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**A Dissertation**  
**for the Degree of Doctor of Philosophy**

**Development of adjuvants using cytokine and polymeric  
particles for efficient mucosal immunization**

점막면역백신의 효율증진을 위한 사이토카인 및 고분자

입자를 이용한 면역보조제의 개발

**February, 2016**

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# 농학 박사 학위 논문

## Development of adjuvants using cytokine and polymeric particles for efficient mucosal immunization

점막면역백신의 효율증진을 위한 사이토카인 및 고분자 입자를 이용한 면역보조제의 개발

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이 논문을 농학 박사 학위 논문으로 제출함.

2015년 12월

서울대학교 대학원 농생명공학부

이혜선

이혜선의 농학 박사학위논문을 인준함.

2016년 1월

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## Summary

Mucosal surfaces are a main portal of entry for not only nutrients but also many pathogens that are the cause of infectious disease in livestock animals. Immunization through mucosal routes may be more effective in terms of inducing protective immunity against mucosal pathogens. In addition, mucosal vaccines can offer needle-free delivery and improve accessibility, safety and cost-effectiveness in farm industry. However, challenge to successful mucosal vaccination include poor induction of mucosal immunity, the availability of safe and effective mucosal adjuvant and delivery systems. Recently, major efforts are made to develop new mucosal vaccines by selecting appropriate antigens with high immunogenicity, designing mucosal route of administration, selecting immune-stimulatory adjuvant molecules and developing appropriate delivery systems.

The aim of the present study is to develop an effective mucosal adjuvant and delivery vehicles to improve efficacy of the livestock mucosal vaccine, which is divided into two parts; i) Development of an oral cytokine vaccine adjuvant which was designed to be produced in *Lactococcus lactis* IL1403, ii) Development of mucoadhesive and mannan decorated thiolated polymeric microspheres, designed to target mucosal antigen presenting cells (APCs) as nasal vaccine delivery vehicles.

In study 1, a recombinant cytokine, IL-6-CKS9, was generated by conjugating an M cell-targeting peptide (CKS9) with c-terminus of the murine

interleukin 6 (IL-6), which facilitated enhancement of mucosal immune response. *Lactococcus lactis* IL1403, a food-grade strain of lactic acid bacteria (LAB) which is widely used in dairy industry, was used as a host cell to express and secrete the IL-6-CKS9 for a mucosal vaccine adjuvant. The recombinant *L. lactis* IL1403 secreting IL-6-CKS9 was orally administered with a model antigen protein, M-BmpB (*Brachyspira* membrane protein B conjugated with CKS9), to BALB/c mice for mucosal immunization. ELISA analyses showed consistent enhancement tendencies in induction of anti-M-BmpB antibody levels both with mucosal (IgA) and systemic (IgG) immune responses in IL-6-CKS9-LAB treated group compared with other groups tested by conducting mice immunization assays. In addition, the oral administration of model protein antigen with live LAB producing IL-6-CKS9 could induce both Th1 and Th2 type immune responses. Collectively, the results showed successful production and secretion of recombinant murine IL-6 with M cell-targeting moiety (IL-6-CKS9) from *L. lactis* IL1403 and demonstrated that the live recombinant LAB producing IL-6-CKS9 could have a potential to be used as an efficient adjuvant for peroral vaccination.

In study 2, mucoadhesive and mannan decorated microspheres, designed to target APC and were evaluated the efficiency of vaccine delivery carrier after intranasal immunization. The aim was to develop mannan-decorated mucoadhesive thiolated hydroxypropylmethyl cellulose phthalate (HPMCP) microspheres (Man-THM) that contain ApxIIA subunit vaccine – an exotoxin fragment, as a candidate for a subunit nasal vaccine against *A.*

*pleuropneumoniae*. For adjuvant activity, mucoadhesive thiolated HPMCP microspheres decorated with mannan could be targeted to the PRRs (pathogen recognition receptors) and mannose receptors (MR) of APCs in the respiratory immune system. The potential adjuvant ability of Man-THM for intranasal immunization was confirmed by *in vitro* and *in vivo* experiments. As following in a mechanistic study using APCs *in vitro*, it was found that Man-THM enhanced receptor-mediated endocytosis by the MR of APCs. *In vivo*, the nasal vaccination of ApxIIA-loaded Man-THM in mice resulted in higher levels of mucosal sIgA and serum IgG than mice immunized with the ApxIIA or ApxIIA-loaded THM due to the specific recognition of the mannan by the MRs on the APCs. Moreover, ApxIIA-containing Man-THM protected immunized mice when challenged with strains of *A. pleuropneumoniae* serotype 5. These results suggest that mucoadhesive Man-THM could be considered as a promising candidate for a nasal vaccine delivery system to elicit systemic and mucosal immunity that can protect from pathogenic bacteria infection.

**Key words:** Mucosal immunization, oral vaccine adjuvant, cytokine, M cell targeting peptide, nasal vaccine delivery carrier, mucoadhesive polymer, mannan

**Student number:** 2010-24116

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## List of Abbreviations

APCs: antigen-presenting cells

APP5: *A. pleuropneumoniae* serotype

ApxIIA: Apx toxin II fragment A

BAL: bronchoalveolar lavage

BALT: bronchoepithelium and lower respiratory tract

BCA: Bicinchoninic acid

CKS9: CKSTHPLSCM (M cell targeting peptide)

CLS: confocal microscopy

CT: cholera toxin

DCs: dendritic cells

DLS: dynamic light scattering

DIC: differential interference contrast

ELISA: enzyme linked immune sorbent assays

FA: Freund's adjuvant

FAE: follicle-associated epithelium

FDCs: follicular dendritic cells

FITC: fluorescein isothiocyanate

GALT: gut-associated lymphoid tissue

HEVs: high endothelial venules

HPMCP: hydroxypropylmethyl cellulose phthalate

HRP: horseradish peroxidase

i.m.: intramuscular

i.n.: intranasal

i.p.: intraperitoneal

LAB: lactic acid bacteria

MALT: mucosa-associated lymphoid tissues

Man-THM: mannan decorated thiolated HPMCP micropsphere

M-BmpB: *Brachyspira* membrane protein B conjugated with CKS9

MR: mannose receptor

mSC: membrane secretory component

NALT: nasopharynx associated lymphoid tissue

PFA: polyformaldehyde

PPs: peyer's patch

PRRs: pathogen recognition receptors

pIgR: polymeric Ig receptor

SIgA: secretory IgA;

SIgM: secretory IgM

TLR: toll like receptor

# Introduction

Mucosal surfaces constitute the largest and most important interface between the body and the outside environment. Most pathogens initiated their infection through access to the mucosal region of body. To maintain normal physiology, the mucosa must be prevented entry and dissemination of dangerous pathogens. Innate immune responses are important in preventing initial infections and adaptive immune responses play a key role in preventing infection from previously encountered pathogens (Pavot et al., 2012).

Mucosal vaccines can induce mucosal immunity, including antigen-specific secretory IgA production, to protect invasion by pathogens and to neutralize toxins at mucosal surfaces. Accordingly, effective mucosal vaccines can protect from pathogens before their invasion across mucosal barriers and neutralize toxins, ingested or generated in the mucosal space by pathogens (Pasetti et al., 2011). Administering vaccines through non-mucosal routes often lead to poor protection against mucosal pathogens, since such vaccines do not generate memory lymphocytes that migrate to mucosal surfaces (Lamm, 1997).

Although mucosal vaccination induces mucosa-tropic memory lymphocytes, few mucosal vaccines have been used clinically. Live vaccine vectors pose safety risks, whereas killed pathogens or molecular antigens are usually weak immunogens when applied to intact mucosa. Adjuvants can boost immunogenicity, however, most conventional mucosal adjuvants have unfavorable safety profiles. For example cholera toxin (CT) can induce efficient

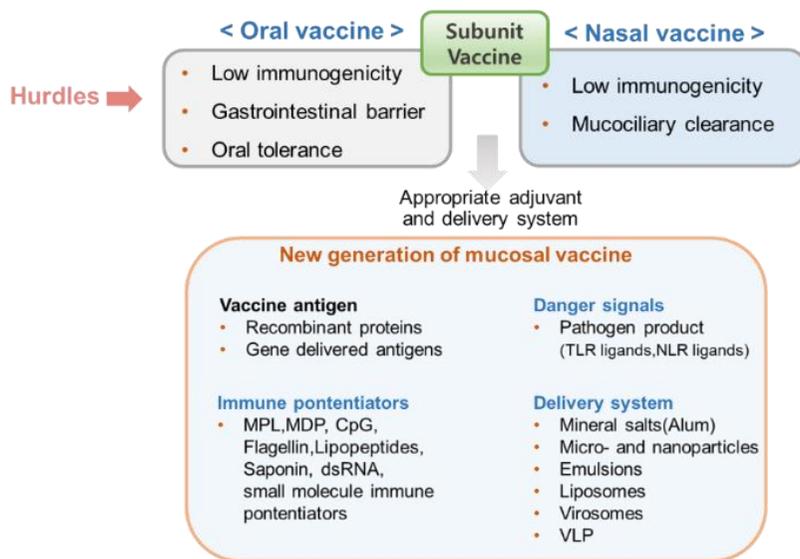
mucosal immune responses to antigens when they are co-administered, even when the antigens by themselves cannot induce good immune responses. However, in clinical trials it has been reported that CT can also induce adverse side-effects (Holmgren et al., 1993). Moreover, the immune mechanisms of protection against many mucosal infections are poorly understood.

Recently, subunit vaccine is an emerging safe vaccine strategy for vaccination because it could avoid potential harmfulness of whole-body vaccine due to renewal of infectivity, however the poor immunogenicity of subunit vaccine is one of its drawbacks to be overcome (Skwarczynski and Toth, 2011). Mucosal immunization with subunit vaccines has unique challenges associated with not only the immunogen but also the route of antigen delivery (Figure 1).

Oral vaccines need to resist harsh pH conditions, such as pH 1–2 in gastric acid in the stomach, and multiple digestive enzymes to reach mucosal immune tissues such as Peyer's patches (PPs) in sufficient amounts. One of the most significant problems associated with oral mucosal vaccines is possible tolerance induction against the orally introduced antigens, because it is generally known that oral administration of soluble proteins dominantly triggers oral tolerance (Cho et al., 2008). Tolerance induction by orally introduced vaccine materials translates into systemic unresponsiveness after challenge infection by the same antigen. To overcome this tolerogenic mucosal environment, many efforts have been made to develop effective mucosal

adjuvants that can stimulate both innate and adaptive immunities and are capable of inducing effective mucosal and systemic immune responses. (Figure 1)

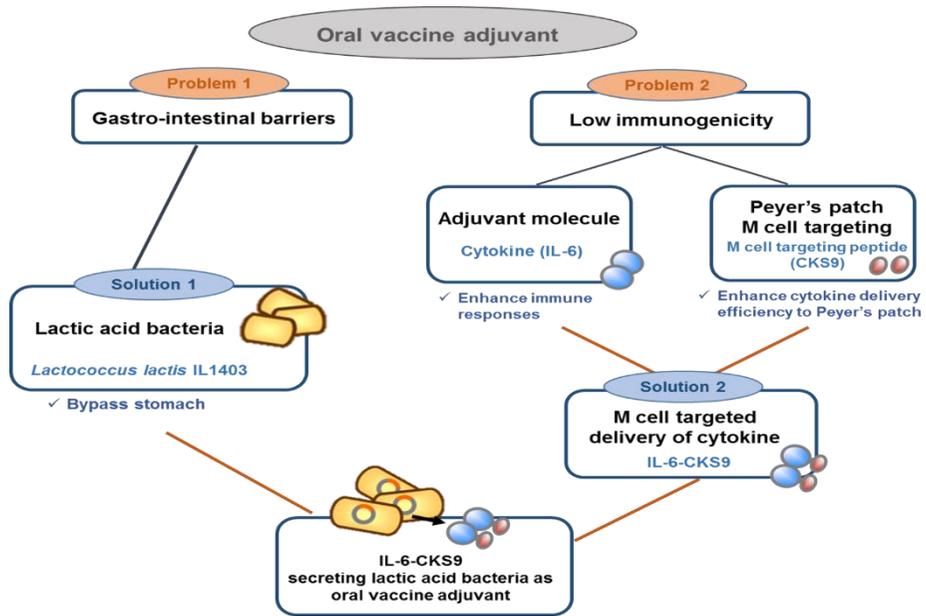
Nasal vaccines need to overcome the poor efficacy of nasally administered vaccines which is caused by the physiology of nasal cavity and the mucociliary clearance of respiratory tract. Increase the residence time of delivered antigen to retain long enough time for the interaction between antigen and the lymphatic system is an important aspect to be considered in development of nasal vaccines (Figure 1).



**Figure 1.** Appropriate adjuvants and delivery systems are needed for mucosal subunit vaccination

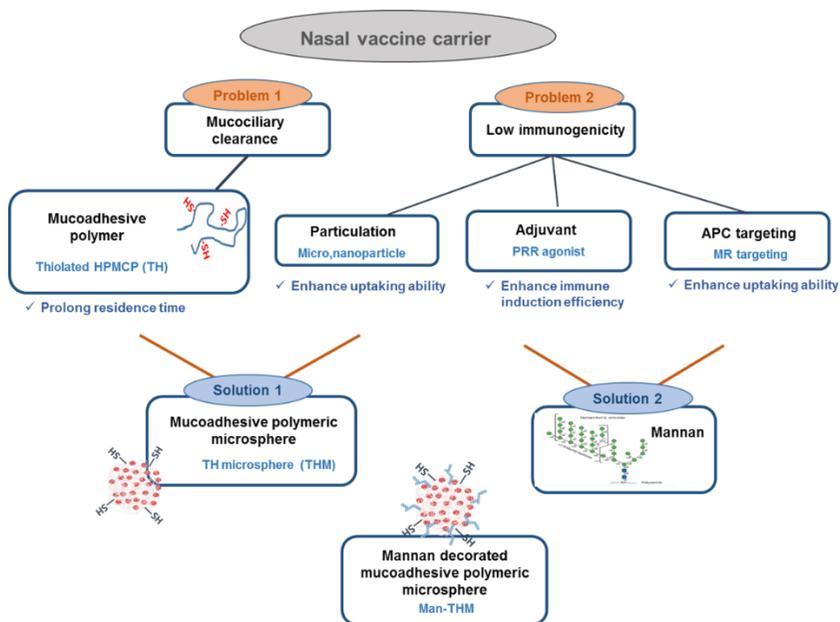
In this study, in order to develop an efficient mucosal vaccination through oral and nasal routes, an oral cytokine vaccine adjuvant named as 'IL-6-CKS9' and nasal vaccine delivery carrier named as 'Man-THM' were developed.

To overcome the main hurdles of oral subunit vaccine (Figure 1), Study 1 was mainly focused on developing vaccine adjuvant. A recombinant cytokine, IL-6-CKS9, was generated by conjugating an M cell-targeting peptide (CKS9) with c-terminus of the murine interleukin 6 (IL-6), which facilitated enhancement of mucosal immune response. *Lactococcus lactis* IL1403, a food-grade strain of lactic acid bacteria (LAB), was used as a host cell to express and secrete the IL-6-CKS9 for a mucosal vaccine adjuvant (Figure 2).



**Figure 2.** Strategy for developing oral vaccine adjuvant (study 1)

To overcome the main hurdles of nasal subunit vaccine (Figure 3), Study 2 was mainly focused on developing an efficient nasal vaccine carrier which was designed to hold mucoadhesive and APCs targeted properties. Mucoadhesive thiolated HPMCP microsphere (Man-THM) were decorated with mannan which could be targeted to PRRs and mannose receptors (MR) of antigen presenting cells (APCs) in respiratory immune system to obtain adjuvant ability (Figure 3).



**Figure 3.** Strategy for developing nasal vaccine carrier (study 2)

# **Chapter 1. Review of Literature**

## **1. Overview of mucosal immune system**

### **1) Mucosal immunity**

Mucosal membranes are huge surface covering the aero-digestive and the urogenital tracts as well as the eye conjunctiva, the inner ear and the ducts of all exocrine glands and they are susceptible to infection by pathogenic microorganisms. The mucosal surfaces of the gastrointestinal and respiratory tracts are the principal portals of entry for most pathogens. Direct inoculation of pathogens into the bloodstream is another important route of infection which goes with systemic immune responses (Holmgren and Czerkinsky, 2005).

Most external mucosal surfaces are distributed with organized follicles and scattered antigen-reactive or sensitized lymphoid elements, including B cells, T lymphocytes, T-cell subsets, plasma cells, and a variety of other cellular elements involved in the induction and maintenance of immune response. A remarkable fact is that a healthy human adult contributes almost 80% of all their immunocytes to mucosal immune system. These immunocytes are distributed in both largest lymphoid organ system and mucosa-associated lymphoid tissues (MALT) (Czerkinsky and Holmgren, 2012).

The MALT is a highly compartmentalized immunological system and it functions independently of systemic immune responses. The immunologic network operating on external mucosal surfaces consists of gut-associated lymphoid tissue (GALT), bronchus-associated lymphoid tissue (BALT), tonsils and nasopharynx associated lymphoid tissue (NALT), male and female genital tracts, mammary glands, and the products of lactation (Table 1). The organized lymphoid follicles in the GALT, NALT and BALT are considered as the principal inductive sites of mucosal immune response and appendix, peritoneal precursor lymphoid cells, and rectal lympho-epithelial tissue (rectal tonsils) also serve as inductive sites of local immune responses (Brandtzaeg et al., 2008).

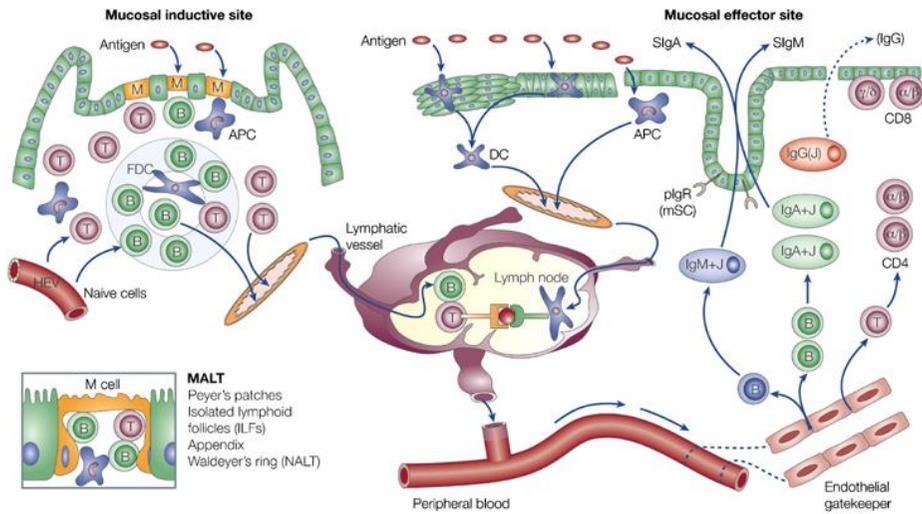
Inductive sites for mucosal immunity are constituted by regional MALT with their B-cell follicles and M-cell (M)-containing follicle-associated epithelium through which exogenous antigens are transported actively to reach APCs, including DCs, macrophages, B cells, and FDCs. In addition, quiescent intra- or sub-epithelial DCs may capture antigens at the effector site and migrate via draining lymphatics to local or regional lymph nodes where they become active APCs, which stimulate T cells for productive or down regulatory immune responses. Naïve B and T cells enter MALT and lymph nodes via HEVs. After being primed to become memory or effector B and T cells, they migrate from MALT and lymph nodes to peripheral blood for subsequent extravasation at mucosal effector sites (Figure 4) (Brandtzaeg, 2009).

The major antibody isotype in external secretions is secretory

immunoglobulin A (SIgA). Approximately 40 mg of IgA per kg of body weight is secreted daily, especially from the gastrointestinal tract, and the total amount of synthesized IgA is almost twice the amount of IgG produced daily in humans.

**Table 1.** Recommended nomenclature for mucosa-associated immune cell compartments (Brandtzaeg et al., 2008)

Preferred abbreviations	Explanations	Note
MALT	Mucosa-associated lymphoid tissue	The principal inductive sites for mucosal immune responses, subdivided according to anatomical location as below
GALT	Gut-associated lymphoid tissue	
NALT	Nasopharynx-associated lymphoid tissue	In humans, NALT consists of the lymphoid tissue of Waldeyer's pharyngeal ring, including the adenoids (the unpaired nasopharyngeal tonsil) and the paired palatine tonsils. Rodents lack tonsils, but have paired NALT structures dorsally in the floor of the nasal cavity
BALT	Bronchus-associated lymphoid tissue	Not generally present in the normal lungs of adult humans
PP	Peyer's patch	
LP	Lamina propria	Refers usually to the connective tissue of gut mucosa, restricted to the stroma above the muscularis mucosae (thus excluding the submucosa), but can also be used in relation to other mucosae
FAE	Follicle-associated epithelium	Covers the domes of MALT structures and contains variable numbers of M cell
ILF	Isolated lymphoid follicle	PPs and ILFs constitute the major part of GALT, but also the appendix is included although functionally less explored
IEL	Surface epithelium	Refers usually to the epithelium of the small intestine where most intraepithelial lymphocytes (IELs) occur
MLN	Mesenteric lymph node	
CLN	Cervical lymph node	Should be specified as deep or superficial



**Figure 4.** Depiction of the human mucosal immune system. (APCs, antigen-presenting cells; DCs, dendritic cells; FDCs, follicular dendritic cells; HEVs, high endothelial venules; MALT, mucosa-associated lymphoid tissue; mSC, membrane secretory component; pIgR, polymeric Ig receptor; SIgA, secretory IgA; SIgM, secretory IgM) (Brandtzaeg, 2009).

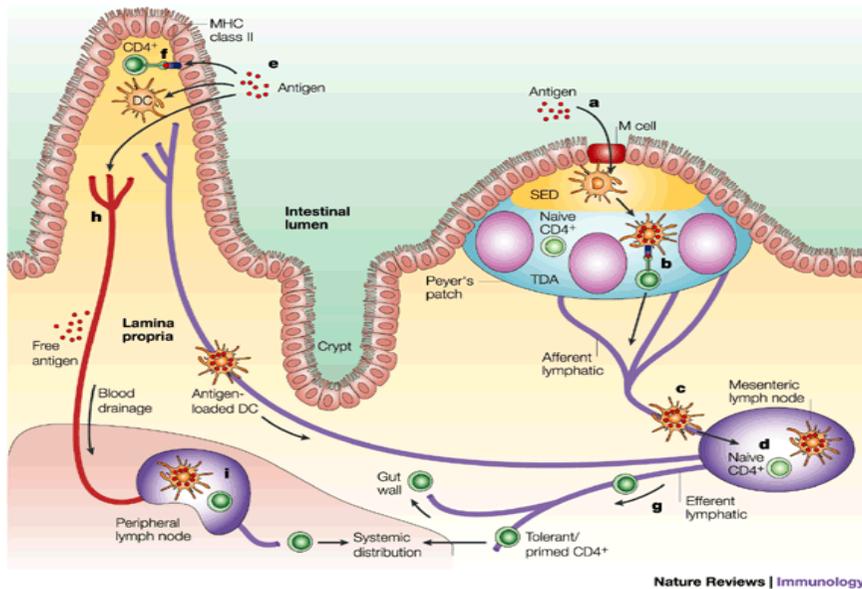
## **2) Gut-associated lymphoid tissue (GALT)**

The gut-associated lymphoid tissue described in the Figure 5 lines the digestive system and has two organizational levels to its structure: one with little organization, characterized by loose clusters of lymphoid cells in the lamina propria of the intestinal villi, and the other with a high level of organization, called Peyer's patches. The so-called intraepithelial lymphocytes (IELs) can be found in the outer mucosal epithelial layer, and the majority of these cells are CD8+ T-lymphocytes. Due to its localization, it is thought that this population of T cells may function to encounter antigens that enter through the intestinal mucous epithelium. The under layer of epithelia is the lamina propria, which contains large numbers of B cells, plasma cells, activated Th cells and macrophages in loose clusters (Mowat, 2003).

Peyer's patches, located in the submucosal layer underneath the lamina propria, contain 30–40 lymphoid follicles organized as macroscopic nodules or aggregates. In a similar way to what happens with lymphoid follicles in other sites, those from mature Peyer's patches can develop into secondary follicles with germinal centers, supported or connected by follicular dendritic cells. Parafollicular T-lymphocyte zones located between the large B-cell follicles present a large number of high endothelium venules, allowing cellular migration and lymphocytes' recirculation. Between the follicle-associated epithelium (FAE) and the organized lymphoid follicle aggregates, there is a more diffuse area known as the sub-epithelial dome (SED). The FAE is the

name given to the mucous membrane overlying the organized lymphoid follicles. The FAE is a small region characterized by the presence of specialized flattened epithelial cells called M cells. Together, the FAE, lymphoid follicles and associated structures form the antigen sampling and inductive sites of the mucosal immune system. It has been widely accepted that M cells are probably playing a key role in mucosal infection and immunity. It is thought that the main role of M cells is the sampling of antigens to transport them across mucosal epithelia to the underlying lymphoid tissues where protective immune responses are generated. In addition, M cells are a common route for complex antigens and pathogen invasion, for example, several invasive *Salmonella* species, *Vibrio cholerae*, *Yersinia* species, *Escherichia coli* and the polio virus. M cells have been identified in the epithelia of a variety of mucosal tissues and within the FAE of a wide variety of animal species, including laboratory animals (mice, rats, rabbits), domestic pets and humans. In mice and humans, M cells reside in about 10% of the FAE in contrast with 50% in the rabbit. In the gut, M cells are easily recognized by the lack of surface microvilli and the normal thick layer of mucus that characterizes the rest of the epithelial cells. Additionally, M cells contain a deep invagination similar to a pocket in the basolateral cytoplasmic membrane that contains one or more lymphocytes and occasional macrophages. The epithelium of the gut intestine provides an effective barrier to the entrance of most pathogens and particulates due to strong connections between epithelial cells called tight junctions. In contrast, the M cells can be exploited by microorganisms as the port of entrance for two reasons:

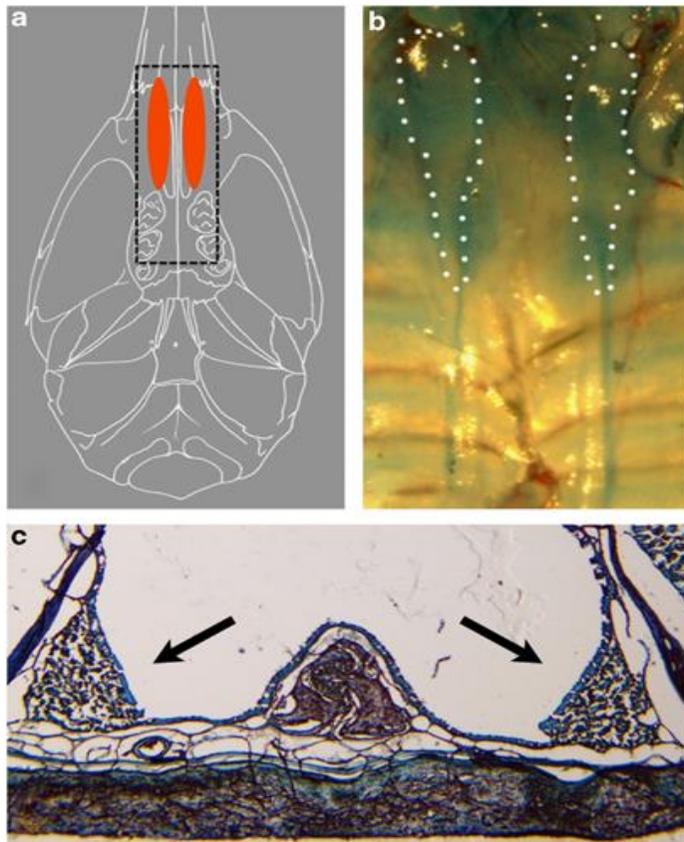
the organisms can adhere with less difficulty to the apical cell membrane, and after that, these agents need only be transported a short distance before reaching the M cell pocket, where by interaction with lymphocytes, the antigens or the particulates gain rapid access to the organized MALT inductive site (Borges et al., 2010).



**Figure 5.** Antigen might enter through the microfold (M) cells in the follicle-associated epithelium (FAE) (a), and after transfer to local dendritic cells (DCs), might then be presented directly to T cells in the Peyer's patch (b). Alternatively, antigen or antigen-loaded DCs from the Peyer's patch might gain access to draining lymph (c), with subsequent T-cell recognition in the mesenteric lymph nodes (MLNs) (d). A similar process of antigen or antigen-presenting cell (APC) dissemination to MLNs might occur if antigen enters through the epithelium covering the villus lamina propria (e), but in this case, there is the further possibility that MHC class II<sup>+</sup> enterocytes might act as local APCs (f). In all cases, the antigen-responsive CD4<sup>+</sup> T cells acquire expression of the  $\alpha 4\beta 7$  integrin and the chemokine receptor CCR9, leave the MLN in the efferent lymph (g) and after entering the bloodstream through the thoracic duct, exit into the mucosa through vessels in the lamina propria. T cells which have recognized antigen first in the MLN might also disseminate from the bloodstream throughout the peripheral immune system. Antigen might also gain direct access to the bloodstream from the gut (h) and interact with T cells in peripheral lymphoid tissues (i) (Mowat, 2003).

### **3) Nasopharynx-associated lymphoid tissue (NALT)**

In rodents, NALT is found on both sides of the nasopharyngeal duct, dorsal to the cartilaginous soft palate, and it is considered to be analogous to Waldeyer's ring in humans (pharyngeal lymphoid tissue that includes adenoid, tubal tonsil, palatine tonsil, lingual tonsil) (Figure 6). NALT is a well-organized structure consisting of B- and T-cell-enriched areas, which are covered by an epithelial layer containing M cells, the so-called follicle-associated epithelium (FAE). The function of these antigen-sampling M cells seems to be similar to those found on the FAE of Peyer's patches. Although NALT and Peyer's patches share certain similarities, the two differ markedly in morphology, lymphoid migration patterns and the binding properties of the high endothelial venules. Additionally, intraepithelial lymphocytes and antigen-presenting cells, including dendritic cells (DCs) and macrophages, can also be found in NALT. Therefore, NALT contains all of the lymphoid cells that are required for the induction and regulation of mucosal immune response to antigens delivered to the nasal cavity (Kiyono and Fukuyama, 2004).



**Figure 6.** Localization of the nasopharynx-associated lymphoid tissue (NALT) on the soft palate. (a) Schematic showing the paired NALT in relation to the palate and the molars. (b) Soft palate from a naive CBA/Ca mouse that was injected Evans blue to visualize the paired NALT (dotted lines). (c) Cross-section of the nasal cavity of a naive C57BL/6 mouse showing the triangular shape of the NALTs (arrows). NALTs are located on either side of the nasal airways, which are fully separated by the nasal septum in the posterior part of the nose. Giemsa-stained frozen section prepared after decalcification of the osseous tissue. Bar=100  $\mu$ m (Nacer et al., 2014).

## **2. Mucosal immunization**

### **1) Strategy for mucosal immunization**

#### **(1) Live attenuated or inactivated vaccines**

Vaccines based on live-attenuated viruses or microbes that have been inactivated by heat or chemicals comprise the majority of licensed vaccines used for the prevention of infectious disease (Chadwick et al., 2010). To date, these are the only vaccines approved for mucosal delivery and the only ones for which efficacy is correlated with effector mucosal immune responses (Ye et al., 2011). For examples, the oral polio vaccine is a live-attenuated vaccine that produces serum antibodies as well as local sIgA in the intestinal mucosa. The mucosal sIgA confers protection from poliovirus entry and multiplication. Several live-attenuated vaccines administered via the mucosal route are licensed for enteric infections such cholera, typhoid, and rotavirus. The success of live-attenuated and inactivated vaccines is attributed to the presentation of multiple immunogens and adjuvanting second signals that combine to elicit strong antibody and cellular responses and long-term memory (Table 2). However, not all viruses can be attenuated, and the risk of reversion can compromise safety, especially for viruses with ill-defined attenuation. Although inactivation of viruses and bacteria is a more generalizable approach and although these vaccines are much safer, inactivated vaccines can exhibit loss of antigens or PAMPs. This loss results in rapid waning of protective immunity

and causes the inactivated vaccines to be less effective than live-attenuated vaccines. Thus, a goal for synthetic or engineered mucosal vaccines is to identify the immunostimulatory factors of attenuated or inactivated microbes.

**Table 2.** Internationally licensed vaccines for human use against mucosal infections (Holmgren and Svennerholm, 2012)

Vaccines against	Administration route	Type
Cholera	Oral	Inactivated <i>V. cholerae</i> bacteria + CTB toxoid
	Oral	Inactivated <i>V. cholerae</i> bacteria
	Oral	Live attenuated <i>V. cholerae</i> O1 bacteria
Typhoid	Oral	Live attenuated <i>S. typhi</i> bacteria
	Parenteral	Purified Vi polysaccharide
Rotavirus	Oral	Live attenuated, mono-valent rotavirus
	Oral	Live attenuated, penta-valent rotaviruses
Influenza	Intranasal	Live attenuated, trivalent influenza viruses
	Intranasal	Similar to above
Polio	Oral	Live attenuated, trivalent polio viruses
	Oral	Live attenuated, bivalent polioviruses
	Oral	Live attenuated, monovalent polioviruses
	Parenteral	Inactivated, trivalent polioviruses Same grown in GMK cells

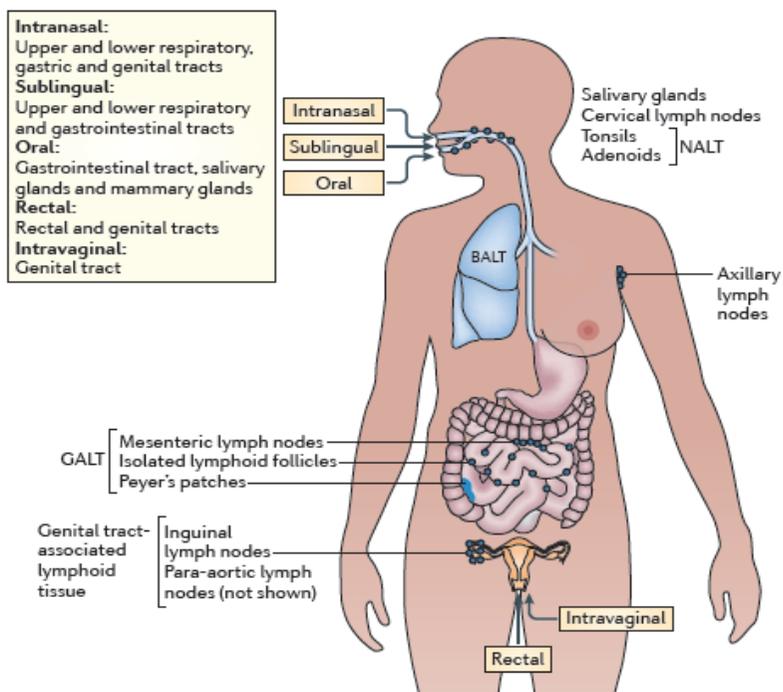
## (2) Subunit vaccines

Vaccines based on pathogen-specific proteins or based on polysaccharides conjugated to proteins or peptides are the second largest category of licensed prophylactic vaccines. In this category the toxoid vaccines, which are typically isolated and inactivated bacterial toxins designed to elicit immunity to the toxic compounds responsible for the disease (Schiller and Lowy, 2015). Examples of toxoid vaccines are the diphtheria toxin vaccine and tetanus toxin vaccine that are administered in combination during childhood as the DTaP (diphtheria, tetanus, pertussis) vaccine (Glanz et al., 2013). Subunit and conjugate vaccines as well as toxoid vaccines are administered primarily by subcutaneous or intramuscular routes and not mucosally, and only serological correlates of antibody protection have been established for these vaccines. One notable exception is DukoralR (Crucell, Netherlands), a vaccine against cholera that is composed of the cholera toxin B subunit and the inactivated strain of *Vibrio cholerae* O1 (Kraehenbuhl and R Neutra, 2013). Oral, but not parenteral, immunization with inactivated whole-cell cholera bacteria together with the cholera toxin B subunit protects against cholera colonization and toxin binding, respectively. Only the orally administered vaccine induces protective mucosal IgA antibodies against the bacterium and its toxin, and provides long-lasting intestinal immunological memory. There are no other examples of successfully licensed subunit vaccines that are administered by mucosal immunization and provide protection. Despite the paucity of successfully licensed subunit and conjugate vaccines for mucosal immunization, several promising studies

highlight the potential and importance of this approach for mucosal vaccine development. Lo et al. have explored the intranasal administration of soluble influenza hemagglutinin protein, which is functionally linked to a targeting peptide that is specific for Claudin-4 on M cells. They found that this soluble fusion protein was able to induce both a specific serum IgG and a mucosal IgA response (Lo et al., 2012). In a recent example by Ye and Zhu et al., a subunit vaccine consisting of the HSV-2 envelope glycoprotein fused to the IgG Fc fragment was delivered intranasally; it elicited systemic as well as mucosal B and T cell responses and conferred protection from intravaginal challenge with HSV-2 (Ye et al., 2011). Furthermore, repeated intravaginal immunization of recombinant HIV-1 envelope protein (gp140), without the use of a mucosal adjuvant, induces systemic and mucosal neutralizing IgG antibodies (Cranage et al., 2010). Mucosal immunization with subunit vaccines has unique challenges associated with not only the immunogen but also the route of antigen delivery. Mucus does not appear to hinder the diffusion of soluble proteins, and transport of large protein antigens should be possible. However, mucosal immunization with protein antigens is limited by the need to protect the protein antigens from degradation by mucosal proteases or commensal microflora. In addition, subunit vaccines are typically poorly immunogenic and require the use of adjuvants to be effective.

## 2) Mucosal vaccine delivery routes

Mucosal vaccination was initially dominated by oral vaccines. The development of intranasal vaccines followed, and today there are many different routes are explored for the delivery of mucosal vaccines, including aerosol inhalation and intravaginal, rectal and sublingual routes (Figure 7).



**Figure 7.** Mucosal immunization routes and compartmentalization of effector functions (Lycke, 2012)

**Table 3.** Comparative anatomical dissemination of secretory immunoglobulin A antibody responses after different routes of immunization (Czerkinsky and Holmgren, 2010)

	Nasal	Sublingual	Oral	Rectal	Vaginal
Upper respiratory	+++	+++	-	-	-
Lower Respiratory	+ to +++	+++	-	-	-
Stomach	-	+ /+++	+ /+++	-	-
Small intestine	-	+++	+++	-	-
Colon	-	?	+	++	-
Rectum	-	?	(+)	+++	-
Reproductive tract	+++	+++	-	-	++ /+++
Blood	+++	+++	+(+)	+(+)	+(+)

## **(1) Oral delivery of vaccine**

Oral delivery of vaccine is an attractive mode of immunization because of its acceptability and its simplicity of administration. The GALT play a central role in the induction of oral tolerance and immunity to infectious agents. In PP, DCs are separated from the intestinal lumen by the follicle-associated epithelium (FAE). It allows transfer of pathogens in lymphoid tissue through Microfold (M) cells. M cells represent a potential portal for oral delivery of peptides and for mucosal vaccination, since they possess a high transcytotic capacity and are able to transport a broad range of materials. Although the mucosal immune system comprises several anatomically remote and functionally distinct compartments, it is firmly established that the oral ingestion of antigens induces humoral and cellular responses not only at the site of antigen exposure but also in other mucosal compartments (Stagg et al., 2003). This is due to the dissemination of antigen-sensitized precursor B and T lymphocytes from the inductive to the effector sites.

Oral vaccines represent the biggest challenge for mucosal vaccine development. This is not only because of the harsh gut environment, which degrades most antigenic epitopes that are delivered in soluble form, but also due to mucosal tolerance, which protects against unwanted immune responses to digested antigens. A plethora of studies have reported success in experimental animals with oral vaccine delivery, although results in ensuing clinical trials have mostly been disappointing (Table 4). Attempts to develop

live attenuated oral vaccines are continuing, but stronger immunogenicity and better stability and safety profiles are required to launch new live mucosal vaccines, and this appears to be difficult to achieve.

**Table 4.** Oral delivery of vaccine (Pavot et al., 2012)

Delivery strategies	Examples	Responses	Model
PLA or PLGA nanoparticles	PLG-encapsulated CS6 antigen from <i>E. coli</i>	– IgA antibody-secreting cell responses ↗ – Serum IgG responses ↗	Human
PLA or PLGA nanoparticles	Lotus tetragonolobus from Winged or Asparagus pea anchored PLGA nanoparticles	– Mucosal and systemic response ↗	Mouse
Liposomes	Plasmid DNA pRc/CMV HBS encoding the small region of hepatitis B surface antigen (HBsAg) into Lipodine liposomes	– IgA responses ↗	Mouse
Liposomes	L-BLP25 (liposomal formulation of BP25 lipopeptide, MPL® and three lipids)	– Median survival time of 4.4 months longer	Human
Bacterial ghost	<i>Escherichia coli</i> O157:H7 bacteria ghosts	– Cellular and humoral immunity ↗	Mouse
Plant lectins/adjuvants	Mistletoe lectin 1, tomato lectin, Phaseolus vulgaris, wheat germ agglutinin (WGA), and Ulex europaeus 1	– Serum and mucosal antibody responses ↗	Mouse
Plant lectins/adjuvants	Rice-based vaccine that expressed cholera toxin B subunit	– Production of specific serum IgG and IgA antibody after three intra nasal or oral doses ↗	Mouse
Transgenic plants (bioreactors)	Plant-based rotavirus VP6	– High titers of anti-VP6 mucosal IgA and serum IgG ↗	Mouse
Transgenic plants (bioreactors)	Recombinant LT-B in transgenic corn	– Both serum IgG anti-LT and numbers of specific antibody secreting cells ↗	Human

↗: Increase

## **(2) Nasal delivery of vaccine**

Intranasal vaccination stimulates immune responses in the nasopharynx-associated lymphoid tissue (NALT) and is effective at inducing systemic and mucosal immunity in the gastric mucosa and the respiratory and genital tracts (Brandtzaeg, 2011). NALT lies in the roof of the nasopharynx in large mammals, at the caudal end of the pharyngeal septum. In rodents, the tissues are paired and lie on either side of dorsal surface of the hard palate. The NALT is easily overlooked, because macroscopically it frequently appears similar to the surrounding nasopharyngeal epithelium. Like GALT in all mammalian species, rodent NALT has a smooth surface of dome epithelium with M cells, while human palatine tonsils and adenoids have deep and branched antigen-retaining crypts with a reticular epithelium containing M cells (Brandtzaeg, 2003).

Intranasal vaccines delivered into the nostrils are an attractive mode of immunization and the nasal mucosa is a practical site for vaccine administration because of the absence of acidity, lack of abundant secreted enzymes and small mucosal surface area that result in a low dose requirement of antigen. Furthermore, the nose is easily accessible, is highly vascularized, can be used for the easy immunization of large population groups. It is well established that nasally administered vaccines can induce both mucosal and systemic immune-responses, especially if the vaccine is based on attenuated live cells or if the antigen is adjuvanted by the use of an immunostimulator or a delivery system. This data were confirmed after nasal immunization of humans against

diphtheria, tetanus (Alpar et al., 2001), influenza (Fiore et al., 2009) and infection with *Streptococcus* mutants (Childers et al., 2006). In addition, it has been described in animal studies, potent responses in the respiratory and genital tracts could be induced by intranasal immunization, as a consequence of the common mucosal immune system (Czerkinsky and Holmgren, 2012). For example, studies with cholera toxin B subunits showed that nasal mucosal immunization produced an exceptionally strong immune response in the respiratory and genito-vaginal tracts (Holmgren and Czerkinsky, 2005).

To be effective following intranasal delivery, the formulation should ensure stability of the antigen, retain long enough time for the antigen to interact with the lymphatic system, stimulate both the innate and cellular systems with or without the use of safe efficacious adjuvants, by targeting specific parts of the immune cells, and provide long-term immunity against the pathogen. Hence, it is of great importance that one considers carefully the choice of an absorption enhancer for a nasally delivered drug that is not readily absorbed especially in terms of potential nasal and systemic toxicity. Intranasal vaccines delivered into the nostrils are an attractive mode of immunization but we have to keep in mind that there are risks of passing into the brain through olfactory nerves and could induce important side effects.

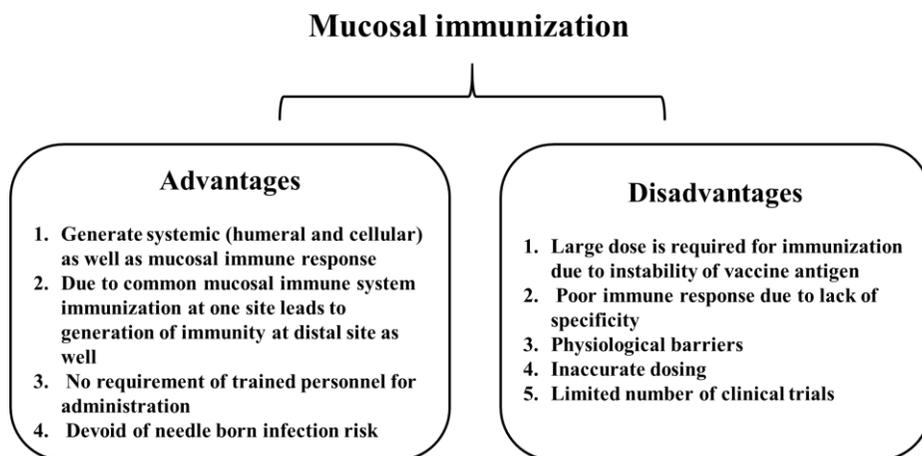
**Table 5.** Nasal delivery of vaccine (Pavot et al., 2012)

Delivery strategies	Examples	Responses	Model
Chitosan	Influenza, pertussis, and diphtheria	– Serum IgG responses similar to and secretory IgA levels ↗	Animals Human
Chitosan	N-trimethyl chitosan (TMC) nanoparticles	– Protection against the appropriate challenge	Mouse
Chitosan	The nasal diphtheria vaccine with chitosan	– Systemic T cell responses ↗ – Antigen-specific IFN-gamma production ↗ – Th2-type responses ↗ – Protective levels of toxin-neutralizing antibodies ↗	Human
Chitosan	Aminated gelatin microspheres (AGMS)	– Nasal absorption of insulin ↗	Rat
Cyclodextrins	Dimethyl-beta-cyclodextrin as adjuvants for nasally applied DT and TT	– Specific serum IgG titres ↗	Mouse
Liposomes	Liposomes incorporated insulin and coated with chitosan and carbapol	– Plasma glucose level up to 2 days ↘	Rat
Nanoparticles	Coated poly(anhydride) nanoparticles with either flagellin from <i>Salmonella enteritidis</i> or mannosamine	– Serum titers of IgG2a and IgG1 ↗ – TH1 and Th2 response ↗	Mouse
Nanoparticles	Nanoencapsulated reporter plasmid (encoding β-galactosidase protein)	– Antibody levels ↗	Mouse
Modified vaccinia virus Ankara (MVA) vector	MVA expressing HIV-1 Env IIIB Ag	– Immune response to the HIV Ag ↗ – Mucosal CD8(+) T cell response in genital tissue and draining lymph nodes ↗ – Mucosal IgA and IgG Abs in vaginal washings ↗ – Specific secretion of beta-chemokines ↗	Mouse

↗: Increase

### 3) Advantage and limitation of mucosal immunization

Mucosal vaccines are considered to be most suitable to combat rising and re-emerging infectious diseases transmitted through mucosal routes. Mucosal immunization induces secretory IgA (sIgA) and serum IgG responses to provide two layers of defense against mucosal pathogens. Some of the important advantages and limitations of mucosal immunization are shown in Figure 8.

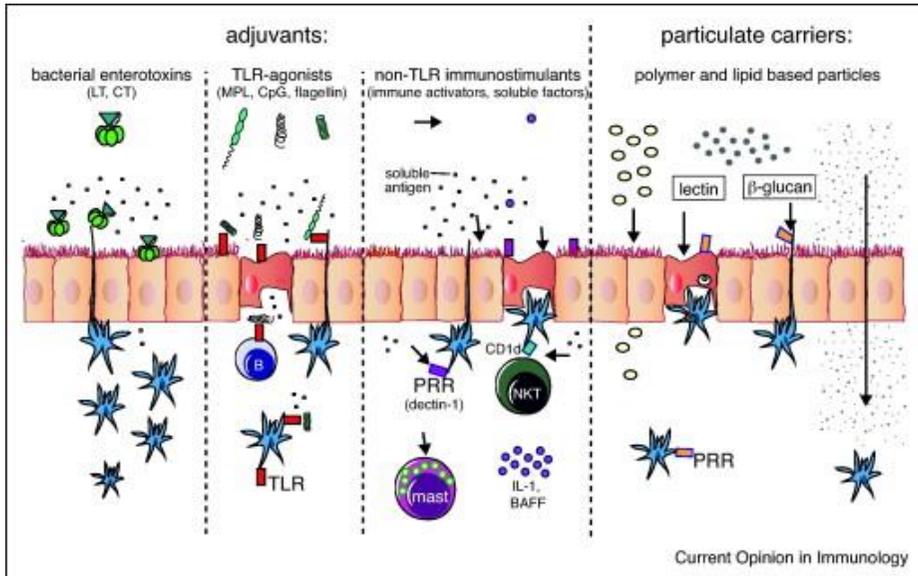


**Figure 8.** Advantages and limitations of mucosal immunization (Mishra et al., 2010)

### **3. Mucosal adjuvant**

#### **1) Adjuvant**

An adjuvant is a vaccine component that, through its capacity to act as an immunostimulant, induces and/or enhances an immune response to co-administered or co-delivered antigens. While there are many classes of adjuvants, not all are effective at promoting mucosal immune responses. In fact, alum, the most common adjuvant used in current human vaccines, is a poor inducer of mucosal immunity. The mucosal adjuvants with the most potential are derived from bacterial enterotoxins, TLR ligands, or small molecule, non-TLR immunostimulants (Figure 9).



**Figure 9.** Enhancing mucosal vaccines through adjuvant and particulate carrier formulations. Mucosal vaccine formulations are designed to enhance delivery and immune responses to antigen. Adjuvants often provide danger signals, which direct and stimulate the immune responses to co-administered soluble antigen. Examples include bacterial enterotoxins, TLR-agonists, and non-TLR immunostimulants. Particulate vaccine carriers facilitate delivery of encapsulated antigen and can promote interactions with immune-responsive cells at mucosal surfaces (Lawson et al., 2011).

## **(1) Bacterial enterotoxins**

Perhaps the best-studied mucosal adjuvants are the bacterially derived ADP-ribosylating enterotoxins, including cholera toxin (CT), heat-labile enterotoxin from *Escherichia coli* (LT), and their mutants or subunits. These enterotoxins promote induction of antigen-specific IgA antibodies and long-lasting memory to co-administered antigens when administered mucosally or transcutaneously (Freytag and Clements, 2005). However, safety issues have prevented full realization of the potential of this class of potent mucosal adjuvants. Intranasal immunization, even with low-toxicity mutants, can induce Bell's palsy (Mutsch et al., 2004), and oral administration with toxin derivatives has either induced unacceptable secretion or poor immunogenicity, as with the B-subunit alone. One promising candidate for oral, sublingual, rectal, or transcutaneous administration is the newly created double mutant LT (R192G/L211A), which in mouse studies results in equivalent antibody and cellular responses to co-administered antigen as does native LT, but without its associated toxicity (Norton et al., 2011). Other researchers are now exploring non-traditional delivery of LT-derived adjuvants, such as transcutaneous, sublingual or intrarectal administration routes (Hervouet et al., 2010). Additionally, a promising LT transcutaneous patch for travelers' diarrhea has been recently evaluated in a Phase II clinical trial (Frech et al., 2008).

## **(2) TLR agonists**

Another class of mucosal adjuvants is Toll-like receptor (TLR) agonists,

reviewed recently by Steinhagen et al. (Steinhagen et al., 2011). This diverse group of ligands activates innate TLR pathogen sensors, promoting intracellular signaling cascades that lead to cytokine secretion and immune cell activation. The first and only current usage of a TLR mucosal adjuvant in the US is in the recently FDA-approved human papillomavirus vaccine, Cervarix<sup>TM</sup>, by GlaxoSmithKline. This formulation incorporates the adjuvant system AS04, which is composed of alum with the TLR4 agonist MPL. MPL promotes a Th1-biased response towards co-administered antigens (Schwarz, 2009). Other MPL formulations for mucosal vaccines continue to be explored. For example, AS01, which contains liposomes, MPL, and saponin, enhanced systemic and mucosal immunity in a HIV vaccine study with non-human primates (Cranage et al., 2011).

Another promising mucosal adjuvant is the TLR9 agonist CpG. These small oligodeoxynucleotide sequences induce strong Th1 responses and have been effective in animal vaccine studies when delivered mucosally (Huang et al., 2008); however, in human subjects CpGs have only been investigated for cancer and systemic immunity in HIV patients or malaria, but not for induction of mucosal immunity.

Recent studies continue to elucidate the mechanisms of action of TLR-targeting adjuvants. Their mucosal efficacy often appears owing to a direct engagement with B-cells or other APCs. Direct TLR-stimulation enhances TI IgA class switching (Katsenelson et al., 2007). *In vitro* application of TLR2

ligands to circulating human B-cells stimulates IgA production while upregulating gut homing markers (Liang et al., 2011). Furthermore, production of autoreactive antibodies by CpG-stimulated B-cells suggests that TLR ligands could be a cause of, or potential therapeutic for, autoimmune diseases (Avalos et al., 2009). Ligand structure can also influence specific APC targeting; for example, CpGs preferentially activate B-cells, macrophages, or DCs depending upon their molecular structure. Lastly, studies have revealed both a TLR9 and TLR4 requirement for antimicrobial, autoimmune, or allograft Th17 responses (Marta et al., 2008), and the induction of IL-17A or Th17 cells after mucosal vaccination with MPL, flagellin, and LPS has been reported.

### **(3) Non-TLR immunostimulants (Immune activators, soluble factors)**

Other adjuvants that have shown evidence of or potential to work mucosally include a variety of small molecule, non-TLR immunostimulants. Many of these molecules activate innate immune sensors on specific cell types.  $\alpha$ -Galactosylceramide, a CD1d ligand and NKT cell activator, induced IgA in mice after an intranasal influenza vaccine (Lee et al., 2011) and an HIV vaccine given orally or intranasally (Courtney et al., 2009), but seems to suppress Th17 responses. Alternatively, there are several fungal or bacterially derived small molecules that will bind to antimicrobial receptors on APCs. For example, a dectin-1 agonist will enhance Th17 responses concurrent with IgA induction (Agrawal et al., 2010). Similarly, mast cell activators can promote Th17 cell development when given intradermally (McGowen et al., 2009) and enhance

B-cell proliferation and IgA production (Merluzzi et al., 2010). Lastly, M-cell targeting ligands fused to antigenic proteins increased M-cell uptake of the antigen and increased systemic and mucosal antibody responses (Kim et al., 2010). Other small molecule adjuvants mimic immune mediators. For example, recombinant IL-1 family cytokines, including IL-1 $\alpha/\beta$ , IL-18, and IL-33, increased IgA and IgG antibodies after an intranasal influenza subunit vaccine (Kayamuro et al., 2010). Similarly, a whole-cell killed *Pseudomonas* vaccine administered with adenovirus overexpressing BAFF enhanced systemic and mucosal antibody levels, reduced bacterial burden, and increased survival after challenge. But as a word of caution, outcomes following human and mouse studies frequently do not agree; an intranasal influenza vaccine, adjuvanted with type I interferon, demonstrated protection in mice but did not induce neutralizing antibodies in human volunteers (Couch et al., 2009).

As the complex nature of mucosal immune induction is understood, the mechanisms of action of current adjuvants are better defined, and promising new mucosal adjuvants are becoming available. With this knowledge, researchers are also exploring adjuvant combinations, including coadministration or molecular fusions (El-Kamary et al., 2010) as well as heterologous prime/boost regimens to optimize mucosal immunity (Glynn et al., 2005).

**Table 6.** Immunological considerations of known mucosal adjuvant (Newsted et al., 2015; Woodrow et al., 2012)

	Mucosal adjuvant	Target	Route of mucosal administration	Antibody	T cells	Cytokines and chemokines	Clinical testing
LT and CT	LT and mutant forms	GM1	Nasal, oral, sublingual, rectal	IgG1, IgG2, IgA	Th1/Th2, CD8	IL-6, IL-8, IL-10, IL-1( $\alpha\beta$ )	Yes
	CT and mutant forms	GM1	Nasal, oral, sublingual, vaginal, rectal	IgA, IgG1, IgE	Th2, CD8	IL-4, IL-5, IL-6, IL-10	Yes
TLR agonists	MPL	TLR4	Nasal, oral, sublingual, vaginal			IL-1, IL-17, IFN- $\gamma$	Yes
	CpG-ODN	TLR9	Nasal, oral, sublingual, vaginal, rectal	IgG2, IgA	Th1/Th2, CD8	IL-6, IL-12, IL-8, RANTES, MIP-1 $\alpha$ , MIP-1 $\beta$ , TNF- $\alpha$ , IFN- $\gamma$	Yes
	poly(I:C)	TLR3	Nasal, sublingual, vaginal				Yes
	Flagellin	TLR5	Nasal	IgG, IgA	Th1/Th2	TNF- $\alpha$ , IFN- $\gamma$ , MIP-2, IL-6	
	Pam <sub>3</sub> CSK <sub>4</sub>	TLR1/2	Nasal, sublingual				Yes
	FSL-1	TLR2/6	Nasal, sublingual, vaginal				
	R848	TLR7/8	Nasal, sublingual, vaginal				Yes
Cytokines	FLT3	Various tissues	Nasal, sublingual				Yes
	IL-1 family of cytokines	Various	Nasal, rectal				Yes
	Mast cell activators	Mast cells, GM1	Nasal				
Novel adjuvant	$\alpha$ -Galactosylceramide	CD1d, NKT cell	Nasal				
	c-di-GMP	Second messenger	Sublingual				

## **2) Carriers for mucosal delivery**

The promise of mucosal vaccines is that they can be designed to recapitulate the earliest cellular interactions with local APCs and mucosal follicles to generate local immune responses. In recent years, it was observed that innovative approaches to engineering delivery carriers to overcome mucosal barriers so that vaccines and adjuvants can be delivered to the oral, nasal, and urogenital mucosa. The carriers can be made of a variety of materials such as lipids, natural or synthetic polymers, inorganic materials to form particles and capsules of controlled size and architecture (Table 7) (Woodrow et al., 2012).

### **(1) Lipid based particles**

#### **(i) Liposomes**

Liposomes consist of a self-assembled phospholipid bilayer encapsulating an aqueous core and they are capable of establishing a single lipid bilayer or multiple bilayers separated by regions of water. There are several advantages to using a liposome-based nanoparticles in vaccine formulations as they trap and hinder drug degradation, they are capable of delivering drugs that possess poor solubility, they reduce the toxicity of drugs by shielding and they alter the targeting and distributing of the drug. These advantages would be especially beneficial for establishing control and utility of vaccine antigens in a mucosal environment. Furthermore, many antigen types can be considered compatible with liposome-based formulations as they can reside in the aqueous core,

peptides and DNA, or they can be embedded into the bilayer through insertion, adsorption or covalent binding. Liposomes are commonly endocytosed by antigen-presenting cells resulting in easy access to the carried antigen – immunomodulators or Toll-like receptors ligands – that promote the adjuvant activity of the liposome and aid in establishing a strong immune response, even with weaker antigens. The enhanced immune response after mucosal administration of antigen-containing liposomal vesicles is most likely attributed to their particulate nature. Fusogenic liposome vesicles are able to effectively merge with epithelia cells of the mucosa, to act as depot to delivery antigen to APCs for processing and presentation, and subsequently cause the induction of humoral immunity (Thérien and Shahum, 1989).

#### **(ii) Immune stimulating complexes (ISCOMs)**

ISCOMs appear as spherical, hollow, rigid, cage-like particles of about 40 nm in diameter with probably most of the antigens exposed on the surface. In many papers the capacity of ISCOMs to boost the immune response has been demonstrated. The applicability of ISCOMs is diverse. Apart from parenteral immunization, ISCOMs have been used for mucosal delivery of antigens. They are able to boost both humoral and cellular responses. Antigens from viral, bacterial and parasitic sources have been studied. ISCOMs are able to generate a broad range of immune responses. These include APC activation, increased MHC II expression on APCs, cytokine induction, especially IL-2 and IFN-gamma, CD4+ T-cell responses and CD8+ CTL responses. The type of immune

response that is generated after immunization with a particular ISCOM vaccine probably also depends on factors like the antigen, route and immunization schedule. This makes it hard to give general rules (Kersten and Crommelin, 2003).

### **(iii) Virus-like particles (VLPs)**

VLP and virosomes constitute a category of subunit vaccines wherein the immunogens are derived from viral components that self-assemble into higher-order three dimensional architectures that preserve the antigenic structure of virus immunogens. VLPs are formed from the self-assembly of one or more viral capsid or envelope proteins that are expressed recombinantly in mammalian or insect cells. The hepatitis B vaccine was the first commercially viable VLP-based vaccine. It is produced from the self-assembly of the hepatitis B surface antigen (HBsAg) expressed recombinantly in yeast cells. The human papillomavirus (HPV) vaccine has been the only other VLP since to be licensed for human use. The human papillomavirus (HPV) vaccine has been the only other VLP since to be licensed for human use. The quadrivalent HPV vaccine from Merck & Co. (GardasilR) is composed of the L1 capsid proteins of HPV-6, -11, -16, and -18 types that are expressed recombinantly in yeast and self-assembled into VLPs (Wheeler et al., 2009). However, VLP technology can be limited by difficulties of scale-up, the need for purification from the expression systems, and the requirement for adjuvants. Only virosomes have been used effectively without additional adjuvants (Moser et al., 2007), so they may be

attractive as mucosal vaccines. Although both VLPs and virosomes have great potential as vaccine carriers, they are harder to formulate and are less reproducible compared with entirely synthetic polymer nanoparticles.

#### **(iv) Archaeosome**

Archaeosome vesicles, composed of polar lipids extract from Archaea, have demonstrated strong systemic adjuvant activities. The mechanism of action of archaeosomes is attributed to MHC class I cross-priming via the phagosome-to-cytosol transporter associated with antigen processing-dependent classical processing pathway, and upregulation of costimulation by APCs without overt inflammatory cytokine production. Furthermore, they facilitate potent CD8<sup>+</sup> T cell memory to co-delivered antigen, comparable in magnitude and quality to live bacterial vaccine vectors. The archaeosomes also demonstrated a capacity to induce humoral as well as cell-mediated immune response in orally immunized mice relative to liposome vaccines, supporting the ability of archaeosome to successfully protect and deliver the antigen to an immune inducing site leading to a robust immune response (Krishnan and Sprott, 2008).

#### **(2) Polymer based particles**

The versatility of polymeric particles for mucosal vaccine design arises from the availability of different polymers and methods for particle synthesis. Common polymer compositions of nanoparticles include biodegradable or bioeliminable synthetic polymers [e.g., polyesters, polyanhydrides, and natural

polymers (chitosan, alginate and albumin), copolymers, and polymer blends (des Rieux et al., 2006). Both synthetic and natural polymer-based micro- and nanoparticle carrier systems are widely used in drug delivery applications and show the greatest versatility for designing effective mucosal vaccines (des Rieux et al., 2006). Particle size, composition (surface chemistry and polymer architecture), and ability to control the presentation of antigen and PAMPs have important roles in immune activation. Size plays a critical role in the amount of antigen that can be delivered as well as the manner in which the antigen is internalized and processed by the mucosal immune system. The selection of polymers and fabrication method can generate nanoparticles with a wide range of sizes and geometries. In addition to size, the surface chemistry and polymer composition of nanoparticles can be specifically engineered to overcome transport barriers, interact with tissues and cells, and promote.

**(i) Poly (esters)**

Poly (esters) are typically composed of lactic acid, glycolic acid, and the copolymers thereof various molecular weight and compositions. Poly (esters) materials have long history of safety records and hence FDA approved its applications in humans in the fields of medical and pharmaceutical. The use of biodegradable microspheres and nanospheres for vaccine development is a major area of activity in the utilization of biodegradable microspheres and nanospheres. PLGA, copolymer of poly(d, l-lactide-co-glycolide), are the most commonly employed polymeric materials and can be prepared by various

techniques such as oil-in-water emulsification, coacervation (polymer phase separation), spray drying and solvent evaporation. PLGA microparticles, used as antigen carriers, enhance antigen recognition and uptake by DCs localized in specialized mucosal-associated lymphoid tissue. (Anderson and Shive, 2012). Moreover, PLGA has an excellent controlled-release profile, excellent toxicological profiles. Woodrow *et al* showed that topical delivery of fluorescently labeled PLGA nanoparticles to the vaginal mucosa resulted in distribution and penetration of the particles throughout the local tissue (Woodrow et al., 2008). Cellular uptake and trafficking likely contributed to the observed tissue distribution. The surfaces of PLGA nanoparticles are easily modified to express tailored physiochemical properties that enhance particle diffusivity through the mucosa and transcytosis by mucosal M cells. One of the most important attributes of PLGA is that vaccine antigens can be encapsulated into the matrix or on the surface of PLGA carriers. Although most applications of PLGA nanoparticles have been in subcutaneous or intramuscular depot injections, many groups have turned their attention to studying PLGA nanoparticles as a possible delivery vehicle for mucosal immunization. Efforts have been made in delineating the optimal conditions for increased uptake of PLGA microspheres across the mucosal barrier (Alpar et al., 2005).

## **(ii) Polyethylenimine (PEI)**

Biodegradable NPs are also extensively explored as carriers for gene therapy. These NPs can improve transfection efficiency in target cells, and reduce

toxicity associated with gene delivery of viral vector. Polyethylenimine (PEI) has been used as a cationic polymer for the formation of complex with negatively charged DNA *in vitro* and *in vivo*. PEI-plasmid DNA complex exhibits extremely high transgene efficiency. This effect is believed to associate with its protection function of PEI against enzymatic degradation of DNA. Another effect of PEI, called 'proton sponge', indicates that pH buffering property of PEI could facilitate DNA endosomal escape. Bivas-Benita *et al.* evaluated the immunogenicity of a DNA vaccine encoding the *Mycobacterium tuberculosis (Mtb)* latency antigen Rv1733c for pulmonary delivery using PLGA-PEI NP. Their results revealed that the complex of PLGA-PEI NPs absorbed with Rv1733c DNA plasmid considerably increase T-cell proliferation and IFN- $\gamma$  production, compared to that given intramuscularly (Bivas-Benita *et al.*, 2009; Mann *et al.*, 2013).

### **(iii) Chitosan**

Chitosan is a linear polysaccharide polymer consisting of randomly distributed  $\beta$ -(1-4)-linked D-glucosamine and N-acetyl-D-glucosamine. Because of its mucoadhesive and permeation properties, chitosan is able to considerably enhance the adsorption and transportation of peptide cross mucosal epithelium. A number of studies have confirmed such capacities of chitosan to facilitate macromolecule transportation across mucosal barrier and to interact with mucosal tissue. There are several researches that show the efficacy of chitosan as an adjuvant and delivery system for mucosal vaccines.

The *Bordetella pertusis* filamentous haemagglutinin and recombinant pertusis toxins have been shown to induce strong antigen-specific systemic and mucosal immune responses after intranasal administration with chitosan. Diphtheria toxin nasally co-administrated with chitosan induced systemic and local immune responses (Kang et al., 2009).

### **(3) Nanoemulsion**

Nanoemulsion technologies are liquid suspensions that exhibit long-term colloidal stability and have been used to encapsulate and deliver vaccines directly onto mucosal surfaces. Nanoemulsion droplets are formed by the dispersion of two immiscible liquids. They range in size from 20 nm to 200 nm, which is similar to the size of opportunistic pathogens, and are readily taken up by mucosal M cells and subsequently presented to APCs. Table 7 presents an overview of the major classes of nanoemulsions. Briefly, water-in-oil emulsions incorporate and deliver hydrophilic drugs much more efficiently than do oil-in-water emulsions, which are used to incorporate and deliver hydrophobic drugs. Single-nanoemulsion technology has been successfully employed in the generation of a hepatitis B vaccine. The HBsAg nanoemulsion system generated a strong immune response, producing high titers of both IgA and IgG; this strong response indicates the usefulness of nanoemulsion for NALT mucosal immunization. Unfortunately, single-nanoemulsion methods have poor controlled-release profiles and may not be able to withstand degradation within mucosal sites other than NALT. Hanson et al. introduced the concept of

a double-emulsion method with good controlled release profiles. Double-emulsion technology has proven useful for delivering vaccines to mucosal surfaces before they are degraded (Hanson et al., 2008). Additionally, double emulsions are more stable and are able to encapsulate antigens without deleterious effects to the antigen during the emulsification process. Nanoemulsion technology has provided a novel delivery method for immunizing the mucosal immune system.

#### **(4) Inorganic nanoparticles**

Many inorganic nanoparticles have been studied for vaccines applications. Their advantages come from their rigid structure and controllable synthesis processes (Kalkanidis et al., 2006). Gold NPs (AuNPs) offer unique characteristics, such as biocompatibility, easy fabrication into different shapes (spherical, rod, cubic, etc.) and sizes (2 – 150 nm), and can be readily surface-modified with carbohydrates (Zhao et al., 2014) making them an attractive choice for mucosal vaccine delivery. Tao *et al.* investigated the potential of a novel influenza A vaccine composed of M2e–AuNPs, conjugated with CpG adjuvants and administered intranasally in mice. It was found that M2e-specific IgG serum antibodies were induced by vaccination containing CpG. Furthermore, mice that received soluble CpG-adjuvanted M2e–AuNP were fully protected against the challenge lethal PR8 (Tao et al., 2014).

## **(5) Live vectors**

Live recombinant bacteria or viral vectors that express heterologous antigens are being extensively investigated in the development of vaccines and it is believed that these will provide an optimum immune response against the expressed antigen. Examples of live vectors being utilized in this way include *Salmonella typhi*, BCG, *Shigella flexnerii*, *Lactococcus lactis*, *Lactobacillus casei*, retroviruses, adenoviral, and so on (Moingeon et al., 2002).

Two attenuated *Salmonella* serovars are the most intensively used bacterial vectors, *Salmonella typhimurium* in mice and *Salmonella typhi* in humans. Harokopakis *et al.* investigated the immunogenicity of a chimeric protein comprising the binding domain of a streptococcal protein adhesin linked to the cholera toxin (CT) A2 and B subunits (CTA2/B) in *S. typhimurium* BRD509 via oral or intranasal immunization of mice (Harokopakis et al., 1997; Mielcarek et al., 2001).

Lactic acid bacteria (LAB) are a diverse group of Gram-positive, nonsporulating, low G+C content bacteria. Many of them have been given generally regarded as safe status. Over the past two decades, intensive genetic and molecular research carried out on LAB, mainly *Lactococcus lactis* and some species of the *Lactobacillus* genus, has revealed new, potential biomedical LAB applications, including the use of LAB as adjuvants, immunostimulators, or therapeutic drug delivery systems, or as factories to produce therapeutic molecules. LAB enable immunization via the mucosal route, which increases

effectiveness against pathogens that use the mucosa as the major route of entry into the human body. Many studies on the effectiveness of various representatives of the LAB group as carriers of heterologous antigens for vaccination were conducted with the highly immunogenic tetanus toxin C-terminal fragment (TTFC) as antigen. This protein fragment has been intensively studied as a replacement for inactivated toxin in the combined diphtheria, tetanus, and pertussis (DTP) vaccine (Grangette et al., 2004).

Adenoviral vectors have received considerable attention for vaccine development because of their high immunogenicity and efficacy. The adenoviral backbone is a type 1 immune adjuvant enhancing the immune response toward the foreign antigen and of all vector platforms, it derives the strongest transgene expression with continuously raised levels of protein *in vivo* for up to 3 weeks. The adenoviral vector triggers potent transgene product-specific CD8<sup>+</sup> T-cell responses and to a lesser extent CD4<sup>+</sup> T-cell responses. Ad5-based vaccines have been developed for several infectious diseases, including malaria, HIV, influenza and Ebola virus disease, with demonstrated safety, immunogenicity and efficacy (Majhen et al., 2014).

**Table 7.** Particulate carriers commonly employed to deliver vaccine antigen to mucosal sites (Woodrow et al., 2012)

	<b>Particulate carrier type</b>	<b>Vaccine characteristics</b>	<b>Advantages</b>	<b>Disadvantages</b>	<b>Route</b>
<b>Lipid based particles</b>	<b>(1) Liposome</b>	Water-insoluble drugs Water-soluble drugs Proteins DNA	Easy surface modification Synthesized from nontoxic material Dual function Wide range of antigen encapsulation	Low antigen loading Low stability	Oral, Nasal
	<b>(2) ISCOMs</b>	Proteins	Adjuvant itself	Reactogenicity	Nasal
	<b>(3) Archaeosome</b>	Proteins	Adjuvant itself	Reactogenicity	Nasal
	<b>(4) Virus like particles</b>	Plasmid DNA Proteins Peptides	Lacks viral genes Highly immunogenic High rate of uptake Undergoes self-assembly	Formulated by recombinant technology	Oral, Nasal
<b>Polymers</b>	<b>(1) Poly (esters)</b>				
	PLGA	Plasmid DNA Protein Peptide Low-molecular-weight molecules	Controlled release Sensitive to environment Stable microenvironment Biocompatible	Low loading efficiency Degradation of antigen during encapsulation	Oral, Nasal, Intravaginal
	PLA	Plasmid DNA Protein Peptide Lipophilic compound	Controlled release Surface easily modified	Low loading efficiency Degradation of antigen during encapsulation	Oral, Nasal
	<b>(2) Polyethylenimine</b>	Plasmid DNA	Efficiently transfected	Cytotoxicity	Nasal, Oral
<b>(3) Chitosan</b>	Plasmid DNA Protein Peptide	Mucoadhesive Non-toxic Bio-degradable facilitate macromolecule transportation across mucosal barrier		Oral, Nasal	

	<b>Particulate carrier type</b>	<b>Vaccine characteristics</b>	<b>Advantages</b>	<b>Disadvantages</b>	<b>Route</b>
<b>Emulsion</b>	<b>(1)Water-in-oil</b>	Th1-stimulating antigens	Slow release of antigen	Reactogenicity	Oral, Nasal
	<b>(2)Oil- in-water</b>	Th2-stimulating antigens	Slow release of antigen	Reactogenicity	Oral, Nasal
<b>Iorganic nanoparticles</b>	<b>AuNPs, Cochleate</b>	Protein	Biocompatibility Easy fabrication into different shapes (spherical, rod, cubic, etc.) Surface easily mofied		Nasal
<b>Live vectors</b>	<b>(1) Bacteria</b> <i>Salmonella typhi</i> , BCG, <i>Shigella flexnerii</i> , <i>Lactococcus lactis</i> ,etc	Recombinant bacteria (vaccine antigen containing)		Safety issue, Transmissibility and longterm persistence in the host	Oral, Nasal
	<b>(2) Viral vector</b> Retroviruses, adenovus			Safety issue Disease-causing potential, Transmissibility and longterm persistence in the host	Oral, Nasal

# **Chapter 2. Recombinant interleukin 6 with M cell-targeting moiety produced in *Lactococcus lactis* IL1403 as a potent mucosal adjuvant for peroral immunization**

## **1. Introduction**

Lactic acid bacteria (LAB) are fascinating recombinant hosts for expression of therapeutic molecules such as antigens, cytokines, and enzymes, *etc.*, because they could survive from stomach acid and protect the producing molecules from enzymatic degradation during passage through the gastrointestinal tract, and LAB themselves only induce minute level of immune responses to host animals following ingestion as ‘generally recognized as safe (GRAS)’ microorganisms. Thus, numerous researches were conducted to use LAB as a live oral delivery vehicle for therapeutic protein molecules and *Lactococcus lactis*, a food-grade strain of lactic acid bacteria (LAB) which is nonpathogenic, noninvasive, and noncolonizing bacteria, might be the most frequently used LAB host for this purpose (Bermúdez-Humarán, 2009; Wells and Mercenier, 2008). For examples, a genetically engineered recombinant *L. lactis* producing TTFC (tetanus toxin fragment C) showed successful results as

an oral vaccine (Grangette et al., 2002). Co-expression of IL-2 or IL-6 with the model antigen TTFC in *L. lactis* and its mucosal administration could enhance mucosal and systemic immune responses in mice (Steidler et al., 1998).

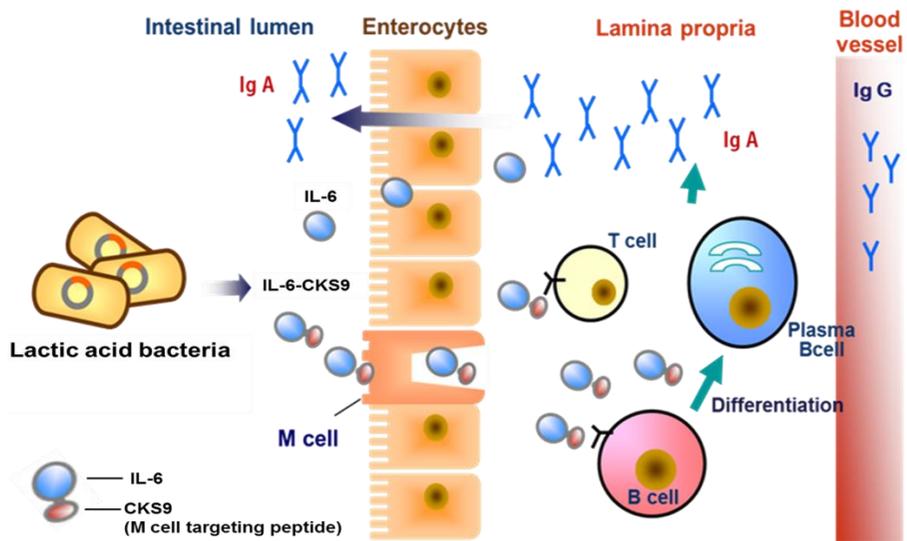
Most animal pathogens initiated their infection through access to the mucosal region of body. Thus, effective mucosal vaccine is necessary in the aspect that it could protect the host body from the pathogens at the initial site of infection by induction of mucosal immunity (Lycke, 2012). However, development of mucosal vaccine, especially oral vaccine, is challenged because of harsh condition of gastrointestinal environment to vaccine stability. Recently, subunit vaccine has been regarded as a safe vaccine strategy to avoid potential harmfulness of whole-body vaccine due to renewal of its infectivity though the poor immunogenicity is one of its drawbacks must be overcome (Coller et al., 2011). Therefore, exploration of the effective mucosal adjuvants is inevitable to improve availability of the subunit vaccine.

There are many classes of adjuvants, but not all are effective in mucosal immune responses. Such as, alum, the most common adjuvant used in current vaccines, is not effective in mucosal immunity. At present, bacterial enterotoxins, CT (cholera toxin), LT (heat-labile enterotoxin), TLR (toll-like receptor) ligands, cytokines, and non-TLR immune stimulants, *etc.* are classified as potential mucosal adjuvants (Lawson et al., 2011). Interleukin 6 (IL-6) is a multifunctional cytokine conducting significant roles in both innate

and adaptive immune responses, which produced by both pro-APCs (B cells, macrophages, and dendritic cells) and non-professional APCs (epithelial, endothelial, and some tumor cells) (Akira, 1993; Taga and Kishimoto, 1997). In innate immune response, as a pro-inflammatory cytokine, it is associated with up regulation of acute phase protein. In adaptive immune response, as a B cell proliferation factor, its function is associated with final differentiation of the plasma B cells (La Flamme et al., 2000). Besides, IL-6 mediates proliferation of lymphocytes, regulation of IL-2 receptor, and Th1-associated cytokine expression (Su et al., 2008). There have been several reports about successful application of IL-6 as a mucosal vaccine adjuvant. As mentioned above, co-expression of IL-6 with TTFC in *L. lactis* and its nasal administration enhanced mucosal and systemic immune responses in mice (Steidler et al., 1998). Co-expression of one of IBV (infectious bronchitis virus) antigen with chicken IL-6 in *Mycoplasma gallisepticum* and its oral vaccination showed specific mucosal immune response in chicken (Shil et al., 2011). Moreover, IL-6 also performed as an effective adjuvant in DNA vaccine for FMDV (foot and mouth disease virus) (Su et al., 2008).

Peyer's patches are 'gut associated lymphoid tissue (GALT)' and M cells are antigen-collecting portals located on the 'follicle associated epithelium (FAE)' of Peyer's patches, which facilitate to transport antigens from gut lumen to underneath mucosal immune system. Thus, M cell-targeting might be one of

the promising strategies to develop an effective oral vaccine (Lelouard et al., 2012). An M cell-targeting peptide ligand, CKS9, consisted of 9-amino acids with cyclic conformation was identified via phage display screening in our previous research (Yoo et al., 2010). In this study, we developed a novel M cell-targeting cytokine adjuvant system for oral vaccine by generation of recombinant *L. lactis* producing murine IL-6 conjugated with CKS9. We hypothesized that this live recombinant LAB could enhance mucosal immunity in gastrointestinal tract by secretion of IL-6 with M cell-targeting moiety and its specific delivery to the mucosal immune system through the M cells, when they orally administered with vaccine. M-BmpB (*Brachyspira* membrane protein B conjugated with CKS9) was adapted as a model antigen in this study. *Brachyspira hyodysenteriae* is one of the pathogenic bacteria causing a mucohemorrhagic dysentery in pigs and the BmpB, *Brachyspira* outer membrane lipoprotein B, was reported as an antigen which could induce protective antibody against *B. hyodysenteriae* for subunit vaccine (Hampson et al., 2000; Lee et al., 2000). This study demonstrated that the construction of cytokine expression vector system for LAB, *in vitro* functional validation of the recombinant cytokine, and *in vivo* efficacy of the recombinant LAB as an oral vaccine adjuvant system.



▪ **Experimental flow chart**

<b>Characterization</b>	<ul style="list-style-type: none"> <li>▪ Vector construction</li> <li>▪ Confirmation of the expression of IL-6s</li> </ul>
<b>In vitro</b>	<ul style="list-style-type: none"> <li>▪ Physiological characterization of recombinant LAB</li> <li>▪ Biological activity validation of the recombinant IL-6s</li> </ul>
<b>In vivo</b>	<ul style="list-style-type: none"> <li>▪ Evaluation of recombinant IL-6s-producing LAB as an adjuvant for mucosal immunity</li> <li>▪ Investigation of the occurrence of oral tolerance</li> </ul>

**Figure 10.** Experimental flow of the study 1

## 2. Materials and methods

### 1) Construction of expression vector system for IL-6s

To construct mouse IL-6 gene expression vector system for *L. lactis*, pILPtuf, in which includes, *tuf*, one of the strongest constitutive promoters (Figure 15), was adapted as a mother plasmid, which was previously constructed by our research group (Kim et al., 2009). We generated the mouse IL-6 gene fragments with or without nucleotide sequences encoding Usp45-secretion signal peptide (Borrero et al., 2011) and the M cell-targeting peptide, CKS9, through the oligonucleotide-assembly PCR using a series of synthetic overlapped oligonucleotides (IDT Inc., Belgium), which were computationally designed as approximately 60-base-pair long, according to a previously described method (Hoover and Lubkowski, 2002). Three resulting gene fragments, Usp45-IL-6 (651 bp), Usp45-CKS9-IL-6 (696 bp), and Usp45-IL-6-CKS9 (699 bp), were digested by *NdeI* and *XhoI* restriction enzyme, then introduced into same restriction enzyme sites of the pILPtufMb plasmid, respectively. The amino acid sequence of mouse IL-6 was obtained from GenBank (NM-031168.1) and codon usage of each gene fragment was optimized to facilitate the translation of eukaryotic cytokine-coding gene (mouse IL-6) in prokaryotic host (*L. lactis* IL1403). A web-based analysis program, DNA work 3.1, was used for oligonucleotide design and codon optimization (Hoover and Lubkowski, 2002). The schematic diagram of the synthetic gene fragments and expression vector

system were shown in Figure 11(A).

## **2) Confirmation of the expression and secretion of IL-6s from LAB**

Each expression vector, pILPtuf-IL-6, pILPtuf-CKS9-IL-6, or pILPtuf-IL-6-CKS9, was transformed into *L. lactis* IL1403 by electroporation, respectively. To confirm the expression and secretion of IL-6s from *L. lactis* IL1403, intracellular protein and secreted protein fractions were separately prepared as previously described (Mathiesen et al., 2009). The expression and secretion of IL-6s from *L. lactis* IL1403 were analyzed by Western blot using rabbit-anti-mouse IL-6 antibody (Millipore, USA). The accumulation levels of secreted IL-6s in culture supernatants were assessed by ELISA kit (R&D systems, USA) with the lapse of time during the cultivation of transformants.

## **3) Biological activity validation of the recombinant IL-6s**

In order to validate biological activity of the recombinant IL-6s secreted from *L. lactis* IL1403, a murine hybridoma cell line, 7TD1 (RIKEN cell bank, Japan), of which proliferation efficiency could be prominently improved under IL-6 signal, was adapted as an *in vitro* model bioassay system in this study (Shil et al., 2011). Briefly, the 7TD1 cells were cultivated with RPMI 1640 media (HyClone, USA) containing 5% (v/v) FBS (GIBCO, USA), 25  $\mu$ M 2-mercaptoethanol, and 0.5 ng/ml of commercial mouse IL-6 (Millipore, USA). Prior to bioassays, the 7TD1 cells were washed with IL-6 free RPMI 1640

media twice and, incubated for an additional 48 h without IL-6. Subsequently the starved 7TD1 cells were divided by aliquots of  $1.0 \times 10^4$  cells, then bioassays were conducted by cultivation of each 7TD1 cell aliquot at various concentrations (0.2 pg/ml, 1 pg/ml, 5 pg/ml, 25 pg/ml, 125 pg/ml or 625 pg/ml) of commercial IL-6 or secreted IL-6s from *L. lactis* IL1403 at 37 °C for 48 h. After incubation, CellTiter96 Aqueous One Solution (Promega, USA) was added to the each well and incubated for an additional 4 h, then optical density of colored solution was measured at 490 nm using a microplate reader (Tecan Infinite® 200 PRO;Mannedorf, Switzerland) and all the assays were performed in triplicate.

#### **4) Closed-ileal loop assay and immunohistochemistry**

Closed-ileal loop and immunohistochemistry assays were conducted to verify M-cell targeting property of the recombinant IL-6s secreted from *L. lactis* IL1403 according to the method previously reported by our research group (Yoo et al., 2010). Briefly, 8 week-old female BALB/c mice (Samtako, Korea) were fasted overnight and anesthetized. Then, abdomen was incised and the mid-ileal region of small intestine was tied in 2 cm length with ligature to form closed-ileal loop containing one or two Peyer's patches. The culture supernatant of recombinant *L. lactis* IL1403 producing IL-6, CKS9-IL-6, or IL-6-CKS9 was prepared in which concentration of the IL-6 could be 5 µg/ml, respectively, by using centrifugal buffer-exchange columns, Centricon

(Millipore, USA). Then, 1 µg of each recombinant IL-6 was injected into the ileal loop. After 1 h incubation, the ileal loops were excised, washed in PBS to remove any free and loosely bound proteins, and then fixed with 4% paraformaldehyde (PFA). Tissue sections were fixed in cold acetone for 5 min and blocked by 3% goat serum, then fluorescent signals of recombinant IL-6s which incorporated within the tissues were detected using rabbit-anti-mouse IL-6 antibody (Abcam, UK) and donkey anti-rabbit IgG antibody conjugated with Alexa 555 (Abcam, UK) for the primary and secondary antibodies, respectively. M cells on Peyer's patch (PP) was labeled by anti-Gp2 (glycoprotein 2) monoclonal antibody-FITC (MBL, Japan) and nuclei were stained by 4', 6-diamidino-2-phenylindole (DAPI) (Sigma, USA).

### **5) Peroral immunization of mice**

Peroral immunization study was conducted with 8 week-old female BALB/c mice (Samtako, Korea) which were assigned in five groups (n=5) to validate the effect of the IL-6-producing LAB as a mucosal vaccine adjuvant. M-BmpB protein was used as a model antigen and prepared by cultivation of the recombinant *E. coli* BL21 harbouring the M-BmpB gene which was previously constructed by our research group (Jiang et al., 2014). Each group of mice was fasted for 6 h before orally administration and immunized on days 0, 7, and 14, respectively: (i) 200 µl of PBS; (ii) 200 µg of purified M-BmpB in 200 µl of PBS; (iii) 200 µg of M-BmpB with 10<sup>9</sup> cells of wild type *L. lactis* IL1403 in

200 µl of PBS; (iv) 200 µg of M-BmpB with  $10^9$  cells of recombinant *L. lactis* IL1403 producing IL-6 in 200 µl of PBS; (v) 200 µg of M-BmpB with  $10^9$  cells of recombinant *L. lactis* IL1403 producing IL-6-CKS9 in 200 µl of PBS. Before 30 min of every peroral immunization, each mouse was gavaged with 500 µl of neutralizing solution (7.5% NaHCO<sub>3</sub>: PBS=1:4) to reduce stomach acidity. Blood and fecal samples were prepared from the each group of immunized mice on day 21 and 28 after primary peroral immunization. Briefly, blood samples were collected from the caudal vein and centrifuged after clotting at 2.000 rpm for 10 min to prepare sera. For fecal sample preparation, several pieces of fecal pellets freshly-taken from each mouse were collected and suspended evenly in PBS by vigorous vortexing in a concentration of 200 mg/ml and centrifuged at 14000 rpm, 4 °C for 15 min, then clarified fecal extracts were obtained. Additionally, mid-ileal region of small intestines (3 cm) of the mice were immediately collected after sacrifice. The small intestines were homogenized in 500 µl of ice-cold PBS. Tissues were removed by centrifugation (14,000 rpm, 10 min and 4 °C), and the supernatants were collected. Blood samples and feces samples were collected at days 21, 28 and intestinal samples were collected at days 28. All the samples were stored at -70 °C until analysis. The immunization and sample collection schedule was depicted in Figure 16(A). All experimental procedures using laboratory animals were approved by the Institutional Animal Care and Use Committee of Seoul National University (Approval Number: SNU 121106-3) and followed the guidelines suggested by the committee.

## **6) ELISA assays**

Enzyme-Linked-Immuno-Sorbent Assays (ELISA) were conducted to assess the levels of antigen-specific Ig Gs (IgG, IgG1, and IgG2a) or IgA in mice sera or fecal samples, respectively. 96-well plates (Thermo) were coated with 2.5 µg of purified M-BmpB in 100 µl of 50 mM carbonate-bicarbonate buffer (pH 9.6) at 4 °C overnight and blocked with 1% BSA at 37 °C for 1 h. Then, appropriately diluted sera or fecal samples were added into wells and incubated at 37 °C for 2 h. After incubation, HRP (Horse Radish Peroxidase)-conjugated goat anti-mouse IgG, IgG1, or IgG2a (Santa Cruz Biotechnology, USA) was treated for sera and HRP-conjugated goat anti IgA (Santa Cruz Biotechnology, USA) was treated for fecal and intestinal samples to each designated well at 37 °C for 1 h, respectively. TMB solution (Sigma-Aldrich, USA) was added to the wells for the HRP substrate, reaction was stopped after 10 min by adding 1 M H<sub>2</sub>SO<sub>4</sub>, and absorbance values were determined using GRL 1000 Microplate Reader (General Labs Diagnostics, USA). Appropriate washing of each well proceeded between the steps with PBS containing 0.05% of Tween 20 during the assays. The ELISA results are expressed as the OD values measured at 450 nm for dilutions of 1:800 for serum, 1:10 for fecal extracts, and 1:100 for intestinal samples.

## **7) ELISPOT assays**

ELISPOT assays were performed to ascertain antigen-specific induction of

cytokines from the lymphocytes in Peyer's patches after peroral immunization in mice. Lymphocytes were isolated from the small intestinal Peyer's patches in mice at 7<sup>th</sup> day after final immunization as described previously (Kim et al., 2010). ELISpot kits (R&D systems, USA) were used for validation of antigen-specific cytokine induction. The isolated lymphocytes were applied to each plate of kit which was pre-coated with antibodies for a cytokine of interest, then stimulated with 100 nM of purified M-BmpB to induce cytokine expression at 37 °C for 36 h. The rest of procedure was followed by manufacturer's manual. The colored spots formed by cytokine production from lymphocytes were counted with ELISPOT reader (Cellular Technology Ltd, USA). Mean numbers were calculated from triplicated ELISPOT assays.

### **8) Investigation of oral tolerance induction**

In order to test the possible oral tolerance induction, peroral immunization was re-conducted with mice according to the method described in above. Orally immunized mice were boosted systematically at 2 weeks after the last immunization by intraperitoneal injection of recombinant M-BmpB (20 µg), then sera and fecal extracts were prepared at 7 days after the boost immunization. The oral immunization, systemic boosting and sample collection schedule was depicted in Figure 18(A). The levels of M-BmpB specific serum IgG and fecal IgA were analyzed through ELISA and then calculated as the reciprocal endpoint titers.

## **9) Statistical analysis**

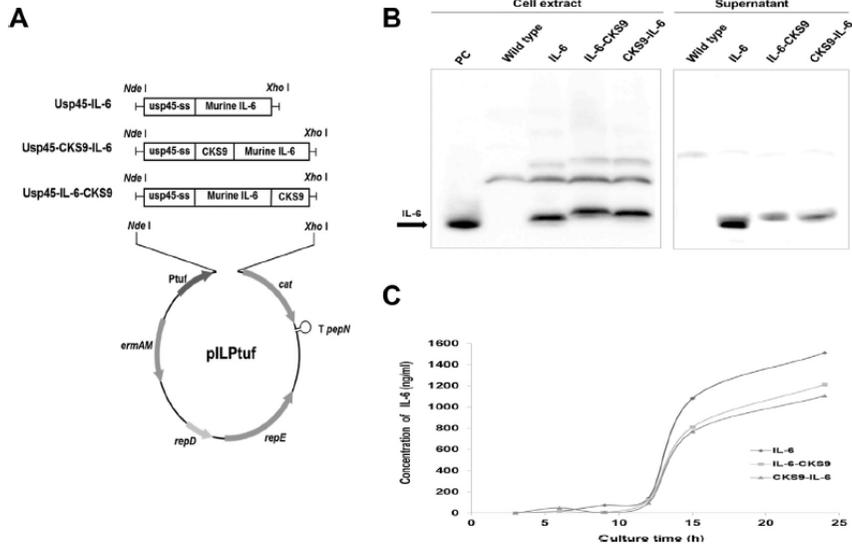
Quantified results were expressed as the mean and standard deviation (SD). Statistical significance was assessed using a one-way analysis of variance (ANOVA) with *post hoc* Tukey Multiple Comparisons test.

### 3. Results

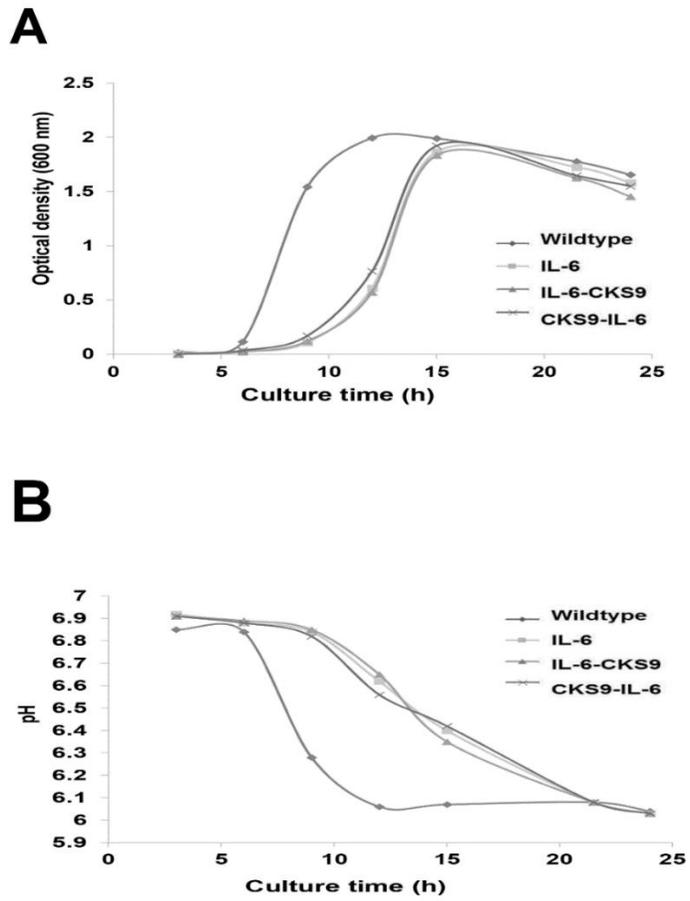
#### 1) Production of recombinant IL-6s from LAB

Each transformant *L. lactis* IL1403 harbouring pILPtuf-IL-6, pILPtuf-CKS9-IL-6, or pILPtuf-IL-6-CKS9 was cultivated separately, and production of the recombinant IL-6s was investigated. The transformant LAB showed retardation in growth [Figure 12(A)] and lactic acid production [Figure 12(B)] during cultivation compared with wild type *L. lactis* IL1403, which was assumed that the production of recombinant protein would be affected as an energy burden to normal physiology in transformant LAB. Western blot analysis [Figure 11(B)] revealed IL-6-specific bands in both cellular extract and culture supernatant fractions from all the transformant LAB. The molecular mass of recombinant IL-6 which detected from the culture supernatant fraction was consistent with control, commercially obtained murine IL-6. However, the recombinant IL-6 from cellular extract fraction showed slightly higher molecular mass compared with control representing unprocessed pre-peptide of Usp45, the protein secretion signal (Borrero et al., 2011). CKS9-IL-6 and IL-6-CKS9 also showed higher molecular masses compared with IL-6 in both intracellular and supernatant fractions due to their conjugation of M cell-targeting moiety on N-terminus or C-terminus of IL-6. Consequently, we could confirm expression and secretion of murine IL-6s as soluble forms from the *L. Lactis* IL1403 as expected. Accumulation of each secreted recombinant IL-6 in culture

supernatant also have been quantitatively monitored during cultivation with the lapse of time [Figure 11(C)]. The recombinant protein accumulation in each transformant culture generally followed growth pattern of its host [Figure 12(A)], which exponentially increased in step with log phase in host growth and reached to over 1 mg/L of culture media within 24 h. Unexpectedly, accumulation of all the recombinant IL-6s continuously increased after stationary phase in host growth. IL-6 showed much higher accumulation level in culture media compared with CKS9-IL-6 and IL-6-CKS9.



**Figure 11.** (A) Schematic diagrams for construction of recombinant IL-6s expression vector system. Ptuf: promoter originated from *tuf* gene; Usp45: protein secretion signal peptide originated from *Usp45* gene; T *pepN*: transcription terminator originated from *pepN* gene; ermAM: erythromycin resistance gene; cat: chloramphenicol resistance gene; repD: replication protein D; repE: replication protein E. (B) Confirmation of expression and secretion of IL-6s from LAB. Western blot analysis. Cellular extracts and concentrated culture supernatants from wild-type and LAB producing recombinant IL-6s were applied to SDS-PAGE, then immunoblotting. PC: Positive control, 100ng of purified commercial IL-6. (C) Accumulation profile of the secreted IL-6s in culture supernatant during cultivation of transformant LAB.

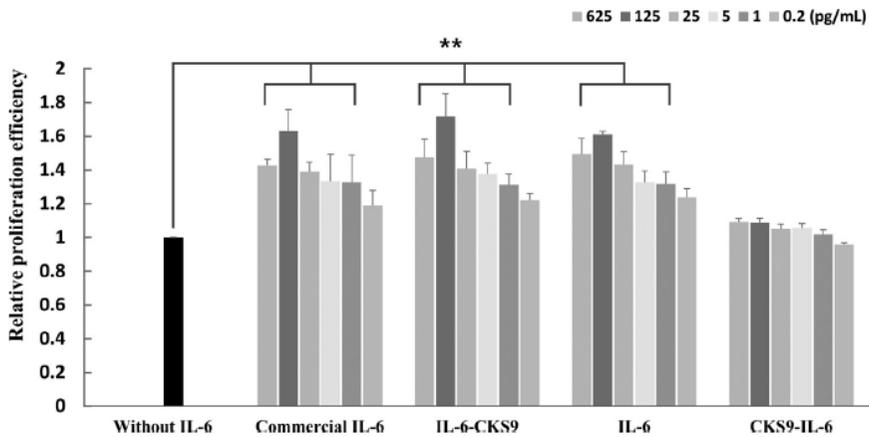


**Figure 12.** Comparison of cultivation profile between wildtype and transformant LABs.

(A) Growth profile, (B) acid production profile.

## **2) Biological activity validation of the recombinant IL-6s**

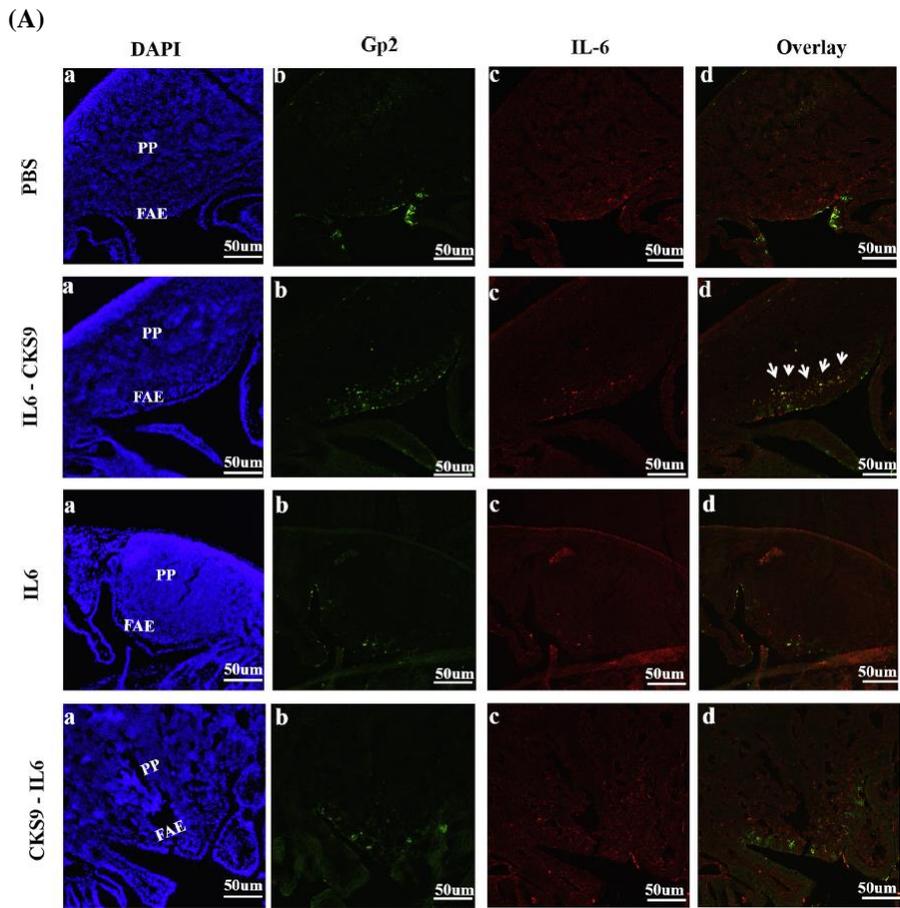
Biological activity of the recombinant IL-6s secreted from LAB were validated using 7TD1 cells having a characteristic of proliferation increment under biologically active IL-6 signals. 7TD1 cells were cultivated with different doses of commercial IL-6 or recombinant IL-6s and the proliferation efficiency of the each treatment of cells was monitored after 48 h. As shown in Figure 13, recombinant IL-6 and IL-6-CKS9 revealed coordinate biological activities with commercial IL-6 and dose dependency for IL-6s was observed on proliferation efficiency among the test groups. Only 1 pg/ml concentration of IL-6s appeared enough to increase proliferation efficiency of the 7TD1 significantly as far as we tested. Interestingly, there was no detectable bioactivity of recombinant CKS9-IL-6, in which M cell-targeting moiety was conjugated on N-terminus of IL-6.



**Figure 13.** Biological activity validation of the secreted IL-6s from LAB. The culture supernatants from LAB producing recombinant IL-6s (IL-6, IL-6-CKS9, and CKS9-IL-6) were diluted in RPMI1640 media and treated to 7TD1 cells at various concentrations. The cell proliferation of 7TD1 cells was measured using MTS assay and the result was given as relative fold compared to the cell proliferation of 7TD1 cells treated without IL-6 as control.

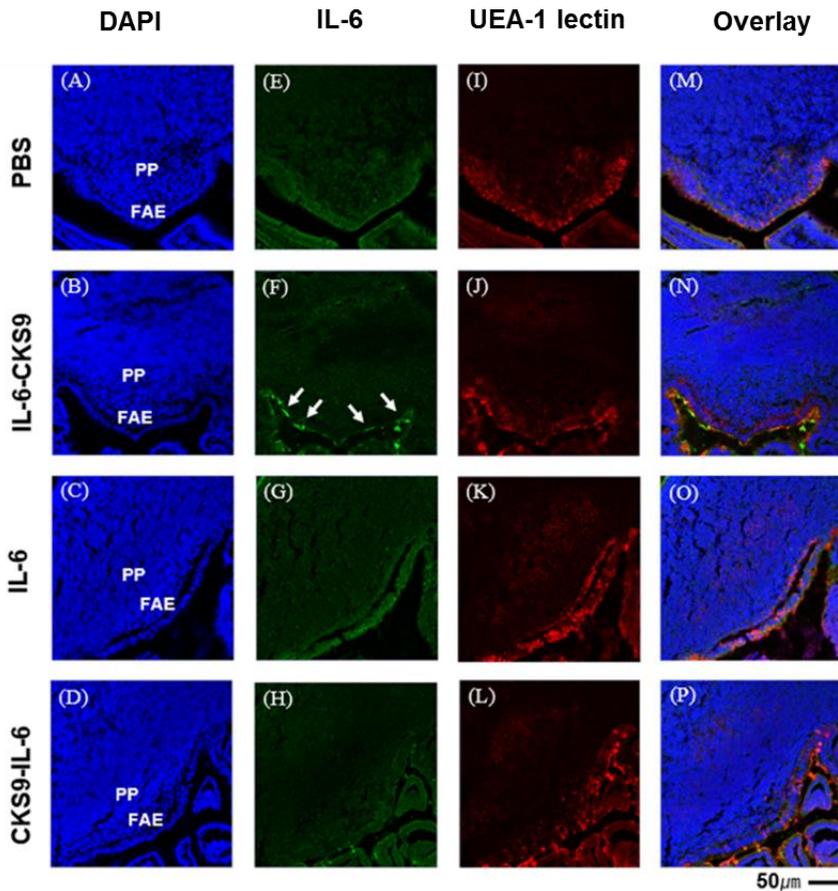
### **3) M cell-targeting ligand mediated delivery of IL-6s to Peyer's patches.**

Closed ileal loop assay and consecutive immunohistochemical analysis were performed to verify whether M cell-targeting moiety, the CKS9 peptide ligand, could facilitate specific delivery of IL-6s to Peyer's patches. After injection of recombinant IL-6, CKS9-IL-6, or IL-6-CKS9 preparation into the lumen of ileal loops, respectively, then we monitored fluorescent signals for IL-6s which would be strongly associated with M cells on Peyer's patch (PP) region (Yoo et al., 2010). A well-known M cell specific antibody, anti-Gp2-FITC, was used for localization of M cells in Peyer's patch. As shown in Figure 14, IL-6-CKS9 treated group only showed apparently higher yellow coincided fluorescent signal in Peyer's patch compared to other groups tested, indicating enhancement of M cell-targeting property of IL-6 by its C-terminal conjugation of CKS9 ligand. On the contrary, CKS9-IL-6, in spite of its N-terminal CKS9 conjugation, showed seldom positive signal for IL-6 with GP2 location in Peyer's patch.



**Figure 14 (A).** M cell-targeting ligand mediated delivery of IL-6s to Peyer's patches. The location of co-localization signals for IL-6s targeting M cells are indicated by white arrows. PP: Peyer's patches; FAE: Follicle associated epithelium.

(B)



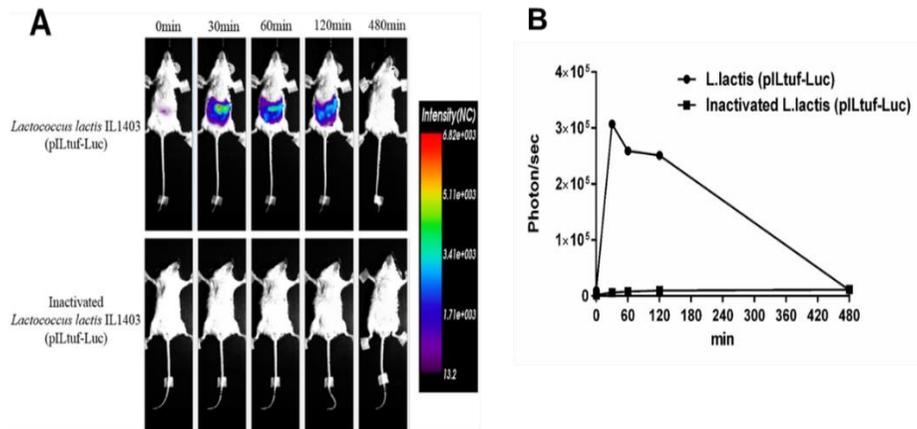
**Figure 14 (B).** M cell-targeting ligand mediated delivery of IL-6s to Peyer's patches. M cell-rich mucus layer region of the FAE on PP was labeled by Ulex Europaeus Agglutinin I (UEA-1) lectin conjugated with tetra-methyl-rhodamine isothiocyanate (red). The location of positive green signals for IL-6s targeting FAE (M cell-rich region) are indicated by white arrows. PP: Peyer's patches; FAE: Follicle associated epithelium.

#### **4) Evaluation of recombinant IL-6s-producing LAB as an adjuvant for mucosal immunity**

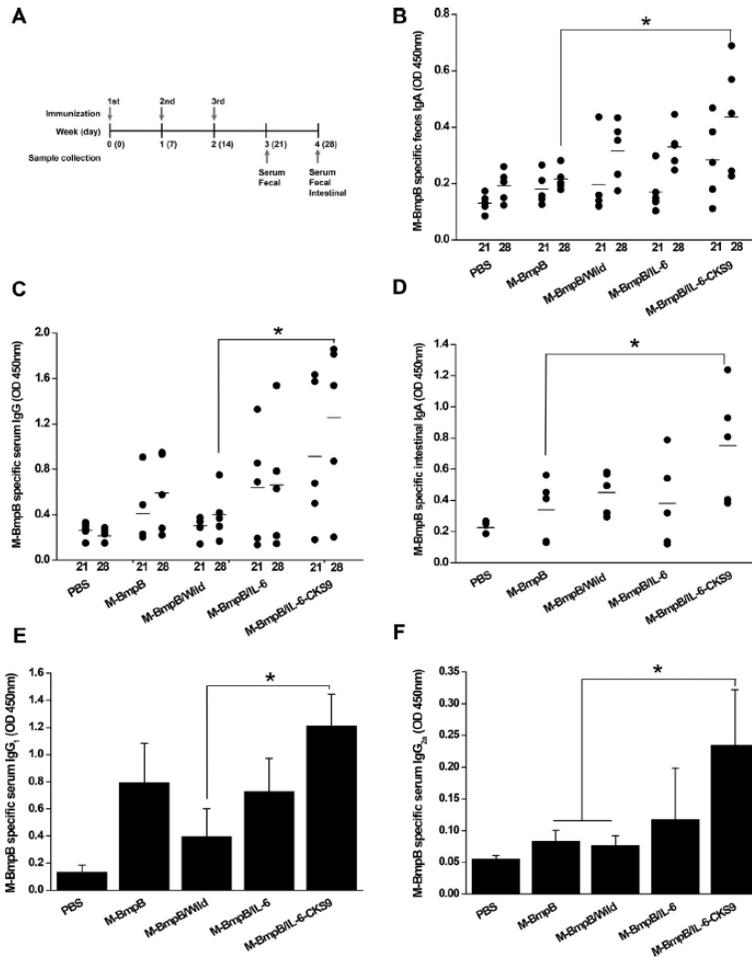
To evaluate the adjuvant effect of IL-6-producing *L. lactis* IL1403 in mucosal immunity, peroral immunization was performed with mice. The model antigen, M-BmpB, was administered alone or in combination with wildtype *L. lactis* IL1403, IL-6-secreting *L. lactis* IL1403 (IL-6-LAB), or IL-6-CKS9-secreting *L. lactis* IL1403 (IL-6-CKS9-LAB). CKS9-IL-6-LAB group was excluded from the immunization because of its low biological activity and targeting efficiency (Figure 13 and 14). At day 21 and 28 after the immunization, anti-M-BmpB-specific IgA or IgG were detected by ELISA from fecal or sera, respectively. As shown in Figure 16(B) and (C), the mice treated with IL-6-CKS9-LAB showed prominently higher tendency in anti-M-BmpB-specific antibody levels for both fecal IgA and serum IgG compared with other groups tested: M-BmpB alone, M-BmpB with wildtype LAB. Similar tendency was also observed in the anti-M-BmpB-IgA levels from intestinal extracts at day 28 [Figure 16(D)]. For the next, we analyzed immune responses in terms of antibody isotypes in sera collected from individual mice at day 28 after peroral immunization, especially with IgG1 and IgG2 which are considered as typical markers for Th2 and Th1 type immune responses, respectively. Serum IgG1 levels in the mice treated with IL-6-CKS9-LAB were significantly higher than BmpB with wildtype LAB treated group [Figure 16(E)] and, with serum IgG2

levels, IL-6-CKS9-LAB treated group showed significantly higher level than BmpB alone and BmpB with wildtype treated group [Figure 16(F)]. Unexpectedly, the statistical significances could not be obtained between IL-6-LAB and IL-6-CKS9-LAB groups in all the immunization results. However, we could observe consistent enhancements in induction of anti-M-BmpB antibody levels both with mucosal (IgA) and systemic immune responses (IgG) in IL-6-CKS9-LAB treated group compared with IL-6-LAB group by conducting two separated mice immunization assays (Figure 16 and 18).

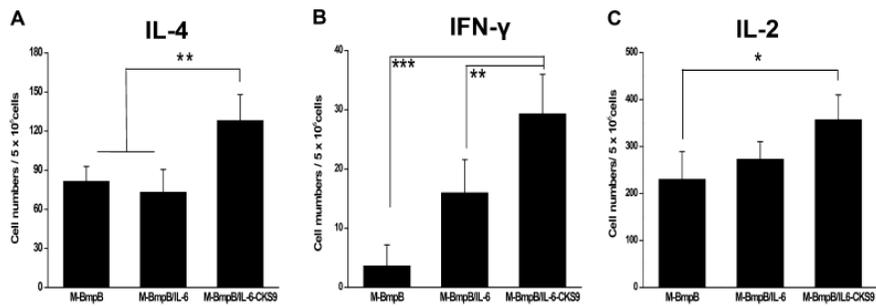
ELISPOT assays showed that lymphocytes secreted significantly higher levels of both Th1 and Th2 type of cytokines, IL-4 and IFN- $\gamma$ , respectively, responding to *in vitro* M-BmpB stimulation in IL-6-CKS9-LAB treated group compared with M-BmpB alone and IL-6-LAB groups [ Figure 17(A) and (B)]. In addition, secretion of IL-2 which is associated with T cell proliferation was also increased in in IL-6-CKS9-LAB treated group [Figure 17(C)].



**Figure 15.** Confirmation of the activity of *tuf* promoter *in vivo* after oral administration of live *Lactococcus lactis* (pILtuf-Luc). (A) Mouse was fed once with  $10^{10}$  CFU of live or heat inactivated ( $50^{\circ}\text{C}$ , 1hour) *L. lactis* (pILtuf-Luc) and 3mg of D-Luciferin substrate dissolve in 200ul saline was injected intragastrically at each time point and the bioluminescent signals were imaged immediately using Optix MX3 imaging system. The related photon signals in body of whole animals at each time point are plotted in (B).



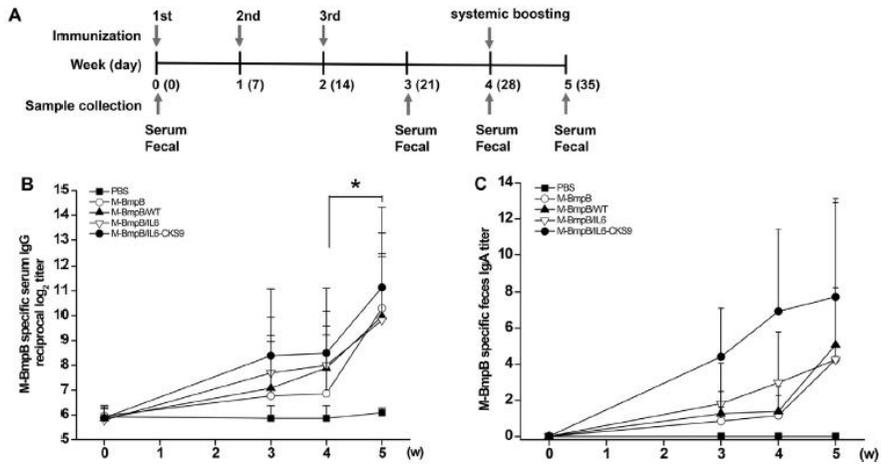
**Figure 16.** *In vivo* validation of LAB producing recombinant IL-6s as oral adjuvants by peroral immunization in mice. (A) Schematic view of immunization and sampling schedule. (B~D) M-BmpB-specific fecal IgA and serum IgG titers at day 21 and 28, intestinal IgA titer at day 28. Each dot denotes anti-M-BmpB antibody titer value from individual mouse and the bar represents average mean (n=5 in each group tested). (E, F) Isotype analysis for Immunoglobulin G (IgG). M-BmpB-specific serum IgG1 (E) and serum IgG2a (F) titer at day 28 after immunization. (n=3 in each group tested, error bar represents standard deviation, \*: p < 0.1, one-way ANOVA).



**Figure 17.** IL-4, IL-2 and IFN- $\lambda$  levels in the peyer's patch of mice after peroral immunization. *In vitro* cytokine induction assay. Lymphocytes secreting IL-4 (A), IFN- $\lambda$  (B), and IL-2 (C) in PPs were measured by ELISPOT assay. (n=3 in each group tested, error bar represents standard deviation, \*: p < 0.1, \*\*: p < 0.05, \*\*\*: p < 0.01, one-way ANOVA).

## **5) Investigation of oral tolerance induction**

A critical point to be cleared before securing the use of an oral vaccine or adjuvant is to insure that the orally-administered antigen does not induce systemic tolerance. In order to determine whether the oral administration of M-BmpB with IL-6-CKS9-LAB adjuvant would induce tolerogenic immune responses, we monitored M-BmpB specific IgG and IgA levels after systemic challenge with M-BmpB to mice which orally immunized with M-BmpB or corresponding IL-6s secreting LABs (Figure 18). As results, the orally immunized group treated with M-BmpB and IL-6-CKS9-LAB adjuvant displayed increased anti-BmpB serum IgG [Figure 18(B)] and fecal IgA [Figure 18(C)] titers after systemic challenge with M-BmpB antigen.



**Figure 18.** Investigation of a tolerogenic immune response against M-BmpB in systemic and mucosal compartments after systemic boost immunization in mice orally immunized with each indicated antigen. (A) Schematic view of oral immunization and sampling schedule, systemic challenging and sampling schedule. (B, C) Serum IgG and fecal IgA was monitored at day 0, 21, 28 and 35. (n=5 in each group tested, error bar represents standard deviation, \*:  $p < 0.1$ , \*\*:  $p < 0.05$ , one-way ANOVA).

#### **4. Discussion**

Protection against orally transmitted infections requires immunity at the site of pathogen entry (Wegmann et al., 2012). However, the induction mechanism for mucosal immune response is different from systemic immune response, thus exploration of effective way to induce mucosal immune response and development of efficient mucosal vaccine system is an important issue (Pavot et al., 2012). Recently, various researches for development of mucosal adjuvants are ongoing to facilitate mucosal vaccination. LT and CT are known to be the most effective mucosal adjuvants which were validated in mice researches in spite of their high cytotoxicity (Da Hora et al., 2011; Fujihashi et al., 2002). Toll like receptor agonists, such as CpG ligands (Fukuyama et al., 2011; Heikenwalder et al., 2004) or cytokines, such as BAFF (TNF- $\alpha$  family) (Tertilt et al., 2009) and IL-6 (Steidler et al., 1998) might be potentially effective mucosal adjuvants. However, low absorption efficiency of such adjuvants into immune system across the mucosal barrier is still challenging problem, especially in peroral vaccination.

The present study examined whether the introduction of an M cell-targeting peptide ligand, CKS9, to a cytokine, IL-6, could facilitate mucosal adjuvant function of the cytokine by increasing its accessibility to immune system across the intestinal mucosal barrier, then effectively induce antigen-specific secretory antibody, IgA, after peroral immunization in mice model. As referred in

‘Introduction’, we expected that orally administered IL-6-producing LAB, especially with M cell-targeting moiety, would be effective to induce mucosal immune response from gastrointestinal tract in peroral vaccination. An edible lactic acid bacteria, *L. lactis* IL1403, served as a live vehicle for the production of the recombinant IL-6 adjuvants and one of the most well-known protein secretion signals in LAB, pre-peptide of Usp45, was employed for the cytokine secretion system in this study (Figure 11). In spite of physiological delay in growth compared with wildtype (Figure 12), all the transformant LAB showed successful expression and secretion of recombinant IL-6s as soluble forms [Figure 11(B)] and secreted IL-6s were accumulated in milligram scale per liter of culture media within 24 h during cultivation. Continuous increment of the target protein accumulation after stationary growth phase of the host LAB was inferred as relatively long half-life of the recombinant IL-6s and considered as a beneficial feature for oral adjuvant [Figure 11(C)].

All the recombinant IL-6s revealed equivalent functional activity with commercial IL-6 except CKS9-IL-6 in which M cell-targeting moiety conjugated with N-terminus of the IL-6 (Figure 13). IL-6 is known as a ‘four-alpha-helix-bundle’ family cytokine and 15~22 of N-terminus amino acid residues forming the first alpha-helix has been identified to be crucial in biological function of this type of cytokines, thus N-terminal modification often causes its loss of function (Simpson et al., 1997). We inferred that N-terminal

introduction of the CKS9 peptide to IL-6 might adversely affect to structural integrity to maintain its functional activity. It is considered as another beneficial feature for oral adjuvant that IL-6 could be functional even at pictogram scale of very low concentration (Figure 13).

Immunohistochemical assays revealed that the IL-6-CKS9, in which M cell-targeting moiety conjugated with C-terminus of the IL-6, only possessed M cell-targeting efficacy among the recombinant IL-6s produced from the transformant LAB (Figure 14). As described previously, M cells are the specialized cell type located on the FAE on Peyer's patches in intestinal epithelia and have a role collecting and delivering gut lumen antigen to mucosal immune system. Thus, M cell-targeting property could be also beneficial for oral adjuvant to facilitate efficient induction of mucosal immune response. We ascertained that the N-terminal introduction of the CKS9 to IL-6 caused adverse effects not only in cytokine function but also in M cell-targeting property.

The efficacy of the LAB producing recombinant IL-6s as oral adjuvants was validated by peroral vaccination with mice using M-BmpB as a model antigen. Especially, only immunized with M-BmpB/IL-6-CKS9-LAB group appears significant difference in serum IgG and fecal/intestinal IgA levels compared with those immunized with M-BmpB alone or M-BmpB/wildtype-LAB groups, however M-BmpB/IL-6-LAB groups showed no significant difference compared with them. Although the statistical significances could not be

obtained between IL-6-LAB and IL-6-CKS9-LAB groups because of individual differences in sympathy responding to immunization among mice, a consistent enhancement was observed in induction of anti-M-BmpB antibody levels both with mucosal (IgA) [Figure 16(B) and (D)] and systemic immune responses (IgG) [Figure 16(C)] in IL-6-CKS9-LAB treated group compared with IL-6-LAB group. The similar higher induction of anti-M-BmpB antibody in IL-6-CKS9-LAB group was reappeared in succeeding mice immunization for tolerance validation [Figure 18(B) and (C)]. The higher tendency in anti-M-BmpB induction than IL-6-LAB group was inferred that the introduction of CKS9 to IL-6 could facilitate its delivery to mucosal immune system across the intestinal barrier especially through the M cells that could induce higher immune response than IL-6 which was without M cell targeting efficiency. Large scale of *in vivo* immunization assay to minimize individual variances among animals would be needed to obtain more definite efficacy of the CKS9 peptide ligand for targeted delivery to M cells in further study. Besides, the group treated M-BmpB with wildtype LAB as an adjuvant showed higher IgA induction tendency compared with the group treated with M-BmpB only, which suggested a synergistic advantage of LAB as a live vehicle for the cytokine adjuvant [Figure 16(B) and (D)].

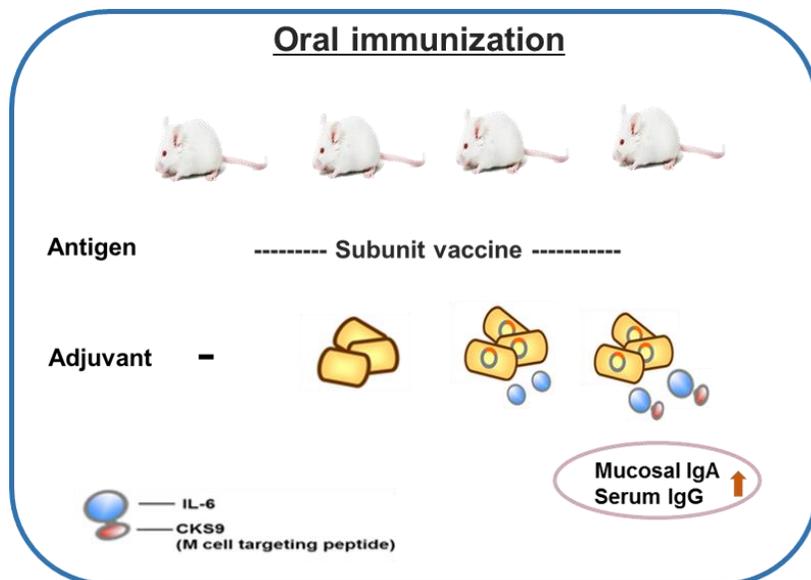
Furthermore, we found that the treatment of IL-6-CKS9-LAB as an oral adjuvant could induce both Th1 and Th2 type immune responses

simultaneously [Figure 16(E) and (F)]. One of the general drawbacks of subunit vaccine is that it could only induce weak Th2 type, humoral immune response, rather than Th1 type, cellular immune response. For the effective defense not only to exogenous pathogen such as bacteria but also to endogenous pathogen such as virus, and in the aspect of immune homeostasis, balanced induction of Th1 and Th2 type immune responses would be important property for vaccine adjuvant (Kidd, 2003). Additionally, we ascertained again that treatment of IL-6-CKS9-LAB could induce both Th1 and Th2 type cytokines, IL-4 and IFN- $\gamma$ , respectively, significantly higher than control groups from the lymphocytes [Figure 17(A) and (B)]. Biological function of IL-6-CKS9-LAB as a cytokine adjuvant was also confirmed by detecting enhancement of the IL-2 induction, which is one of the crucial cytokines associated with proliferation of lymphocytes [Figure 17(C)].

Finally, we investigated oral tolerogenic response for our vaccine strategy, which is one of the most important issues in oral vaccine research. The characteristics of the IL-6-CKS9-LAB-adjuvanted immune responses, both with systemic IgG [Figure 18(B)] and mucosal IgA [Figure 18(C)], were maintained after systemic challenge by M-BmpB antigen and, consequently, represent the non-tolerance response against M-BmpB antigen.

## 5. Conclusion

We designed a novel cytokine conjugated with M cell-targeting moiety (IL-6-CKS9) could be applied as an oral adjuvant and our results are firstly validated that M cell-targeting strategy for IL-6 could enhance induction of antigen-specific antibody in both mucosal and systemic immune response after peroral vaccination. Our results also suggest that cytokine-producing lactic acid bacteria would have a great potential as an effective and convenient oral adjuvant for development of efficient oral vaccine system.



**Figure 19.** Schematic illustration for the result of the study 1

# **Chapter 3. Nasal immunization with mannan-decorated mucoadhesive HPMCP microspheres containing ApxIIA toxin induces protective immunity against challenge infection with *Actinobacillus pleuropneumoniae* in mice**

## **1. Introduction**

The nasal route holds great promise for vaccine delivery because of the organization of the respiratory lymphoid tissue (Lycke, 2012). The respiratory mucosa is the first site of contact for inhaled antigens and the nasal associated lymphoid tissue (NALT) and inducible bronchus-associated lymphoid tissue (iBALT) at the base of the nasal cavity and large airway are very important in the defense of mucosal surfaces (Tacchi et al., 2014). In addition, the intranasal administration of an antigen not only induces systemic immune responses but also induces mucosal responses (Vicente et al., 2013). However, the administration of subunit vaccines alone seldom leads to a protective antibody response because the residence time of soluble antigens in the respiratory mucosa is limited, which results in very small doses of antigen reaching the

antigen-presenting cells of the sub-epithelial region (Bento et al., 2015). Moreover, subunit vaccines are often poorly immunogenic as they lack the necessary “danger signals” to activate dendritic cells (DC) and subsequently, T cells (Nochi et al., 2010).

The encapsulation of the antigen into polymeric particles has been studied as a promising approach to improve the transport of antigens across the respiratory mucosa (Rajapaksa and Lo, 2010). In addition, the increased residence time of particulate delivery systems at the mucosal surface may facilitate the increased uptake of such agents. To this end, mucoadhesive polymers provide a strategy that can help increase residence time and hence the uptake of particulate vaccines when administered by the nasal or pulmonary routes (Pawar et al., 2013). Recently, a new generation of synthetic polymers known as thiomers or thiolated polymers have been shown to have strong mucoadhesive properties due to their binding with the cysteine-rich subdomains of mucus glycoprotein (Bernkop-Schnürch et al., 2001; Bernkop-Schnürch et al., 2004). In this work, thiolated HPMCP (TH), previously verified as having mucoadhesive properties (Singh et al., 2015), was used as a nasal subunit vaccine carrier.

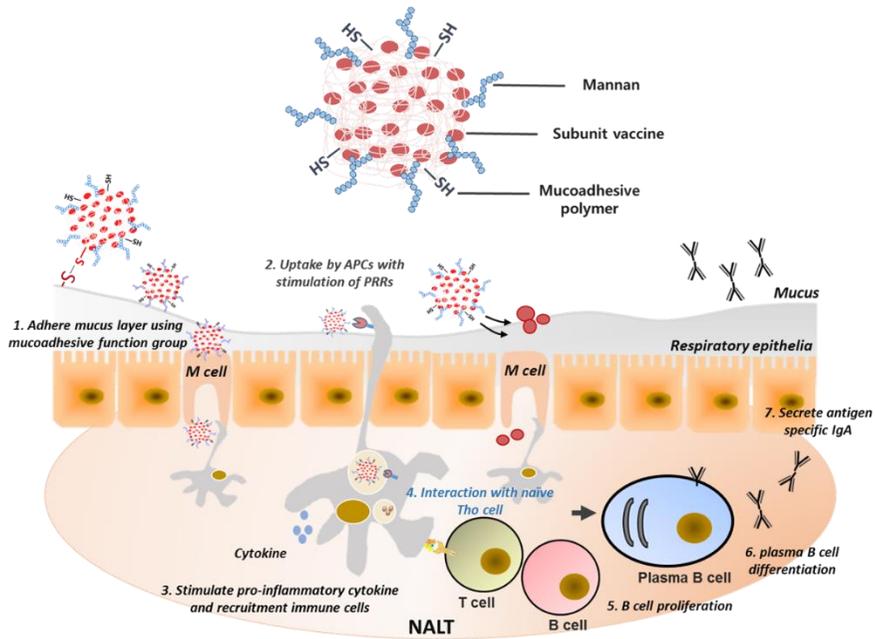
Another major hurdle in the development of intranasal vaccines is poor immunogenicity of vaccine subunits due to the lack of “danger signals” that can activate APCs. The development of effective targeted particulate vaccine formulations combined with strong immunomodulation should improve the

efficacy of non-living subcomponent mucosal vaccines (Carapau et al., 2013). Mannan isolated from the cell wall of *Saccharomyces cerevisiae* is one of the natural PAMPs (pathogen associated molecular patterns) of APCs and is a ligand of the mannose receptor (MR) widely expressed on APCs (Jiang et al., 2008; van de Veerdonk et al., 2009). Mannan can induce the production of pro-inflammatory cytokines and up-regulate the expression of co-stimulatory molecules in these cells, thereby activating both innate and adaptive immunity (Stambas et al., 2002a; Stambas et al., 2002b). Moreover, targeting antigens to the endocytic receptors of APCs is an attractive strategy for enhancing the efficacy of vaccines (Jiang et al., 2009). It has been demonstrated that using mannan to target the delivery of antigens to the MR effectively induces cellular and humoral immune responses (Chavez-Santoscoy et al., 2012; Cruz et al., 2012; Jones et al., 2015).

*Actinobacillus pleuropneumoniae* is known to cause contagious porcine pleuropneumoniae. Among the many virulence factors of *A. pleuropneumoniae*, Apx toxins, which act to create pores in the host cell membrane, are known to be substantially involved in the pathogenesis of pleuropneumoniae (Shin et al., 2005). The importance of Apx toxins as vaccine candidates has been demonstrated in many studies. In particular, ApxII toxin is a promising vaccine candidate because nearly all serotypes of *A. pleuropneumoniae* (with the exception of serotypes 10 and 14) express ApxII antigen (Seo et al., 2013). In

order to develop an efficient intranasal vaccine against *A. pleuropneumoniae* in the swine farm industry, ApxIIA, which has previously been confirmed to possess immunogenicity in swine, was adapted as a model antigen in our study (Seo et al., 2013).

The aim of the present work was to explore the potential of a novel Man-THM vehicle for the intranasal administration of ApxIIA as a vaccine against *A. pleuropneumoniae*. For this purpose, Man-THM was prepared by the double emulsion method and the surface of the microsphere was decorated with mannan. After optimization of the preparation method and characterization of the obtained ApxIIA-loaded Man-THM, the physiochemical properties of the Man-THM as a nasal delivery carrier were evaluated by *in vitro* and *in vivo* assays. After intranasal immunization, the immune adjuvant ability of the formulation of mannan-decorated THM with ApxIIA vaccine was investigated by measuring the systemic and secretory antibodies in the serum and mucosal secretions. Furthermore, assays were performed after *A. pleuropneumoniae* serotype 5 challenge in mice immunized with the ApxIIA-loaded Man-THM.



### Experimental flow chart

Characterization	<ul style="list-style-type: none"> <li>Synthesis of thiolated HPMCP (TH)</li> <li>Preparation of THM, Man-THM</li> <li>Physiochemical characterization of microspheres (size, morphology)</li> </ul>
<i>In vitro</i>	<ul style="list-style-type: none"> <li>Confirm decoration of mannan in Man-THM</li> <li><i>In vitro</i> uptake study with APCs</li> </ul>
<i>In vivo</i>	<ul style="list-style-type: none"> <li>Evaluation of Man-THM as a nasal vaccine carrier</li> <li>Challenging assay</li> </ul>

**Figure 20.** Experimental flow of the study 2

## **2. Materials and methods**

### **1) Materials**

HPMCP was kindly provided by Shin-Etsu Chemical Co., Ltd. (Tokyo, Japan). Dimethyl sulfoxide (DMSO), *N,N'*-dicyclohexylcarbodiimide (DCC), *N*-hydroxysuccinimide (NHS), L-cysteine hydrochloride monohydrate, poly(vinyl alcohol) (PVA), Pluronic® F-127, dichloromethane, 4',6-diamidino-2-phenylindole dilactate (DAPI), carbonate-bicarbonate buffer capsule, fluorescein isothiocyanate (FITC), and mannan derived from *Saccharomyces cerevisiae* were purchased from Sigma-Aldrich (St. Louis, MO, USA). RPMI 1640 media, DMEM media, and FBS were purchased from Gibco (Waltham, MA, USA). Bicinchoninic acid (BCA) protein assay reagents (A and B) were purchased from Thermo Scientific Pierce (Rockford, IL, USA). Horseradish peroxidase (HRP)-conjugated goat anti-mouse IgA, IgG, IgG1, and IgG2a antibody were purchased from Santa Cruz Biotechnology (Dallas, TX, USA). All other materials were of analytical reagent grade.

### **2) Cell lines and experimental animals**

RAW 264.7 murine macrophage and Jaws II murine dendritic cell lines were obtained from American Type Culture Collection (Manassas, VA, USA). 6 week-old BALB/c female mice (Samtako Inc, Korea) were used in the immunization experiments and all experimental procedures were approved by

the Institutional Animal Care and Use Committee of Seoul National University (Approval Number: SNU 140811-2) and followed the guidelines suggested by the committee.

### **3) Synthesis of thiolated HPMCP (TH)**

TH was synthesized according to previously reported methods (Quan et al., 2008). Briefly, 4 g of HPMCP was dissolved in 100 ml DMSO and the carboxylic acid moieties of the polymer were activated by DCC (9 g) and NHS (5 g) with constant stirring at room temperature for 24 h under nitrogenous condition. The dicyclohexylurea as a by-product was removed by filtration and the filtrate was further reacted with L-cysteine hydrochloride (0.4 g) for 48 h under similar conditions. The reaction mixture was filtered to remove by-products and the filtrate was dialyzed initially against DMSO, and then against distilled water to remove the unbound L-cysteine. Finally, the product was lyophilized and stored at -20 °C until use. The conjugation of L-cysteine was confirmed by <sup>1</sup>H NMR spectroscopy (Avance 600, Bruker, Germany).

### **4) Preparation of ApxIIA-loaded THM and ApxIIA-loaded Man-THM**

#### **(1) Isolation and purification of ApxIIA**

Recombinant ApxIIA was produced using an *E. coli* expression system, pQE (Qiagen GmbH, Hilden, Germany) and ApxIIA was induced and purified according to the methods described in a previous study (Shin et al., 2011).

Briefly, when the culture reached an optical density of 0.6 nm (OD 0.6 nm), isopropyl  $\beta$ -D-1-thiogalactopyranoside (IPTG, 1 mM) was added and then cultured continuously for 4 h. The harvested cells were re-suspended in lysis buffer (20 mM Tris–hydrogen chloride, 500 mM sodium chloride, 8 M urea, 40 mM imidazole, pH 7.0) and nickel–nitrilotriacetic acid (Ni-NTA) chelate affinity chromatography was then performed according to the manufacturer’s instruments. The bound protein was eluted with elution buffer (20 mM Tris–hydrogen chloride, 500 mM sodium chloride, 8 M urea, 500 mM imidazole, pH 7.0).

## (2) Preparation of ApxIIA-loaded THM and ApxIIA-loaded Man-THM

20 mg/ml ApxIIA was stabilized with Pluronic F-127 solution (1 wt.-%) to form an internal aqueous phase ( $W_1$ ). 0.2 ml of  $W_1$  (4 mg ApxIIA) was emulsified with an organic phase (O) consisting of 100 mg of TH dissolved in 5 ml of dichloromethane (DCM) using an ultrasonic processor (Sonics, Vibra cells™) (2 output watts) for 1 min to form a primary emulsion ( $W_1/O$ ). The prepared primary emulsion ( $W_1/O$ ) was added drop by drop to 50 ml of 1 wt.-% PVA solution ( $W_2$ ) to create an external aqueous phase or was added to a mixture of 0.25 wt.-% PVA and 0.5 wt.-% mannan for Man-THM. A homogenizer (Ultra-Turrax™ HomogenizerT25, IKA) was then used at 11,000 rpm for 4 min to form a  $W_1/O/W_2$  emulsion, which was then stirred with a magnetic stirrer for 2–3 h at room temperature to allow the solvent to evaporate. ApxIIA-loaded

THM and ApxIIA-loaded Man-THM were collected and washed 3 times with distilled water after centrifugation at 14,000 rpm for 10 min at 4 °C. The microspheres were lyophilized and finally stored at -70 °C for later characterization. The unloaded THM and Man-THM were prepared with similar methods.

## **5) Characterization of the microspheres**

### **(1) Size and surface morphology**

The particle size distribution of the microspheres was measured using an electrophoretic light scattering spectrophotometer (ELS-8000, Otsuka Electronics Ltd., Osaka, Japan) with an argon laser beam at 488 nm and 25 °C. The microspheres were placed on a stud and were gold-coated using Sputter Coater (BAL-TEC/SCD 005) and observed using a field emission scanning electron microscopy (AURIGA, Jena, Germany).

### **(2) Confirmation and quantification of mannan decoration in the THM**

To confirm mannan-coating in the Man-THM, FITC was conjugated with mannan. Briefly, 100 mg of mannan dissolved in 1 ml of DW was slowly mixed with 5 mg of FITC dissolved in 1 ml of DMSO. After stirring for 4 h at room temperature in dark conditions, the reaction product was dropped into 8 ml of

ethanol to remove the unreacted FITC. The mannan-FITC conjugate that precipitated was washed with ethanol and collected by centrifugation at 14,000 rpm 3 times for 10 min. FITC-mannan-decorated THM was visualized by confocal laser microscope (SP8 X STED, Leica, Wetzlar, Germany).

To quantify the FITC-mannan decoration in the THM, FITC-mannan was extracted by dissolving FITC-Man-THM in the DMSO. The fluorescence of extracted FITC-mannan from FITC-Man-THM was quantified using FITC-mannan standard curve.

## **6) *In vitro* studies**

(1) *In vitro* release of APXIIA from APXIIA-loaded THM and APXIIA-loaded Man-THM

10 mg of APXIIA-loaded THM and APXIIA-loaded Man-THM suspended in 1 ml of PBS (pH 7.4) in 1.5 ml microtubes were agitated for up to 24 h at 37 °C and 100 rpm using a shaking incubator. A 100 µl aliquot was withdrawn from the release medium at each time point and the amount of released APXIIA was determined as the cumulative release (wt. -%) against incubation time by the Micro BCA protein assay method.

(2) Uptake of microspheres by the APCs

For the uptake of the microspheres, RAW 264.7 and JAWS II cells were

seeded into 6-well plates (Costar, IL, USA) at  $1 \times 10^6$  cells/well. After the cells reached 80% confluence, the medium was changed with 3 mg/ml of mannan for 20 min before the addition of microspheres to block MR. Normal or MR-blocked cells were changed with the media with a suspension of OVA-FITC-loaded THM and OVA-FITC-loaded Man-THM at a microsphere concentration of 0.2 mg/ml for 1 and 2 h. After incubation, the microsphere suspension was removed and the wells were washed three times with 1 ml of PBS to remove the traces of microspheres left in the wells. The cells were then detached from the culture dishes and flow cytometry analysis was carried out to determine the uptake of microspheres by the cells.

### (3) Internalization of microspheres

The phagocytosis of microspheres by the macrophages and DCs was confirmed by confocal laser microscope (SP8 X STED, Leica).  $1 \times 10^6$  cells per well were cultured in 35 mm cover glass bottom dishes (SPL Life Science, Korea) two days prior to feeding with FITC-OVA-loaded microspheres at a concentration of 0.2 mg/ml. After incubation at 37 °C for 2 h, non-phagocytized microspheres were removed by washing three times with PBS and fixed with 4% paraformaldehyde at room temperature for 10 min. The nucleus was stained by Hoechst 33342 nucleic acid stain (Invitrogen, MA, USA).

### **7) *In vivo* imaging of the intranasal administration of the microspheres**

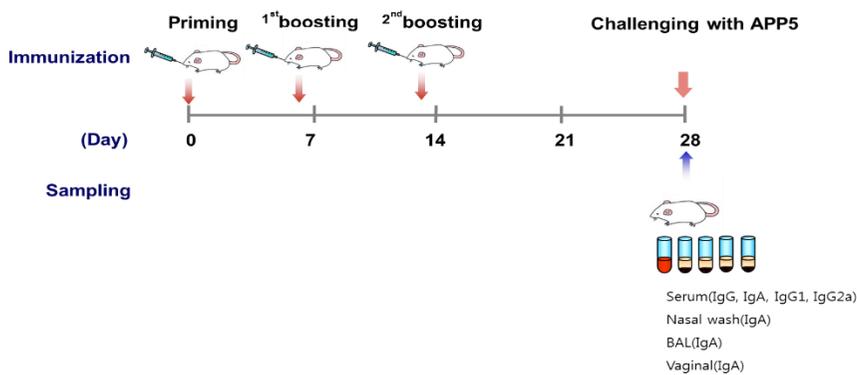
For the *in vivo* fluorescence study, cy5.5-conjugated OVA was used. Cy5.5-conjugated OVA and the same amount of cy5.5-conjugated OVA-loaded THM and MAN-THM were intranasally administrated to 6 week-old BALB/c mice. The fluorescence was monitored at 10 min, 30 min, 60 min, 120 min, 180 min, and 240 min. The fluorescence images were acquired under an excitation of 695 nm using the Optix-MX3 (Advanced Research Technologies, Canada). The fluorescence intensities in the regions of interest (ROI) were analyzed from the fluorescence images.

### **8) *In vivo* immunization studies**

#### **(1) Immunization of mice**

The nasal immunization study was conducted with 6 week-old female BALB/c mice assigned to five groups (n=5). The nasal administration groups were immunized 3 times on days 0, 7, and 14 by dropping 15  $\mu$ l of PBS containing APXIIA (5  $\mu$ g)- or APXIIA (5  $\mu$ g)-loaded THM, or APXIIA (5  $\mu$ g)-loaded Man-THM into the nostrils during inhalation under anesthesia. Mice receiving intraperitoneal administrations (i.p.) were immunized at day 0 with 5  $\mu$ g of APXIIA emulsified with complete Freund's adjuvant, and subsequently received booster immunizations twice at days 7 and 14 with the same amount of antigen emulsified with incomplete Freund's adjuvant. The

serum, nasal wash, bronchoalveolar lavage (BAL), and vaginal wash collections were performed on day 28 (Figure 21). Briefly, blood samples were collected from the facial vein and centrifuged after clotting at 2,000 rpm for 10 min for sera preparation. BAL samples were harvested from the lower respiratory tract of the mice as described previously (Mizuno et al., 2006). Briefly, after the bronchi were exposed from the neck, a truncated needle with a syringe containing 400  $\mu$ l PBS was inserted through a small pore on each bronchus. Mucus exudation was harvested with a back-and-forth motion of the injector, and the supernatants were collected by centrifugation (3,000 rpm and 10 min at 4 °C). The nasal washes were collected by 300  $\mu$ l PBS through the trachea toward the nose. For vaginal wash preparation, the vagina was washed 15 times with 150  $\mu$ l of PBS and the obtained fluids were collected by centrifugation (14,000 rpm, 10 min at 4 °C). All of the samples were stored at -70 °C until analysis.



**Figure 21.** Schematic representation of immunization schedule

## (2) Measurement of APXIIA-specific antibodies

The levels of APXIIA-specific IgG, IgA, IgG1, and IgG2a antibodies in serum, as well as sIgA antibodies in nasal wash, BAL, and vaginal wash were determined by ELISA, as described previously (Li et al., 2015). Briefly, 96-well plates (Thermo, USA) were coated with 0.1 µg of APXIIA in 100 µl of 50 mM carbonate-bicarbonate buffer (pH 9.6) at 4 °C overnight and blocked with 1 wt.-% BSA at 37 °C for 1 h. Then, serially diluted serum, and appropriately diluted nasal wash, BAL, and vaginal wash were added into the wells and incubated at 37 °C for 1 h. After incubation, HRP (horse radish peroxidase)-conjugated goat anti-mouse IgA, IgG, IgG1, or IgG2a were added to the designated wells at 37 °C for 1 h. Appropriate washing of each well was performed between each step with PBS containing 0.05% of Tween 20. TMB solution (Sigma-Aldrich, USA) was added to the wells for the HRP substrate, the reaction was stopped after 10 min by adding 2 M H<sub>2</sub>SO<sub>4</sub>, and absorbance was measured at 450 nm using a microplate reader (Infinite® 200 PRO, USA). For serum IgG, IgG1, IgG2a and IgA the ELISA results are expressed as the endpoint titer and for BAL, nasal wash, and vaginal IgA, the results are expressed as the OD value measured at 450 nm (dilutions of 1:500 for BAL IgA, 1:10 for nasal wash IgA, 1:100 for vaginal IgA).

## 9) Bacterial challenge

(1) Growth of *A. pleuropneumoniae* serotype 5 (APP5) and intranasal challenge in mice

The *A. pleuropneumoniae* strains used for this study were serotype 5, Korean isolates. The bacteria were cultivated at 37 °C in chocolate agar and broth with  $\beta$ -NAD (10  $\mu$ g/ml). The minimum lethal dose was determined after challenge infection with APP5 in the nasal cavity of the mice. APP5 was grown until the optical density of 0.6 nm (OD 0.6 nm) was 0.5, reaching a concentration of  $5 \times 10^7$  colony-forming units (CFUs) per ml. The bacteria were then harvested through centrifugation at 8,000 rpm and washed with PBS. The pellets were re-suspended with PBS into various dilutions, and the bacteria were introduced into the snout of the mice anesthetized by Zoletil (Virbac Laboratories, Carros, France) and Rompun (Bayer Korea, Ansan, Korea). For the intranasal challenge of immunized mice, a MLD (minimum lethal dose) of  $5 \times 10^7$  CFU/mouse was used for APP5. Challenged mice were observed for 4 days.

(2) Calculation of bacteria from the lung tissue

After challenging the immunized mice with APP5, the lung tissue was surgically removed just before the death of the challenged mice. The lungs were fragmented into small pieces and then ground with a homogenizer. The

tissue mixture was spread on BHI agar plates with  $\beta$ -NAD (10  $\mu$ g/ml) after serial 10-fold dilutions with PBS. The plates were incubated at 37 °C overnight and the number of colonies was counted.

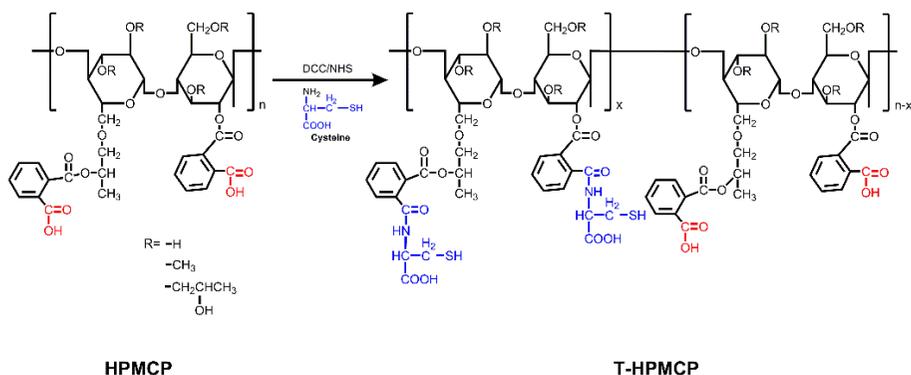
#### **10) Statistical analysis**

Quantified results were expressed as the mean and standard deviation (SD). Statistical significance was assessed using a one-way analysis of variance (ANOVA) with the post-hoc Tukey multiple comparisons test.

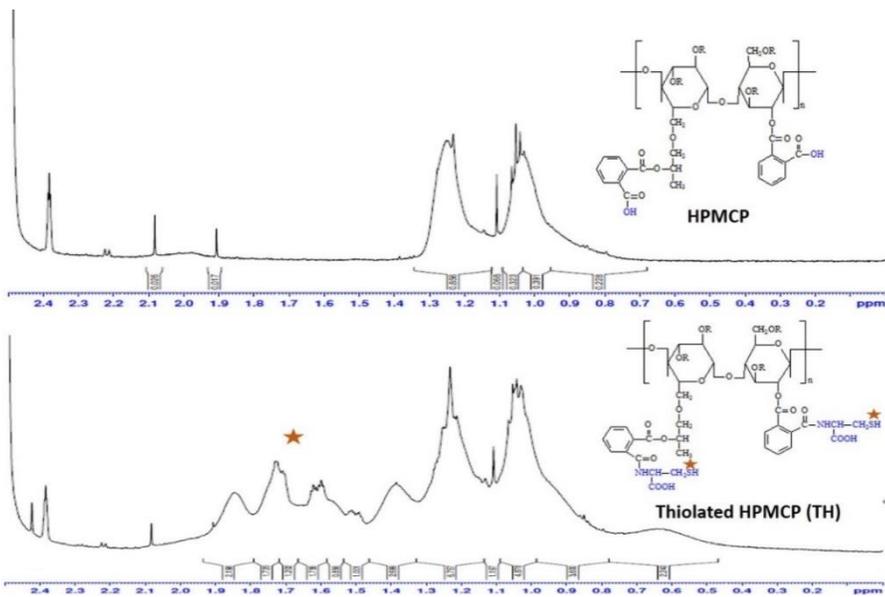
### 3. Results and Discussion

#### 1) Preparation of TH, THM, Man-THM, ApxIIA-loaded THM, and ApxIIA-loaded Man-THM

TH was synthesized by amide bond formation between the carboxylic acid groups of HPMCP and the primary amino groups of the cysteine using a DCC/NHS-activated coupling reaction, as shown in Figure 22. The coupling of cysteine with HPMCP was confirmed by <sup>1</sup>H-NMR by observing the appearance of the thiol proton resonance peak at 1.6 ppm (Figure 23). It was found that approximately 11.4 mol.-% of cysteine was conjugated with HPMCP. THM, Man-THM, ApxIIA-loaded THM, and ApxIIA-loaded Man-THM were prepared using the double emulsion method, as described in the Materials and Methods.



**Figure 22.** The reaction scheme for the synthesis of thiolated HPMCP (TH)



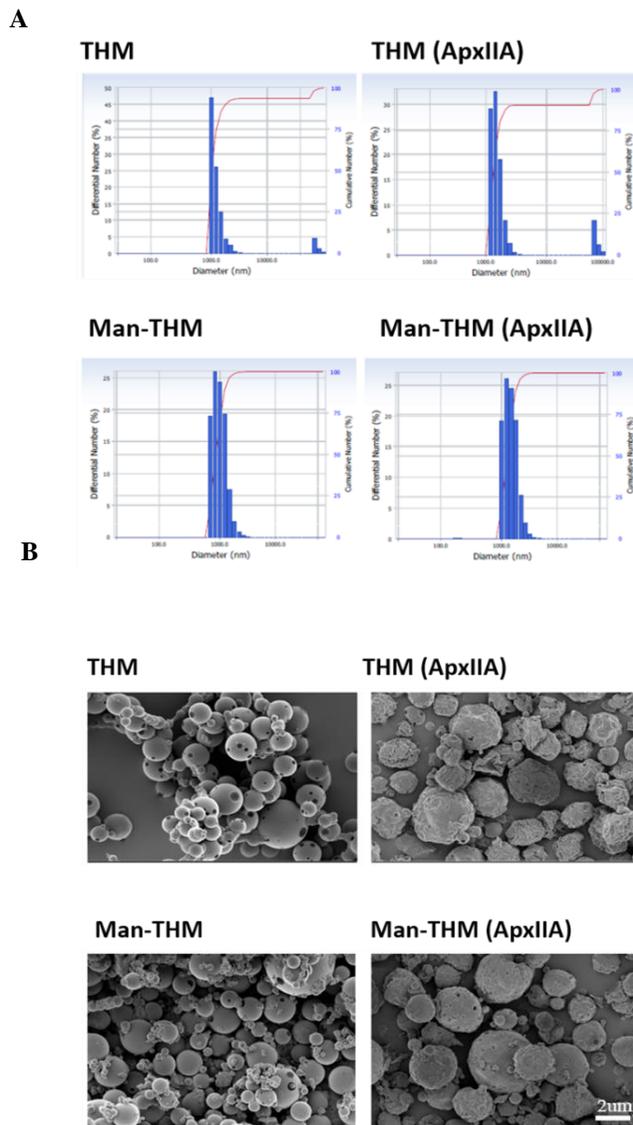
**Figure 23.** Confirmation of the synthesis of TH by  $^1\text{H}$  NMR (DMSO- $d_6$ , 600 MHz). Conjugation of the thiol group in HPMCP is depicted by the protons-S (H) of the cysteine in the NMR spectrum.

## 2) Characterization of THM and Man-THM

### (1) Size and surface morphology

The sizes of THM and Man-THM as measured by DLS were  $1.38\pm 0.33\ \mu\text{m}$  and  $1.21\pm 0.31\ \mu\text{m}$ , respectively, as shown in Figure 24(A). The decoration of THM with mannan did not result in much change in sizes due to the small amount of coated mannan. The sizes of the ApxIIA-loaded THM and ApxIIA-loaded Man-THM were  $1.53\pm 0.33\ \mu\text{m}$  and  $1.66\pm 0.33\ \mu\text{m}$ , respectively, slightly larger than the unloaded microparticles. The morphologies of the THM and Man-THM as observed by SEM are shown in Figure 24(B). The THM were spherical in shape and decoration with the mannan did not influence the spherical shape of the microparticles. Recently, many studies have demonstrated the relationship between particle size and mucociliary clearance in the airways (Möller et al., 2006; Usmani et al., 2005). One study showed that mucociliary clearance removed all particles larger than  $6\ \mu\text{m}$  from the airways within 24 h, while particles  $6\ \mu\text{m}$  or smaller were retained for more than 24 h (Henning et al., 2010). The size of the microspheres also affects uptake by the APCs. It has already been reported that DCs take up particles with sizes in the range of nanometers to micrometers. Particles smaller than  $5\ \mu\text{m}$  can be taken up by DCs and are transferred to the local lymph nodes and spleen, stimulating both local and systemic immune responses; DCs do not take up particles larger than  $10\ \mu\text{m}$  (Oechslein et al., 1996). The mean sizes of the microspheres in this

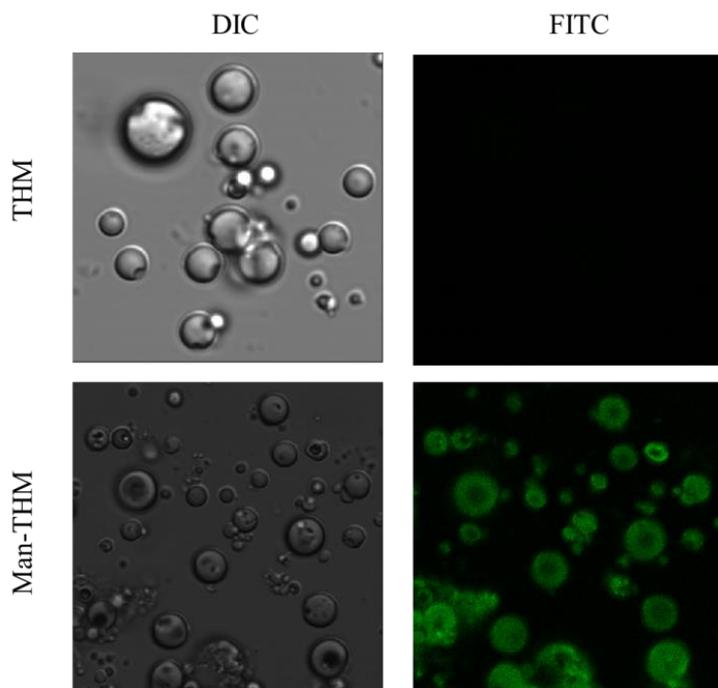
study were less than 3  $\mu\text{m}$ , which is similar to the sizes of the bacteria that the immune system has evolved to combat. It is reasonable to assume that our microspheres would be internalized through phagocytosis by APCs, which would then mature and migrate to the nearby lymph nodes (Champion et al.,2008).



**Figure 24.** (A) Particle size distributions of THM, Man-THM, ApxIIA-loaded THM, and ApxIIA-loaded Man-THM. (B) SEM photographs of THM, Man-THM, ApxIIA-loaded THM, and ApxIIA-loaded Man-THM.

## (2) Confirmation of the mannan surface decoration in Man-THM

The surface decoration of THM with mannan molecules was evaluated by confocal microscopy, as shown in Figure 25. FITC-conjugated mannan was used to create FITC-Man-THM. The confocal images indicated that the surface of the Man-THM was homogeneously coated with mannan due to the mannan's amphiphilic property, which functions as a stabilizer during the formation of microspheres through  $W_1/O/W_2$  double emulsion. Through quantification of the amount of FITC-mannan decorating in THM, almost 0.5 –wt% of FITC-mannan was detected from the FITC-Man-THM.

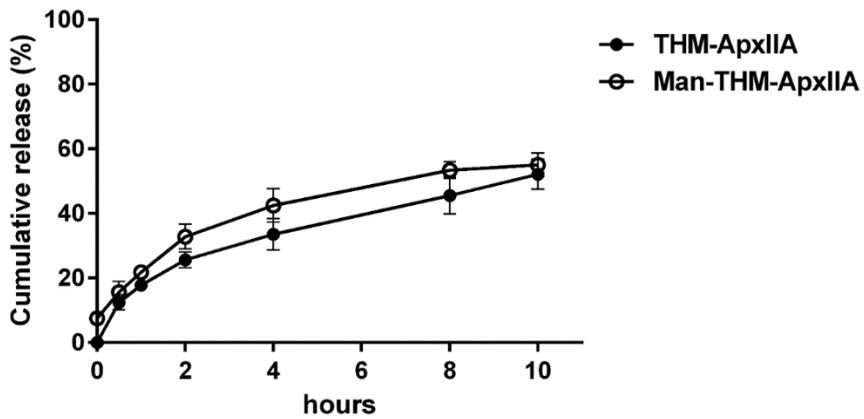


**Figure 25.** Confirmation of the surface decoration of mannan in Man-THM. FITC-conjugated mannan was used to create the FITC-Man-THM and the surface fluorescent signal was monitored by CLSM. First column: image by differential interference contrast (DIC) microscopy; second column: fluorescence by confocal laser scanning (CLS) microscopy.

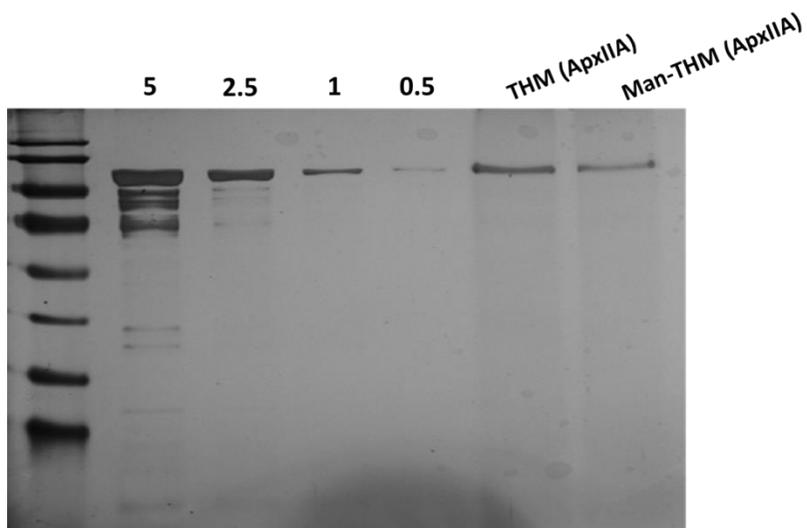
### 3) *In vitro* studies

#### (1) *In vitro* release of ApxIIA from ApxIIA-loaded THM and ApxIIA-loaded Man-THM

The *in vitro* release behaviors of ApxIIA-loaded THM and Man-THM were studied in PBS (pH 7.4) (Figure 26). The release profiles of ApxIIA were presented as a percentage of the amount of ApxIIA released from the microparticles with respect to the total amount of ApxIIA encapsulated. The results indicated that approximately 50 wt.-% of the ApxIIA was rapidly released from the ApxIIA-loaded THM and Man-THM within 10 h, showing similar patterns of release. Distinct from low molecular weight drugs, proteins possess secondary, tertiary, and even quaternary structures with labile bonds between the side chains of chemically reactive groups. Disruption of these structures or modifications of these side chains can lead to a loss of protein or vaccine activity (Jiang et al., 2014). Thus, ensuring the stability of the antigen during its loading process into the microparticles is important for retaining its antigenicity. The structural integrity of ApxIIA was evaluated by SDS-PAGE before and after loading onto THM and Man-THM. As shown in Figure 27, the band patterns of the ApxIIA released from the ApxIIA-loaded THM and Man-THM were similar to that of native ApxIIA, indicating that the ApxIIA was not structurally altered during antigen loading.



**Figure 26.** Release of ApxIIA from ApxIIA -loaded THM and ApxIIA-loaded Man-THM at pH 7.4 and 37°C (n = 3, error bar represents standard deviations).

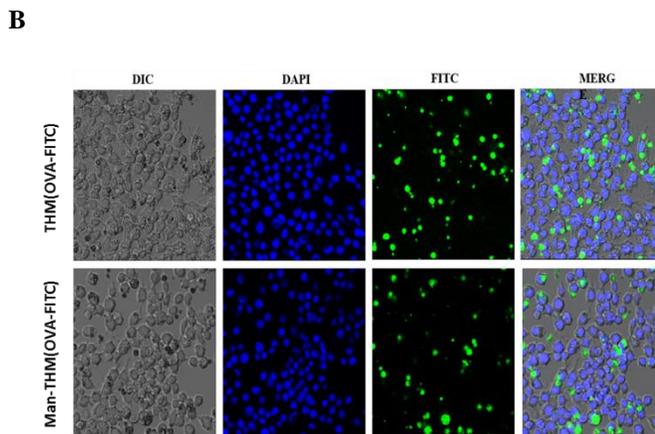
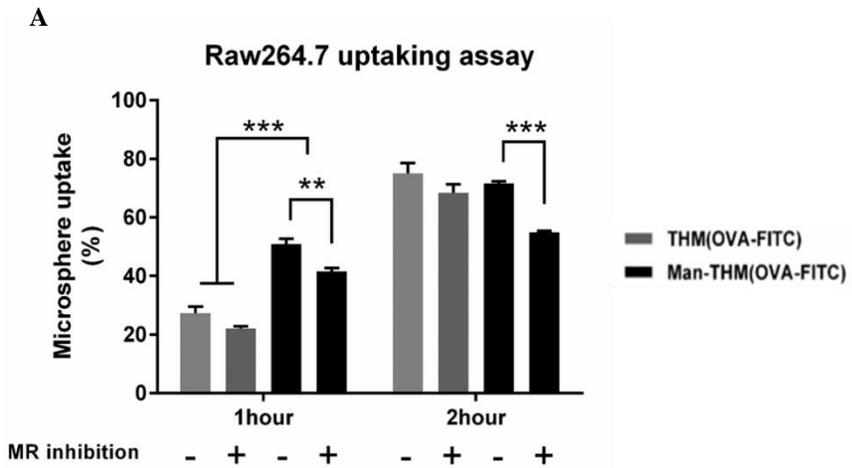


**Figure 27.** Evaluation of the structural integrity of ApxIIA released from the ApxIIA-loaded THM and Man-THM by SDS-PAGE. Lane 1: marker, Lane 2, 3, 4, and 5: 5, 2.5, 1, and 0.5ug of ApxIIA, respectively, Lane 6: ApxIIA released from the ApxIIA-loaded THM, Lane 7: ApxIIA released from the ApxIIA-loaded Man-THM.

## (2) Cellular uptake of microspheres by RAW264.7

The targeted delivery of microspheres to APCs via MR was determined by observing the uptake of microspheres by APCs. The analysis of the cellular uptake of microspheres was performed in RAW 264.7 using FITC-labeled OVA-loaded THM and FITC-labeled OVA-loaded Man-THM. The uptake of microspheres by RAW 264.7 cells was observed by FACS analysis according to incubation time and depended on the blockade of MR. Figure 28(A) shows the uptake of microspheres by RAW 264.7 at 37 °C for 1 and 2 h. The MR-targeted Man-THM exhibited significantly higher uptake efficiency for RAW 264.7 cells at 1 h post-treatment; however, there were no statistical differences between THM and Man-THM at 2 h due to the non-specific uptake of particles by RAW 264.7. In addition, the blockade of MR in RAW 264.7 by pre-treatment with mannan resulted in a significant decrease in the Man-THM uptake at both 1 and 2 h, indicating that the uptake of Man-THM was mediated by interactions between mannan and MR.

In order to confirm microsphere uptake, confocal laser scanning microscopy was also performed. Figure 28(B) shows the internalization of the microspheres by RAW 264.7 at 37 °C after 2 h of treatment. It was found that the internalization efficiency of THM and Man-THM by RAW 264.7 cell lines at 2 h was similarly consistent with the results of the cellular uptake of the microspheres observed by FACS.

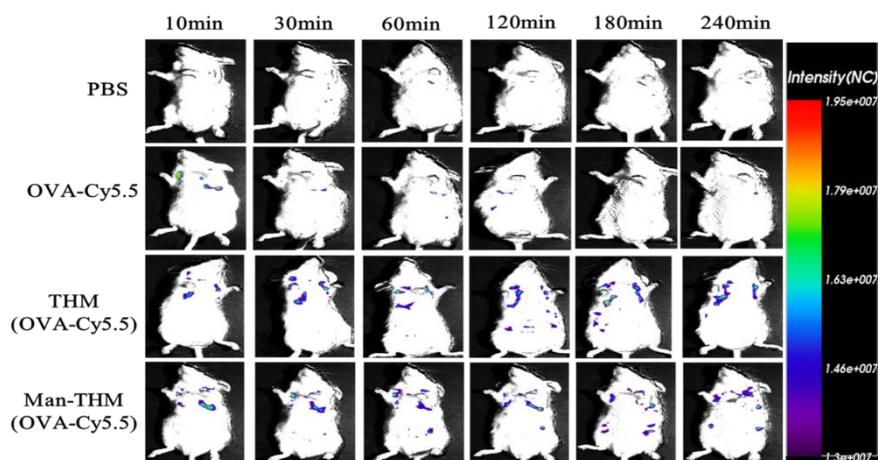


**Figure 28.** (A) Measurement of microsphere uptake by RAW 264.7 using FACS. Uptake of OVA-FITC-loaded THM and OVA-FITC loaded Man-THM by RAW 264.7 in 1 h and 2 h with and without MR inhibition at 37 °C (n = 3, error bar represents standard deviation; \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.005, one-way ANOVA). (B) Confocal microscopic images of RAW 264.7 cells after 2 h culture with OVA-loaded THM and OVA-loaded Man-THM at 37 °C.

#### **4) *In vivo* imaging of the intranasal administration of the microspheres**

The primary function of the mucosal layer is to protect the lungs by trapping and removing foreign particles by the mucociliary escalator, which causes trapped particles to be coughed up out of the lungs. The development of an efficient intranasal vaccine depends on the vaccine's ability to maintain adequate contact time in the respiratory mucosa, allowing for interaction with the respiratory immune cells. In a previous study, we confirmed that thiolated HPMCP (TH) exhibited a 1.72-fold greater mucus adhesion ability than HPMCP upon contact with a mucin layer. In this study, we recruited TH microspheres as intranasal delivery vehicles. To confirm the performance of intranasal THM-delivered antigen in the respiratory mucosa, we evaluated antigen signaling in the respiratory system after the intranasal administration of the microspheres. *In vivo* optical molecular imaging (Optix-MX3) clearly showed that Cy5.5-OVA-loaded THM and Cy5.5-OVA-loaded Man-THM were retained in the respiratory mucosa 4 h after nasal administration. In contrast, the majority of free Cy5.5-OVA had disappeared from the respiratory mucosa within 1 h after nasal administration (Figure 29). These results indicated that the mucoadhesive ability of antigen-loaded THM and Man-THM efficiently prolonged their retention time in the respiratory mucosa and increased the chance of uptake by APCs. In addition, signals were detected from the distal secondary lymphoid tissues including the spleen, implying that APCs

internalized antigen-loaded THM and Man-THM from the respiratory system and migrated into the secondary lymphoid tissue to induce systemic and distal mucosal immune responses.



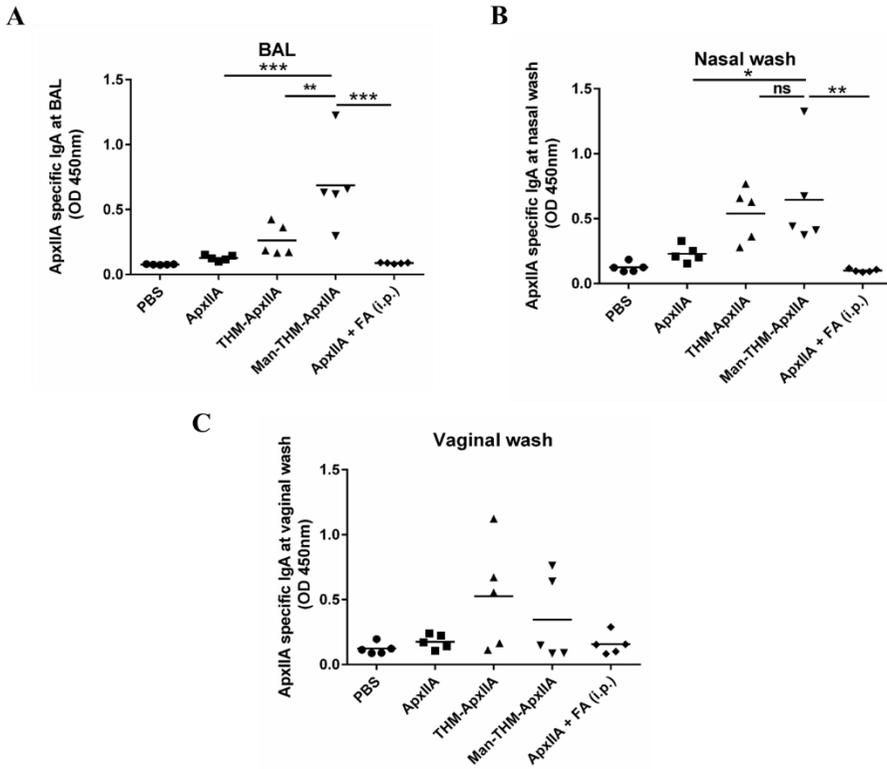
**Figure 29.** Cy5.5-conjugated OVA was used. Cy5.5-conjugated OVA and the same amount of cy5.5-conjugated OVA loaded onto THM and MAN-THM were intranasally administered to 6 week-old BALB/c mice. The fluorescence was monitored at 10 min, 30 min, 60 min, 120 min, 180 min, and 240 min. The fluorescence images were acquired under an excitation of 695 nm using the Optix-MX3 (Advanced Research Technologies, Canada). The fluorescence intensities in the regions of interest (ROI) were analyzed from the fluorescence images.

## 5) *In vivo* immunization

### (1) ApxIIA-specific mucosal immune responses

Based on the results of the microsphere characterization, we hypothesized that the mucoadhesive microspheres would have prolonged residence time in the respiratory mucosa, and that MR-targeting ligands could enhance the delivery of Ag into mucosal APCs and thus promote the induction of mucosal immune responses. To validate our hypothesis, we evaluated immunizations with the intranasal administration of ApxIIA alone, ApxIIA-loaded THM, and ApxIIA-loaded Man-THM, and also evaluated the level of ApxIIA-specific mucosal immune responses in the BAL and nasal wash. Figure 30 shows the levels of anti-ApxIIA sIgA in the various mucosal tissues after nasal vaccination with different formulations. The mice immunized with ApxIIA-loaded Man-THM showed higher levels of anti-ApxIIA sIgA antibody in the BAL [Figure 30(A)] compared with those immunized with the ApxIIA-loaded THM and native ApxIIA. The levels of anti-ApxIIA sIgA antibody in the nasal wash [Figure 30(B)] showed a similar pattern with the anti-ApxIIA IgG antibody response, although no significance difference between THM and Man-THM was observed. The local and distal production of sIgA antibodies are the most important characteristics of nasally administered vaccines. Notably, our results indicated that intranasally delivered particulate vaccines could induce anti-ApxIIA sIgA antibody in the vagina [Figure 30(C)]. These results

indicated that nasally administrated particulate vaccines efficiently induced antibody responses at both local and distal mucosal sites, which can defend the body against pathogens from other mucosal sites such as the vagina. Conversely, i.p. vaccination with ApxIIA could not induce sIgA responses at both local and distal mucosal sites, proving the importance of mucosal vaccines to induce mucosal immune responses.

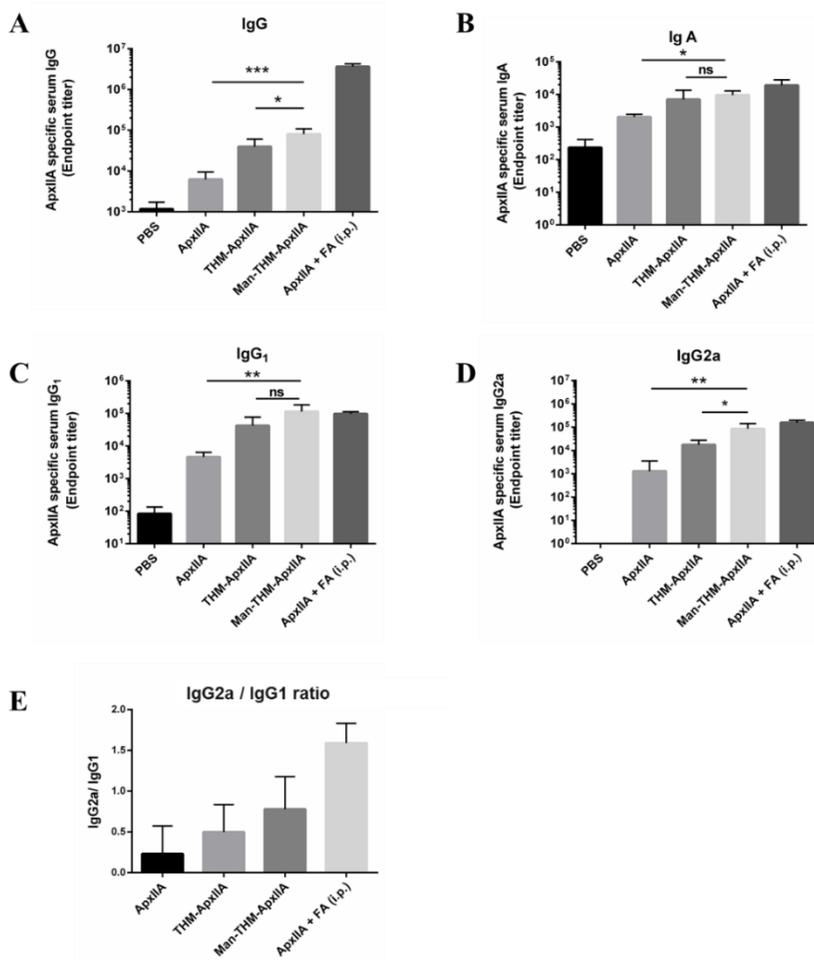


**Figure 30.** ApxIIA-specific IgA performance in the mucosal sites at 4 weeks post-immunization. ApxIIA-specific brochealveolar lavage (A), nasal wash (B), vaginal wash (C), IgA levels in mice immunized with the indicated formulations were analyzed by ELISA and then calculated by optical density (450 nm) (n = 5, error bars represent standard deviations; \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.005, one-way ANOVA).

## (2) ApxIIA-specific serum IgG (IgG1 and IgG2a) responses

The levels of ApxIIA-specific serum isotypes of IgG, IgA, IgG1, and IgG2a are shown in Figure 31. Serum-derived IgG contributes to the immune defense of the lower respiratory tract (Renegar et al., 2004), and the production of IgG1 and IgG2a isotypes is associated with Th2 type and Th1-type immune responses, respectively (Dobrovolskaia and McNeil, 2007). The mice immunized with ApxIIA-loaded Man-THM displayed significantly higher IgG titers than those receiving ApxIIA-loaded THM and ApxIIA. However, significance differences in serum IgA was not found between the THM and Man-THM groups [Figure 31(A) and (B)]. The level of ApxIIA-specific IgG2a was the most significantly enhanced of the IgG isotypes, although the level of the other isotypes of ApxIIA-specific IgG also increased in response to the nasal administration of ApxIIA-loaded Man-THM [Figure 31(C) and (D)]. Furthermore, in order to understand the characteristics of the immune responses induced by the nasal immunization of ApxIIA-loaded THM and Man-THM, the ratio of IgG2a and IgG1 was analyzed as an indicator of Th1 or Th2 bias in the immune response [Figure 31(E)]. As shown in the figure, the ratio of IgG2a and IgG1 was increased in the particulate vaccine groups compared with the native antigen groups. Additionally, the ApxIIA-loaded Man-THM group showed a higher IgG2a and IgG1 ratio than the ApxIIA-loaded THM group, implying a Th1 bias in the immune response. This suggests that the enhanced ApxIIA-

specific systemic immune response provoked by nasal immunization with ApxIIA-loaded Man-THM was due to the enhancement of the Th1 immune response.



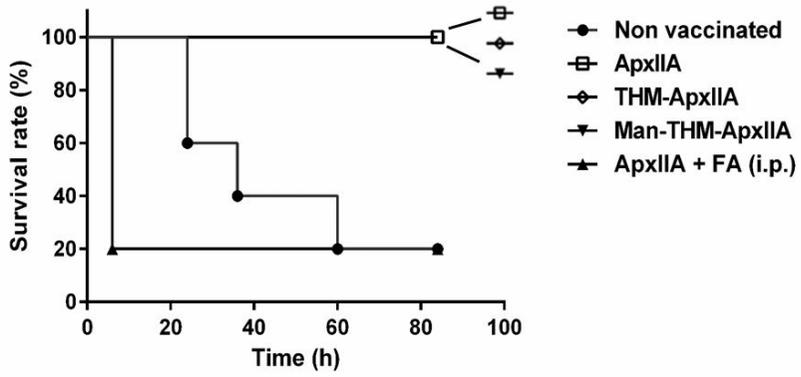
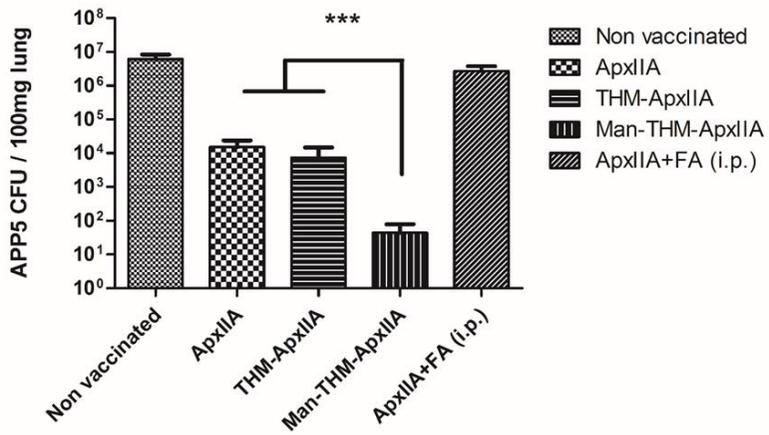
**Figure 31.** ApxIIA-specific systemic immune responses at 4 weeks post-immunization. ApxIIA-specific serum IgG (A), serum IgA (B), serum IgG1 (C), serum IgG2A (D), and IgG2a, IgG1 ratios (E) in mice nasally immunized with the indicated formulations were analyzed by ELISA assay (n = 5, error bars represent standard deviations; \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.005, one-way ANOVA).

## 6) Challenge assay

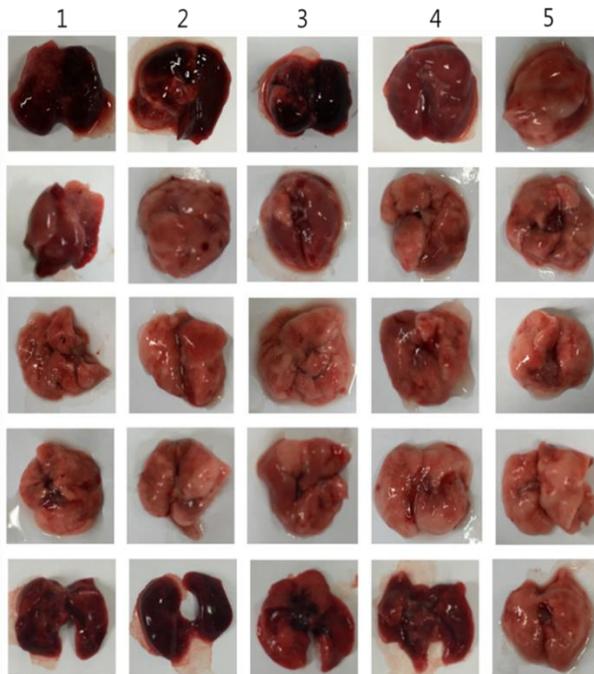
We next investigated if the enhanced mucosal and systemic immune response induced by the nasal administration of ApxIIA-loaded Man-THM could establish protective immunity against bacterial infection in a murine model (Figure 32). We inoculated  $5 \times 10^7$  CFU of *A. pleuropneumoniae* serotype 5 intranasally and monitored the survival of the mice for 96 h. As shown in the Figure 32(A), mice in the unvaccinated control group started to die at 24 h after the infectious challenge, and 80% of the mice died within 60 h. Conversely, all mice intranasally vaccinated with ApxIIA, ApxIIA-loaded THM, and ApxIIA-loaded Man-THM survived the challenge. Surprisingly, 80% of the mice intraperitoneally immunized with ApxIIA died within 12 h after challenge. The lower body weights of the mice in the i.p. group (data not shown) due to the use of the Freud adjuvant may explain why these mice could not overcome the high burden of bacteria as compared to mice with higher body weights.

In order to confirm the clearance of ApxIIA by the protective immune response, we harvested the lung tissue of the surviving mice at 96 h or immediately from the mice that died before 96 h [Figure 32(C)], and counted the number of bacteria in the lung tissue homogenates [Figure 32(B)]. The number of bacteria measured in the lung tissue homogenates prepared from mice in the ApxIIA-loaded Man-THM group was approximately 100-fold lower than those from the ApxIIA and ApxIIA-loaded THM groups, although

all mice from the three groups survived the infectious challenge. We concluded that ApxIIA-loaded Man-THM enhanced the systemic and mucosal immune responses against ApxIIA, which enhanced the bacterial clearance ability of the mice challenged with *A. pleuropneumoniae*, thus improving survival.

**A****B**

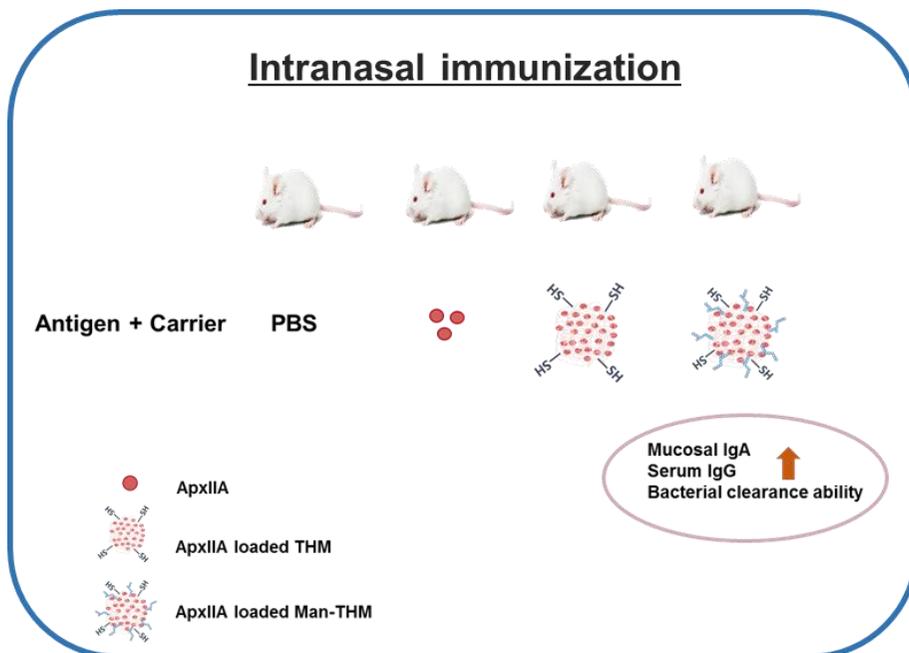
C



**Figure 32.** Induction of protective immunity after intranasal challenge with *A. pleuropneumoniae*. (A) 14 days after the last immunization, 5 mice per group were challenged intranasally with a mean lethal dose ( $5 \times 10^7$  CFU) of *A. pleuropneumoniae*, with the survival rate (%) monitored for an additional 4 days. (B) The number of residual bacteria was counted per 100 mg fresh lung tissue weight from each mice per group. ( $p < 0.05$ ,  $**p < 0.01$ ,  $***p < 0.005$ , one-way ANOVA) (C) The lungs were characterized before becoming homogenates and after bacteria challenge.

#### 4. Conclusion

The development of an effective nasal vaccine requires appropriate carriers and an adjuvant system that protects antigens from rapid nasal clearance by the mucociliary apparatus and efficiently delivers antigens to APCs. In this study, mannan-decorated mucoadhesive microspheres (Man-THM) were successfully prepared and their targeted delivery to the MR on APCs was evaluated *in vitro* and *in vivo*. It was found that intranasal vaccination with ApxIIA-loaded Man-THM in mice induced enhanced mucosal sIgA and serum antibody (IgG and IgA) responses as compared to ApxIIA-loaded THM due to specific interactions between the mannan on the Man-THM and the MR on APCs. Furthermore, to assess the protective efficacy of these vaccines, mice were vaccinated with ApxIIA-loaded Man-THM and challenged with *A. pleuropneumoniae*. The adjuvant effect of Man-THM was clearly demonstrated by the 100% survival rate and enhanced bacterial clearance, proving successful protection from *A. pleuropneumoniae*. Our results strongly support the efficacy of Man-THM as an adjuvant carrier system for the effective induction of systemic and mucosal immune responses.



**Figure 33.** Schematic illustration for the result of the study

## **Chapter 4. Overall conclusion**

The mucosa is a primary target for infection and, at the same time, the first line of defense against many pathogens in which secretory IgA is a main

protective component of the immune system. Mucosal vaccines, in contrast to injected vaccines, have been reported to provide additional secretory IgA mediated protection against pathogens at the mucosal site of entry. One of the most important virtues of mucosal vaccination is, while injected vaccines generally fails to do so, capable of inducing protective immune responses both in the mucosal and systemic immune compartments. However, there are several very serious concerns about mucosal vaccines. Mucosa-administered antigens are generally less immunogenic and physical barrier of mucosa also is an obstacle that to be overcome. In this regard, induction of mucosal immunity through vaccination is a rather difficult task, and potent mucosal adjuvants and special delivery systems are required for successful mucosal vaccination.

The aim of the study was development of mucosal adjuvant using cytokine and polymeric particles for efficient mucosal immunization. In order to achieve this purpose, adjuvant systems were designed according to the coping strategies

against the hurdles in developing subunit vaccine (Figure 1-3).

Study 1 was mainly focused on developing oral cytokine adjuvant. A recombinant cytokine, IL-6-CKS9, was generated by conjugating an M cell-targeting peptide (CKS9) with c-terminus of the murine interleukin 6 (IL-6), which facilitated enhancement of mucosal immune response. *Lactococcus lactis* IL1403, a food-grade strain of lactic acid bacteria (LAB) which is widely used in dairy industry, was used as a host cell to express and secreted the IL-6-CKS9 for a mucosal vaccine adjuvant. The recombinant *L. lactis* IL1403 secreted IL-6-CKS9 was orally administered with a model antigen protein, M-BmpB (*Brachyspira* membrane protein B conjugated with CKS9), to BALB/c mice for mucosal immunization. ELISA analyses showed consistent enhancement tendencies in induction of anti-M-BmpB antibody levels both with mucosal (IgA) and systemic (IgG) immune responses in IL-6-CKS9-LAB treated group compared with other groups tested by conducting mice immunization assays. In addition, the oral administration of model protein antigen with live LAB producing IL-6-CKS9 could induce both Th1 and Th2 type immune responses. Collectively, the results showed successful production and secretion of recombinant murine IL-6 with M cell-targeting moiety (IL-6-CKS9) from *L. lactis* IL1403 and demonstrated that the live recombinant LAB producing IL-6-CKS9 could have a potential to be used as an efficient adjuvant for peroral vaccination.

Study 2 was mainly focused on developing an efficient nasal vaccine carrier. The nasal route for vaccine delivery by particles has attracted considerable interest, although challenges such as the rapid mucociliary clearance in the respiratory mucosa and the low immunogenicity of subunit vaccine still remain (Figure 3). Recently, a new generation of synthetic polymers known as thiolated polymers, such as thiolated hydroxypropylmethyl cellulose phthalate (HPMCP), have been shown to have strong mucoadhesive properties due to their binding with the cysteine-rich subdomains of mucus glycoprotein in the mucosal site. So, thiolated HPMCP (TH) was applied to make microspheres as a nasal vaccine carrier for prolonging the residence time in the respiratory mucosa to make interaction time of vaccine molecules and antigen presenting cells (APCs) longer. Moreover, another major hurdle in the development of intranasal vaccines is poor immunogenicity of vaccine subunits due to the lack of “danger signals” that can activate APCs. To this end, study 2 was aimed to develop mannan-decorated mucoadhesive THM (Man-THM) as an adjuvant system for nasal vaccine. Mannan is one of the natural PAMPs (pathogen associated molecular patterns) of APCs and is a ligand of the mannose receptor (MR) widely expressed on APCs. Mannan can induce the production of pro-inflammatory cytokines and up-regulate the expression of co-stimulatory molecules in these cells, thereby activating both innate and adaptive immunity. The aim of study 2 was to explore the potential of a novel Man-THM vehicle for the intranasal administration of ApxIIA as a vaccine against *A.*

*pleuropneumoniae* which is known to cause contagious porcine pleuropneumoniae. ApxIIa-loaded Man-THM was prepared by the double emulsion solvent evaporation method and using the mixture of 0.25wt.-% PVA and 0.5wt.-% mannan ( $W_2$  phase) to decorate the surface of microspheres with mannan. After optimization of the preparation method and characterization of the obtained ApxIIA-loaded Man-THM, the physiochemical properties of the Man-THM as a nasal delivery carrier were evaluated by *in vitro* and *in vivo* assays. The surface of the Man-THM was homogeneously coated with mannan due to the mannan's amphiphilic property, which functions as a stabilizer during the formation of microspheres through  $W_1/O/W_2$  double emulsion. In a mechanistic study using APCs *in vitro*, it was found that Man-THM enhanced receptor-mediated endocytosis by MR of APCs. *In vivo*, the nasal vaccination of ApxIIA-loaded Man-THM in mice resulted in higher levels of mucosal sIgA and serum IgG than mice in the ApxIIA and ApxIIA-loaded THM groups due to the specific recognition of the mannan by the MRs on the APCs. Moreover, ApxIIA-containing Man-THM protected immunized mice when challenged with strains of *A. pleuropneumoniae* serotype 5. The number of bacteria measured in the lung tissue homogenates prepared from mice in the ApxIIA-loaded Man-THM group was approximately 100-fold lower than those from the ApxIIA and ApxIIA-loaded THM groups. The results showed that ApxIIA-loaded Man-THM enhanced the systemic and mucosal immune responses against ApxIIA, which enhanced the bacterial clearance ability of the mice

challenged with *A. pleuropneumoniae*, thus improving survival. The results strongly support the efficacy of Man-THM as an adjuvant carrier system for the effective induction of systemic and mucosal immune responses.

In conclusion, adjuvant system using cytokine and polymeric particles were applied to oral and nasal subunit vaccine, respectively in the study 1 and 2. An oral cytokine vaccine adjuvant named as 'IL6-CKS9' and nasal vaccine delivery carrier named as 'Man-THM' were developed and evaluated its adjuvant efficiency through *in vitro* and *in vivo*. Although there is much work to be done before these systems are applied for livestock, however, 'IL6-CKS9' and 'Man-THM' have potential to be safe and efficient adjuvant systems for mucosal vaccine.

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## Summary in Korean

점막표면은 대부분 병원균이 체내로 침입하는 주요 관문이며 이는 가축에게 있어서 전염병을 일으키는 주요 원인경로이기도 하다. 때문에 점막을 통하여 면역반응을 일으켜 점막에서의 방어체계인 sIgA를 생성하여 병원체가 감염하는 경로인 점막에서 초도에 방어를 함으로써 병원체의 감염을 막는 것이 굉장히 중요하다. 점막면역은 전신면역을 일으키는 근육주사와는 달리 주사바늘에 의한 공포심이 가해지지 않고 또한 접근성이 좋고 안전하여 개발만 된다면 경제적으로도 저렴하여 농가에서 사용하기 용이한 장점이 있다. 하지만 점막을 통하여 일으키는 면역은 면역유도능력이 전신면역이 일으키는 것에 비하여 낮으며 그 면역원성을 높여줄 수 있는 안전하고 효과적인 점막백신 어주변트와 전달체가 아직 용이하게 사용되지 못할뿐더러 아직 전반적인 점막면역에 대한 학설이 완벽하지 못한 것 또한 한가지 원인으로 꼽을 수 있다. 최근 들어 효과적인 점막면역백신을 개발하기 위하여 면역원성이 높은 항원을 선별하는 것에 대한 연구, 효과적인 투여경로의 디자인, 점막면역을 자극 할 수 있는 어주변트 물질에 대한 탐색, 그리고 점막으로의 백신전달시스템에 대한 연구가 활발히 진행 중에 있다.

본 연구의 목적은 가축 점막면역 백신 개발 특히 경구백신과 비강백신의

개발에 있어서 백신의 효율을 높이고자 하는 목적으로 신형 경구백신 어주번트의 개발과와 비강백신 전달체 개발에 대한 것으로서 연구 1은 사이토카인(IL-6)을 *Lactococcus lactis* IL1403에서 생산하여 경구용 백신 어주번트로 활용한 것에 대한 연구이고, 연구 2는 점막점착성 고분자 전달체에 mannan을 코팅시켜 항원제시세포를 표적 할 수 있는 비강백신 전달체에 대한 내용이다.

먼저, 연구 1은 재조합 IL-6-CKS9 사이토카인, 즉 M 세포 표적 펩타이드(CKS9)를 IL-6의 C 말단에 연결된 형태로 구성하여 점막면역반응을 증진시킬 수 있는 어주번트로 개발하고자 하였다. *Lactococcus lactis* IL1403는 유업에 널리 사용되고 있는 식품성 유산균의 한 종류로써 IL-6-CKS9을 발현 및 분비시키는 숙주세포로 사용하였으며 이 IL-6-CKS9을 생산할 수 있는 재조합 *L. lactis* IL1403을 경구백신 어주번트로 개발하였다. IL-6-CKS9 생산 재조합 *L. lactis* IL1403을 모델 항원 단백질인 M-BmpB(*Brachyspira* 막 단백질 B에 CKS9을 conjugation 시킨 형태)를 BALB/c 마우스에 경구로 함께 투여하여 면역을 시켰다. 경구면역 후 ELISA 분석을 통하여 소장과 분변에서의 점막 IgA와 혈중 IgG가 IL6-CKS9-유산균을 어주번트로 활용한 그룹에서 기타 그룹에 비해 높은 역가를 나타냈으며 이는 두 번의 독립적인 면역실험에서 같은 경향으로 보이는 것으로 확인이 되었다. 뿐만 아니라 혈중 BmpB 특이적 IgG1과 IgG2a의 역가 측정을 통하여 IL6-CKS9-유산균을 경구백신 어주번트로 사용하였을 경우, Th1 타입의 면역반응과 Th2 타입의 면역반응을 동시에

증진하는 것으로 확인이 되었다. 결론적으로 M 세포 표적 펩타이드가 도입된 IL-6 (IL6-CKS9)를 성공적으로 *L. lactis* IL1403에서 발현 및 분비를 시켰으며 경구백신 실험을 통하여 IL-6-CKS9 분비 유산균을 어주번트로 활용하였을 경우 점막 IgA 및 혈중 IgG의 생성을 증진시켰으므로 IL6-CKS9-유산균의 경구백신 어주번트로써의 가능성을 확인하였다.

연구 2는 점막점착성능력과 항원제시세포를 표적 할 수 있는 능력을 지닌 비강백신 전달체를 개발하고 그것의 효과를 비강면역을 통하여 검증한 것에 관한 내용이다. 비강을 통하여 점막면역을 일으키는 것은 다른 점막면역경로에 비해 효과적이어서 점막면역에서 가장 많은 각광을 받고 있다. 하지만 효과적인 비강백신을 개발함에 있어서 호흡기 점막의 점액섬모청소(mucocilliary clearance)로 인해 투여한 항원물질이 비강에서의 머무르는 시간이 짧은 문제점과 서브유닛 백신으로써의 면역원성이 낮은 문제점은 극복해야 할 난관이다. 하여 연구 2에서는 mannan을 점막점착성 HPMCP microsphere (THM)에 코팅을 시켜 Man-THM을 구성함으로써 점막점착성 능력으로 mucocilliary clearance를 극복하고 또 mannan의 도입을 통하여 항원제시세포를 표적 할 수 있는 전달체를 구축하였다. *A. pleuropneumoniae*를 방어할 수 있는 exotoxin fragment인 ApxIIA를 모델 항원 단백질로 사용하였고 Man-THM 에 ApxIIA를 담지하였다. Man-THM은 mannan이 코팅되어 있어 호흡기 항원제시세포의 PRRs(pathogen recognition receptors)를 표적할 수 있을 뿐만 아니라 mannose receptor(MR)를 표적할 수 있어서 어주번트로 작용할 수 있다.

Man-THM의 비강면역 전달체로써의 어주변트 기능은 *in vitro*와 *in vivo*에서 확인하였다. 먼저 *in vitro*에서 Man-THM 이 APCs에서 mannose receptor를 통한 receptor-mediated endocytosis를 하는 것을 검증하였고 *in vivo*에서 ApxIIA를 담지 한 Man-THM을 BALB/C 마우스에 비강을 통하여 면역을 시킨 후 호흡기 점막에서 분비되는 IgA와 혈중 IgG측정을 통하여 ApxIIA를 담지한 Man-THM이 ApxIIA 단독으로 혹은 ApxIIA를 THM에 담지하여 면역을 시킨 그룹에 비해 높은 항체역가를 나타내는 것을 확인 하였다. 뿐만 아니라 비강면역 후, *A. pleuropneumoniae* serotype 5를 비강을 통하여 공격 접종하였을 때 효과적인 방어효과를 나타내는 것을 확인하였고 ApxIIA를 담지한 Man-THM 그룹이 기타 그룹에 비하여 폐 내에 잔존하고 있는 *A. pleuropneumoniae* 수가 유의적으로 감소하는 것 확인하였다. 결론적으로 항원제시세포 표적형 점막점착성 Man-THM이 효과적으로 비강면역을 유도를 증진시키는 것을 확인함으로써 비강백신 전달체로써의 잠재성이 있음을 확인하였다.

# **Appendix. Mannan-decorated thiolated Eudragit microspheres for targeting antigen presenting cells via nasal vaccination**

## **1. Introduction**

Mucosal immunity is mediated by secretory immunoglobulin A (sIgA) antibody, which prevents pathogen attachment from mucosal epithelia (e.g. respiratory tract, vaginal tract and gastro-intestinal tract) and clear pathogens before they invade or infect the underlying tissue (Holmgren and Czerkinsky, 2005). Thus, an effective mucosal vaccine is necessary in the aspect that it can protect the host body from the pathogens at the initial site of infection by induction of mucosal immunity. Moreover, mucosal immunization frequently results in the stimulation of both mucosal and systemic immunity (Czerkinsky and Holmgren, 2012). Among mucosal immunizations, intranasal administration is a promising vaccination route because it induces potent cross-protective immune responses both in the respiratory tracts and the genital tracts (Lycke, 2012).

For an effective delivery of subunit vaccine through intranasal route, two major limitations need to be overcome. One is the rapid clearance of vaccines by normal mucociliary process and the other one is the weak immune response induced due to inefficient antigen presentation by immune cells. To overcome these limitations, the strategy of subunit vaccine formulations for nasal vaccination should be designed to retain the vaccine carrier in the nasal cavity for longer duration for sufficient delivery through NALT (Hagenaars et al., 2010) and to target the immune cells using a ligand in the vaccine carrier for efficient uptake by APCs (Foged et al., 2002).

In recent years, several particulate delivery systems have been developed for nasal delivery of subunit vaccines rather than soluble subunit antigens due to their several physicochemical advantages like particle size, zeta potential, hydrophobicity, tight junction opening properties and receptor-binding characteristic (Vajdy and O'Hagan, 2001). However, many vaccine delivery particles have little affinity with the nasal epithelium, and they are usually cleared within minutes by ciliary movement in the nasal cavity (Illum, 2003). The first major hurdle - rapid nasal clearance of vaccine in nasal epithelium, can be achieved by mucoadhesive polymeric carriers which can extend the nasal residence time efficiently (Amidi et al., 2006; Khutoryanskiy, 2011; Rahamatullah Shaikh et al., 2011). Recently, a new generation of synthetic polymers, called as thiomers or thiolated polymers, have been reported for their

strong mucoadhesive properties due to binding with the cysteine-rich subdomains of mucus glycoprotein (Bernkop-Schnürch et al., 2001; Bernkop-Schnürch et al., 2004). In this work, thiolated Eudragit L-100 was used as nasal subunit vaccine carrier because it holds both pH-sensitive and mucoadhesive features (Islam et al., 2011; Lee et al., 2011; Lee et al., 2012).

Another major hurdle to be overcome is the poor immunogenicity of subunit vaccine caused by inefficient antigen presentation. Targeting antigens to endocytic receptors on professional APCs represent an attractive strategy to enhance the efficacy of vaccine. Mannose receptor (MR) is a member of C-type lectin receptor widely expressed on APCs such as DCs and macrophages (Taylor et al., 2005) which is important for antigen internalization. Mannan (polymer of mannose units) isolated from the cell wall of *Saccharomyces cerevisiae*, is one of the natural ligands that has strong affinity for MR (Taylor et al., 1990; Vinogradov et al., 1998). Moreover, several approaches that target delivery of antigens to MR have demonstrated effective induction of cellular and humoral immune responses (Cui and Mumper, 2002; Gu et al., 1998; Keler et al., 2004; Sasaki et al., 1997; Tacke et al., 2007; Vaughan et al., 2000).

In this study, we made a surface modification in mucoadhesive polymer, thiolated Eudragit L-100 (TE), with MR targeting ligand, as a vaccine carrier for nasal vaccine delivery. Mannan-decorated TE microparticles were prepared and characterized based on their size, morphology, vaccine releasing pattern,

MR targeting efficiency and the APCs activating efficiency. Finally, through intranasal immunization, the immune adjuvant ability of the formulation of Mannan-decorated TE microparticles with OVA as a model vaccine was investigated by measuring specific systemic and secretory antibodies in serum and mucosal secretions.

## **2. Materials and methods**

### **1) Materials**

Eudragit L-100 was purchased from Evonik Industries AG (Essen, Germany), mannan derived from *Saccharomyces cerevisiae* and ovalbumin (OVA) were purchased from Sigma (MO, USA), RPMI 1640 media, DMEM media and FBS were purchased from Gibco (MA, USA). All other materials were of analytical reagent grade.

### **2) Cell lines and experimental animals**

Raw 264.7 murine macrophage and Jaws II murine dendritic cell lines were obtained from American Type Culture Collection (VA, USA). Six-week-old BALB/c female mice (Samtako Inc, Korea) were used in immunization experiment and all experimental procedures were approved by the Institutional Animal Care and Use Committee of Seoul National University (Approval Number: SNU 140328-7) and followed the guidelines suggested by the committee.

### **3) Synthesis of thiolated Eudragit-L100 (TE)**

TE was synthesized according to method reported previously (Quan et al., 2008). Briefly, Eudragit L-100 was dissolved in dimethyl sulfoxide (DMSO) and the carboxylic acid moieties of the polymer were activated by *N, N'*-

dicyclohexyl carbodiimide (DCC) and *N*-hydroxyl succinimide (NHS) for one day before L-cysteine hydrochloride was added. The reaction mixture was incubated at room temperature for two days under nitrogen to avoid the oxidation of sulphhydryl groups by atmospheric oxygen. To remove the unbounded L-cysteine hydrochloride after reaction, the thiolated polymer was initially dialyzed against DMSO for one day, and then against distilled water for two days. After dialysis, the polymer solution was freeze-dried and the conjugate was stored in air tight containers at -20 °C until further use. The amount of cysteine in the conjugate was calculated by measurement of <sup>1</sup>H nuclear magnetic resonance (<sup>1</sup>H NMR) (Avance™ 600, Germany).

#### **4) Preparation of OVA-loaded TE microspheres (TEM) and OVA-loaded Man-TE microspheres (Man-TEM)**

50 mg/ml OVA was stabilized with Pluronic F-127 solution (1wt-%) to form an internal aqueous phase (W<sub>1</sub>). 0.2 ml of W<sub>1</sub> (10 mg OVA) was emulsified with an organic phase using an ultrasonic processor (Sonics, Vibra cells™) (2 output watts) for 1 min to form primary emulsion (W<sub>1</sub>/O). The organic phase (O) was consisted of 200 mg of TE dissolved in 5 ml of dichloromethane (DCM). The prepared primary emulsion (W<sub>1</sub>/O) was added drop by drop to 50 ml of 1 wt-% poly (vinyl alcohol) (PVA) solution (W<sub>2</sub>) as an external aqueous phase or mixture of 0.25 wt-% PVA and 0.5 wt-% mannan for Man-TEM, respectively. A homogenizer (Ultra-Turrax™ HomogenizerT25, IKA) was used

to continue emulsification at 11,000 rpm for 4 min to form  $W_1/O/W_2$  emulsion, which was then stirred with magnetic stirrer for 2–3 h at room temperature to allow the solvent to evaporate. OVA-loaded TEM and OVA-loaded Man-TEM were then collected and washed 3 times with distilled water after centrifugation at 14,000 rpm for 10 min at 4 °C. Followed by washing, the microspheres were lyophilized and finally stored at –70 °C to characterize them later. The unloaded TEM and Man-TEM were also prepared according to similar method. As a fact, it was not possible to make Eudragit microspheres because the Eudragit is not soluble in dichloromethane (DCM), which was used in dissolving thiolated Eudragit and resulted in non-comparison of immune activity between non-mucoadhesive microparticles and mucoadhesive ones.

## **5) Characterization of microspheres**

### **(1) Size and surface morphology**

The particle size distribution of the microspheres was measured using an electrophoretic light scattering spectrophotometer (ELS-8000, Otsuka Electronics Ltd., Japan). Prior to measuring, dispersing the freeze dried microspheres into sterile water, followed by vigorous vortexing and pipetting and then measuring the size distribution. To measure surface morphology of microspheres, the freeze dried microspheres were placed on a stud and were gold-coated using Sputter Coater (BAL-TEC/SCD 005), and observed using a field emission scanning electron microscopy (AURIGA, Germany).

## (2) Confirmation of decoration of mannan in TEM

To confirm mannan-coating in Man-TEM, FITC was conjugated with mannan. Briefly, 100 mg of mannan dissolve in 1 ml of DW was slowly mixed with 5 mg of FITC dissolve in 1 ml of DMSO. After stirring for 4 h at room temperature in dark condition, the reaction product was dropped into 8 ml of ethanol to remove the unreacted FITC. Mannan-FITC conjugate thus precipitated was washed and collected by centrifugation at 14,000 rpm for 10 min for 3 times. FITC-mannan decorated microspheres were prepared according to procedure described in the method 2.4 and FITC-mannan-decorated TEM were visualized by differential interference contrast microscope (DIC) and confocal laser microscope (SP8 X STED, Leica).

## 6) *In vitro* studies

### (1) Loading efficiency and loading content

The loading efficiency and loading content of microspheres were calculated using the following equations:

Loading efficiency (%)

$$= \frac{\text{amount of protein initially used} - \text{unloaded free protein}}{\text{amount of protein initially used}} \times 100\%$$

$$\text{Loading content (\%)} = \frac{\text{amount of protein in microspheres}}{\text{amount of microspheres}} \times 100\%$$

The loading efficiency and loading content of OVA into the microspheres were determined by quantifying the unloaded OVA in the supernatant using Micro Bicinchoninic Acid (BCA) protein assay method. The samples were analyzed in triplicate for each formulation studied.

(2) *In vitro* release of OVA from OVA-loaded TEM and OVA-loaded Man-TEM

10 mg of OVA-loaded TEM and OVA-loaded Man-TEM suspended in 1 ml of PBS (pH 7.4) into 1.5 ml microtubes were agitated up to 24 h at 37 °C with 100 rpm using shaking incubator. 100 µl of aliquot was withdrawn from the release medium and replaced by equal volume of PBS at each time point and the amount of released OVA was determined as cumulative release (wt. - %) against incubation times by Micro BCA protein assay method.

(3) Uptake of microspheres by APCs

For uptake of microspheres, RAW 264.7 cells were seeded into 6-well plates (Costar, IL, USA) at  $1 \times 10^6$  cells/well. After the cells reached 80% confluence, the medium was changed with 3 mg/ml of mannan for 20 min before the addition of microspheres to block MR (Jiang et al., 2008). Normal or MR-blocked cells were changed with the media with suspension of OVA-FITC-loaded TEM and OVA-FITC-loaded Man-TEM at a microsphere concentration of 0.2 mg/ml for 1 and 2 h. After incubation, the microsphere suspension was

removed and the wells were washed three times with 1 ml of PBS to remove the traces of microspheres left in the wells. After then, cells were detached from culture dishes and fluorescence-activated cell sorting (FACS) (BD biosciences, San Jose, CA) analysis was carried out to determine the uptake of microspheres by cells, with 10,000 cells measured routinely in each sample. The percentage of cells with internalized microspheres was calculated by the number of fluorescence events of FL1-height (FL1-H) signals.

#### (4) Internalization of microspheres

Phagocytosis of microspheres by macrophages and DCs was confirmed by confocal laser microscope (SP8 X STED, Leica).  $1 \times 10^6$  cells per well were cultured in 35 mm cover glass bottom dishes (SPL Life Science, Korea) two days prior to feeding with FITC-OVA-loaded microspheres at a concentration of 0.2 mg/ml. After incubation at 37 °C for 2 h, non-phagocytized microspheres were removed by washing three times with PBS and fixed with 4% paraformaldehyde at room temperature for 10 min. The nucleus was stained by Hoechst 33342 nucleic acid stain (Invitrogen, MA, USA).

### **7) *In vivo* studies**

#### (1) Immunization of mice

Nasal immunization study was conducted with 6 week-old female BALB/c mice which were assigned in five groups (n=5). Nasal administration groups

were immunized 4 times on days 0, 7, 14 and 21 respectively by dropping of 20  $\mu$ l PBS containing OVA (20  $\mu$ g) or OVA (20  $\mu$ g) -loaded TEM and OVA (20  $\mu$ g)-loaded Man-TEM into the nostrils during inhalation under anesthesia. Intramuscular administrations were immunized 3 times on day 0, 7 and 14 respectively by injection of 50  $\mu$ l PBS containing 20  $\mu$ g of OVA. Collection of serum, nasal wash, bronchoalveolar lavage (BAL), vaginal wash and intestinal wash were performed at day 42. Briefly, blood samples were collected from the caudal vein and centrifuged after clotting at 2,000 rpm for 10 min to prepare sera. BAL samples were harvested from the low respiratory tract of mice as described previously (Mizuno et al., 2006). Briefly, after the bronchi were exposed from the neck, a truncated needle with a syringe containing 400  $\mu$ l PBS was inserted through a small pore on each bronchus. Mucus exudation was harvested by a back and forth motion of the injector, and the supernatants were collected by centrifugation (3,000 rpm, 10 min and 4 °C). The nasal washes were collected by 300  $\mu$ l PBS through the trachea toward the nose. For vaginal wash sample preparation, after pipetting through the vaginal with 150  $\mu$ l of PBS for 15 times and the obtained fluids were collected by centrifugation (14,000 rpm, 10 min and 4 °C). For intestinal wash sample preparation, mid-ileal region of small intestines (3 cm) of the mice were immediately collected after sacrifice. The small intestines were homogenized in 500  $\mu$ l of ice-cold PBS and then tissues were removed by centrifugation (14,000 rpm, 10 min and 4 °C) and the supernatants were collected. All the samples were stored

at  $-70\text{ }^{\circ}\text{C}$  until analysis.

## (2) Measurement of OVA-specific antibodies

OVA-specific IgG, IgA, IgG1 and IgG2a antibodies in serum, sIgA antibodies in nasal wash, BAL, vaginal wash and intestinal wash were determined by the ELISA described previously (Kang et al., 2007). Briefly, 96-well plates (Thermo, USA) were coated with  $2.5\text{ }\mu\text{g}$  of OVA in  $100\text{ }\mu\text{l}$  of  $50\text{ mM}$  carbonate-bicarbonate buffer (pH 9.6) at  $4\text{ }^{\circ}\text{C}$  overnight and blocked with 1wt-% BSA at  $37\text{ }^{\circ}\text{C}$  for 1 h. Then, diluted serum, nasal wash, BAL, vaginal wash and intestinal wash were added into wells and incubated at  $37\text{ }^{\circ}\text{C}$  for 1 h. After incubation, HRP (horse radish peroxidase)-conjugated goat anti-mouse IgA, IgG, IgG1, or IgG2a (Santa Cruz Biotechnology, USA) were treated to each designated well at  $37\text{ }^{\circ}\text{C}$  for 1 h, respectively. TMB solution (Sigma-Aldrich, USA) was added to the wells for the HRP substrate, reaction was stopped after 10 min by adding  $2\text{ M H}_2\text{SO}_4$ , and absorbance was measured at  $450\text{ nm}$  using microplate reader (Infinite® 200 PRO, USA). Appropriate washing of each well was proceeded between the steps with PBS containing 0.05% of Tween 20 during the assays. The ELISA results are expressed as the OD value measured at  $450\text{ nm}$  for dilutions of 1:200,000 for serum IgG and IgG1, 1:5,000 for serum IgG2a and IgA, 1:500 for BAL IgA, 1: 10 for nasal wash IgA, 1:100 for vaginal IgA, 1:50 for intestinal IgA.

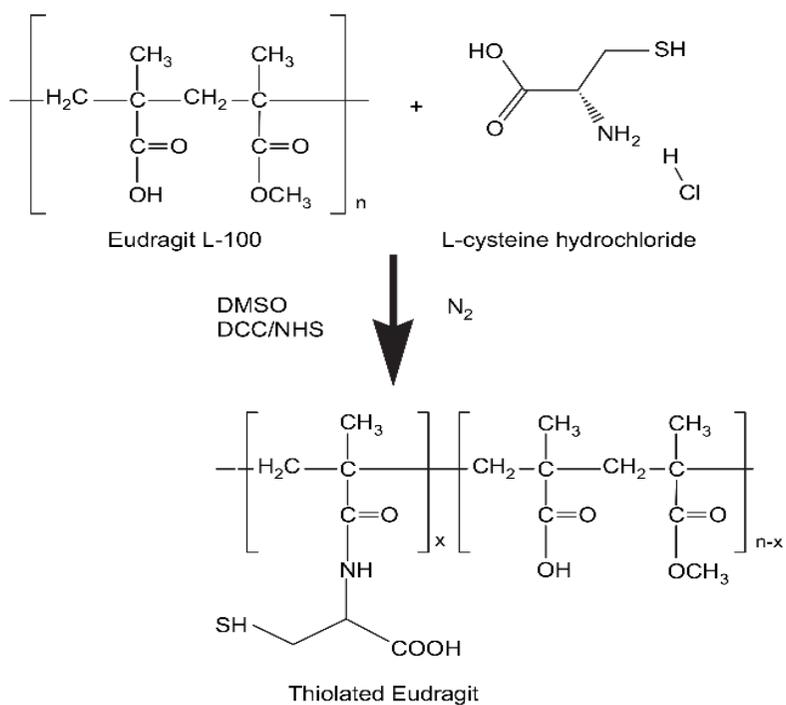
## **8) Statistical analysis**

Quantified results were expressed as the mean and standard deviation (SD). Statistical significance was assessed using a one-way analysis of variance (ANOVA) with post hoc Tukey Multiple Comparisons test.

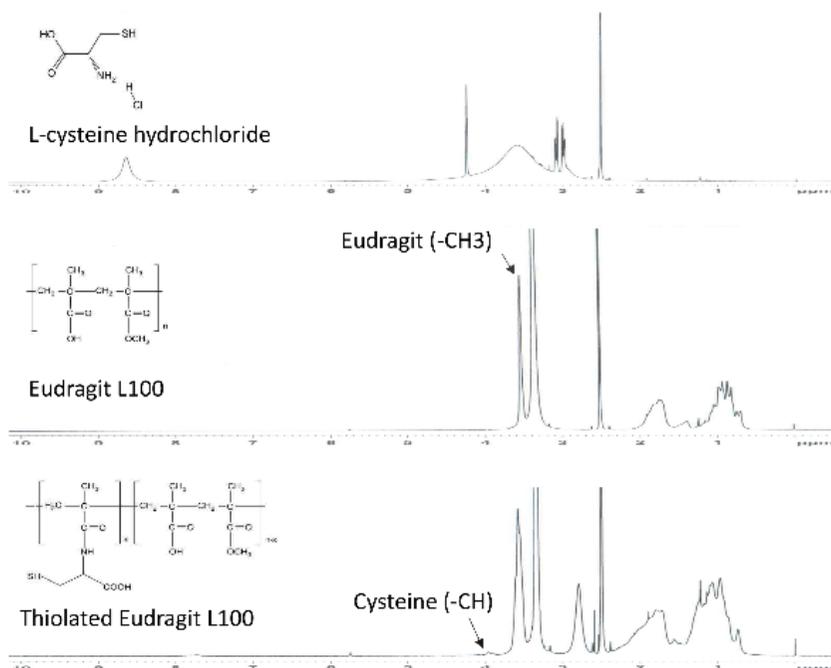
### **3. Results and discussion**

#### **1) Preparation of TE, TEM, Man-TEM, OVA-loaded TEM and OVA-loaded Man-TEM**

The reaction scheme for synthesis of TE is shown in Figure 34. The <sup>1</sup>H NMR of cysteine, Eudragit L-100 and TE are represented in Figure 35. It was found that about 5.4 mol.-% of cysteine was conjugated with Eudragit L-100. TEM, Man-TEM, OVA-loaded TEM and OVA-loaded Man-TEM were prepared using double emulsion method described in materials and methods.



**Figure 34.** Reaction scheme for the synthesis of TE



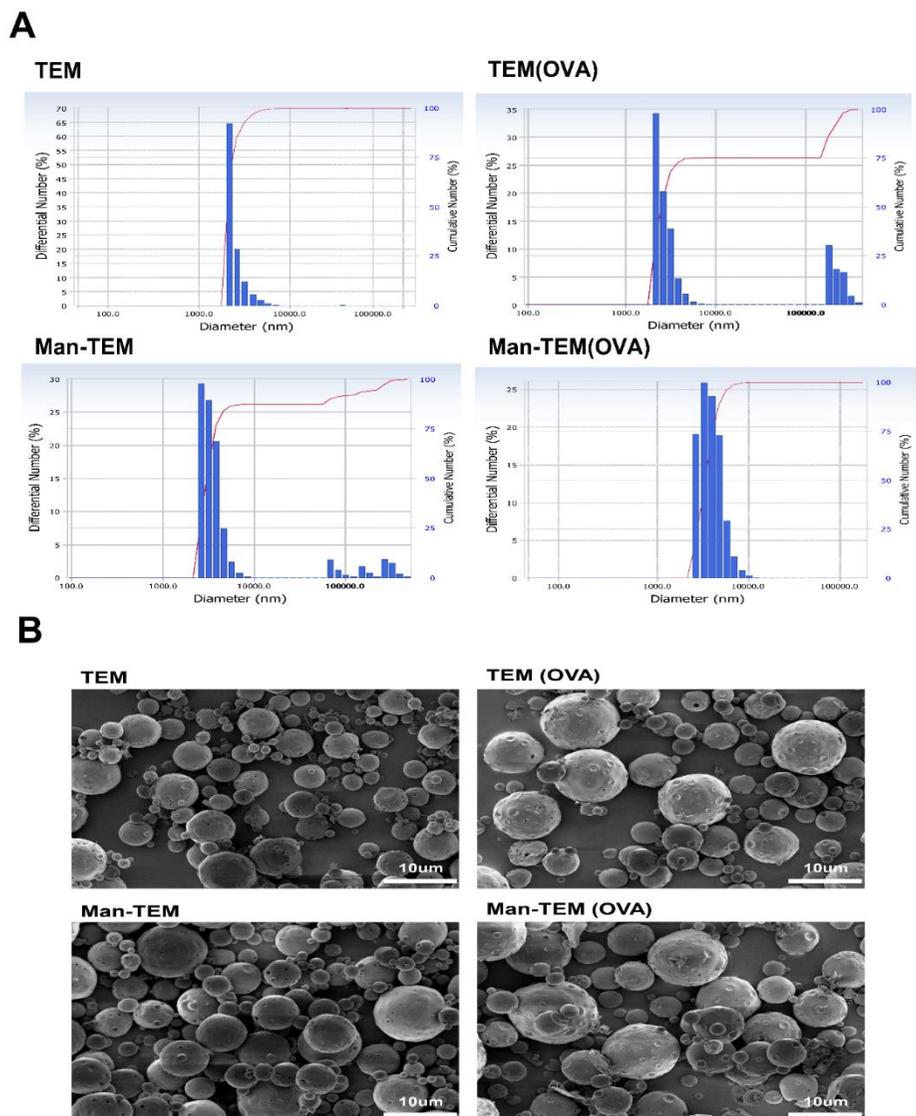
**Figure 35.** <sup>1</sup>H NMR spectra of cysteine, Eudragit L-100 and thiolated Eudragit L-100

## 2) Characterization of TEM and Man-TEM

### (1) Size and surface morphology

The sizes of TEM and Man-TEM measured by DLS are shown in Figure 36(A). The sizes of TEM and Man-TEM were  $2856.9 \pm 757.1$  nm and  $3386.6 \pm 827.8$  nm respectively, without much change in sizes after decoration of TEM with mannan due to small amount mannan coating. Similarly, the sizes of OVA-loaded TEM and OVA-loaded Man-TEM were slightly bigger than those of TEM and Man-TEM, respectively. The sizes of OVA-loaded TEM and OVA-loaded Man-TEM were  $3043.4.9 \pm 732.3$  nm and  $4431.9 \pm 1334.1$  nm. The morphology of TEM and Man-TEM observed by SEM are shown in Figure 36(B). TEM and Man-TEM have spherical shapes, with no change in shape after decoration with mannan. Recently, studies have demonstrated a significant correlation between physical particle size and retaining times in the human airways: with 24 h mucociliary clearance removed all particles larger than  $6 \mu\text{m}$  from the human airways, while particles with  $6 \mu\text{m}$  or smaller retained more than 24 h (Henning et al., 2010). Besides, it has been shown that microspheres' sizes influence their uptake by APCs and microfold cells (M cells). Particle smaller than  $5 \mu\text{m}$  may be transferred to the draining lymph nodes and spleen and stimulate both mucosal and systemic immune responses while particles in the range of  $5\text{-}10 \mu\text{m}$  tend to remain in Peyer's patches to stimulate primarily a mucosal immune response. Particles larger than  $10 \mu\text{m}$  are not likely to be taken

up at all (Oechslein et al., 1996). In this study, the mean size of microspheres was less than 5  $\mu\text{m}$ , and it is considered that this particular range of particle size can be internalized through phagocytosis by APCs playing a crucial role in initiating immune responses (Champion et al., 2008). Since the microparticles have similar size to the pathogen to which the immune system has evolved to combat, they are taken up like a pathogen by the DCs, which then mature and migrate to the nearby lymph nodes.

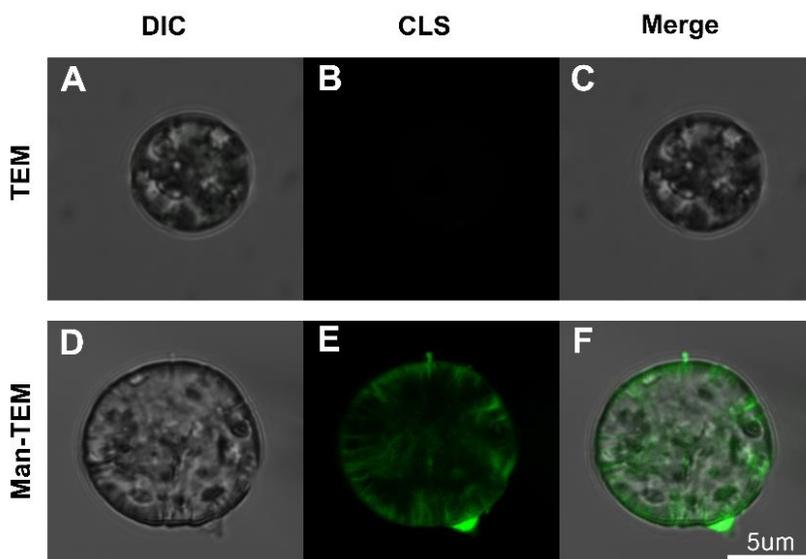


**Figure 36.** Size and morphology of microspheres. (A) Particle size distributions of TEM, Man-TEM, OVA-loaded TEM and OVA-loaded Man-TEM. (B) SEM photographs of TEM, Man-TEM, OVA-loaded TEM and OVA-loaded Man-TEM

( $\times 3,000$ ). Bar represents 10  $\mu\text{m}$ .

## (2) Confirmation of the surface decoration of mannan in Man-TEM

The surface decoration of TEM with mannan molecule was evaluated by confocal microscopy as shown in Figure 37. FITC-conjugated mannan was used for making FITC-Man-TEM. The confocal images indicated that the surface of the Man-TEM was homogeneously coated with mannan, as a stabilizer due to amphiphilic property of mannan, during making microspheres through  $W_1/O/W_2$  double emulsion. But, it was not possible to assay the amount of decorated mannan by elemental analysis or elemental spectroscopy for chemical analysis due to the small amount of mannan in the Man-TEM.



**Figure 37.** Confirmation of the surface decoration of mannan in Man-TEM. FITC-conjugated mannan was applied for making FITC-Man-TEM and the surface fluorescent signal was monitored by CLSM. First column: image by differential interference contrast (DIC) microscopy; second column: fluorescence by confocal laser scanning (CLS) microscopy and third column: combined image of DIC and CLS. Scale bar represents 5  $\mu\text{m}$ .

### 3) *In vitro* studies

(1) Loading efficiency and loading content of OVA loaded TEM and OVA loaded Man-TEM

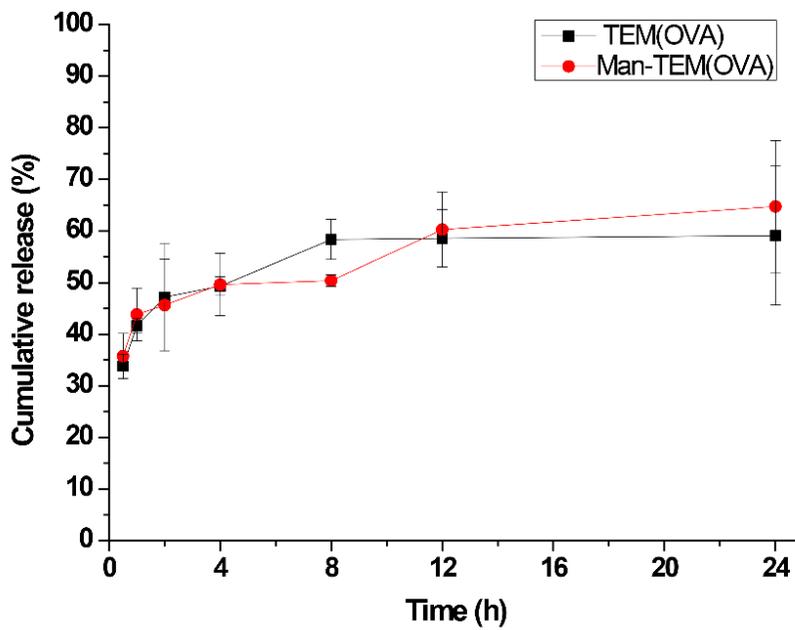
The loading efficiency and loading content of OVA in the OVA loaded TEM and OVA loaded Man-TEM are shown in Table 8. The loading efficiency and loading content of OVA in the TEM (OVA) were 53.93% and 2.70% by weight respectively, whereas the loading efficiency and loading content of OVA in the Man-TEM (OVA) were 50.50% and 2.52% by weight respectively, suggesting that there were no significant differences in the loading efficiency and loading content of OVA between the TEM and Man-TEM.

**Table 8.** Loading efficiency and loading content of OVA loaded TEM and OVA-loaded Man-TEM

<b>Microspheres</b>	<b>Loading efficiency (%)</b>	<b>Loading content (%)</b>
TEM (OVA)	53.93 ± 5.20	2.70 ± 0.26
Man-TEM(OVA)	50.50 ± 2.48	2.52 ± 0.12

(2) *In vitro* release of OVA from OVA-loaded TEM and OVA-loaded Man-TEM

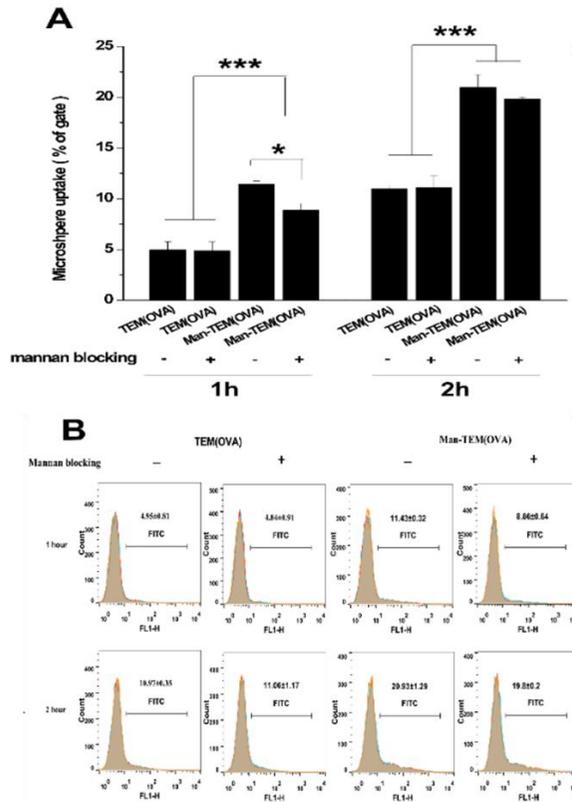
*In vitro* OVA release from the OVA-loaded TEM and OVA-loaded Man-TEM were performed in PBS (pH 7.4) incubated at 37 °C with mild agitation of 100 rpm and the amount of released OVA was evaluated by micro-BCA assay. The results represented in Figure 38 indicated that almost 45 wt-% of OVA was rapidly released from the OVA-loaded TEM and OVA-loaded Man-TEM within 2 h with not much difference in release pattern. We used Eudragit L-100, an anionic copolymer based on methacrylic acid and methylmethacrylate, which has pH sensitive property with a fast dissolution above pH 6.0. Thiolated Eudragit L-100 (TE) was modified from Eudragit L-100 with thiol groups using cysteine through amide conjugation, but it maintains the fast dissolution above pH 7.4. There has been a great deal of research on the relationship between microsphere and its residence time in nasal cavity. Jaganathan and Vyas's research team has been evaluated the nasal clearance rate of microsphere in rabbit nasal cavity using a surface modified PLGA microspheres which average size were less than 10 µm. Considering the average residence time of microspheres in nasal cavity is about 2 h (Jaganathan and Vyas, 2006), rapid release of OVA from the microspheres within 2 h might provide the antigens to APCs efficiently.



**Figure 38.** Release of OVA from OVA-loaded TEM and OVA-loaded Man-TEM at pH 7.4 and 37 °C (n = 3, error bar represents standard deviation).

### (3) Uptake of microspheres by APCs

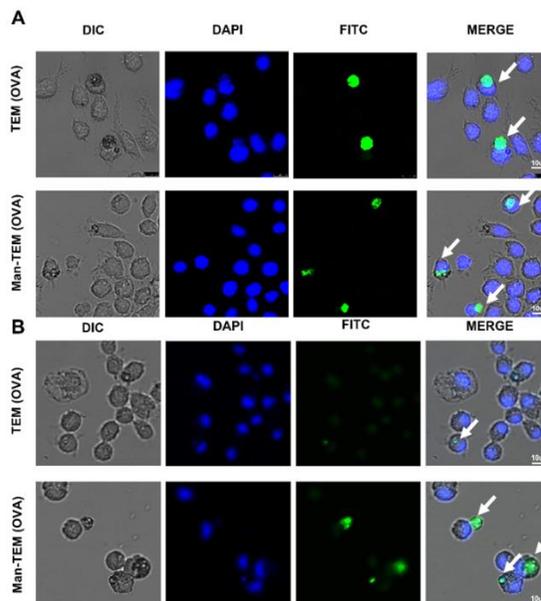
Cellular uptake of vaccines by the delivery system is very important for the clinical application of vaccines. Design of receptor-mediated endocytosis for targeting APCs using mannan as a specific ligand is one of approaches to enhance immune responses because the mannan has strong affinity with mannose receptors of APCs (Taylor et al., 1990). *In vitro* uptake assay was performed with two kinds of APCs - one is JAWS II and the other is RAW 264.7. The uptake of microspheres was checked in terms of incubation time and competition assay. Figure 39 shows uptake of microspheres by RAW 264.7 at 37 °C for 1 h and 2 h. The results indicated that the uptake of Man-TEM by both cell lines was much more than that of TEM with an increase of the uptake at 1 h and 2 h due to the specific interaction between mannan in the Man-TEM microsphere and mannann receptors of both cells. Importantly, the uptake of Man-TEM by both cell lines at 1 h decreased by pretreatment of mannan due to the blocking of mannann receptors by mannan whereas the uptake of TEM by both cells was not affected by pretreatment of mannan, indicating that the Man-TEM was uptaken by mannann receptor-mediated mechanism. However, the uptake of Man-TEM at first 1 h was not fully inhibited even after the treatment of mannan to block the mannann receptors. Similarly, there was no significant difference in the uptake of Man-TEM with or without blocking at 2 h, probably due to the depletion of mannann concentration in the cell media as the cells consume it.



**Figure 39.** Measurement of microsphere uptake by APCs using flow cytometry. Uptake of OVA-FITC-loaded TEM and OVA-FITC loaded Man-TEM by RAW 264.7 in 1 h and 2 h with or without MR inhibition respectively at 37 °C. (A) The impacts of microsphere uptake were displayed as percent of cells according to the histogram data of FACS analysis (B). (B) Histogram represented data. All histograms results are from one representative experiment of three independent experiments. Panel numbers indicate the mean percentages of positive cells (microsphere uptake) of respective samples. (n = 3, error bar represents standard deviation; \*p < 0.1, \*\*p < 0.05, \*\*\*p < 0.01, one-way ANOVA).

#### (4) Internalization of microspheres

To confirm the phagocytosis ability of mannan-decorated mucoadhesive microspheres by APCs, we treated FITC-labelled OVA-loaded microspheres to APCs. Figure 40 shows internalization of microspheres by RAW 264.7 and JAWS II cells at 37 °C after 2 h of treatment. It was found that more internalization of Man-TEM by both cell lines was observed than that of TEM, consistent with the results of cellular uptake of the microspheres observed by FACS.



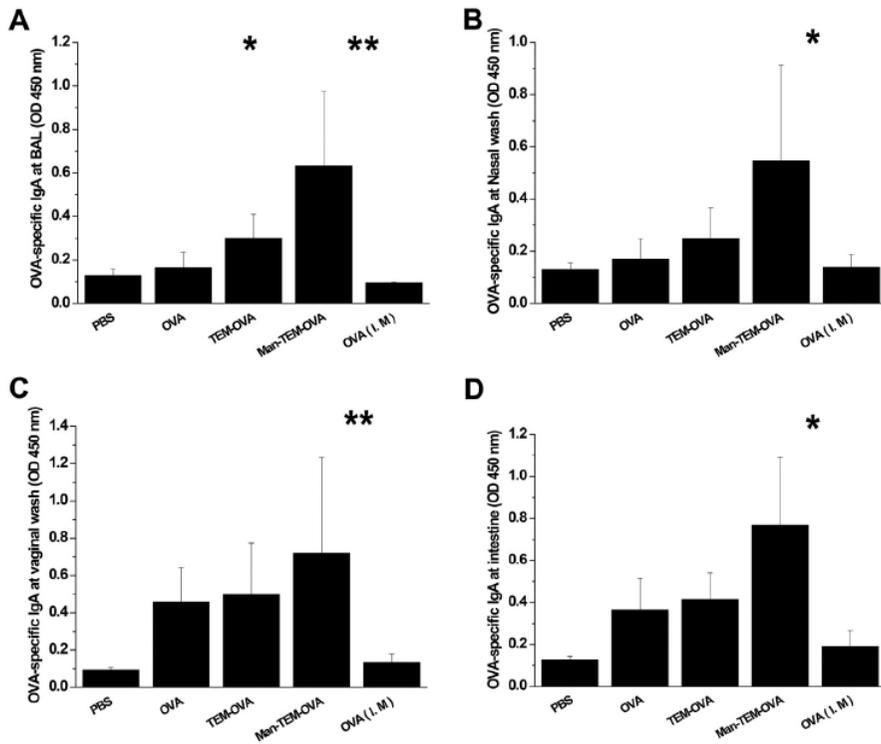
**Figure 40.** Confocal microscopic images of RAW 264.7 cells (A) and JAWS II cells (B) after 2 h culture with of OVA-loaded TEM and OVA-loaded Man-TEM at 37 °C. (Green: OVA-FITC loaded microsphere, blue: staining of nuclei with DAPI).

#### **4) *In vivo* immunization**

##### (1) OVA-specific mucosal immune responses

The main purpose of this study was to investigate the immune response of the vaccine released from the vaccine-loaded Man-TEM after nasal delivery, because the induction of immune responses by delivered vaccine play a major role in immunological defense at mucosal site (Neuhaus et al., 2014). Figure 41 shows anti-OVA sIgA values in the various mucosal sites after nasal vaccination with different formulations. The mice treated with OVA-loaded Man-TEM showed higher titers of anti-OVA sIgA antibody responses in the mucosal sites (BAL) compared with those treated with the OVA solution or intramuscularly delivered OVA. Particularly, mice treated with the OVA-loaded Man-TEM in the BAL displayed 3.8 fold higher IgA titer compared to those treated with the OVA solution, suggesting that mucoadhesive and mannan decorated Man-TEM enhanced the uptake of antigens through the respiratory epithelium due to the prolonged disposition of antigens in the respiratory cavity by the mucoadhesive property of the TE while mannan in Man-TEM enhanced uptake of vaccines due to the targeting to MR receptors in APCs. Regarding to use mouse model, it is often difficult to distinguish the immune responses induced by nasal delivery compared to these induced by the swallowing of the vaccine or the inadvertent channeling of the vaccine to the lung. So, it is inevitable that a significant amount of nasal-administered

microspheres might enter the lungs and it might contribute to the immune response in the lung. Unfortunately, we could not find significant differences between OVA loaded TEM and soluble OVA group. Moreover, the local and distal production of sIgA antibodies are the most important characteristics of nasally immunized vaccine. Our results indicated that nasally administrated particulates with vaccine induced immune responses efficiently at both local and distal mucosal sites, which can defect the pathogens invaded from other mucosal sites like vaginal or oral route. In contrast, intramuscularly vaccinated OVA group could not induce significant sIgA titer at local and distal mucosal sites compared to other nasally administrated groups.

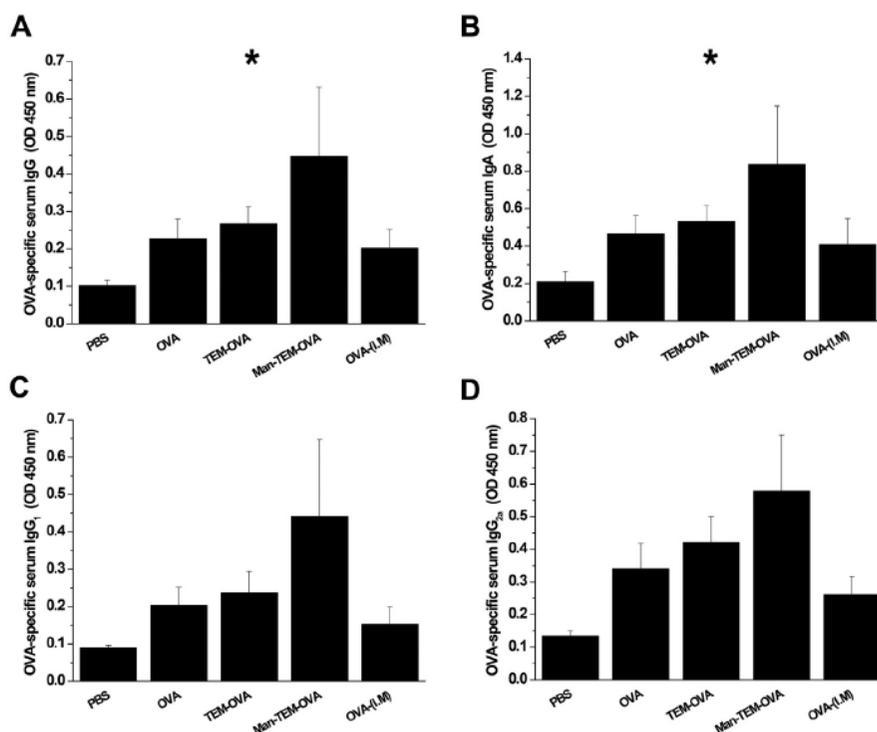


**Figure 41.** OVA-specific IgA performance in mucosal site at 6 weeks post immunization. OVA specific brochealveolar lavage (A), nasal wash (B), vaginal wash (C) and intestinal wash (D) IgA levels in mice immunized with each indicated formulations were analyzed by ELISA and then calculated as optical density (450 nm) (n = 5, error bar represents standard deviation; \*p < 0.1, \*\*p < 0.05, \*\*\*p < 0.01, one-way ANOVA).

## (2) OVA-specific serum IgG (IgG1 and IgG2a) responses

Serum isotype values of IgG, IgA, IgG1 and IgG2a are shown in Figure 42. Serum derived IgG also contributes to in lower respiratory tract immune defense (Renegar et al., 2004) and the production of the IgG1 isotype is associated with a Th2 type response and the IgG2a is associated with a Th1-type response (Dobrovolskaia and McNeil, 2007). The mice treated with OVA-loaded Man-TEM displayed higher titers of anti-OVA IgG and anti-OVA IgA compared to those treated with the soluble OVA, which is similar to the production of sIgA. In particular, mice treated with the OVA-loaded Man-TEM displayed 1.9 fold higher titers in IgG and 1.7 fold higher titers in IgA compared to those treated with the OVA solution. The anti-OVA IgG1 and IgG2a antibody responses in serum after nasal immunization are also shown in Figure 42(C) and 42(D). The anti-OVA IgG1 titers of mice treated with the OVA-loaded Man-TEM in serum 6 weeks after the first immunization were 2.14 and 1.86 fold higher compared to those treated with the OVA and OVA-loaded TEM, respectively, although there was no significant difference between them. Also, the anti-OVA IgG2a titers of mice treated with the OVA-loaded Man-TEM in serum displayed similar results as the anti-OVA IgG1, suggesting that these OVA-loaded Man-TEM appear to induce both Th1 and Th2 immune responses *in vivo*. The results of this study demonstrated that the nasal immunization of mice with OVA-loaded Man-TEM elevated immune

responses owing to mucoadhesive property of the thiolated Eudragit and enhanced APCs targeting by the mannan.



**Figure 42.** OVA-specific systemic immune responses at 6 weeks post immunization. OVA specific serum IgG (A), serum IgA (B), serum IgG1 (C) and serum IgG2A (D) levels in mice nasally immunized with each indicated formulations were analyzed by ELISA and then calculated as optical density (450 nm) (n = 5, error bar represents standard deviation; \*p < 0.1, \*\*p < 0.05, \*\*\*p < 0.01, one-way ANOVA).

#### **4. Conclusion**

Development of an effective nasal vaccine requires appropriate carriers or adjuvant systems that protect antigens from rapid nasal clearance by mucociliary apparatus and require to deliver antigens efficiently for alarming APCs. In this study, mucoadhesive microspheres decorated with mannan molecules (Man-TEM) were successfully prepared and evaluated *in vitro* and *in vivo* for vaccine delivery to mannose receptors of APCs. It was found that OVA-loaded Man-TEM were specifically bound with mannose receptors on murine macrophages and dendritic cells *in vitro* and induced more mucosal sIgA and serum antibody (IgG and IgA) than OVA-loaded TEM upon intranasal vaccination in mouse due to a specific interaction between mannose in the Man-TEM and mannose receptors on APCs. The intranasal immunization results strongly support the efficacy of Man-TEM as an adjuvant-carrier system for the effective induction of specific immune response.

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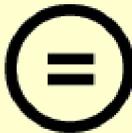
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**A Dissertation**  
**for the Degree of Doctor of Philosophy**

**Development of adjuvants using cytokine and polymeric  
particles for efficient mucosal immunization**

점막면역백신의 효율증진을 위한 사이토카인 및 고분자

입자를 이용한 면역보조제의 개발

**February, 2016**

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**Department of Agricultural Biotechnology**  
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# 농학박사학위논문

## Development of adjuvants using cytokine and polymeric particles for efficient mucosal immunization

점막면역백신의 효율증진을 위한 사이토카인 및 고분자 입자를 이용한 면역보조제의 개발

지도교수 최윤재

이 논문을 농학 박사 학위 논문으로 제출함.

2015년 12월

서울대학교 대학원 농생명공학부

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이혜선의 농학 박사학위논문을 인준함.

2016년 1월

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## Summary

Mucosal surfaces are a main portal of entry for not only nutrients but also many pathogens that are the cause of infectious disease in livestock animals. Immunization through mucosal routes may be more effective in terms of inducing protective immunity against mucosal pathogens. In addition, mucosal vaccines can offer needle-free delivery and improve accessibility, safety and cost-effectiveness in farm industry. However, challenge to successful mucosal vaccination include poor induction of mucosal immunity, the availability of safe and effective mucosal adjuvant and delivery systems. Recently, major efforts are made to develop new mucosal vaccines by selecting appropriate antigens with high immunogenicity, designing mucosal route of administration, selecting immune-stimulatory adjuvant molecules and developing appropriate delivery systems.

The aim of the present study is to develop an effective mucosal adjuvant and delivery vehicles to improve efficacy of the livestock mucosal vaccine, which is divided into two parts; i) Development of an oral cytokine vaccine adjuvant which was designed to be produced in *Lactococcus lactis* IL1403, ii) Development of mucoadhesive and mannan decorated thiolated polymeric microspheres, designed to target mucosal antigen presenting cells (APCs) as nasal vaccine delivery vehicles.

In study 1, a recombinant cytokine, IL-6-CKS9, was generated by conjugating an M cell-targeting peptide (CKS9) with c-terminus of the murine

interleukin 6 (IL-6), which facilitated enhancement of mucosal immune response. *Lactococcus lactis* IL1403, a food-grade strain of lactic acid bacteria (LAB) which is widely used in dairy industry, was used as a host cell to express and secrete the IL-6-CKS9 for a mucosal vaccine adjuvant. The recombinant *L. lactis* IL1403 secreting IL-6-CKS9 was orally administered with a model antigen protein, M-BmpB (*Brachyspira* membrane protein B conjugated with CKS9), to BALB/c mice for mucosal immunization. ELISA analyses showed consistent enhancement tendencies in induction of anti-M-BmpB antibody levels both with mucosal (IgA) and systemic (IgG) immune responses in IL-6-CKS9-LAB treated group compared with other groups tested by conducting mice immunization assays. In addition, the oral administration of model protein antigen with live LAB producing IL-6-CKS9 could induce both Th1 and Th2 type immune responses. Collectively, the results showed successful production and secretion of recombinant murine IL-6 with M cell-targeting moiety (IL-6-CKS9) from *L. lactis* IL1403 and demonstrated that the live recombinant LAB producing IL-6-CKS9 could have a potential to be used as an efficient adjuvant for peroral vaccination.

In study 2, mucoadhesive and mannan decorated microspheres, designed to target APC and were evaluated the efficiency of vaccine delivery carrier after intranasal immunization. The aim was to develop mannan-decorated mucoadhesive thiolated hydroxypropylmethyl cellulose phthalate (HPMCP) microspheres (Man-THM) that contain ApxIIA subunit vaccine – an exotoxin fragment, as a candidate for a subunit nasal vaccine against *A.*

*pleuropneumoniae*. For adjuvant activity, mucoadhesive thiolated HPMCP microspheres decorated with mannan could be targeted to the PRRs (pathogen recognition receptors) and mannose receptors (MR) of APCs in the respiratory immune system. The potential adjuvant ability of Man-THM for intranasal immunization was confirmed by *in vitro* and *in vivo* experiments. As following in a mechanistic study using APCs *in vitro*, it was found that Man-THM enhanced receptor-mediated endocytosis by the MR of APCs. *In vivo*, the nasal vaccination of ApxIIA-loaded Man-THM in mice resulted in higher levels of mucosal sIgA and serum IgG than mice immunized with the ApxIIA or ApxIIA-loaded THM due to the specific recognition of the mannan by the MRs on the APCs. Moreover, ApxIIA-containing Man-THM protected immunized mice when challenged with strains of *A. pleuropneumoniae* serotype 5. These results suggest that mucoadhesive Man-THM could be considered as a promising candidate for a nasal vaccine delivery system to elicit systemic and mucosal immunity that can protect from pathogenic bacteria infection.

**Key words:** Mucosal immunization, oral vaccine adjuvant, cytokine, M cell targeting peptide, nasal vaccine delivery carrier, mucoadhesive polymer, mannan

**Student number:** 2010-24116

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## List of Abbreviations

APCs: antigen-presenting cells

APP5: *A. pleuropneumoniae* serotype

ApxIIA: Apx toxin II fragment A

BAL: bronchoalveolar lavage

BALT: bronchoepithelium and lower respiratory tract

BCA: Bicinchoninic acid

CKS9: CKSTHPLSCM (M cell targeting peptide)

CLS: confocal microscopy

CT: cholera toxin

DCs: dendritic cells

DLS: dynamic light scattering

DIC: differential interference contrast

ELISA: enzyme linked immune sorbent assays

FA: Freund's adjuvant

FAE: follicle-associated epithelium

FDCs: follicular dendritic cells

FITC: fluorescein isothiocyanate

GALT: gut-associated lymphoid tissue

HEVs: high endothelial venules

HPMCP: hydroxypropylmethyl cellulose phthalate

HRP: horseradish peroxidase

i.m.: intramuscular

i.n.: intranasal

i.p.: intraperitoneal

LAB: lactic acid bacteria

MALT: mucosa-associated lymphoid tissues

Man-THM: mannan decorated thiolated HPMCP micropsphere

M-BmpB: *Brachyspira* membrane protein B conjugated with CKS9

MR: mannose receptor

mSC: membrane secretory component

NALT: nasopharynx associated lymphoid tissue

PFA: polyformaldehyde

PPs: peyer's patch

PRRs: pathogen recognition receptors

pIgR: polymeric Ig receptor

SIgA: secretory IgA;

SIgM: secretory IgM

TLR: toll like receptor

# Introduction

Mucosal surfaces constitute the largest and most important interface between the body and the outside environment. Most pathogens initiated their infection through access to the mucosal region of body. To maintain normal physiology, the mucosa must be prevented entry and dissemination of dangerous pathogens. Innate immune responses are important in preventing initial infections and adaptive immune responses play a key role in preventing infection from previously encountered pathogens (Pavot et al., 2012).

Mucosal vaccines can induce mucosal immunity, including antigen-specific secretory IgA production, to protect invasion by pathogens and to neutralize toxins at mucosal surfaces. Accordingly, effective mucosal vaccines can protect from pathogens before their invasion across mucosal barriers and neutralize toxins, ingested or generated in the mucosal space by pathogens (Pasetti et al., 2011). Administering vaccines through non-mucosal routes often lead to poor protection against mucosal pathogens, since such vaccines do not generate memory lymphocytes that migrate to mucosal surfaces (Lamm, 1997).

Although mucosal vaccination induces mucosa-tropic memory lymphocytes, few mucosal vaccines have been used clinically. Live vaccine vectors pose safety risks, whereas killed pathogens or molecular antigens are usually weak immunogens when applied to intact mucosa. Adjuvants can boost immunogenicity, however, most conventional mucosal adjuvants have unfavorable safety profiles. For example cholera toxin (CT) can induce efficient

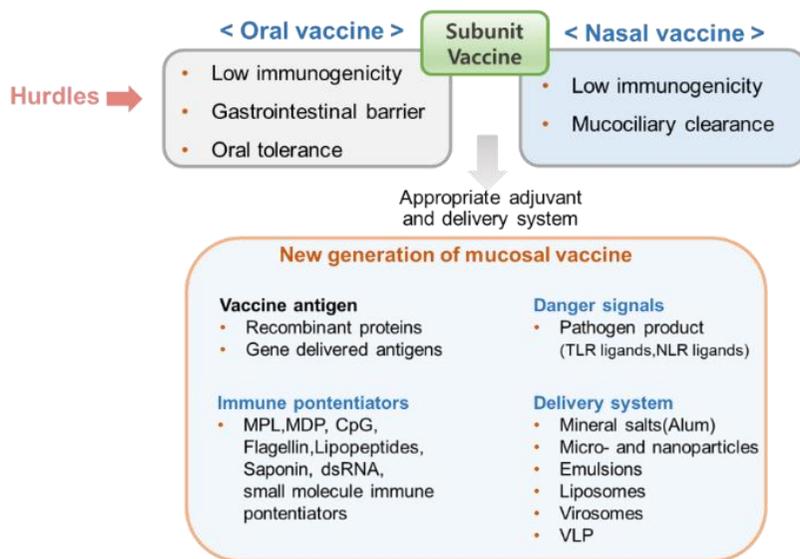
mucosal immune responses to antigens when they are co-administered, even when the antigens by themselves cannot induce good immune responses. However, in clinical trials it has been reported that CT can also induce adverse side-effects (Holmgren et al., 1993). Moreover, the immune mechanisms of protection against many mucosal infections are poorly understood.

Recently, subunit vaccine is an emerging safe vaccine strategy for vaccination because it could avoid potential harmfulness of whole-body vaccine due to renewal of infectivity, however the poor immunogenicity of subunit vaccine is one of its drawbacks to be overcome (Skwarczynski and Toth, 2011). Mucosal immunization with subunit vaccines has unique challenges associated with not only the immunogen but also the route of antigen delivery (Figure 1).

Oral vaccines need to resist harsh pH conditions, such as pH 1–2 in gastric acid in the stomach, and multiple digestive enzymes to reach mucosal immune tissues such as Peyer's patches (PPs) in sufficient amounts. One of the most significant problems associated with oral mucosal vaccines is possible tolerance induction against the orally introduced antigens, because it is generally known that oral administration of soluble proteins dominantly triggers oral tolerance (Cho et al., 2008). Tolerance induction by orally introduced vaccine materials translates into systemic unresponsiveness after challenge infection by the same antigen. To overcome this tolerogenic mucosal environment, many efforts have been made to develop effective mucosal

adjuvants that can stimulate both innate and adaptive immunities and are capable of inducing effective mucosal and systemic immune responses. (Figure 1)

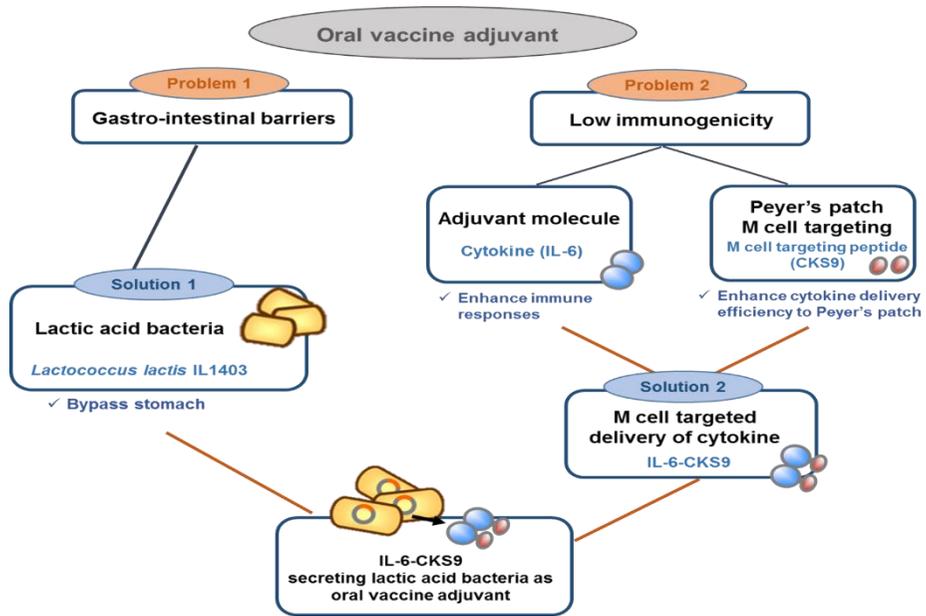
Nasal vaccines need to overcome the poor efficacy of nasally administered vaccines which is caused by the physiology of nasal cavity and the mucociliary clearance of respiratory tract. Increase the residence time of delivered antigen to retain long enough time for the interaction between antigen and the lymphatic system is an important aspect to be considered in development of nasal vaccines (Figure 1).



**Figure 1.** Appropriate adjuvants and delivery systems are needed for mucosal subunit vaccination

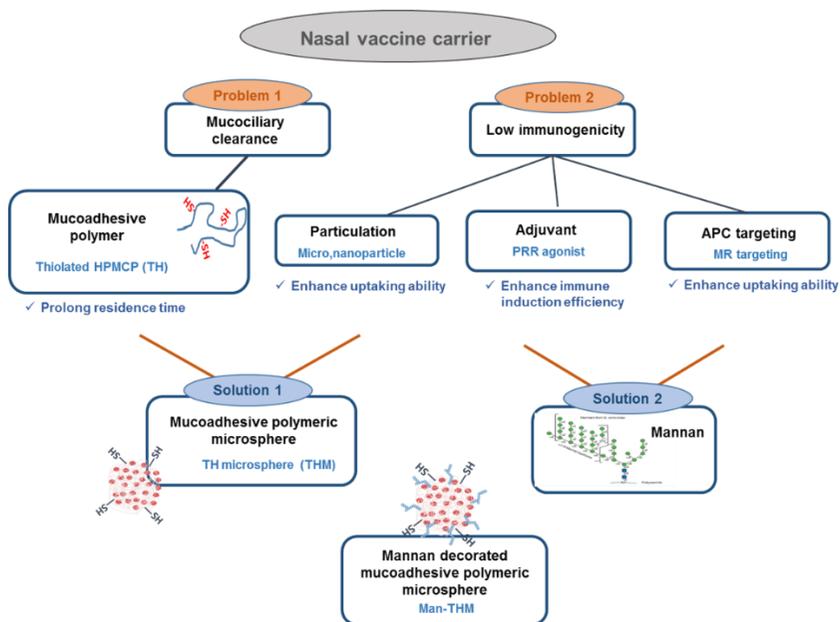
In this study, in order to develop an efficient mucosal vaccination through oral and nasal routes, an oral cytokine vaccine adjuvant named as 'IL-6-CKS9' and nasal vaccine delivery carrier named as 'Man-THM' were developed.

To overcome the main hurdles of oral subunit vaccine (Figure 1), Study 1 was mainly focused on developing vaccine adjuvant. A recombinant cytokine, IL-6-CKS9, was generated by conjugating an M cell-targeting peptide (CKS9) with c-terminus of the murine interleukin 6 (IL-6), which facilitated enhancement of mucosal immune response. *Lactococcus lactis* IL1403, a food-grade strain of lactic acid bacteria (LAB), was used as a host cell to express and secrete the IL-6-CKS9 for a mucosal vaccine adjuvant (Figure 2).



**Figure 2.** Strategy for developing oral vaccine adjuvant (study 1)

To overcome the main hurdles of nasal subunit vaccine (Figure 3), Study 2 was mainly focused on developing an efficient nasal vaccine carrier which was designed to hold mucoadhesive and APCs targeted properties. Mucoadhesive thiolated HPMCP microsphere (Man-THM) were decorated with mannan which could be targeted to PRRs and mannose receptors (MR) of antigen presenting cells (APCs) in respiratory immune system to obtain adjuvant ability (Figure 3).



**Figure 3.** Strategy for developing nasal vaccine carrier (study 2)

# **Chapter 1. Review of Literature**

## **1. Overview of mucosal immune system**

### **1) Mucosal immunity**

Mucosal membranes are huge surface covering the aero-digestive and the urogenital tracts as well as the eye conjunctiva, the inner ear and the ducts of all exocrine glands and they are susceptible to infection by pathogenic microorganisms. The mucosal surfaces of the gastrointestinal and respiratory tracts are the principal portals of entry for most pathogens. Direct inoculation of pathogens into the bloodstream is another important route of infection which goes with systemic immune responses (Holmgren and Czerkinsky, 2005).

Most external mucosal surfaces are distributed with organized follicles and scattered antigen-reactive or sensitized lymphoid elements, including B cells, T lymphocytes, T-cell subsets, plasma cells, and a variety of other cellular elements involved in the induction and maintenance of immune response. A remarkable fact is that a healthy human adult contributes almost 80% of all their immunocytes to mucosal immune system. These immunocytes are distributed in both largest lymphoid organ system and mucosa-associated lymphoid tissues (MALT) (Czerkinsky and Holmgren, 2012).

The MALT is a highly compartmentalized immunological system and it functions independently of systemic immune responses. The immunologic network operating on external mucosal surfaces consists of gut-associated lymphoid tissue (GALT), bronchus-associated lymphoid tissue (BALT), tonsils and nasopharynx associated lymphoid tissue (NALT), male and female genital tracts, mammary glands, and the products of lactation (Table 1). The organized lymphoid follicles in the GALT, NALT and BALT are considered as the principal inductive sites of mucosal immune response and appendix, peritoneal precursor lymphoid cells, and rectal lympho-epithelial tissue (rectal tonsils) also serve as inductive sites of local immune responses (Brandtzaeg et al., 2008).

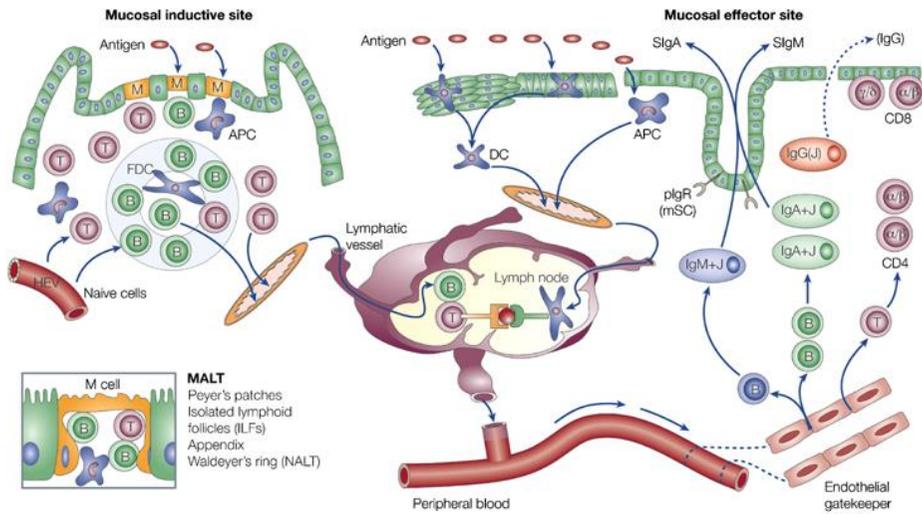
Inductive sites for mucosal immunity are constituted by regional MALT with their B-cell follicles and M-cell (M)-containing follicle-associated epithelium through which exogenous antigens are transported actively to reach APCs, including DCs, macrophages, B cells, and FDCs. In addition, quiescent intra- or sub-epithelial DCs may capture antigens at the effector site and migrate via draining lymphatics to local or regional lymph nodes where they become active APCs, which stimulate T cells for productive or down regulatory immune responses. Naïve B and T cells enter MALT and lymph nodes via HEVs. After being primed to become memory or effector B and T cells, they migrate from MALT and lymph nodes to peripheral blood for subsequent extravasation at mucosal effector sites (Figure 4) (Brandtzaeg, 2009).

The major antibody isotype in external secretions is secretory

immunoglobulin A (SIgA). Approximately 40 mg of IgA per kg of body weight is secreted daily, especially from the gastrointestinal tract, and the total amount of synthesized IgA is almost twice the amount of IgG produced daily in humans.

**Table 1.** Recommended nomenclature for mucosa-associated immune cell compartments (Brandtzaeg et al., 2008)

Preferred abbreviations	Explanations	Note
MALT	Mucosa-associated lymphoid tissue	The principal inductive sites for mucosal immune responses, subdivided according to anatomical location as below
GALT	Gut-associated lymphoid tissue	
NALT	Nasopharynx-associated lymphoid tissue	In humans, NALT consists of the lymphoid tissue of Waldeyer's pharyngeal ring, including the adenoids (the unpaired nasopharyngeal tonsil) and the paired palatine tonsils. Rodents lack tonsils, but have paired NALT structures dorsally in the floor of the nasal cavity
BALT	Bronchus-associated lymphoid tissue	Not generally present in the normal lungs of adult humans
PP	Peyer's patch	
LP	Lamina propria	Refers usually to the connective tissue of gut mucosa, restricted to the stroma above the muscularis mucosae (thus excluding the submucosa), but can also be used in relation to other mucosae
FAE	Follicle-associated epithelium	Covers the domes of MALT structures and contains variable numbers of M cell
ILF	Isolated lymphoid follicle	PPs and ILFs constitute the major part of GALT, but also the appendix is included although functionally less explored
IEL	Surface epithelium	Refers usually to the epithelium of the small intestine where most intraepithelial lymphocytes (IELs) occur
MLN	Mesenteric lymph node	
CLN	Cervical lymph node	Should be specified as deep or superficial



**Figure 4.** Depiction of the human mucosal immune system. (APCs, antigen-presenting cells; DCs, dendritic cells; FDCs, follicular dendritic cells; HEVs, high endothelial venules; MALT, mucosa-associated lymphoid tissue; mSC, membrane secretory component; pIgR, polymeric Ig receptor; SIgA, secretory IgA; SIgM, secretory IgM) (Brandtzaeg, 2009).

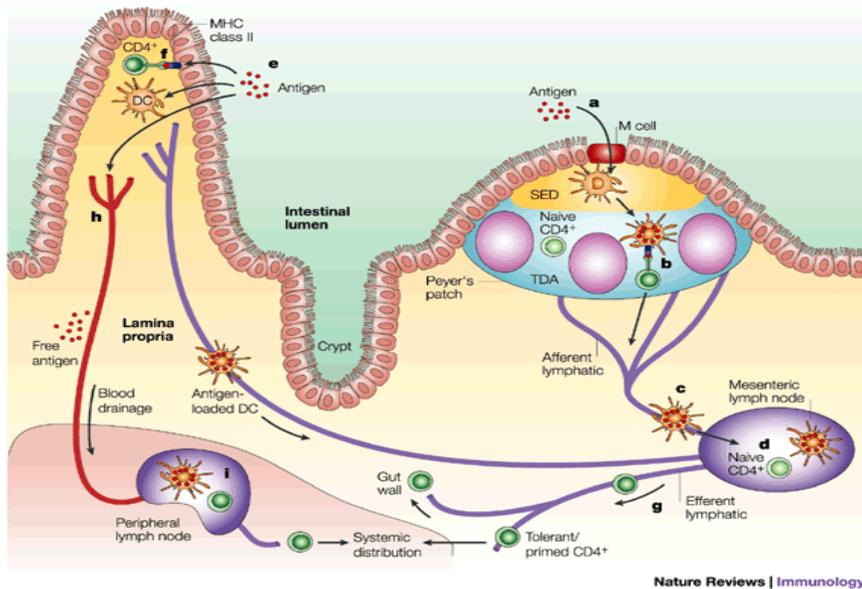
## **2) Gut-associated lymphoid tissue (GALT)**

The gut-associated lymphoid tissue described in the Figure 5 lines the digestive system and has two organizational levels to its structure: one with little organization, characterized by loose clusters of lymphoid cells in the lamina propria of the intestinal villi, and the other with a high level of organization, called Peyer's patches. The so-called intraepithelial lymphocytes (IELs) can be found in the outer mucosal epithelial layer, and the majority of these cells are CD8+ T-lymphocytes. Due to its localization, it is thought that this population of T cells may function to encounter antigens that enter through the intestinal mucous epithelium. The under layer of epithelia is the lamina propria, which contains large numbers of B cells, plasma cells, activated Th cells and macrophages in loose clusters (Mowat, 2003).

Peyer's patches, located in the submucosal layer underneath the lamina propria, contain 30–40 lymphoid follicles organized as macroscopic nodules or aggregates. In a similar way to what happens with lymphoid follicles in other sites, those from mature Peyer's patches can develop into secondary follicles with germinal centers, supported or connected by follicular dendritic cells. Parafollicular T-lymphocyte zones located between the large B-cell follicles present a large number of high endothelium venules, allowing cellular migration and lymphocytes' recirculation. Between the follicle-associated epithelium (FAE) and the organized lymphoid follicle aggregates, there is a more diffuse area known as the sub-epithelial dome (SED). The FAE is the

name given to the mucous membrane overlying the organized lymphoid follicles. The FAE is a small region characterized by the presence of specialized flattened epithelial cells called M cells. Together, the FAE, lymphoid follicles and associated structures form the antigen sampling and inductive sites of the mucosal immune system. It has been widely accepted that M cells are probably playing a key role in mucosal infection and immunity. It is thought that the main role of M cells is the sampling of antigens to transport them across mucosal epithelia to the underlying lymphoid tissues where protective immune responses are generated. In addition, M cells are a common route for complex antigens and pathogen invasion, for example, several invasive *Salmonella* species, *Vibrio cholerae*, *Yersinia* species, *Escherichia coli* and the polio virus. M cells have been identified in the epithelia of a variety of mucosal tissues and within the FAE of a wide variety of animal species, including laboratory animals (mice, rats, rabbits), domestic pets and humans. In mice and humans, M cells reside in about 10% of the FAE in contrast with 50% in the rabbit. In the gut, M cells are easily recognized by the lack of surface microvilli and the normal thick layer of mucus that characterizes the rest of the epithelial cells. Additionally, M cells contain a deep invagination similar to a pocket in the basolateral cytoplasmic membrane that contains one or more lymphocytes and occasional macrophages. The epithelium of the gut intestine provides an effective barrier to the entrance of most pathogens and particulates due to strong connections between epithelial cells called tight junctions. In contrast, the M cells can be exploited by microorganisms as the port of entrance for two reasons:

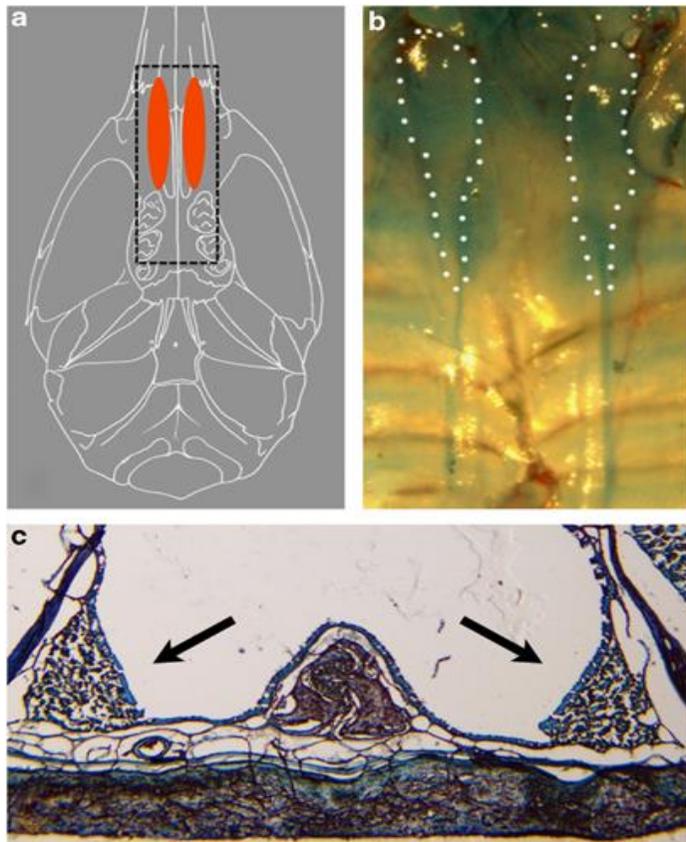
the organisms can adhere with less difficulty to the apical cell membrane, and after that, these agents need only be transported a short distance before reaching the M cell pocket, where by interaction with lymphocytes, the antigens or the particulates gain rapid access to the organized MALT inductive site (Borges et al., 2010).



**Figure 5.** Antigen might enter through the microfold (M) cells in the follicle-associated epithelium (FAE) (a), and after transfer to local dendritic cells (DCs), might then be presented directly to T cells in the Peyer's patch (b). Alternatively, antigen or antigen-loaded DCs from the Peyer's patch might gain access to draining lymph (c), with subsequent T-cell recognition in the mesenteric lymph nodes (MLNs) (d). A similar process of antigen or antigen-presenting cell (APC) dissemination to MLNs might occur if antigen enters through the epithelium covering the villus lamina propria (e), but in this case, there is the further possibility that MHC class II<sup>+</sup> enterocytes might act as local APCs (f). In all cases, the antigen-responsive CD4<sup>+</sup> T cells acquire expression of the  $\alpha 4\beta 7$  integrin and the chemokine receptor CCR9, leave the MLN in the efferent lymph (g) and after entering the bloodstream through the thoracic duct, exit into the mucosa through vessels in the lamina propria. T cells which have recognized antigen first in the MLN might also disseminate from the bloodstream throughout the peripheral immune system. Antigen might also gain direct access to the bloodstream from the gut (h) and interact with T cells in peripheral lymphoid tissues (i) (Mowat, 2003).

### **3) Nasopharynx-associated lymphoid tissue (NALT)**

In rodents, NALT is found on both sides of the nasopharyngeal duct, dorsal to the cartilaginous soft palate, and it is considered to be analogous to Waldeyer's ring in humans (pharyngeal lymphoid tissue that includes adenoid, tubal tonsil, palatine tonsil, lingual tonsil) (Figure 6). NALT is a well-organized structure consisting of B- and T-cell-enriched areas, which are covered by an epithelial layer containing M cells, the so-called follicle-associated epithelium (FAE). The function of these antigen-sampling M cells seems to be similar to those found on the FAE of Peyer's patches. Although NALT and Peyer's patches share certain similarities, the two differ markedly in morphology, lymphoid migration patterns and the binding properties of the high endothelial venules. Additionally, intraepithelial lymphocytes and antigen-presenting cells, including dendritic cells (DCs) and macrophages, can also be found in NALT. Therefore, NALT contains all of the lymphoid cells that are required for the induction and regulation of mucosal immune response to antigens delivered to the nasal cavity (Kiyono and Fukuyama, 2004).



**Figure 6.** Localization of the nasopharynx-associated lymphoid tissue (NALT) on the soft palate. (a) Schematic showing the paired NALT in relation to the palate and the molars. (b) Soft palate from a naive CBA/Ca mouse that was injected Evans blue to visualize the paired NALT (dotted lines). (c) Cross-section of the nasal cavity of a naive C57BL/6 mouse showing the triangular shape of the NALTs (arrows). NALTs are located on either side of the nasal airways, which are fully separated by the nasal septum in the posterior part of the nose. Giemsa-stained frozen section prepared after decalcification of the osseous tissue. Bar=100  $\mu$ m (Nacer et al., 2014).

## **2. Mucosal immunization**

### **1) Strategy for mucosal immunization**

#### **(1) Live attenuated or inactivated vaccines**

Vaccines based on live-attenuated viruses or microbes that have been inactivated by heat or chemicals comprise the majority of licensed vaccines used for the prevention of infectious disease (Chadwick et al., 2010). To date, these are the only vaccines approved for mucosal delivery and the only ones for which efficacy is correlated with effector mucosal immune responses (Ye et al., 2011). For examples, the oral polio vaccine is a live-attenuated vaccine that produces serum antibodies as well as local sIgA in the intestinal mucosa. The mucosal sIgA confers protection from poliovirus entry and multiplication. Several live-attenuated vaccines administered via the mucosal route are licensed for enteric infections such cholera, typhoid, and rotavirus. The success of live-attenuated and inactivated vaccines is attributed to the presentation of multiple immunogens and adjuvanting second signals that combine to elicit strong antibody and cellular responses and long-term memory (Table 2). However, not all viruses can be attenuated, and the risk of reversion can compromise safety, especially for viruses with ill-defined attenuation. Although inactivation of viruses and bacteria is a more generalizable approach and although these vaccines are much safer, inactivated vaccines can exhibit loss of antigens or PAMPs. This loss results in rapid waning of protective immunity

and causes the inactivated vaccines to be less effective than live-attenuated vaccines. Thus, a goal for synthetic or engineered mucosal vaccines is to identify the immunostimulatory factors of attenuated or inactivated microbes.

**Table 2.** Internationally licensed vaccines for human use against mucosal infections (Holmgren and Svennerholm, 2012)

Vaccines against	Administration route	Type
Cholera	Oral	Inactivated <i>V. cholerae</i> bacteria + CTB toxoid
	Oral	Inactivated <i>V. cholerae</i> bacteria
	Oral	Live attenuated <i>V. cholerae</i> O1 bacteria
Typhoid	Oral	Live attenuated <i>S. typhi</i> bacteria
	Parenteral	Purified Vi polysaccharide
Rotavirus	Oral	Live attenuated, mono-valent rotavirus
	Oral	Live attenuated, penta-valent rotaviruses
Influenza	Intranasal	Live attenuated, trivalent influenza viruses
	Intranasal	Similar to above
Polio	Oral	Live attenuated, trivalent polio viruses
	Oral	Live attenuated, bivalent polioviruses
	Oral	Live attenuated, monovalent polioviruses
	Parenteral	Inactivated, trivalent polioviruses Same grown in GMK cells

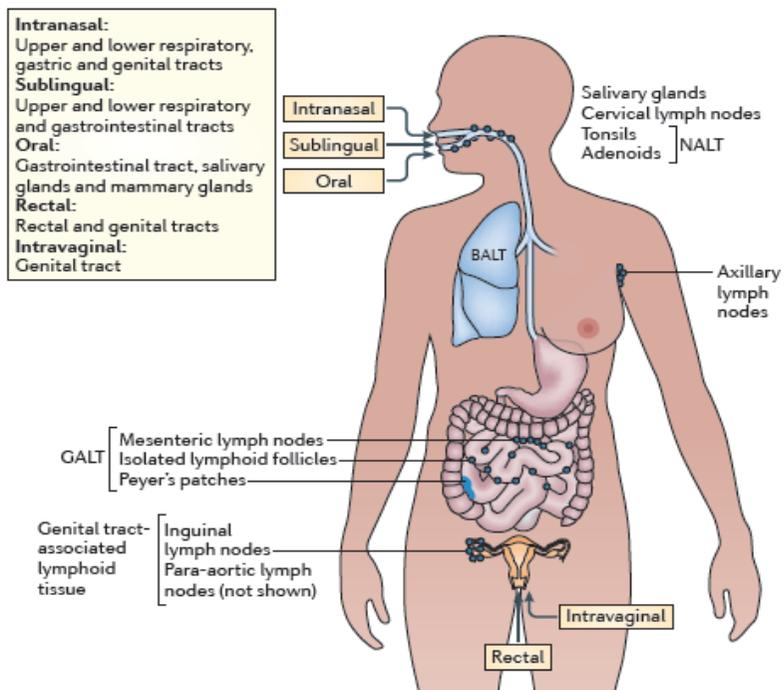
## (2) Subunit vaccines

Vaccines based on pathogen-specific proteins or based on polysaccharides conjugated to proteins or peptides are the second largest category of licensed prophylactic vaccines. In this category the toxoid vaccines, which are typically isolated and inactivated bacterial toxins designed to elicit immunity to the toxic compounds responsible for the disease (Schiller and Lowy, 2015). Examples of toxoid vaccines are the diphtheria toxin vaccine and tetanus toxin vaccine that are administered in combination during childhood as the DTaP (diphtheria, tetanus, pertussis) vaccine (Glanz et al., 2013). Subunit and conjugate vaccines as well as toxoid vaccines are administered primarily by subcutaneous or intramuscular routes and not mucosally, and only serological correlates of antibody protection have been established for these vaccines. One notable exception is DukoralR (Crucell, Netherlands), a vaccine against cholera that is composed of the cholera toxin B subunit and the inactivated strain of *Vibrio cholerae* O1 (Kraehenbuhl and R Neutra, 2013). Oral, but not parenteral, immunization with inactivated whole-cell cholera bacteria together with the cholera toxin B subunit protects against cholera colonization and toxin binding, respectively. Only the orally administered vaccine induces protective mucosal IgA antibodies against the bacterium and its toxin, and provides long-lasting intestinal immunological memory. There are no other examples of successfully licensed subunit vaccines that are administered by mucosal immunization and provide protection. Despite the paucity of successfully licensed subunit and conjugate vaccines for mucosal immunization, several promising studies

highlight the potential and importance of this approach for mucosal vaccine development. Lo et al. have explored the intranasal administration of soluble influenza hemagglutinin protein, which is functionally linked to a targeting peptide that is specific for Claudin-4 on M cells. They found that this soluble fusion protein was able to induce both a specific serum IgG and a mucosal IgA response (Lo et al., 2012). In a recent example by Ye and Zhu et al., a subunit vaccine consisting of the HSV-2 envelope glycoprotein fused to the IgG Fc fragment was delivered intranasally; it elicited systemic as well as mucosal B and T cell responses and conferred protection from intravaginal challenge with HSV-2 (Ye et al., 2011). Furthermore, repeated intravaginal immunization of recombinant HIV-1 envelope protein (gp140), without the use of a mucosal adjuvant, induces systemic and mucosal neutralizing IgG antibodies (Cranage et al., 2010). Mucosal immunization with subunit vaccines has unique challenges associated with not only the immunogen but also the route of antigen delivery. Mucus does not appear to hinder the diffusion of soluble proteins, and transport of large protein antigens should be possible. However, mucosal immunization with protein antigens is limited by the need to protect the protein antigens from degradation by mucosal proteases or commensal microflora. In addition, subunit vaccines are typically poorly immunogenic and require the use of adjuvants to be effective.

## 2) Mucosal vaccine delivery routes

Mucosal vaccination was initially dominated by oral vaccines. The development of intranasal vaccines followed, and today there are many different routes are explored for the delivery of mucosal vaccines, including aerosol inhalation and intravaginal, rectal and sublingual routes (Figure 7).



**Figure 7.** Mucosal immunization routes and compartmentalization of effector functions (Lycke, 2012)

**Table 3.** Comparative anatomical dissemination of secretory immunoglobulin A antibody responses after different routes of immunization (Czerkinsky and Holmgren, 2010)

	Nasal	Sublingual	Oral	Rectal	Vaginal
Upper respiratory	+++	+++	-	-	-
Lower Respiratory	+ to +++	+++	-	-	-
Stomach	-	+ /+++	+ /+++	-	-
Small intestine	-	+++	+++	-	-
Colon	-	?	+	++	-
Rectum	-	?	(+)	+++	-
Reproductive tract	+++	+++	-	-	++ /+++
Blood	+++	+++	+(+)	+(+)	+(+)

## **(1) Oral delivery of vaccine**

Oral delivery of vaccine is an attractive mode of immunization because of its acceptability and its simplicity of administration. The GALT play a central role in the induction of oral tolerance and immunity to infectious agents. In PP, DCs are separated from the intestinal lumen by the follicle-associated epithelium (FAE). It allows transfer of pathogens in lymphoid tissue through Microfold (M) cells. M cells represent a potential portal for oral delivery of peptides and for mucosal vaccination, since they possess a high transcytotic capacity and are able to transport a broad range of materials. Although the mucosal immune system comprises several anatomically remote and functionally distinct compartments, it is firmly established that the oral ingestion of antigens induces humoral and cellular responses not only at the site of antigen exposure but also in other mucosal compartments (Stagg et al., 2003). This is due to the dissemination of antigen-sensitized precursor B and T lymphocytes from the inductive to the effector sites.

Oral vaccines represent the biggest challenge for mucosal vaccine development. This is not only because of the harsh gut environment, which degrades most antigenic epitopes that are delivered in soluble form, but also due to mucosal tolerance, which protects against unwanted immune responses to digested antigens. A plethora of studies have reported success in experimental animals with oral vaccine delivery, although results in ensuing clinical trials have mostly been disappointing (Table 4). Attempts to develop

live attenuated oral vaccines are continuing, but stronger immunogenicity and better stability and safety profiles are required to launch new live mucosal vaccines, and this appears to be difficult to achieve.

**Table 4.** Oral delivery of vaccine (Pavot et al., 2012)

Delivery strategies	Examples	Responses	Model
PLA or PLGA nanoparticles	PLG-encapsulated CS6 antigen from <i>E. coli</i>	– IgA antibody-secreting cell responses ↗ – Serum IgG responses ↗	Human
PLA or PLGA nanoparticles	Lotus tetragonolobus from Winged or Asparagus pea anchored PLGA nanoparticles	– Mucosal and systemic response ↗	Mouse
Liposomes	Plasmid DNA pRc/CMV HBS encoding the small region of hepatitis B surface antigen (HBsAg) into Lipodine liposomes	– IgA responses ↗	Mouse
Liposomes	L-BLP25 (liposomal formulation of BP25 lipopeptide, MPL® and three lipids)	– Median survival time of 4.4 months longer	Human
Bacterial ghost	<i>Escherichia coli</i> O157:H7 bacteria ghosts	– Cellular and humoral immunity ↗	Mouse
Plant lectins/adjuvants	Mistletoe lectin 1, tomato lectin, Phaseolus vulgaris, wheat germ agglutinin (WGA), and Ulex europaeus 1	– Serum and mucosal antibody responses ↗	Mouse
Plant lectins/adjuvants	Rice-based vaccine that expressed cholera toxin B subunit	– Production of specific serum IgG and IgA antibody after three intra nasal or oral doses ↗	Mouse
Transgenic plants (bioreactors)	Plant-based rotavirus VP6	– High titers of anti-VP6 mucosal IgA and serum IgG ↗	Mouse
Transgenic plants (bioreactors)	Recombinant LT-B in transgenic corn	– Both serum IgG anti-LT and numbers of specific antibody secreting cells ↗	Human

↗: Increase

## **(2) Nasal delivery of vaccine**

Intranasal vaccination stimulates immune responses in the nasopharynx-associated lymphoid tissue (NALT) and is effective at inducing systemic and mucosal immunity in the gastric mucosa and the respiratory and genital tracts (Brandtzaeg, 2011). NALT lies in the roof of the nasopharynx in large mammals, at the caudal end of the pharyngeal septum. In rodents, the tissues are paired and lie on either side of dorsal surface of the hard palate. The NALT is easily overlooked, because macroscopically it frequently appears similar to the surrounding nasopharyngeal epithelium. Like GALT in all mammalian species, rodent NALT has a smooth surface of dome epithelium with M cells, while human palatine tonsils and adenoids have deep and branched antigen-retaining crypts with a reticular epithelium containing M cells (Brandtzaeg, 2003).

Intranasal vaccines delivered into the nostrils are an attractive mode of immunization and the nasal mucosa is a practical site for vaccine administration because of the absence of acidity, lack of abundant secreted enzymes and small mucosal surface area that result in a low dose requirement of antigen. Furthermore, the nose is easily accessible, is highly vascularized, can be used for the easy immunization of large population groups. It is well established that nasally administered vaccines can induce both mucosal and systemic immune-responses, especially if the vaccine is based on attenuated live cells or if the antigen is adjuvanted by the use of an immunostimulator or a delivery system. This data were confirmed after nasal immunization of humans against

diphtheria, tetanus (Alpar et al., 2001), influenza (Fiore et al., 2009) and infection with *Streptococcus* mutants (Childers et al., 2006). In addition, it has been described in animal studies, potent responses in the respiratory and genital tracts could be induced by intranasal immunization, as a consequence of the common mucosal immune system (Czerkinsky and Holmgren, 2012). For example, studies with cholera toxin B subunits showed that nasal mucosal immunization produced an exceptionally strong immune response in the respiratory and genito-vaginal tracts (Holmgren and Czerkinsky, 2005).

To be effective following intranasal delivery, the formulation should ensure stability of the antigen, retain long enough time for the antigen to interact with the lymphatic system, stimulate both the innate and cellular systems with or without the use of safe efficacious adjuvants, by targeting specific parts of the immune cells, and provide long-term immunity against the pathogen. Hence, it is of great importance that one considers carefully the choice of an absorption enhancer for a nasally delivered drug that is not readily absorbed especially in terms of potential nasal and systemic toxicity. Intranasal vaccines delivered into the nostrils are an attractive mode of immunization but we have to keep in mind that there are risks of passing into the brain through olfactory nerves and could induce important side effects.

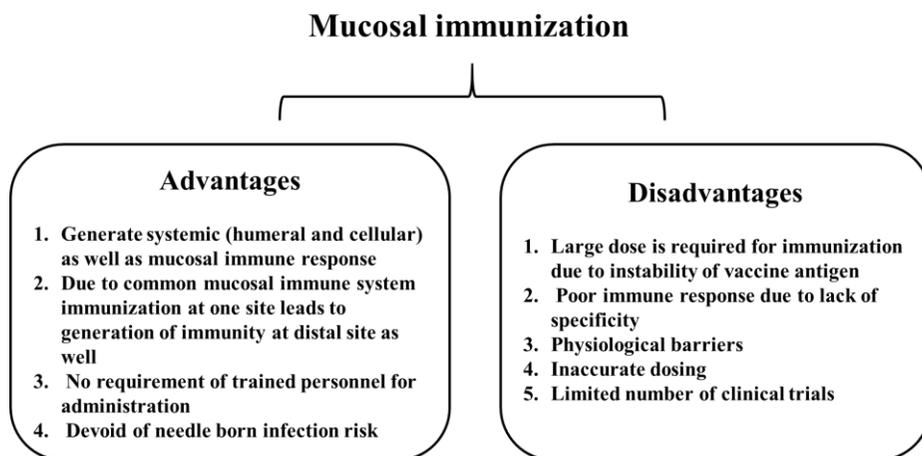
**Table 5.** Nasal delivery of vaccine (Pavot et al., 2012)

Delivery strategies	Examples	Responses	Model
Chitosan	Influenza, pertussis, and diphtheria	– Serum IgG responses similar to and secretory IgA levels ↗	Animals Human
Chitosan	N-trimethyl chitosan (TMC) nanoparticles	– Protection against the appropriate challenge	Mouse
Chitosan	The nasal diphtheria vaccine with chitosan	– Systemic T cell responses ↗ – Antigen-specific IFN-gamma production ↗ – Th2-type responses ↗ – Protective levels of toxin-neutralizing antibodies ↗	Human
Chitosan	Aminated gelatin microspheres (AGMS)	– Nasal absorption of insulin ↗	Rat
Cyclodextrins	Dimethyl-beta-cyclodextrin as adjuvants for nasally applied DT and TT	– Specific serum IgG titres ↗	Mouse
Liposomes	Liposomes incorporated insulin and coated with chitosan and carbapol	– Plasma glucose level up to 2 days ↘	Rat
Nanoparticles	Coated poly(anhydride) nanoparticles with either flagellin from Salmonella enteritidis or mannosamine	– Serum titers of IgG2a and IgG1 ↗ – TH1 and Th2 response ↗	Mouse
Nanoparticles	Nanoencapsulated reporter plasmid (encoding β-galactosidase protein)	– Antibody levels ↗	Mouse
Modified vaccinia virus Ankara (MVA) vector	MVA expressing HIV-1 Env IIIB Ag	– Immune response to the HIV Ag ↗ – Mucosal CD8(+) T cell response in genital tissue and draining lymph nodes ↗ – Mucosal IgA and IgG Abs in vaginal washings ↗ – Specific secretion of beta-chemokines ↗	Mouse

↗: Increase

### 3) Advantage and limitation of mucosal immunization

Mucosal vaccines are considered to be most suitable to combat rising and re-emerging infectious diseases transmitted through mucosal routes. Mucosal immunization induces secretory IgA (sIgA) and serum IgG responses to provide two layers of defense against mucosal pathogens. Some of the important advantages and limitations of mucosal immunization are shown in Figure 8.

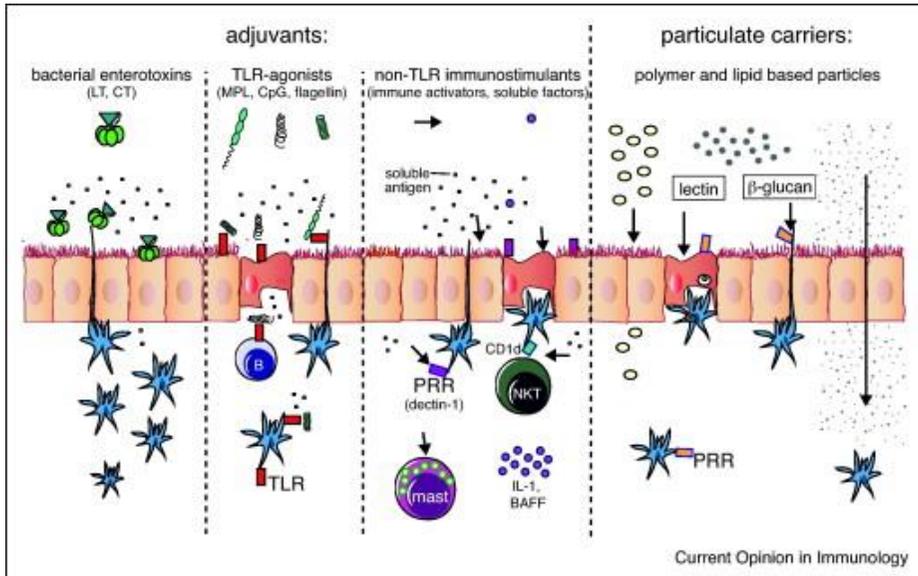


**Figure 8.** Advantages and limitations of mucosal immunization (Mishra et al., 2010)

### **3. Mucosal adjuvant**

#### **1) Adjuvant**

An adjuvant is a vaccine component that, through its capacity to act as an immunostimulant, induces and/or enhances an immune response to co-administered or co-delivered antigens. While there are many classes of adjuvants, not all are effective at promoting mucosal immune responses. In fact, alum, the most common adjuvant used in current human vaccines, is a poor inducer of mucosal immunity. The mucosal adjuvants with the most potential are derived from bacterial enterotoxins, TLR ligands, or small molecule, non-TLR immunostimulants (Figure 9).



**Figure 9.** Enhancing mucosal vaccines through adjuvant and particulate carrier formulations. Mucosal vaccine formulations are designed to enhance delivery and immune responses to antigen. Adjuvants often provide danger signals, which direct and stimulate the immune responses to co-administered soluble antigen. Examples include bacterial enterotoxins, TLR-agonists, and non-TLR immunostimulants. Particulate vaccine carriers facilitate delivery of encapsulated antigen and can promote interactions with immune-responsive cells at mucosal surfaces (Lawson et al., 2011).

## **(1) Bacterial enterotoxins**

Perhaps the best-studied mucosal adjuvants are the bacterially derived ADP-ribosylating enterotoxins, including cholera toxin (CT), heat-labile enterotoxin from *Escherichia coli* (LT), and their mutants or subunits. These enterotoxins promote induction of antigen-specific IgA antibodies and long-lasting memory to co-administered antigens when administered mucosally or transcutaneously (Freytag and Clements, 2005). However, safety issues have prevented full realization of the potential of this class of potent mucosal adjuvants. Intranasal immunization, even with low-toxicity mutants, can induce Bell's palsy (Mutsch et al., 2004), and oral administration with toxin derivatives has either induced unacceptable secretion or poor immunogenicity, as with the B-subunit alone. One promising candidate for oral, sublingual, rectal, or transcutaneous administration is the newly created double mutant LT (R192G/L211A), which in mouse studies results in equivalent antibody and cellular responses to co-administered antigen as does native LT, but without its associated toxicity (Norton et al., 2011). Other researchers are now exploring non-traditional delivery of LT-derived adjuvants, such as transcutaneous, sublingual or intrarectal administration routes (Hervouet et al., 2010). Additionally, a promising LT transcutaneous patch for travelers' diarrhea has been recently evaluated in a Phase II clinical trial (Frech et al., 2008).

## **(2) TLR agonists**

Another class of mucosal adjuvants is Toll-like receptor (TLR) agonists,

reviewed recently by Steinhagen et al. (Steinhagen et al., 2011). This diverse group of ligands activates innate TLR pathogen sensors, promoting intracellular signaling cascades that lead to cytokine secretion and immune cell activation. The first and only current usage of a TLR mucosal adjuvant in the US is in the recently FDA-approved human papillomavirus vaccine, Cervarix<sup>TM</sup>, by GlaxoSmithKline. This formulation incorporates the adjuvant system AS04, which is composed of alum with the TLR4 agonist MPL. MPL promotes a Th1-biased response towards co-administered antigens (Schwarz, 2009). Other MPL formulations for mucosal vaccines continue to be explored. For example, AS01, which contains liposomes, MPL, and saponin, enhanced systemic and mucosal immunity in a HIV vaccine study with non-human primates (Cranage et al., 2011).

Another promising mucosal adjuvant is the TLR9 agonist CpG. These small oligodeoxynucleotide sequences induce strong Th1 responses and have been effective in animal vaccine studies when delivered mucosally (Huang et al., 2008); however, in human subjects CpGs have only been investigated for cancer and systemic immunity in HIV patients or malaria, but not for induction of mucosal immunity.

Recent studies continue to elucidate the mechanisms of action of TLR-targeting adjuvants. Their mucosal efficacy often appears owing to a direct engagement with B-cells or other APCs. Direct TLR-stimulation enhances TI IgA class switching (Katsenelson et al., 2007). *In vitro* application of TLR2

ligands to circulating human B-cells stimulates IgA production while upregulating gut homing markers (Liang et al., 2011). Furthermore, production of autoreactive antibodies by CpG-stimulated B-cells suggests that TLR ligands could be a cause of, or potential therapeutic for, autoimmune diseases (Avalos et al., 2009). Ligand structure can also influence specific APC targeting; for example, CpGs preferentially activate B-cells, macrophages, or DCs depending upon their molecular structure. Lastly, studies have revealed both a TLR9 and TLR4 requirement for antimicrobial, autoimmune, or allograft Th17 responses (Marta et al., 2008), and the induction of IL-17A or Th17 cells after mucosal vaccination with MPL, flagellin, and LPS has been reported.

### **(3) Non-TLR immunostimulants (Immune activators, soluble factors)**

Other adjuvants that have shown evidence of or potential to work mucosally include a variety of small molecule, non-TLR immunostimulants. Many of these molecules activate innate immune sensors on specific cell types.  $\alpha$ -Galactosylceramide, a CD1d ligand and NKT cell activator, induced IgA in mice after an intranasal influenza vaccine (Lee et al., 2011) and an HIV vaccine given orally or intranasally (Courtney et al., 2009), but seems to suppress Th17 responses. Alternatively, there are several fungal or bacterially derived small molecules that will bind to antimicrobial receptors on APCs. For example, a dectin-1 agonist will enhance Th17 responses concurrent with IgA induction (Agrawal et al., 2010). Similarly, mast cell activators can promote Th17 cell development when given intradermally (McGowen et al., 2009) and enhance

B-cell proliferation and IgA production (Merluzzi et al., 2010). Lastly, M-cell targeting ligands fused to antigenic proteins increased M-cell uptake of the antigen and increased systemic and mucosal antibody responses (Kim et al., 2010). Other small molecule adjuvants mimic immune mediators. For example, recombinant IL-1 family cytokines, including IL-1 $\alpha/\beta$ , IL-18, and IL-33, increased IgA and IgG antibodies after an intranasal influenza subunit vaccine (Kayamuro et al., 2010). Similarly, a whole-cell killed *Pseudomonas* vaccine administered with adenovirus overexpressing BAFF enhanced systemic and mucosal antibody levels, reduced bacterial burden, and increased survival after challenge. But as a word of caution, outcomes following human and mouse studies frequently do not agree; an intranasal influenza vaccine, adjuvanted with type I interferon, demonstrated protection in mice but did not induce neutralizing antibodies in human volunteers (Couch et al., 2009).

As the complex nature of mucosal immune induction is understood, the mechanisms of action of current adjuvants are better defined, and promising new mucosal adjuvants are becoming available. With this knowledge, researchers are also exploring adjuvant combinations, including coadministration or molecular fusions (El-Kamary et al., 2010) as well as heterologous prime/boost regimens to optimize mucosal immunity (Glynn et al., 2005).

**Table 6.** Immunological considerations of known mucosal adjuvant (Newsted et al., 2015; Woodrow et al., 2012)

	Mucosal adjuvant	Target	Route of mucosal administration	Antibody	T cells	Cytokines and chemokines	Clinical testing
LT and CT	LT and mutant forms	GM1	Nasal, oral, sublingual, rectal	IgG1, IgG2, IgA	Th1/Th2, CD8	IL-6, IL-8, IL-10, IL-1( $\alpha\beta$ )	Yes
	CT and mutant forms	GM1	Nasal, oral, sublingual, vaginal, rectal	IgA, IgG1, IgE	Th2, CD8	IL-4, IL-5, IL-6, IL-10	Yes
TLR agonists	MPL	TLR4	Nasal, oral, sublingual, vaginal			IL-1, IL-17, IFN- $\gamma$	Yes
	CpG-ODN	TLR9	Nasal, oral, sublingual, vaginal, rectal	IgG2, IgA	Th1/Th2, CD8	IL-6, IL-12, IL-8, RANTES, MIP-1 $\alpha$ , MIP-1 $\beta$ , TNF- $\alpha$ , IFN- $\gamma$	Yes
	poly(I:C)	TLR3	Nasal, sublingual, vaginal				Yes
	Flagellin	TLR5	Nasal	IgG, IgA	Th1/Th2	TNF- $\alpha$ , IFN- $\gamma$ , MIP-2, IL-6	
	Pam <sub>3</sub> CSK <sub>4</sub>	TLR1/2	Nasal, sublingual				Yes
	FSL-1	TLR2/6	Nasal, sublingual, vaginal				
	R848	TLR7/8	Nasal, sublingual, vaginal				Yes
Cytokines	FLT3	Various tissues	Nasal, sublingual				Yes
	IL-1 family of cytokines	Various	Nasal, rectal				Yes
	Mast cell activators	Mast cells, GM1	Nasal				
Novel adjuvant	$\alpha$ -Galactosylceramide	CD1d, NKT cell	Nasal				
	c-di-GMP	Second messenger	Sublingual				

## **2) Carriers for mucosal delivery**

The promise of mucosal vaccines is that they can be designed to recapitulate the earliest cellular interactions with local APCs and mucosal follicles to generate local immune responses. In recent years, it was observed that innovative approaches to engineering delivery carriers to overcome mucosal barriers so that vaccines and adjuvants can be delivered to the oral, nasal, and urogenital mucosa. The carriers can be made of a variety of materials such as lipids, natural or synthetic polymers, inorganic materials to form particles and capsules of controlled size and architecture (Table 7) (Woodrow et al., 2012).

### **(1) Lipid based particles**

#### **(i) Liposomes**

Liposomes consist of a self-assembled phospholipid bilayer encapsulating an aqueous core and they are capable of establishing a single lipid bilayer or multiple bilayers separated by regions of water. There are several advantages to using a liposome-based nanoparticles in vaccine formulations as they trap and hinder drug degradation, they are capable of delivering drugs that possess poor solubility, they reduce the toxicity of drugs by shielding and they alter the targeting and distributing of the drug. These advantages would be especially beneficial for establishing control and utility of vaccine antigens in a mucosal environment. Furthermore, many antigen types can be considered compatible with liposome-based formulations as they can reside in the aqueous core,

peptides and DNA, or they can be embedded into the bilayer through insertion, adsorption or covalent binding. Liposomes are commonly endocytosed by antigen-presenting cells resulting in easy access to the carried antigen – immunomodulators or Toll-like receptors ligands – that promote the adjuvant activity of the liposome and aid in establishing a strong immune response, even with weaker antigens. The enhanced immune response after mucosal administration of antigen-containing liposomal vesicles is most likely attributed to their particulate nature. Fusogenic liposome vesicles are able to effectively merge with epithelia cells of the mucosa, to act as depot to delivery antigen to APCs for processing and presentation, and subsequently cause the induction of humoral immunity (Thérien and Shahum, 1989).

**(ii) Immune stimulating complexes (ISCOMs)**

ISCOMs appear as spherical, hollow, rigid, cage-like particles of about 40 nm in diameter with probably most of the antigens exposed on the surface. In many papers the capacity of ISCOMs to boost the immune response has been demonstrated. The applicability of ISCOMs is diverse. Apart from parenteral immunization, ISCOMs have been used for mucosal delivery of antigens. They are able to boost both humoral and cellular responses. Antigens from viral, bacterial and parasitic sources have been studied. ISCOMs are able to generate a broad range of immune responses. These include APC activation, increased MHC II expression on APCs, cytokine induction, especially IL-2 and IFN-gamma, CD4+ T-cell responses and CD8+ CTL responses. The type of immune

response that is generated after immunization with a particular ISCOM vaccine probably also depends on factors like the antigen, route and immunization schedule. This makes it hard to give general rules (Kersten and Crommelin, 2003).

### **(iii) Virus-like particles (VLPs)**

VLP and virosomes constitute a category of subunit vaccines wherein the immunogens are derived from viral components that self-assemble into higher-order three dimensional architectures that preserve the antigenic structure of virus immunogens. VLPs are formed from the self-assembly of one or more viral capsid or envelope proteins that are expressed recombinantly in mammalian or insect cells. The hepatitis B vaccine was the first commercially viable VLP-based vaccine. It is produced from the self-assembly of the hepatitis B surface antigen (HBsAg) expressed recombinantly in yeast cells. The human papillomavirus (HPV) vaccine has been the only other VLP since to be licensed for human use. The human papillomavirus (HPV) vaccine has been the only other VLP since to be licensed for human use. The quadrivalent HPV vaccine from Merck & Co. (GardasilR) is composed of the L1 capsid proteins of HPV-6, -11, -16, and -18 types that are expressed recombinantly in yeast and self-assembled into VLPs (Wheeler et al., 2009). However, VLP technology can be limited by difficulties of scale-up, the need for purification from the expression systems, and the requirement for adjuvants. Only virosomes have been used effectively without additional adjuvants (Moser et al., 2007), so they may be

attractive as mucosal vaccines. Although both VLPs and virosomes have great potential as vaccine carriers, they are harder to formulate and are less reproducible compared with entirely synthetic polymer nanoparticles.

#### **(iv) Archaeosome**

Archaeosome vesicles, composed of polar lipids extract from Archaea, have demonstrated strong systemic adjuvant activities. The mechanism of action of archaeosomes is attributed to MHC class I cross-priming via the phagosome-to-cytosol transporter associated with antigen processing-dependent classical processing pathway, and upregulation of costimulation by APCs without overt inflammatory cytokine production. Furthermore, they facilitate potent CD8+ T cell memory to co-delivered antigen, comparable in magnitude and quality to live bacterial vaccine vectors. The archaeosomes also demonstrated a capacity to induce humoral as well as cell-mediated immune response in orally immunized mice relative to liposome vaccines, supporting the ability of archaeosome to successfully protect and deliver the antigen to an immune inducing site leading to a robust immune response(Krishnan and Sprott, 2008).

#### **(2) Polymer based particles**

The versatility of polymeric particles for mucosal vaccine design arises from the availability of different polymers and methods for particle synthesis. Common polymer compositions of nanoparticles include biodegradable or bioeliminable synthetic polymers [e.g., polyesters, polyanhydrides, and natural

polymers (chitosan, alginate and albumin), copolymers, and polymer blends (des Rieux et al., 2006). Both synthetic and natural polymer-based micro- and nanoparticle carrier systems are widely used in drug delivery applications and show the greatest versatility for designing effective mucosal vaccines (des Rieux et al., 2006). Particle size, composition (surface chemistry and polymer architecture), and ability to control the presentation of antigen and PAMPs have important roles in immune activation. Size plays a critical role in the amount of antigen that can be delivered as well as the manner in which the antigen is internalized and processed by the mucosal immune system. The selection of polymers and fabrication method can generate nanoparticles with a wide range of sizes and geometries. In addition to size, the surface chemistry and polymer composition of nanoparticles can be specifically engineered to overcome transport barriers, interact with tissues and cells, and promote.

**(i) Poly (esters)**

Poly (esters) are typically composed of lactic acid, glycolic acid, and the copolymers thereof various molecular weight and compositions. Poly (esters) materials have long history of safety records and hence FDA approved its applications in humans in the fields of medical and pharmaceutical. The use of biodegradable microspheres and nanospheres for vaccine development is a major area of activity in the utilization of biodegradable microspheres and nanospheres. PLGA, copolymer of poly(d, l-lactide-co-glycolide), are the most commonly employed polymeric materials and can be prepared by various

techniques such as oil-in-water emulsification, coacervation (polymer phase separation), spray drying and solvent evaporation. PLGA microparticles, used as antigen carriers, enhance antigen recognition and uptake by DCs localized in specialized mucosal-associated lymphoid tissue. (Anderson and Shive, 2012). Moreover, PLGA has an excellent controlled-release profile, excellent toxicological profiles. Woodrow *et al* showed that topical delivery of fluorescently labeled PLGA nanoparticles to the vaginal mucosa resulted in distribution and penetration of the particles throughout the local tissue (Woodrow et al., 2008). Cellular uptake and trafficking likely contributed to the observed tissue distribution. The surfaces of PLGA nanoparticles are easily modified to express tailored physiochemical properties that enhance particle diffusivity through the mucosa and transcytosis by mucosal M cells. One of the most important attributes of PLGA is that vaccine antigens can be encapsulated into the matrix or on the surface of PLGA carriers. Although most applications of PLGA nanoparticles have been in subcutaneous or intramuscular depot injections, many groups have turned their attention to studying PLGA nanoparticles as a possible delivery vehicle for mucosal immunization. Efforts have been made in delineating the optimal conditions for increased uptake of PLGA microspheres across the mucosal barrier (Alpar et al., 2005).

#### **(ii) Polyethylenimine (PEI)**

Biodegradable NPs are also extensively explored as carriers for gene therapy. These NPs can improve transfection efficiency in target cells, and reduce

toxicity associated with gene delivery of viral vector. Polyethylenimine (PEI) has been used as a cationic polymer for the formation of complex with negatively charged DNA *in vitro* and *in vivo*. PEI-plasmid DNA complex exhibits extremely high transgene efficiency. This effect is believed to associate with its protection function of PEI against enzymatic degradation of DNA. Another effect of PEI, called ‘proton sponge’, indicates that pH buffering property of PEI could facilitate DNA endosomal escape. Bivas-Benita *et al.* evaluated the immunogenicity of a DNA vaccine encoding the *Mycobacterium tuberculosis (Mtb)* latency antigen Rv1733c for pulmonary delivery using PLGA-PEI NP. Their results revealed that the complex of PLGA-PEI NPs absorbed with Rv1733c DNA plasmid considerably increase T-cell proliferation and IFN- $\gamma$  production, compared to that given intramuscularly (Bivas-Benita *et al.*, 2009; Mann *et al.*, 2013).

### **(iii) Chitosan**

Chitosan is a linear polysaccharide polymer consisting of randomly distributed  $\beta$ -(1-4)-linked D-glucosamine and N-acetyl-D-glucosamine. Because of its mucoadhesive and permeation properties, chitosan is able to considerably enhance the adsorption and transportation of peptide cross mucosal epithelium. A number of studies have confirmed such capacities of chitosan to facilitate macromolecule transportation across mucosal barrier and to interact with mucosal tissue. There are several researches that show the efficacy of chitosan as an adjuvant and delivery system for mucosal vaccines.

The *Bordetella pertusis* filamentous haemagglutinin and recombinant pertusis toxins have been shown to induce strong antigen-specific systemic and mucosal immune responses after intranasal administration with chitosan. Diphtheria toxin nasally co-administrated with chitosan induced systemic and local immune responses (Kang et al., 2009).

### **(3) Nanoemulsion**

Nanoemulsion technologies are liquid suspensions that exhibit long-term colloidal stability and have been used to encapsulate and deliver vaccines directly onto mucosal surfaces. Nanoemulsion droplets are formed by the dispersion of two immiscible liquids. They range in size from 20 nm to 200 nm, which is similar to the size of opportunistic pathogens, and are readily taken up by mucosal M cells and subsequently presented to APCs. Table 7 presents an overview of the major classes of nanoemulsions. Briefly, water-in-oil emulsions incorporate and deliver hydrophilic drugs much more efficiently than do oil-in-water emulsions, which are used to incorporate and deliver hydrophobic drugs. Single-nanoemulsion technology has been successfully employed in the generation of a hepatitis B vaccine. The HBsAg nanoemulsion system generated a strong immune response, producing high titers of both IgA and IgG; this strong response indicates the usefulness of nanoemulsion for NALT mucosal immunization. Unfortunately, single-nanoemulsion methods have poor controlled-release profiles and may not be able to withstand degradation within mucosal sites other than NALT. Hanson et al. introduced the concept of

a double-emulsion method with good controlled release profiles. Double-emulsion technology has proven useful for delivering vaccines to mucosal surfaces before they are degraded (Hanson et al., 2008). Additionally, double emulsions are more stable and are able to encapsulate antigens without deleterious effects to the antigen during the emulsification process. Nanoemulsion technology has provided a novel delivery method for immunizing the mucosal immune system.

#### **(4) Inorganic nanoparticles**

Many inorganic nanoparticles have been studied for vaccines applications. Their advantages come from their rigid structure and controllable synthesis processes (Kalkanidis et al., 2006). Gold NPs (AuNPs) offer unique characteristics, such as biocompatibility, easy fabrication into different shapes (spherical, rod, cubic, etc.) and sizes (2 – 150 nm), and can be readily surface-modified with carbohydrates (Zhao et al., 2014) making them an attractive choice for mucosal vaccine delivery. Tao *et al.* investigated the potential of a novel influenza A vaccine composed of M2e–AuNPs, conjugated with CpG adjuvants and administered intranasally in mice. It was found that M2e-specific IgG serum antibodies were induced by vaccination containing CpG. Furthermore, mice that received soluble CpG-adjuvanted M2e–AuNP were fully protected against the challenge lethal PR8 (Tao et al., 2014).

## **(5) Live vectors**

Live recombinant bacteria or viral vectors that express heterologous antigens are being extensively investigated in the development of vaccines and it is believed that these will provide an optimum immune response against the expressed antigen. Examples of live vectors being utilized in this way include *Salmonella typhi*, BCG, *Shigella flexnerii*, *Lactococcus lactis*, *Lactobacillus casei*, retroviruses, adenoviral, and so on (Moingeon et al., 2002).

Two attenuated *Salmonella* serovars are the most intensively used bacterial vectors, *Salmonella typhimurium* in mice and *Salmonella typhi* in humans. Harokopakis *et al.* investigated the immunogenicity of a chimeric protein comprising the binding domain of a streptococcal protein adhesin linked to the cholera toxin (CT) A2 and B subunits (CTA2/B) in *S. typhimurium* BRD509 via oral or intranasal immunization of mice (Harokopakis et al., 1997; Mielcarek et al., 2001).

Lactic acid bacteria (LAB) are a diverse group of Gram-positive, nonsporulating, low G+C content bacteria. Many of them have been given generally regarded as safe status. Over the past two decades, intensive genetic and molecular research carried out on LAB, mainly *Lactococcus lactis* and some species of the *Lactobacillus* genus, has revealed new, potential biomedical LAB applications, including the use of LAB as adjuvants, immunostimulators, or therapeutic drug delivery systems, or as factories to produce therapeutic molecules. LAB enable immunization via the mucosal route, which increases

effectiveness against pathogens that use the mucosa as the major route of entry into the human body. Many studies on the effectiveness of various representatives of the LAB group as carriers of heterologous antigens for vaccination were conducted with the highly immunogenic tetanus toxin C-terminal fragment (TTFC) as antigen. This protein fragment has been intensively studied as a replacement for inactivated toxin in the combined diphtheria, tetanus, and pertussis (DTP) vaccine (Grangette et al., 2004).

Adenoviral vectors have received considerable attention for vaccine development because of their high immunogenicity and efficacy. The adenoviral backbone is a type 1 immune adjuvant enhancing the immune response toward the foreign antigen and of all vector platforms, it derives the strongest transgene expression with continuously raised levels of protein *in vivo* for up to 3 weeks. The adenoviral vector triggers potent transgene product-specific CD8<sup>+</sup> T-cell responses and to a lesser extent CD4<sup>+</sup> T-cell responses. Ad5-based vaccines have been developed for several infectious diseases, including malaria, HIV, influenza and Ebola virus disease, with demonstrated safety, immunogenicity and efficacy (Majhen et al., 2014).

**Table 7.** Particulate carriers commonly employed to deliver vaccine antigen to mucosal sites (Woodrow et al., 2012)

	<b>Particulate carrier type</b>	<b>Vaccine characteristics</b>	<b>Advantages</b>	<b>Disadvantages</b>	<b>Route</b>
<b>Lipid based particles</b>	<b>(1) Liposome</b>	Water-insoluble drugs Water-soluble drugs Proteins DNA	Easy surface modification Synthesized from nontoxic material Dual function Wide range of antigen encapsulation	Low antigen loading Low stability	Oral, Nasal
	<b>(2) ISCOMs</b>	Proteins	Adjuvant itself	Reactogenicity	Nasal
	<b>(3) Archaeosome</b>	Proteins	Adjuvant itself	Reactogenicity	Nasal
	<b>(4) Virus like particles</b>	Plasmid DNA Proteins Peptides	Lacks viral genes Highly immunogenic High rate of uptake Undergoes self-assembly	Formulated by recombinant technology	Oral, Nasal
<b>Polymers</b>	<b>(1) Poly (esters)</b>				
	PLGA	Plasmid DNA Protein Peptide Low-molecular-weight molecules	Controlled release Sensitive to environment Stable microenvironment Biocompatible	Low loading efficiency Degradation of antigen during encapsulation	Oral, Nasal, Intravaginal
	PLA	Plasmid DNA Protein Peptide Lipophilic compound	Controlled release Surface easily modified	Low loading efficiency Degradation of antigen during encapsulation	Oral, Nasal
	<b>(2) Polyethylenimine</b>	Plasmid DNA	Efficiently transfected	Cytotoxicity	Nasal, Oral
<b>(3) Chitosan</b>	Plasmid DNA Protein Peptide	Mucoadhesive Non-toxic Bio-degradable facilitate macromolecule transportation across mucosal barrier		Oral, Nasal	

	Particulate carrier type	Vaccine characteristics	Advantages	Disadvantages	Route
<b>Emulsion</b>	(1)Water-in-oil	Th1-stimulating antigens	Slow release of antigen	Reactogenicity	Oral, Nasal
	(2)Oil- in-water	Th2-stimulating antigens	Slow release of antigen	Reactogenicity	Oral, Nasal
<b>Iorganic nanoparticles</b>	AuNPs, Cochleate	Protein	Biocompatibility Easy fabrication into different shapes (spherical, rod, cubic, etc.) Surface easily mofied		Nasal
<b>Live vectors</b>	(1) <b>Bacteria</b> <i>Salmonella typhi</i> , BCG, <i>Shigella flexnerii</i> , <i>Lactococcus lactis</i> ,etc	Recombinant bacteria (vaccine antigen containing)		Safety issue, Transmissibility and longterm persistence in the host	Oral, Nasal
	(2) <b>Viral vector</b> Retroviruses, adenovus			Safety issue Disease-causing potential, Transmissibility and longterm persistence in the host	Oral, Nasal

# **Chapter 2. Recombinant interleukin 6 with M cell-targeting moiety produced in *Lactococcus lactis* IL1403 as a potent mucosal adjuvant for peroral immunization**

## **1. Introduction**

Lactic acid bacteria (LAB) are fascinating recombinant hosts for expression of therapeutic molecules such as antigens, cytokines, and enzymes, *etc.*, because they could survive from stomach acid and protect the producing molecules from enzymatic degradation during passage through the gastrointestinal tract, and LAB themselves only induce minute level of immune responses to host animals following ingestion as ‘generally recognized as safe (GRAS)’ microorganisms. Thus, numerous researches were conducted to use LAB as a live oral delivery vehicle for therapeutic protein molecules and *Lactococcus lactis*, a food-grade strain of lactic acid bacteria (LAB) which is nonpathogenic, noninvasive, and noncolonizing bacteria, might be the most frequently used LAB host for this purpose (Bermúdez-Humarán, 2009; Wells and Mercenier, 2008). For examples, a genetically engineered recombinant *L. lactis* producing TTFC (tetanus toxin fragment C) showed successful results as

an oral vaccine (Grangette et al., 2002). Co-expression of IL-2 or IL-6 with the model antigen TTFC in *L. lactis* and its mucosal administration could enhance mucosal and systemic immune responses in mice (Steidler et al., 1998).

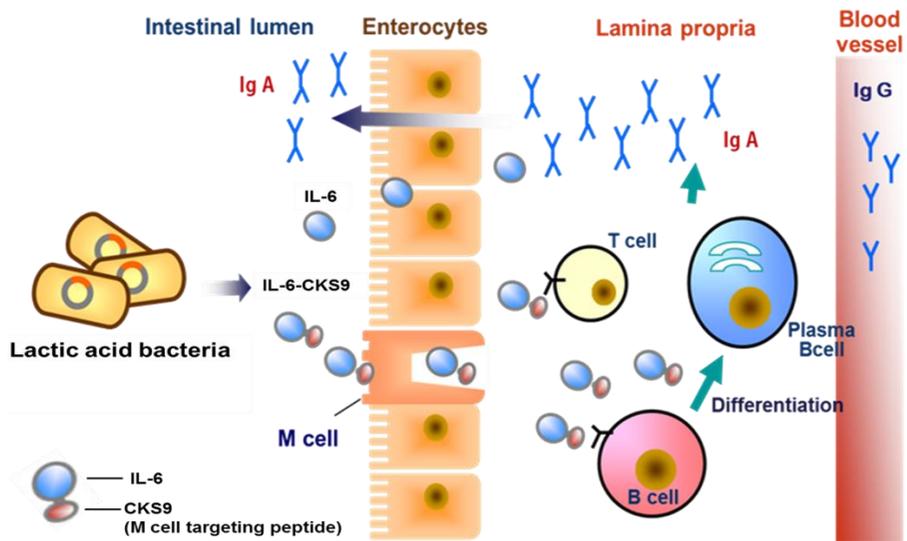
Most animal pathogens initiated their infection through access to the mucosal region of body. Thus, effective mucosal vaccine is necessary in the aspect that it could protect the host body from the pathogens at the initial site of infection by induction of mucosal immunity (Lycke, 2012). However, development of mucosal vaccine, especially oral vaccine, is challenged because of harsh condition of gastrointestinal environment to vaccine stability. Recently, subunit vaccine has been regarded as a safe vaccine strategy to avoid potential harmfulness of whole-body vaccine due to renewal of its infectivity though the poor immunogenicity is one of its drawbacks must be overcome (Coller et al., 2011). Therefore, exploration of the effective mucosal adjuvants is inevitable to improve availability of the subunit vaccine.

There are many classes of adjuvants, but not all are effective in mucosal immune responses. Such as, alum, the most common adjuvant used in current vaccines, is not effective in mucosal immunity. At present, bacterial enterotoxins, CT (cholera toxin), LT (heat-labile enterotoxin), TLR (toll-like receptor) ligands, cytokines, and non-TLR immune stimulants, *etc.* are classified as potential mucosal adjuvants (Lawson et al., 2011). Interleukin 6 (IL-6) is a multifunctional cytokine conducting significant roles in both innate

and adaptive immune responses, which produced by both pro-APCs (B cells, macrophages, and dendritic cells) and non-professional APCs (epithelial, endothelial, and some tumor cells) (Akira, 1993; Taga and Kishimoto, 1997). In innate immune response, as a pro-inflammatory cytokine, it is associated with up regulation of acute phase protein. In adaptive immune response, as a B cell proliferation factor, its function is associated with final differentiation of the plasma B cells (La Flamme et al., 2000). Besides, IL-6 mediates proliferation of lymphocytes, regulation of IL-2 receptor, and Th1-associated cytokine expression (Su et al., 2008). There have been several reports about successful application of IL-6 as a mucosal vaccine adjuvant. As mentioned above, co-expression of IL-6 with TTFC in *L. lactis* and its nasal administration enhanced mucosal and systemic immune responses in mice (Steidler et al., 1998). Co-expression of one of IBV (infectious bronchitis virus) antigen with chicken IL-6 in *Mycoplasma gallisepticum* and its oral vaccination showed specific mucosal immune response in chicken (Shil et al., 2011). Moreover, IL-6 also performed as an effective adjuvant in DNA vaccine for FMDV (foot and mouth disease virus) (Su et al., 2008).

Peyer's patches are 'gut associated lymphoid tissue (GALT)' and M cells are antigen-collecting portals located on the 'follicle associated epithelium (FAE)' of Peyer's patches, which facilitate to transport antigens from gut lumen to underneath mucosal immune system. Thus, M cell-targeting might be one of

the promising strategies to develop an effective oral vaccine (Lelouard et al., 2012). An M cell-targeting peptide ligand, CKS9, consisted of 9-amino acids with cyclic conformation was identified via phage display screening in our previous research (Yoo et al., 2010). In this study, we developed a novel M cell-targeting cytokine adjuvant system for oral vaccine by generation of recombinant *L. lactis* producing murine IL-6 conjugated with CKS9. We hypothesized that this live recombinant LAB could enhance mucosal immunity in gastrointestinal tract by secretion of IL-6 with M cell-targeting moiety and its specific delivery to the mucosal immune system through the M cells, when they orally administered with vaccine. M-BmpB (*Brachyspira* membrane protein B conjugated with CKS9) was adapted as a model antigen in this study. *Brachyspira hyodysenteriae* is one of the pathogenic bacteria causing a mucohemorrhagic dysentery in pigs and the BmpB, *Brachyspira* outer membrane lipoprotein B, was reported as an antigen which could induce protective antibody against *B. hyodysenteriae* for subunit vaccine (Hampson et al., 2000; Lee et al., 2000). This study demonstrated that the construction of cytokine expression vector system for LAB, *in vitro* functional validation of the recombinant cytokine, and *in vivo* efficacy of the recombinant LAB as an oral vaccine adjuvant system.



▪ **Experimental flow chart**

<b>Characterization</b>	<ul style="list-style-type: none"> <li>▪ Vector construction</li> <li>▪ Confirmation of the expression of IL-6s</li> </ul>
<b>In vitro</b>	<ul style="list-style-type: none"> <li>▪ Physiological characterization of recombinant LAB</li> <li>▪ Biological activity validation of the recombinant IL-6s</li> </ul>
<b>In vivo</b>	<ul style="list-style-type: none"> <li>▪ Evaluation of recombinant IL-6s-producing LAB as an adjuvant for mucosal immunity</li> <li>▪ Investigation of the occurrence of oral tolerance</li> </ul>

**Figure 10.** Experimental flow of the study 1

## 2. Materials and methods

### 1) Construction of expression vector system for IL-6s

To construct mouse IL-6 gene expression vector system for *L. lactis*, pILPtuf, in which includes, *tuf*, one of the strongest constitutive promoters (Figure 15), was adapted as a mother plasmid, which was previously constructed by our research group (Kim et al., 2009). We generated the mouse IL-6 gene fragments with or without nucleotide sequences encoding Usp45-secretion signal peptide (Borrero et al., 2011) and the M cell-targeting peptide, CKS9, through the oligonucleotide-assembly PCR using a series of synthetic overlapped oligonucleotides (IDT Inc., Belgium), which were computationally designed as approximately 60-base-pair long, according to a previously described method (Hoover and Lubkowski, 2002). Three resulting gene fragments, Usp45-IL-6 (651 bp), Usp45-CKS9-IL-6 (696 bp), and Usp45-IL-6-CKS9 (699 bp), were digested by *NdeI* and *XhoI* restriction enzyme, then introduced into same restriction enzyme sites of the pILPtufMb plasmid, respectively. The amino acid sequence of mouse IL-6 was obtained from GenBank (NM-031168.1) and codon usage of each gene fragment was optimized to facilitate the translation of eukaryotic cytokine-coding gene (mouse IL-6) in prokaryotic host (*L. lactis* IL1403). A web-based analysis program, DNA work 3.1, was used for oligonucleotide design and codon optimization (Hoover and Lubkowski, 2002). The schematic diagram of the synthetic gene fragments and expression vector

system were shown in Figure 11(A).

## **2) Confirmation of the expression and secretion of IL-6s from LAB**

Each expression vector, pILPtuf-IL-6, pILPtuf-CKS9-IL-6, or pILPtuf-IL-6-CKS9, was transformed into *L. lactis* IL1403 by electroporation, respectively. To confirm the expression and secretion of IL-6s from *L. lactis* IL1403, intracellular protein and secreted protein fractions were separately prepared as previously described (Mathiesen et al., 2009). The expression and secretion of IL-6s from *L. lactis* IL1403 were analyzed by Western blot using rabbit-anti-mouse IL-6 antibody (Millipore, USA). The accumulation levels of secreted IL-6s in culture supernatants were assessed by ELISA kit (R&D systems, USA) with the lapse of time during the cultivation of transformants.

## **3) Biological activity validation of the recombinant IL-6s**

In order to validate biological activity of the recombinant IL-6s secreted from *L. lactis* IL1403, a murine hybridoma cell line, 7TD1 (RIKEN cell bank, Japan), of which proliferation efficiency could be prominently improved under IL-6 signal, was adapted as an *in vitro* model bioassay system in this study (Shil et al., 2011). Briefly, the 7TD1 cells were cultivated with RPMI 1640 media (HyClone, USA) containing 5% (v/v) FBS (GIBCO, USA), 25  $\mu$ M 2-mercaptoethanol, and 0.5 ng/ml of commercial mouse IL-6 (Millipore, USA). Prior to bioassays, the 7TD1 cells were washed with IL-6 free RPMI 1640

media twice and, incubated for an additional 48 h without IL-6. Subsequently the starved 7TD1 cells were divided by aliquots of  $1.0 \times 10^4$  cells, then bioassays were conducted by cultivation of each 7TD1 cell aliquot at various concentrations (0.2 pg/ml, 1 pg/ml, 5 pg/ml, 25 pg/ml, 125 pg/ml or 625 pg/ml) of commercial IL-6 or secreted IL-6s from *L. lactis* IL1403 at 37 °C for 48 h. After incubation, CellTiter96 Aqueous One Solution (Promega, USA) was added to the each well and incubated for an additional 4 h, then optical density of colored solution was measured at 490 nm using a microplate reader (Tecan Infinite® 200 PRO;Mannedorf, Switzerland) and all the assays were performed in triplicate.

#### **4) Closed-ileal loop assay and immunohistochemistry**

Closed-ileal loop and immunohistochemistry assays were conducted to verify M-cell targeting property of the recombinant IL-6s secreted from *L. lactis* IL1403 according to the method previously reported by our research group (Yoo et al., 2010). Briefly, 8 week-old female BALB/c mice (Samtako, Korea) were fasted overnight and anesthetized. Then, abdomen was incised and the mid-ileal region of small intestine was tied in 2 cm length with ligature to form closed-ileal loop containing one or two Peyer's patches. The culture supernatant of recombinant *L. lactis* IL1403 producing IL-6, CKS9-IL-6, or IL-6-CKS9 was prepared in which concentration of the IL-6 could be 5 µg/ml, respectively, by using centrifugal buffer-exchange columns, Centricon

(Millipore, USA). Then, 1 µg of each recombinant IL-6 was injected into the ileal loop. After 1 h incubation, the ileal loops were excised, washed in PBS to remove any free and loosely bound proteins, and then fixed with 4% paraformaldehyde (PFA). Tissue sections were fixed in cold acetone for 5 min and blocked by 3% goat serum, then fluorescent signals of recombinant IL-6s which incorporated within the tissues were detected using rabbit-anti-mouse IL-6 antibody (Abcam, UK) and donkey anti-rabbit IgG antibody conjugated with Alexa 555 (Abcam, UK) for the primary and secondary antibodies, respectively. M cells on Peyer's patch (PP) was labeled by anti-Gp2 (glycoprotein 2) monoclonal antibody-FITC (MBL, Japan) and nuclei were stained by 4', 6-diamidino-2-phenylindole (DAPI) (Sigma, USA).

### **5) Peroral immunization of mice**

Peroral immunization study was conducted with 8 week-old female BALB/c mice (Samtako, Korea) which were assigned in five groups (n=5) to validate the effect of the IL-6-producing LAB as a mucosal vaccine adjuvant. M-BmpB protein was used as a model antigen and prepared by cultivation of the recombinant *E. coli* BL21 harbouring the M-BmpB gene which was previously constructed by our research group (Jiang et al., 2014). Each group of mice was fasted for 6 h before orally administration and immunized on days 0, 7, and 14, respectively: (i) 200 µl of PBS; (ii) 200 µg of purified M-BmpB in 200 µl of PBS; (iii) 200 µg of M-BmpB with 10<sup>9</sup> cells of wild type *L. lactis* IL1403 in

200 µl of PBS; (iv) 200 µg of M-BmpB with  $10^9$  cells of recombinant *L. lactis* IL1403 producing IL-6 in 200 µl of PBS; (v) 200 µg of M-BmpB with  $10^9$  cells of recombinant *L. lactis* IL1403 producing IL-6-CKS9 in 200 µl of PBS. Before 30 min of every peroral immunization, each mouse was gavaged with 500 µl of neutralizing solution (7.5% NaHCO<sub>3</sub>: PBS=1:4) to reduce stomach acidity. Blood and fecal samples were prepared from the each group of immunized mice on day 21 and 28 after primary peroral immunization. Briefly, blood samples were collected from the caudal vein and centrifuged after clotting at 2,000 rpm for 10 min to prepare sera. For fecal sample preparation, several pieces of fecal pellets freshly-taken from each mouse were collected and suspended evenly in PBS by vigorous vortexing in a concentration of 200 mg/ml and centrifuged at 14,000 rpm, 4 °C for 15 min, then clarified fecal extracts were obtained. Additionally, mid-ileal region of small intestines (3 cm) of the mice were immediately collected after sacrifice. The small intestines were homogenized in 500 µl of ice-cold PBS. Tissues were removed by centrifugation (14,000 rpm, 10 min and 4 °C), and the supernatants were collected. Blood samples and feces samples were collected at days 21, 28 and intestinal samples were collected at days 28. All the samples were stored at -70 °C until analysis. The immunization and sample collection schedule was depicted in Figure 16(A). All experimental procedures using laboratory animals were approved by the Institutional Animal Care and Use Committee of Seoul National University (Approval Number: SNU 121106-3) and followed the guidelines suggested by the committee.

## **6) ELISA assays**

Enzyme-Linked-Immuno-Sorbent Assays (ELISA) were conducted to assess the levels of antigen-specific Ig Gs (IgG, IgG1, and IgG2a) or IgA in mice sera or fecal samples, respectively. 96-well plates (Thermo) were coated with 2.5 µg of purified M-BmpB in 100 µl of 50 mM carbonate-bicarbonate buffer (pH 9.6) at 4 °C overnight and blocked with 1% BSA at 37 °C for 1 h. Then, appropriately diluted sera or fecal samples were added into wells and incubated at 37 °C for 2 h. After incubation, HRP (Horse Radish Peroxidase)-conjugated goat anti-mouse IgG, IgG1, or IgG2a (Santa Cruz Biotechnology, USA) was treated for sera and HRP-conjugated goat anti IgA (Santa Cruz Biotechnology, USA) was treated for fecal and intestinal samples to each designated well at 37 °C for 1 h, respectively. TMB solution (Sigma-Aldrich, USA) was added to the wells for the HRP substrate, reaction was stopped after 10 min by adding 1 M H<sub>2</sub>SO<sub>4</sub>, and absorbance values were determined using GRL 1000 Microplate Reader (General Labs Diagnostics, USA). Appropriate washing of each well proceeded between the steps with PBS containing 0.05% of Tween 20 during the assays. The ELISA results are expressed as the OD values measured at 450 nm for dilutions of 1:800 for serum, 1:10 for fecal extracts, and 1:100 for intestinal samples.

## **7) ELISPOT assays**

ELISPOT assays were performed to ascertain antigen-specific induction of

cytokines from the lymphocytes in Peyer's patches after peroral immunization in mice. Lymphocytes were isolated from the small intestinal Peyer's patches in mice at 7<sup>th</sup> day after final immunization as described previously (Kim et al., 2010). ELISpot kits (R&D systems, USA) were used for validation of antigen-specific cytokine induction. The isolated lymphocytes were applied to each plate of kit which was pre-coated with antibodies for a cytokine of interest, then stimulated with 100 nM of purified M-BmpB to induce cytokine expression at 37 °C for 36 h. The rest of procedure was followed by manufacturer's manual. The colored spots formed by cytokine production from lymphocytes were counted with ELISPOT reader (Cellular Technology Ltd, USA). Mean numbers were calculated from triplicated ELISPOT assays.

### **8) Investigation of oral tolerance induction**

In order to test the possible oral tolerance induction, peroral immunization was re-conducted with mice according to the method described in above. Orally immunized mice were boosted systematically at 2 weeks after the last immunization by intraperitoneal injection of recombinant M-BmpB (20 µg), then sera and fecal extracts were prepared at 7 days after the boost immunization. The oral immunization, systemic boosting and sample collection schedule was depicted in Figure 18(A). The levels of M-BmpB specific serum IgG and fecal IgA were analyzed through ELISA and then calculated as the reciprocal endpoint titers.

## **9) Statistical analysis**

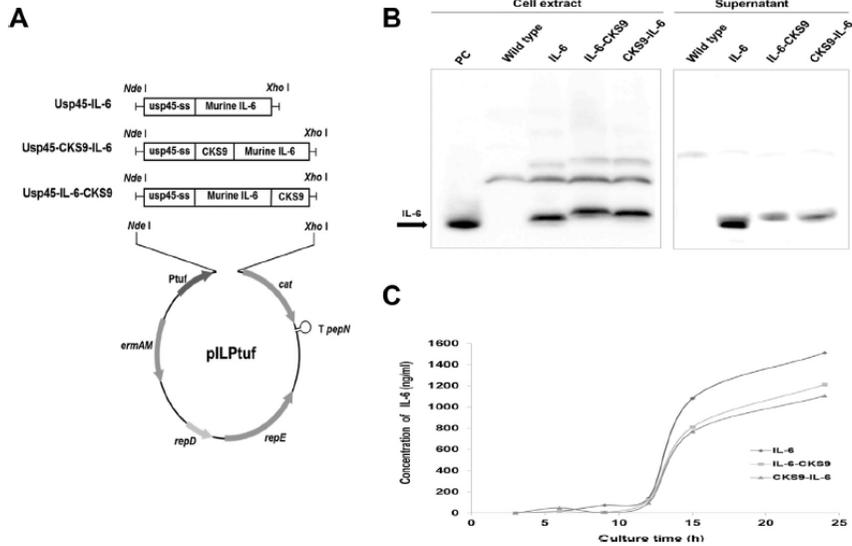
Quantified results were expressed as the mean and standard deviation (SD). Statistical significance was assessed using a one-way analysis of variance (ANOVA) with *post hoc* Tukey Multiple Comparisons test.

### 3. Results

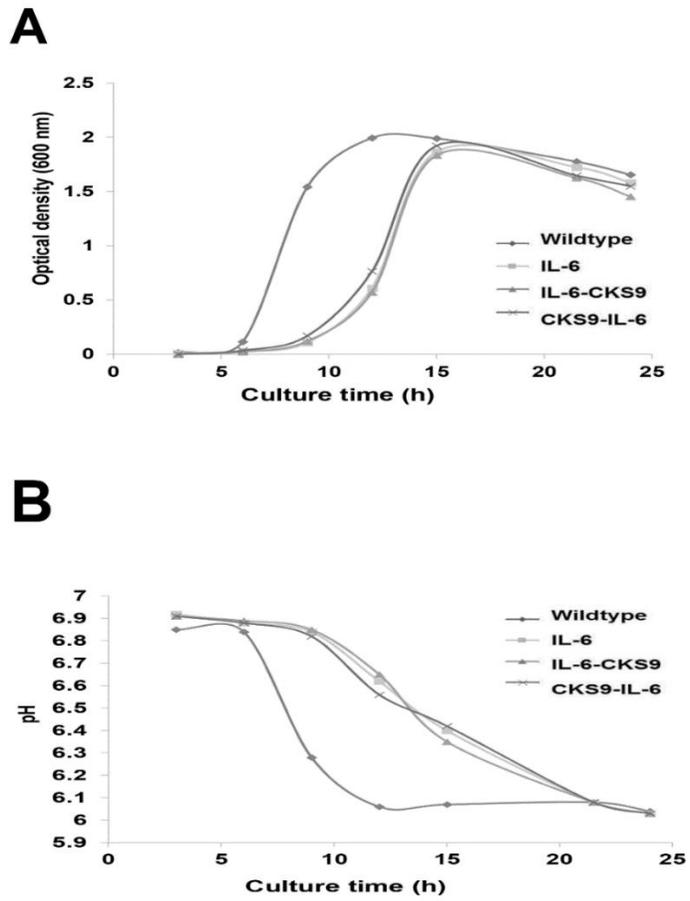
#### 1) Production of recombinant IL-6s from LAB

Each transformant *L. lactis* IL1403 harbouring pILPtuf-IL-6, pILPtuf-CKS9-IL-6, or pILPtuf-IL-6-CKS9 was cultivated separately, and production of the recombinant IL-6s was investigated. The transformant LAB showed retardation in growth [Figure 12(A)] and lactic acid production [Figure 12(B)] during cultivation compared with wild type *L. lactis* IL1403, which was assumed that the production of recombinant protein would be affected as an energy burden to normal physiology in transformant LAB. Western blot analysis [Figure 11(B)] revealed IL-6-specific bands in both cellular extract and culture supernatant fractions from all the transformant LAB. The molecular mass of recombinant IL-6 which detected from the culture supernatant fraction was consistent with control, commercially obtained murine IL-6. However, the recombinant IL-6 from cellular extract fraction showed slightly higher molecular mass compared with control representing unprocessed pre-peptide of Usp45, the protein secretion signal (Borrero et al., 2011). CKS9-IL-6 and IL-6-CKS9 also showed higher molecular masses compared with IL-6 in both intracellular and supernatant fractions due to their conjugation of M cell-targeting moiety on N-terminus or C-terminus of IL-6. Consequently, we could confirm expression and secretion of murine IL-6s as soluble forms from the *L. Lactis* IL1403 as expected. Accumulation of each secreted recombinant IL-6 in culture

supernatant also have been quantitatively monitored during cultivation with the lapse of time [Figure 11(C)]. The recombinant protein accumulation in each transformant culture generally followed growth pattern of its host [Figure 12(A)], which exponentially increased in step with log phase in host growth and reached to over 1 mg/L of culture media within 24 h. Unexpectedly, accumulation of all the recombinant IL-6s continuously increased after stationary phase in host growth. IL-6 showed much higher accumulation level in culture media compared with CKS9-IL-6 and IL-6-CKS9.



**Figure 11.** (A) Schematic diagrams for construction of recombinant IL-6s expression vector system. Ptuf: promoter originated from *tuf* gene; Usp45: protein secretion signal peptide originated from *Usp45* gene; T *pepN*: transcription terminator originated from *pepN* gene; ermAM: erythromycin resistance gene; cat: chloramphenicol resistance gene; repD: replication protein D; repE: replication protein E. (B) Confirmation of expression and secretion of IL-6s from LAB. Western blot analysis. Cellular extracts and concentrated culture supernatants from wild-type and LAB producing recombinant IL-6s were applied to SDS-PAGE, then immunoblotting. PC: Positive control, 100ng of purified commercial IL-6. (C) Accumulation profile of the secreted IL-6s in culture supernatant during cultivation of transformant LAB.

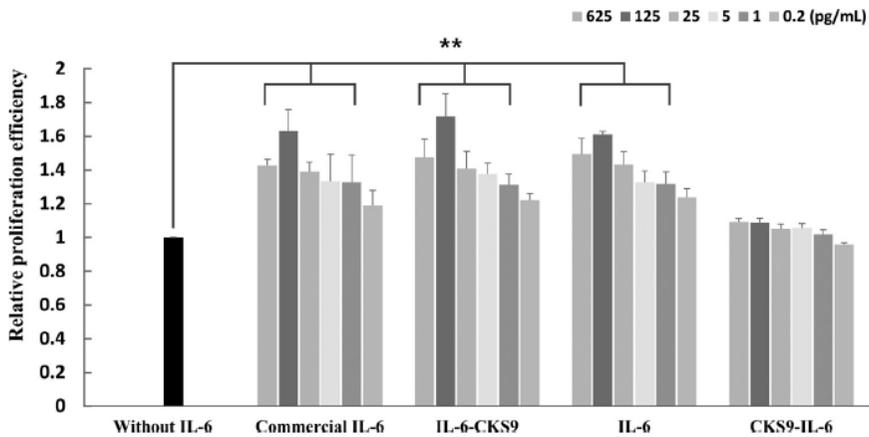


**Figure 12.** Comparison of cultivation profile between wildtype and transformant LABs.

(A) Growth profile, (B) acid production profile.

## **2) Biological activity validation of the recombinant IL-6s**

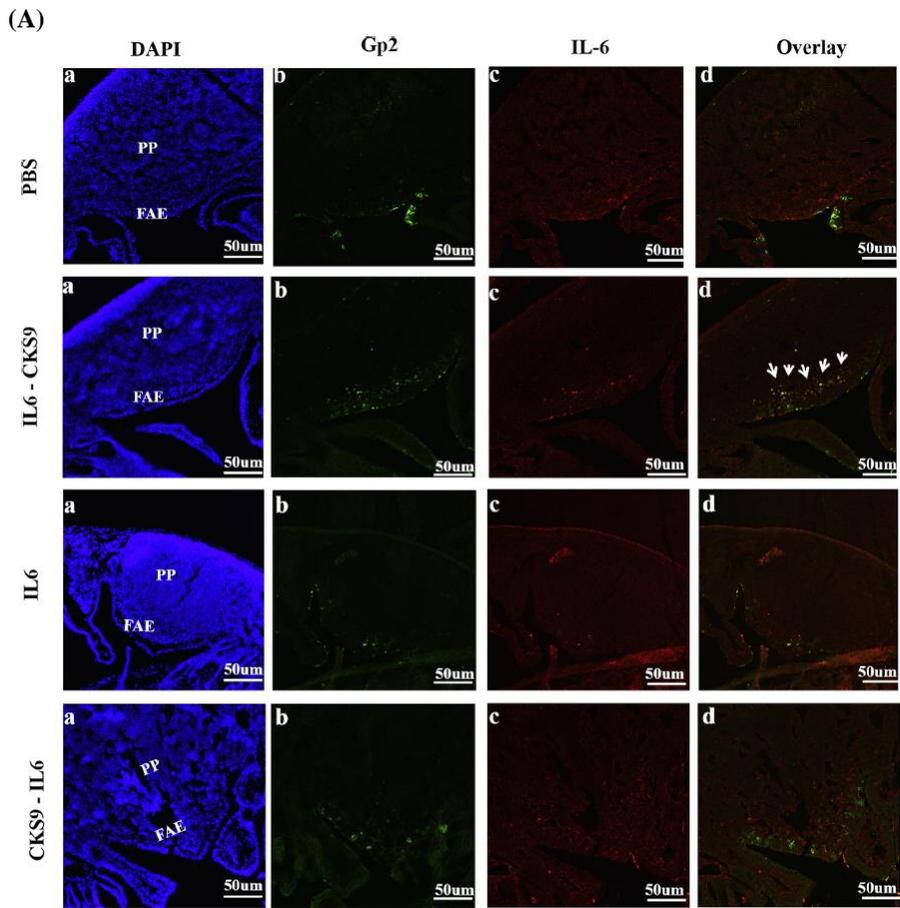
Biological activity of the recombinant IL-6s secreted from LAB were validated using 7TD1 cells having a characteristic of proliferation increment under biologically active IL-6 signals. 7TD1 cells were cultivated with different doses of commercial IL-6 or recombinant IL-6s and the proliferation efficiency of the each treatment of cells was monitored after 48 h. As shown in Figure 13, recombinant IL-6 and IL-6-CKS9 revealed coordinate biological activities with commercial IL-6 and dose dependency for IL-6s was observed on proliferation efficiency among the test groups. Only 1 pg/ml concentration of IL-6s appeared enough to increase proliferation efficiency of the 7TD1 significantly as far as we tested. Interestingly, there was no detectable bioactivity of recombinant CKS9-IL-6, in which M cell-targeting moiety was conjugated on N-terminus of IL-6.



**Figure 13.** Biological activity validation of the secreted IL-6s from LAB. The culture supernatants from LAB producing recombinant IL-6s (IL-6, IL-6-CKS9, and CKS9-IL-6) were diluted in RPMI1640 media and treated to 7TD1 cells at various concentrations. The cell proliferation of 7TD1 cells was measured using MTS assay and the result was given as relative fold compared to the cell proliferation of 7TD1 cells treated without IL-6 as control.

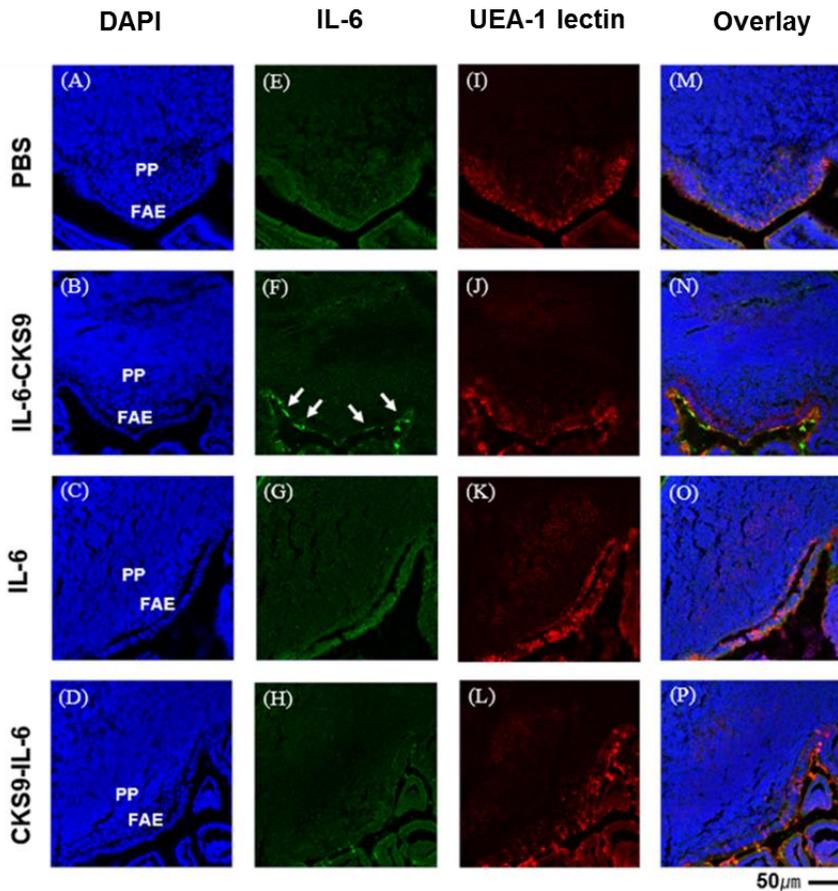
### **3) M cell-targeting ligand mediated delivery of IL-6s to Peyer's patches.**

Closed ileal loop assay and consecutive immunohistochemical analysis were performed to verify whether M cell-targeting moiety, the CKS9 peptide ligand, could facilitate specific delivery of IL-6s to Peyer's patches. After injection of recombinant IL-6, CKS9-IL-6, or IL-6-CKS9 preparation into the lumen of ileal loops, respectively, then we monitored fluorescent signals for IL-6s which would be strongly associated with M cells on Peyer's patch (PP) region (Yoo et al., 2010). A well-known M cell specific antibody, anti-Gp2-FITC, was used for localization of M cells in Peyer's patch. As shown in Figure 14, IL-6-CKS9 treated group only showed apparently higher yellow coincided fluorescent signal in Peyer's patch compared to other groups tested, indicating enhancement of M cell-targeting property of IL-6 by its C-terminal conjugation of CKS9 ligand. On the contrary, CKS9-IL-6, in spite of its N-terminal CKS9 conjugation, showed seldom positive signal for IL-6 with GP2 location in Peyer's patch.



**Figure 14 (A).** M cell-targeting ligand mediated delivery of IL-6s to Peyer's patches. The location of co-localization signals for IL-6s targeting M cells are indicated by white arrows. PP: Peyer's patches; FAE: Follicle associated epithelium.

(B)



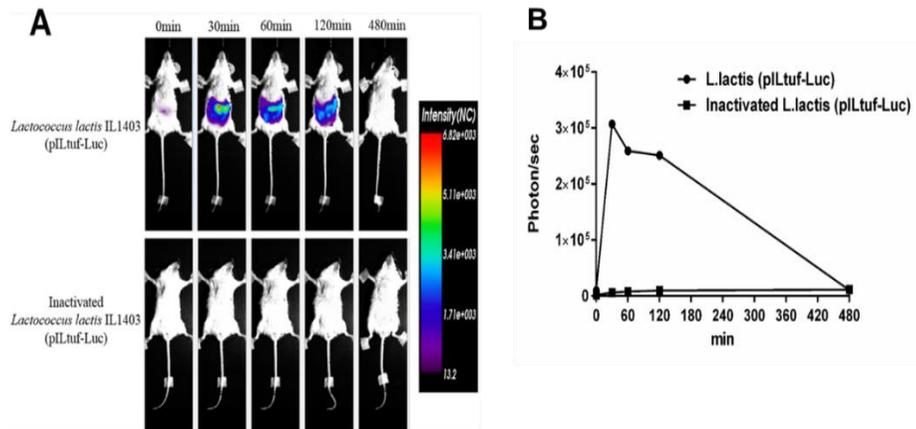
**Figure 14 (B).** M cell-targeting ligand mediated delivery of IL-6s to Peyer's patches. M cell-rich mucus layer region of the FAE on PP was labeled by Ulex Europaeus Agglutinin I (UEA-1) lectin conjugated with tetra-methyl-rhodamine isothiocyanate (red). The location of positive green signals for IL-6s targeting FAE (M cell-rich region) are indicated by white arrows. PP: Peyer's patches; FAE: Follicle associated epithelium.

#### **4) Evaluation of recombinant IL-6s-producing LAB as an adjuvant for mucosal immunity**

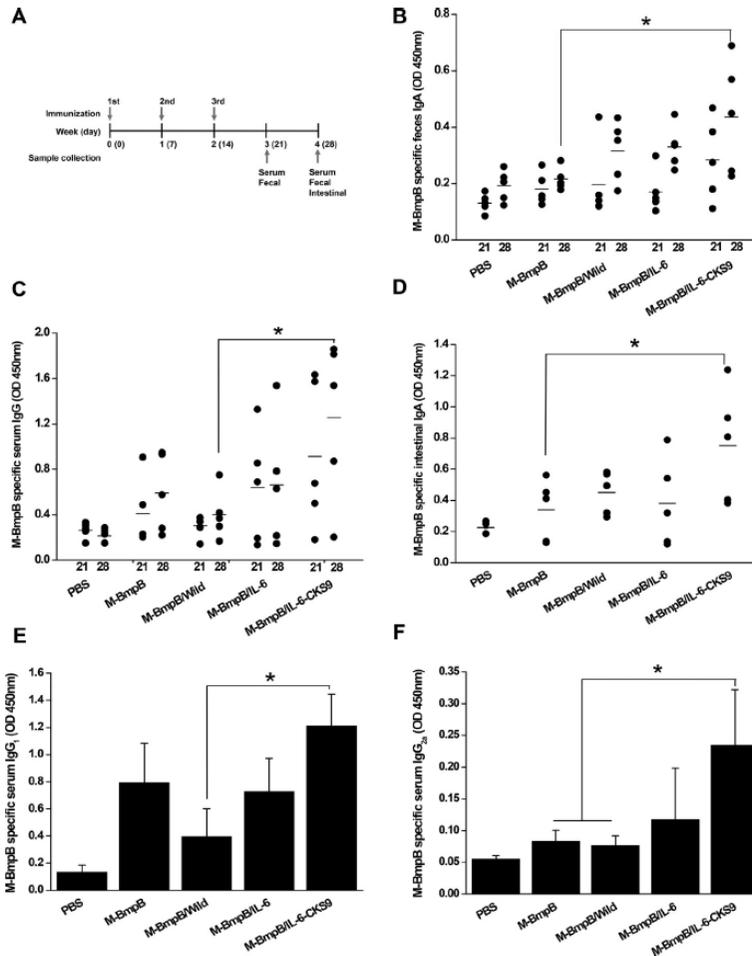
To evaluate the adjuvant effect of IL-6-producing *L. lactis* IL1403 in mucosal immunity, peroral immunization was performed with mice. The model antigen, M-BmpB, was administered alone or in combination with wildtype *L. lactis* IL1403, IL-6-secreting *L. lactis* IL1403 (IL-6-LAB), or IL-6-CKS9-secreting *L. lactis* IL1403 (IL-6-CKS9-LAB). CKS9-IL-6-LAB group was excluded from the immunization because of its low biological activity and targeting efficiency (Figure 13 and 14). At day 21 and 28 after the immunization, anti-M-BmpB-specific IgA or IgG were detected by ELISA from fecal or sera, respectively. As shown in Figure 16(B) and (C), the mice treated with IL-6-CKS9-LAB showed prominently higher tendency in anti-M-BmpB-specific antibody levels for both fecal IgA and serum IgG compared with other groups tested: M-BmpB alone, M-BmpB with wildtype LAB. Similar tendency was also observed in the anti-M-BmpB-IgA levels from intestinal extracts at day 28 [Figure 16(D)]. For the next, we analyzed immune responses in terms of antibody isotypes in sera collected from individual mice at day 28 after peroral immunization, especially with IgG1 and IgG2 which are considered as typical markers for Th2 and Th1 type immune responses, respectively. Serum IgG1 levels in the mice treated with IL-6-CKS9-LAB were significantly higher than BmpB with wildtype LAB treated group [Figure 16(E)] and, with serum IgG2

levels, IL-6-CKS9-LAB treated group showed significantly higher level than BmpB alone and BmpB with wildtype treated group [Figure 16(F)]. Unexpectedly, the statistical significances could not be obtained between IL-6-LAB and IL-6-CKS9-LAB groups in all the immunization results. However, we could observe consistent enhancements in induction of anti-M-BmpB antibody levels both with mucosal (IgA) and systemic immune responses (IgG) in IL-6-CKS9-LAB treated group compared with IL-6-LAB group by conducting two separated mice immunization assays (Figure 16 and 18).

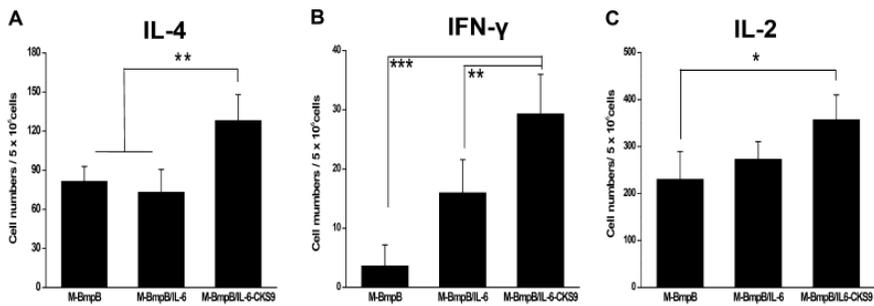
ELISPOT assays showed that lymphocytes secreted significantly higher levels of both Th1 and Th2 type of cytokines, IL-4 and IFN- $\gamma$ , respectively, responding to *in vitro* M-BmpB stimulation in IL-6-CKS9-LAB treated group compared with M-BmpB alone and IL-6-LAB groups [ Figure 17(A) and (B)]. In addition, secretion of IL-2 which is associated with T cell proliferation was also increased in in IL-6-CKS9-LAB treated group [Figure 17(C)].



**Figure 15.** Confirmation of the activity of *tuf* promoter *in vivo* after oral administration of live *Lactococcus lactis* (pILtuf-Luc). (A) Mouse was fed once with  $10^{10}$  CFU of live or heat inactivated ( $50^{\circ}\text{C}$ , 1hour) *L. lactis* (pILtuf-Luc) and 3mg of D-Luciferin substrate dissolve in 200ul saline was injected intragastrically at each time point and the bioluminescent signals were imaged immediately using Optix MX3 imaging system. The related photon signals in body of whole animals at each time point are plotted in (B).



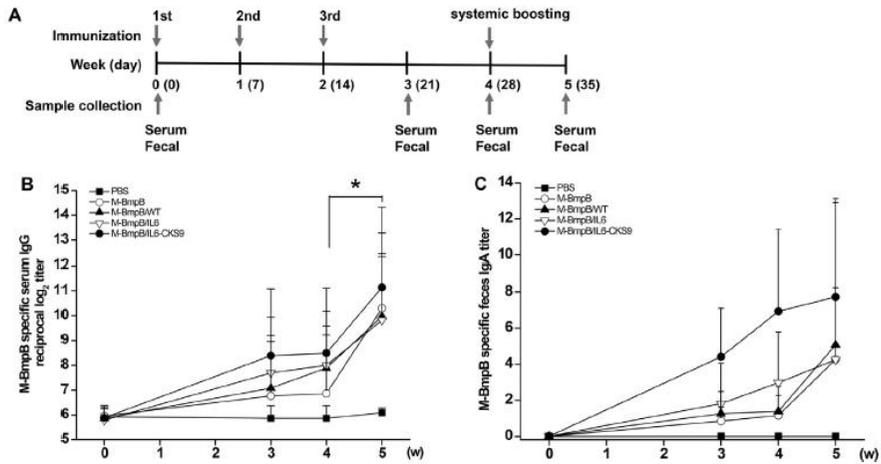
**Figure 16.** *In vivo* validation of LAB producing recombinant IL-6s as oral adjuvants by peroral immunization in mice. (A) Schematic view of immunization and sampling schedule. (B~D) M-BmpB-specific fecal IgA and serum IgG titers at day 21 and 28, intestinal IgA titer at day 28. Each dot denotes anti-M-BmpB antibody titer value from individual mouse and the bar represents average mean (n=5 in each group tested). (E, F) Isotype analysis for Immunoglobulin G (IgG). M-BmpB-specific serum IgG1 (E) and serum IgG2a (F) titer at day 28 after immunization. (n=3 in each group tested, error bar represents standard deviation, \*: p < 0.1, one-way ANOVA).



**Figure 17.** IL-4, IL-2 and IFN- $\lambda$  levels in the peyer's patch of mice after peroral immunization. *In vitro* cytokine induction assay. Lymphocytes secreting IL-4 (A), IFN- $\lambda$  (B), and IL-2 (C) in PPs were measured by ELISPOT assay. (n=3 in each group tested, error bar represents standard deviation, \*: p < 0.1, \*\*: p < 0.05, \*\*\*: p < 0.01, one-way ANOVA).

## **5) Investigation of oral tolerance induction**

A critical point to be cleared before securing the use of an oral vaccine or adjuvant is to insure that the orally-administered antigen does not induce systemic tolerance. In order to determine whether the oral administration of M-BmpB with IL-6-CKS9-LAB adjuvant would induce tolerogenic immune responses, we monitored M-BmpB specific IgG and IgA levels after systemic challenge with M-BmpB to mice which orally immunized with M-BmpB or corresponding IL-6s secreting LABs (Figure 18). As results, the orally immunized group treated with M-BmpB and IL-6-CKS9-LAB adjuvant displayed increased anti-BmpB serum IgG [Figure 18(B)] and fecal IgA [Figure 18(C)] titers after systemic challenge with M-BmpB antigen.



**Figure 18.** Investigation of a tolerogenic immune response against M-BmpB in systemic and mucosal compartments after systemic boost immunization in mice orally immunized with each indicated antigen. (A) Schematic view of oral immunization and sampling schedule, systemic challenging and sampling schedule. (B, C) Serum IgG and fecal IgA was monitored at day 0, 21, 28 and 35. (n=5 in each group tested, error bar represents standard deviation, \*:  $p < 0.1$ , \*\*:  $p < 0.05$ , one-way ANOVA).

#### **4. Discussion**

Protection against orally transmitted infections requires immunity at the site of pathogen entry (Wegmann et al., 2012). However, the induction mechanism for mucosal immune response is different from systemic immune response, thus exploration of effective way to induce mucosal immune response and development of efficient mucosal vaccine system is an important issue (Pavot et al., 2012). Recently, various researches for development of mucosal adjuvants are ongoing to facilitate mucosal vaccination. LT and CT are known to be the most effective mucosal adjuvants which were validated in mice researches in spite of their high cytotoxicity (Da Hora et al., 2011; Fujihashi et al., 2002). Toll like receptor agonists, such as CpG ligands (Fukuyama et al., 2011; Heikenwalder et al., 2004) or cytokines, such as BAFF (TNF- $\alpha$  family) (Tertilt et al., 2009) and IL-6 (Steidler et al., 1998) might be potentially effective mucosal adjuvants. However, low absorption efficiency of such adjuvants into immune system across the mucosal barrier is still challenging problem, especially in peroral vaccination.

The present study examined whether the introduction of an M cell-targeting peptide ligand, CKS9, to a cytokine, IL-6, could facilitate mucosal adjuvant function of the cytokine by increasing its accessibility to immune system across the intestinal mucosal barrier, then effectively induce antigen-specific secretory antibody, IgA, after peroral immunization in mice model. As referred in

‘Introduction’, we expected that orally administered IL-6-producing LAB, especially with M cell-targeting moiety, would be effective to induce mucosal immune response from gastrointestinal tract in peroral vaccination. An edible lactic acid bacteria, *L. lactis* IL1403, served as a live vehicle for the production of the recombinant IL-6 adjuvants and one of the most well-known protein secretion signals in LAB, pre-peptide of Usp45, was employed for the cytokine secretion system in this study (Figure 11). In spite of physiological delay in growth compared with wildtype (Figure 12), all the transformant LAB showed successful expression and secretion of recombinant IL-6s as soluble forms [Figure 11(B)] and secreted IL-6s were accumulated in milligram scale per liter of culture media within 24 h during cultivation. Continuous increment of the target protein accumulation after stationary growth phase of the host LAB was inferred as relatively long half-life of the recombinant IL-6s and considered as a beneficial feature for oral adjuvant [Figure 11(C)].

All the recombinant IL-6s revealed equivalent functional activity with commercial IL-6 except CKS9-IL-6 in which M cell-targeting moiety conjugated with N-terminus of the IL-6 (Figure 13). IL-6 is known as a ‘four-alpha-helix-bundle’ family cytokine and 15~22 of N-terminus amino acid residues forming the first alpha-helix has been identified to be crucial in biological function of this type of cytokines, thus N-terminal modification often causes its loss of function (Simpson et al., 1997). We inferred that N-terminal

introduction of the CKS9 peptide to IL-6 might adversely affect to structural integrity to maintain its functional activity. It is considered as another beneficial feature for oral adjuvant that IL-6 could be functional even at pictogram scale of very low concentration (Figure 13).

Immunohistochemical assays revealed that the IL-6-CKS9, in which M cell-targeting moiety conjugated with C-terminus of the IL-6, only possessed M cell-targeting efficacy among the recombinant IL-6s produced from the transformant LAB (Figure 14). As described previously, M cells are the specialized cell type located on the FAE on Peyer's patches in intestinal epithelia and have a role collecting and delivering gut lumen antigen to mucosal immune system. Thus, M cell-targeting property could be also beneficial for oral adjuvant to facilitate efficient induction of mucosal immune response. We ascertained that the N-terminal introduction of the CKS9 to IL-6 caused adverse effects not only in cytokine function but also in M cell-targeting property.

The efficacy of the LAB producing recombinant IL-6s as oral adjuvants was validated by peroral vaccination with mice using M-BmpB as a model antigen. Especially, only immunized with M-BmpB/IL-6-CKS9-LAB group appears significant difference in serum IgG and fecal/intestinal IgA levels compared with those immunized with M-BmpB alone or M-BmpB/wildtype-LAB groups, however M-BmpB/IL-6-LAB groups showed no significant difference compared with them. Although the statistical significances could not be

obtained between IL-6-LAB and IL-6-CKS9-LAB groups because of individual differences in sympathy responding to immunization among mice, a consistent enhancement was observed in induction of anti-M-BmpB antibody levels both with mucosal (IgA) [Figure 16(B) and (D)] and systemic immune responses (IgG) [Figure 16(C)] in IL-6-CKS9-LAB treated group compared with IL-6-LAB group. The similar higher induction of anti-M-BmpB antibody in IL-6-CKS9-LAB group was reappeared in succeeding mice immunization for tolerance validation [Figure 18(B) and (C)]. The higher tendency in anti-M-BmpB induction than IL-6-LAB group was inferred that the introduction of CKS9 to IL-6 could facilitate its delivery to mucosal immune system across the intestinal barrier especially through the M cells that could induce higher immune response than IL-6 which was without M cell targeting efficiency. Large scale of *in vivo* immunization assay to minimize individual variances among animals would be needed to obtain more definite efficacy of the CKS9 peptide ligand for targeted delivery to M cells in further study. Besides, the group treated M-BmpB with wildtype LAB as an adjuvant showed higher IgA induction tendency compared with the group treated with M-BmpB only, which suggested a synergistic advantage of LAB as a live vehicle for the cytokine adjuvant [Figure 16(B) and (D)].

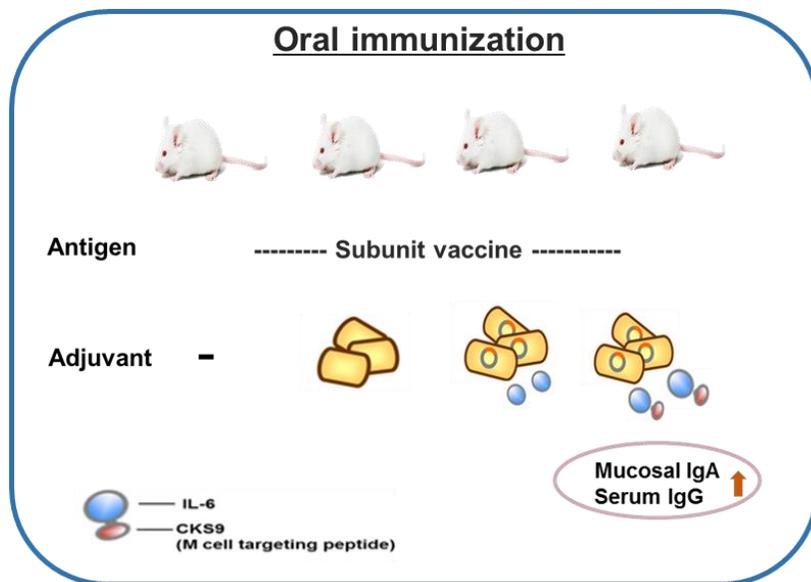
Furthermore, we found that the treatment of IL-6-CKS9-LAB as an oral adjuvant could induce both Th1 and Th2 type immune responses

simultaneously [Figure 16(E) and (F)]. One of the general drawbacks of subunit vaccine is that it could only induce weak Th2 type, humoral immune response, rather than Th1 type, cellular immune response. For the effective defense not only to exogenous pathogen such as bacteria but also to endogenous pathogen such as virus, and in the aspect of immune homeostasis, balanced induction of Th1 and Th2 type immune responses would be important property for vaccine adjuvant (Kidd, 2003). Additionally, we ascertained again that treatment of IL-6-CKS9-LAB could induce both Th1 and Th2 type cytokines, IL-4 and IFN- $\gamma$ , respectively, significantly higher than control groups from the lymphocytes [Figure 17(A) and (B)]. Biological function of IL-6-CKS9-LAB as a cytokine adjuvant was also confirmed by detecting enhancement of the IL-2 induction, which is one of the crucial cytokines associated with proliferation of lymphocytes [Figure 17(C)].

Finally, we investigated oral tolerogenic response for our vaccine strategy, which is one of the most important issues in oral vaccine research. The characteristics of the IL-6-CKS9-LAB-adjuvanted immune responses, both with systemic IgG [Figure 18(B)] and mucosal IgA [Figure 18(C)], were maintained after systemic challenge by M-BmpB antigen and, consequently, represent the non-tolerance response against M-BmpB antigen.

## 5. Conclusion

We designed a novel cytokine conjugated with M cell-targeting moiety (IL-6-CKS9) could be applied as an oral adjuvant and our results are firstly validated that M cell-targeting strategy for IL-6 could enhance induction of antigen-specific antibody in both mucosal and systemic immune response after peroral vaccination. Our results also suggest that cytokine-producing lactic acid bacteria would have a great potential as an effective and convenient oral adjuvant for development of efficient oral vaccine system.



**Figure 19.** Schematic illustration for the result of the study 1

# **Chapter 3. Nasal immunization with mannan-decorated mucoadhesive HPMCP microspheres containing ApxIIA toxin induces protective immunity against challenge infection with *Actinobacillus pleuropneumoniae* in mice**

## **1. Introduction**

The nasal route holds great promise for vaccine delivery because of the organization of the respiratory lymphoid tissue (Lycke, 2012). The respiratory mucosa is the first site of contact for inhaled antigens and the nasal associated lymphoid tissue (NALT) and inducible bronchus-associated lymphoid tissue (iBALT) at the base of the nasal cavity and large airway are very important in the defense of mucosal surfaces (Tacchi et al., 2014). In addition, the intranasal administration of an antigen not only induces systemic immune responses but also induces mucosal responses (Vicente et al., 2013). However, the administration of subunit vaccines alone seldom leads to a protective antibody response because the residence time of soluble antigens in the respiratory mucosa is limited, which results in very small doses of antigen reaching the

antigen-presenting cells of the sub-epithelial region (Bento et al., 2015). Moreover, subunit vaccines are often poorly immunogenic as they lack the necessary “danger signals” to activate dendritic cells (DC) and subsequently, T cells (Nochi et al., 2010).

The encapsulation of the antigen into polymeric particles has been studied as a promising approach to improve the transport of antigens across the respiratory mucosa (Rajapaksa and Lo, 2010). In addition, the increased residence time of particulate delivery systems at the mucosal surface may facilitate the increased uptake of such agents. To this end, mucoadhesive polymers provide a strategy that can help increase residence time and hence the uptake of particulate vaccines when administered by the nasal or pulmonary routes (Pawar et al., 2013). Recently, a new generation of synthetic polymers known as thiomers or thiolated polymers have been shown to have strong mucoadhesive properties due to their binding with the cysteine-rich subdomains of mucus glycoprotein (Bernkop-Schnürch et al., 2001; Bernkop-Schnürch et al., 2004). In this work, thiolated HPMCP (TH), previously verified as having mucoadhesive properties (Singh et al., 2015), was used as a nasal subunit vaccine carrier.

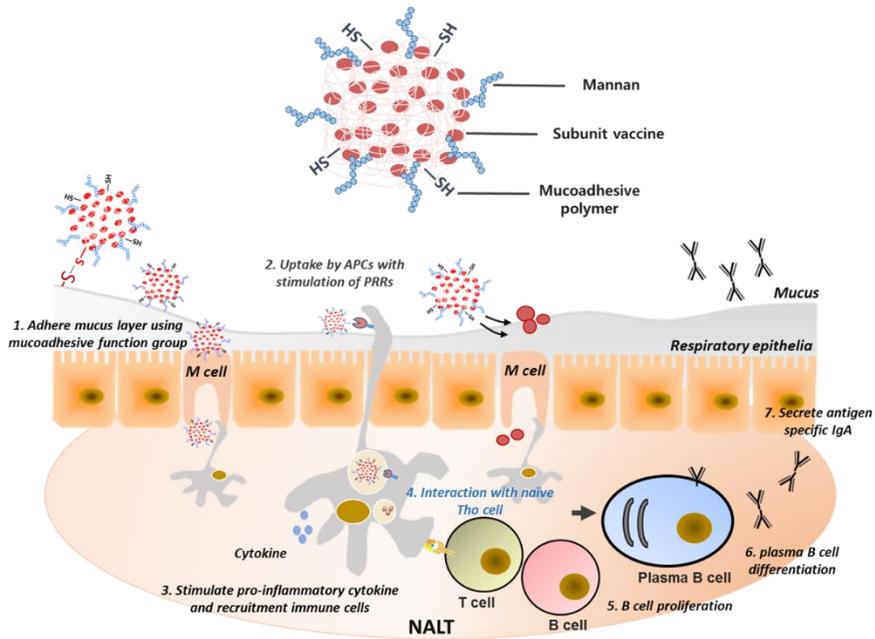
Another major hurdle in the development of intranasal vaccines is poor immunogenicity of vaccine subunits due to the lack of “danger signals” that can activate APCs. The development of effective targeted particulate vaccine formulations combined with strong immunomodulation should improve the

efficacy of non-living subcomponent mucosal vaccines (Carapau et al., 2013). Mannan isolated from the cell wall of *Saccharomyces cerevisiae* is one of the natural PAMPs (pathogen associated molecular patterns) of APCs and is a ligand of the mannose receptor (MR) widely expressed on APCs (Jiang et al., 2008; van de Veerdonk et al., 2009). Mannan can induce the production of pro-inflammatory cytokines and up-regulate the expression of co-stimulatory molecules in these cells, thereby activating both innate and adaptive immunity (Stambas et al., 2002a; Stambas et al., 2002b). Moreover, targeting antigens to the endocytic receptors of APCs is an attractive strategy for enhancing the efficacy of vaccines (Jiang et al., 2009). It has been demonstrated that using mannan to target the delivery of antigens to the MR effectively induces cellular and humoral immune responses (Chavez-Santoscoy et al., 2012; Cruz et al., 2012; Jones et al., 2015).

*Actinobacillus pleuropneumoniae* is known to cause contagious porcine pleuropneumoniae. Among the many virulence factors of *A. pleuropneumoniae*, Apx toxins, which act to create pores in the host cell membrane, are known to be substantially involved in the pathogenesis of pleuropneumoniae (Shin et al., 2005). The importance of Apx toxins as vaccine candidates has been demonstrated in many studies. In particular, ApxII toxin is a promising vaccine candidate because nearly all serotypes of *A. pleuropneumoniae* (with the exception of serotypes 10 and 14) express ApxII antigen (Seo et al., 2013). In

order to develop an efficient intranasal vaccine against *A. pleuropneumoniae* in the swine farm industry, ApxIIA, which has previously been confirmed to possess immunogenicity in swine, was adapted as a model antigen in our study (Seo et al., 2013).

The aim of the present work was to explore the potential of a novel Man-THM vehicle for the intranasal administration of ApxIIA as a vaccine against *A. pleuropneumoniae*. For this purpose, Man-THM was prepared by the double emulsion method and the surface of the microsphere was decorated with mannan. After optimization of the preparation method and characterization of the obtained ApxIIA-loaded Man-THM, the physiochemical properties of the Man-THM as a nasal delivery carrier were evaluated by *in vitro* and *in vivo* assays. After intranasal immunization, the immune adjuvant ability of the formulation of mannan-decorated THM with ApxIIA vaccine was investigated by measuring the systemic and secretory antibodies in the serum and mucosal secretions. Furthermore, assays were performed after *A. pleuropneumoniae* serotype 5 challenge in mice immunized with the ApxIIA-loaded Man-THM.



▪ **Experimental flow chart**

<b>Characterization</b>	<ul style="list-style-type: none"> <li>▪ Synthesis of thiolated HPMCP (TH)</li> <li>▪ Preparation of THM, Man-THM</li> <li>▪ Physicochemical characterization of microspheres (size, morphology)</li> </ul>
<b>In vitro</b>	<ul style="list-style-type: none"> <li>▪ Confirm decoration of mannan in Man-THM</li> <li>▪ <i>In vitro</i> uptake study with APCs</li> </ul>
<b>In vivo</b>	<ul style="list-style-type: none"> <li>▪ Evaluation of Man-THM as a nasal vaccine carrier</li> <li>▪ Challenging assay</li> </ul>

**Figure 20.** Experimental flow of the study 2

## **2. Materials and methods**

### **1) Materials**

HPMCP was kindly provided by Shin-Etsu Chemical Co., Ltd. (Tokyo, Japan). Dimethyl sulfoxide (DMSO), *N,N'*-dicyclohexylcarbodiimide (DCC), *N*-hydroxysuccinimide (NHS), L-cysteine hydrochloride monohydrate, poly(vinyl alcohol) (PVA), Pluronic® F-127, dichloromethane, 4',6-diamidino-2-phenylindole dilactate (DAPI), carbonate-bicarbonate buffer capsule, fluorescein isothiocyanate (FITC), and mannan derived from *Saccharomyces cerevisiae* were purchased from Sigma-Aldrich (St. Louis, MO, USA). RPMI 1640 media, DMEM media, and FBS were purchased from Gibco (Waltham, MA, USA). Bicinchoninic acid (BCA) protein assay reagents (A and B) were purchased from Thermo Scientific Pierce (Rockford, IL, USA). Horseradish peroxidase (HRP)-conjugated goat anti-mouse IgA, IgG, IgG1, and IgG2a antibody were purchased from Santa Cruz Biotechnology (Dallas, TX, USA). All other materials were of analytical reagent grade.

### **2) Cell lines and experimental animals**

RAW 264.7 murine macrophage and Jaws II murine dendritic cell lines were obtained from American Type Culture Collection (Manassas, VA, USA). 6 week-old BALB/c female mice (Samtako Inc, Korea) were used in the immunization experiments and all experimental procedures were approved by

the Institutional Animal Care and Use Committee of Seoul National University (Approval Number: SNU 140811-2) and followed the guidelines suggested by the committee.

### **3) Synthesis of thiolated HPMCP (TH)**

TH was synthesized according to previously reported methods (Quan et al., 2008). Briefly, 4 g of HPMCP was dissolved in 100 ml DMSO and the carboxylic acid moieties of the polymer were activated by DCC (9 g) and NHS (5 g) with constant stirring at room temperature for 24 h under nitrogenous condition. The dicyclohexylurea as a by-product was removed by filtration and the filtrate was further reacted with L-cysteine hydrochloride (0.4 g) for 48 h under similar conditions. The reaction mixture was filtered to remove by-products and the filtrate was dialyzed initially against DMSO, and then against distilled water to remove the unbound L-cysteine. Finally, the product was lyophilized and stored at -20 °C until use. The conjugation of L-cysteine was confirmed by <sup>1</sup>H NMR spectroscopy (Avance 600, Bruker, Germany).

### **4) Preparation of ApxIIA-loaded THM and ApxIIA-loaded Man-THM**

#### **(1) Isolation and purification of ApxIIA**

Recombinant ApxIIA was produced using an *E. coli* expression system, pQE (Qiagen GmbH, Hilden, Germany) and ApxIIA was induced and purified according to the methods described in a previous study (Shin et al., 2011).

Briefly, when the culture reached an optical density of 0.6 nm (OD 0.6 nm), isopropyl  $\beta$ -D-1-thiogalactopyranoside (IPTG, 1 mM) was added and then cultured continuously for 4 h. The harvested cells were re-suspended in lysis buffer (20 mM Tris–hydrogen chloride, 500 mM sodium chloride, 8 M urea, 40 mM imidazole, pH 7.0) and nickel–nitrilotriacetic acid (Ni-NTA) chelate affinity chromatography was then performed according to the manufacturer’s instruments. The bound protein was eluted with elution buffer (20 mM Tris–hydrogen chloride, 500 mM sodium chloride, 8 M urea, 500 mM imidazole, pH 7.0).

## (2) Preparation of ApxIIA-loaded THM and ApxIIA-loaded Man-THM

20 mg/ml ApxIIA was stabilized with Pluronic F-127 solution (1 wt.-%) to form an internal aqueous phase ( $W_1$ ). 0.2 ml of  $W_1$  (4 mg ApxIIA) was emulsified with an organic phase (O) consisting of 100 mg of TH dissolved in 5 ml of dichloromethane (DCM) using an ultrasonic processor (Sonics, Vibra cells™) (2 output watts) for 1 min to form a primary emulsion ( $W_1/O$ ). The prepared primary emulsion ( $W_1/O$ ) was added drop by drop to 50 ml of 1 wt.-% PVA solution ( $W_2$ ) to create an external aqueous phase or was added to a mixture of 0.25 wt.-% PVA and 0.5 wt.-% mannan for Man-THM. A homogenizer (Ultra-Turrax™ HomogenizerT25, IKA) was then used at 11,000 rpm for 4 min to form a  $W_1/O/W_2$  emulsion, which was then stirred with a magnetic stirrer for 2–3 h at room temperature to allow the solvent to evaporate. ApxIIA-loaded

THM and ApxIIA-loaded Man-THM were collected and washed 3 times with distilled water after centrifugation at 14,000 rpm for 10 min at 4 °C. The microspheres were lyophilized and finally stored at -70 °C for later characterization. The unloaded THM and Man-THM were prepared with similar methods.

## **5) Characterization of the microspheres**

### **(1) Size and surface morphology**

The particle size distribution of the microspheres was measured using an electrophoretic light scattering spectrophotometer (ELS-8000, Otsuka Electronics Ltd., Osaka, Japan) with an argon laser beam at 488 nm and 25 °C. The microspheres were placed on a stud and were gold-coated using Sputter Coater (BAL-TEC/SCD 005) and observed using a field emission scanning electron microscopy (AURIGA, Jena, Germany).

### **(2) Confirmation and quantification of mannan decoration in the THM**

To confirm mannan-coating in the Man-THM, FITC was conjugated with mannan. Briefly, 100 mg of mannan dissolved in 1 ml of DW was slowly mixed with 5 mg of FITC dissolved in 1 ml of DMSO. After stirring for 4 h at room temperature in dark conditions, the reaction product was dropped into 8 ml of

ethanol to remove the unreacted FITC. The mannan-FITC conjugate that precipitated was washed with ethanol and collected by centrifugation at 14,000 rpm 3 times for 10 min. FITC-mannan-decorated THM was visualized by confocal laser microscope (SP8 X STED, Leica, Wetzlar, Germany).

To quantify the FITC-mannan decoration in the THM, FITC-mannan was extracted by dissolving FITC-Man-THM in the DMSO. The fluorescence of extracted FITC-mannan from FITC-Man-THM was quantified using FITC-mannan standard curve.

## **6) *In vitro* studies**

(1) *In vitro* release of APXIIA from APXIIA-loaded THM and APXIIA-loaded Man-THM

10 mg of APXIIA-loaded THM and APXIIA-loaded Man-THM suspended in 1 ml of PBS (pH 7.4) in 1.5 ml microtubes were agitated for up to 24 h at 37 °C and 100 rpm using a shaking incubator. A 100 µl aliquot was withdrawn from the release medium at each time point and the amount of released APXIIA was determined as the cumulative release (wt. -%) against incubation time by the Micro BCA protein assay method.

(2) Uptake of microspheres by the APCs

For the uptake of the microspheres, RAW 264.7 and JAWS II cells were

seeded into 6-well plates (Costar, IL, USA) at  $1 \times 10^6$  cells/well. After the cells reached 80% confluence, the medium was changed with 3 mg/ml of mannan for 20 min before the addition of microspheres to block MR. Normal or MR-blocked cells were changed with the media with a suspension of OVA-FITC-loaded THM and OVA-FITC-loaded Man-THM at a microsphere concentration of 0.2 mg/ml for 1 and 2 h. After incubation, the microsphere suspension was removed and the wells were washed three times with 1 ml of PBS to remove the traces of microspheres left in the wells. The cells were then detached from the culture dishes and flow cytometry analysis was carried out to determine the uptake of microspheres by the cells.

### (3) Internalization of microspheres

The phagocytosis of microspheres by the macrophages and DCs was confirmed by confocal laser microscope (SP8 X STED, Leica).  $1 \times 10^6$  cells per well were cultured in 35 mm cover glass bottom dishes (SPL Life Science, Korea) two days prior to feeding with FITC-OVA-loaded microspheres at a concentration of 0.2 mg/ml. After incubation at 37 °C for 2 h, non-phagocytized microspheres were removed by washing three times with PBS and fixed with 4% paraformaldehyde at room temperature for 10 min. The nucleus was stained by Hoechst 33342 nucleic acid stain (Invitrogen, MA, USA).

### **7) *In vivo* imaging of the intranasal administration of the microspheres**

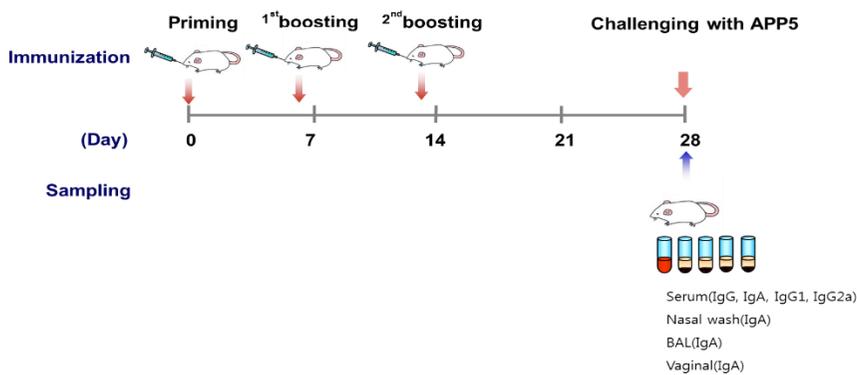
For the *in vivo* fluorescence study, cy5.5-conjugated OVA was used. Cy5.5-conjugated OVA and the same amount of cy5.5-conjugated OVA-loaded THM and MAN-THM were intranasally administrated to 6 week-old BALB/c mice. The fluorescence was monitored at 10 min, 30 min, 60 min, 120 min, 180 min, and 240 min. The fluorescence images were acquired under an excitation of 695 nm using the Optix-MX3 (Advanced Research Technologies, Canada). The fluorescence intensities in the regions of interest (ROI) were analyzed from the fluorescence images.

### **8) *In vivo* immunization studies**

#### **(1) Immunization of mice**

The nasal immunization study was conducted with 6 week-old female BALB/c mice assigned to five groups (n=5). The nasal administration groups were immunized 3 times on days 0, 7, and 14 by dropping 15  $\mu$ l of PBS containing APXIIA (5  $\mu$ g)- or APXIIA (5  $\mu$ g)-loaded THM, or APXIIA (5  $\mu$ g)-loaded Man-THM into the nostrils during inhalation under anesthesia. Mice receiving intraperitoneal administrations (i.p.) were immunized at day 0 with 5  $\mu$ g of APXIIA emulsified with complete Freund's adjuvant, and subsequently received booster immunizations twice at days 7 and 14 with the same amount of antigen emulsified with incomplete Freund's adjuvant. The

serum, nasal wash, bronchoalveolar lavage (BAL), and vaginal wash collections were performed on day 28 (Figure 21). Briefly, blood samples were collected from the facial vein and centrifuged after clotting at 2,000 rpm for 10 min for sera preparation. BAL samples were harvested from the lower respiratory tract of the mice as described previously (Mizuno et al., 2006). Briefly, after the bronchi were exposed from the neck, a truncated needle with a syringe containing 400  $\mu$ l PBS was inserted through a small pore on each bronchus. Mucus exudation was harvested with a back-and-forth motion of the injector, and the supernatants were collected by centrifugation (3,000 rpm and 10 min at 4 °C). The nasal washes were collected by 300  $\mu$ l PBS through the trachea toward the nose. For vaginal wash preparation, the vagina was washed 15 times with 150  $\mu$ l of PBS and the obtained fluids were collected by centrifugation (14,000 rpm, 10 min at 4 °C). All of the samples were stored at -70 °C until analysis.



**Figure 21.** Schematic representation of immunization schedule

## (2) Measurement of APXIIA-specific antibodies

The levels of APXIIA-specific IgG, IgA, IgG1, and IgG2a antibodies in serum, as well as sIgA antibodies in nasal wash, BAL, and vaginal wash were determined by ELISA, as described previously (Li et al., 2015). Briefly, 96-well plates (Thermo, USA) were coated with 0.1 µg of APXIIA in 100 µl of 50 mM carbonate-bicarbonate buffer (pH 9.6) at 4 °C overnight and blocked with 1 wt.-% BSA at 37 °C for 1 h. Then, serially diluted serum, and appropriately diluted nasal wash, BAL, and vaginal wash were added into the wells and incubated at 37 °C for 1 h. After incubation, HRP (horse radish peroxidase)-conjugated goat anti-mouse IgA, IgG, IgG1, or IgG2a were added to the designated wells at 37 °C for 1 h. Appropriate washing of each well was performed between each step with PBS containing 0.05% of Tween 20. TMB solution (Sigma-Aldrich, USA) was added to the wells for the HRP substrate, the reaction was stopped after 10 min by adding 2 M H<sub>2</sub>SO<sub>4</sub>, and absorbance was measured at 450 nm using a microplate reader (Infinite® 200 PRO, USA). For serum IgG, IgG1, IgG2a and IgA the ELISA results are expressed as the endpoint titer and for BAL, nasal wash, and vaginal IgA, the results are expressed as the OD value measured at 450 nm (dilutions of 1:500 for BAL IgA, 1:10 for nasal wash IgA, 1:100 for vaginal IgA).

## 9) Bacterial challenge

(1) Growth of *A. pleuropneumoniae* serotype 5 (APP5) and intranasal challenge in mice

The *A. pleuropneumoniae* strains used for this study were serotype 5, Korean isolates. The bacteria were cultivated at 37 °C in chocolate agar and broth with  $\beta$ -NAD (10  $\mu$ g/ml). The minimum lethal dose was determined after challenge infection with APP5 in the nasal cavity of the mice. APP5 was grown until the optical density of 0.6 nm (OD 0.6 nm) was 0.5, reaching a concentration of  $5 \times 10^7$  colony-forming units (CFUs) per ml. The bacteria were then harvested through centrifugation at 8,000 rpm and washed with PBS. The pellets were re-suspended with PBS into various dilutions, and the bacteria were introduced into the snout of the mice anesthetized by Zoletil (Virbac Laboratories, Carros, France) and Rompun (Bayer Korea, Ansan, Korea). For the intranasal challenge of immunized mice, a MLD (minimum lethal dose) of  $5 \times 10^7$  CFU/mouse was used for APP5. Challenged mice were observed for 4 days.

(2) Calculation of bacteria from the lung tissue

After challenging the immunized mice with APP5, the lung tissue was surgically removed just before the death of the challenged mice. The lungs were fragmented into small pieces and then ground with a homogenizer. The

tissue mixture was spread on BHI agar plates with  $\beta$ -NAD (10  $\mu$ g/ml) after serial 10-fold dilutions with PBS. The plates were incubated at 37 °C overnight and the number of colonies was counted.

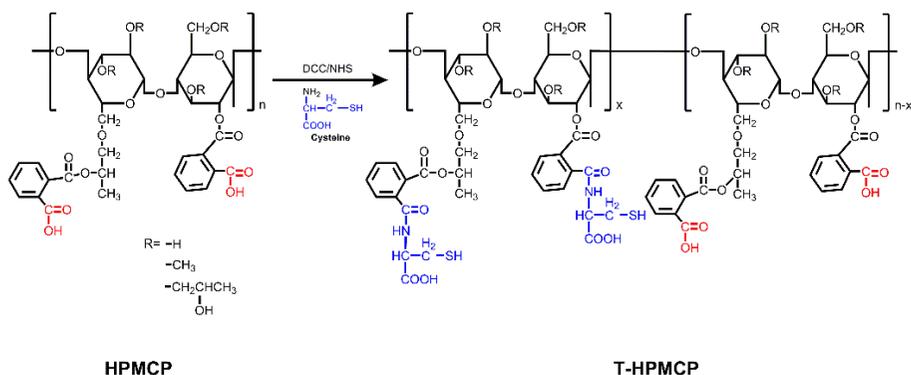
#### **10) Statistical analysis**

Quantified results were expressed as the mean and standard deviation (SD). Statistical significance was assessed using a one-way analysis of variance (ANOVA) with the post-hoc Tukey multiple comparisons test.

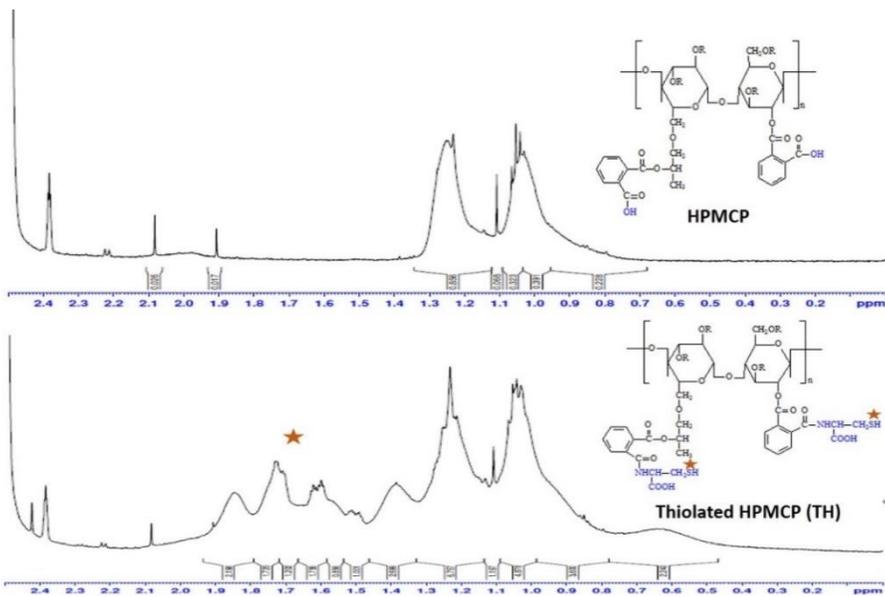
### 3. Results and Discussion

#### 1) Preparation of TH, THM, Man-THM, ApxIIA-loaded THM, and ApxIIA-loaded Man-THM

TH was synthesized by amide bond formation between the carboxylic acid groups of HPMCP and the primary amino groups of the cysteine using a DCC/NHS-activated coupling reaction, as shown in Figure 22. The coupling of cysteine with HPMCP was confirmed by <sup>1</sup>H-NMR by observing the appearance of the thiol proton resonance peak at 1.6 ppm (Figure 23). It was found that approximately 11.4 mol.-% of cysteine was conjugated with HPMCP. THM, Man-THM, ApxIIA-loaded THM, and ApxIIA-loaded Man-THM were prepared using the double emulsion method, as described in the Materials and Methods.



**Figure 22.** The reaction scheme for the synthesis of thiolated HPMCP (TH)



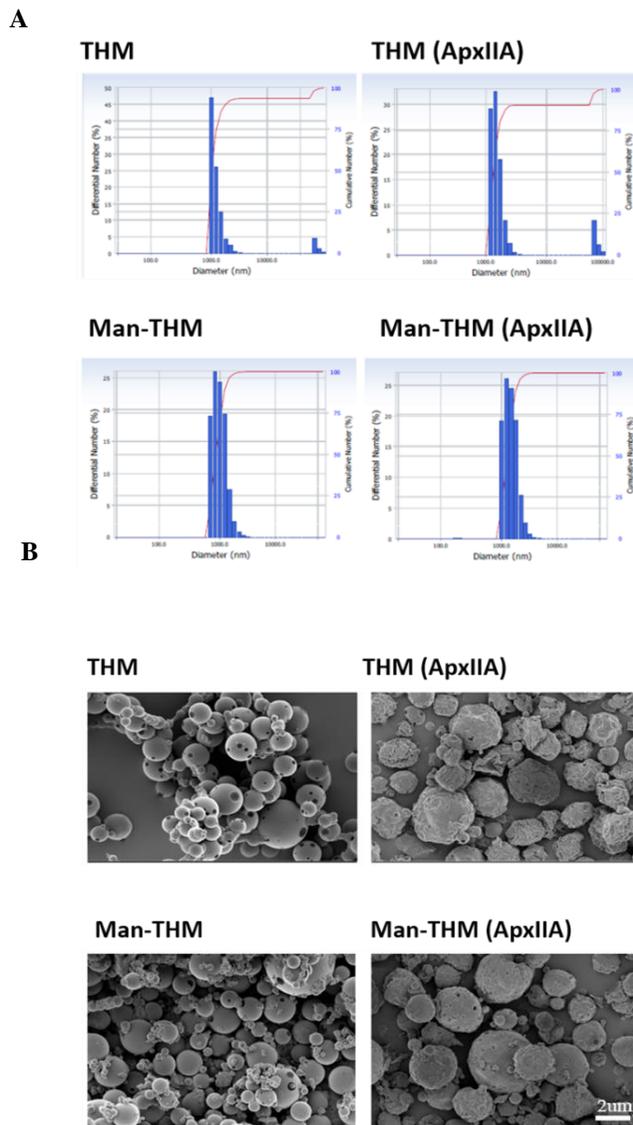
**Figure 23.** Confirmation of the synthesis of TH by <sup>1</sup>H NMR (DMSO-d<sub>6</sub>, 600 MHz). Conjugation of the thiol group in HPMCP is depicted by the protons-S (H) of the cysteine in the NMR spectrum.

## 2) Characterization of THM and Man-THM

### (1) Size and surface morphology

The sizes of THM and Man-THM as measured by DLS were  $1.38\pm 0.33\ \mu\text{m}$  and  $1.21\pm 0.31\ \mu\text{m}$ , respectively, as shown in Figure 24(A). The decoration of THM with mannan did not result in much change in sizes due to the small amount of coated mannan. The sizes of the ApxIIA-loaded THM and ApxIIA-loaded Man-THM were  $1.53\pm 0.33\ \mu\text{m}$  and  $1.66\pm 0.33\ \mu\text{m}$ , respectively, slightly larger than the unloaded microparticles. The morphologies of the THM and Man-THM as observed by SEM are shown in Figure 24(B). The THM were spherical in shape and decoration with the mannan did not influence the spherical shape of the microparticles. Recently, many studies have demonstrated the relationship between particle size and mucociliary clearance in the airways (Möller et al., 2006; Usmani et al., 2005). One study showed that mucociliary clearance removed all particles larger than  $6\ \mu\text{m}$  from the airways within 24 h, while particles  $6\ \mu\text{m}$  or smaller were retained for more than 24 h (Henning et al., 2010). The size of the microspheres also affects uptake by the APCs. It has already been reported that DCs take up particles with sizes in the range of nanometers to micrometers. Particles smaller than  $5\ \mu\text{m}$  can be taken up by DCs and are transferred to the local lymph nodes and spleen, stimulating both local and systemic immune responses; DCs do not take up particles larger than  $10\ \mu\text{m}$  (Oechslein et al., 1996). The mean sizes of the microspheres in this

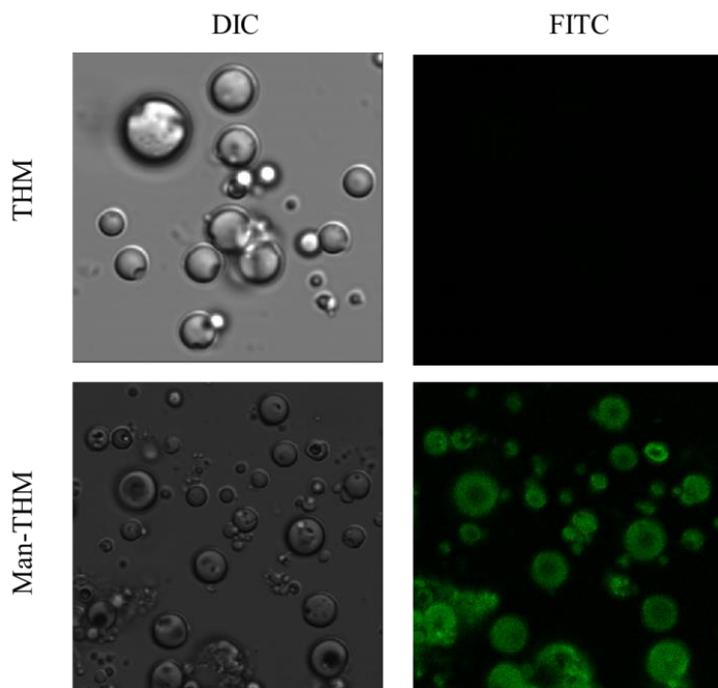
study were less than 3  $\mu\text{m}$ , which is similar to the sizes of the bacteria that the immune system has evolved to combat. It is reasonable to assume that our microspheres would be internalized through phagocytosis by APCs, which would then mature and migrate to the nearby lymph nodes (Champion et al.,2008).



**Figure 24.** (A) Particle size distributions of THM, Man-THM, ApxIIA-loaded THM, and ApxIIA-loaded Man-THM. (B) SEM photographs of THM, Man-THM, ApxIIA-loaded THM, and ApxIIA-loaded Man-THM.

## (2) Confirmation of the mannan surface decoration in Man-THM

The surface decoration of THM with mannan molecules was evaluated by confocal microscopy, as shown in Figure 25. FITC-conjugated mannan was used to create FITC-Man-THM. The confocal images indicated that the surface of the Man-THM was homogeneously coated with mannan due to the mannan's amphiphilic property, which functions as a stabilizer during the formation of microspheres through  $W_1/O/W_2$  double emulsion. Through quantification of the amount of FITC-mannan decorating in THM, almost 0.5 –wt% of FITC-mannan was detected from the FITC-Man-THM.

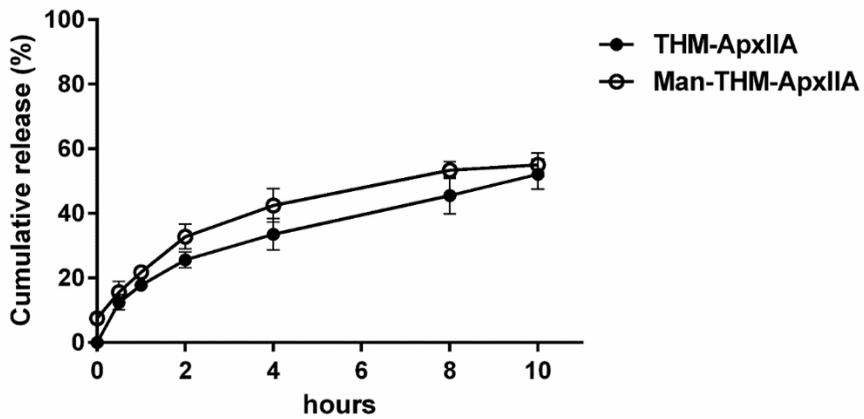


**Figure 25.** Confirmation of the surface decoration of mannan in Man-THM. FITC-conjugated mannan was used to create the FITC-Man-THM and the surface fluorescent signal was monitored by CLSM. First column: image by differential interference contrast (DIC) microscopy; second column: fluorescence by confocal laser scanning (CLS) microscopy.

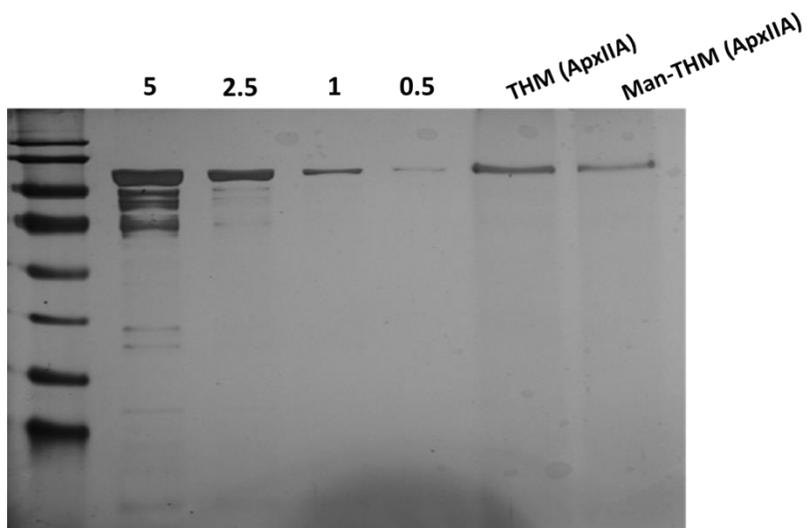
### 3) *In vitro* studies

#### (1) *In vitro* release of ApxIIA from ApxIIA-loaded THM and ApxIIA-loaded Man-THM

The *in vitro* release behaviors of ApxIIA-loaded THM and Man-THM were studied in PBS (pH 7.4) (Figure 26). The release profiles of ApxIIA were presented as a percentage of the amount of ApxIIA released from the microparticles with respect to the total amount of ApxIIA encapsulated. The results indicated that approximately 50 wt.-% of the ApxIIA was rapidly released from the ApxIIA-loaded THM and Man-THM within 10 h, showing similar patterns of release. Distinct from low molecular weight drugs, proteins possess secondary, tertiary, and even quaternary structures with labile bonds between the side chains of chemically reactive groups. Disruption of these structures or modifications of these side chains can lead to a loss of protein or vaccine activity (Jiang et al., 2014). Thus, ensuring the stability of the antigen during its loading process into the microparticles is important for retaining its antigenicity. The structural integrity of ApxIIA was evaluated by SDS-PAGE before and after loading onto THM and Man-THM. As shown in Figure 27, the band patterns of the ApxIIA released from the ApxIIA-loaded THM and Man-THM were similar to that of native ApxIIA, indicating that the ApxIIA was not structurally altered during antigen loading.



**Figure 26.** Release of ApxIIA from ApxIIA -loaded THM and ApxIIA-loaded Man-THM at pH 7.4 and 37°C (n = 3, error bar represents standard deviations).

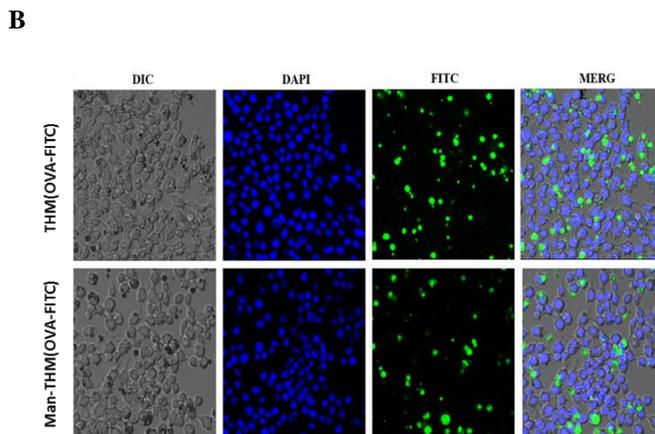
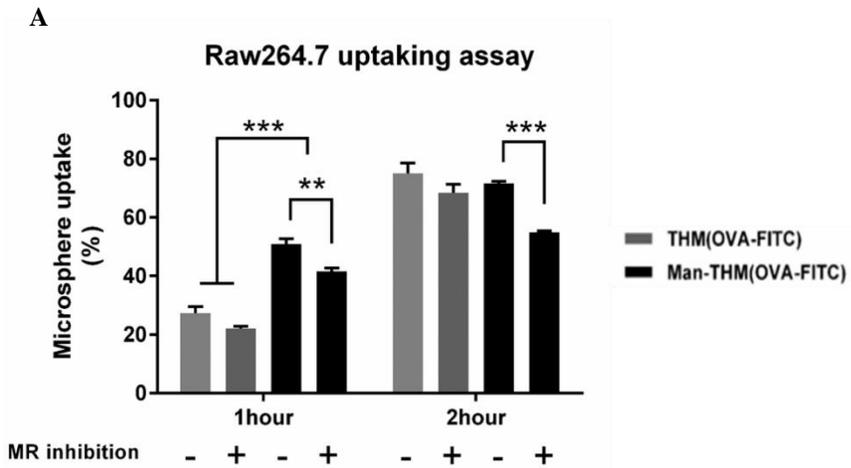


**Figure 27.** Evaluation of the structural integrity of ApxIIA released from the ApxIIA-loaded THM and Man-THM by SDS-PAGE. Lane 1: marker, Lane 2, 3, 4, and 5: 5, 2.5, 1, and 0.5ug of ApxIIA, respectively, Lane 6: ApxIIA released from the ApxIIA-loaded THM, Lane 7: ApxIIA released from the ApxIIA-loaded Man-THM.

## (2) Cellular uptake of microspheres by RAW264.7

The targeted delivery of microspheres to APCs via MR was determined by observing the uptake of microspheres by APCs. The analysis of the cellular uptake of microspheres was performed in RAW 264.7 using FITC-labeled OVA-loaded THM and FITC-labeled OVA-loaded Man-THM. The uptake of microspheres by RAW 264.7 cells was observed by FACS analysis according to incubation time and depended on the blockade of MR. Figure 28(A) shows the uptake of microspheres by RAW 264.7 at 37 °C for 1 and 2 h. The MR-targeted Man-THM exhibited significantly higher uptake efficiency for RAW 264.7 cells at 1 h post-treatment; however, there were no statistical differences between THM and Man-THM at 2 h due to the non-specific uptake of particles by RAW 264.7. In addition, the blockade of MR in RAW 264.7 by pre-treatment with mannan resulted in a significant decrease in the Man-THM uptake at both 1 and 2 h, indicating that the uptake of Man-THM was mediated by interactions between mannan and MR.

In order to confirm microsphere uptake, confocal laser scanning microscopy was also performed. Figure 28(B) shows the internalization of the microspheres by RAW 264.7 at 37 °C after 2 h of treatment. It was found that the internalization efficiency of THM and Man-THM by RAW 264.7 cell lines at 2 h was similarly consistent with the results of the cellular uptake of the microspheres observed by FACS.

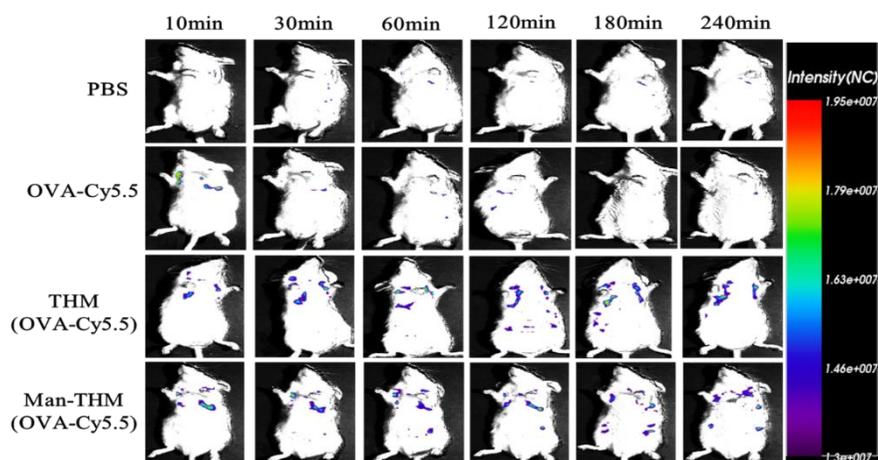


**Figure 28.** (A) Measurement of microsphere uptake by RAW 264.7 using FACS. Uptake of OVA-FITC-loaded THM and OVA-FITC loaded Man-THM by RAW 264.7 in 1 h and 2 h with and without MR inhibition at 37 °C (n = 3, error bar represents standard deviation; \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.005, one-way ANOVA). (B) Confocal microscopic images of RAW 264.7 cells after 2 h culture with OVA-loaded THM and OVA-loaded Man-THM at 37 °C.

#### **4) *In vivo* imaging of the intranasal administration of the microspheres**

The primary function of the mucosal layer is to protect the lungs by trapping and removing foreign particles by the mucociliary escalator, which causes trapped particles to be coughed up out of the lungs. The development of an efficient intranasal vaccine depends on the vaccine's ability to maintain adequate contact time in the respiratory mucosa, allowing for interaction with the respiratory immune cells. In a previous study, we confirmed that thiolated HPMCP (TH) exhibited a 1.72-fold greater mucus adhesion ability than HPMCP upon contact with a mucin layer. In this study, we recruited TH microspheres as intranasal delivery vehicles. To confirm the performance of intranasal THM-delivered antigen in the respiratory mucosa, we evaluated antigen signaling in the respiratory system after the intranasal administration of the microspheres. *In vivo* optical molecular imaging (Optix-MX3) clearly showed that Cy5.5-OVA-loaded THM and Cy5.5-OVA-loaded Man-THM were retained in the respiratory mucosa 4 h after nasal administration. In contrast, the majority of free Cy5.5-OVA had disappeared from the respiratory mucosa within 1 h after nasal administration (Figure 29). These results indicated that the mucoadhesive ability of antigen-loaded THM and Man-THM efficiently prolonged their retention time in the respiratory mucosa and increased the chance of uptake by APCs. In addition, signals were detected from the distal secondary lymphoid tissues including the spleen, implying that APCs

internalized antigen-loaded THM and Man-THM from the respiratory system and migrated into the secondary lymphoid tissue to induce systemic and distal mucosal immune responses.



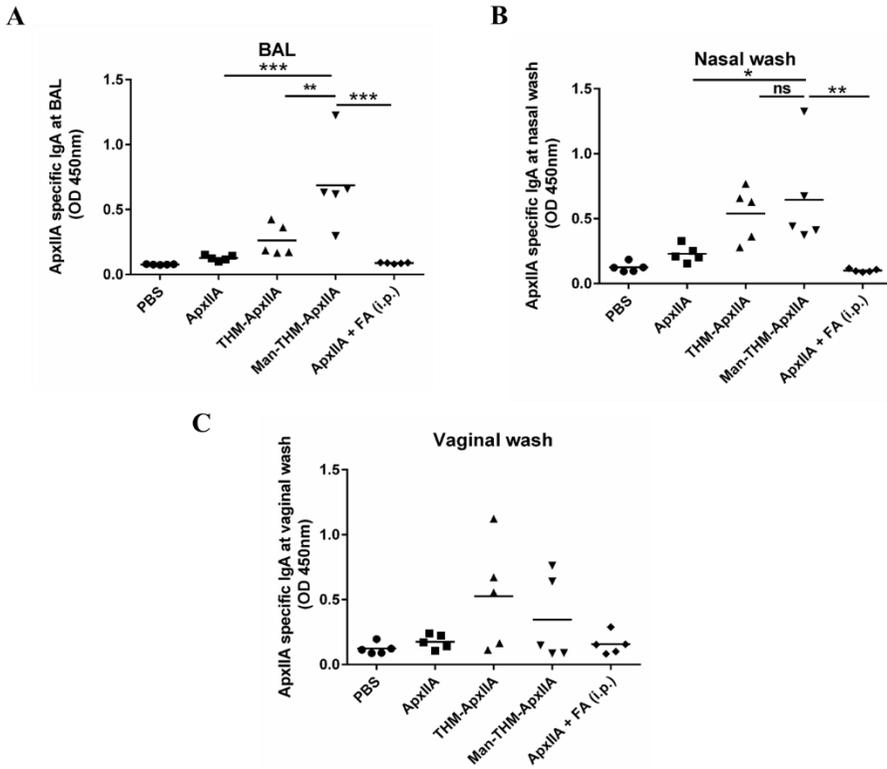
**Figure 29.** Cy5.5-conjugated OVA was used. Cy5.5-conjugated OVA and the same amount of cy5.5-conjugated OVA loaded onto THM and MAN-THM were intranasally administered to 6 week-old BALB/c mice. The fluorescence was monitored at 10 min, 30 min, 60 min, 120 min, 180 min, and 240 min. The fluorescence images were acquired under an excitation of 695 nm using the Optix-MX3 (Advanced Research Technologies, Canada). The fluorescence intensities in the regions of interest (ROI) were analyzed from the fluorescence images.

## 5) *In vivo* immunization

### (1) ApxIIA-specific mucosal immune responses

Based on the results of the microsphere characterization, we hypothesized that the mucoadhesive microspheres would have prolonged residence time in the respiratory mucosa, and that MR-targeting ligands could enhance the delivery of Ag into mucosal APCs and thus promote the induction of mucosal immune responses. To validate our hypothesis, we evaluated immunizations with the intranasal administration of ApxIIA alone, ApxIIA-loaded THM, and ApxIIA-loaded Man-THM, and also evaluated the level of ApxIIA-specific mucosal immune responses in the BAL and nasal wash. Figure 30 shows the levels of anti-ApxIIA sIgA in the various mucosal tissues after nasal vaccination with different formulations. The mice immunized with ApxIIA-loaded Man-THM showed higher levels of anti-ApxIIA sIgA antibody in the BAL [Figure 30(A)] compared with those immunized with the ApxIIA-loaded THM and native ApxIIA. The levels of anti-ApxIIA sIgA antibody in the nasal wash [Figure 30(B)] showed a similar pattern with the anti-ApxIIA IgG antibody response, although no significance difference between THM and Man-THM was observed. The local and distal production of sIgA antibodies are the most important characteristics of nasally administered vaccines. Notably, our results indicated that intranasally delivered particulate vaccines could induce anti-ApxIIA sIgA antibody in the vagina [Figure 30(C)]. These results

indicated that nasally administered particulate vaccines efficiently induced antibody responses at both local and distal mucosal sites, which can defend the body against pathogens from other mucosal sites such as the vagina. Conversely, i.p. vaccination with ApxIIA could not induce sIgA responses at both local and distal mucosal sites, proving the importance of mucosal vaccines to induce mucosal immune responses.

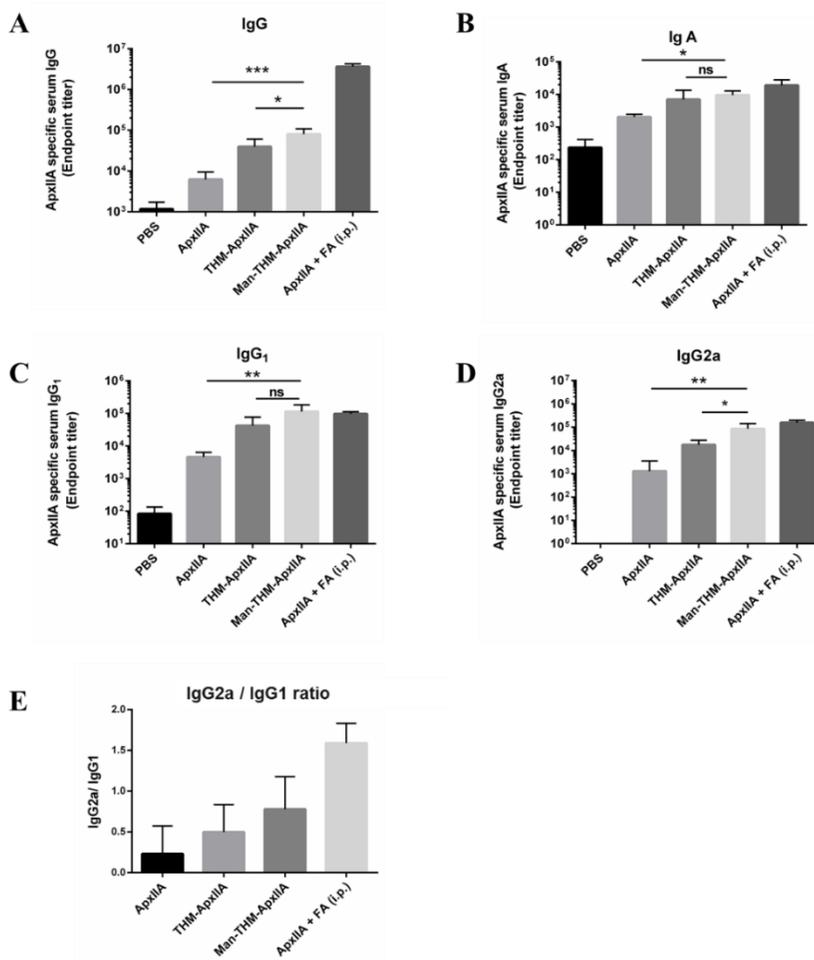


**Figure 30.** ApxIIA-specific IgA performance in the mucosal sites at 4 weeks post-immunization. ApxIIA-specific brochealveolar lavage (A), nasal wash (B), vaginal wash (C), IgA levels in mice immunized with the indicated formulations were analyzed by ELISA and then calculated by optical density (450 nm) (n = 5, error bars represent standard deviations; \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.005, one-way ANOVA).

## (2) ApxIIA-specific serum IgG (IgG1 and IgG2a) responses

The levels of ApxIIA-specific serum isotypes of IgG, IgA, IgG1, and IgG2a are shown in Figure 31. Serum-derived IgG contributes to the immune defense of the lower respiratory tract (Renegar et al., 2004), and the production of IgG1 and IgG2a isotypes is associated with Th2 type and Th1-type immune responses, respectively (Dobrovolskaia and McNeil, 2007). The mice immunized with ApxIIA-loaded Man-THM displayed significantly higher IgG titers than those receiving ApxIIA-loaded THM and ApxIIA. However, significance differences in serum IgA was not found between the THM and Man-THM groups [Figure 31(A) and (B)]. The level of ApxIIA-specific IgG2a was the most significantly enhanced of the IgG isotypes, although the level of the other isotypes of ApxIIA-specific IgG also increased in response to the nasal administration of ApxIIA-loaded Man-THM [Figure 31(C) and (D)]. Furthermore, in order to understand the characteristics of the immune responses induced by the nasal immunization of ApxIIA-loaded THM and Man-THM, the ratio of IgG2a and IgG1 was analyzed as an indicator of Th1 or Th2 bias in the immune response [Figure 31(E)]. As shown in the figure, the ratio of IgG2a and IgG1 was increased in the particulate vaccine groups compared with the native antigen groups. Additionally, the ApxIIA-loaded Man-THM group showed a higher IgG2a and IgG1 ratio than the ApxIIA-loaded THM group, implying a Th1 bias in the immune response. This suggests that the enhanced ApxIIA-

specific systemic immune response provoked by nasal immunization with ApxIIA-loaded Man-THM was due to the enhancement of the Th1 immune response.



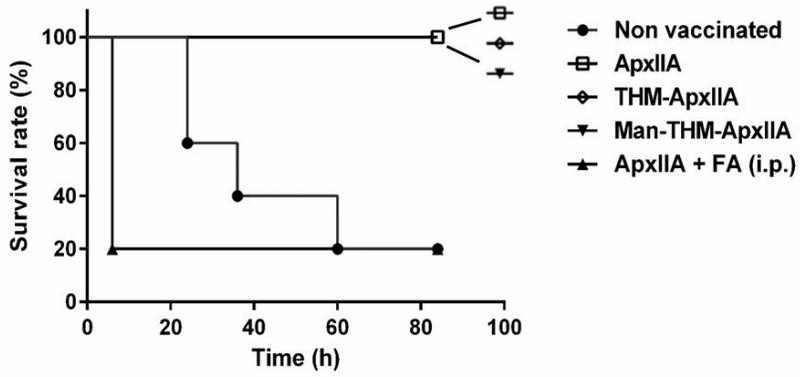
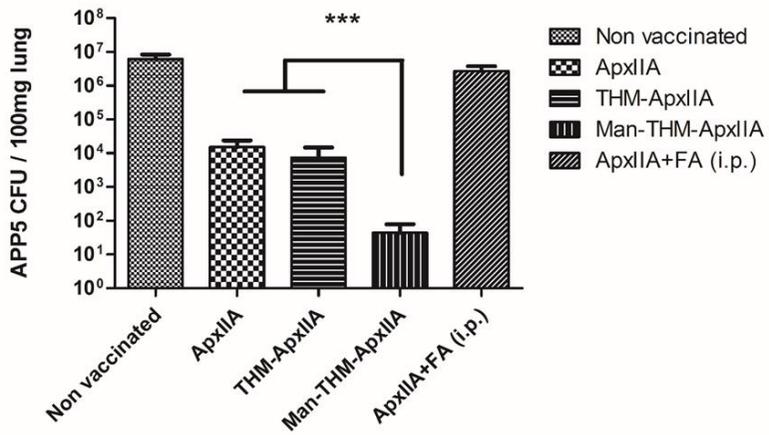
**Figure 31.** ApxIIA-specific systemic immune responses at 4 weeks post-immunization. ApxIIA-specific serum IgG (A), serum IgA (B), serum IgG1 (C), serum IgG2A (D), and IgG2a, IgG1 ratios (E) in mice nasally immunized with the indicated formulations were analyzed by ELISA assay (n = 5, error bars represent standard deviations; \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.005, one-way ANOVA).

## 6) Challenge assay

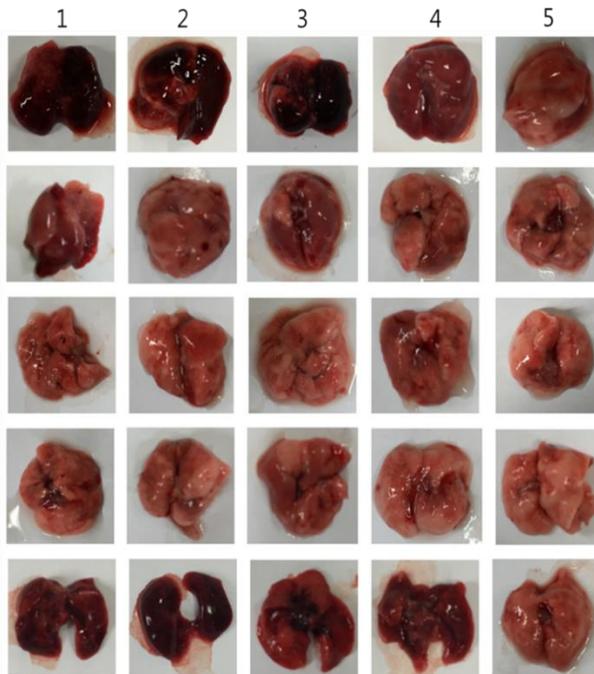
We next investigated if the enhanced mucosal and systemic immune response induced by the nasal administration of ApxIIA-loaded Man-THM could establish protective immunity against bacterial infection in a murine model (Figure 32). We inoculated  $5 \times 10^7$  CFU of *A. pleuropneumoniae* serotype 5 intranasally and monitored the survival of the mice for 96 h. As shown in the Figure 32(A), mice in the unvaccinated control group started to die at 24 h after the infectious challenge, and 80% of the mice died within 60 h. Conversely, all mice intranasally vaccinated with ApxIIA, ApxIIA-loaded THM, and ApxIIA-loaded Man-THM survived the challenge. Surprisingly, 80% of the mice intraperitoneally immunized with ApxIIA died within 12 h after challenge. The lower body weights of the mice in the i.p. group (data not shown) due to the use of the Freud adjuvant may explain why these mice could not overcome the high burden of bacteria as compared to mice with higher body weights.

In order to confirm the clearance of ApxIIA by the protective immune response, we harvested the lung tissue of the surviving mice at 96 h or immediately from the mice that died before 96 h [Figure 32(C)], and counted the number of bacteria in the lung tissue homogenates [Figure 32(B)]. The number of bacteria measured in the lung tissue homogenates prepared from mice in the ApxIIA-loaded Man-THM group was approximately 100-fold lower than those from the ApxIIA and ApxIIA-loaded THM groups, although

all mice from the three groups survived the infectious challenge. We concluded that ApxIIA-loaded Man-THM enhanced the systemic and mucosal immune responses against ApxIIA, which enhanced the bacterial clearance ability of the mice challenged with *A. pleuropneumoniae*, thus improving survival.

**A****B**

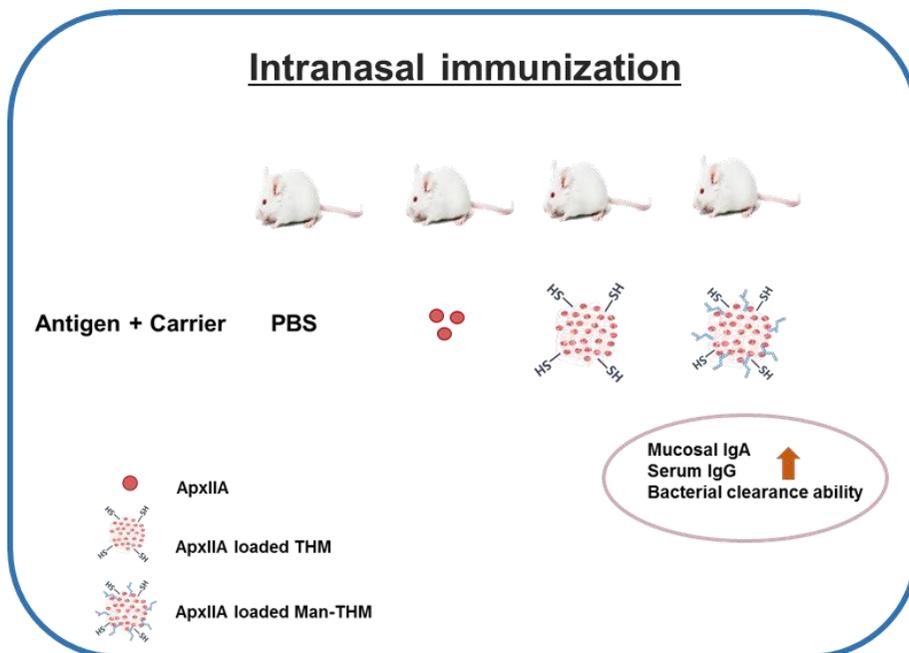
C



**Figure 32.** Induction of protective immunity after intranasal challenge with *A. pleuropneumoniae*. (A) 14 days after the last immunization, 5 mice per group were challenged intranasally with a mean lethal dose ( $5 \times 10^7$  CFU) of *A. pleuropneumoniae*, with the survival rate (%) monitored for an additional 4 days. (B) The number of residual bacteria was counted per 100 mg fresh lung tissue weight from each mice per group. ( $p < 0.05$ ,  $**p < 0.01$ ,  $***p < 0.005$ , one-way ANOVA) (C) The lungs were characterized before becoming homogenates and after bacteria challenge.

#### 4. Conclusion

The development of an effective nasal vaccine requires appropriate carriers and an adjuvant system that protects antigens from rapid nasal clearance by the mucociliary apparatus and efficiently delivers antigens to APCs. In this study, mannan-decorated mucoadhesive microspheres (Man-THM) were successfully prepared and their targeted delivery to the MR on APCs was evaluated *in vitro* and *in vivo*. It was found that intranasal vaccination with ApxIIA-loaded Man-THM in mice induced enhanced mucosal sIgA and serum antibody (IgG and IgA) responses as compared to ApxIIA-loaded THM due to specific interactions between the mannan on the Man-THM and the MR on APCs. Furthermore, to assess the protective efficacy of these vaccines, mice were vaccinated with ApxIIA-loaded Man-THM and challenged with *A. pleuropneumoniae*. The adjuvant effect of Man-THM was clearly demonstrated by the 100% survival rate and enhanced bacterial clearance, proving successful protection from *A. pleuropneumoniae*. Our results strongly support the efficacy of Man-THM as an adjuvant carrier system for the effective induction of systemic and mucosal immune responses.



**Figure 33.** Schematic illustration for the result of the study

## **Chapter 4. Overall conclusion**

The mucosa is a primary target for infection and, at the same time, the first line of defense against many pathogens in which secretory IgA is a main

protective component of the immune system. Mucosal vaccines, in contrast to injected vaccines, have been reported to provide additional secretory IgA mediated protection against pathogens at the mucosal site of entry. One of the most important virtues of mucosal vaccination is, while injected vaccines generally fails to do so, capable of inducing protective immune responses both in the mucosal and systemic immune compartments. However, there are several very serious concerns about mucosal vaccines. Mucosa-administered antigens are generally less immunogenic and physical barrier of mucosa also is an obstacle that to be overcome. In this regard, induction of mucosal immunity through vaccination is a rather difficult task, and potent mucosal adjuvants and special delivery systems are required for successful mucosal vaccination.

The aim of the study was development of mucosal adjuvant using cytokine and polymeric particles for efficient mucosal immunization. In order to achieve this purpose, adjuvant systems were designed according to the coping strategies

against the hurdles in developing subunit vaccine (Figure 1-3).

Study 1 was mainly focused on developing oral cytokine adjuvant. A recombinant cytokine, IL-6-CKS9, was generated by conjugating an M cell-targeting peptide (CKS9) with c-terminus of the murine interleukin 6 (IL-6), which facilitated enhancement of mucosal immune response. *Lactococcus lactis* IL1403, a food-grade strain of lactic acid bacteria (LAB) which is widely used in dairy industry, was used as a host cell to express and secreted the IL-6-CKS9 for a mucosal vaccine adjuvant. The recombinant *L. lactis* IL1403 secreted IL-6-CKS9 was orally administered with a model antigen protein, M-BmpB (*Brachyspira* membrane protein B conjugated with CKS9), to BALB/c mice for mucosal immunization. ELISA analyses showed consistent enhancement tendencies in induction of anti-M-BmpB antibody levels both with mucosal (IgA) and systemic (IgG) immune responses in IL-6-CKS9-LAB treated group compared with other groups tested by conducting mice immunization assays. In addition, the oral administration of model protein antigen with live LAB producing IL-6-CKS9 could induce both Th1 and Th2 type immune responses. Collectively, the results showed successful production and secretion of recombinant murine IL-6 with M cell-targeting moiety (IL-6-CKS9) from *L. lactis* IL1403 and demonstrated that the live recombinant LAB producing IL-6-CKS9 could have a potential to be used as an efficient adjuvant for peroral vaccination.

Study 2 was mainly focused on developing an efficient nasal vaccine carrier. The nasal route for vaccine delivery by particles has attracted considerable interest, although challenges such as the rapid mucociliary clearance in the respiratory mucosa and the low immunogenicity of subunit vaccine still remain (Figure 3). Recently, a new generation of synthetic polymers known as thiolated polymers, such as thiolated hydroxypropylmethyl cellulose phthalate (HPMCP), have been shown to have strong mucoadhesive properties due to their binding with the cysteine-rich subdomains of mucus glycoprotein in the mucosal site. So, thiolated HPMCP (TH) was applied to make microspheres as a nasal vaccine carrier for prolonging the residence time in the respiratory mucosa to make interaction time of vaccine molecules and antigen presenting cells (APCs) longer. Moreover, another major hurdle in the development of intranasal vaccines is poor immunogenicity of vaccine subunits due to the lack of “danger signals” that can activate APCs. To this end, study 2 was aimed to develop mannan-decorated mucoadhesive THM (Man-THM) as an adjuvant system for nasal vaccine. Mannan is one of the natural PAMPs (pathogen associated molecular patterns) of APCs and is a ligand of the mannose receptor (MR) widely expressed on APCs. Mannan can induce the production of pro-inflammatory cytokines and up-regulate the expression of co-stimulatory molecules in these cells, thereby activating both innate and adaptive immunity. The aim of study 2 was to explore the potential of a novel Man-THM vehicle for the intranasal administration of ApxIIA as a vaccine against *A.*

*pleuropneumoniae* which is known to cause contagious porcine pleuropneumoniae. ApxIIa-loaded Man-THM was prepared by the double emulsion solvent evaporation method and using the mixture of 0.25wt.-% PVA and 0.5wt.-% mannan ( $W_2$  phase) to decorate the surface of microspheres with mannan. After optimization of the preparation method and characterization of the obtained ApxIIA-loaded Man-THM, the physiochemical properties of the Man-THM as a nasal delivery carrier were evaluated by *in vitro* and *in vivo* assays. The surface of the Man-THM was homogeneously coated with mannan due to the mannan's amphiphilic property, which functions as a stabilizer during the formation of microspheres through  $W_1/O/W_2$  double emulsion. In a mechanistic study using APCs *in vitro*, it was found that Man-THM enhanced receptor-mediated endocytosis by MR of APCs. *In vivo*, the nasal vaccination of ApxIIA-loaded Man-THM in mice resulted in higher levels of mucosal sIgA and serum IgG than mice in the ApxIIA and ApxIIA-loaded THM groups due to the specific recognition of the mannan by the MRs on the APCs. Moreover, ApxIIA-containing Man-THM protected immunized mice when challenged with strains of *A. pleuropneumoniae* serotype 5. The number of bacteria measured in the lung tissue homogenates prepared from mice in the ApxIIA-loaded Man-THM group was approximately 100-fold lower than those from the ApxIIA and ApxIIA-loaded THM groups. The results showed that ApxIIA-loaded Man-THM enhanced the systemic and mucosal immune responses against ApxIIA, which enhanced the bacterial clearance ability of the mice

challenged with *A. pleuropneumoniae*, thus improving survival. The results strongly support the efficacy of Man-THM as an adjuvant carrier system for the effective induction of systemic and mucosal immune responses.

In conclusion, adjuvant system using cytokine and polymeric particles were applied to oral and nasal subunit vaccine, respectively in the study 1 and 2. An oral cytokine vaccine adjuvant named as 'IL6-CKS9' and nasal vaccine delivery carrier named as 'Man-THM' were developed and evaluated its adjuvant efficiency through *in vitro* and *in vivo*. Although there is much work to be done before these systems are applied for livestock, however, 'IL6-CKS9' and 'Man-THM' have potential to be safe and efficient adjuvant systems for mucosal vaccine.

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## Summary in Korean

점막표면은 대부분 병원균이 체내로 침입하는 주요 관문이며 이는 가축에게 있어서 전염병을 일으키는 주요 원인경로이기도 하다. 때문에 점막을 통하여 면역반응을 일으켜 점막에서의 방어체계인 sIgA를 생성하여 병원체가 감염하는 경로인 점막에서 초도에 방어를 함으로써 병원체의 감염을 막는 것이 굉장히 중요하다. 점막면역은 전신면역을 일으키는 근육주사와는 달리 주사바늘에 의한 공포심이 가해지지 않고 또한 접근성이 좋고 안전하여 개발만 된다면 경제적으로도 저렴하여 농가에서 사용하기 용이한 장점이 있다. 하지만 점막을 통하여 일으키는 면역은 면역유도능력이 전신면역이 일으키는 것에 비하여 낮으며 그 면역원성을 높여줄 수 있는 안전하고 효과적인 점막백신 어주변트와 전달체가 아직 용이하게 사용되지 못할뿐더러 아직 전반적인 점막면역에 대한 학설이 완벽하지 못한 것 또한 한가지 원인으로 꼽을 수 있다. 최근 들어 효과적인 점막면역백신을 개발하기 위하여 면역원성이 높은 항원을 선발하는 것에 대한 연구, 효과적인 투여경로의 디자인, 점막면역을 자극 할 수 있는 어주변트 물질에 대한 탐색, 그리고 점막으로의 백신전달시스템에 대한 연구가 활발히 진행 중에 있다.

본 연구의 목적은 가축 점막면역 백신 개발 특히 경구백신과 비강백신의

개발에 있어서 백신의 효율을 높이고자 하는 목적으로 신형 경구백신 어주번트의 개발과와 비강백신 전달체 개발에 대한 것으로서 연구 1은 사이토카인(IL-6)을 *Lactococcus lactis* IL1403에서 생산하여 경구용 백신 어주번트로 활용한 것에 대한 연구이고, 연구 2는 점막점착성 고분자 전달체에 mannan을 코팅시켜 항원제시세포를 표적 할 수 있는 비강백신 전달체에 대한 내용이다.

먼저, 연구 1은 재조합 IL-6-CKS9 사이토카인, 즉 M 세포 표적 펩타이드(CKS9)를 IL-6의 C 말단에 연결된 형태로 구성하여 점막면역반응을 증진시킬 수 있는 어주번트로 개발하고자 하였다. *Lactococcus lactis* IL1403는 유업에 널리 사용되고 있는 식품성 유산균의 한 종류로써 IL-6-CKS9을 발현 및 분비시키는 숙주세포로 사용하였으며 이 IL-6-CKS9을 생산할 수 있는 재조합 *L. lactis* IL1403을 경구백신 어주번트로 개발하였다. IL-6-CKS9 생산 재조합 *L. lactis* IL1403을 모델 항원 단백질인 M-BmpB(*Brachyspira* 막 단백질 B에 CKS9을 conjugation 시킨 형태)를 BALB/c 마우스에 경구로 함께 투여하여 면역을 시켰다. 경구면역 후 ELISA 분석을 통하여 소장과 분변에서의 점막 IgA와 혈중 IgG가 IL6-CKS9-유산균을 어주번트로 활용한 그룹에서 기타 그룹에 비해 높은 역가를 나타냈으며 이는 두 번의 독립적인 면역실험에서 같은 경향으로 보이는 것으로 확인이 되었다. 뿐만 아니라 혈중 BmpB 특이적 IgG1과 IgG2a의 역가 측정을 통하여 IL6-CKS9-유산균을 경구백신 어주번트로 사용하였을 경우, Th1 타입의 면역반응과 Th2 타입의 면역반응을 동시에

증진하는 것으로 확인이 되었다. 결론적으로 M 세포 표적 펩타이드가 도입된 IL-6 (IL6-CKS9)를 성공적으로 *L. lactis* IL1403에서 발현 및 분비를 시켰으며 경구백신 실험을 통하여 IL-6-CKS9 분비 유산균을 어주번트로 활용하였을 경우 점막 IgA 및 혈중 IgG의 생성을 증진시킴으로써 IL6-CKS9-유산균의 경구백신 어주번트로써의 가능성을 확인하였다.

연구 2는 점막점착성능력과 항원제시세포를 표적 할 수 있는 능력을 지닌 비강백신 전달체를 개발하고 그것의 효과를 비강면역을 통하여 검증한 것에 관한 내용이다. 비강을 통하여 점막면역을 일으키는 것은 다른 점막면역경로에 비해 효과적이어서 점막면역에서 가장 많은 각광을 받고 있다. 하지만 효과적인 비강백신을 개발함에 있어서 호흡기 점막의 점액섬모청소(mucocilliary clearance)로 인해 투여한 항원물질이 비강에서의 머무르는 시간이 짧은 문제점과 서브유닛 백신으로써의 면역원성이 낮은 문제점은 극복해야 할 난관이다. 하여 연구 2에서는 mannan을 점막점착성 HPMCP microsphere (THM)에 코팅을 시켜 Man-THM을 구성함으로써 점막점착성 능력으로 mucocilliary clearance를 극복하고 또 mannan의 도입을 통하여 항원제시세포를 표적 할 수 있는 전달체를 구축하였다. *A. pleuropneumoniae*를 방어할 수 있는 exotoxin fragment인 ApxIIA를 모델 항원 단백질로 사용하였고 Man-THM 에 ApxIIA를 담지하였다. Man-THM은 mannan이 코팅되어 있어 호흡기 항원제시세포의 PRRs(pathogen recognition receptors)를 표적할 수 있을 뿐만 아니라 mannose receptor(MR)를 표적할 수 있어서 어주번트로 작용할 수 있다.

Man-THM의 비강면역 전달체로써의 어주변트 기능은 *in vitro*와 *in vivo*에서 확인하였다. 먼저 *in vitro*에서 Man-THM 이 APCs에서 mannose receptor를 통한 receptor-mediated endocytosis를 하는 것을 검증하였고 *in vivo*에서 ApxIIA를 담지 한 Man-THM을 BALB/C 마우스에 비강을 통하여 면역을 시킨 후 호흡기 점막에서 분비되는 IgA와 혈중 IgG측정을 통하여 ApxIIA를 담지한 Man-THM이 ApxIIA 단독으로 혹은 ApxIIA를 THM에 담지하여 면역을 시킨 그룹에 비해 높은 항체역가를 나타내는 것을 확인 하였다. 뿐만 아니라 비강면역 후, *A. pleuropneumoniae* serotype 5를 비강을 통하여 공격 접종하였을 때 효과적인 방어효과를 나타내는 것을 확인하였고 ApxIIA를 담지한 Man-THM 그룹이 기타 그룹에 비하여 폐 내에 잔존하고 있는 *A. pleuropneumoniae* 수가 유의적으로 감소하는 것 확인하였다. 결론적으로 항원제시세포 표적형 점막점착성 Man-THM이 효과적으로 비강면역을 유도를 증진시키는 것을 확인함으로써 비강백신 전달체로써의 잠재성이 있음을 확인하였다.

# **Appendix. Mannan-decorated thiolated Eudragit microspheres for targeting antigen presenting cells via nasal vaccination**

## **1. Introduction**

Mucosal immunity is mediated by secretory immunoglobulin A (sIgA) antibody, which prevents pathogen attachment from mucosal epithelia (e.g. respiratory tract, vaginal tract and gastro-intestinal tract) and clear pathogens before they invade or infect the underlying tissue (Holmgren and Czerkinsky, 2005). Thus, an effective mucosal vaccine is necessary in the aspect that it can protect the host body from the pathogens at the initial site of infection by induction of mucosal immunity. Moreover, mucosal immunization frequently results in the stimulation of both mucosal and systemic immunity (Czerkinsky and Holmgren, 2012). Among mucosal immunizations, intranasal administration is a promising vaccination route because it induces potent cross-protective immune responses both in the respiratory tracts and the genital tracts (Lycke, 2012).

For an effective delivery of subunit vaccine through intranasal route, two major limitations need to be overcome. One is the rapid clearance of vaccines by normal mucociliary process and the other one is the weak immune response induced due to inefficient antigen presentation by immune cells. To overcome these limitations, the strategy of subunit vaccine formulations for nasal vaccination should be designed to retain the vaccine carrier in the nasal cavity for longer duration for sufficient delivery through NALT (Hagenaars et al., 2010) and to target the immune cells using a ligand in the vaccine carrier for efficient uptake by APCs (Foged et al., 2002).

In recent years, several particulate delivery systems have been developed for nasal delivery of subunit vaccines rather than soluble subunit antigens due to their several physicochemical advantages like particle size, zeta potential, hydrophobicity, tight junction opening properties and receptor-binding characteristic (Vajdy and O'Hagan, 2001). However, many vaccine delivery particles have little affinity with the nasal epithelium, and they are usually cleared within minutes by ciliary movement in the nasal cavity (Illum, 2003). The first major hurdle - rapid nasal clearance of vaccine in nasal epithelium, can be achieved by mucoadhesive polymeric carriers which can extend the nasal residence time efficiently (Amidi et al., 2006; Khutoryanskiy, 2011; Rahamatullah Shaikh et al., 2011). Recently, a new generation of synthetic polymers, called as thiomers or thiolated polymers, have been reported for their

strong mucoadhesive properties due to binding with the cysteine-rich subdomains of mucus glycoprotein (Bernkop-Schnürch et al., 2001; Bernkop-Schnürch et al., 2004). In this work, thiolated Eudragit L-100 was used as nasal subunit vaccine carrier because it holds both pH-sensitive and mucoadhesive features (Islam et al., 2011; Lee et al., 2011; Lee et al., 2012).

Another major hurdle to be overcome is the poor immunogenicity of subunit vaccine caused by inefficient antigen presentation. Targeting antigens to endocytic receptors on professional APCs represent an attractive strategy to enhance the efficacy of vaccine. Mannose receptor (MR) is a member of C-type lectin receptor widely expressed on APCs such as DCs and macrophages (Taylor et al., 2005) which is important for antigen internalization. Mannan (polymer of mannose units) isolated from the cell wall of *Saccharomyces cerevisiae*, is one of the natural ligands that has strong affinity for MR (Taylor et al., 1990; Vinogradov et al., 1998). Moreover, several approaches that target delivery of antigens to MR have demonstrated effective induction of cellular and humoral immune responses (Cui and Mumper, 2002; Gu et al., 1998; Keler et al., 2004; Sasaki et al., 1997; Tacke et al., 2007; Vaughan et al., 2000).

In this study, we made a surface modification in mucoadhesive polymer, thiolated Eudragit L-100 (TE), with MR targeting ligand, as a vaccine carrier for nasal vaccine delivery. Mannan-decorated TE microparticles were prepared and characterized based on their size, morphology, vaccine releasing pattern,

MR targeting efficiency and the APCs activating efficiency. Finally, through intranasal immunization, the immune adjuvant ability of the formulation of Mannan-decorated TE microparticles with OVA as a model vaccine was investigated by measuring specific systemic and secretory antibodies in serum and mucosal secretions.

## **2. Materials and methods**

### **1) Materials**

Eudragit L-100 was purchased from Evonik Industries AG (Essen, Germany), mannan derived from *Saccharomyces cerevisiae* and ovalbumin (OVA) were purchased from Sigma (MO, USA), RPMI 1640 media, DMEM media and FBS were purchased from Gibco (MA, USA). All other materials were of analytical reagent grade.

### **2) Cell lines and experimental animals**

Raw 264.7 murine macrophage and Jaws II murine dendritic cell lines were obtained from American Type Culture Collection (VA, USA). Six-week-old BALB/c female mice (Samtako Inc, Korea) were used in immunization experiment and all experimental procedures were approved by the Institutional Animal Care and Use Committee of Seoul National University (Approval Number: SNU 140328-7) and followed the guidelines suggested by the committee.

### **3) Synthesis of thiolated Eudragit-L100 (TE)**

TE was synthesized according to method reported previously (Quan et al., 2008). Briefly, Eudragit L-100 was dissolved in dimethyl sulfoxide (DMSO) and the carboxylic acid moieties of the polymer were activated by *N, N'*-

dicyclohexyl carbodiimide (DCC) and *N*-hydroxyl succinimide (NHS) for one day before L-cysteine hydrochloride was added. The reaction mixture was incubated at room temperature for two days under nitrogen to avoid the oxidation of sulphhydryl groups by atmospheric oxygen. To remove the unbounded L-cysteine hydrochloride after reaction, the thiolated polymer was initially dialyzed against DMSO for one day, and then against distilled water for two days. After dialysis, the polymer solution was freeze-dried and the conjugate was stored in air tight containers at -20 °C until further use. The amount of cysteine in the conjugate was calculated by measurement of <sup>1</sup>H nuclear magnetic resonance (<sup>1</sup>H NMR) (Avance™ 600, Germany).

#### **4) Preparation of OVA-loaded TE microspheres (TEM) and OVA-loaded Man-TE microspheres (Man-TEM)**

50 mg/ml OVA was stabilized with Pluronic F-127 solution (1wt-%) to form an internal aqueous phase (W<sub>1</sub>). 0.2 ml of W<sub>1</sub> (10 mg OVA) was emulsified with an organic phase using an ultrasonic processor (Sonics, Vibra cells™) (2 output watts) for 1 min to form primary emulsion (W<sub>1</sub>/O). The organic phase (O) was consisted of 200 mg of TE dissolved in 5 ml of dichloromethane (DCM). The prepared primary emulsion (W<sub>1</sub>/O) was added drop by drop to 50 ml of 1 wt-% poly (vinyl alcohol) (PVA) solution (W<sub>2</sub>) as an external aqueous phase or mixture of 0.25 wt-% PVA and 0.5 wt-% mannan for Man-TEM, respectively. A homogenizer (Ultra-Turrax™ HomogenizerT25, IKA) was used

to continue emulsification at 11,000 rpm for 4 min to form  $W_1/O/W_2$  emulsion, which was then stirred with magnetic stirrer for 2–3 h at room temperature to allow the solvent to evaporate. OVA-loaded TEM and OVA-loaded Man-TEM were then collected and washed 3 times with distilled water after centrifugation at 14,000 rpm for 10 min at 4 °C. Followed by washing, the microspheres were lyophilized and finally stored at –70 °C to characterize them later. The unloaded TEM and Man-TEM were also prepared according to similar method. As a fact, it was not possible to make Eudragit microspheres because the Eudragit is not soluble in dichloromethane (DCM), which was used in dissolving thiolated Eudragit and resulted in non-comparison of immune activity between non-mucoadhesive microparticles and mucoadhesive ones.

## **5) Characterization of microspheres**

### **(1) Size and surface morphology**

The particle size distribution of the microspheres was measured using an electrophoretic light scattering spectrophotometer (ELS-8000, Otsuka Electronics Ltd., Japan). Prior to measuring, dispersing the freeze dried microspheres into sterile water, followed by vigorous vortexing and pipetting and then measuring the size distribution. To measure surface morphology of microspheres, the freeze dried microspheres were placed on a stud and were gold-coated using Sputter Coater (BAL-TEC/SCD 005), and observed using a field emission scanning electron microscopy (AURIGA, Germany).

## (2) Confirmation of decoration of mannan in TEM

To confirm mannan-coating in Man-TEM, FITC was conjugated with mannan. Briefly, 100 mg of mannan dissolve in 1 ml of DW was slowly mixed with 5 mg of FITC dissolve in 1 ml of DMSO. After stirring for 4 h at room temperature in dark condition, the reaction product was dropped into 8 ml of ethanol to remove the unreacted FITC. Mannan-FITC conjugate thus precipitated was washed and collected by centrifugation at 14,000 rpm for 10 min for 3 times. FITC-mannan decorated microspheres were prepared according to procedure described in the method 2.4 and FITC-mannan-decorated TEM were visualized by differential interference contrast microscope (DIC) and confocal laser microscope (SP8 X STED, Leica).

## 6) *In vitro* studies

### (1) Loading efficiency and loading content

The loading efficiency and loading content of microspheres were calculated using the following equations:

Loading efficiency (%)

$$= \frac{\text{amount of protein initially used} - \text{unloaded free protein}}{\text{amount of protein initially used}} \times 100\%$$

$$\text{Loading content (\%)} = \frac{\text{amount of protein in microspheres}}{\text{amount of microspheres}} \times 100\%$$

The loading efficiency and loading content of OVA into the microspheres were determined by quantifying the unloaded OVA in the supernatant using Micro Bicinchoninic Acid (BCA) protein assay method. The samples were analyzed in triplicate for each formulation studied.

(2) *In vitro* release of OVA from OVA-loaded TEM and OVA-loaded Man-TEM

10 mg of OVA-loaded TEM and OVA-loaded Man-TEM suspended in 1 ml of PBS (pH 7.4) into 1.5 ml microtubes were agitated up to 24 h at 37 °C with 100 rpm using shaking incubator. 100 µl of aliquot was withdrawn from the release medium and replaced by equal volume of PBS at each time point and the amount of released OVA was determined as cumulative release (wt. - %) against incubation times by Micro BCA protein assay method.

(3) Uptake of microspheres by APCs

For uptake of microspheres, RAW 264.7 cells were seeded into 6-well plates (Costar, IL, USA) at  $1 \times 10^6$  cells/well. After the cells reached 80% confluence, the medium was changed with 3 mg/ml of mannan for 20 min before the addition of microspheres to block MR (Jiang et al., 2008). Normal or MR-blocked cells were changed with the media with suspension of OVA-FITC-loaded TEM and OVA-FITC-loaded Man-TEM at a microsphere concentration of 0.2 mg/ml for 1 and 2 h. After incubation, the microsphere suspension was

removed and the wells were washed three times with 1 ml of PBS to remove the traces of microspheres left in the wells. After then, cells were detached from culture dishes and fluorescence-activated cell sorting (FACS) (BD biosciences, San Jose, CA) analysis was carried out to determine the uptake of microspheres by cells, with 10,000 cells measured routinely in each sample. The percentage of cells with internalized microspheres was calculated by the number of fluorescence events of FL1-height (FL1-H) signals.

#### (4) Internalization of microspheres

Phagocytosis of microspheres by macrophages and DCs was confirmed by confocal laser microscope (SP8 X STED, Leica).  $1 \times 10^6$  cells per well were cultured in 35 mm cover glass bottom dishes (SPL Life Science, Korea) two days prior to feeding with FITC-OVA-loaded microspheres at a concentration of 0.2 mg/ml. After incubation at 37 °C for 2 h, non-phagocytized microspheres were removed by washing three times with PBS and fixed with 4% paraformaldehyde at room temperature for 10 min. The nucleus was stained by Hoechst 33342 nucleic acid stain (Invitrogen, MA, USA).

### **7) *In vivo* studies**

#### (1) Immunization of mice

Nasal immunization study was conducted with 6 week-old female BALB/c mice which were assigned in five groups (n=5). Nasal administration groups

were immunized 4 times on days 0, 7, 14 and 21 respectively by dropping of 20  $\mu$ l PBS containing OVA (20  $\mu$ g) or OVA (20  $\mu$ g) -loaded TEM and OVA (20  $\mu$ g)-loaded Man-TEM into the nostrils during inhalation under anesthesia. Intramuscular administrations were immunized 3 times on day 0, 7 and 14 respectively by injection of 50  $\mu$ l PBS containing 20  $\mu$ g of OVA. Collection of serum, nasal wash, bronchoalveolar lavage (BAL), vaginal wash and intestinal wash were performed at day 42. Briefly, blood samples were collected from the caudal vein and centrifuged after clotting at 2,000 rpm for 10 min to prepare sera. BAL samples were harvested from the low respiratory tract of mice as described previously (Mizuno et al., 2006). Briefly, after the bronchi were exposed from the neck, a truncated needle with a syringe containing 400  $\mu$ l PBS was inserted through a small pore on each bronchus. Mucus exudation was harvested by a back and forth motion of the injector, and the supernatants were collected by centrifugation (3,000 rpm, 10 min and 4 °C). The nasal washes were collected by 300  $\mu$ l PBS through the trachea toward the nose. For vaginal wash sample preparation, after pipetting through the vaginal with 150  $\mu$ l of PBS for 15 times and the obtained fluids were collected by centrifugation (14,000 rpm, 10 min and 4 °C). For intestinal wash sample preparation, mid-ileal region of small intestines (3 cm) of the mice were immediately collected after sacrifice. The small intestines were homogenized in 500  $\mu$ l of ice-cold PBS and then tissues were removed by centrifugation (14,000 rpm, 10 min and 4 °C) and the supernatants were collected. All the samples were stored

at  $-70\text{ }^{\circ}\text{C}$  until analysis.

## (2) Measurement of OVA-specific antibodies

OVA-specific IgG, IgA, IgG1 and IgG2a antibodies in serum, sIgA antibodies in nasal wash, BAL, vaginal wash and intestinal wash were determined by the ELISA described previously (Kang et al., 2007). Briefly, 96-well plates (Thermo, USA) were coated with  $2.5\text{ }\mu\text{g}$  of OVA in  $100\text{ }\mu\text{l}$  of  $50\text{ mM}$  carbonate-bicarbonate buffer (pH 9.6) at  $4\text{ }^{\circ}\text{C}$  overnight and blocked with 1wt-% BSA at  $37\text{ }^{\circ}\text{C}$  for 1 h. Then, diluted serum, nasal wash, BAL, vaginal wash and intestinal wash were added into wells and incubated at  $37\text{ }^{\circ}\text{C}$  for 1 h. After incubation, HRP (horse radish peroxidase)-conjugated goat anti-mouse IgA, IgG, IgG1, or IgG2a (Santa Cruz Biotechnology, USA) were treated to each designated well at  $37\text{ }^{\circ}\text{C}$  for 1 h, respectively. TMB solution (Sigma-Aldrich, USA) was added to the wells for the HRP substrate, reaction was stopped after 10 min by adding  $2\text{ M H}_2\text{SO}_4$ , and absorbance was measured at  $450\text{ nm}$  using microplate reader (Infinite® 200 PRO, USA). Appropriate washing of each well was proceeded between the steps with PBS containing 0.05% of Tween 20 during the assays. The ELISA results are expressed as the OD value measured at  $450\text{ nm}$  for dilutions of 1:200,000 for serum IgG and IgG1, 1:5,000 for serum IgG2a and IgA, 1:500 for BAL IgA, 1: 10 for nasal wash IgA, 1:100 for vaginal IgA, 1:50 for intestinal IgA.

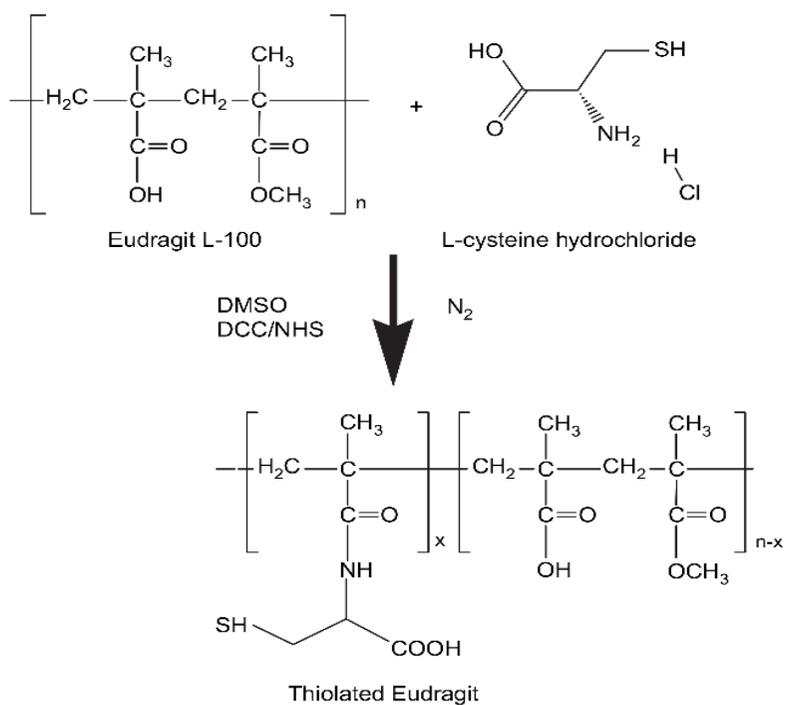
## **8) Statistical analysis**

Quantified results were expressed as the mean and standard deviation (SD). Statistical significance was assessed using a one-way analysis of variance (ANOVA) with post hoc Tukey Multiple Comparisons test.

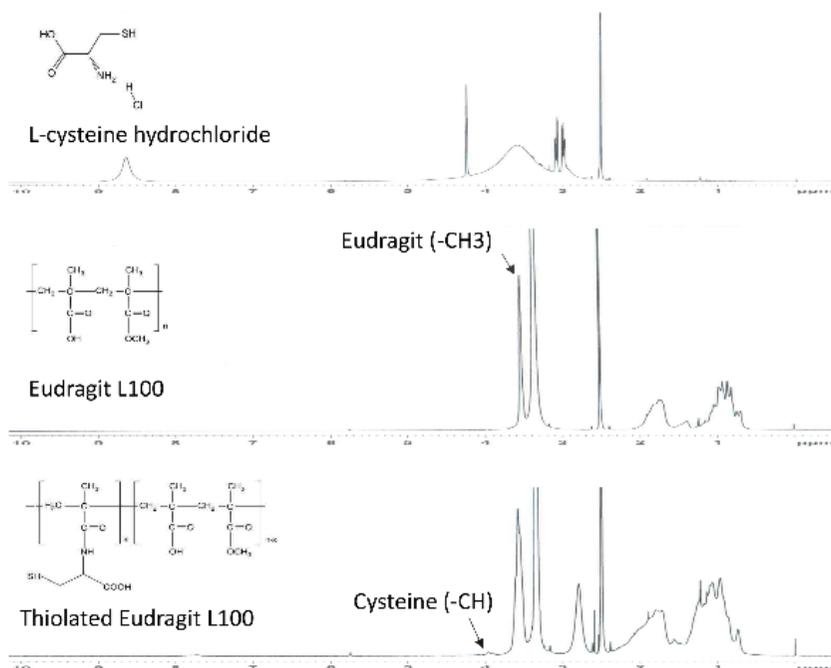
### **3. Results and discussion**

#### **1) Preparation of TE, TEM, Man-TEM, OVA-loaded TEM and OVA-loaded Man-TEM**

The reaction scheme for synthesis of TE is shown in Figure 34. The  $^1\text{H}$  NMR of cysteine, Eudragit L-100 and TE are represented in Figure 35. It was found that about 5.4 mol.-% of cysteine was conjugated with Eudragit L-100. TEM, Man-TEM, OVA-loaded TEM and OVA-loaded Man-TEM were prepared using double emulsion method described in materials and methods.



**Figure 34.** Reaction scheme for the synthesis of TE



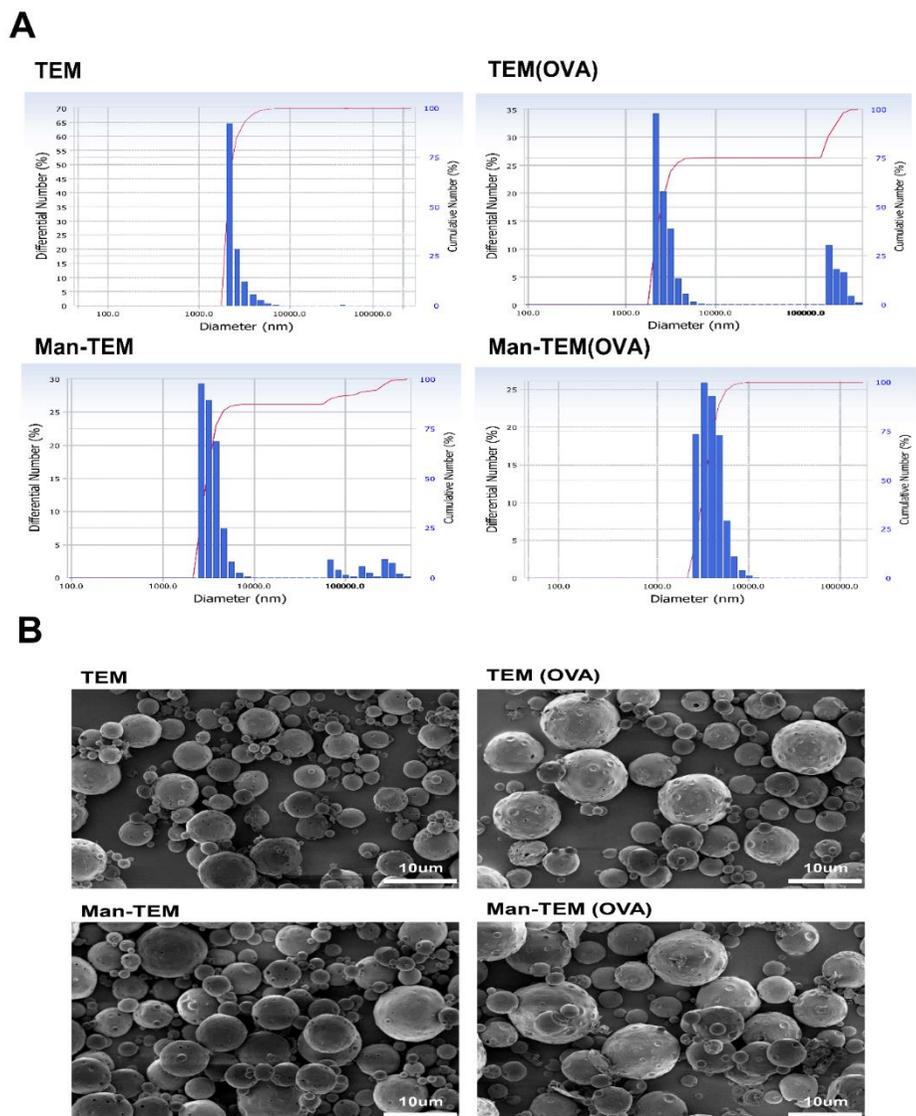
**Figure 35.** <sup>1</sup>H NMR spectra of cysteine, Eudragit L-100 and thiolated Eudragit L-100

## 2) Characterization of TEM and Man-TEM

### (1) Size and surface morphology

The sizes of TEM and Man-TEM measured by DLS are shown in Figure 36(A). The sizes of TEM and Man-TEM were  $2856.9 \pm 757.1$  nm and  $3386.6 \pm 827.8$  nm respectively, without much change in sizes after decoration of TEM with mannan due to small amount mannan coating. Similarly, the sizes of OVA-loaded TEM and OVA-loaded Man-TEM were slightly bigger than those of TEM and Man-TEM, respectively. The sizes of OVA-loaded TEM and OVA-loaded Man-TEM were  $3043.4.9 \pm 732.3$  nm and  $4431.9 \pm 1334.1$  nm. The morphology of TEM and Man-TEM observed by SEM are shown in Figure 36(B). TEM and Man-TEM have spherical shapes, with no change in shape after decoration with mannan. Recently, studies have demonstrated a significant correlation between physical particle size and retaining times in the human airways: with 24 h mucociliary clearance removed all particles larger than  $6 \mu\text{m}$  from the human airways, while particles with  $6 \mu\text{m}$  or smaller retained more than 24 h (Henning et al., 2010). Besides, it has been shown that microspheres' sizes influence their uptake by APCs and microfold cells (M cells). Particle smaller than  $5 \mu\text{m}$  may be transferred to the draining lymph nodes and spleen and stimulate both mucosal and systemic immune responses while particles in the range of  $5\text{-}10 \mu\text{m}$  tend to remain in Peyer's patches to stimulate primarily a mucosal immune response. Particles larger than  $10 \mu\text{m}$  are not likely to be taken

up at all (Oechslein et al., 1996). In this study, the mean size of microspheres was less than 5  $\mu\text{m}$ , and it is considered that this particular range of particle size can be internalized through phagocytosis by APCs playing a crucial role in initiating immune responses (Champion et al., 2008). Since the microparticles have similar size to the pathogen to which the immune system has evolved to combat, they are taken up like a pathogen by the DCs, which then mature and migrate to the nearby lymph nodes.

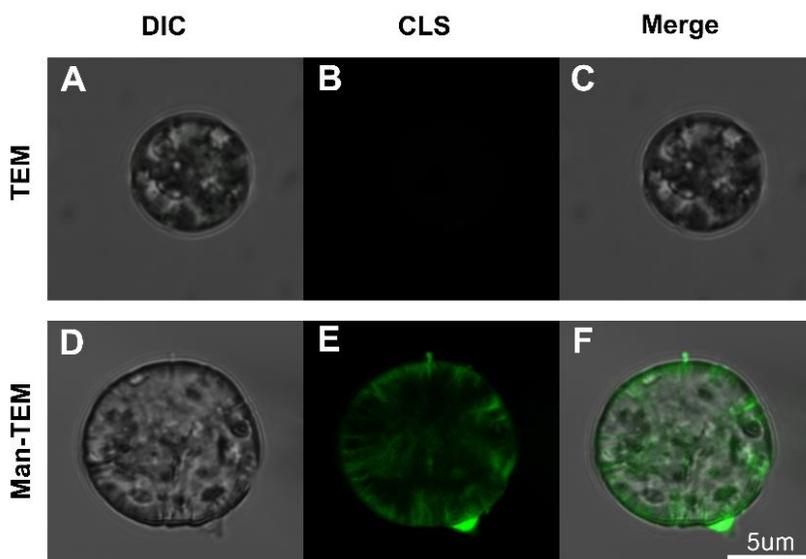


**Figure 36.** Size and morphology of microspheres. (A) Particle size distributions of TEM, Man-TEM, OVA-loaded TEM and OVA-loaded Man-TEM. (B) SEM photographs of TEM, Man-TEM, OVA-loaded TEM and OVA-loaded Man-TEM

( $\times 3,000$ ). Bar represents 10  $\mu\text{m}$ .

## (2) Confirmation of the surface decoration of mannan in Man-TEM

The surface decoration of TEM with mannan molecule was evaluated by confocal microscopy as shown in Figure 37. FITC-conjugated mannan was used for making FITC-Man-TEM. The confocal images indicated that the surface of the Man-TEM was homogeneously coated with mannan, as a stabilizer due to amphiphilic property of mannan, during making microspheres through  $W_1/O/W_2$  double emulsion. But, it was not possible to assay the amount of decorated mannan by elemental analysis or elemental spectroscopy for chemical analysis due to the small amount of mannan in the Man-TEM.



**Figure 37.** Confirmation of the surface decoration of mannan in Man-TEM. FITC-conjugated mannan was applied for making FITC-Man-TEM and the surface fluorescent signal was monitored by CLSM. First column: image by differential interference contrast (DIC) microscopy; second column: fluorescence by confocal laser scanning (CLS) microscopy and third column: combined image of DIC and CLS. Scale bar represents 5  $\mu\text{m}$ .

### 3) *In vitro* studies

(1) Loading efficiency and loading content of OVA loaded TEM and OVA loaded Man-TEM

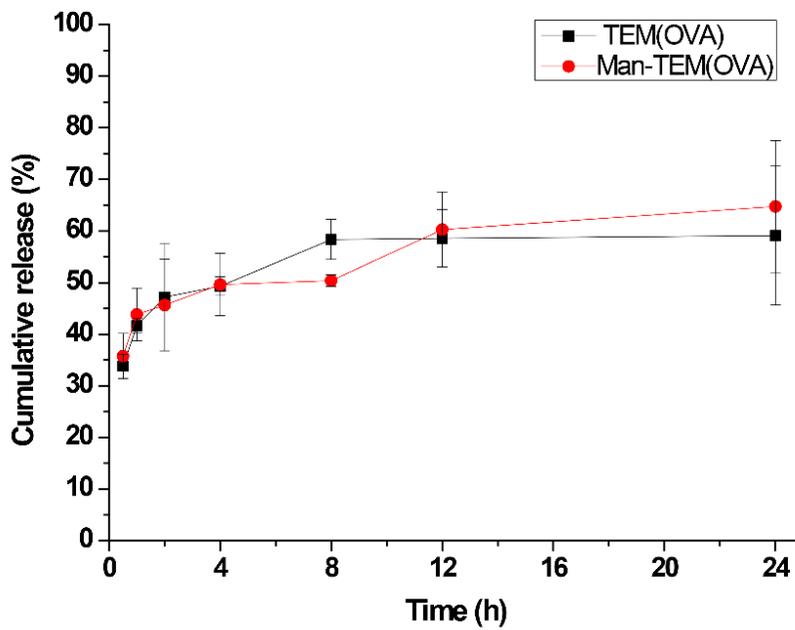
The loading efficiency and loading content of OVA in the OVA loaded TEM and OVA loaded Man-TEM are shown in Table 8. The loading efficiency and loading content of OVA in the TEM (OVA) were 53.93% and 2.70% by weight respectively, whereas the loading efficiency and loading content of OVA in the Man-TEM (OVA) were 50.50% and 2.52% by weight respectively, suggesting that there were no significant differences in the loading efficiency and loading content of OVA between the TEM and Man-TEM.

**Table 8.** Loading efficiency and loading content of OVA loaded TEM and OVA-loaded Man-TEM

<b>Microspheres</b>	<b>Loading efficiency (%)</b>	<b>Loading content (%)</b>
TEM (OVA)	53.93 ± 5.20	2.70 ± 0.26
Man-TEM(OVA)	50.50 ± 2.48	2.52 ± 0.12

(2) *In vitro* release of OVA from OVA-loaded TEM and OVA-loaded Man-TEM

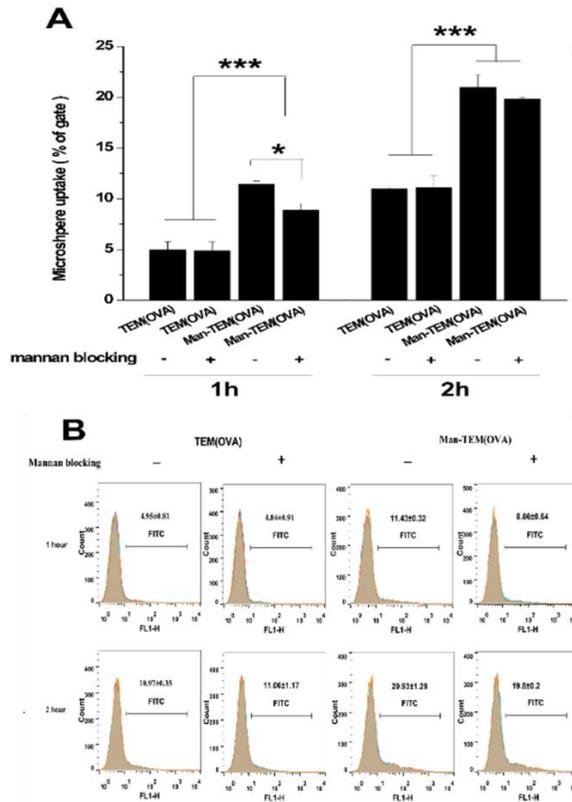
*In vitro* OVA release from the OVA-loaded TEM and OVA-loaded Man-TEM were performed in PBS (pH 7.4) incubated at 37 °C with mild agitation of 100 rpm and the amount of released OVA was evaluated by micro-BCA assay. The results represented in Figure 38 indicated that almost 45 wt-% of OVA was rapidly released from the OVA-loaded TEM and OVA-loaded Man-TEM within 2 h with not much difference in release pattern. We used Eudragit L-100, an anionic copolymer based on methacrylic acid and methylmethacrylate, which has pH sensitive property with a fast dissolution above pH 6.0. Thiolated Eudragit L-100 (TE) was modified from Eudragit L-100 with thiol groups using cysteine through amide conjugation, but it maintains the fast dissolution above pH 7.4. There has been a great deal of research on the relationship between microsphere and its residence time in nasal cavity. Jaganathan and Vyas's research team has been evaluated the nasal clearance rate of microsphere in rabbit nasal cavity using a surface modified PLGA microspheres which average size were less than 10 µm. Considering the average residence time of microspheres in nasal cavity is about 2 h (Jaganathan and Vyas, 2006), rapid release of OVA from the microspheres within 2 h might provide the antigens to APCs efficiently.



**Figure 38.** Release of OVA from OVA-loaded TEM and OVA-loaded Man-TEM at pH 7.4 and 37 °C (n = 3, error bar represents standard deviation).

### (3) Uptake of microspheres by APCs

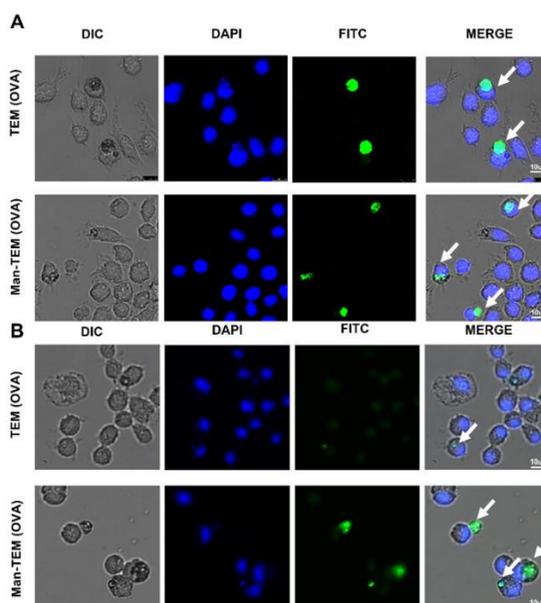
Cellular uptake of vaccines by the delivery system is very important for the clinical application of vaccines. Design of receptor-mediated endocytosis for targeting APCs using mannan as a specific ligand is one of approaches to enhance immune responses because the mannan has strong affinity with mannose receptors of APCs (Taylor et al., 1990). *In vitro* uptake assay was performed with two kinds of APCs - one is JAWS II and the other is RAW 264.7. The uptake of microspheres was checked in terms of incubation time and competition assay. Figure 39 shows uptake of microspheres by RAW 264.7 at 37 °C for 1 h and 2 h. The results indicated that the uptake of Man-TEM by both cell lines was much more than that of TEM with an increase of the uptake at 1 h and 2 h due to the specific interaction between mannan in the Man-TEM microsphere and mannann receptors of both cells. Importantly, the uptake of Man-TEM by both cell lines at 1 h decreased by pretreatment of mannan due to the blocking of mannann receptors by mannan whereas the uptake of TEM by both cells was not affected by pretreatment of mannan, indicating that the Man-TEM was uptaken by mannann receptor-mediated mechanism. However, the uptake of Man-TEM at first 1 h was not fully inhibited even after the treatment of mannan to block the mannann receptors. Similarly, there was no significant difference in the uptake of Man-TEM with or without blocking at 2 h, probably due to the depletion of mannann concentration in the cell media as the cells consume it.



**Figure 39.** Measurement of microsphere uptake by APCs using flow cytometry. Uptake of OVA-FITC-loaded TEM and OVA-FITC loaded Man-TEM by RAW 264.7 in 1 h and 2 h with or without MR inhibition respectively at 37 °C. (A) The impacts of microsphere uptake were displayed as percent of cells according to the histogram data of FACS analysis (B). (B) Histogram represented data. All histograms results are from one representative experiment of three independent experiments. Panel numbers indicate the mean percentages of positive cells (microsphere uptake) of respective samples. (n = 3, error bar represents standard deviation; \*p < 0.1, \*\*p < 0.05, \*\*\*p < 0.01, one-way ANOVA).

#### (4) Internalization of microspheres

To confirm the phagocytosis ability of mannan-decorated mucoadhesive microspheres by APCs, we treated FITC-labelled OVA-loaded microspheres to APCs. Figure 40 shows internalization of microspheres by RAW 264.7 and JAWS II cells at 37 °C after 2 h of treatment. It was found that more internalization of Man-TEM by both cell lines was observed than that of TEM, consistent with the results of cellular uptake of the microspheres observed by FACS.



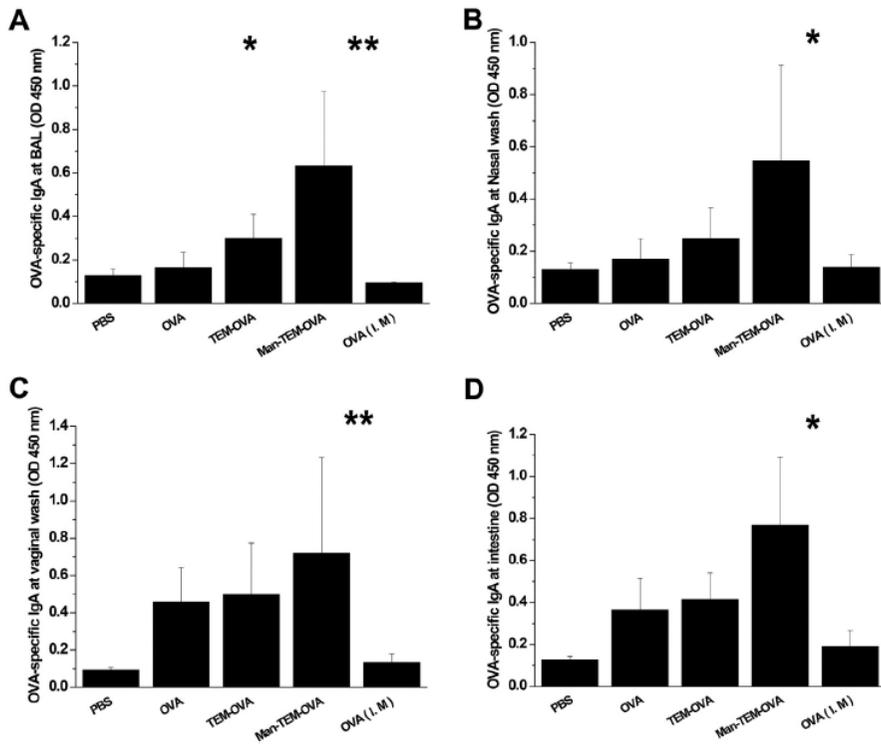
**Figure 40.** Confocal microscopic images of RAW 264.7 cells (A) and JAWS II cells (B) after 2 h culture with of OVA-loaded TEM and OVA-loaded Man-TEM at 37 °C. (Green: OVA-FITC loaded microsphere, blue: staining of nuclei with DAPI).

#### **4) *In vivo* immunization**

##### (1) OVA-specific mucosal immune responses

The main purpose of this study was to investigate the immune response of the vaccine released from the vaccine-loaded Man-TEM after nasal delivery, because the induction of immune responses by delivered vaccine play a major role in immunological defense at mucosal site (Neuhaus et al., 2014). Figure 41 shows anti-OVA sIgA values in the various mucosal sites after nasal vaccination with different formulations. The mice treated with OVA-loaded Man-TEM showed higher titers of anti-OVA sIgA antibody responses in the mucosal sites (BAL) compared with those treated with the OVA solution or intramuscularly delivered OVA. Particularly, mice treated with the OVA-loaded Man-TEM in the BAL displayed 3.8 fold higher IgA titer compared to those treated with the OVA solution, suggesting that mucoadhesive and mannan decorated Man-TEM enhanced the uptake of antigens through the respiratory epithelium due to the prolonged disposition of antigens in the respiratory cavity by the mucoadhesive property of the TE while mannan in Man-TEM enhanced uptake of vaccines due to the targeting to MR receptors in APCs. Regarding to use mouse model, it is often difficult to distinguish the immune responses induced by nasal delivery compared to these induced by the swallowing of the vaccine or the inadvertent channeling of the vaccine to the lung. So, it is inevitable that a significant amount of nasal-administered

microspheres might enter the lungs and it might contribute to the immune response in the lung. Unfortunately, we could not find significant differences between OVA loaded TEM and soluble OVA group. Moreover, the local and distal production of sIgA antibodies are the most important characteristics of nasally immunized vaccine. Our results indicated that nasally administrated particulates with vaccine induced immune responses efficiently at both local and distal mucosal sites, which can defect the pathogens invaded from other mucosal sites like vaginal or oral route. In contrast, intramuscularly vaccinated OVA group could not induce significant sIgA titer at local and distal mucosal sites compared to other nasally administrated groups.

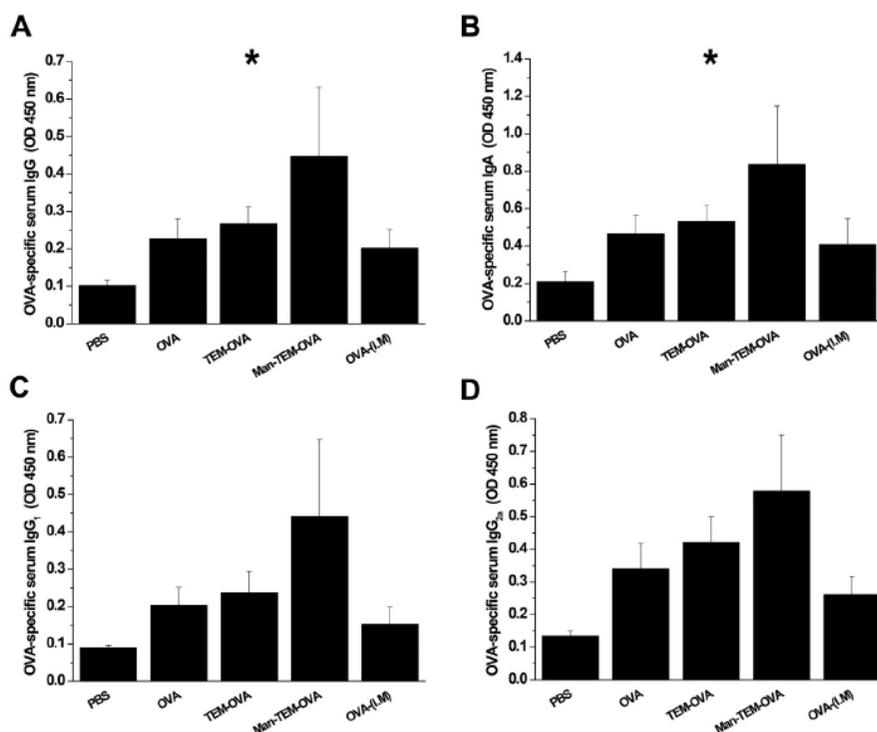


**Figure 41.** OVA-specific IgA performance in mucosal site at 6 weeks post immunization. OVA specific brochealveolar lavage (A), nasal wash (B), vaginal wash (C) and intestinal wash (D) IgA levels in mice immunized with each indicated formulations were analyzed by ELISA and then calculated as optical density (450 nm) (n = 5, error bar represents standard deviation; \*p < 0.1, \*\*p < 0.05, \*\*\*p < 0.01, one-way ANOVA).

## (2) OVA-specific serum IgG (IgG1 and IgG2a) responses

Serum isotype values of IgG, IgA, IgG1 and IgG2a are shown in Figure 42. Serum derived IgG also contributes to in lower respiratory tract immune defense (Renegar et al., 2004) and the production of the IgG1 isotype is associated with a Th2 type response and the IgG2a is associated with a Th1-type response (Dobrovolskaia and McNeil, 2007). The mice treated with OVA-loaded Man-TEM displayed higher titers of anti-OVA IgG and anti-OVA IgA compared to those treated with the soluble OVA, which is similar to the production of sIgA. In particular, mice treated with the OVA-loaded Man-TEM displayed 1.9 fold higher titers in IgG and 1.7 fold higher titers in IgA compared to those treated with the OVA solution. The anti-OVA IgG1 and IgG2a antibody responses in serum after nasal immunization are also shown in Figure 42(C) and 42(D). The anti-OVA IgG1 titers of mice treated with the OVA-loaded Man-TEM in serum 6 weeks after the first immunization were 2.14 and 1.86 fold higher compared to those treated with the OVA and OVA-loaded TEM, respectively, although there was no significant difference between them. Also, the anti-OVA IgG2a titers of mice treated with the OVA-loaded Man-TEM in serum displayed similar results as the anti-OVA IgG1, suggesting that these OVA-loaded Man-TEM appear to induce both Th1 and Th2 immune responses *in vivo*. The results of this study demonstrated that the nasal immunization of mice with OVA-loaded Man-TEM elevated immune

responses owing to mucoadhesive property of the thiolated Eudragit and enhanced APCs targeting by the mannan.



**Figure 42.** OVA-specific systemic immune responses at 6 weeks post immunization. OVA specific serum IgG (A), serum IgA (B), serum IgG1 (C) and serum IgG2A (D) levels in mice nasally immunized with each indicated formulations were analyzed by ELISA and then calculated as optical density (450 nm) (n = 5, error bar represents standard deviation; \*p < 0.1, \*\*p < 0.05, \*\*\*p < 0.01, one-way ANOVA).

#### **4. Conclusion**

Development of an effective nasal vaccine requires appropriate carriers or adjuvant systems that protect antigens from rapid nasal clearance by mucociliary apparatus and require to deliver antigens efficiently for alarming APCs. In this study, mucoadhesive microspheres decorated with mannan molecules (Man-TEM) were successfully prepared and evaluated *in vitro* and *in vivo* for vaccine delivery to mannose receptors of APCs. It was found that OVA-loaded Man-TEM were specifically bound with mannose receptors on murine macrophages and dendritic cells *in vitro* and induced more mucosal sIgA and serum antibody (IgG and IgA) than OVA-loaded TEM upon intranasal vaccination in mouse due to a specific interaction between mannose in the Man-TEM and mannose receptors on APCs. The intranasal immunization results strongly support the efficacy of Man-TEM as an adjuvant-carrier system for the effective induction of specific immune response.

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