Review Article

Induction of Specific CD8+ T Cells against Intracellular Bacteria by CD8+ T-Cell-Oriented Immunization Approaches

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For protection against intracellular bacteria such as *Mycobacterium tuberculosis* and *Listeria monocytogenes*, the cellular arm of adaptive immunity is necessary. A variety of immunization methods have been evaluated and are reported to induce specific CD8+ T cells against intracellular bacterial infection. Modified BCG vaccines have been examined to enhance CD8+ T-cell responses. Naked DNA vaccination is a promising strategy to induce CD8+ T cells. In addition to this strategy, live attenuated intracellular bacteria such as *Shigella, Salmonella*, and *Listeria* have been utilized as carriers of DNA vaccines in animal models. Vaccination with dendritic cells pulsed with antigenic peptides or the cells introduced antigen genes by virus vectors such as retroviruses is also a powerful strategy. Furthermore, vaccination with recombinant lentivirus has been attempted to induce specific CD8+ T cells. Combinations of these strategies (prime-boost immunization) have been studied for the efficient induction of intracellular bacteria-specific CD8+ T cells.

1. Introduction

The types of effective immune responses against infectious diseases depend on the location of the pathogens responsible. Generally extracellular pathogens are vulnerable to antibody-mediated effector mechanisms. On the other hand, protection against intracellular pathogens depends on the induction of specific cell-mediated immunity [1, 2]. Induction of effective resistance to infection depends on vaccines with the capacity of eliciting certain effectors.

In this review, we focused on strategies for the induction of CD8+ T cells against intracellular bacterial infections. CD8+ cytotoxic T-lymphocytes (CTL) are the main effectors against bacteria, such as *Rickettsia* or *Listeria monocytogenes*, located in the cytoplasm of host cells, while CD4+ type 1 helper T (Th1) cells play a pivotal role in the protection against infections caused by intracellular bacteria, such as *Mycobacterium* or *Salmonella*, located in vacuolar compartments. Many reports have indicated that in addition to Th1 cells, CD8+ CTL are also important for protection against these bacteria. After CD8+ CTL are antigen-presented, they directly kill the infected cells with an oriented release of granules like perforin and granzymes, as well as by granule-independent mechanisms (reviewed in [3]). Therefore, the induction of the bacteria-specific CD8+ T cells at an appropriate timing and magnitude is a key factor for protection against infections. Attenuated vaccines have been used for the induction of cellular immunity including that of CD8+ T cells. However, a variety of immunization methods have been recently reported to effectively induce specific CD8+ T cells. We reviewed methods to induce CD8+ T cells specific for intracellular pathogenic bacteria with emphasis on our efforts to induce CD8+ T cells specific for *Mycobacterium tuberculosis* and *L. monocytogenes*.

2. Intracellular Bacteria

Several bacteria have evolved in their mechanisms that allow them to survive in the host cells. These bacteria are considered intracellular based on their localization within the host cells, and are further categorized based on several criteria: bacteria such as *Chlamydia* and *Rickettsia* that cannot survive

Table 1: Intracellular bacteria.

Intracytosolic bacteria
Listeria monocytogenes *Shigella flexneri**
Rickettsia prowaseki
Intravascular bacteria
Mycobacterium spp. (*M. tuberculosis, M. bovis, M. avium*)
Nocardia asteroides
Legionella pneumophila, Chlamydia trachomatis
Intralysosomal bacteria
Salmonella enterica Typhimurium*, *Salmonella enterica* Typhi
*Yersinia enterocolitica**, *Coxiella burnetii*

Asterisks indicate bacteria utilized as carrier for DNA vaccine.

outside host cells are called obligate intracellular bacteria. On the other hand, facultative intracellular bacteria such as *Salmonella*, *Mycobacteria*, *Shigella*, and *Listeria* can survive outside of host cells.

Intracellular bacteria are also divided into three different groups depending on their intracellular niche (Table 1, reviewed in [4]) as follows: (1) cytoplasmic bacteria, which exit the phagosome and reside in the host cell cytoplasm: (2) intravascular bacteria, which persist in nonacidic vacuoles that have little interaction with the endosomal system of the host cells: and (3) intralysosomal bacteria, which persist in acidic, hydrolytic compartments that interact with the endosomal network of host cells.

(1) *Cytoplasmic Bacteria.* *L. monocytogenes* is a typical cytoplasmic, gram-positive, facultative intracellular bacterium. The *L. monocytogenes* infection system in mice has been studied and regarded as an excellent model system for intracellular bacterial infections [2, 5]. This 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 it is capable of escaping from the phagocytic vesicles into the cytoplasm of the host cells with the help of listeriolysin O (LLO; Hly), 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 in listerial infections and to play a critical role in protective immunity.

(2) *Intravascular Bacteria.* Bacteria such as *Mycobacteria* and *Nocardia* have been shown to block the normal maturation steps of phagosomes of host cells. In other words, these bacteria inhibit the phagosome-lysosome fusion after being phagocytosed. Vacuoles containing *Mycobacteria* do not acidify below pH 6.2 to 6.5 and exhibit paucity of the vacuolar proton ATPase, which is responsible for the acidification of endosomal and lysosomal compartments. For protection against *M. tuberculosis* infection, both CD4+ T cells and CD8+ T cells have been shown to be critical [1, 2].

(3) *Intralysosomal Bacteria.* *Salmonella* has been reported to belong to the intralysosomal bacteria. More than 2000 serotypes of *Salmonella* have been described. DNA homology analysis revealed that the genus consists of two species: *S. enterica* and *S. bongori*. *S. enterica* is further subdivided into six subgroups both phenotypically and genetically. Bacteria that belong to *S. enterica* are often briefly designated as *S.* Typhimurium and *S.* Enteritidis based on their serotypes for convenience sake. *Salmonella* penetrates through M cells into the Peyer's patches where they are phagocytosed by the underlying macrophages. The precise nature of *Salmonella*-containing vacuoles is controversial and is dependent on investigators and the types of the cells infected by *Salmonella*, but, the vacuoles inhabited by *Salmonella* have characteristics consistent with those that are late endosomal or lysosomal in nature. *Salmonella* vacuoles in macrophages are subject to be acidified by the fusion with lysosomes. Although the acidification of these vacuoles is partially reduced in those containing live *Salmonella*, the pH of the vacuoles containing live bacteria is still relatively acidic. *Yersinia* also belongs to this category. After passing through M cells, they are engulfed by macrophages and carried to the mesenteric lymph nodes where replication occurs.

CD8+ T cells have been considered to be critical for protection against these intracellular bacterial infections, especially, intracytosolic and intravascular bacterial infections. Intracellular bacteria themselves have been used as attenuated bacterial vaccines and also as carriers of DNA vaccines.

3. Antigen Processing and Presentation Required for CTL Induction

Both CTL and helper T cells have the same T-cell receptor molecules on their surfaces, apart from CD8 and CD4 molecules on their surfaces, respectively. Antigens (antigenic peptides) in association with MHC class I molecules on the surface of antigen-presenting cells (APC) are presented to CD8+ CTL. Furthermore, antigens in association with MHC class II molecules are presented to CD4+ Th cells. Therefore, the efficient induction of CTL and Th cells requires efficient presentation of antigenic molecules via MHC class I and II antigen processing and presentation pathways, respectively (reviewed in [6]).

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 CD8+ CTL, antigenic peptides must be presented on MHC class I molecules on the surface of professional APC that possess special accessory molecules. In general, proteins located in the cytoplasm of APC (endogenous antigens) are processed with the proteasome complex and selected peptides go into the endoplasmic reticulum (ER) through transporters associated with antigen processing (TAP) molecules. Antigenic peptides of 8 to 10 amino acid residues bind to the groove of MHC class I molecules in the ER after which they travel to the cell surface and are presented to CD8+ T cells (reviewed in [7]).

A new type of antigen presentation pathway to induce CD8+ T cells against intravesicular bacteria such as *M. tuberculosis* has been proposed [8, 9]. Intracellular bacteria induce apoptosis in infected macrophages after they are

phagocytosed. The formed apoptotic vesicles are captured by dendritic cells (DC) and induce CD8$^+$ T cells. This cross-priming pathway explains how intravesicular bacteria induce CD8$^+$ T cells.

4. Attenuated Bacteria Vaccination

Mycobacterium bovis bacillus Calmette-Guérin (BCG) is the only approved vaccine to date against tuberculosis (TB) and the most widely distributed attenuated bacterial vaccine [10, 11]. Despite BCG is among the most widely used vaccines throughout the world, TB still poses a serious global health threat. Whereas BCG is believed to protect newborn and young children against early manifestations of TB, its efficacy against pulmonary TB in adults is still a subject of debate [12] and was reported to wane with time since vaccination [13]. Moreover, the viable nature of BCG makes it partly unsafe in case of immunocompromised individuals. This highlights the need to develop a more effective, safe, and reliable vaccine against TB [14]. One of weak points of BCG vaccination is that the vaccination cannot induce strong CD8$^+$ T-cell responses. In contrast to *M. tuberculosis*, growth of BCG is not affected in mice lacking β2-microglobulin [15, 16]. The weak CD8$^+$ T-cell responses by BCG vaccination may be caused by weak invasiveness of BCG compared with *M. tuberculosis*. In order to enhance CD8$^+$ T-cell responses by BCG, Kaufmann's group reported recombinant BCG strain harboring listeriolysin O (LLO; LLO) derived from *L. monocytogenes* [17]. LLO is a pore-forming sulfhydryl-activated cytolysin and is essential for the release of *L. monocytogenes* from phagosomal vacuoles into the cytoplasm of host cells. Although recombinant *hly*$^+$ BCG strain expressing LLO did not egress into the cytoplasmic compartment of host cells, it improved MHC class I presentation of cophagocytosed ovalbumin as compared with wild-type BCG strain. Further, they developed *urease C*-deficient *hly*$^+$ BCG [18]. Urease C deficiency inhibits to increase an intraphagosomal pH and facilitates LLO activity. They found that LLO promotes antigen translocation into the cytoplasm and enhance not only CD8$^+$ T-cell responses via cross-priming mechanisms, but also the apoptosis of infected macrophages. One of other well-studied recombinant BCG is BCG overexpressing antigen 85B (Ag85B) gene (rBCG30) [19]. Ag85B is one of the major secreted proteins in *Mycobacteria* and the over-expressing BCG elicits more effective protective immunity against *M. tuberculosis* challenge.

In addition to recombinant BCG, a variety of live attenuated *M. tuberculosis* strains have been reported. Several strategies are being pursued to develop the attenuated strains, which include auxotrophs (strains that are able to grow only in supplementation of particular nutrients) and mutants that have deletions in virulence genes such as RD1 (region of deletion-1) genes.

Recombinant attenuated *Salmonella* strains have also been examined for vaccines against *M. tuberculosis*. Wang et al. [20] reported that orogastrical immunization of BALB/c mine by a live attenuated *Salmonella* Typhimurium strain harboring the *M. tuberculosis* ESAT6 (early secreted antigenic target 6-kDa protein)-Ag85B fusion gene or

(a) Gene gun system
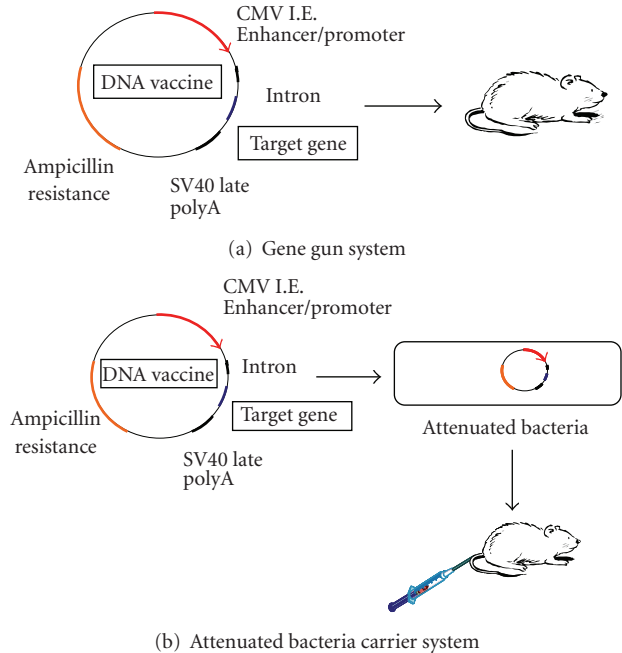

(b) Attenuated bacteria carrier system

Figure 1: DNA vaccination system. (a) Naked DNA vaccination. Eukaryotic expression plasmids that have a strong enhancer/promoter such as cytomegalovirus immediate-early promoter/enhancer are used as DNA vaccines. Naked DNA vaccination carried out by needle injection (intramuscular, subcutaneous), gene gun bombardment, or topical application to skin. (b) Live attenuated bacteria carrier DNA vaccination. Live attenuated intracellular bacteria, such as *Listeria*, *Shigella*, or *Salmonella*, are used as carriers of DNA vaccines. They were given orally or parenterally (needle injection).

combination of this vaccine with BCG vaccine induced strong Ag85B-specific mucosal humoral and cellular immune responses including effector CD8$^+$ T-cell responses and exerted high protective efficacy in mice against *M. tuberculosis* challenge. Cross-priming mechanisms may occur for the CD8$^+$ T-cell responses by these recombinant *Salmonella* vaccines.

5. DNA Vaccination

DNA vaccination is a method by which target antigen genes are directly introduced into host cells. The vaccination strategy is categorized into two groups (Figure 1). One is the so-called naked DNA vaccination. Eukaryotic expression plasmids encoding target antigen genes are used for this strategy. Immunization methods for the naked DNA vaccines are intramuscular injections, gene gun bombardment of DNA-coated gold particles into the epidermis, intradermal DNA immunization [21], and topical application of DNA vaccines [22]. The other is carrier-mediated DNA vaccination. Liposomes, microparticle encapsulation, and attenuated bacteria have been examined as carriers of DNA (reviewed in [23, 24]). Here we briefly review naked DNA vaccination and attenuated bacteria carrier DNA vaccination for the induction of CD8$^+$ T cells specific for intracellular bacterial infections.

5.1. Naked DNA Vaccination. Genetic immunization with naked DNA has been shown to efficiently induce cellular as well as humoral immune responses. The reason why this method produces an efficient induction of immune responses is due to the fact that it involves efficient antigen presentation through DC [25, 26]. It is of particular interest that the amount of DNA required by gene gun DNA immunization is 100 to 1000 times less DNA than that by muscle DNA inoculation to generate equivalent antibody responses [27]. Muscle DNA immunization raises predominant Th1 responses, while gene gun DNA immunization is apt to produce type 2 helper T (Th2) responses [28]. This difference is considered to be mainly due to the difference in (1) the amount of antigen produced from the plasmids and (2) the amount of CpG motif present in plasmid DNA vaccines. In addition, gene gun DNA immunization has brought about highly reproducible and reliable results in antibody production and the induction of specific CD8+ CTL and interferon-γ (IFN-γ) production from immune splenocytes [29].

Codon usage is a problem for the effective induction of specific immune responses by DNA vaccination against pathogenic bacteria. We constructed a plasmid DNA vaccine harboring a wild-type DNA sequence of a dominant CTL epitope of *L. monocytogenes* derived from LLO 91–99 (GYKDGNEYI). We then attempted to immunize mice with the DNA vaccine by intramuscular injection. However, this vaccine could not clearly induce LLO 91–99-specific CTL in BALB/c mice [30]. A reason for the induction failure may be the difference in codon usage between mammalian cells and *L. monocytogenes*. The *L. monocytogenes* genome is highly A+T-rich. In contrast, the mammalian genome is G+C-rich. This difference may affect the efficiency of *L. monocytogenes* gene expression in mammalian cells. To address this difference, we examined a DNA vaccine using the LLO 91–99 gene whose codons were optimized to those of the mammalian cells. The codon-optimized DNA vaccine gave an excellent specific CD8+ CTL induction by intramuscular immunization [30]. We further evaluated the codon optimization effect on CTL induction using the DNA vaccine [31]. In this previously performed study, using mammalian culture cells, we analyzed the translation efficiency of several genes composed of different levels of optimization to mammalian cells but encoding an identical CTL epitope derived from *L. monocytogenes* (LLO 91–99) and showed that the codon optimization level of the genes is not precisely proportional to, but correlates well with the translation efficiency in mammalian cells. These results also correlated well with the induction level of specific CTL response in vivo [31].

Several studies have been performed on the efficient induction of CTL of a particular specificity. We have demonstrated that the minigene DNA vaccine encoding only a dominant CTL epitope of *L. monocytogenes* (LLO 91–99) was effective for inducing CTL in vivo by gene gun DNA immunization [30]. This result suggests that the DNA vaccine plasmids are directly taken up by APC, which present target peptides to T cells by DNA immunization. Injection of a single CTL epitope minigene DNA generates a single CTL epitope peptide, which is supposed to enter the ER with the help of TAP molecules. However, Cho et al. [32] suggested that cross-priming is a predominant mechanism for inducing CD8+ T cell responses in gene gun DNA immunization. Some CTL epitopes have been modified to have greater immunogenic capacity by substituting several amino acid residues (epitope enhancement) [33].

As one particular approach for the efficient induction of a CD8+ T-cell subset, Wolkers et al. [34] showed that the carboxy-terminal fusion of a CTL epitope to a carrier protein of foreign origin efficiently induced CD8+ CTL. They constructed DNA vaccines by encoding a carboxy-terminal fusion of CTL epitopes (NP 366–374 derived from influenza virus or E7 49–57 derived from human papilloma virus) into green fluorescent protein (GFP) and showed that the DNA vaccines induced a much larger clonal size of antigen-specific CD8+ CTL by intramuscular immunization compared to the clonal size induced by these epitope minigene DNA vaccines. The purpose of the GFP fusion strategy was to provide CD4+ T-cell help by recognizing CD4+ T-cell epitopes in GFP protein. Maecker et al. [35] also showed that CTL induction by both intramuscular and intradermal DNA administration is dependent upon the generation of CD4+ T-cell help via the class II MHC-dependent pathway. Our results showed that CTL minigene DNA vaccination by gene gun DNA immunization induced specific CTL without any CD4+ T-cell help [36]. We speculate that the method of naked DNA immunization (needle injection or gene gun injection) determines requirement for CD4+ T-cell help.

Several studies have attempted to produce multimerized CTL epitope DNA vaccines (polyepitope DNA vaccines). This vaccine was first evaluated by Whitton et al. [37]. They generated a recombinant vaccinia virus system for the expression of CTL-epitope minigenes tandemly fused in a "string-of-beads" manner and showed that this vaccine can induce CD8+ CTL specific for each different epitope and protect vaccinated animals against infections. Subsequently, Thomson et al. [38] constructed a DNA vaccine plasmid containing 10 contiguous minimal CTL epitopes, which were restricted by five MHC alleles derived from five viruses (influenza virus, adenovirus, murine cytomegalovirus, Sendai virus, and lymphocytic chorimeningitis virus), a murine malaria parasite (*Plasmodium berghei*), and a tumor model antigen (ovalbumin). They administered in mice with the plasmid by intramuscular injection or gene gun-mediated intradermal injection and showed that the DNA vaccination successfully induced each epitope-specific CTL activity. Results of our single CTL-epitope DNA vaccine showed that a single dominant CTL epitope is sufficient for the induction of protective immunity [39], suggesting that selecting the most effective CTL epitope for each pathogen is critical for the efficacy of DNA vaccines.

Although some reports have suggested that the flanking sequences of a CTL epitope are important for the precise processing of the CTL epitope in vivo and that some CTL epitopes will interfere with other epitope function (Del Val et al. [40]), a majority of reports have shown that immunization with multimerized CTL epitope DNA without any spacer successfully induces CTL specific for each CTL epitope.

However, some reports (e.g., Velders et al. [41]) suggested the importance of defined flanking sequences around epitopes and the addition of ubiquitin. Ishioka et al. [42] evaluated minigene DNA vaccines encoding multiple HLA-restricted CTL epitopes employing HLA class I-transgenic mice. Such studies are useful as pilot experiments to evaluate DNA vaccines before attempting human studies.

We have used this naked DNA vaccination for identifying immunodominant CD8$^+$ T-cell epitopes of *M. tuberculosis* antigens [43–45]. We have used gene gun DNA immunization because it is highly reproducible and efficiently induces CD8$^+$ T cells [28]. After immunization, immune spleen cells were examined for their responses to overlapping peptides covering full-length proteins by measuring IFN-γ levels by enzyme-linked immunosorbent assay or by counting the numbers of IFN-γ-secreting cells by enzyme-linked immunospot assay. We combined these methods with computer algorithms, such as BIMAS [46] and SYFPEITHI [47], to predict T-cell epitopes. These programs were helpful for reducing the amino acid region of the bona fide T-cell epitope. However, the algorithms are still not perfect for accurate identification of T-cell epitopes at this time. A peptide that shows the highest score in these algorithms is not necessarily the best T-cell epitope. Experimental validation is definitely necessary to determine actual CD8$^+$ T-cell epitopes.

5.2. Live Attenuated Bacteria Carrier DNA Vaccination. Live attenuated bacteria, particularly intracellular bacteria, have been examined as carriers of DNA vaccines [48]. Advantages of these vaccination systems include (1) possible mucosal route of immunization, (2) propensity to infect APC, (3) relative ease of genetic manipulation, (4) adjuvant effects of carrier bacteria, (5) possible amplification of DNA vaccine plasmids in vivo, and (6) simplicity of handling and stocking. *Salmonella*, *Listeria*, and *Shigella* have been mainly examined for this purpose.

5.2.1. Shigella as a Carrier of DNA Vaccines. The first reported DNA vaccine-carrying bacterium was *Shigella*. Sizemore et al. [49, 50] showed that the strain *S. flexneri* 2a 15D harboring a plasmid expressing the *lacZ* reporter gene, which is controlled by an in vivo-induced promoter, elicited modest antibody and cellular immune responses against the reporter protein. The *Shigella* strain 15D (a derivative of the wild-type *S. flexneri* 2a strain 2457T) harbors a deletion mutation in the *asd* gene encoding aspartate β-semialdehyde dehydrogenase, an essential enzyme required for synthesizing the bacterial cell wall constituent diaminopimelic acid (DAP). The strain 15D retains invasiveness for mammalian cells but cannot survive in the absence of DAP supplementation in vivo. The use of an invasive yet nonreplicating attenuated vector such as 15D may be suitable for delivering plasmid DNA vaccines to mucosal lymphoid tissues. This study was supported by experiments in mice intranasally immunized with the strain 15D expressing measles virus envelope protein or nucleoprotein (NP) by Fennelly et al. [51]. They showed that mice vaccinated with the strain 15D harboring plasmid vectors encoding different measles

virus antigens induced a vigorous antigen-specific response against measles virus. They observed the production of measles virus protein-specific CD8$^+$ T cells and IFN-γ responses, as well as modest production of specific serum antibodies.

5.2.2. Salmonella as a Carrier of DNA Vaccines. An attenuated *Salmonella* strain widely used is the *S.* Typhimurium *aroA* strain [52], which interferes with the biosynthesis of aromatic amino acids. Darji et al. [53] reported that orally administered attenuated *S.* Typhimurium *aroA* carrying plasmids containing the coding sequence of β-galactosidase (β-gal) of *Escherichia coli*, or truncated forms of ActA or LLO of *L. monocytogenes* driven by eukaryotic promoters induce efficient humoral and cellular immune responses. Immunization of *Salmonella* carrying a LLO-encoding expression plasmid elicited protective immunity against a lethal dose of *L. monocytogenes* challenge. As *Salmonella* is reported to induce apoptosis when it enters macrophages, bystander DC may capture the DNA vaccine plasmid through the phagocytosis of *Salmonella*-infected apoptotic cells [54]. In addition to oral *Salmonella* DNA vaccine administration, the nasal route of administration has also been examined. Darji et al. [55] compared the oral and nasal administration of *Salmonella* harboring a eukaryotic expression plasmid encoding β-gal. They showed that both routes could induce systemic T-cell responses but nasal administration was clearly inferior to oral administration. This may be due to the lower number of bacteria that could be applied nasally.

Several investigators have improved the *Salmonella* carrier by introducing genes conferring invasiveness. Introduction of the LLO gene of *L. monocytogenes* into the *S.* Typhimurium $\triangle aroA$ strain resulted in enhanced plasmid delivery [56].

5.2.3. Listeria as a Carrier of DNA Vaccines. The ability of *L. monocytogenes* to enter the host cytoplasm after phagocytosis and deliver plasmid DNA directly into the cytoplasm makes it an attractive DNA delivery platform for inducing cellular immune responses.

Hense et al. [57] evaluated *Listeria* as a vehicle for gene transfer using a variety of cell lines. They observed gene transfer into host cells after treating cells infected with plasmid-carrying *Listeria* with tetracycline, a bacteriostatic antibiotic. They speculated that the metabolic block by tetracycline treatment makes these bacteria susceptible to cellular defense mechanisms and induces release of plasmid into the host cell cytoplasm. They reported that bacterial properties required for the delivery of eukaryotic expression plasmids were strictly dependent on the ability of the bacteria to both invade eukaryotic cells and egress from the vacuole into the cytoplasm of the infected host cells. Dietrich et al. [58] reported on the DNA vaccination system of an attenuated self-destructing *L. monocytogenes* strain by demonstrating the feasibility of the system in the cell culture system using a deletion mutant of *L. monocytogenes* $\Delta 2$ that lacks the entire lecithinase operon including the virulence-associated genes *actA*, *mpl*, and *plcB* [59]. This strain can infect macrophages and replicate in the cytoplasm but cannot spread to adjacent

cells. This attenuated mutant was introduced with a plasmid containing a gene for the lysis protein PLY118 of the listerial bacteriophage A118. PLY118 expression was controlled by the *actA* promoter, which is active when *L. monocytogenes* is in the host cell cytoplasm. Thus, this *L. monocytogenes* mutant escapes from the phagosome and then lyses when the PLY118 gene is expressed in the cytoplasm. Autolysis of the *L. monocytogenes* mutant apparently releases the plasmid DNA into the host cell cytoplasm, allowing the expression of the transgene in the host cells. We applied this system for DNA vaccines against *M. tuberculosis* by constructing self-destructing attenuated *L. monocytogenes* Δ2 strains carrying eukaryotic expression plasmids for the mycobacterial antigen 85 complex (Ag85A and Ag85B) and MPT51 [60]. Intravenous immunization of BALB/c mice by these *Listeria*-carrying DNA vaccines elicited significant protective responses against virulent *M. tuberculosis*.

However, these plasmids are lost from the carrier *Listeria* in vivo [61]. Pilgrim et al. [62] modified the *Listeria* system in order to stabilize the plasmid in the *L. monocytogenes* carrier strain. They constructed an *L. monocytogenes* strain that has the chromosomal deletion region compassing the *trpS* gene (encoding tryptophanyl-tRNA synthetase) and also the *actA* gene. Since the *trpS* gene is essential for bacterial viability, the *trpS*-deleted *Listeria* can maintain itself only in the presence of plasmids carrying the *trpS* gene. They constructed DNA vaccine plasmids containing the *trpS* gene in addition to the listerial autolysis cassette consisting of the lysis gene of phage A118 (ply118) under the control of the *actA* promoter, which is activated only in the cytoplasm of infected mammalian host cells. They reported no plasmid loss for more than 50 generations of *Listeria*. This new *Listeria*-carrying DNA vaccine allows cell-to-cell spread, which was much more efficient in DNA delivery than the nonspreading counterparts like the Δ2 listerial strain.

6. Dendritic Cell Vaccination

DC are the most powerful APC that initiate the primary immune response. They capture pathogens and apoptotic cells at the portal of entry sites in the body and then they migrate to regional lymphoid organs where they present antigens to naive T cells [63]. DC have a distinct ability to prime naive T cells. Therefore, DC-based vaccines have been powerful for tumors and infectious diseases.

DC vaccines have been examined for efficacy as vaccines against infectious diseases as well as cancer. There are several strategies for using DC as vaccines against intracellular bacteria, including ex vivo pulses with bacteria or bacterial antigens or the transfer of genes encoding antigens to DC. McShane et al. [64] showed that for immunization with DC pulsed with CD4+- or CD8+ T-cell epitope peptides in the *M. tuberculosis* antigen 85A (Ag85A), copresentation of both epitope peptides on the same DC was required for protection. Badovinac et al. [65] showed that vaccination with LLO 91–99 peptide-coated DC generated CD8+ T cells with the phenotype and function of memory cells in a short time (4–6 days after immunization) and that the early memory CD8+ T cells underwent vigorous secondary expansion in response to a variety of booster immunizations leading to elevated numbers of effector and memory T cells and enhanced protective immunity against *Listeria* challenge infection.

Retroviral transduction is advantageous for long-term antigen presentation in vivo because the transgene integrates into the chromosome leading to gene expression throughout the life of the cell and its progeny. In our previous study [66], we showed that DC vaccination retrovirally transduced with a minimal CTL epitope derived from *L. monocytogenes* successfully induced the specific CTL and protective immunity against lethal listerial challenge. We also found that the retrovirally transduced DC vaccine was more effective than a CTL epitope peptide-pulsed DC vaccine or a minigene DNA vaccine for eliciting protective immunity. We also evaluated retrovirally transduced DC vaccination with the *M. tuberculosis*-derived Ag85A gene [67]. The results indicated that DC vaccination successfully induced specific cellular immunity, including immune responses of CD4+ T cells and CD8+ CTL, as well as specific antibody responses. In the system, the de novo synthesized Ag85A proteins in the Ag85A gene-transduced DC are processed via the MHC class I pathway to induce specific CD8+ T cells. Specific CD4+ T-cell responses to the proteins may also be evoked through the uptake of the secreted proteins by APC or direct antigen presentation by Ag85A gene-transduced DC.

These results showed that DC vaccination efficiently induces CD8+ CTL, which have the capacity to protect vaccinated animals from pathogenic bacteria. DC vaccination would not be feasible for preventing acute infectious diseases because it is laborious and costly; however, DC vaccination could be a promising strategy against serious chronic infectious diseases.

7. Recombinant Virus Vaccination

Recombinant viral vector systems for gene therapy have been developed and their efficacy has been examined in gene delivery to DC and in direct immunization. Adenoviral vectors have been shown to deliver antigen genes to DC. For example, Wang et al. [68] reported that single intranasal immunization of BALB/c mice with Ag85A recombinant human adenovirus (type 5; Ad5) induced Ag85A-specific CD8+ and CD4+ T-cell responses and also provided protection against intranasal inhalation of *M. tuberculosis*. However, preexisting immunity against viral proteins expressed by the vector prevents effective immunization [69]. Therefore, immunogenicity of the rare adenovirus serotype 35 (Ad35) combined with Ad5 fiber knob (Ad35k5) were examined, and Ad5 fiber knob was found to be important for the immunogenicity in mice and Rhesus monkeys [70].

Retroviral vectors based on murine leukemia virus have been employed to express antigens in vivo. Splenic DC were found to contain injected proviral DNA and were able to efficiently present antigens to T cells [71], but the retroviral vectors only infect dividing cells and do not infect nondividing cells including DC. Therefore, antigen expression and succeeding immune responses would be limiting.

Lentiviral vectors have been shown to efficiently transduce a variety of nondividing cells [72, 73]. In addition, lentiviral vectors pseudotyped with minimal filovirus envelopes have been reported to increase gene transfer in murine lungs [74]. Third-generation self-inactivating lentiviral vectors have been excellent viral vectors because of their advanced safety profile and the presumed absence of preexisting antivector immunity, allowing in vivo administraion.

We showed that third-generation lentivirus vectors, which express the *M. tuberculosis* MPT51 antigen, efficiently induced cell-mediated immunity against pulmonary tuberculosis with intratracheal instillation [75]. We also showed that a single intratracheal MPT51 lentivirus administration was effective for inducing antigen-specific CD8$^+$ T-cell responses in the lung. Esslinger et al. [76] showed that lentiviral vector injection into the footpad of mice was capable of transducing regional DC which appeared in the draining lymph nodes and in the spleen. They showed that in vivo administration of lentivector was superior to the transfer of transduced DC or peptide/adjuvant vaccination in terms of both amplitude and longevity of the resultant CTL response. These results confirm the effectiveness of the lentiviral vector system for mucosal T cell-based vaccination.

8. Improvement in Immunization Regimen: Prime-Boost Immunization

Evaluation of vaccination has indicated that the repeated injection of the same vaccine has a limitation in terms of its overall immunological effects. Especially, DNA immunization has been reported to induce considerably strong immunological responses in the rodents, but not in the primates including human [77].

Instead of the repeated injection of the same vaccine, the heterologous prime-boost regimen including DNA vaccination, which is primed with naked DNA vaccination and boosted with recombinant viral vectors such as vaccinia virus and adenovirus, has been shown to evoke superior levels of immunity to DNA vaccine or recombinant virus alone [78]. The relatively low-level but persistent expression of immunogenic proteins in vivo by naked DNA vaccines has been suggested to be important for priming immunological responses and inducing enhanced cellular immunity [78]. Interestingly, Eo et al. [79] reported that mucosal immunological responses were optimal when animals were primed with the recombinant vaccinia virus vector and boosted with a naked DNA vaccine, which is an opposite approach compared to the regimen for systemic immunological responses. Strong immunization by recombinant virus vaccines may be necessary to give enough priming effects in the mucous membrane.

A variety of prime-boost regimens have been examined for *M. tuberculosis* infection [80]. Many investigators examined the regimens in which priming with DNA vaccines and boosting with other immunization strategies. Tanghe et al. [81] immunized C57BL/6 mice first with Ag85A DNA vaccine and then with recombinant Ag85A proteins and

showed that this regimen induced stronger specific IFN-γ responses and better protective immunity against *M. tuberculosis* i. v. challenge compared to Ag85A DNA vaccine alone. Similarly, ESAT6 protein boosting immunization increased immune responses by ESAT6 DNA vaccines [82]. McShane et al. [83] showed that priming immunization with ESAT6 and MPT63 DNA vaccines and boosting with modified vaccinia virus Ankara (MVA) harboring Ag85A gene (MVA85A) induced protective immunity against *M. tuberculosis* infection compared to BCG immunization in mice. Feng et al. [84] showed that priming with Ag85B DNA vaccine and boosting with BCG vaccine strengthened protective immunity against *M. tuberculosis* induced by BCG vaccine alone in mice. Skinner et al. [85] also reported that priming with ESAT6 and Ag85A DNA vaccines and boosting with BCG vaccine enhanced specific IFN-γ production from immune splenocytes compared with that by the DNA vaccine or BCG vaccine alone in mice. Furthermore, Romano et al. [86] showed that immunization of BALB/c mice with Ag85A DNA vaccine first and boosting with BCG vaccine induced stronger protective immunity against *M. tuberculosis* challenge than that by Ag85A DNA vaccine alone. These results demonstrated that DNA vaccine priming and BCG vaccine boosting enhanced immune responses induced by BCG vaccine alone.

The regimens in which BCG vaccine was used as a priming vaccine also have been tried. As the BCG vaccine has been injected to people all over the world, this regimen seems to be reasonable. Priming with BCG vaccine and intranasal boosting with MVA85A in mice enhanced Ag85A-specific CD4$^+$ and CD8$^+$ T-cell responses and strengthened protective immunity against aerosol *M. tuberculosis* challenge infection in mice [87]. This regimen was reported in humans. McShane et al. [88] reported that in volunteers who had been vaccinated 0.5–38 years previously with BCG, vaccination with MVA85A induced substantially higher levels of antigen-specific IFN-γ-secreting T cells and that at 24 weeks after vaccination, these levels were 5–30 times greater than in vaccinees administered a single BCG vaccination. Santosuosso et al. [89] reported that intranasal immunization of mice with recombinant Ag85A-expressing adenovirus after subcutaneous BCG immunization augmented Ag85A-specific CD4$^+$ and CD8$^+$ T-cell responses in the lung and protective immunity against intratracheal *M. tuberculosis* infection.

WHO have showed a list of TB vaccine candidates (TB vaccine pipeline: http://www.stoptb.org/retooling/). These TB vaccine strategies are based on the prime-boost regimens and the vaccine candidates are categorized into three vaccine groups, namely, (1) priming vaccines, (2) boosting vaccines, and (3) therapeutic vaccines after *M. tuberculosis* infection. Reports on the prime-boost regimens by these TB vaccine candidates have been publishing. Tchilian et al. [90] reported that priming with $\Delta ureC\,hly^+$ BCG and boosting with MVA85A induced protective immunity against *M. tuberculosis* infection in mice. The protective effects were much higher in $\Delta ureC\,hly^+$ BCG vaccination than that in parental BCG vaccination. MVA85A boost immunization enhanced Ag85A-specific T-cell responses, but did not affect bacterial numbers in the lung after *M. tuberculosis* aerosol infection.

9. Conclusions

A variety of immunization methods have been evaluated and reported to induce specific CD8$^+$ T cells against intracellular bacterial infections. Modified BCG vaccines have been examined to enhance CD8$^+$ T-cell responses. Naked DNA vaccination is a promising strategy to induce CD8$^+$ T cells. This method has a variety of advantages over conventional attenuated bacterial vaccination, including a relatively easy design and construction using recombinant DNA technology, relatively low cost, high stability, and safety. In addition to the naked DNA vaccination strategy, live attenuated intracellular bacteria such as *Shigella*, *Salmonella*, and *Listeria* have also been utilized as carriers of DNA vaccines in animal models. Furthermore, a strong and immediate induction of specific CD8$^+$ T cells is expected with DC vaccination. DC are the most potent antigen-presenting cells in the body. Immunization of DC pulsed with antigenic peptides or introduced with antigen genes by viral vectors such as retroviruses is a powerful strategy. Recombinant virus vaccination, especially recombinant lentivirus vaccination, has also been utilized to induce specific CD8$^+$ T cells. These strategies and combinations of different strategies (prime-boost immunization) have been examined for the efficient induction of intracellular bacteria-specific CD8$^+$ T cells.

Acknowledgments

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