and CD8$^+$ T cells. IFN-γ produced from them has been considered to be important as biomarker of TB. Induction of appropriate immune responses against protective Mtb antigens in appropriate stages is necessary for preventing TB. Identification of protective Mtb antigens and the T-cell epitopes are critical for clarification of kinetics of TB and development of effective TB vaccines. A variety of TB vaccine strategies have been examined including rBCG and DNA vaccines. Prime-boost strategies with combination of different TB vaccines are promising for prophylactic TB vaccines. Therapeutic TB vaccines for latent TB have also been examined.

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