Comparison of DNA delivery systems for vaccination against intracellular bacteria

vorgelegt von
M. Sc.
Nayoung Kim
aus Seoul, Korea

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Vorsitzender: Prof. Dr. Ulf Stahl
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Berichter: Prof. Dr. Stefan H. E. Kaufmann

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1. Introduction

1.1. DNA vaccine development against tuberculosis

1.1.1 Vaccines

Vaccines are amongst the most powerful developments in modern medical science and a cost-effective way for disease prevention. The incidences of diseases such as smallpox, diphtheria, measles, mumps, pertussis, rubella, poliomyelitis, tetanus, and hepatitis B have declined dramatically as vaccinations have become common. Smallpox was eradicated in 1979, and efforts are currently under way to eradicate or eliminate three vaccine-preventable diseases – polio, measles, and maternal and neonatal tetanus (State of the world's vaccines and immunization. WHO ISBN92/4/154623/9. World Health Organization. Geneva. 2003). However, no vaccine is 100% effective and live attenuated vaccines can be detrimental in immunocompromised individuals. Many of current common vaccines are live attenuated, for instance, tuberculosis, typhoid, measles, and mumps (Zinkernagel, 2003; Welsh et al., 2004). Furthermore, there is a critical need for vaccines against diseases which cause over 5 millions of deaths throughout the world every year, for example, malaria, tuberculosis (TB), and acquired immune deficiency syndrome (AIDS). There is no vaccine against malaria or AIDS. Vaccines againnst pneumococcal disease, meningococcal disease, and rotavirus diarrhea are also required urgently in developing countries (State of the world's vaccines and immunization. WHO ISBN92/4/154623/9. World Health Organization. Geneva. 2003). It should be noted that most unsuccessful vaccines require T cell-mediated immunity,

which is difficult to achieve with conventional vaccines. Vaccines that do not work satisfactorily and do not induce long-term protection include vaccination against TB, leprosy, and most classical parasitic infections, such as malaria, leishmaniasis and schistosomiasis, but also against some viral infections, including herpes, papilloma, and human immunodeficiency viruses (HIV). The efficient control of virtually all these agents requires T cell-mediated effector mechanisms in addition to protective antibodies (Zinkernagel, 2003).

The concept of vaccination originated in the prevention from infectious disease by E. Jenner in the late 18th century. In modern medicine, vaccinology has been enlarging its scope to cover not only prophylactic but also therapeutic vaccines against cancer, allergy, autoimmune diseases as well as infectious diseases including bacterial and viral diseases by novel achievements in science and technology (Moingeon et al., 2003). There are two main categories of vaccines: whole organism vaccines and subunit vaccines. Whole organism vaccines include inactivated/killed, live attenuated, and recombinant vaccines. Genetic engineering technology provides a new way to produce deletion or other mutant strains and recombinant strains as vaccine candidates. Subunit vaccines include proteins, peptides, DNAs, polysccharides, and toxoids, and the development of subunit vaccine are accelerated by genetic engineering technology, enlarged knowledge of immunology, genomics, and proteomics. There are several features that vaccines should accomplish: safety, sustained pathogen-specific protection, induction of neutralizing antibodies/protective T cell responses, and practical considerations (Duclos, 2004; Bonhoeffer et al., 2004) (Table 1).

Table 1 Features of effective vaccines

Safe	Vaccine must not itself cause illness or death
Protective	Vaccine must protect against illness resulting from exposure to live pathogen
Sustained protection	Protection against illness must last for several years
Induces neutralizing antibody	Some pathogens (such as poliovirus) infect cells that cannot be replaced (e.g. neurons). Neutralizing antibody is essential to prevent infection of such cells.
Induces protective T cells	Some pathogens, particularly intracellular, are more effectively dealt with by cell-mediated responses.
Practical considerations	Low cost per dose, biological stability, ease of administration, few side-effect.

(adapted from C. Janeway. Immunobiology. 5th ed.)

1.1.2 Tuberculosis as a global health problem

Tuberculosis (TB) is a major global health problem with two million deaths and 8.8 million new cases in 2002. The global incidence rate of TB was growing at approximately 1.1% per year, and the number of cases at 2.4% year (Global tuberculosis control: WHO report 2004. WHO/HTM/TB/2004.331. World Health Organization, Geneva, 2004). In many cases, TB is a curable disease with a complex, and long-term regimen of drug treatment. Drug-resistant strains of *Mycobacterium tuberculosis*, which is the major causative agent of TB, develop as a consequence of inconsistent or partial treatment. Rapid global dissemination of the W-Beijing family strains, notable multi-drug-resistant strains, is an emerging public health threat (Bifani et al., 2002). More than 50 million people are already infected with multi-drug resistant strains. TB and HIV/AIDS form a lethal combination, each speeding the other pathogen's progress. Fifteen millions are already coinfected, and it results in extra 0.5

million deaths. The emergence of multi-drug-resistant strains of *M. tuberculosis* and the susceptibility of patients infected with HIV to TB have fueled the spread of the disease.

Currently only one vaccine is available. *Mycobacterium bovis* Bacille Calmette-Guérin (BCG) is an attenuated strain derived from *M. bovis* and developed by Calmette and Guérin in the early 19th century (Kaufmann, 2001). Although BCG prevents disseminated TB in newborns, it fails to protect against the most common form of the disease, pulmonary tuberculosis in adults (Kaufmann, 2001). Protective efficacy is variable (ranging from 1-80%) against adult pulmonary disease and wanes with time (Colditz et al., 1994). Furthermore, different BCG strains showed different protection rates and different levels of immune responses in mice (Lagranderie et al., 1996). Strikingly, some strains, such as the Prague and Japanese strains, were unable to protect mice against a secondary mycobacterial challenge (Lagranderie et al., 1996). Protection from TB is associated with the maintenance of a strong cell-mediated response to infection involving both cluster of differentiation (CD) 4$^+$ and CD8$^+$ T cells and the ability to respond with T helper type 1 (Th 1) type cytokines, particularly Interferon-γ (IFN-γ) (Dupuis et al., 2000; Flynn and Chan, 2001). BCG vaccination induces IFN-γ-secreting T cells, predominately of the CD4$^+$ T cell phenotype (Lalvani et al., 1998; Goonetilleke et al., 2003). Recent studies suggest that BCG delivered parenterally may fail to induce T cell immune responses in the lung mucosa, which are considered critical for protection against pulmonary disease (Gallichan and Rosenthal, 1996; Belyakov et al., 1999). However, the basis of the variability is still uncertain. Therefore, the development of a novel, more

effective vaccine is urgently required.

1.1.3 Novel TB vaccine development

Three broad approaches in vaccine development against TB are being pursued: whole bacterial vaccines, subunit vaccines, and combination vaccines (Britton and Palendira, 2003; Kaufmann, 2000). The first approach includes either attenuated strains of *M. tuberculosis* produced by random mutagenesis or targeted deletion of putative virulence factors, or by genetic manipulation of BCG to express new antigens or cytokines. Live bacterial vaccines have the advantage that many antigens can act together to induce maximum protection, but in the case of TB pathogens, there are serious safety concerns. A modified BCG overexpressing the 30 kDa protein Ag85A, a major secreting protein of *M. tuberculosis* is in phase I trial since Jan. 2004 (Hoag, 2004; Horwitz and Harth, 2003). This is the first TB vaccine candidate showing evidence of higher potency than the current BCG vaccine in preclinical trials. Recombinant BCG into which the region of deletion-1 (RD1) locus was reintroduced also showed enhanced protection against *M. tuberculosis* (Pym et al., 2003). In an effort to improve access to the major histocompatibility complex (MHC) pathway of antigen processing, recombinant BCG strains were generated which secrete a hemolytic fusion protein containing listeriolysin O (LLO) of *Listeria monocytogenes* (Hess et al., 1998) and proved enhanced vaccine potency *in vivo* (Grode et al., manuscript in preparation).

The second approach utilizes non-viable subunit vaccines to deliver immunodominant mycobacterial antigens. Both protein and DNA vaccines can induce partial protection against experimental tuberculosis infection in mice

(Britton and Palendira, 2003). Protein subunit vaccines mostly induce humoral immunity, which is less significant for protection against intracellular bacteria. In contrast, DNA vaccines can be expressed, processed in the cell, and presented by MHC molecules to the cell surface. This mechanism enables to mimic the antigen presentation process of intracellular bacteria and to induce cell-mediated immune responses including both $CD4^+$ and $CD8^+$ T cell immune responses. The genes encoding Antigen 85A/B, ESAT-6, HSP65, and many others have been exploited as subunit vaccine candidates (Huygen et al., 1996; Tascon et al., 1996; Lowrie et al., 1997) but still protection levels with DNA vaccination against tuberculosis has been generally less effective than BCG vaccination alone (Britton and Palendira, 2003). DNA vaccine encoding Hsp 65 also showed therapeutic effect in mice (Lowrie et al., 1999) but prophylactic vaccination is considered to be the most cost-effective way to control TB.

The third approach includes tests of immunomodulatory adjuvants and prime-boost protocols. For example, a subunit vaccine can be used as a boost vaccine. Several prime-boost strategies have been tested: DNA-protein, DNA-recombinant virus expressing the same respective antigens, DNA-BCG, and BCG-DNA (Tanghe et al., 2001; McShane et al., 2001; Feng et al., 2001; Goonetilleke et al., 2003; Mollenkopf et al., submitted).

1.1.4 DNA vaccine development

During the past decade, DNA vaccination has been increasingly employed in an attempt to achieve simpler, safer, and more effective vaccination protocols. DNA vaccination involves inoculation with an expression vector that encodes

an antigenic protein. The encoded antigen is then produced *in situ* and elicits an immune response. Since the idea of DNA vaccines was proposed, studies have shown immunogenecity or protective efficacy of DNA vaccines for a variety of disease targets, including cancer, allergy, autoimmune diseases, bacterial diseases, and viral diseases. DNA vaccines against intracellular organisms that require cell-mediated immunity, such as the agent of TB, malaria, leishmaniasis, hepatitis, and AIDS, would be highly desirable as well as those against cancer. Immunization of various species (ranging from mice to human) with unique plasmid DNA constructs encoding foreign proteins has resulted in immune responses to antigens derived from a variety of infectious agents, including influenza (Ulmer et al., 1993; Fynan et al., 1993), HIV (Wang et al., 1994), rabies (Xiang et al., 1994), hepatitis B and C (Major et al., 1995; Sallberg et al., 1997), malaria (Wang, 1998), and mycobacteria (Tascon et al., 1996; Huygen et al., 1996).

Exogenous antigens provided by killed/inactivated pathogens, recombinant protein, or protein derived from live vaccines are taken up by antigen presenting cells by phagocytosis or endocytosis and are presented by MHC class II molecules to stimulate $CD4^+$ T cells, which can help to generate effective antibody responses. In contrast, MHC class I molecules associate with antigenic peptides synthesized within the cytoplasm of the cells and are elicited by live or DNA vaccines (Gurunathan et al., 2000). DNA vaccination favors a Th 1 response. The predominant immunoglobulin (Ig) isotype detected after DNA vaccination is IgG2a (Roman et al., 1997). It was also shown that the frequency of cytolytic T lymphocyte (CTL) precursors in mice that were vaccinated with plasmid DNA encoding a Sendai virus nucleoprotein

were comparable to those elicited by live Sendai virus infection (Chen et al., 1998). Until now, Th 1 responses and CTLs induced by DNA vaccination have been shown in various bacterial and viral systems (Fu et al., 1997; Seaman et al., 2004; Cho et al., 2001).

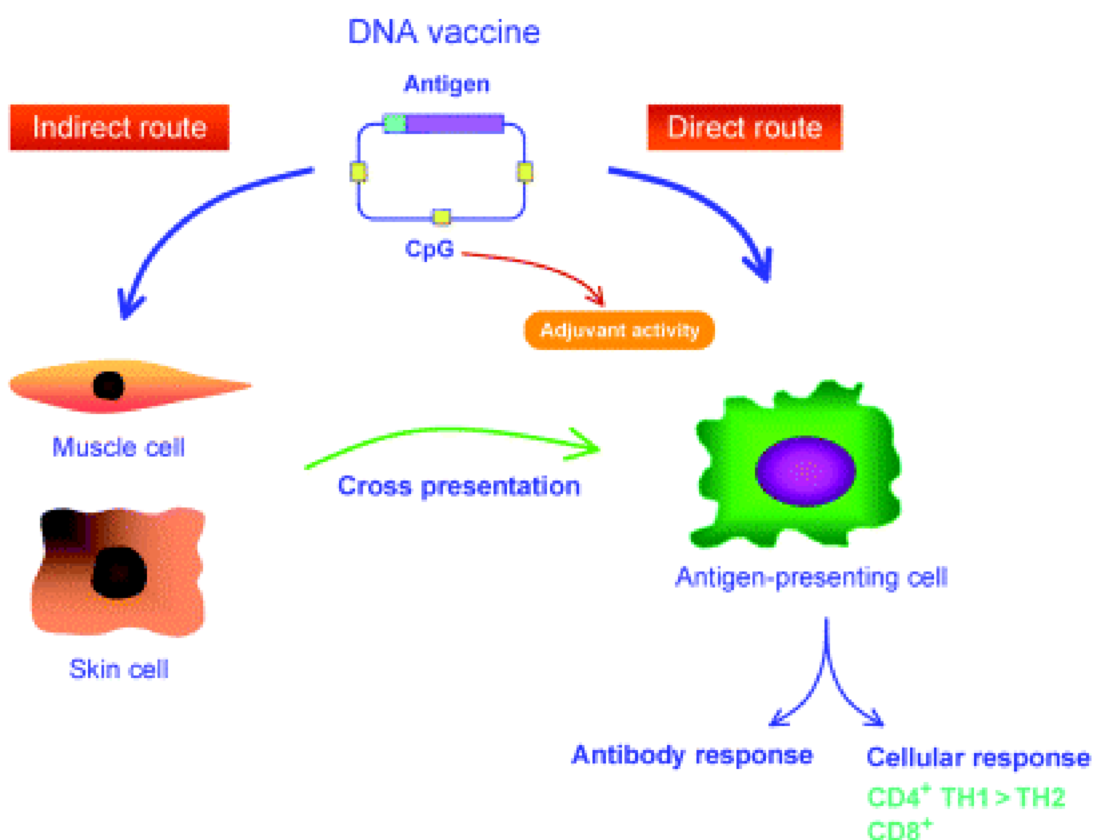

Fig. 1 Mode of action of DNA vaccines. Following injection of an antigen-encoding plasmid, transfected muscle or skin cells act as antigen depots. Transfer to antigen-presenting cells occurs via cross-presentation. Direct transfection of antigen-presenting cells can also occur. CpG sequences in the plasmid DNA stimulate the innate immune system. The outcome is induction of all arms of the immune response. (Adapted from Stevenson, 2004. DNA vaccines and adjuvants. Immunol. Rev. 199:5-8)

There are at least 3 mechanisms by which the antigen encoded by plasmid DNA is processed and presented to elicit an immune response (Fig. 1): direct transfection of bone-marrow derived APCs (Iwasaki et al., 1997; Doe et al.,

1996), direct transfection of somatic cells (Agadjanyan et al., 1999), and cross-priming (Ulmer et al., 1996). Some evidence suggests that bone marrow-derived APCs, but not somatic cells, directly induce immune responses after DNA vaccination (Iwasaki et al., 1997; Doe et al., 1996). However, because somatic cells such as myocytes or keratinocytes constitute the predominant cell populations transfected after DNA inoculation via muscle or skin injection, respectively, these cells may serve as a reservoir for antigen. Thus, somatic cells can be important in the induction of immune responses via cross-priming and may play a role in augmenting and/or maintaining the response (Gurunathan et al., 2000).

In 1990, it was shown that direct intramuscular inoculation of plasmid DNA, so-called 'naked DNA' encoding several different reporter genes, could induce protein expression in muscle cells (Wolff et al., 1990). The first demonstration of protective efficacy of a DNA vaccine in an animal model was reported in the influenza model in 1993 (Ulmer et al., 1993) and the induction of an antigen specific immune response was shown for the first time in humans by a malaria DNA vaccine in 1998 (Wang et al., 1998).

Ideally, DNA vaccine candidates should be pathogen-specific and immunogenic and should not induce any detrimental response to the host. The vectors for DNA vaccines usually contain viral promoters for transcription in mammalian cells and bacterial unmethylated cytidine-phosphate-guanosine (CpG) motifs in bacterial plasmid backbone, which activate B cells and dendritic cells (DCs) through Toll-like receptor 9 (TLR9) in human to produce Th 1 cytokines and promote priming and differectiation of Th 1 T cells (Sato et al., 1996; Ahmad-Nejad et al., 2002; Tascon et al., 2000). The TLRs are

pattern recognition receptors like MMR, that enable macrophages and dendritic cells to recognize bacteria, thus ensuring that an appropriate immune response is generated to defend against the particular pathogen causing infection (Medzhitov and Janeway, Jr., 1998).

There have been tremendous efforts to improve the efficacy of DNA vaccines, for example, developing new viral or non-viral vectors, insertion of gene regulatory elements in the plasmid backbone, and exchanging codon-usages to frequently used ones in mammals (Doria-Rose and Haigwood, 2003). Other approaches are construction of plasmids for co-expression of cytokines or costimulatory molecules, co-immunization of plasmids containing genes encoding cytokines or costimulatory molecules, and application of cytokines or CpG motifs as immunoadjuvants. These molecules can influence not only the magnitude but also the type of immune response induced by DNA vaccines (Scheerlinck, 2001). The usage of cytokines, costimulatory molecules, their genes, and other immunomodulatory agents may increase these safety concerns and has to be dealt with very carefully since all cytokines exhibit dose-dependent toxicity.

Several major safety concerns were identified by the Food and Drug Administration, U. S. A. (Smith and Klinman, 2001): the possibility that DNA vaccination could stimulate the production of autoantibodies against plasmid DNA, potentially inducing or accelerating the development of systemic autoimmune diseases; the possible induction of a local inflammatory response against organ-specific autoimmunity; the development of tolerance rather than immunity to the encoded antigen, putting vaccine recipients at increased risk from infection; polarization of the hosts cytokine response profile owing to

CpG motifs present in the plasmid backbone; and/or the potential for integration of the plasmid DNA vaccine into the genome of host cells. So these need to be controlled and avoided.

1.1.5 DNA vaccine carrier systems

DNA vaccines have enormous advantages: they are economical, relatively safe, easy to handle, and stable at room temperature. These characteristics are particularly beneficial to use in less developed countries where the majority of infectious disease incidences are reported. However, low efficiency of DNA delivery is one of the disadvantages of naked DNA. Thus effective DNA delivery systems can enhance cellular uptake of DNA, facilitate intracellular targeting of DNA to cytoplasm or nucleus, and reduce the amount of DNA required. The reduction of the amount of DNA also reduces the possible risk of integration and any potentially harmful side effects.

Several methods have been reported to improve DNA delivery: gene-gun, liposomes and lipids, proteins, biodegradable polymers, and attenuated bacteria. Gene-gun is a gas-driven biolistic bombardment device that propels gold particles coated with plasmid DNAs directly in an intradermal way. Initially, the DNA delivered by gene-gun was known to induce predominantly B cell immune responses and Th 2 immune responses (Fynan et al., 1993) but recently there are a few reports that claimed that gene-gun methods could also induce Th 1 immune responses and CTLs (Trimble et al., 2003) though inconsistently (Bartholdy et al., 2003).

Liposomes are bilayered membranes consisting of amphiphathic molecules such as phospholipids, forming unilayered or multilayered vesicles. Cationic

liposomes have been used for in vitro transfection for a long time, and showed improved TB DNA vaccine efficacy by formulation in cationic lipids (D'Souza et al., 2002). Although many lipid particles and oil-in-water emulsions proved too toxic for widespread use in humans, a squalene oil-in-water emulsion, MF59, was developed without the presence of additional immunostimulatory adjuvants, which proved to be a potent adjuvant with an acceptable safety profile (De Donato et al., 1999).

Virus-like particles (VLPs) from various viruses, and histone-like protein (TmHU) from hyperthermostable eubacterium *Thermotoga maritima* have shown the potential of effective DNA delivery *in vitro* and/or *in vivo*. Envelope proteins (VP1) from murine polyoma virus can self-assemble into particles. Virus-like particles have also been used as a gene delivery vehicle for long-term expression of a reporter gene with small amounts (5 μg) of DNA in mice (Krauzewicz et al., 2000). In many studies, VLPs have been designed to express pathogen-specific antigens. For example, a candidate vaccine against human papilloma virus (HPV) based on VLP composed of the L1 capsid protein is likely to be available in the near future (Koutsky et al., 2002). Histone-like protein showed a capability as an efficient mediator for transfection of eukaryotic cells *in vitro* and *in vivo* (Esser et al., 2000). In that report, 5 μg of DNA with TmHU protein successfully expressed a reporter gene in mice. Recombinant hepatitis B surface antigen (HBsAg) is used as a hepatitis B vaccine and suggested as a subunit vaccine carrier (Singh and O'Hagan, 2002).

Microparticles or biodegradable polymers, such as poly(lactide-co-glycolide)s (PLGs) have been used in humans for many years as suture material and as

controlled-release drug delivery systems for proteins, peptides, oligonucleotides, and drugs. Biodegradable particles also appear to have significant potential as a vehicle for DNA vaccines (Hedley et al., 1998); Jung et al., 2000; Singh et al., 2000). PLG is biocompatible, biodegradable, and non-immunogenic, thus PLG particles can be repeatedly administered. Absorption of these particles into cells depends on the size of particles, and DNA loading capacity depends on the positive charge and the pH. There are two kinds of particles: One is a surface-loading type, and the other is an encapsulating type. The particles can be applied by intramuscular, subcutaneous, oral, intranasal, or intravaginal route.

Attenuated strains of invasive bacteria *Shigella flexneri, Salmonella typhimurium*, and *Listeria monocytogenes* have been used for the delivery of plasmid DNA (Sizemore et al., 1997; Darji et al., 1997; Dietrich et al., 1998). Attenuated bacteria also can deliver DNA into host cells but express heterologous proteins at higher levels than conventional DNA vaccines.

1.2. Pathology of and immunity to tuberculosis

1.2.1 *Mycobacterium tuberculosis*

Mycobacteria are rod-shaped, aerobic, non-spore forming, non motile bacteria and called acid-fast bacilli because they do not stain readily by Gram staining, but once stained they resist decolorization by acid or alcohol despite being categorized as gram-positive bacteria. Because mycobacteria grow 20 to 100 times slower than other bacteria, it takes 4-6 weeks to obtain a colony of *M. tuberculosis* for drug sensitivity studies. The *Mycobacterium* genus has a cell wall of unique composition due to the dominant presence of mycolic acids that

make up more than 50% of its dry weight. The genome of *M. tuberculosis* has been sequenced and shown to be 4.41 Mb in size and to contain about 4000 protein-coding genes of which 52% can be assigned a function (Cole et al., 1998). Only 376 putative proteins share no homology with known proteins and presumably are unique to *M. tuberculosis* (Camus et al., 2002).

1.2.2 Pathology of and immunity to tuberculosis in human

The main route of infection for the tubercle bacillus is the respiratory tract. The bacteria are inhaled in airborne droplets that proceed distally to the lung to establish an infection (Kaufmann, 2001). After entering the lung, the first cell type encountered by the bacteria is the alveolar macrophage, which has the microbicidal armory to destroy most potential invaders. The immune response is initiated when *M. tuberculosis* arrives in the alveolar space, where it encounters alveolar macrophages. However, the tubercle bacillus has the extraordinary ability to persist and even to replicate in this extremely hostile environment, where most other pathogens perish. *M. tuberculosis* resides in phagosomes, which are not acidified into lysosomes (Clemens, 1996). Inhibition of acidification has been associated with urease secreted by mycobacteria and with uptake of mycobacteria by complement- or mannose-binding receptors rather than Fc receptors (Schlesinger, 1993). The inhibition of phagosomal acidification occurs by accumolation of a proton-ATPase (Schaible et al., 1998). Residing in the early recycling endosome, *M. tuberculosis* has ready to access to iron, which is essential for intracellular survival (Schaible et al., 2002). The pathogenicity of *M. tuberculosis* has been attributed to several cell wall components, for example, cord factor, a surface

glycolipid and lipoarabinomannan (LAM). Interestingly, the macrophage mannose receptor (MMR) binds the virulent *M. tuberculosis* strains H37Rv and Erdman but not the avirulent strain H37Ra, although both strains contain the same amount of terminal dimannosyl residues (Schlesinger et al., 1996; Schlesinger et al., 1994). A major heteropolysaccharide, LAM inhibits macrophage activation by IFN-γ, and induces macrophages to secret tumor necrosis factor-α (TNF-α) and interleukin-10 (IL-10) (Barnes et al., 1992). Lectin DC-specific intercellular adhesion molecule-3 grabbing nonintegrin (DC-SIGN) is also known as a *M. tuberculosis* receptor on human DCs (Tailleux et al., 2003). The tubercle bacillus and its cell wall glycolipid lipoarabinomannan seem to bind to and to induce, via DC-SIGN, an intracellular signal leading to IL-10 production, which in turn could impair activation of protective T cell responses directed against *M. tuberculosis* (Kaufmann and Schaible, 2003). Ingestion of *M. tuberculosis* by macrophages is also believed to depend on the engagement of TLRs, TLR2 and TLR4. However, the role of TLRs are still controversial since there are contradictory results from TLR2- or 4-deficient mice (Abel et al., 2002; Shim et al., 2003; Reiling et al., 2002).

The bacteria enter the parenchyma and can replicate within the alveolar macrophages or in resident lung macrophages. The signals induced result in migration of monocyte-derived macrophages and resident DCs to the focal site of infection in the lungs. Immunohistochemical, electron microscopic, and flow cytometric analyses showed that *M. bovis* BCG purified protein derivative (PPD) beads mobilized CD11c$^+$ DCs of comparable maturation. Transfer of DCs from PPD antigen-challenged lungs conferred a Th 1 anamnestic

cytokine response in recipients (Chiu et al., 2004). Once there, $CD4^+$ and $CD8^+$ T cells are primed against mycobacterial antigens. Primed T cells expand and migrate back to the lungs and then through the lung tissue to the focus of infection, presumably in response to signals such as chemokines produced by or in response to infected cells (Gonzalez-Juarrero and Orme, 2001).

Formation of compact granulomas that contain the pathogen at these sites begins with the accumulation of macrophages at sites of bacterial implantation and multiplication (Dannenberg, Jr., 1989). The migration of macrophages and T cells, as well as B cells, to the site of infection culminates in the formation of a granuloma, a characteristic feature of tuberculosis. In addition to T cells and macrophages, the granuloma consists of other host cells including B cells, dendritic cells, endothelial cells, and fibroblasts (Gonzalez-Juarrero and Orme, 2001). The granuloma can later show central caseous necrosis and give rise to cavities, although this does not occur in all cases of disease. A key aspect of granuloma formation is the development of fibrosis within the granuloma and in surrounding parenchyma, which produces macroscopic nodules (tubercles). The massive activation of macrophages that occurs within tubercles often results in the concentrated release of lytic enzymes (Converse et al., 1996; Chandrasekhar and Mukherjee, 1990). These enzymes destroy nearby healthy cells, resulting in circular regions of necrotic tissue which eventually form a lesion with caseous consistency. As these caseous lesions heal, they become calcified and are readily visible on X-rays, where they are called Ghon complexes. In adults, the disease advances as a necrotizing pneumonic process that can involve bronchioles and result in the spread of

infection to other areas of the lungs (North and Jung, 2004).

Tuberculosis immunity relies mainly on cell-mediated immunity rather than humoral immunity. The acquired cellular immune response to *M. tuberculosis* is complex. $CD4^+$ and $CD8^+$ T cells, as well as unconventional T cells such as $\gamma\delta$ T cells and CD1-restricted $CD4^-CD8^-$ or $CD4^+/CD8^+$ single positive $\alpha\beta$ T cell subsets, and natural killer (NK) cells are involved (Shen et al., 2002; Schaible et al., 2000; Suzuki et al., 1986) but generally $CD4^+$ T cells play a central role in protection. Interferon-γ is a key cytokine in the immune response against *M. tuberculosis* (Flynn et al., 1993). This is demonstrated by the considerably increased risk of TB patients with reduced cell-mediated immunity, such as those infected with HIV or individuals undergoing immunosuppressive therapy, compared with patients with defective humoral immunity, such as those with multiple myeloma, who show no increased predisposition to TB (Cohen et al., 1987). The patients who were deficient IL12Rc signaling and IFN-γ production suffered from severe mycobacterial and Samonella infections (de Jong et al., 1998).

The macrophage has multiple functions in TB, including antigen processing and presentation, and effector cell functions. Ingestion of *M. tuberculosis* by macrophages triggers, via NF-κB activation, transcription of numerous macrophage genes including those that code for proinflammatory cytokines and chemokines. The infected macrophage releases IL-12 and IL-18, which stimulate T lymphocytes, predominantly $CD4^+$ T cells to release IFN-γ (Wang et al., 1999). However, at least in the mouse model of infection, *M. tuberculosis* has the ability to evade the onslaught of innate immunity, as

virulent bacilli replicate exponentially within mouse lung during the first few weeks after infection, yet after the onset of acquired immunity, the growth of the bacilli plateaus (Hingley-Wilson et al., 2003).

Protective acquired immunity to *M. tuberculosis* is dominated by $CD4^+$ and $CD8^+$ T cells with the Th 1 cytokine profile (Flynn and Chan, 2001). The importance of the Th 1 cytokines IFN-γ and IL-12 is supported by the high susceptibility to mycobacterial infection of individuals with defects in IL-12, its receptor, and the IFN-γ receptor (Doffinger et al., 2002; Fieschi et al., 2003; Lichtenauer-Kaligis et al., 2003). The primary producers of IFN-γ are $CD4^+$ and $CD8^+$ T cells and NK cells. It is also noteworthy that humans with TB can generate a Th 1 response to *M. tuberculosis*, as evidenced by the presence in their blood and lungs of $CD4^+$ and $CD8^+$ T cells capable of responding specifically to *M. tuberculosis* antigens by replicating and synthesizing IFN-γ and other Th 1 cytokines, such as IL-12 and IL-18 in vitro (Arend et al., 2000; Ulrichs et al., 2000; Lalvani et al., 2001). At least some of the $CD8^+$ T cells, γδ T cells, and CD1 restricted T cells secrete perforin and granulysin in human which apparently kills mycobacteria within macrophages directly (Stenger et al., 1997; Ernst et al., 2000). T cells with specificity for mycobacterial glycolipids presented by CD1 (CD1a, b, and c) molecules seem to have a unique role in human tuberculosis. CD1 molecules are abundantly expressed on DCs but not on macrophages. Generally CD1-glycolipid-specific T cells produce IFN-γ and express cytolytic activity (Schaible et al., 2000).

1.2.3 Pathology of and immunity to tuberculosis in animal models

Animal models are essential for investigating the immune response in vivo

where the ability to control infection can be assessed in a physiological setting, after the selective removal of one or more components of the host response suspected of being involved. Much of what is known about the immunology of TB has come from studies of immunity to TB in mice, although the histopathology of TB in the rabbit and guinea pig is more human-like than in the mouse during early stages of infection. Additionally, the costs are relatively low, and there are more experimental tools and strains available including transgenic mice and gene knock-out mice.

Tuberculosis in mice is also mostly a lung disease, progressive and lethal, in spite of the generation of Th 1-mediated immunity (North and Jung, 2004). Mice deficient in CD4 T cells showed impaired ability to control infection and died of tuberculosis (Caruso et al., 1999). These mice showed deficiency in early production of IFN-γ in the lung and macrophage activation (Caruso et al., 1999). The development of and bacterial containment in granulomatous lesions was markedly impaired in TcR-β-/-, and less severely affected in TcR-δ-/- mutants. Mycobacteria-induced IFN-γ production by spleen cells in vitro was almost abolished in TcR-β-/- and virtually unaffected in TcR-δ-/- mice. (Ladel et al., 1995). A recent comparative study of targeted gene-deleted mice incapable of making αβ T cells, MHC class I (β2m), MHC class II, or γδ T cells showed that both $CD4^+$ and $CD8^+$ αβ T cells contribute to the ability of mice to inhibit *M. tuberculosis* growth from about day 20 post infection initiated with 100 colony-forming unit (CFU) of *M. tuberculosis* via the respiratory route. In contrast, mice incapable of generating γδ T cells were identical to wild-type mice in their ability to control infection and survival (Mogues et al., 2001). Whereas in the absence of MHC class I-dependent immunity, lung infection

progressed to a 1 log higher level than in wild-type mice and was controlled at a stationary level for a long period of time, in the absence of MHC class II dependent immunity, infection remained progressive and was lethal (Mogues et al., 2001). However, the data obtained in mice that are genetically deficient for β2m or transporter associated with antigen processing 1 (TAP1) gene clearly demonstrate an important role for $CD8^+$ T cells in protection (Behar et al., 1999; Flynn et al., 1992). The increased susceptibility of β2m-KO mice over MHC class I (K^bD^b)-KO mice was due to defective iron metabolism, and iron overload represented an exacerbating cofactor for TB (Schaible et al., 2002). $CD8^+$ T cells are apparently required to control TB in the latent phase (van Pinxteren et al., 2000) and require IFN-γ (Tascon et al., 1998). $CD8^+$ T cells also participate in the memory immune response to *M. tuberculosis* in mice (Serbina and Flynn, 2001). CD1d - restricted T cells, responding to mycobacterial glycolopids such as glucosemonomycolate, LAM, and isoprenoids, also participate in optimal protection through DCs as antigen presenting cells expressing CD1 molecules (Schaible et al., 2000).

There are some recent supportive results on macrophages as the effectors of Th 1 immunity after activation by IFN-γ. Nitric oxide is one of the major antimicrobial defense molecules of macrophages. It is generated from L-arginine by action of the inducible isoform of nitric oxide synthase 2 (NOS2). The other antimicrobial defense mechanism is based on reactive oxygen, which is generated by the transfer of an electron from NADPH to molecular oxygen by NADPH-oxydase. The NOS2-deficient mice showed impaired protection from *M. tuberculosis* infection but NADPH-oxidase-deficient mice were only slightly susceptible and only virulent strains of *M. tuberculosis* could

overcome the growth inhibitory action of a Th1-dependent, NOS2-independent mechanism of defense (Macmicking et al., 1997; Jung et al., 2002; Cooper et al., 2000). Protective immune response dominantly depends on Th 1 cytokines as revealed in IFN-γ or IL-12 deficient mice (Cooper et al., 1993). IFN-γ works synergistically with TNF-α in activating macrophages. Gene knock-out mice deficient in TNFR1 showed exacerbated TB, and granuloma formation was impaired (Flynn et al., 1995). $CD4^+$ T cells also produce lymphotoxin α, which participates in protection against *M. tuberculosis* (Roach et al., 2001). Therefore the whole range of acquired immunity is required for optimal protection from *M. tuberculosis* infection.

1.3. Experimental listeriosis

1.3.1 *Listeria monocytogenes*

Intracellular bacteria do not only include *M. tuberculosis* but also *Listeria monocytogenes*, *Salmonella* species, *Legionella pneumophila*, other *Mycobacterium* species, and many others (Fig. 2). Many of them are pathogenic (Schaible et al., 1999). *L. monocytogenes* is a gram-positive, motile facultative intracellular bacterium that causes severe food-borne infections, i. e. listeriosis. Pregnant women, their neonates, and immunosuppressed individuals are particularly susceptible to severe Listeria infection. The bacteria tolerate high concentrations of salt, and relatively low pH, and are able to multiply at refrigeration temperatures.

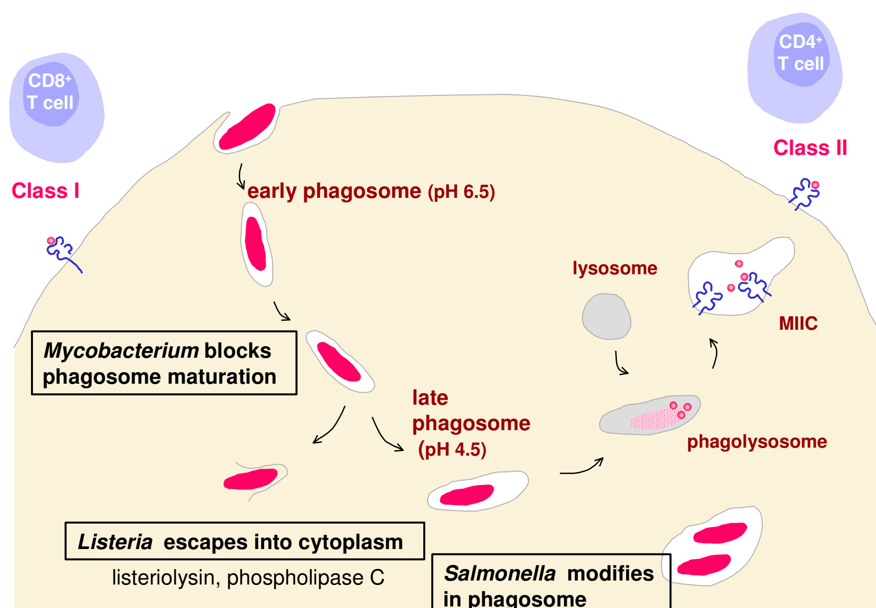

Fig. 2 Antigen presentation and intracellular bacteria (Adapted from L. Grode).

Virulence factors of *L. monocytogenes* are very well known. Upon phagocytosis by macrophages, *L. monocytogenes* escapes the vacuole by secreting listeriolysin O (LLO), an essential virulence factor (Mengaud et al., 1987; Bielecki et al., 1990). The protein LLO is a sulfhydril-activated, pore-forming cytolysin, which is active at the low pH existing in the phagosome and promotes evasion from the phagosome into the cytoplasmic compartment. Cell culture studies of the effects of hly gene, encoding LLO, showed that LLO is required for the survival and proliferation of *L. monocytogenes* within macrophages and non-professional phagocytes (Portnoy et al., 1988; Kuhn et al., 1988). The locus, including the *hly* (*lisA*) gene is a 9 kb virulence gene cluster that is involved in functions essential to intracellular survival (Bieleki et al., 1990). A PEST sequence (P, Pro; E, Glu; S, Ser; T, Thr) close to N-terminus of LLO is essential for the virulence and intracellular

compartmentalization of this pathogen (Decatur and Portnoy, 2000). In the cytosol, *L. monocytogenes* expresses ActA, which polymerizes actin, enabling bacterial mobility and cell-to-cell spread (Kocks et al., 1992) While both ActA and LLO-deficient bacteria are avirulent upon inoculation of mice, ActA-deficient bacteria induce long-term, CD8 T cell-mediated protective immunity while LLO-deficient bacteria do not (Goossens and Milon, 1992; Bouwer et al., 1994). Two different phospholipase C molecules (PlcA, and PlcB) contribute to the escape of *L. monocytogenes* into cytoplasm and to cell-to-cell spread (Marquis et al., 1995). Internalins (InlA, and InlB) and a secreted 60 kDa protein (p60) encoded by *iap* gene are involved in invasion of non-phagocytic host cells (Dramsi et al., 1995; Gaillard et al., 1994; Kuhn and Goebel, 1989). The internalin locus encodes the first invasin described in a gram-positive bacterium, implicated in internalization by cells that are not usually phagocytic, such as epithelial and endothelial cells and hepatocytes (Gaillard et al., 1996). Deletion of the gene encoding p60 in *L. monocytogenes* led to abnormal cell division and loss of actin-based mobility (Pilgrim et al., 2003).

1.3.2 Pathology of and immunity to listeriosis

Perinatal listeriosis mainly results from invasion of the fetus via the placenta and develops as chorioamnionitis. Its consequence is abortion, usually from 5 months of gestation onwards, or the birth of a baby or stillborn fetus with generalized infection, a clinical syndrome known as granulomatosis infantiseptica and characterized by the presence of pyogranulomatous microabscesses disseminated over the body and a high mortality (Klatt et al., 1986). *L. monocytogenes* is one of the three principal causes of bacterial

meningitis in neonates.

The gastrointestinal tract is thought to be the primary site of entry of *L. monocytogenes* into the adult host. In the initial stages, the bacteria were detected mostly in the absorptive epithelial cells of the apical area of the villi, whereas in later phases most were inside macrophages of the stroma of the villi (Racz et al., 1972). The bacteria that cross the intestinal barrier are carried by the lymph or blood to the mesenteric lymph nodes, the spleen, and the liver. Experimental infections of mice via the intravenous route have shown that *L. monocytogenes* bacteria are rapidly cleared from the bloodstream by neutrophils and resident macrophages in the spleen and liver but not all bacteria are destroyed by tissue macrophages, and the surviving bacteria start to grow for 2 to 5 days in mouse organs (Conlan and North, 1991). The principal sites of bacterial multiplication in the liver are the hepatocytes. During the early steps of liver colonization, polymorphonuclear neutrophils are recruited at the sites of infection, forming discrete microabscesses. Two to four days after infection, neutrophils are gradually replaced by blood-derived mononuclear cells together with lymphocytes to form the characteristic granulomas. Between days 5 and 7 post infection, *L. monocytogenes* bacteria start to disappear from mouse organs until their sterile clearance as a result of IFN-γ-mediated macrophage activation and the induction of an acquired immune response primarily mediated by $CD8^+$ lymphocytes, which together destroy infected cells (Harty et al., 1992; (Kaufmann, 1993). These CD8 T cells are focused on listerial epitopes present in secreted virulence-associated proteins, such as lysteriolysin O (LLO), metalloprotease, and 60 kb protein, p60 (Vazquez-Boland et al., 2001).

Acquired immunity against *L. monocytogenes* is entirely cell-mediated and largely dependent on $CD8^+$ T cells, but antibodies play little or no role in immunity. Protection against *L. monocytogenes* also involves a vigorous Th 1-biased $CD4^+$ T cell response and innate immunity such as the activation of IFN-γ-producing NK cells in response to IL-12 and TNF-α secretion by infected macrophages.

Innate immunity to *L. monocytogenes* plays a crucial role in controlling the initial bacterial burden, allowing time for acquired immune responses to develop and confer sterilizing immunity (Unanue, 1997). Several lines of investigation have implicated monocyte recruitment in early defense against infection. Antibody-mediated blockade of CD11b or deficiency of the CCR2 chemokine receptor markedly enhances susceptibility to *L. monocytogenes* infection by preventing monocyte recruitment to infected tissues (Rosen et al., 1989; Kurihara et al., 1997). The proinflammatory response induced by *L. monocytogenes* in the liver may be initiated by the interaction of the bacteria with receptors, such as TLRs. Human TLR2 promotes monocyte activation by *L. monocytogenes* (Flo et al., 2000). However, MyD88-deficient mice, which are largely defective in TLR signaling, are fully proficient to mount a protective acquired immune response to *L. monocytogenes* despite their increased susceptibility to primary infection (Seki et al., 2002; Edelson and Unanue, 2002; Way et al., 2003). $TLR2^{-/-}$ mice did not show increased susceptibility to *L. monocytogenes*, either (Edelson and Unanue, 2002).

Invasion of host cell cytosol by LLO and phosphilipases provides an essential stimulus that promotes the development of protective adaptive immunity

against *L. monocytogenes* (Serbina et al., 2003). T cell-mediated immune responses become operative 4-5 days following bacterial inoculation and are essential for complete clearance of *L. monocytogenes* from infected mice (Busch and Pamer, 1999; Bhardwaj et al., 1998). Experiments using MHC class I (β2m)$^-$ and MHC class II$^-$ deficient mice demonstrated that CD8$^+$ T cells are the principle T cell effectors for host defense to *L. monocytogenes* (Ladel et al., 1994). Mice lacking IFN-γ, TNF, or their specific receptors have inadequate innate immune responses and are highly susceptible to infection with virulent *L. monocytogenes* (Huang et al., 1993; Pfeffer et al., 1993; Rothe et al., 1993). Adoptive transfer experiments using CD8$^+$ T cells derived from IFN-γ deficient mice, however, demonstrated that IFN-γ production by CD8$^+$ T cells is not the sole effector mechanism (Harty et al., 1992; Harty and Bevan, 1995). It is noteworthy that IFN-γ is also produced by NK cells, especially in the early phase of infection (Nishibori et al., 1996). There are 2 major cytolytic mechanisms of CD8$^+$ T cells; perforin-dependent and Fas/FasL-dependent. Both perforin deficient mice and Fas-decifient mice showed higher susceptibility to *L. monocytogenes* but perforin-mediated mechanisms are more significant early during primary infection, whereas Fas/FasL-mediated mechanisms appear more significant late during primary infection (Kagi et al., 1994; Jensen et al., 1998).

Experimental murine listeriosis has been studied for the past 4 decades to examine basic aspects of innate and acquired cellular immunity to intracellular bacteria. Critical features of the murine model are that it yields rapid and quantitative results, either by enumeration of colony forming units (CFU) in the

liver and spleen or by determination of a lethal infection dose (Portnoy et al., 2002). Median survival times of mice are about 250 days for resistant mice (C57BL/6 and BALB/c) and about 100 days for susceptible mice (DBA/2, C3H, CBA, and 129Sv) after infection of 100 CFU of *M. tuberculosis* by aerosol, but survival results can be assessed with a lethal dose of *L. monocytogenes* in less than 10 days. Well-characterized antigens and epitopes also provide defined scopes to investigate antigen-specific immune responses to intracellular bacteria. For instance, large numbers of CTL responding to *L. monocytogenes* infection in BALB/c mice are specific for immunodominant epitope LLO_{91-99} presented by the $H2\text{-}K^{d}$ MHC class I molecule (Pamer et al., 1991). Two other epitopes, mpl_{84-92} and $p60_{449-457}$, elicit subdominant T cell responses, while another epitope, $p60_{217-225}$, elicits an intermediate response (Pamer, 1994; Busch et al., 1997). Less information is available concerning MHC class II-restricted epitopes. Only the $H2\text{-}E^{k}$-restricted peptide $LLO_{215-234}$ and the $H2\text{-}A^{d}$-restricted peptide $p60_{301-312}$ have been elucidated in some detail (Geginat et al., 1998).

1.4. Selection of the target genes for TB DNA vaccine

The state-of-the-art of genomics and proteomics has brought changes in vaccinology. Particularly, it has greatly benefited the rational selection of effective DNA vaccine candidates. The current vaccine against TB, *M. bovis* BCG, is closely related to *M. tuberculosis* (>90% DNA homology) but a large number of deletion mutations in many open reading frames (ORFs) has been discovered, and 16 regions of deletion (RDs) encoding 129 ORFs were reported so far, for example, a gene encoding the early secretory antigenic

target of 6 kDa (ESAT-6) (Behr et al., 1999). Out of them, 39 ORFs are missing in all BCG strains. There are 376 putative, unique proteins in *M. tuberculosis* that share no homology with known proteins (Camus et al., 2002). By comparative proteome analysis based on two-dimensional electrophoresis of *M. tuberculosis* H37Rv and *M. bovis* BCG, 39 *M. tuberculosis*-specific secreting proteins were identified (Mattow et al., 2001). Out of these genes, effective DNA vaccine candidates can be selected.

The genes encoding Antigen 85A/B, ESAT-6, HSP65, and many others have been exploited as subunit vaccine candidates (Huygen et al., 1996; Lowrie et al., 1997; Tascon et al., 1996) but still protection levels with DNA vaccination against challenge with *M. tuberculosis* has been generally less effective than BCG vaccination alone (Britton and Palendira, 2003). Ergo, heterologous prime-boost strategies would be worth adopting.

Three novel DNA vaccine candidates were selected by Mollenkopf et al. (Mollenkopf et al., submitted), based on 2D-electrophoresis analysis to select *M. tuberculosis*-specific secreting protein (Mattow et al., 2001). The proteoms of *M. tuberculosis* H37Rv was compared with those of *M. bovis* BCG Chicago. These are Rv3407, Rv2520c, and Rv1511. Rv3407 is a 300 bp non-essential gene (Sassetti et al., 2003), and encodes a 99 a. a. conserved hypothetical protein of unknown function. Rv2520c is a 228 bp non-essential gene (Sassetti et al., 2003), and encodes a 72 a. a. possible conserved membrane protein of unknown function. Rv1511 or *gmdA* is a 1023 bp non-essential gene (Lamichhane et al., 2003), and encodes a 340 a. a. GDP-mannose 4, 6 dehydratase, which is probably involved in nucleotide-sugar metabolism.

1.5. Specific aims of the study

The goal of this study was to compare DNA delivery systems for vaccination against intracellular bacteria. Specifically, this study was performed to select the most potent DNA vaccine candidates against *L. monocytogenes* as an experimental model system and against *M. tuberculosis*, to detect antigen-specific immune response thereby, to compare the different DNA vaccine carrier systems in listeriosis model, to find the most effective one, and to apply the results to TB.

To select the most potent DNA vaccine candidate, plasmid DNAs encoding wild-type LLO, p60, or mutant LLO against *L. monocytogenes* and plasmid DNA encoding Rv1511, Rv2520, and Rv3407 against *M. tuberculosis* were tested by protection assays. To compare the efficiency of DNA vaccine delivery, PLG stabilized with PVA, PLG stabilized with CTAB, a novel encapsulating particle, VLP, and TmHU were tested because these DNA carriers showed improved DNA transfer of reporter genes *in vitro* and/or *in vivo*, and were regarded relatively safe compared with lipid DNA carriers. The antigen specific immune responses were determined by flow cytometry with MHC class I/peptide tetramers, IFN-γ intracellular flow cytometry, IFN-γ ELISpot, IgG1/IgG2a ELISA, and CTL assays.

2. Materials and Methods

2.1. Materials

2.1.1 Mice

Female BALB/c mice (6-8 week-old) were purchased from the Federal Institute for Risk Assessment, Berlin, Germany and maintained under specific-pathogen-free conditions in the animal facilities at the Federal Institute for Risk Assessment, Berlin, Germany, or in the animal facilities of the Max-Planck-Institute for Infection Biology, Berlin, Germany. All animal experiments were performed in accordance with German and institutional animal care guidelines.

2.1.2 Cell lines

Mastocytoma cell line, P815 was obtained from the American Type Culture Collection (ATCC, Manassas, VA, U.S.A.) and cultured in RPMI 1640 (Biochrom, Berlin, Germany) supplemented with 10% fetal calf serum (FCS) (Biochrom, Berlin, Germany), 100 U/ml penicillin (Biochrom, Berlin, Germany), 100 U/ml streptomycin (Biochrom, Berlin, Germany), 4 mM L-Glutamine (Gibco, Karlsruhe, Germany), and 5 µM 2-Mercaptoethanol (ME) (Gibco, Karlsruhe, Germany), named complete RPMI 1640, in a fully humidified incubator in 5% CO_2 at 37°C. Macrophage/monocyte cell line, J774A.1 was obtained from ATCC and cultured in Dulbeco's modified Eagle's medium (DMEM) (Biochrom, Berlin, Germany) supplemented with 10% FCS, 100 U/ml penicillin, 100 U/ml streptomycin, and 4 mM L-Glutamine (Gibco, Karlsruhe,

Germany) in a humidified incubator in 10% CO_2 at 37°C.

2.1.3 Bacteria and virus

Listeria monocytogenes EGD strain Sv 1/2a was originally obtained from G. B. Mackaness. The bacteria were grown in Luria-Bertani (LB) broth (Difco, Heidelberg, Germany) without any antibiotics to an OD_{600} of 0.6, harvested by centrifugation, and stored as stock in final 10% glycerol in LB at –80ºC. The next day, one stock was thawed, plated onto LB agar plates, and colony-forming units (CFU) were assessed.

Mycobacterium tuberculosis H37Rv strain was grown in Middlebrook 7H9 broth (Difco, Heidelberg, Germany) containing 0.05% Tween 80, supplemented with albumin-dextrose complex, and stored in aliquots at –70°C.

Mycobacterium bovis BCG Danish 1331 (Statens Serum Institute, Copenhagen, Denmark) was cultured in Dubos broth base (Difco, Heidelberg, Germany) supplemented with Dubos medium albumin (Difco, Heidelberg, Germany) at 37°C. A mid-logarithmic culture was aliquoted and stored at -80°C before use. Mycobacterial cell stocks were plated onto Middlebrook 7H11 agar plates supplemented with oleic acid albumin-dextrose complex (Difco, Heidelberg, Germany) and CFU were assessed.

Lymphocytic choriomeningitis virus (LCMV) WE strain was originally obtained from Dr. F. Lehmann-Grube (Schwenk et al., 1971) and grown in L929 fibroblast cells.

2.1.4 Plasmid DNAs

Plasmid DNAs encoding p60 named pCiap, listeriolysin O (LLO) named

pClisA, and non-hemolytic, mutant LLO named pChly492 were constructed by Fensterle et al. (Fensterle et al., 1999) and J. Hess et al. (Hess et al., 2000) (Fig. 3). Briefly, wild-type LLO gene and p60 gene of *L. monocytogenes* without the bacterial signal sequence were amplified by polymerase chain reaction (PCR) and inserted into *EcoR*I/*Xba*I site and *Xho*I/*Xba*I site of pCI mammalian expression vector (Promega, Madison, WI, U.S.A.), respectively. *L. monocytogenes* strain BUG337 encoding an LLO version with a single amino acid (a. a.) exchange at the a. a. position 492 (Trp-492-Ala) was kindly provided by Dr. P. Cossart (Michel et al., 1990). The mutant LLO gene was amplified from genomic DNA of *L. monocytogenes* strain BUG337 by PCR, and integrated into *Xho*I/*Xba*I site of pCI vector, which is named pChly492. The gene map of pChly492 is the same as pClisA.

Plasmid DNAs encoding *M. tuberculosis* specifically expressed genes were constructed by Mollenkopf et al. (Mollenkopf et al, submitted) (Fig. 4). Briefly, *M. tuberculosis* specific genes were determined by comparative proteomics comparing *M. tuberculosis* strain H37Rv and strain Erdmann with *M. bovis* BCG strain Chicago and strain Copenhagen using two-dimensional gel electrophoresis, mass spectrometry, and N-terminal sequencing techniques (Mollenkopf et al., 2002). Three TB DNA vaccine candidate genes, Rv1511, Rv2520, and Rv3407 were selected by preliminary DNA vaccination experiments and amplified from *M. tuberculosis* strain H37Rv genomic DNA by PCR and cloned into *BamH*I sites of pCMVtpa4 mammalian expression vector containing an ER-targeting leader sequence, tpa4, which is originally the human tissue plasminigen activator signal sequence (Weiss et al., 1999). This sequence improves induction of immune responses and protection with

DNA vaccine against *M. tuberculosis* (Delogu et al., 2002; Li et al., 1999). This pCMVtpa4 vector was a kind gift from Dr. J. Ulmer, Chiron, U.S.A..

All molecular biological techniques followed standard techniques and enzymes were purchased from New England Biolabs (Frankfurt am Main, Germany). All plasmid DNAs for DNA vaccination were prepared using Qiagen Endotoxin-Free Plasmid Purification kit (Qiagen, Hilden, Germany) following manufacturer's instruction. After purification, the DNAs were digested by proper restriction enzymes to confirm by 1 % agarose gel electrophoresis in 1 X TAE buffer. For example, pCI vector was digested by *Nde*I, pCiap was digested by *Hpa*I, and pClisA and pChly492 were digested by *Nhe*I to yield fragments of 1542 b. p. and 2466 b. p. bands from pCI, 1211 b.p. and 4165 b.p. bands from pCiap, 1320 b.p. and 4191 b.p. from pCliaA and pChly492.

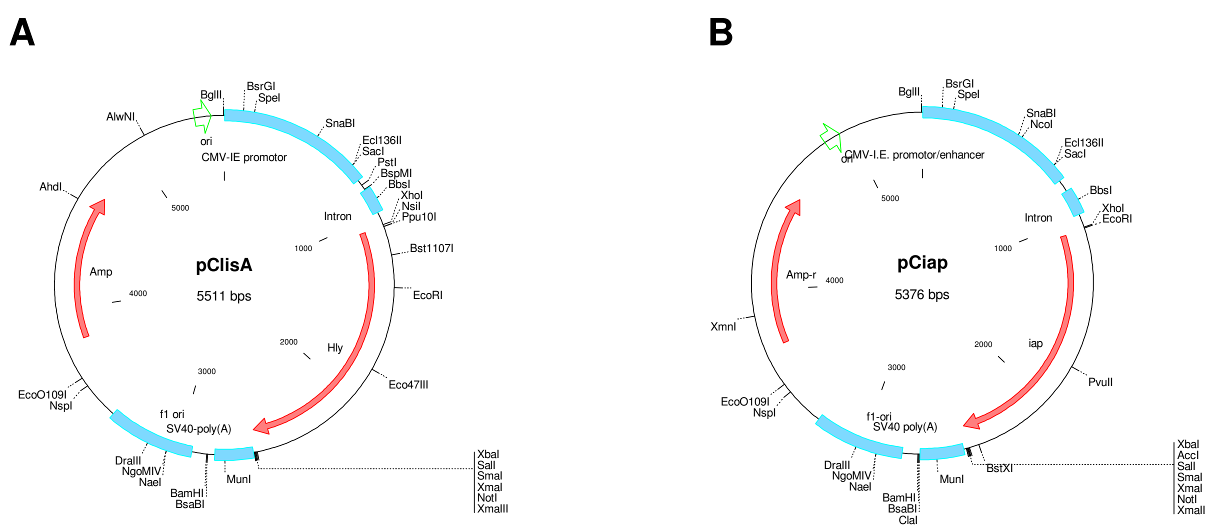

Fig. 3 Plasmid DNAs encoding *L. monocytogenes* genes. Immunodominant antigens of *L. monocytogenes* were inserted into pCI mammalian expression vector under a CMV promoter. A. Plasmid pClisA encodes listeriolysin A antigen. Plasmid pChly492 encoding mutant LLO has the same gene map. B. Plasmid pCiap encodes p60 antigen.

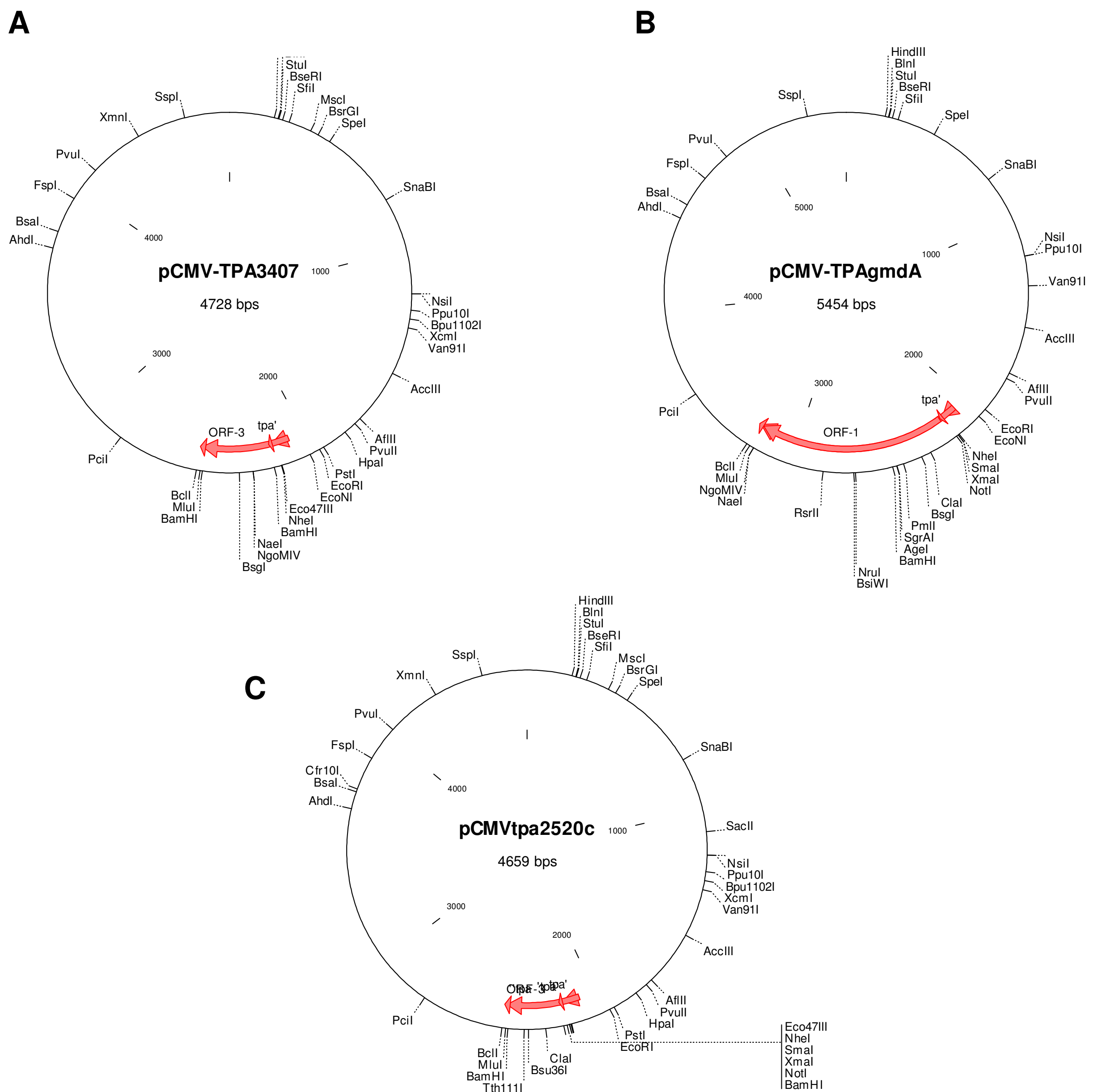

Fig. 4 Plasmid DNA encoding *M. tuberculosis* specifically expressed genes. *M. tuberculosis*-specifically expressed genes were inserted into mammalian expression vector under a CMV promoter to be fused to downstream of tpa4, the ER-targeting sequence. A. Plasmid pCMV-TPA3407 encodes Rv3407 gene. B. Plasmid pCMV-TPAgmdA encodes Rv1511 gene. C. Plasmid pCMV-TPA2520c encodes Rv2520 gene.

2.1.5 Peptides

The LLO-derived peptide corresponding to a. a. 91-99 (single-letter code, GYKDGNEY), immunodominant epitope restricted by MHC class I molecule, H2-K^d (Pamer et al., 1991) and the p60-derived peptide corresponding to a. a. 217-225 (KYGVSQDI), immunodominant epitope restricted by H2-K^d (Pamer, 1994) were synthesized by Gerini Biotools (Berlin, Germany).

The Rv1511-derived peptide (GYVKFDQRYL) corresponding to a putative epitope restricted by H2-K^d and the Rv3407-derived peptides (IPARRPQNL, RPQNLLDVT) corresponding to putative epitopes restricted by H2-L^d were synthesized as described above. The putative epitopes and affinities to class I MHC molecules were predicted by computer programs, MAPPP (MHC-I Antigenic Peptide Processing Prediction) (http://www.mpiib-berlin.mpg.de/MAPPP/) (Hakenberg et al., 2003; Mollenkopf et al., submitted) and FragPredict, provided by Max-Planck-Institute for Infection Biology, Berlin, Germany (Nussbaum et al., 2003).

The LCMV $NP_{118-126}$ (RPQASGVYM) peptide, representing a H-2^d–restricted T cell epitope, was kindly provided by Dr. P. Aichele (Aichele et al., 1990).

All peptides were synthesized by Gerini Biotools (Berlin, Germany) and stored at –20ºC as stocks resolved in sterile distilled water at 1mg/ml.

2.1.6 DNA carrier systems

Three different kinds of DNA vaccine delivery systems were used in this study: cationic poly(lactic-co-glycolic acid) (PLG) microparticles, histone-like protein from hyperthermostable eubacterium *Thermotoga maritima* (TmHU), and

virus-like particles, VP1 protein from mouse polyoma virus (VLP).

Cationic PLG particles were produced by a reaction with RG 502H (Boeringer Ingelheim, Ingelheim, Germany) and polyethylenimin (BASF, Ludwigshafen, Germany), stabilized with hexadecyltrimethyl ammonium bromide (CTAB) (Fluka, Steinheim, Germany) or with polyvinylalcohol (PVA) (Hoechst, Frankfurt am Main, Germany) (Singh et al., 2000a). The size of particles was 830.63 nm ± 36.2. Particles were resolved in sterile endotoxin-free distilled water (DW), sonicated for 30 seconds, and added to a DNA solution at a ratio of 1:100 = DNA: PLG (weight/weight) in 100µl of sterile, endotoxin-free DW per injection. The mixture of DNA and PLG was incubated at 4ºC overnight and then injected intramuscularly into mice. For encapsulating particles, novel amine-modified polyesters were made to encapsulate DNA at a ratio of 5:1 (w/w) in 20 µl/application, and applied intranasally. All particles were kindly provided by Prof. Dr. T. Kissel, University of Marburg, Marburg, Germany.

Histone-like protein from *Thermotoga maritima* (TmHU) and VP1 protein from mouse polyoma virus (VLP) were purified and kindly supplied by Dr. J. Hess, November AG, Erlangen, Germany. TmHU protein was mixed with DNA at a ratio of 1:1.2=DNA: protein (w/w) in 100µl of sterile, endotoxin-free distilled water (DW) per injection and injected subcutaneously (s. c.) into mice. The VP1 protein was mixed with DNA at a ratio of 1:1 (w/w) in 100µl of sterile, endotoxin-free DW per injection, incubated for 15 min at room temperature, and then injected s. c. into mice.

2.1.7 Antibodies and tetramers

Rat immunoglobulin (Ig), anti-mouse CD8α monoclonal antibody (mAb) (clone

YTS169), anti-mouseCD4 mAb (clone YTS191.1), anti-mouse CD62L mAb (clone Mel14), anti-mouse IFN-γ (clone XMG1.2 and clone R4), and anti-mouse IgG Fc Rc (clone 2.4G2) (Karpovsky et al., 1984) were purified from rat serum or hybridoma supernatants with protein G sepharose or purchased from Becton Dickinson (B & D, Heidelberg, Germany). Antibodies were cyanine 5 (Cy5)-, phycoerythrin (PE)-, or fluorescein-5-isothiocyanate (FITC)-conjugated according to standard protocols.

A modified form of the full-length cDNA of the H2-K^d H chain and human β2m were kindly provided by Dr. E. Pamer (Altman et al., 1996; Busch and Pamer, 1998). Tetrameric H2-K^d/peptide complexes were generated following the protocols described in Busch and Pamer, 1998. In brief, a specific biotinylation site was inserted to the COOH terminus of truncated H2-K^d heavy chain (no transmembrane region, truncation after a. a. 284). This fusion protein and β2m were expressed in large amounts as recombinant proteins in *Escherichia coli* using the isopropyl-β-d-thiogalactopyranoside (IPTG)-inducible pET3a vector system (Novagen, Madison, U.S.A.) and *Escherichia coli* strain BL21 (DE3) (Novagen, Madison, U.S.A.) as an expression host. Purified heavy chain and β2m were dissolved in 8 M urea and diluted into refolding buffer containing high concentration of synthetic peptides (60 μM) to generate monomeric, soluble H2-K^d-peptide complexes. MHC-peptide complexes were purified by gel filtration over a Superdex 200HR column (Pharmacia Biotech AB, Piscataway, NJ., U.S.A.), and in vitro biotinylated for 12 hrs at 20°C in the presence of 15 μg biotin operon repressor protein A (BirA) (Avidity, Boulder, CO., U.S.A.), 80 μM biotin, 10 mM ATP, 10 mM MgOAc, 20 mM bicine, and 10 mM Tris-HCL (pH 8.3). To remove free biotin, MHC complexes were again

purified by gel filtration, and then tetramerized by addition of PE-conjugated streptavidin (Molecular Probes, Eugene, OR., U.S.A.) at a molar ratio of 4:1. Tetramers were purified by gel filtration over a Superdex 200HR column and stored at 3-5 mg/ml at 4°C in 1X PBS containing 0.02% sodium azide, 1 µg/ml leupeptin, and 0.5 mM EDTA.

2.1.8 Buffers

Carbonate buffer, pH9.7

15mM Na2CO3

35mMNaHCO3

TAE buffer

36mM Tris-HCl

30mMNa2HPO4/NaH2PO4

Phosphate buffered saline (PBS), pH 7. 4

8 g/l NaCl

0. 2 g/l KCl

0. 2 g/l KH_2PO_4

1. 3 g/l Na_2HPO_4

Refolding buffer pH8.0

100mM Tris-HCl

400mM L-arginine-HCl

1mM NaEDTA

5mM red. gluthione

2.2. Methods

2.2.1 Vaccination

Sex- and age-matched BALB/c mice were immunized with 10 or 100 µg of naked DNA or with 10 µg of DNA with each carrier in 100 µl of volume intramuscularly (i.m.), subcutaneously (s.c.), or intranasally 3 times at 3 weeks intervals (Fig. 3). As positive control, sublethal dose (1 X 10^3 or 5 X 10^2) of *L. monocytogenes* EGD strain or 1 X 10^6 *M. bovis* BCG Danish 1331 was injected intravenously (i.v.) into mice at the same time as the prime vaccination.

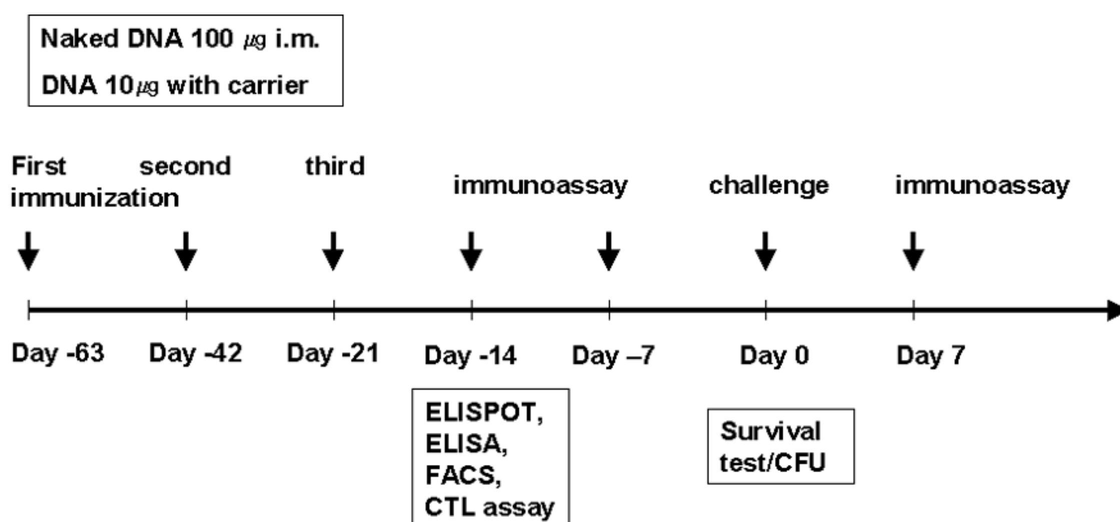

Fig. 5 Experimental scheme Mice were vaccinated with 10 or 100 µg of naked DNA or with 10 µg of DNA with each carrier in 100 µl i. m., s. c., or i. n. 3 times at 3 weeks intervals. Immunoassays from mice vaccinated with *L. monocytogenes* genes were performed from day –14, one week after the last boost and those from mice vaccinated with *M. tuberculosis* genes were performed from day 7, 4 weeks after the last boost.

2.2.2 Protection assay

Mice vaccinated with DNA encoding *L. monocytogenes* genes were

challenged i.v. with a lethal dose ($1x10^4$ or $5x10^4$) of *L. monocytogenes* strain EGD in 100µl of sterile PBS, at day 0, 3 weeks after the last boost. Survival was checked daily until day 10 post infection.

For protection assays against *M. tuberculosis*, mice were challenged with an aerosol generated from a 10 ml, single-cell suspension containing a total of 1×10^8 CFU of *M. tuberculosis* strain H37Rv, using a Middlebrook Airborne Infection Apparatus (Middlebrook, Terre Haute, IN, U.S.A.), at day 0 (Grode et al. submitted). These aerosol doses delivered 100-200 live bacilli to the lungs of each animal. At days 30, 60, and 90 post infection, lungs, livers, and spleens were aseptically removed from infected mice to assess CFU. Serial dilutions from organ homogenates were plated onto Middlebrook 7H11 agar plates supplemented with oleic acid albumin-dextrose complex (Difco, Heidelberg, Germany) and were incubated at 37°C for 3-4 weeks.

Naïve mice as negative controls and mice of positive control groups as described above were also challenged in the same way as experimental groups. Statistics were assessed by t-test or logrank test using Graph Pad Prism software.

2.2.3 Cytokine ELISpot assay

Enzyme-linked immunospot assays (ELISpot) were performed to determine the number of antigen specific T cells secreting IFN-γ (Miyahira et al., 1995). Spleens were removed from mice aseptically and prepared as single cell suspension by passing through a stainless-steel mesh. Red blood cells were removed by low osmotic pressure shock. One day before the assay, 96-well nitrocellulose plates (Millititer HA; Millipore, Bedford, MA, U. S. A.) were

coated with 5 µg/ml of the anti-mouse IFNγ mAb (clone R4) in 100 µl of carbonate buffer, pH 9.6, per well. After overnight incubation at 4℃, the plates were washed twice with PBS and blocked for 2 hrs at 37℃ with 100 µl of 1% bovine serum albumin (BSA) (Sigma-Aldrich, Munich, Germany) in PBS per well. Splenocytes ($1x10^5$ or $5x10^5$ cells/well) were added in 100 µl complete RPMI 1640 media per well. P815 cells were coated with 10^{-6}M of designated peptides at 37℃ for 2 hrs and then washed twice with complete RPMI 1640. On the other hand, J774A.1 cells were incubated with 10ug/ml of *L. monocytogenes* crude extracts or *M. tuberculosis* crude extracts at 37℃ for 2 hrs. Otherwise, autolougous splenocytes were incubated with peptides or bacterial crude extract. P815 cells, J774A.1 cells, or autologous splenocytes (10^5 cells/well) with or without antigen were added into wells containing responder-splenocytes in 100 µl of complete RPMI 1640. After 20-24 hrs incubation at 37℃, 5% CO_2, the plates were washed 10 times with 0.05% Tween-20 in PBS (washing buffer). To detect IFNγ secreting cells, 0.25 µg/ml biotinylated anti-mouse IFNγ mAb (clone XMG1.2) in 100 µl washing buffer per well was added and incubated at 37℃ for 2 hrs. The plates were washed 10 times in washing buffer, and incubated for 1 h at 37℃ in 0.05 µg/ml alkaline phosphatase-coupled streptavidin (Dinova, Hamburg, Germany) in 100 µl of 1 X PBS per well. After 5 washes, spots of IFNγ-secreting cells were visualized by adding 50 µl of the ready-to-use substrate 5-Bromo-4-Chloro-3-Indolyl Phosphate/Nitro Blue Tetrazolium (BCIP/NBT) (Sigma-Aldrich, Munich, Germany) dissolved in water. The reaction was stopped after 15 min at 37℃ by washing several times with distilled water. After drying, spots were counted under a dissecting microscope at 3-fold magnification. The frequency of

antigen-specific T cells is expressed as the number of spots of IFNγ-secreting cells per $1x10^5$ or $5x10^5$ splenocytes.

2.2.4 Flow cytometry

Single cell suspensions from spleens were prepared as described above and stained for fluorescence-activated cell sorting (FACS). Before intracellular IFN-γ staining, $2x10^6$ splenocytes per reaction were incubated with 10^{-6} M of designated peptides at 37°C for 5 hrs and washed once with 1X PBS. During the last 4 hrs of incubation, 10 µg/ml Brefeldin A (Sigma-Aldrich, Munich, Germany) was added. Thereafter, the cells were resuspended, incubated on ice with 10 µg/ml anti-mouse IgG Fc receptor (Rc) (clone 2.4G2), 10 µg/ml rat IgG, and 50 µg/ml unconjugated strepavidin (Molecular Probes, Eugene, OR, U.S.A.) in 100 µl of 1X PBS for 5 min, and extracellularly stained with Cy5-conjugated anti-mouse CD8α mAb (clone YTS169) and PE-conjugated anti-mouse CD4 mAb (clone YTS191.1) for 20 min. The cells were washed with 1X PBS and fixed with 1% paraformaldehyde (PFA) (Sigma-Aldrich, Munich, Germany) for 20 min at room temperature. After fixation, the cells were washed with 0.1% BSA in 1X PBS. The cells were perforated with 0.5% saponin, 0.1% BSA, blocked with 10 µg/ml anti-mouse IgG Fc Rc (clone 2.4G2), 10 µg/ml Rat IgG (Dianova, Hamburg, Germany), and 50 µg/ml unconjugated strepavidin (Molecular Probes, Eugene, OR, U.S.A.) in 100 µl of 1X PBS for 10min and intracellularly stained with FITC-conjugated anti-mouse IFN-γ mAb (clone XMG1.2).

For tetramer analyses, $1x10^6$ splenocytes were blocked as described above, stained on ice with Cy5-conjugated anti-mouse CD8α mAb (clone 169), FITC-

conjugated anti-mouse CD62L mAb (clone Mel. 14), and PE-conjugated H2-K^d/peptide tetramers for 60min, and washed with 1X PBS.

Cells were analyzed using a FACSCalibur™ and the CELLQuest™ software (B & D, Mountain View, CA, U.S.A.).

2.2.5 Measurement of CTL activity

The activity of antigen specific CTL was detected by peptide-loaded target cells stained with 5- (and 6-) carboxyfluorescein diacetate succinimidylester (CFSE), which is a highly stable amine-reactive reagent readily incorporated into cells. When membrane damage occurs, the dye is almost instantaneously lost and the cells are no longer able to take up or retain the charged dye. Several reports have shown that CFSE-prelabeled target cells can be successfully evaluated to detect cytolysis (Sheehy et al., 2001). Splenocytes from naïve mice were prepared as described above and washed twice with ice-cold PBS. The cells were resuspended at a concentration of $2x10^7$ cells/ml, and stained with a low concentration (0.25 µM) of CFSE (Molecular Probes, Eugene, OR, U.S.A.) or with a high concentration (5 µM) of CFSE for 4 min. After labeling, complete RPMI 1640 medium was added to stop the reaction, and splenocytes labeled with a high concentration of CFSE were loaded with designated peptides at 37°C for 1-2 hrs. The two populations of different CFSE intensities were mixed at a 1:1 ratio, and $6x10^7$ cells adoptively transferred into each recipient mouse (Aichele et al., 1990). The recipient mice were primed with 200 plaque-forming units (pfu) of LCMV intraperitoneally or $5x10^2$ *L. monocytogenes* i.v. at designated days before analysis. After 24 hrs, recipient mice were sacrificed, and splenocytes were analyzed by FACS

(FACScalibur, B & D, Mountain View, CA, U.S.A.).

2.2.6 ELISA

Enzyme-linked immunoabsorbent assay (ELISA) was performed to determine whether Th 1 or Th 2 immune responses are dominantly induced by DNA vaccination. IgG1 antibody responses indicate Th 2 immune response and IgG2a antibody responses indicate Th 1 immune response. Briefly, 96 well microtiter plates (Nunc) were coated with 10 µg/ml of *L. monocytogenes* crude extract in 0.1M bicarbonate buffer overnight, washed five times in washing buffer, blocked with PBS, 0.05% Tween 20, 1% BSA at 37°C for 2 hrs, and washed again. Blood sera were collected from mice, pooled, and serially diluted in PBS, and added into ELISA plates as triplicates. As positive control, mouse IgG1 Ab (clone 25D1) and mouse IgG2a Ab (clone F23.1) was used and as negative control, BSA was used at the concentration of 100 µg/ml in PBS. The plates were incubated at 37°C for 1 hr, washed, and 2nd Abs added, anti-mouse IgG1 Ab (clone X56) and anti-mouse IgG2a (clone R19-15) (PharMingen) at the concentration of 0.5µg/ml in 100µl/well of PBS. After incubation at 37°C for 1 hr, the plates were washed, and 50 µl/well of 1mg/ml p-nitrophenyl phosphate solution (Sigma) added. After incubation at room temperature for 20 min, 50 µl/well of 0.5M EDTA, pH 8 was added to stop the coloring reaction. The intensity of the reaction was measured at OD405 by SpectraMax250 (Molecular Devices) and SoftmaxPro software.

3. Results

3.1. DNA vaccination in the listeriosis model

3.1.1 Verification of plasmid DNAs for vaccination.

The plasmid DNAs encoding p60 named pCiap, wild-type LLO named pClisA, and non-hemolytic, mutant LLO named pChly492 for DNA vaccination were purified and confirmed by the sizes of the DNA fragments after restriction enzyme digestion in 1 % agarose gel electrophoresis (Fig. 6). The gene maps of the DNAs are displayed in Fig. 3. The pCI vector was digested with *Nde*I, pCiap was digested with *Hpa*I, and pClisa and pChly492 were digested with *Nhe*I. The enzymes were chosen to distinguish each vector by different sizes of DNA fragments. The pCI vector (Lane 1) revealed a 1542 b. p. band and a 2466 b. p. fragment after digestion with *Nde*I, pCiap (Lane 2) revealed a 1211 b.p. and a 4165 b.p. fragment with *Hpa*I digestion, and pClisA and pChly492 (Lane 3 and 4) revealed a 1320 b.p. and a 4191 b.p. fragment with *Nhe*I digestion. All fragments' sizes were correct and the sequence of pChly492 was confirmed by DNA sequencing. The result of sequencing displayed 3 additional unexpected mutations regardless of the mutation of a. a. 492 (Trp-Ala). These mutations are located in a.a. 64 (Tyr-Cys), a. a. 160 (Gly-Ser), and a. a. 197 (no a. a. change) and not located in any known functional domains, such as PEST sequence (a.a. 32-50), and immunodominant epitopes.

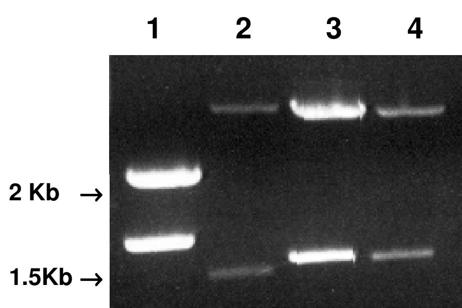

Fig. 6 The DNAs encoding *L. monocytogenes* genes. The pCI vector was digested with *Nde*I, pCiap was digested with *Hpa*I, and pClisa and pChly492 were digested with *Nhe*I. The pCI vector (Lane 1) revealed a 1542 b.p. and a 2466 b.p. fragments, pCiap (Lane 2) revealed a 1211 b.p. and a 4165 b.p. fragments, and pClisA and pChly492 (Lane 3 and 4) revealed a 1320 b.p. and a 4191 b.p. fragments.

3.1.2 Protection against *L. monocytogenes* by DNA vaccination and comparison of DNA vaccine delivery systems

In order to determine which plasmid DNA shows the best protection and which infection dose would be appropriate to reveal the improvement of DNA vaccine efficacy by DNA vaccine carriers, preliminary protection assays were performed with 100 µg of naked DNA upon infection with 1xLD50 of *L. monocytogenes*. As described in Materials and Methods, sex- and age-matched BALB/c mice were injected with 100 µg naked DNA i. m. 3 times at 3 weeks intervals. Three weeks after the last boost, mice were infected with 1xLD50 of *L. monocytogenes*. Groups of 6 mice were vaccinated with vector control as mock, with plasmid DNA encoding p60, with plasmid DNA encoding wild-type LLO, or with plasmid DNA encoding non hemolytic, less virulent mutant LLO. As positive control, a group of 6 mice was immunized with a sublethal dose of *L. monocytogenes* at the time point of priming. The plasmid

DNA encoding mutant LLO induced the best protection against *L. monocytogenes* infection. The protection was as efficient as after a primary *L. monocytogenes* infection of sublethal dose. All mice survived and protection was statistically significant compared to mock treated mice by logrank test using Prism software. The summary of logrank test was * (P=0.0190). The difference of the survival curves between naïve and mutant LLO was also statistically significant (**, P=0.0043). The second best plasmid DNA as a vaccine candidate was the one encoding p60. The summary of the logrank test was * (P=0.0345), compared with naïve, but not significant compared with mock treated mice (P=0.1151) (Fig. 7). Mice injected with vector control also showed slightly increased survival, but not significant. The infection dose for challenge was increased for further experiments for the test of DNA carriers because the improvement of efficacy by DNA carrier systems could not be detected with the infection dose of 1xLD50.

First, the efficacy of 10 µg of naked DNA and DNA with PLG stabilized with

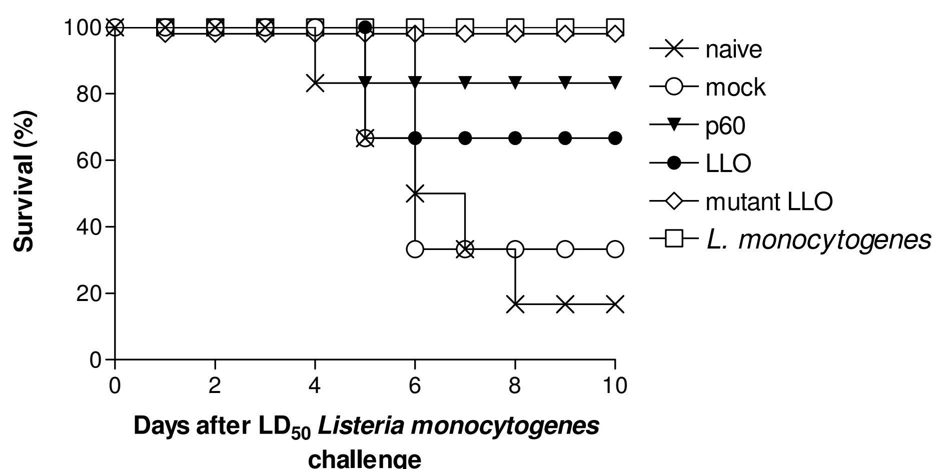

Fig. 7 Survival curves of BALB/c mice immunized with 100µg of naked DNA from 1xLD_{50} *L. monocytogenes* challenge. Difference of survival of mice vaccinated upon 100 µg of naked DNA encoding mutant LLO or with sublethal dose of *L. monocytogenes* from that of mock treated mice is statictically significant (*, P=0.0190). Statistics were assessed by logrank test using Graph Pad Prism software.

CTAB were compared. The vaccination procotols were the same as before but the infection dose for challenge was 5 times LD_{50}. Difference of survival of mice immunized with 10 µg of naked DNA encoding mutant LLO from that of mock treated mice was not statistically significant (P=0.5143). Difference of survival of mice immunized with sublethal dose of *L. monocytogenes* from that of mock treated mice was significant (*, P=0.0185) (Fig. 8, A). Difference of survival of mice immunized with mutant LLO/PLG from that of mock treated mice was not significant (P=0.2029) but the difference of survival curves between mutant LLO and naïve was significant (**, P=0.0015). Difference of survival of mice immunized with sublethal dose of *L. monocytogenes* from that of mock treated mice was significant (*, P=0.0178), and the difference between *L. monocytogenes* immunization and naive was also significant (***, P=0.0006) (Fig. 8, B). Difference of survival of mice immunized with mutant LLO/PLG from that of mutant LLO was not significant (P=0.4441) (Fig. 8, A and B). The PLG stabilized with CTAB could not significantly improve the efficacy of DNA vaccine compared with same amount of naked DNA. Four mice out of 60 mice died after vaccination with DNA and particles. In contrast, no mouse died after vaccination with naked DNA. From this experiment, the DNA encoding mutant LLO was also confirmed as the best DNA vaccine candidate against *L. monocytogenes.*

The purpose of this study was not only to improve protection and immune response against intracellular bacteria but also to reduce the amount of DNA required for vaccination. Thus, 100 µg of naked DNA was compared with 10 µg of DNA and carriers. To clarify the improvement, the infection dose for challenge was increased up to $10xLD_{50}$. The vaccination protocols were the

same as before. Survival tests were performed with mice vaccinated with 10 µg of naked DNA i. m., with 100 µg of naked DNA i. m., with 10 µg of DNA and PLG stabilized with PVA i. m., with 10 µg of DNA and VLP s. c., with 10 µg of DNA and TmHU s. c., or with 10 µg of DNA and newly developed encapsulating particles intranasally. As a result, 100 µg of naked DNA encoding mutant LLO showed 50 % protection from *L. monocytogenes* infection (*, P=0.0498) but no particle improved the efficacy of DNA vaccine significantly (Fig. 9).

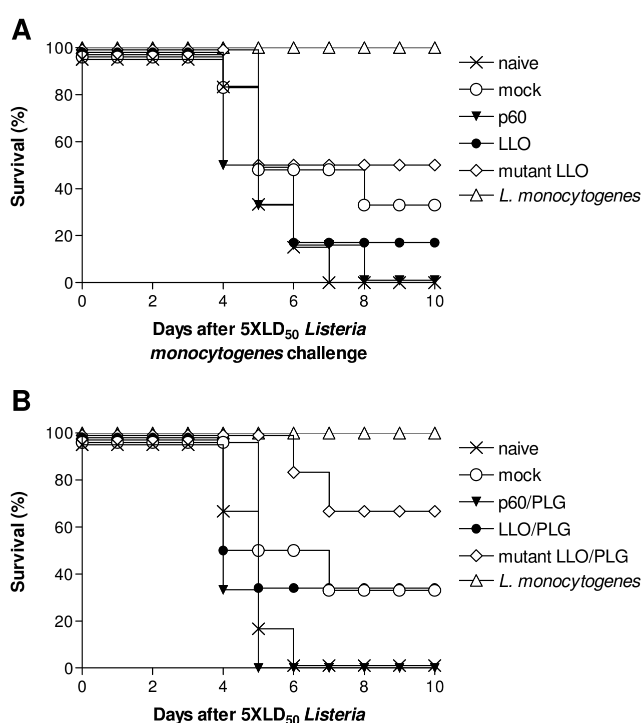

Fig.8 Survival curves of BALB/c mice immunized with 10 µg of naked DNA or with 10 µg of DNA and PLG. A. Intramuscular immunization with 10 µg of naked DNA. Difference of survival of mice immunized with mutant LLO from that of mock treated mice was not statistically significant (P=0.2029). Difference of survival of mice immunized with sublethal dose of *L. monocytogenes* from that of mock was significant (*, P=0.0178). B. Intramuscular immunization with 10 µg DNA absorbed onto 1 mg of PLG stabilized with CTAB. Difference of survival of mice immunized with mutant LLO/PLG from that of mock treated mice was not significant (P=0.5143). Difference of survival of mice immunized with sublethal dose of *L. monocytogenes* from that of mock treated mice was significant (*, P=0.0185). Difference of survival of mice immunized with mutant LLO/PLG from that of mutant LLO was not significant (P=0.4441). Statistics were assessed by logrank test using Graph Pad Prism software.

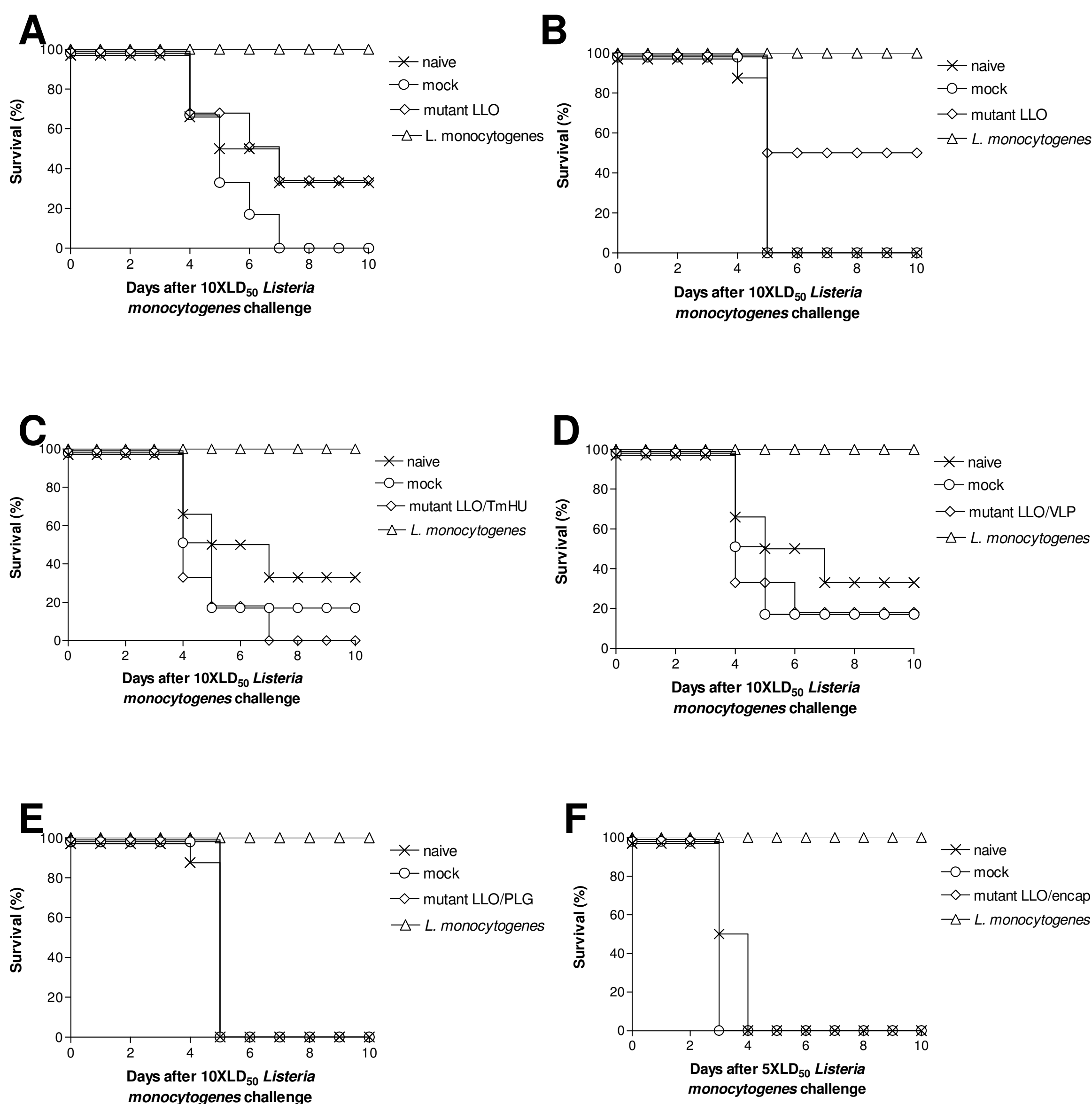

Fig. 9 Survival curves of mice immunized with 10 µg of naked DNA (A), 100 µg of naked DNA (B), 10 µg of DNA and TmHU (C), 10 µg of DNA and VLP (D), 10 µg of DNA and PLG stabilized with PVA (E), and 10 µg of DNA and encapsulating particle (F). Difference of survival of mice vaccinated with 100 µg of naked DNA encoding mutant LLO from that of mock treated mice was statistically significant (*, P=0.0498) and that of *L. monocytogenes* was also significant (***, P=0.0009) (B). Statistics were assessed by logrank test using Graph Pad Prism software.

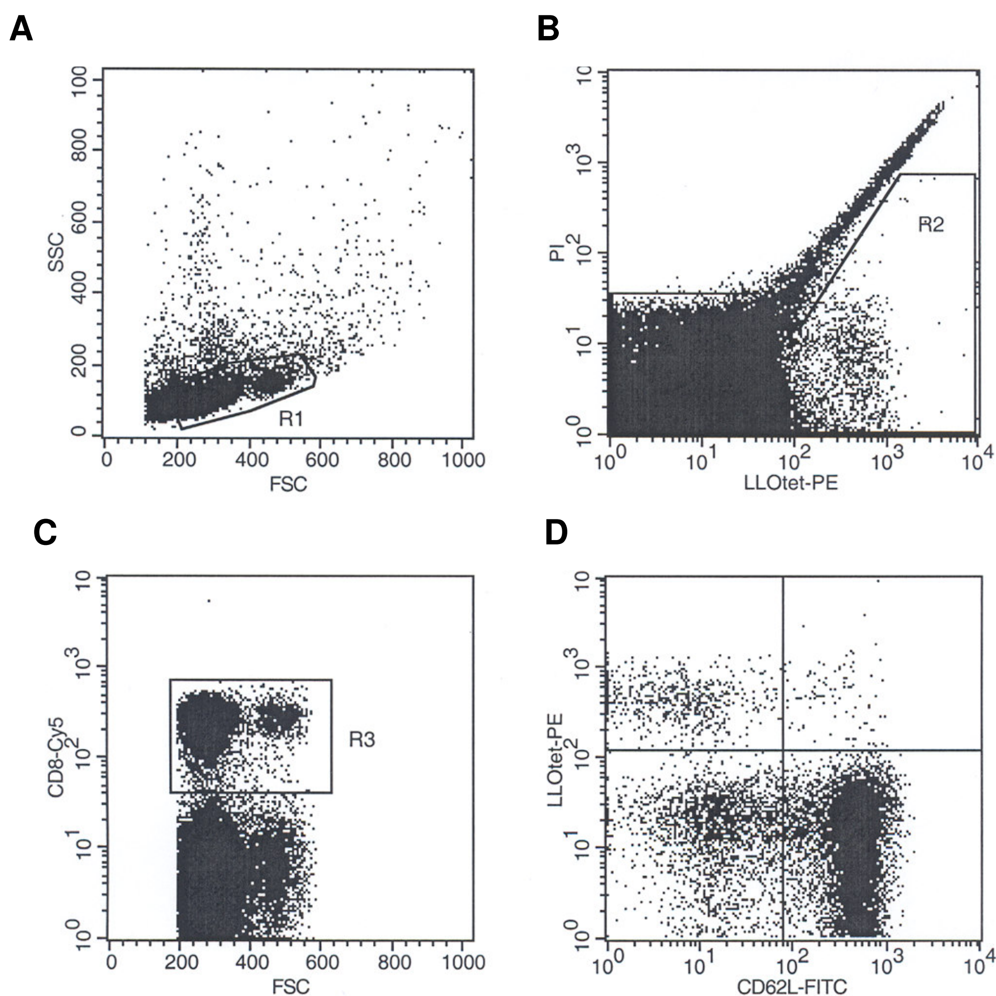

Fig. 10 Gating for flow cytometric analysis with MHC/peptide tetramers. The splenocytes were gated by FSC/SSC scatters (A), PI^- to exclude dead cells (B), and $CD8^+$ cells (C). Finally, LLO tetramer/CD62L dot plot was displayed (D).

3.1.3 Antigen-specific $CD8^+$ T cells induced by DNA vaccination against *L. monocytogenes*

In order to determine antigen-specific immune response induced by DNA vaccination, flow cytometric analysis with MHC class I / LLO_{91-99} peptide tetramer was performed. The preparation and staining protocol is described in Materials and Methods. The splenocytes were gated by FSC/SSC scatters

(Fig. 10, A), PI- cells to exclude dead cells (Fig. 10, B), and $CD8^+$ cells (Fig. 10, C). Finally, LLO tetramer/CD62L dot plots were displayed (Fig. 10, D). The cell surface marker, CD62L is known as an effector/memory cell marker. When the T cell is activated, CD62L expression level decreases and afterwards, the expression level is restored again during memory phase (Busch and Pamer, 1999).

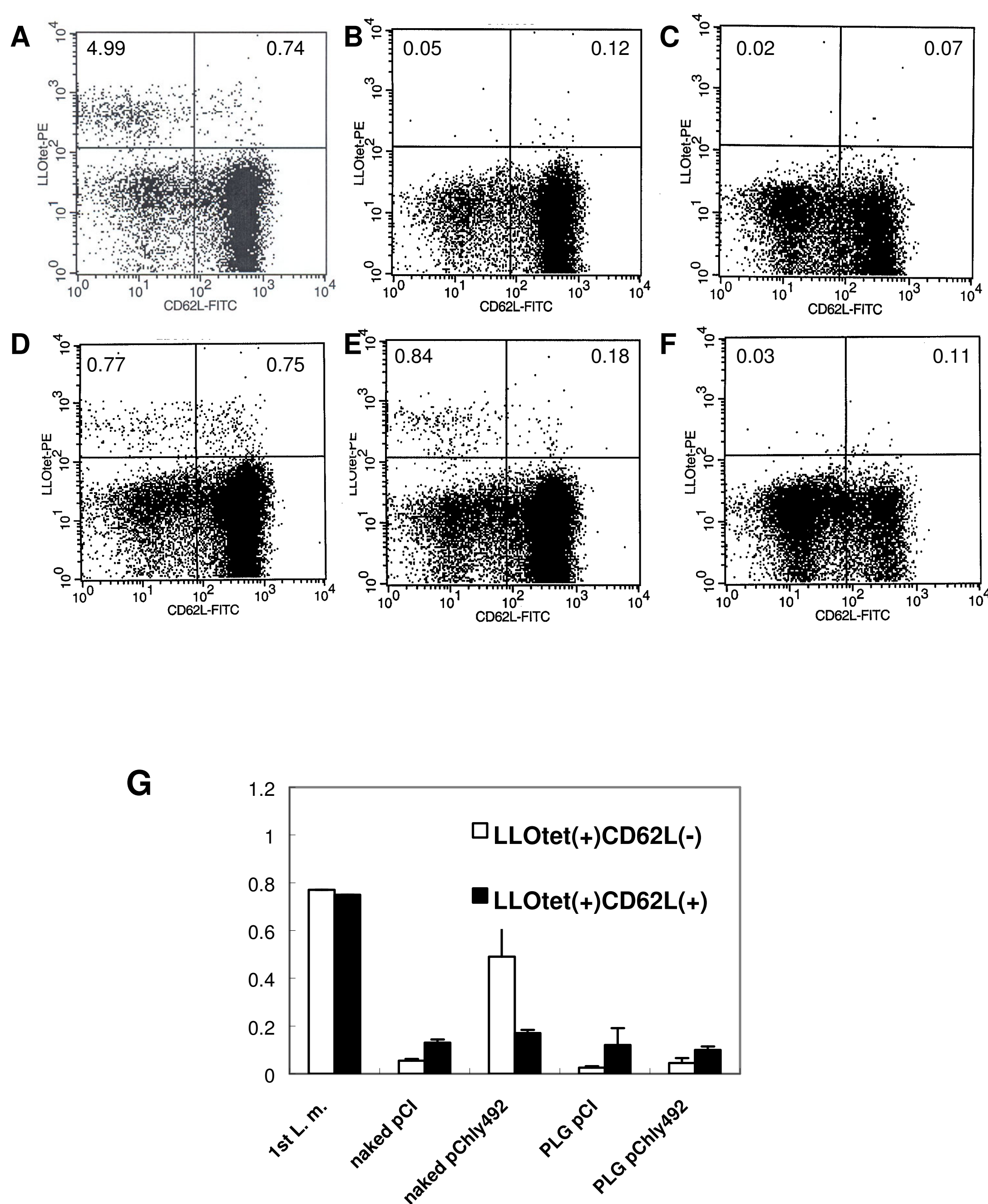

Fig. 11 Antigen-specific CD8$^+$ T cells induced by DNA vaccination. Splenocytes were prepared from a mouse infected secondary with *L. monocytogenes* 5 days before analysis (A), from mice injected with 100 µg of naked DNA of empty vector (B), from mice injected with 10 µg of DNA of empty vector and PLG stabilized with PVA (C), from a mouse immunized with sublethal dose of *L. monocytogenes* 7 weeks before analysis (D), from mice vaccinated with 100 µg of naked DNA encoding mutant LLO (E), and from mice vaccinated with 10 µg of DNA and PLG stabilized with PVA (F). Splenocytes were prepared 5 days after the last boost. The cells were gated as in Fig. 10. The results were summarized in a gragh (G). Values represent averages from 2 mice except that of one *L. monocytogenes* infected mouse. Error bars represent standard deviation.

Groups of 2 mice were vaccinated with 100 µg of naked DNA or with 10 µg of DNA and PLG stabilized with PVA as described above. Mice were sacrificed and were analyzed 5 days after the last boost. As positive control, two mice were immunized with a sublethal dose $5x10^2$ CFU of *L. monocytogenes* 7 weeks before analysis, and one of them was infected secondarily with $1x10^4$ *L. monocytogenes* 5 days before analysis, while the other one was regarded as long-term immunity positive control. As expected, splenocytes from secondary infection showed markedly expanded tetramer positive cell population (Fig. 11, A) and those of primary infection as long-term immunity positive control also showed significant tetramer positive cell population (Fig. 11, D). Both of vector control showed background level of tetramer positive cell populations (Fig. 11, B, C) and mice vaccinated with 10 µg of DNA encoding mutant LLO and PLG also showed no significant tetramer positive cell population (Fig. 11, F). On the other hand, vaccination of 100 µg of DNA encoding mutant LLO induced increased tetramer positive cell population (Fig. 11, E). The tetramer positive and CD62L negative cell population was 0.49% on average and it was 8.9 fold increased (Fig. 11, G). Interestingly, one out of 2 mice showed dramatically increased tetramer positive cell population (0.84%) but the other one showed less (0.14%). $Tetramer^+$ $CD62L^-$ cell populations were 87% out of total $tetramer^+$ cell population of secondary infection at day 5, 82% out of that of DNA vaccination 5 days after the last boost, and 51% out of that of long-term immunity. Thus, 5 days after the last boost, many antigen-specific T cells induced by DNA vaccination were activated. There exist tetramer positive cell populations after 7 days and 10 days after the last boost (Table 2). As conclusion of this experiment, DNA vaccination could induce antigen-specific

immune response in the listeriosis model.

		LLO tetramer positive cell population (%)					
		1st infection (6 wks before the last boost)	2nd infection	100 µg naked vector	100 µg naked Mutant LLO	10 µg DNA/PLG vector	10 µg DNA/PLG mutant LLO
Days after the last boost/ 2nd infection	5	1.52*	5.73*	0.185	0.66	0.145	0.145
	7	2.8*	16.5*	2.08	4.23	1.745	0.91
	10	1.225		0.145	0.965	0.185	0145

Table 2. Kinetics of LLO_{91-99} tetramer positive populations from mice vaccinated with DNA. * data from one mouse. All other values represent averages from 2 mice.

3.1.4 IFN-γ secretion induced by DNA vaccination against *L. monocytogenes*

Since IFN-γ is known as a key cytokine for protection against intracellular bacteria, IFN-γ secreting splenocytes were detected by ELISpot assay as described above. Groups of 3 mice were sacrificed 7 days after the last boost and splenocytes were prepared and incubated with P815 cells (Fig. 12) or autologous splenocytes (Fig. 13) loaded with/without LLO_{91-99} peptides or $p60_{217-225}$ peptides onto ELISpot plates for 24 hrs. These peptides are immunodominant MHC class I H2-K^d-restricted epitopes. Splenocytes from mice vaccinated with 100 µg of naked DNA encoding mutant LLO showed IFN-γ secreting cells after restimulation with LLO_{91-99} peptides, but those of p60 and wild-type LLO did not show increased levels of IFN-γ secreting

splenocytes after restimulation with $p60_{217-225}$ peptides or LLO_{91-99} peptides, respectively. The LLO_{91-99} peptide-specific IFN-γ secreting cell population induced by vaccination with DNA encoding mutant LLO was 17 IFN-γ secreting splenocytes/10^5 cells, while that of vector control was 10.6 IFN-γ secreting splenocytes/10^5 cells, The increase of IFN-γ secreting cells by DNA encoding mutant LLO was up to 70% (Fig. 12). However, the LLO_{91-99} peptide-specific IFN-γ secreting splenocyte population was not detected by vaccination with 10 μg of DNA and PLG stabilized with PVA (Fig. 13). The IFN-γ secreting cell population from mice vaccinated with 100 μg of naked DNA encoding mutant LLO was 26.5/10^5 cells, while that of vector control was 13/10^5 cells. The increase was 50%. As positive control, a mouse was immunized with sublethal dose of *L. monocytogenes* 7 weeks before analysis, and the mouse exhibited high levels of IFN-γ-secreting splenocytes (67.5/10^5). This result is similar to Fig. 12 and consistent with FACS data in Fig. 11. Splenocytes from mice vaccinated with 10 μg of DNA and TmHU or VLP were also tested by ELISpot assay, but failed to show significant level of antigen-specific IFN-γ secreting cell population (data not shown). These results suggest plasmid DNA mutant LLO could induce antigen-specific MHC class I-restricted $CD8^+$ T cell immune response, but plasmid DNA encoding p60 or wild-type LLO failed to induce detectable amounts of antigen-specific MHC class I-restricted $CD8^+$ T cell immune responses. Additionally, no DNA carrier improved DNA vaccination to induce detectable level of antigen-specific immune responses. These results are in line with the protection results.

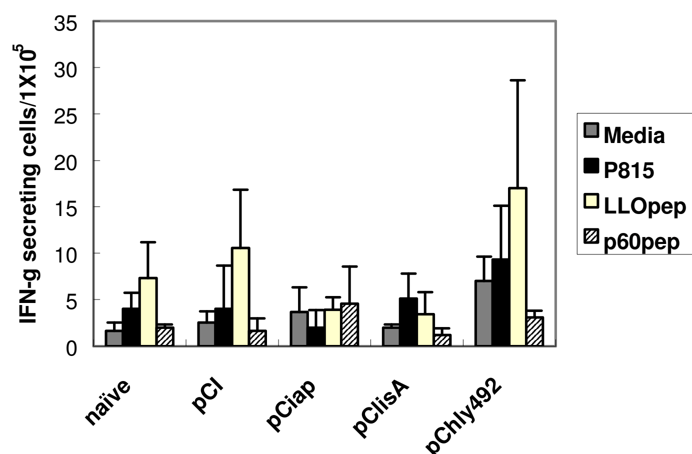

Fig. 12. Frequencies of antigen-specific IFN-γ secreting splenocytes by DNA vaccination. Groups of 3 mice were vaccinated with pCI as vehicle control, with pCiap encoding p60, with pClisA encoding wild-type LLO, or with pChly492 encoding mutant LLO. All vaccination doses were 100 µg of naked DNA. Splenocytes were prepared 7 days after the last boost and MHC class I-restricted LLO_{91-99} or $p60_{217-225}$ peptide-specific IFN-γ secretion determined by ELISpot assay. Values represent means from 3, and error bars represent standard deviation.

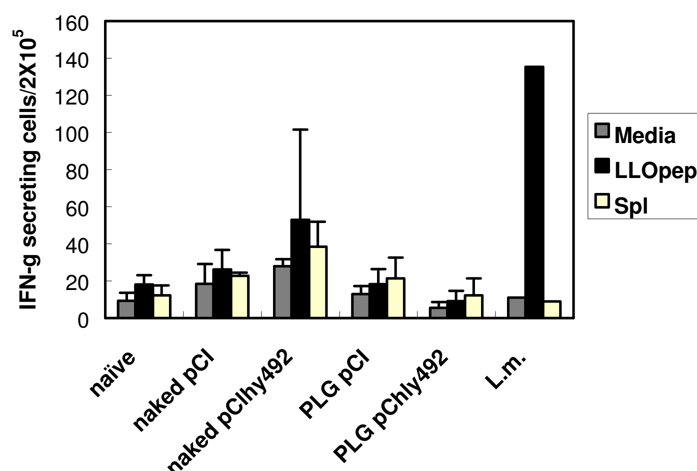

Fig. 13 Frequencies of antigen-specific IFN-γ secreting splenocytes by DNA vaccination. Groups of 2 mice were vaccinated with 100 µg of pCI as vehicle control, with 100 µg of pChly492 encoding mutant LLO, with 10 µg of pCI and PLG stabilized with PVA, or 10 µg of pChly492 and PLG stabilized with PVA. Splenocytes were prepared 7 days after the last boost and MHC class I-restricted LLO_{91-99} peptide-specific IFN-γ secretion determined by ELISpot assay. As positive control, one mouse was immunized with a sublethal dose of *L. monocytogenes*. Values represent means from 2 mice except for positive control, and error bars represent standard deviations.

3.1.5 Th 1/Th 2 immune response

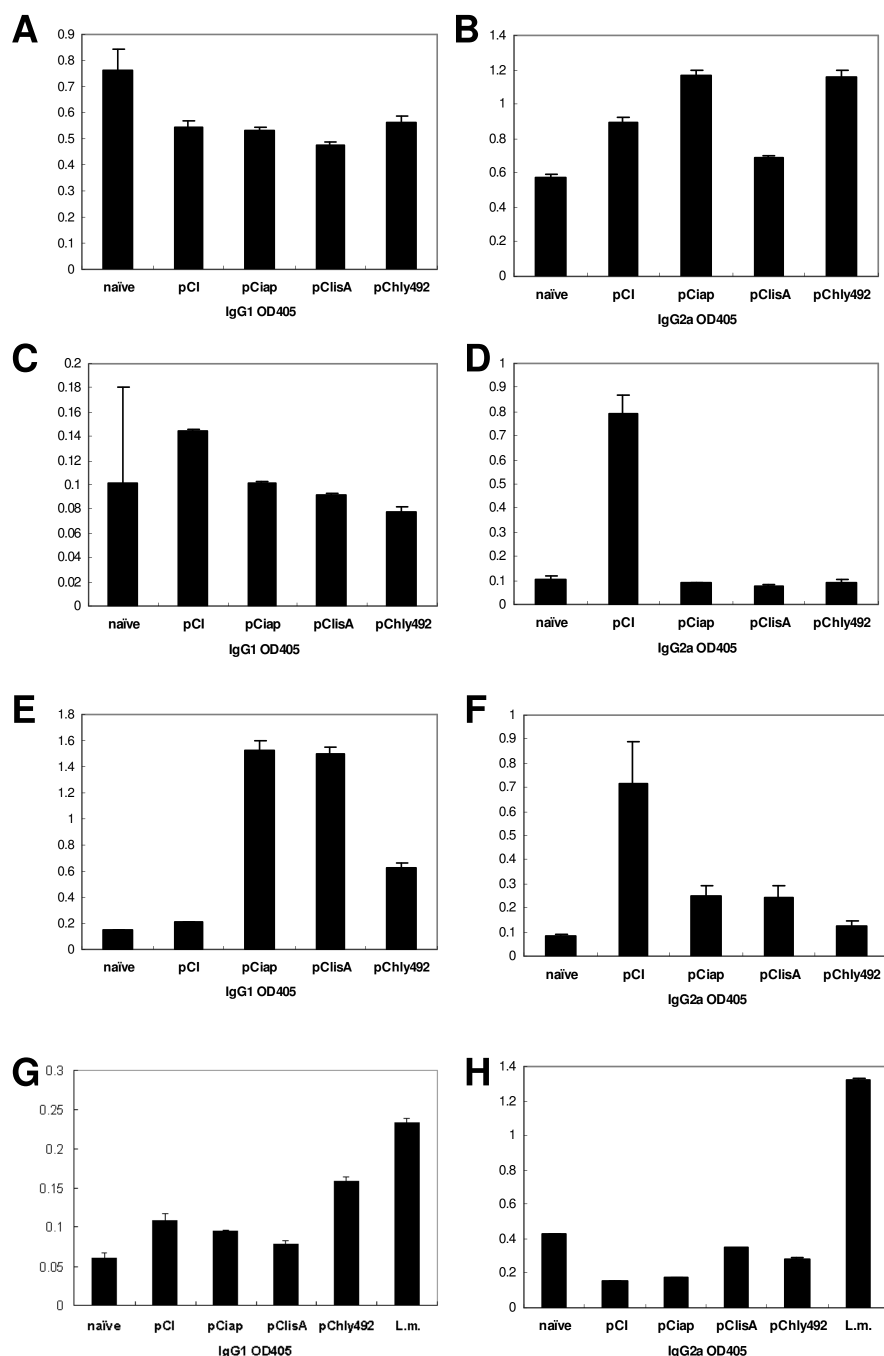

Fig. 14 Comparison of antibody subclasses induced by DNA vaccination. Blood sera were colleted at day 0 from mice vaccinated with 100 µg of naked DNA (A, B), from mice vaccinated with 10 µg of DNA and TmHU (C, D), from mice vaccinated with 10 µg of DNA and VLP (E, F), from mice vaccinated with 10 µg of DNA and PLG stabilized with PVA (G, H). Sera were pooled from groups of three mice and put into plates as triplicates. To detect IgG1 and IgG2a antibody titer, ELISA assay was performed. All dilution factors were 64 except in G and H (128). Left panels (A, C, E, G) show IgG1 titers, and right panels (B, D, F, H) show IgG2a titers. Abscissas indicate OD405. Values represent means from triplicates, and error bars represent standard deviations.

For protection and immunity against intracellular bacteria, the Th 1 T cell immune response plays a pivotal role. To determine the balance between Th 1/Th 2 immune responses, ELISA assays were performed to detect serum IgG1 and IgG2a antibody titers as described above. IgG1 antibody response indicates Th 2 immune response, and IgG2a antibody response indicates Th 1 immune response. Blood sera were colleted from mice vaccinated with 100 μg of naked DNA, from mice vaccinated with 10 μg of DNA and TmHU, from mice vaccinated with 10 μg of DNA and VLP, and from mice vaccinated with 10 μg of DNA and PLG stabilized with PVA at day 0, 3 weeks after the last boost. As positive control, sera were collected from mice immunized with a sublethal dose of *L. monocytogenes* 9 weeks before analysis. As expected, mice vaccinated with 100 μg of naked DNA encoding mutant LLO exhibited high titer of IgG2a and interestingly, DNA encoding p60 also induced high IgG2a production (Fig. 14, B). DNA encoding p60 protected mice the second best from *L. monocytogenes* infection. On the IgG1/IgG2a antibody titer from mice vaccinated with 10 μg of DNA and PLG, PLG is not protein and simply 10 μg of DNA may not be sufficient to induce significant Th 1 immune response.

3.1.6 Failure to detect CTL activity

In vivo killing assay using CFSE labeled target cells was adopted to detect CTL activity. This method is established to give quantitative and qualitative results of actual in vivo killing and little spontaneous leakage (Aichele et al., 1997; Sheehy et al., 2001).

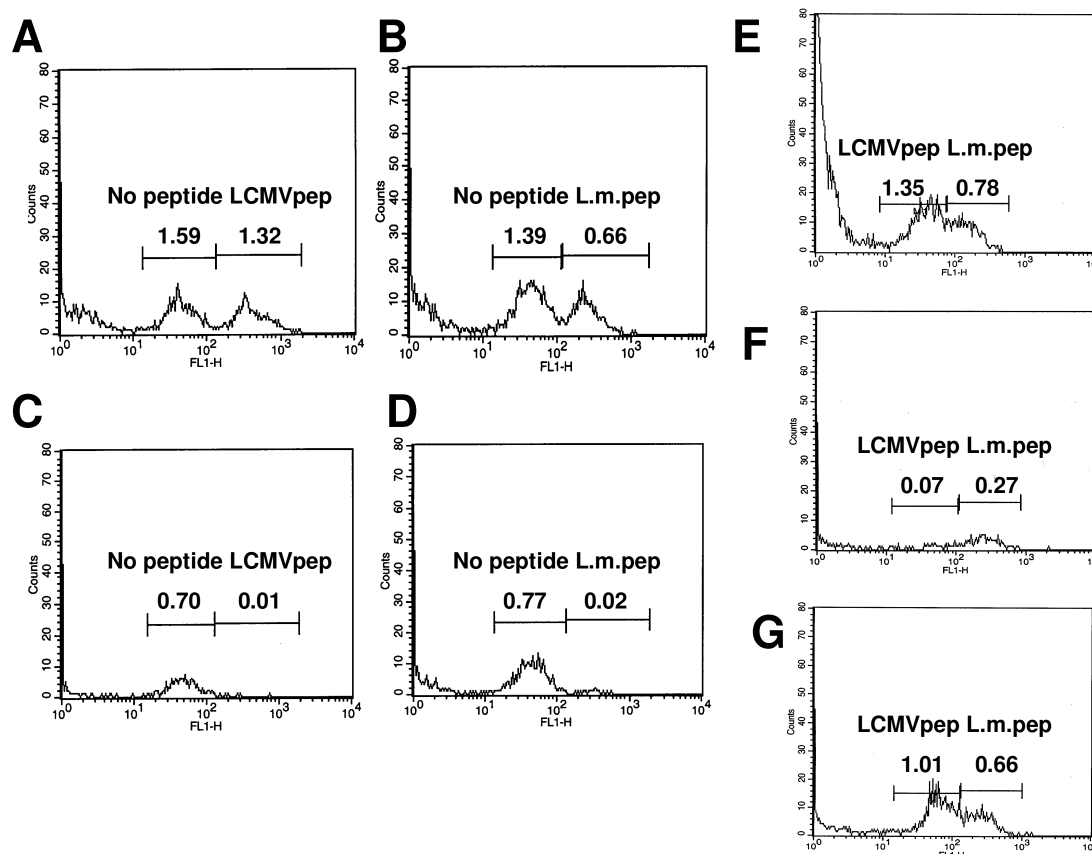

Fig. 15 CTL activities in LCMV-primed or *L. monocytogenes*-primed mice Mice were primed with LCMV (C, F) or with *L. monocytogenes* (D, G), and in vivo killing assay was performed at day 9 (A, B, C, D) and day 24 (E, F, G) post infection. Naïve mice were used as negative control (A, B, E).

Mice were primed with 200 pfu of LCMV or $5x10^2$ *L. monocytogenes* and 9 days or 24 days after the priming, splenocytes from naïve mice were loaded with LCMV-derived MHC class I-restricted peptide or with *L. monocytogenes* derived MHC class I-restricted peptide, labeled with CFSE, and transferred into primed mice i.v. respectively, as described above. At day 9 post infection, splenocytes loaded with antigen-specific peptides were killed in both LCMV-primed mice and *L. monocytogenes*-primed mice (Fig. 15, C, D), but at day 24, splenocytes loaded with antigen-specific peptides were killed only in LCMV-

primed mice (Fig. 15, F), while *L. monocytogenes*-primed mice failed to kill splenocytes loaded with antigen-specific peptides (Fig. 15, G). Naïve mice did not show cytotoxic activity (Fig. 15, A, B, E).

3.2. DNA vaccination against *M. tuberculosis*

3.2.1 Protection against *M. tuberculosis* by naked DNA or by DNA with PLG

Sex- and age- matched BALB/c mice were vaccinated with 10 µg of DNA and PLG stabilized PVA 3 times at 3 weeks intervals. As positive control, 1×10^6 CFU *M. bovis* BCG Danish strain were injected i.v.. Mice were challenge-infected with 100-200 *M. tuberculosis* H37Rv by aerosol 3 weeks after the last boost. At day 30 and 60 post infection, lungs and spleens were removed from groups of five mice, and bacterial loads were assessed. At day 30, mice vaccinated with Rv3407 DNA and PLG showed about 0.3 $\log_{10}$ decreased CFU, and at day 60, about 0.5 $\log_{10}$ CFU decreased, but the difference was statistically not significant (Fig. 16). At day 30, P value between vector control and Rv3407 was 0.1152, and at day 60, P=0.1490. This result also suggested Rv3407 DNA is the better DNA vaccine candidate than Rv2520 and Rv1511, but in a previously report with 100 µg of naked DNA, Rv1511 also showed significant protection against *M. tuberculosis* (Mollenkopf et al., submitted). The DNA vaccine efficacy of 10 µg of DNA and PLG was compared with that of 100 µg of naked DNA (Fig. 17). At day 30, CFU from mice vaccinated with 100 µg of naked Rv3407 DNA was approximately 5.5 $\log_{10,}$ that of vector control (tpa) was approximately 5.9 $\log_{10,}$ but the difference was statistically not significant (P=0.0952). Mice vaccinated with 10 µg of DNA and PLG also showed similar level of decreased CFU. However, this effect did not last until

day 90 post infection.

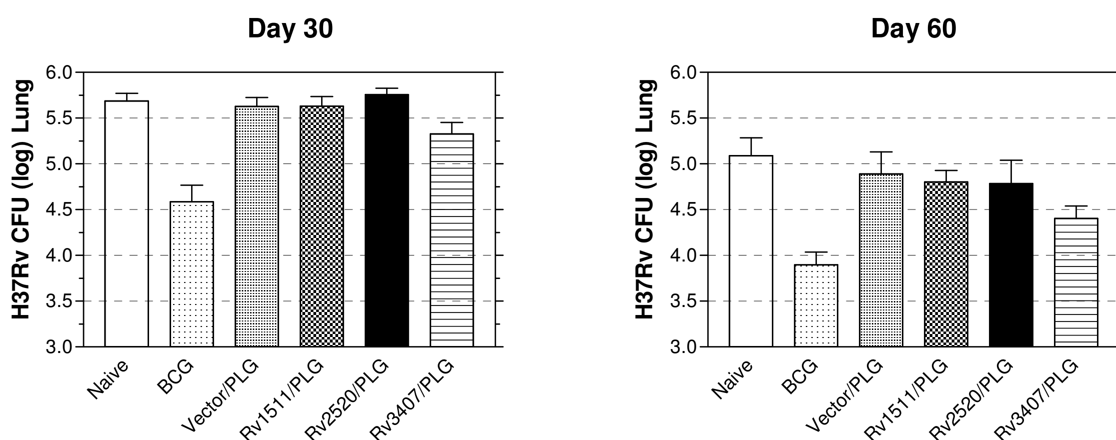

Fig. 16 Protection of BALB/c mice immunized with 10 μg of DNA and PLG stabilized with PVA from *M. tuberculosis* infection. Data represent the mean number of CFU/lung in $\log_{10}$ values for groups of five, and error bars represent standard deviation. Statistics were assessed by t test (Mann-Whitney test) using Prism program.

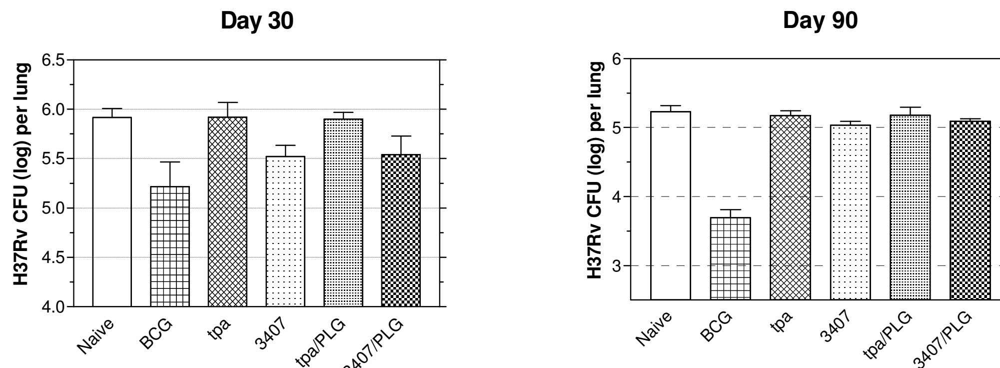

Fig. 17 Protection of BALB/c mice immunized with 100 μg of naked DNA or with 10 μg of DNA/PLG from *M. tuberculosis* infection. Data represent the mean number of CFU/lung in $\log_{10}$ values and error bars represent standard deviation. At day 30, the difference of CFU from mice vaccinated with vector control (tpa) and Rv3407 was not significant (P=0.0952) by t test (Mann-Whitney test) using Prism program.

3.2.2 Antigen-specific CD8$^+$ T cells induced by DNA vaccination against *M. tuberculosis*

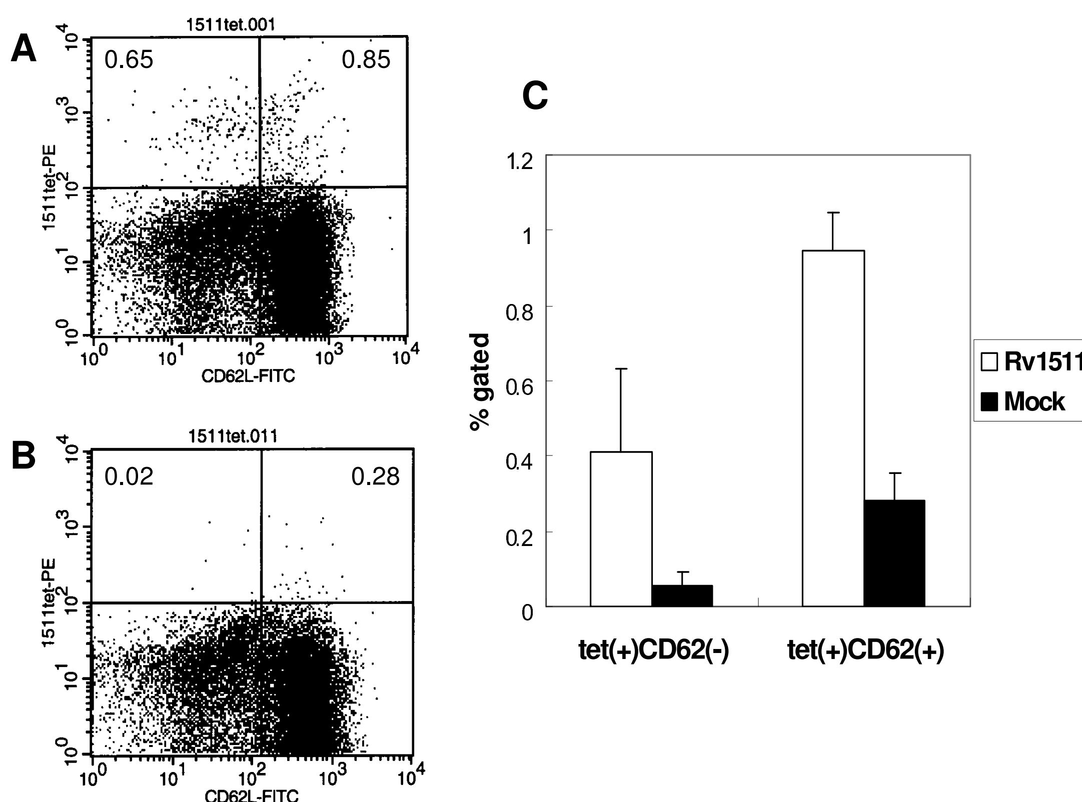

Fig. 18 Antigen-specific CD8$^+$ T cells induced by DNA vaccination A. Splenocytes from mice vaccinated with 100 µg of naked Rv1511 DNA, B. Splenocytes from mice vaccinated with 100 µg of naked empty vector DNA (Mock). The dot plots showed CD8+ living cells. C. Summary of tetramer analysis. Values represent averages from 3 mice and error bars represent standard deviations.

In order to detect antigen-specific immune response induced by DNA vaccination, flow cytometric analysis with MHC class I Hd-K^d/ Rv1511-derived putative epitope tetramer was performed. Mice were vaccinated with 100 µg of naked Rv1511 DNA as described above. The preparation and staining

protocol was described in Materials and Methods. The splenocytes were gated by FSC/SSC scatters (Fig. 10, A), PI negative cells to exclude dead cells (Fig. 10, B), and $CD8^+$ cells (Fig. 10, C). Finally, LLO tetramer/CD62L dot plots were displayed (Fig. 10, D). At day 7, 4 weeks after the last boost, the tetramer positive cell population from mice vaccinated with 100 µg of naked Rv1511 DNA was 1.36% and that of vehicle control (mock) 0.34%. The tetramer positive cell population was 4 fold increased by DNA vaccination (Fig. 18). Therefore, antigen-specific immune responses could be induced by DNA vaccination against *M. tuberculosis*.

3.2.3 IFN-γ secretion induced by DNA vaccination against *M. tuberculosis*

In order to detect antigen-specific IFN-γ secreting cells induced by DNA vaccination, ELISpot assays were performed. Groups of three mice were vaccinated with 100 µg of naked tpa DNA as vehicle control, with 100 µg of naked Rv3407 DNA, with 10 µg of tpa DNA and PLG stabilized with PVA, and with 10 µg of Rv3407 DNA and PLG. Splenocytes were prepared at day –15, 1 week after the last boost (Fig. 19 A, C, E) and at day 30, 7.5 weeks after the last boost (Fig. 19 B, D, F), stimulated with media only (Fig. 19 A, B), with *M. tuberculosis* crude extract (Fig. 19 C, D), or with the mixture of 2 different Rv3407-derived putative MHC class I epitope peptides (Fig. 19 E, F) for 3 days, and antigen-specific IFN-γ secretion was determined by ELISpot assay as described above. At day –15, mice vaccinated with 10 µg of Rv3407 DNA and PLG showed 21.1 IFN-γ secreting splenocytes/$1x10^5$ cells responding to Rv3407-derived epitopes when the splenocytes were incubated in media without antigen, while mice vaccinated with vector control showed 2.2 IFN-γ

secreting splenocytes/1x10^5. However, average background level of spots was 5/1x10^5 cells (Fig. 19, A). At day –15, mice vaccinated with 10 μg of Rv3407 DNA and PLG showed 22.2 IFN-γ secreting splenocytes/1x10^5 cells responding to *M. tuberculosis* crude extract when the splenocytes were incubated in media containing *M. tuberculosis* crude extract, while mice vaccinated with vector control showed 8.9 IFN-γ secreting splenocytes/1x10^5 cells (Fig. 19, C). However, Rv3407-derived peptide specific IFN-γ secreting cells were not increased (Fig. 19, E) and IFN-γ secreting splenocytes by 100 μg of naked Rv3407 DNA were not increased, either.

At day 30, mice vaccinated with 100 μg of naked Rv3407 DNA showed 25.8 IFN-γ secreting splenocytes/1x10^5 cells responding to *M. tuberculosis* crude extract, while mice vaccinated with vector control showed 14.4 IFN-γ secreting splenocytes/1x10^5, which is 79% increased. IFN-γ secreting cells induced by 100 μg of naked Rv3407 DNA also were 85% increased responding to Rv3407-derived peptides, when the splenocytes were incubated in media only (Fig. 19, B). Mice vaccinated with 100 μg of naked Rv3407 DNA showed 36.7 IFN-γ secreting splenocytes/1x10^5 cells responding to *M. tuberculosis* crude extract when the splenocytes were incubated in media containing *M. tuberculosis* crude extract, while mice vaccinated with vector control showed 22 IFN-γ secreting splenocytes/1x10^5 (Fig. 19, D). Mice vaccinated with 100 μg of naked Rv3407 DNA showed 38.3 IFN-γ secreting splenocytes/1x10^5 cells responding to Rv3407-derived peptides when the splenocytes were incubated in media containing *M. tuberculosis* crude extract, while mice vaccinated with vector control showed 5.6 IFN-γ secreting splenocytes/1x10^5 (Fig. 19, F). However, 10 μg of DNA and PLG failed to induce significant IFN-γ

secreting cells at day 30. Ergo, these results suggested DNA vaccination could induce antigen-specific IFN-γ secretion and PLG might improve the induction of immune response at early time point but it did not last longer.

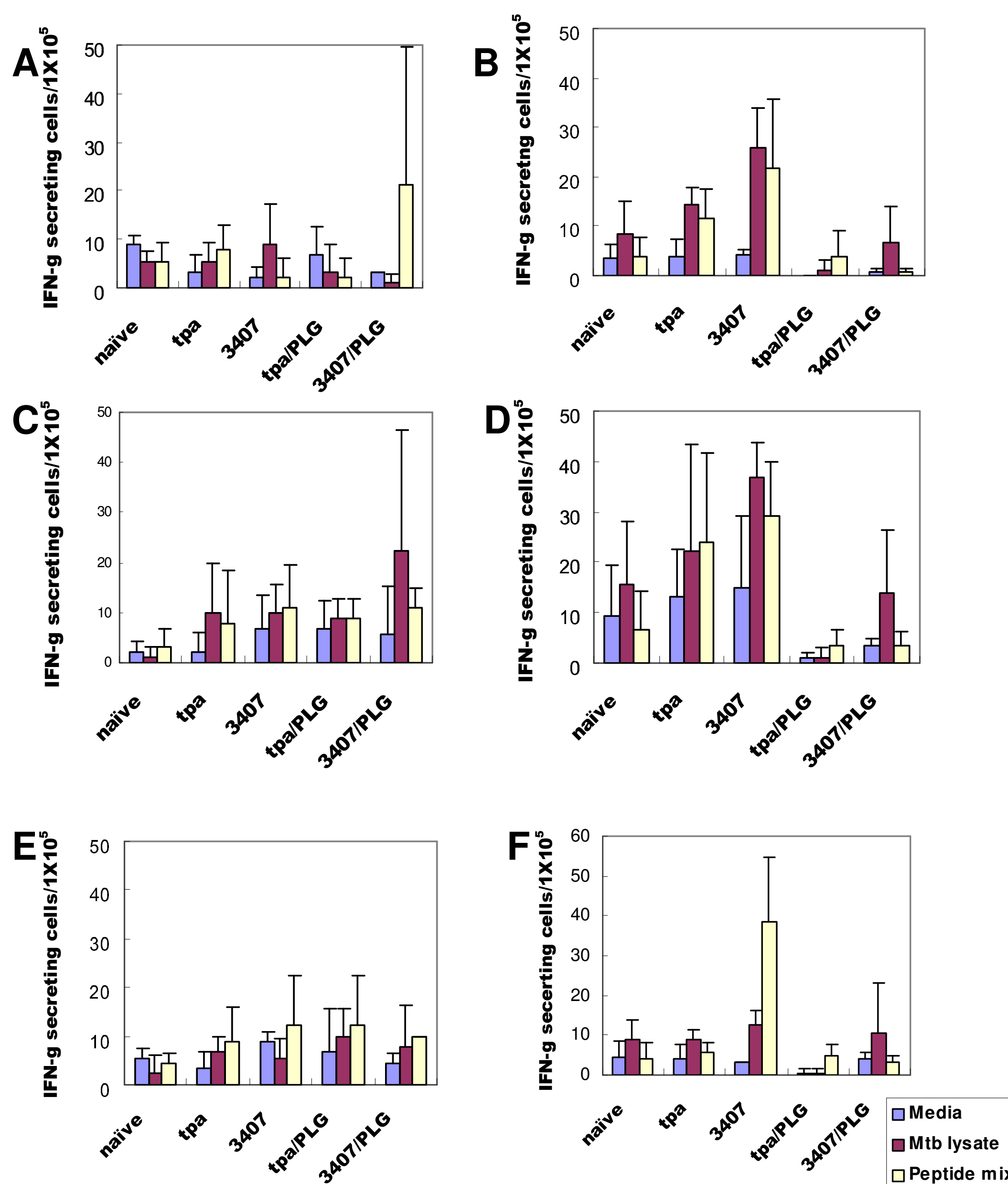

Fig.19 Frequencies of antigen-specific IFN-γ secreting splenocytes by DNA vaccination. Groups of 3 mice were vaccinated with 100 µg of naked tpa DNA as vehicle control, with 100 µg of naked Rv3407 DNA, with 10 µg of tpa DNA and PLG, and with 10 µg of Rv3407 DNA and PLG. Splenocytes were prepared at day –15, 1 weeks after the last boost (A, C, E) and at day 30, 7.5 weeks after the last boost (B, D, F), stimulated with media only (A, B), with *M. tuberculosis* crude extract (C, D), or with Rv3407-derived putative epitope peptide (E, F) for 3 days, and antigen-specific IFN-γ secretion was determined by ELISpot assay. Values represent means from 3, and error bars represent standard deviation.

4. Discussion

Tuberculosis (TB) which is mainly caused by *M. tuberculosis*, is still a major health problem with two million deaths and 8.8 million new cases annually (Global tuberculosis control: WHO report 2004. WHO/HTM/TB/2004.331. World Health Organization, Geneva, 2004). Although *M. bovis* BCG, the current vaccine against TB, prevents disseminated TB in newborns, it fails to protect against the most common form of the disease, pulmonary TB in adults. Therfore, a novel vaccine against TB is urgently needed. DNA vaccines can induce antigen-specific Th 1 $CD4^+$ and $CD8^+$ T cell responses required to protect mammals from intracellular bacterial infection, and they have several advantages: they are economical, relatively safe, easy to handle, and stable at room temperature. However, one of the disadvantages of DNA vaccine is the low efficiency of DNA delivery. Ergo, effective DNA delivery systems can reduce the amount of DNA required, reduce safety concerns, and at the same time improve protection and immune responses.

This study was performed to select the most potent DNA vaccine candidates against *L. monocytogenes* as an experimental model system and against *M. tuberculosis*, to analyze antigen-specific immune responses to compare the different DNA vaccine carrier systems in the listeriosis model, to identify the most effective one, and to apply the results to *M. tuberculosis* infection. The plasmid DNAs encoding mutant LLO (Trp492Ala) of *L. monocytogenes* and encoding Rv3407 of *M. tuberculosis* were selected as DNA vaccine candidates because they induced protection in mice most effectively from *L. monocytogenes* and *M. tuberculosis*, respectively, and also induced antigen-

specific $CD8^+$ T cell immune responses and IFN-γ production, which play a pivotal role in the control of intracellular bacteria. Three different kinds of biodegrabable particles and two different proteins were evaluated to select the most effective DNA delivery system to reduce the amount of DNA required for DNA vaccination, but no DNA carrier with 10μg of DNA could exceed the potency of 100 μg of naked DNA alone.

4.1. Selection of DNA vaccine candidates

In order to select the most effective DNA vaccine candidates against *L. monocytogenes*, the plasmid DNAs encoding p60, wild-type LLO, and mutant LLO (Trp492Ala) were compared. The proteins, p60 and LLO are well known virulence factors and dominant antigens of *L. monocytogenes* (Mengaud et al., 1987; Kuhn and Goebel, 1989) and mutant LLO (Trp492Ala) is non hemolytic and less virulent (Michel et al., 1990). Plasmid DNA encoding p60, LLO (Fensterle et al., 1999), or mutant LLO (Cornell et al., 1999) under CMV promoter proved to provide protective immunity in mice against *L. monocytogenes.*

Sex- and age-matched BALB/c mice were vaccinated with naked DNA or with DNA and carrier 3 times at 3 weeks interval. The vaccination protocol was optimized in previous reports (Fensterle et al., 1999; Mollenkopf et al., submitted). The potency of three plasmid DNAs encoding *L. monocytogenes* genes were compared by protection assay to check survival, and the plasmid DNA encoding mutant LLO showed the best protection against *L. monocytogenes* infection at different infection doses with or without DNA carrier. The second best one was the plasmid DNA encoding p60. The

plasmid DNA encoding wild-type LLO did not show significant protection at high infection doses. This result is consistent with a previous report (Cornell et al., 1999). Interestingly, vector control itself improved protection slightly. This seems to be due to the CpG motif effects (Elkins et al., 1999). For further experiments, the plasmid DNA encoding mutant LLO was selected as the best DNA vaccine candidate.

To develop a novel DNA vaccine against *M. tuberculosis*, three genes were selected by Mollenkopf et al. (Mollenkopf et al., submitted), based on 2D-electrophoresis analysis to select *M. tuberculosis*-specific secreting protein (Mattow et al., 2001). They are Rv3407, Rv2520c, and Rv1511. Rv3407 is a 300 bp non essential gene (Sassetti et al., 2003), and encodes a 99 a. a. conserved hypothetical protein with unknown function. Rv2520c is a 228 bp non essential gene (Sassetti et al., 2003), and encodes a 72 a. a. putative conserved membrane protein with unknown function. Rv1511 or *gmdA* is a 1023 bp non essential gene (Lamichhane et al., 2003), and encodes a 340 a. a. GDP-mannose 4, 6 dehydratase, which is probably involved in nucleotide-sugar metabolism.

The 3 plasmid DNAs encoding *M. tuberculosis* fused with tpa4 under the CMV promoter were compared by protection assays to assess the CFUs, since TB is a chronic disease. The ER-targeting leader sequence, tpa4, representing the human tissue plasminogen activator signal sequence, has been demonstrated to improve induction of immune response and protection with DNA vaccines against *M. tuberculosis* (Delogu et al., 2002; Li et al., 1999). Mice were vaccinated with 10 μg of DNA loaded onto PLG stabilized with PVA and infected with 100-200 *M. tuberculosis* H37Rv by aerosol as decribed

above. The Rv3407 DNA induced protection at day 30 and 60 post infection, but Rv2520 and Rv1511 failed to protect mice from *M. tuberculosis* infection. Surprisingly, the protection by 10 µg of Rv3407 DNA loaded onto PLG was comparable to that of 100 µg of naked DNA at day 30, but DNA vaccination of Rv3407 with or without PLG did not induce protection against *M. tuberculosis* infection significantly at day 90. In contrast to that of *L. monocytogenes* infection, CpG motifs in empty vector did not improve protection against *M. tuberculosis*. Bacterial CpG motifs stimulate protection against *L. monocytogenes* (Elkins et al., 1999) but they fail to enhance the protective efficacy of subunit vaccine against *M. tuberculosis* (Hsieh et al., 2004). The results in this dissertation support these reports.

4.2. Comparison of DNA delivery systems for vaccination

To compare the efficiency of DNA vaccine delivery, PLG stabilized with PVA, PLG stabilized with CTAB, a novel encapsulating particle, VLP, and TmHU were tested because these DNA carriers showed improved DNA transfer of reporter genes *in vitro* and/or *in vivo*, and were regarded relatively safe compared with lipid DNA carriers.

The efficiency of DNA vaccine delivery systems was evaluated by protection assay with 10 µg of DNA at high infection dose of *L. monocytogenes* to verify the results. One biodegradable particle, PLG stabilized with CTAB displayed slightly increased protection compared with 10 µg of naked DNA, but was not statistically significant. All other DNA carriers, i. e. PLG stabilized with PVA, encapsulating biodegradable particle, VLP, and TmHU failed to improve protection at all compared with 100 µg of naked DNA.

The amount of DNA used in prevous reports varies (ranging 10 ng-150 μg) (O'Hagan et al., 2001; Cui and Mumper, 2002; Briones et al., 2001; Singh et al., 2000b). Most reports described that DNA delivery systems improved protection or immune responses. In these studies, DNAs and carriers were compared with the same amount of naked DNA, not with an optimized vaccination protocol established. They focused on the improvement of protection or immune response, not on the reduction of the amount of DNA required for DNA vaccination, but the reduction of the amount of DNA is important to reduce safety concerns and costs. Numerous factors might affect optimal dose for DNA vaccination in addition to the delivery efficiency of DNA carriers: expression efficiency of vector, which is dependent on promoter, stability of mRNA and protein produced by DNA vaccine, immunogenicity of antigen, prime-boost strategies (how many times and how often to administer), the way of administration (i.m., s.c., intradermal, intranasal, or oral), and infection system (Gurunathan et al., 2000; Colosimo et al., 2000). Furthermore, there are many different kinds of biodegradable particles, for example, PLG stabilized with PVA and PLG stabilized with CTAB, and many different methods of production of biodegrabable particles. Therefore, the results in previous reports are hardly comparable to the results in this study.

In a recent interesting report, 150 μg of *M. leprae* Hsp65-encoding plasmid DNA was loaded onto PLG particles in two different ways: addition of DNA after polymerization of PLG and addition of DNA at the beginning of reaction (Johansen et al., 2003). These products were administered s.c. once while 150 μg of naked DNA was administered i.m. three times. As a result, neither

DNA loaded onto PLG particles improved protection, nor immune response compared with 3 times vaccination of naked DNA. Interestingly, when DNA was added at the beginning of reaction, the vaccine efficacy was higher than when DNA was added after polymerization although both of them were lower than that of 3 times administration of naked DNA (Johansen et al., 2003). The PLG particles used in this dissertation were mixed with DNA after polymerization.

Additionally, the DNA loading capacity of PLG is quite low (20-50%) (Briones et al., 2001) and PLG stabilized PVA prodeuced thick precipitates so much that some of the precipitates could not pass through the needle. So the actual amount of DNA loaded onto PLG to inject might be less than expected.

In many reports, reporter genes, such as β-galatosidase and green fluorescent protein (GFP), were used to assess the potential of DNA delivery but the actual pathogen for DNA vaccine was not tested (Cui et al., 2003; Cui and Mumper, 2003; McKeever et al., 2002; Stern et al., 2003). For example, TnHU proved the potential of DNA delivery *in vitro* and *in vivo* using *lacZ* gene as a reporter gene (Esser et al., 2000) but no more papers have come out of this.

Many methods to improve DNA vaccine delivery have been proposed, including new biodegradable particles, PLG bound immunogenic lipid or other immunomodulator, for instance, cholera toxin and lipid A, and antigen-expressing VLP. Many trials did not prove to be efficient and safe enough for use in humans. In another recent report, vaccine efficacy of 30 μg of mycobacterial Hsp65-encoding DNA loaded onto PLG particles with or without trehalose dimicolate (TDM) once was compared with that of 100 μg of naked

DNA three times (Lima et al., 2003). Without TDM, DNA loaded PLG could not improve vaccine efficacy significantly (Lima et al., 2003). TDM did not prove safe enough. Cationic PLG particles formulated with CTAB was one of the attempts to improve the DNA delivery efficacy, and the particles enhanced transfection efficiency up to 100 fold *in vitro* (C. Oster et al., personal comminication), but 4 out of 60 mice died after administration of the particle while no mice died from naked DNA vaccination or DNA and other carriers. The reason of the death is unclear, but CTAB is a strong detergent and safety of PLG with CTAB *in vivo* is not proven yet. However, a recent paper showed enhanced protective efficacy of DNA vaccine encoding Ag85A by absorption onto cationic PLG particles (Mollenkopf et al., 2004)

The results from ELISA assay implicated another problem with the use of proteins as DNA vaccine delivery system. The IgG1/IgG2a antibody titers in blood sera from mice vaccinated with DNA and VLP or with DNA and TmHU were seriously biased. Since VLP and TmHU themselves are proteins, they elicit Th 2 and B cell immune responses after repeated administrations and might induce non-specific immune responses or cross-priming, too (Skoberne et al., 2002). Finally, many of the DNA delivery systems are not practically easy to handle, nor stable at room temperature, and some of them are more expensive than DNA. For example, usually, proteins should be purified and stored at low temperature and the purification process takes time, money, and expertise.

In conclusion, there are many kinds of DNA delivery systems, methods of production of DNA delivery systems, and DNA vaccination protocols, so it is very difficult to identify the best DNA vaccine delivery system. They should be

compared using same plasmid DNA and specific infection system. Above all, development of more efficient and safer DNA delivery system for DNA vaccination is still required and on the other hand, the improvement of DNA vaccine itself is also worth taking into consideration.

4.3. Immune responses induced by DNA vaccination

Vaccines should elicit pathogen-specific immune responses. To determine antigen-specific $CD8^+$ T cells induced by DNA vaccination against *L. monocytogenes*, tetramer analysis was performed using MHC class I H2-K^d/LLO $_{91\text{-}99}$ tetramers. These tetramers stain antigen-specific $CD8^+$ T cell populations in a quantitative way (Busch and Pamer, 1998; Busch and Pamer, 1999). As expected, mice at day 5 post secondary infection showed greatly expanded $CD8^+$ $tetramer^+$ cell polulation and mice 7 weeks after the primary infection also showed significant $CD8^+$ $tetramer^+$ cell population. Mice vaccinated with 100 µg of naked DNA encoding mutant LLO showed significant level of CD8+ tetramer+ cells comparable to long term immune T cells after *L. monocytogenes* infection. These data imply that 5 days after the last boost, many antigen-specific T cells induced by DNA vaccination were still activated. However, mice vaccinated with 10 µg of DNA and PLG did not elicit measurable $CD8^+$ $tetramer^+$ cells. There exist tetramer positive cell populations after 7 days and 10 days after the last boost. These results suggested DNA vaccination elicited antigen-specific CD8+ T cells.

In order to determine antigen-specific CD8+ T cells induced by DNA vaccination against *M. tuberculosis*, a putative epitope from Rv1511 (GYVKFDQRYL) and one from Rv3407 (IPARRPQNL) and their affinity to

MHC class I molecule were predicted by computer programs, MAPPP (MHC-I Antigenic Peptide Processing Prediction) (Hakenberg et al., 2003) and FragPredict, provided by Max-Planck-Institute for Infection Biology, Berlin, Germany (http://www.mpiib-berlin.mpg.de/MAPPP/) (Mollenkopf et al., submitted; Nussbaum et al., 2003) and two MHC class I/peptide tetramers were produced as described above. At day 7, 4 weeks after the last boost, mice vaccinated with 100 µg of naked Rv1511 DNA showed increased $CD8^+$ $tetramer^+$ cell population, but at day 10, 20, 30, and 60, there was no detectable tetramer+ cell population any more (data not shown). Mice vaccinated with 100 µg of naked Rv3407 DNA were also tested to detect the $tetramer^+$ cell population by MHC class I/putative epitope of Rv3407, but there was no detectable tetramer+ cell population at day 14, 20, 30, and 60 (data not shown). There are two possibilities to explain this observation: one is that the $tetramer^+$ cell population might be so rapidly retracted that it is not detectable at day 10 any more, and the other and more likely one is that the epitope prediction or affinity prediction might not be acrurate enough. The affinity of MHC class I/peptide was predicted only by computer program, and no more information is available. Thus, the half-life of MHC class I/peptide might be too short to be used after several days, the putative epitope from Rv3407 might not be immunodominant, or the affinity of peptide from Rv3407 to MHC class I might be much weaker than predicted.

It is generally believed that IFN-γ is a key cytokine in the immune response against intracellular bacteria. Therefore, ELISpot assay was performed to detect IFN-γ secreting cells. The results showed that DNA vaccination with the

plasmid DNA encoding mutant LLO and the Rv3407 DNA induced IFN-γ secreting cells responding to MHC class I-restricted peptides and to bacterial proteins. The DNA vaccination with 100 µg of naked DNA encoding mutant LLO successfully induced IFN-γ, but 10 µg of DNA and PLG did not. Remarkably, DNA vaccination with 10 µg of Rv3407 DNA and PLG induced IFN-γ secreting splenocytes at the early phase, one week after the last boost, but not at the late phase, 7 weeks after the last boost. In contrast, DNA vaccination with 100 µg of naked Rv3407 DNA did not induce IFN-γ secreting splenocytes at the early phase, but did so at the late phase. Generally, these results are consistent with protection data. Vector control also induced slightly increased levels of IFN-γ secreting splenocytes, which implied innate immunity was elicited by CpG motifs.

On the other hand, IFN-γ intracellular staining for FACS analysis was performed, too, but no significant population of CD8+ IFN-γ+ cells was detectable although mice infected with *L. monocytogenes* 7 weeks before analysis showed increased CD8+ IFN-γ+ cell populations (data not shown). There are many kinds of cell types producing IFN-γ, including NK cells, NK T cells, and macrophages as well as Th 1 CD4+ cells. In addition, several recent reports suggested DNA vaccination induced protective immune responses against *L. monocytogenes* without IFN-γ (Barry et al., 2003; Yoshida et al., 2001) and antigen presentation was efficient without IFN-γ (Skoberne and Geginat, 2002). Taken together, the source of IFN-γ secreted after intracellular bacterial infection and the role of IFN-γ in immunity against intracellular bacteria should be investigated in more detail.

To protect mice from intracellular bacteria, $CD8^+$ T cells play an important role. The protection against *L. monocytogenes* is dependent mostly on $CD8^+$ T cells. BCG vaccination induces predominantly $CD4^+$ T cell immune response but optimal protection against *M. tuberculosis* requires both $CD4^+$ and $CD8^+$ T cell immune responses (Kaufmann, 2001; Goonetilleke et al., 2003). Therefore, CTL assays were performed. Firstly, conventional ^{51}Cr-release assays were performed but failed to detect significant CTL activity. One of the reasons was high spontaneous release (data not shown). Secondly, an in vivo killing assay using CFSE labeled target cells was adopted to detect CTL activity. This method is established to give quantitative and qualitative results of actual in vivo killing and less spontaneous leakage (Aichele et al., 1997; Sheehy et al., 2001). The activity of antigen specific CTL was detected by peptide-loaded target cells stained with 5- (and 6-) carboxyfluorescein diacetate succinimidylester (CFSE), which is a highly stable amine-reactive reagent readily incorporated into cells. When membrane damage occurs, the dye is almost instantaneously lost and the cells are no longer able to take up or retain the charged dye. Several reports have shown that CFSE-prelabeled target cells can be successfully evaluated to detect cytolysis (Sheehy et al., 2001). The antigen-specific CTL activity was successfully detected 9 days after both primary infection of *L. monocytogenes* and LCMV. However, 24 days after primary infection, only LCMV-primed mice showed significant CTL activity. Similar results showed that rapid protection *in vivo* is long-lived after immunization with LCMV but short-lived after vaccination with *L. monocytogenes* (Ochsenbein et al., 1999). Therefore, the results imply CTL activity induced by intracellular bacteria is rapidly retracted and the detection

of CTL activity of long term immunity against intracellular bacteria requires much more sensitive methods.

4.4. Conclusion

Several studies have demonstrated that DNA vaccines elicit $CD8^+$ T cell as well as $CD4^+$ T cell responses, but effective DNA vaccine delivery systems can enhance cellular uptake of DNA vaccines and/or facilitate intracellular targeting of DNA to the cytoplasm and nucleus, and also reduce the amount of DNA required. BALB/c mice were vaccinated with 100 µg of naked DNA encoding p60, wild-type LLO, or non-hemolytic mutant LLO under the control of a CMV promoter or 10 µg of DNA with 10 µg of VLP, 12 µg of TmHU, or 1 mg of PLG i. m. 3 times at 3 weeks intervals. Vaccination with naked DNA encoding mutant LLO protected mice efficiently against *L. monocytogenes* but no delivery system significantly improved the efficacy of DNA vaccine. IFN-γ secreting CD8+ splenocytes responding to LLO_{91-99} peptide were determined by ELISpot assay and MHC class I-restricted, LLO_{91-99} peptide specific $CD8^+$ splenocytes were assessed by the tetramer technique.

These results suggest that DNA encoding mutant LLO is a potent vaccine candidate against *L. monocytogenes* and that DNA vaccination can induce antigen-specific immune responses. More sensitive techniques are required to detect memory/effector immune responses elicited by intracellular bacterial antigens. Since memory/effector cells from intracellular bacterial pathogens were short lived, and DNA vaccines express just one antigen or a few antigens at relatively low levels, there might be natural limitations to detect memory/effector cells induced by DNA vaccination against intracellular

bacteria. In addition, critical reviews and optimized comparisons are required to use DNA vaccine carrier systems.

As previously reported, DNA vaccine candidates were identified by comparing the proteomes of *M. tuberculosis* H37Rv and *M. bovis* BCG Chicago, and DNA vaccination with Rv3407 DNA provided protection against *M. tuberculosis* H37Rv by aerosol at post-infection day 30 and 60. DNA vaccination with Rv3407 DNA induced IFN-γ secreting $CD8^+$ splenocytes responding to peptides of putative epitopes derived from Rv3407 as determined by ELISpot. The DNA vaccination also induced MHC class I-restricted, putative epitope-specific $CD8^+$ splenocytes determined by FACS analysis with Rv1511 peptide/H-$2K^d$ tetramers. Vaccination with 10 μg of DNA loaded onto PLG also showed protection but antigen-specific immune response could not be detected.

These results suggest that Rv3407 is the best DNA vaccine candidate against *M. tuberculosis*, and DNA vaccine encoding a *M. tuberculosis*-specifically expressed gene can induce protection against *M. tuberculosis* and antigen-specific immune response, but awaits improvement.

The improvement of DNA vaccine itself is worth taking into consideration. There have been several efforts to enhance the efficacy of DNA vaccines: developing new viral or non-viral vectors, insertion of gene regulatory elements in plasmid backbone, and exchanging codon usages to frequently used in mammals (Doria-Rose and Haigwood, 2003). There are reports on enhanced immunogenicity against *M. tuberculosis* by new vectors, for example, alphavirus plasmid replicon (Kirman et al., 2003) and recombinant

modified vaccinia virus Ankara (Goonetilleke et al., 2003). Fusion to tpa4 signal sequence or ubiquitin intracellular targeting sequence also improved vaccine efficacy against *M. tuberculosis* (Delogu et al., 2002; Li et al., 1999). Optimization of codon usage of DNA vaccine was reported to enhance effective immune response against *L. monocytogenes* (Uchijima et al., 1998). A linear minilalistic vecotor (MIDGE) is also available to reduce any side-effect owing to bacterial plasmid backbones, including CpG motif effect (Lopez-Fuertes et al., 2002). Construction of DNA encoding fusion protein of different antigens and combinations of different DNA vaccines, so-called DNA vaccine cocktail could be considered, too (Delogu et al., 2002).

Protection levels with DNA vaccination against challenge with *M. tuberculosis* have been generally less effective than BCG vaccination alone. Thus it would also be worthwhile taking prime-boost strategies. The prime-boost strategies are effective at generating high levels of T cell memory (Ramshaw and Ramsay, 2000). This approach is being tested in humans using a DNA vaccine against malaria (Moorthy et al., 2004), and has shown promising results against HIV (Takeda et al., 2003).

Several prime-boost strategies using DNA vaccine have been tested: DNA-protein, DNA-recombinant virus expressing the same respective antigens, DNA-BCG, and BCG-DNA against *M. tuberculosis* (Britton and Palendira, 2003). Recently, several studies have demonstrated the efficacy of prime-boost vaccination strategies in generating cellular immunity to *M. tuberculosis* (McShane et al., 2001; Tanghe et al., 2001). Especially, vaccination of DNA encoding Ag85A as prime and BCG as boost markedly improved protection (Feng et al., 2001) and mice that had been intranasally vaccinated with BCG

and then boosted with a recombinant vacinia virus expressing Ag85A had remarkable reduction in bacterial load in the lungs following aerosol challenge with *M. tuberculosis* (Goonetilleke, et al., 2003). The 3 plasmid DNAs used in this study was already tested the efficacy as a boost vaccine after BCG prime (Mollenkopf et al., submitted). Especially, BCG prime-Rv3407 DNA boost vaccination showed superior protection to BCG alone. Since huge populations in the world were previously administered with BCG, the prime-boost strategy of BCG as prime and DNA vaccine as boost is regarded as a more important and practical approach. In addition, recombinant BCG expressing *M. tuberculosis* antigens and deletion mutant *M. tuberculosis* as TB vaccine candidates have the dangerous potential to become more virulent, and repeated administration of DNA vaccines also have potentially harmful side effects to induce autoimmunity or immunity against the vectors. In comparison, the prime-boost strategies with BCG and DNA vaccines have the advantage of less safety concerns. Since BCG elicits mostly $CD4^+$ immune responses, subunit vaccines which can induce $CD8^+$ immune responses would be particularly advantageous. Ergo, the next step to improve TB vaccine should be to adopt prime-boost strategies.

5. Summary

L. monocytogenes induces an acute course of infection in mice and the immune response is mediated by $CD4^+$ Th1 cells and $CD8^+$ cytotoxic T cells. Protection is mostly mediated by $CD8^+$ T cells, which are directed against two immunodominant antigens, LLO and p60. Several studies have demonstrated that DNA vaccines elicit $CD8^+$ as well as $CD4^+$ T-cell responses, but low efficiency of DNA delivery is one of the disadvantages of DNA vaccines. The purpose of this thesis was to compare different DNA vaccine carrier systems, to identify the most effective one(s), and to apply the results to TB vaccination. I vaccinated BALB/c mice with 100 µg of naked DNA encoding p60, wild-type LLO, or non-hemolytic mutant LLO under the control of a CMV promoter or 10 µg of DNA with 10 µg of VLP, 12 µg of TmHU, or 1 mg of PLG i. m. 3 times at 3 weeks intervals. Vaccination with naked DNA encoding mutant LLO protected mice efficiently against *L. monocytogenes* but none of the delivery systems tested significantly improved the efficacy of 10 µg of DNA vaccine compared with vaccination with 100 µg of naked DNA. IFN-γ secreting $CD8^+$ splenocytes responding to $LLO_{91\text{-}99}$ peptide were determined by ELISpot assay and MHC class I-restricted, $LLO_{91\text{-}99}$ peptide specific $CD8^+$ splenocytes were assessed by the tetramer technique. These results suggest that DNA encoding mutant LLO is a promising vaccine candidate against *L. monocytogenes* and that DNA vaccination can induce antigen-specific immune responses. However, further optimization of these carrier systems is required before application to DNA vaccination. DNA vaccination with Rv3407 DNA provided protection against *M. tuberculosis* H37Rv aerosol infection. DNA vaccination with antigen Rv1511 or Rv3407 induced IFN-γ secreting $CD8^+$ splenocytes responding to peptides of putative epitopes derived from Rv1511 or Rv3407, respectively, as determined by ELISpot. The DNA vaccination also induced MHC class I-restricted, putative epitope-specific $CD8^+$ splenocytes as determined by FACS analysis with Rv1511 peptide/H-$2K^d$ tetramers. Vaccination with 10 µg of Rv3407 DNA loaded onto PLG also showed partial protection. These results suggest that DNA vaccines encoding *M. tuberculosis*-specific genes can induce pathogen-specific protection against *M. tuberculosis*, but await further improvement.

6. Zusammenfassung

Die Listeriose ist eine akute Infektion, und die Immunantwort wird durch $CD4^+$ Th1 Zellen und $CD8^+$ zytotoxische T Zellen vermittelt. Der Infektionsschutz wird überwiegend durch $CD8^+$ getragen, die mit zwei immundominanten Antigenen, LLO und p60, reagieren. Verschiedene Studien haben gezeigt, dass DNA Impfungen sowohl $CD8^+$ T Zell- als auch $CD4^+$ T-Zell-Antworten erzeugen, aber die geringe Effizienz der DNA-Aufnahme ist einer der Nachteile von DNA-Impfungen. Ziel dieser Arbeit ist es, durch den Vergleich verschiedener DNA Trägersysteme das effektivste System zu identifizieren, und die erhobenen Resultate auf die Tuberkulose-Impfung zu übertragen. Ich habe BALB/c Mäuse mit 100 µg reiner DNA, die p60, Wildtyp LLO oder mutiertes, nicht hämolytisches LLO unter der Kontrolle eines CMV Promotors kodiert, geimpft, oder mit 10 µg DNA zusammen mit 10 µg VLP, 12 µg TmHU, oder 1 mg PLG. Die Impfung mit reiner, für mutiertes LLO kodierender DNA schützte die Mäuse effizient gegen *L. monocytogenes*, aber keines der untersuchten Transportsysteme verbesserte die Effizienz der DNA Impfung (10 µg) verglichen mit der Gabe von 100 µg reiner DNA. IFN-γ sezernierende $CD8^+$ Milzzellen, die auf das $LLO_{91\text{-}99}$ Peptid reagierten, wurden durch ELISpot Tests bestimmt, und MHC Klasse I-restringierte, $LLO_{91\text{-}99}$ Peptid-spezifische $CD8^+$ Milzzellen mittels Tetramer Färbung untersucht. Die Ergebnisse legen nahe, dass modifiziertes LLO ein wirkungsvoller Impfstoff-Kandidat gegen *L. monocytogenes* ist, und dass dieser DNA-Impfstoff antigen-spezifische Immunantworten induziert. Weitere Optimierung ist jedoch notwendig, bevor DNA-Impfstoff-Träger-Systeme eingesetzt werden können. DNA-Impfung mit dem Antigen Rv3407 erzeugte Schutz gegen Aerosol-Infektion mit *M. tuberculosis* H37Rv. Die Mäuse wurden mit 100 µg DNA durch drei i. m. Injektionen in dreiwöchigen Intervallen geimpft. DNA Impfung mit den Antigenen Rv1511 oder Rv3407 induzierte IFN-γ sekretierende $CD8^+$ Milzzellen, die auf die Peptide der putativen dominanten Epitope von Rv1511 und Rv3407 reagierten, wie durch ELISpot Test nachgewiesen. Die DNA Impfung induzierte auch MHC Klasse I-restringierte, epitopspezifische $CD8^+$ Milzzellen, die mittels FACS Analyse mit Rv1511 Peptid/H-$2K^d$ Tetrameren bestimmt wurden. Eine Impfung mit 10 µg Rv3407 DNA, die auf PLG aufgebracht war, induzierte Protektion. Diese Ergebnisse lassen darauf schließen, dass ein DNA-Impfstoff, welcher ein *M. tuberculosis*-spezifisches Gen kodiert, antigen-spezifischen Schutz gegenüber *M. tuberculosis* vermitteln kann, jedoch einer weiteren Optimierung bedarf.

7. References

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8. Abbreviations

Ab antibody

Ag antigen

AIDS acquired immune deficiency syndrome

β2m beta 2-microglobulin

BCG bacille Calmette-Guérin

BCIP/NBT 5-Bromo-4-Chloro-3-Indolyl Phosphate/Nitro Blue Tetrazolium

BSA bovine serum albumin

CD cluster of differentiation

CFSE 5- (and 6-) carboxyfluorescein diacetate succinimidylester

CFU colony forming unit

CTAB hexadecyltrimethyl ammonium bromide

CTL cytolytic T lymphocyte

Cy5 cyanine 5

DMEM Dulbeco's modified Eagle's medium

DC dendritic cells

DC-SIGN DC-specific intercellular adhesion molecule-3 grabbing nonintegrin

DW distilled water

ELISA enzyme-linked immunoabsorbent assay

ELISpot enzyme-linked immunospot assays

FACS fluorescence-activated cell sorter

FITC fluorescein-5-isothiocyatate

HIV human immunodeficiency virus

HPV human papilloma virus

Ig immunoglobulin

IFN interferon

IL interleukin

i.m. intramuscular

IPTG isopropyl-β-d-thiogalactopyranoside

i.v. intravenous

LAM lipoarabinomannan

LCMV lymphocytic choriomeningitis virus

LLO lysteriolysin O

mAb monoclonal antibody

ME mercaptoethanol

MHC major histocompatibility complex

MMR macrophage mannose receptor

NK natural killer

PBS phosphate buffered saline

PE phycoerythrin

PFA paraformaldehyde

PFU plaque forming unit

PLG poly(lactide-co-glycolide)

PPD purified protein derivative

PVA polyvinylalcohol

s.c. subcutaneous

TAP-1 transporter associated with antigen processing 1

TB tuberculosis

TDM trehalose dimicolate

Th 1 T helper type 1

TLR Toll-like receptor

TmHU histone-like protein from *Thermotoga maritima*

TNF tumor necrosis factor

VLP virus-like particle

w/w weight/weight

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10. Curriculum Vitae

Name: (First Name) **Nayoung** (Last Name) **Kim**
Date of Birth: 6, July, 1970
Place of Birth: Seoul, Korea
Nationality: Republic of Korea (South Korea)
Address: (Residence) Ahornallee 40/41 D-14050 Berlin, Germany
(Lab) Max-Planck Institute for Infection Biology, Department of Immunology, Schumannstr. 21/22, D-10117 Berlin, Germany
Telephone Number: (Office) +49-(0)30-28460-562
(Lab) +49-(0)30-28460-545
(FAX) +49-(0)30-28460-505
(Mobile) +49-(0)174-5186687
E.mail address: kim@mpiib-berlin.mpg.de
naykim@hanmail.net

Education

2004. Ph.D. candidate

Feb.1997. Department of Molecular Biology, College of Natural Sciences, Seoul National University (finished the course work of Ph.D. program)

Feb.1995. Department of Molecular Biology, College of Natural Sciences, Seoul National University (M.Sc.)

Feb.1993. Department of Molecular Biology, College of Natural Sciences, Seoul National University (B.Sc.)

Feb.1989. Sacred Heart Girls' High School

Work Experience

Aug.2001 – present. Max-Planck Institute for Infection Biology, Department of Immunology (Ph. D. Student)

Oct.2000 - Jun. 2001. Microarray Center, Biomedlab Institute, Biomedlab Co. (Senior Researcher)

Nov.1999 - May.2000. Department of Pathology, College of Medicine,

Seoul National University (Researcher)

Mar.1996 - Aug.1996. Department of Molecular Biology, College of Natural Sciences, Seoul National University (Teaching Assistant)

Jan.1993 - Oct.1999. Laboratory of Molecular Immunology, Institute of Molecular Biology and Genetics, Seoul National University (Graduate Student)