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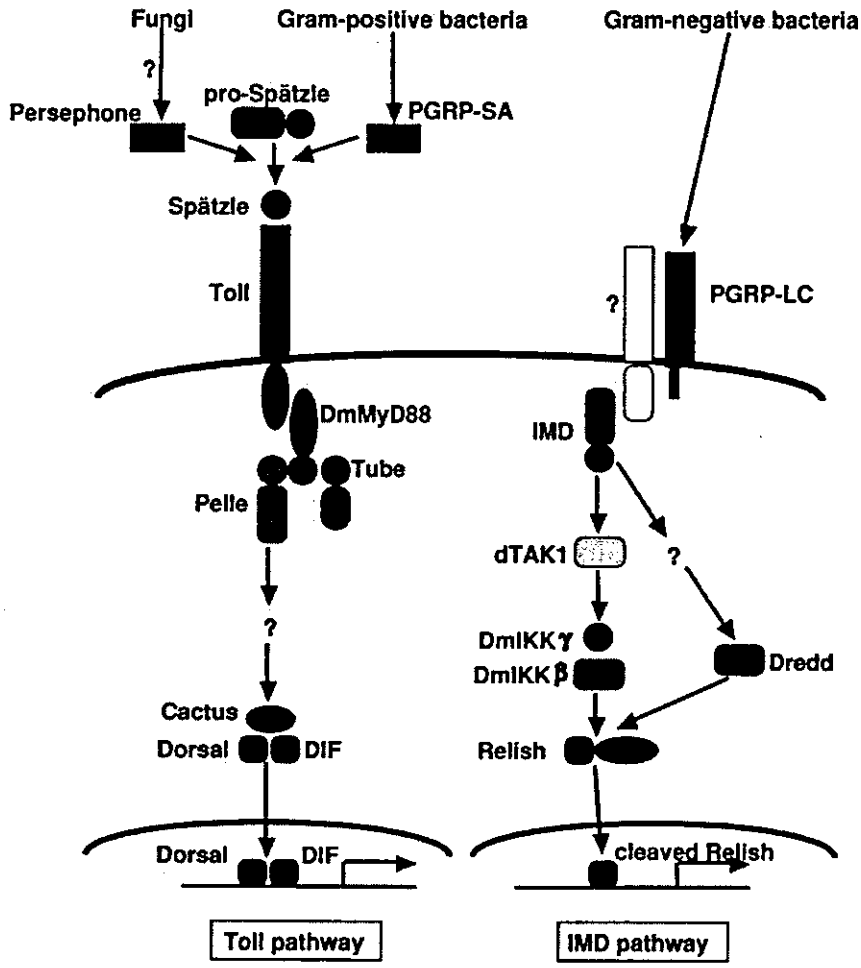

Figure 1 Host defense responses in drosophila. In drosophila, the Toll and IMD pathways confer host defense against pathogen invasion. The Toll pathway regulates production of antimicrobial peptides against fungi and Gram-positive bacteria. PGRP-SA is essential for activation of the Toll pathway in response to Gram-negative bacteria. Persephone is involved in activation of the Toll pathway in response to fungi. PGRP-LC recognizes the invasion of Gram-negative bacteria and is required for activation of the IMD pathway, which is essential for anti-Gram negative bacterial responses.

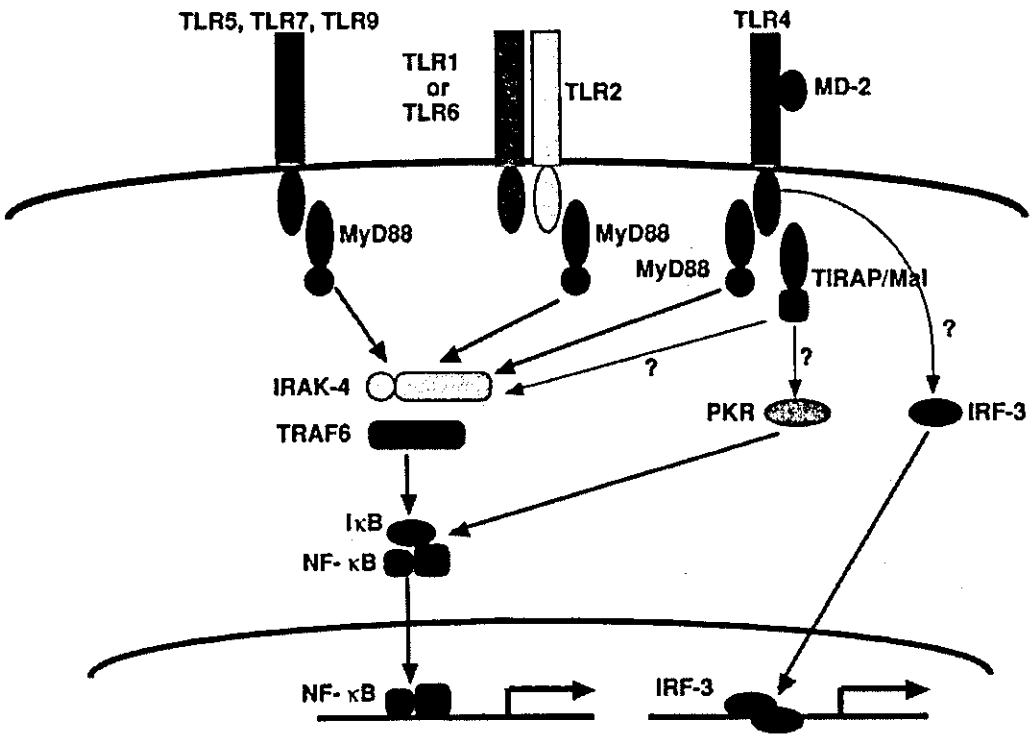

Figure 4 Toll-like receptor (TLR) signaling pathway. TLRs recognize specific patterns of microbial components. MyD88 is an essential adaptor for all TLRs and is critical to the inflammatory response. In the case of the TLR4-mediated pathway, lipopolysaccharide (LPS)-induced activation of signaling molecules such as IRF-3, PKR, MAP kinase, and NF-kB has been reported, indicating the presence of the MyD88-independent pathway. TIRAP/Mal was identified as a component specifically involved in TLR4-mediated signaling.

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8 Induction of antigen-specific T-cell subsets by DNA vaccination

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Abstract

DNA vaccines have advantages over other types of vaccines that the vaccines can induce strong cellular immune responses, namely cytotoxic T lymphocytes (CTL) and helper T lymphocytes (Th). Therefore, DNA vaccines are considered to be promising vaccines as an alternative to attenuated live vaccines. So far, various DNA vaccines have been generated and tried to induce a particular cellular immune responses by virtue of recombinant DNA technology. Following factors should be taken into consideration in designing DNA vaccine plasmids for efficient transcription and translation of target genes; (1) choice of the strong eukaryotic promoter; (2) inclusion of the Kozak consensus translational initiation sequence; (3) codon optimization of target genes to mammalian genes. Various DNA vaccine strategies for induction of specific CTL have been reported and shown their efficacy. These DNA vaccines were designed to have following features; (1) CTL epitope minigene, (2) protein targeting to the endoplasmic reticulum by the

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eukaryotic signal sequence, (3) fusion with b2-microglobulin, and (4) ubiquitination of target genes. In addition, multimerized CTL epitope DNA vaccines have been reported. For induction of specific Th, (1) Th epitope/class II associated invariant chain peptide (CLIP)-exchanged type DNA vaccine; (2) DNA vaccines possessing endosomal/lysosomal targeting signals; (3) DNA vaccines for expression of Th epitope inserted in an immunoglobulin molecule, have been examined and also shown to be effective. In order to induce strong immune responses by DNA vaccines, the immunization route and the immunization regimen such as heterologous "prime-boost" regimen also should be considered.

Introduction

We have been exposed to increasing danger of a variety of infectious diseases. So, the development of effective vaccination strategies against them is the exigencies of the times. The type of effective immune response against infectious diseases depends on the location of pathogens responsible. For example, extracellularly-located pathogens are, in general, vulnerable to antibody-mediated effector mechanisms. On the other hand, protection against intracellularly-located pathogens depends on induction of specific cell-mediated immunity [1-3]. Two major arms of cellular immunity can come into play in the protection. Cytotoxic T lymphocytes (CTL) are a main effector against pathogens located in the cytoplasm of host cells, such as viruses, *Rickettsia* spp. or *Listeria monocytogenes*, while type 1 helper T cells (Th1) play a pivotal role in the protection against infections with intracellular pathogens located in vacuolar compartments, such as *Mycobacteria* spp. or *Salmonella* spp. Therefore, effective resistance to infection depends on the vaccines capable of inducing certain effectors effectively. Among a variety of vaccination strategies tried so far, DNA vaccines have potential advantages over other type of vaccines that the vaccines can induce suitable cellular immune responses with enormous flexibility. A large body of reports has accumulated about DNA immunization for induction of particular cellular immunity [4-7]. Both cellular immune responses have been shown to be effectively induced with DNA vaccines. Here, we review the strategies to induce specific T-cell subsets by naked DNA immunization with an emphasis on vaccines against intracellular pathogens.

Murine infection of listeria monocytogenes: A model system of DNA vaccine study against intracellular pathogens

We have been working on DNA vaccines against *Listeria monocytogenes* as a model of intracellular bacteria. *L. monocytogenes* is a gram-positive facultative intracellular bacterium. Murine *L. monocytogenes* infection system has been studied as a good model system for intracellular bacteria infection [2, 8]. The bacterium has been known to induce major histocompatibility complex (MHC) class I-restricted $CD8^+$ T-cell responses in addition to MHC class II-restricted $CD4^+$ T-cell responses since the bacterium is capable of escaping from phagocytic vesicles into the cytoplasm of the host cells, thereby introducing the bacterial proteins into the MHC class I antigen processing pathway. Both $CD8^+$ CTL and $CD4^+$ Th1 have been shown to be amplified at listerial infection and to play a critical role in the protective immunity by experiments of depletion and adoptive transfer of specific T-cell subsets [9-11] or by analyses of mutant mice with a genetic defect in β2-microglobulin or H2-Aβ gene [12, 13].

So far, several T-cell epitopes (MHC-binding antigenic peptides) in listerial antigens have been reported (Fig. 1). Pamer and colleagues [14, 15] have reported four different *L. monocytogenes* epitopes presented by MHC class I (H2-K^d) molecules to CTL; those are listeriolysin O (LLO) 91-99, p60 (murein hydrolase) 217-225, p60 449-457, and mpl (metalloprotease) 84-92. Two of these four epitopes, LLO 91-99 and p60 217-225, have been demonstrated to induce dominant immune responses. First identified MHC class II binding peptide in *L. monocytogenes* is LLO 215-226, an H2-E^k binding peptide [16, 17]. Then, p60 301-312 was identified as an H2-A^d binding peptide [18]. Recently, Geginat et al. [19] tried to identify $CD4^+$ and $CD8^+$ T cell epitopes in LLO and p60 molecules based on the screening of peptide spot libraries with splenocytes derived from *L. monocytogenes*-infected BALB/c and C57BL/6 mice. They confirmed all known $CD4^+$ and $CD8^+$ T cell epitopes in LLO and p60 molecules and additionally identified six new $H2^d$-, and six new $H2^b$-restricted T cell epitopes, containing $H2^b$-restricted LLO 190-201, LLO 318-329 ($CD4^+$ T cell epitopes) and LLO 296-304 ($CD8^+$ T cell epitopes). The adoptive transfer of LLO 91-99-specific $CD8^+$ CTL [20] or p60 301-312-specific $CD4^+$ Th [18] conferred protection against *L. monocytogenes* lethal infection, suggesting that induction of T cells specific to these T cell epitopes is prerequisite to the protection against listerial challenge. We have tried construction of DNA vaccines which are able to induce only a particular T-cell subset against these T-cell epitopes derived from *L. monocyotogenes* and examined their efficacy.

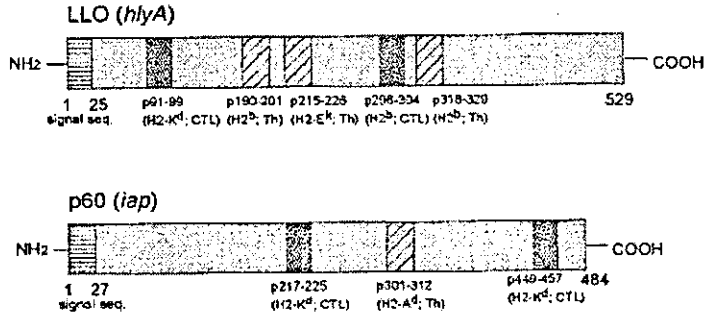

Figure 1. Major protective antigens of *Listeria monocytogenes*, listeriolysin O (LLO) and p60. LLO and p60 molecules of *L. monocytogenes* have been shown to be major protective antigens. So far, several $CD8^+$ CTL and $CD4^+$ Th epitopes in these antigens have been reported.

Antigen recognition by T cells

As mentioned before, T cells which play a pivotal role in adaptive immunity, are divided into two main categories. These are CTL and Th. Both groups have T-cell receptor molecules on their surface in common, but CTL and Th in general have CD8 molecules and CD4 molecules on their surface, respectively. $CD8^+$ CTL are presented antigens (antigenic peptides) in association with MHC class I molecules on the surface of antigen-presenting cells (APC). And $CD4^+$ Th are presented them in association with MHC class II molecules. Therefore, efficient induction of CTL and Th requires efficient presentation of antigenic molecules through MHC class I and MHC class II antigen processing and presentation pathway, respectively.

Antigen processing and presentation pathway through MHC molecules has been described in excellent review papers such as [21]. But, let us review it here briefly because it is important to understand the mechanisms of T-cell subset-oriented DNA vaccines (Fig. 2). MHC class I molecules have been shown to be expressed in almost all somatic cells except for neurons and germ cells. In order to prime CTL, antigenic peptides must be presented on MHC class I molecules on the surface of professional APC which possess special accessory molecules. In general, proteins located in the cytoplasm of the APC (endogenous antigens) are processed with the proteasome complex and selected peptides go into the endoplasmic reticulum (ER) through TAP (transporters associated with antigen processing) molecules. Then, antigenic peptides

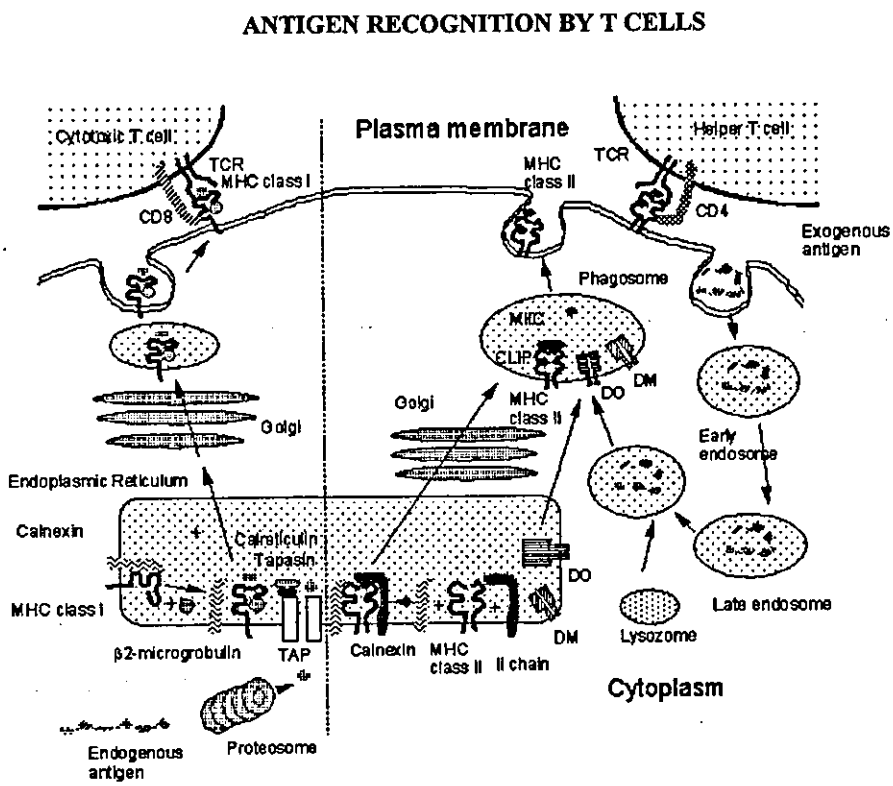

Figure 2. Antigen processing and presentation pathways through MHC class I and class II molecules. Endogenous antigens are thought to be degraded by large, ATP-dependent proteasome complex. The resulting peptides are then translocated into the lumen of the ER by TAP transporter. In the ER, antigenic peptides bind to the groove of MHC class I molecules. The peptide-MHC class I complexes are transported through the Golgi to the cell surface. Exogenous antigens are phagocytosed by phagocytes such as macrophages. Then, the antigens are degraded into peptides in endosome/lysosome compartments. MHC class II molecules associate with Ii molecules in the ER and the complex moves to endosome/lysosome compartments. Antigenic peptides bind to MHC class II molecules in the compartment named MIIC with the help of DM and DO molecules. The peptide-MHC class II complexes are then displayed on the cell surface.

are supposed to bind to the groove of MHC class I molecules in the ER. Therefore, utilization of DNA vaccines expressed only MHC class I binding peptide (CTL epitope) can bypass this antigen processing steps. On the contrary, MHC class II molecules are expressed only in professional APC having specific antigen presenting capacity, such as macrophages, dendritic cells, and B cells. Basically, MHC class II molecules are able to present antigenic peptides derived from the outside of the APC (exogenous antigens). Exogenous proteins are phagocytosed into APC and localized in the vesicles called phagosomes separated from the cytoplasm by lipid bilayer membranes. Then, phagosomes fuse with lysosomes, which are also vesicular compartments full of peptidases. After the fusion, exogenous proteins are degraded into smaller pieces of peptides. MHC class II molecules associate with invariant chain (Ii) molecules in the ER and the complex go to the endosomes by virtue of endosomal targeting signals located in the cytoplasmic regions of Ii molecules and also in MHC class II molecules. In the endosomes, Ii molecules are gradually degraded and only a small portion designated class II associated Ii peptides (CLIP), which is located in the groove of MHC class II molecules, remained. Then, antigenic peptides exchange with CLIP with the help of MHC class II-like molecules, H2-DM and H2-DO (mice), or HLA-DM and HLA-DO (humans) under the influence of local acidic pH and finally go into the groove of MHC class II molecules. Then, they are finally presented to $CD4^+$ T cells on the surface of APC. Therefore, targeting of antigens into endosomal compartments is a key factor to facilitating induction of antigen-specific $CD4^+$ T cells. Next, we would like to summarize DNA-mediated immunization briefly and then review the trials of induction of both T-cell subsets by DNA immunization including our studies.

Outline of DNA-mediated immunization

Wolff et al. [22] first reported that intramuscular injection of plasmid DNA allows the expression of plasmid-encoded proteins in the tissues in vivo at 1990. Then, Tang et al. [23] showed that injection of plasmid DNA directly into skin with gene gun bombardment effectively induces specific antibody production in 1992. Subsequently, Ulmer et al. [24] showed that intramuscular injection of plasmid DNA encoding influenza A nucleoprotein (NP) induces the generation of NP-specific CTL and protection from a challenge with a heterologous strain of influenza A virus. Since these early important studies, DNA vaccines have been studied intensively [4-7]. DNA vaccines are considered to be promising vaccines as alternatives to attenuated live vaccines, as DNA vaccines are capable of eliciting cellular immunity as well as antibodies.

Fig. 3 indicates a typical plasmid utilized for DNA vaccines. DNA vaccines are composed of (1) an antigen-encoding gene whose expression is driven by (2) a strong eukaryotic promoter such as cytomegalovirus immediate-early promoter/enhancer (CMV I.E. enhancer/promoter). And the plasmid possesses (3) a polyadenylation termination sequences such as the sequence derived from simian virus 40 (SV40) or bovine growth hormone (BGH) gene and (4) a prokaryotic selective marker such as ampicillin resistance gene to facilitate selection of *Escherichia coli* having the plasmid. In addition, plasmids for DNA vaccines should contain special nucleotide sequences for enhancing the immunogenicity; an unmethylated cytidine-phosphate-guanosine (CpG) dinucleotide with appropriate flanking regions. In mice, the optimal flanking region is

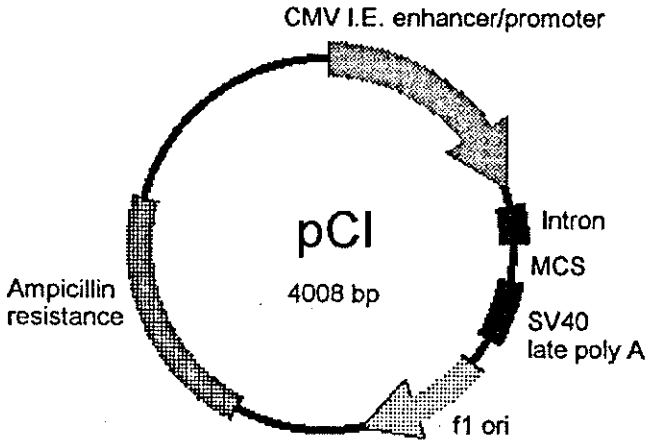

Figure 3. Plasmid for DNA vaccines. The basic mammalian expression plasmids available commercially can be usable as DNA vaccines. The salient features of these plasmids include: (1) a prokaryotic origin of replication (*ori*) and a prokaryotic selectable marker (e.g., ampicillin resistance gene) for providing replication and maintenance functions in *E. coli*; (2) a strong eukaryotic promoter/enhancer capable of driving transcription; (3) an intron (optional); (4) a polyadenylation signal (e.g., SV40 late poly A); (5) a multicloning site (MCS) for convenient subcloning of target genes. A typical mammalian expression plasmid, pCI (Promega), which we utilized as a DNA vaccine backbone plasmid in our studies, is shown here.

composed of two 5' purines and two 3' pyrimidines [25]. Ampicillin resistance gene contains the CpG, but kanamycin resistance gene does not have it [26]. The CpG motif stimulates the innate immune system through Toll-like receptor 9 to produce a series of immunomodulatory cytokines such as interleukin-12 and interferon (IFN)-γ, which promote the development of Th1 cells [27-29].

Major immunization methods for DNA vaccines tried so far are, (1) intramuscular injection into the hid leg quadriceps or tibialis anterior and (2) gene gun bombardment of DNA-coated gold particles into the epidermis. In addition, (3) intradermal DNA immunization [30] and (4) topical application of DNA vaccines [31] have been also reported to be able to induce immunization effects. Furthermore, several "carrier"-mediated DNA vaccine administration methods have been reported. They contain, (5) liposomes, (6) microparticle encapsulation, and (7) attenuated bacteria. These methods are briefly reviewed in [6].

In the intramuscular immunization, primary cells that plasmid DNA is transferred into are considered as muscle cells. As the cells are not professional APC, the mechanisms of DNA vaccines has been controversial. But now, bone marrow-derived APC has been suggested to be involved in antigen presentation in DNA vaccines [32, 33].

It is of particular interest that gene gun DNA immunization requires 100- to 1,000-fold less DNA than muscle DNA inoculation to generate equivalent antibody responses [34, 35]. In addition, gene gun DNA immunization appeared to bring about highly reproducible and reliable results in antibody production and induction of specific CTL and IFN-γproduction from immune splenocytes [36].

It has been suggested that muscle DNA immunization raised predominant Th1 responses, while gene gun DNA immunization produces Th2 responses [37]. These differences are considered to be mainly due to the differences of plasmid amounts used for vaccination. The difference of the plasmid amounts affects (1) amounts of antigen produced from the plasmids, (2) amounts of CpG motifs present in plasmid DNA vaccines.

Optimization in DNA vaccine design

First, we would like to review the general strategies to optimize DNA vaccination in following sections (1)-(4). Then, we will focus on the plasmid design for CTL-oriented and Th-oriented DNA vaccines in section (5).

(1) Choice of eukaryotic promoter

Cheng et al. [38] assessed the activities of five viral and five cellular promoters in different rat tissues by using gene gun bombardment. Their results demonstrated that CMV I.E. enhancer/promoter activity was consistently the highest in each tissues. Hence, the promoter has been used intensively for DNA vaccines. For the specific expression in muscle cells, desmin promoter, which works specifically in muscle cells, was also used for DNA vaccination with intramuscular injection [39].

(2) Inclusion of the Kozak consensus translational initiation sequence

For the efficient translation of target genes, a Kozak consensus translational initiation sequence around ATG start codon (CCA/GCCATG) have been shown to be important [40]. An et al. [41] suggested that minigene DNA vaccines must be designed with the Kozak consensus sequence. In addition, any "ATG" sequences in the 5' untranslated region of antigen genes should be removed as the sequence may work as the translation start codon. Also, the distance between the promoter and the open reading frame may influence the expression level of antigen genes.

(3) Codon optimization

Interspecies difference of codon usage is one of the major obstacles for effective induction of specific immune responses against pathogens by DNA vaccination. When genes derived from pathogens such as bacteria, protozoa, and some viruses, codon usage is one of problems for the expression in eukaryotic cells. We constructed a plasmid DNA vaccine harboring wild-type DNA sequence of *L. monocytogenes* LLO 91-99 (p91wt) and tried immunization of mice with the DNA vaccine by intramuscular injection. However, we could not induce LLO 91-99-specific CTL in BALB/c mice [42]. One of the reasons we failed the induction may be difference of the codon usage between mammalian cells and *L. monocytogenes*. *L. monocytogenes* genome is A+T-rich. In contrast, mammalian genome is G+C-rich. That difference may affect the efficacy of *L. monocytogenes* gene expression in mammalian cells. So, we constructed a DNA vaccine using LLO 91-99 gene, whose codons were optimized to those of the mammalian cells (p91mam). The DNA vaccine gave an excellent CTL induction in intramuscular immunization [42]. We further evaluated the "codon optimization effect" on CTL induction by the DNA vaccine [43]. In that study, we analyzed in mammalian cultured cells, the translational efficiency of several genes composed of different levels