A Dissertation

for the Degree of Doctor of Philosophy

Effects of fusion adjuvants to enhance immunogenicity of porcine epidemic diarrhea virus subunit vaccine

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Summary

Porcine epidemic diarrhea (PED) is one of the most catastrophic swine diseases characterized by severe diarrhea, vomiting, dehydration and even death. The mortality rate is as high as 80-100 % especially in neonatal piglets, which has potential to cause in tremendous economic burden to swine farms. Since, 1992, the PED outbreaks have been occurring every year in the South Korea. This outbreak became an endemic, which resulted substantial economic losses to Korean domestic swine industry. Since, early 2000, PED prevention program using inactivated and modified attenuated vaccines has been adopted, which derived a decline of PED virus (PEDV) associated diarrheal disease outbreak. However, the highest mortality of pigs from PEDV infection was recorded in the year 2018, demanding a development of an effective PED vaccine. The most recently used PED vaccines are live attenuated vaccine (LAV) or inactivated form, which carries issues related to its safety and cost-effectiveness. Especially, the effectiveness of some vaccines has become controversial after an outbreak of PED in the United States, which consequently stressed the demand for the development of a novel PED vaccine.

Subunit vaccines have many advantages over traditional LAVs, including safety, simplicity and mass production through *Escherichia. coli* (*E. coli*) system. In particular, the cost-effectiveness of subunit vaccines are essential interests in association with the economic objective of the livestock industry. However,

antigen candidates of PED subunit vaccine, such as S0, have been expressed as insoluble aggregates in E. coli, which are not suitable to be used as a vaccine. In order to overcome the low solubility and immunogenicity of the PED subunit vaccine, introduction of a molecular fusion adjuvant was necessary.

In the study I, the bacterial flagellin, *Vibrio vulnificus* FlaB, was introduced to S0, a truncated region of PEDV spike protein, and its conjugation effect on the enhancement of solubility and immunogenicity was examined. From previous researches, the conjugation effect of flagellin has been known to be varied depending on the target antigen or the direction of its conjugation, therefore flagellin was conjugated to the C- or N-terminal of S0 in order to generate S0-F and F-S0, respectively.

The flagellin conjugation to C-terminus of S0 displayed improved the solubility around 59 % in *E. coli* expression system compared to generic condition. In particular, flagellin conjugation improved the solubility of recombinant proteins up to 99 %, regardless of its fusion direction in the presence of trigger factor chaperone *tig*. Particularly, flagellin conjugated to the N-terminus of S0 (F-S0) induced significantly higher S0-specific antibodies in serum compared to other recombinant antigens including the flagellin conjugated to the C-terminus of S0 (S0-F) in mice. Therefore, it is evident that the superior ability of S0-F to activate the NLRC4/NAIP5 pathway may interfere with flagellin's adjuvancy and reduce serum S0-specific IgG despite of its advantage in a conjugated form.

In the study II, the conjugation effect of C4d as well as C3d on the solubility and immunogenicity was investigated. The complement fragment C4d was selected as a novel fusion adjuvant candidate, since it has high frequency of T cell epitope in its intra-structure evaluated through *in silico* study. C3d is a split product derived from the complement C3 protein. It has been proved that the human C3d has unusually high frequency of T cell epitopes. C3ds are responsible for many heterogeneous MHC class II molecules in human, and there are circulating memory type of autoreactive helper T cells that recognize these peptide:MHC class II complexes. These characteristics leads C3d to be utilized as a potent fusion adjuvant by donating T cell epitopes to antigen up-taking cells when it is conjugated with target antigen.

To explore another novel adjuvant, which act as C3d, various serum protein candidates were evaluated through in silico study to identify T cell epitope frequencies. Among serum protein candidates, C4d, a split fragment derived from C4, was selected as a novel fusion adjuvant. Following the selection, C3d or C4d was conjugated in tandem repeats with S0, to generate S0-mC3d1, S0-mC3d2 (two tandem repeats), S0-mC3d3 (three tandem repeats), S0-mC4d1, and S0-mC4d2. The conjugation of C4d to S0 improved the solubility of the recombinant proteins regardless of the existence of chaperone *tig*, while the conjugation of C3d had no effect on the solubility enhancement. Thus, both C3d and C4d conjugated to S0 (S0-mC3d1, S0-mC3d2, S0-mC3d3, S0-mC4d1, and S0-mC4d2) induced significantly higher S0-specific antibodies in serum

compared to S0 alone in mice. In addition, the involvement of mC4d-specific autoreactive T cell in the context the adjuvant effect of mC4d has been also confirmed. In contrast, C3d or C4d alone also elicited an adjuvant effect.

In study III, the recombinant protein candidates from the study I and II were compared at the same time to select the best suitable PED subunit vaccine protein in terms of immunogenicity. F-S0, S0-mC3d2 and S0-mC4d2 were selected as fusion adjuvant candidates considering their high immunogenicity. mC3d and mC4d was used as non-conjugated mixture control and also selected as adjuvant candidates as they have greater yield compare to other fusion proteins.

The adjuvanticity after intramuscular administration, all of the recombinant protein candidates induced high level of S0-specific antibody titers in serum compare to control group. From the production yield data, mC3d and mC4d were finally selected as the best immunogenic adjuvant for systemic PED subunit vaccine. Meanwhile, S0-mC4d2 displayed the highest S0-specific IgA titers at various mucosal areas compare to the other recombinant proteins after intranasal administration. Therefore, from the mucosal IgA data results, S0-mC4d2 was finally selected as the best candidate for the mucosal PED subunit vaccine. Meanwhile, following intranasal administration, mC3d or mC4d had no effect on humoral immune response contrast to intramuscular administration. Therefore, T cell epitope donation due to the conjugation of mC4d with target antigen S0 could be responsible for the enhancement of IgA in mucosal fluids,

which demonstrates that mC4d-specific T cell was inferred to be involved in the adjuvant effect of mC4d conjugation.

In this study, the best recombinant proteins for PED subunit vaccine has been selected. In particular, the novel complement fragment adjuvant C4d could be applied to not only to PEDV antigen, but also to various antigens for other vaccines. These findings provide critical insights for the development of a novel PED vaccine and the immunotherapeutics based on flagellin or complement fragments.

Key words: Porcine epidemic diarrhea, PED virus, PED vaccine, Recombinant protein, Fusion adjuvant, Subunit vaccine, Flagellin, Complement fragment, C3d, C4d

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List of Abbreviations

3C1: Poliovirus 3C-like proteinase

APC: Antigen presenting cell

BAL: Bronchoalveolar lavage

CFA: Complete freund's adjuvant

CTL: Cytotoxic T lymphocyte

DAMP: Damage associated molecular pattern

DC: Dendritic cell

E: Envelope protein

ELISA: Enzyme-linked immunosorbent assay

ERGIC: ER-Golgi intermediate compartment

FBS: Fetal bovine serum

Gfl: Growth factor-like motif

GMP: Good manufacturing practice

Hel: helicase motif

Hib: Haemophilus influenzae type b

HRP: Horseradish peroxidase

IB: Inclusion body

IF: Immunofluorescence

IFA: Incomplete freund's adjuvant

IHC: Immunohistochemistry

IL: Interleukin

IM: Intramuscular

IMD: Imidazole

IN: Intranasal

IPTG: Isopropyl-β-D-thiogalactopyranoside

IS: Insoluble fraction

LAV: Live attenuated vaccine

LB broth: Luria-bertani broth

LPS: Lipopolysaccharide

LTB: Heat-labile enterotoxin B

M: Membrane protein

Mb: Metal ion binding domain

MBL: Mannose binding lectin 2

MCP: Monocyte chemoattractant protein

MHC: Major histocampatibility complex

N: Nucleocapsid protein

NSP: Non-structural protein

OD: Optical density

ORF: Open reading frame

PAMP: Pathogen associated molecular pattern

pAPN: Porcine aminopeptidase N

PBS: Phosphate buffered saline

PCR: Polymerase chain reaction

PED: Porcine epidemic diarreha

PEDV: Porcine epidemic diarreha virus

Plp: Papain-like proteinase

PRR: Pattern recognition receptor

RdRp: RNA-dependent RNA polymerase domain

RNP: Ribonucleoprotein

RTC: Transcription complex

RT-PCR: Reverse-transcription polymerase chain reaction

S: Spike protein

SDS-PAGE: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

Th: Helper T cell

TLR: Toll-like receptor

TMB: Tetramethylbenzidine

TNF: Tumor necrosis factor

UTR: Untranslated region

VEE: Venezuelan equine encephalitis

VLP: Virus-like particle

VN: Virus neutralization

Review of Literature

1. Porcine epidemic diarrhea

1) Characteristics of porcine epidemic diarrhea virus

(1) Genetic structure and characteristics of PEDV

PEDV is the member of the genus *Alphacoronavirus* in the family of *Coronaviridae*. The PEDV is an enveloped virus ranges from 90 to 190 nm diameter in size (Song et al., 2015). The genome of the PEDV is composed of a single-stranded positive-sense RNA with approximately 28,000 bases in size (Chen et al., 2010;Song et al., 2015). The PEDV genome consists of 7 open reading frames (ORFs) that encode three non-structural proteins (ORF1a, ORF1b and ORF3) and four structural proteins (ORF2, ORF4-6) between 5'-end and 3'-end untranslated regions (UTRs) (Figure 1) (Kocherhans et al., 2001).

The large ORF1a and 1b cover about 70% at the 5′-end of genome for polymerase genes. The ORF1a translates for a replicase polyprotein 1a (pp1a) which encodes several functional motifs including growth factor-like motif (Gfl), papain-like proteinase (Plp), X domain (X) and poliovirus 3C-like proteinase (3C1), whereas the ORF1b encodes metal ion binding domain (Mb), a RNA-dependent RNA polymerase domain (RdRp) and a helicase motif (Hel). A group of 16 non-structural proteins (nsp1-16) is produced from the post-translational cleavage of pp1a and pp1ab, a frameshift polyprotein of pp1a, by internal

proteases (Sekhon et al., 2016). The ORF3 is located between S and E structural protein and encodes a glycoprotein gp3 which is related to virulence of the PEDV as an ion channel (Wang et al., 2012).

A set of 4 genes encode structural proteins: 150-220 kDa glycosylated spike protein (S), 7 kDa envelope protein (E), 20-30 kDa membrane protein (M) and 58 kDa nucleocapsid protein (N) (Horzinek et al., 1988). The S protein is a major type I transmembrane glycoprotein on virion surface. It can be divided into S1 (1-789aa) and S2 (790- 1,383aa) domains which are responsible for the cellular receptor binding and membrane fusion of the virus, respectively (Lee et al., 2011). The E protein is a hydrophobic transmembrane protein which plays a major role on the morphogenesis and viral assembly. The M protein is the most abundant protein in the PEDV envelope and has a potential role in viral assembly. The N protein is a basic phosphoprotein associated with the genome which provides a structural basis for the helical nucleocapsid. It also antagonizes beta interferon production by host cells to circumvent the host's antiviral immunity (Ding et al., 2014).

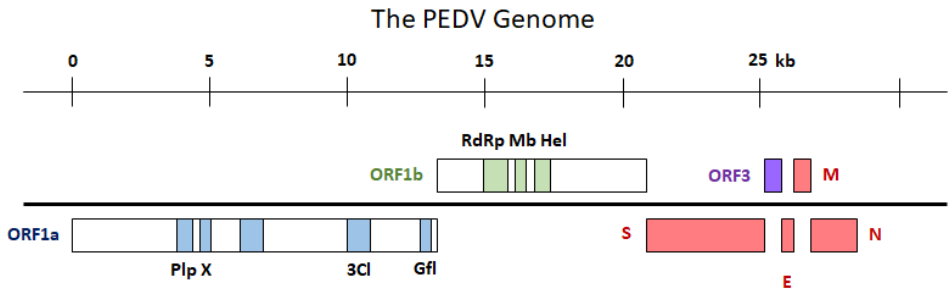
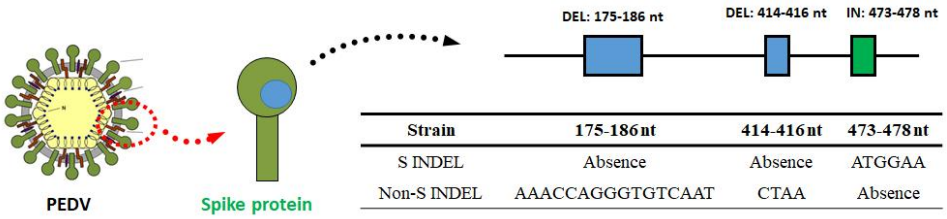


Figure 1. Schematic representations of PEDV genome organization.

A schematic view of the genome of PEDV which consists of 7 ORFs that represented as boxes: ORF1a, ORF1b, S (spike protein), ORF3, E (envelope protein), M (membrane protein), N (nucleocapsid protein). Plp, papain-like proteinase; 3C1, poliovirus 3C-like proteinase (3C1); Gfl, growth factor-like domain; RdPp, RNA-dependent RNA polymerase; Mb, metal ion-binding-domain; Hel, helicase.

(2) PEDV strains

PEDVs exhibit significant genetic diversities throughout the world. The categorization of global PEDV strains into classical, S INDEL strains, and emerging non-S INDEL strains was proposed based on their virulence in piglets and features of the spike (S) gene (Lin et al., 2016a). Generally, S INDEL strains have deletion at 175-186 nt, 414-416 nt and insertion at 473-478 nt in the S gene compare to non-S INDEL strains. Strains with either insertions or deletions in the S gene as the classical CV777 strain that have appeared since the 1970s are considered S INDEL (Wang et al., 2016) while strains without insertions or deletions in the S gene are called non-S INDEL. Non-S INDEL strain, also referred to as 'US prototype' is the highly virulent PEDV related with severe PED outbreaks worldwide (Vlasova et al., 2014). In contrast, S INDEL strain is milder PEDV variant in terms of lower mortality. As non-S INDEL strains are the strains associated with pandemic PED outbreaks and the strains in current circulation (Jarvis et al., 2016), special measurements to protect pigs from these strains are necessary.



Strain	175-186nt	414-416nt	473-478nt
S INDEL	Absence	Absence	ATGGAA
Non-S INDEL	AAACCAGGGTGTCAAT	CTAA	Absence

Figure 2. Insertion or deletion sequences between S INDEL and non-S INDEL strains.

Part of the S genes sequence between S INDEL and non-S INDEL strains were compared. S INDEL strains have deletion at 175-186 nt, 414-416 nt and insertion at 473-478 nt at the same time compare to non-S INDEL strains.

(3) Life cycle of PEDV

PEDV has a confined tissue tropism and replicates efficiently in enterocytes and epithelial cells of porcine small intestine. Porcine aminopeptidase N (pAPN), the cellular receptor for PEDV (Li et al., 2007) which is mainly expressed on the surface of epithelial cells of small intestine, is recognized by the N-terminal region of the PEDV spike protein S1 domain (Lee et al., 2011). The viral genome is released into the cytoplasm after the fusion of the plasma membrane with viral envelope through S protein, and subsequently translated to produce ppla and pp1ab immediately. These polyproteins are cut into 16 non-structural proteins which comprises the replication and transcription complex (RTC) that participates in the minus-strand RNA synthesis using genomic RNA. Both minus strands of sub- and full genomic length are produced and utilized to synthesize sg mRNAs and full-length genomic RNA. The envelope S, M and E proteins are produced and transported into the ER and displayed in the Golgi apparatus. The helical ribonucleoprotein (RNP) complexes are formed as a result of the interactions between the N protein and newly synthesized genomic RNA. At the ER-Golgi intermediate compartment (ERGIC), the progeny virion is assembled by budding of the preformed RNP, and subsequently released by the exocytosis-like vesicles with the plasma membrane (Figure 3) (Lee, 2015).

PEDV destroys target enterocytes as a consequence of massive necrosis and apoptosis, subsequently leads to villous atrophy, vacuolation and a severe reduction of enzymatic activity, all of which are responsible for the interruption

of digestion and absorption of electrolytes and nutrients. This sequence of events consequently causes watery diarrhea followed by dehydration in piglets (Kim and Lee, 2014).

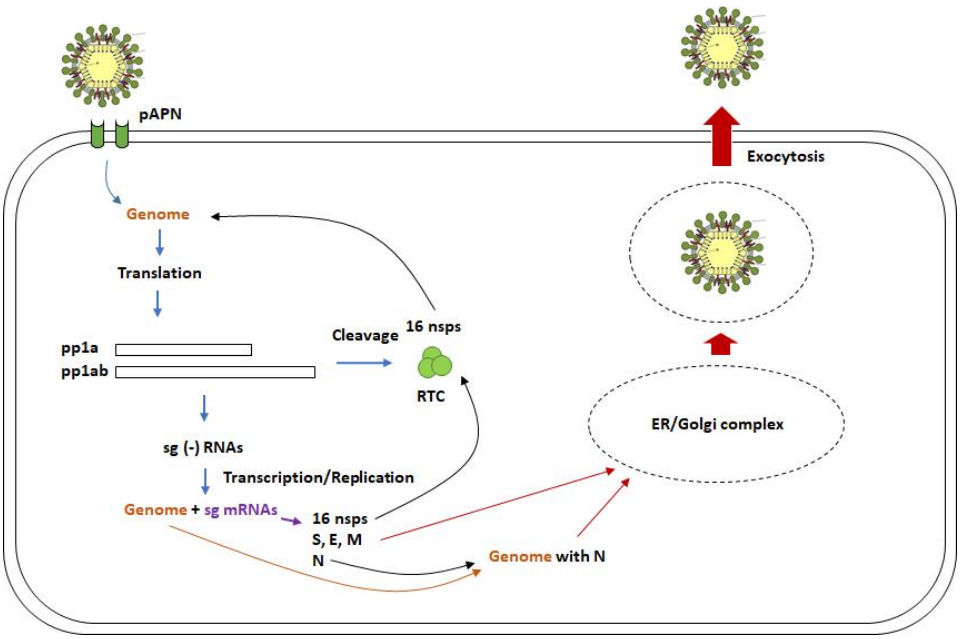


Figure 3. Life cycle of PEDV.

Overview of the PEDV replication cycle.

2) Symptoms and epidemiology of PED

(1) Clinical symptoms

PED is a one type of viral enteritis in swine which is caused by PEDV. Diarrhea is the major symptom of PED in consistent with the name of the disease. PED presents with other diverse clinical signs, which include vomiting, dehydration, weight loss, and anorexia in addition to diarrhea (Pensaert and De Bouck, 1978). Though the PEDV can infect any age of swine, the severity differs according to age (Shibata et al., 2000). Importantly, neonatal pigs are vulnerable for PEDV infection because it commonly induces death of piglets as a result of watery diarrhea and dehydration. Indeed, over 1,000,000 piglets have died from PEDV infection with a death rate as high as 90 % (Sun et al., 2012). Such significant rate of morbidity and mortality in piglets is related with huge economic losses in swine industry.

(2) Epidemiology

PED was first recorded in Europe in 1971 (Oldham, 1972). The coronavirus-like strain CV777, the prototype strain of PEDV, was isolated from swine showing diarrhea in a PED outbreak in Belgium in 1976 (Pensaert and De Bouck, 1978). PEDV spread throughout Europe during the 1970s and 1980s. The number of PED outbreaks, however, reduced markedly in the region during the 1980s and 1990s. Since the 1980s, only a few severe outbreaks have been reported in Europe, however, PEDV spread to Asia and became endemic in pigs in Asian countries, such as Korea, Japan, Taiwan, China and Thailand (Song et al., 2015). Until 2013, PED was thought to have been confined to Asian countries. In 2013, however, an outbreak of PEDV infection occurred for the first time in the United States and PEDV had spread to Canada and Mexico within 1 year (Vlasova et al., 2014). Subsequently, US-like PEDVs emerged or re-emerged in some Asian and Europe countries across the Pacific Ocean, also within 1 year of the US outbreak (Lin et al., 2014;Lin et al., 2016a). The schematic view of the disease spread upon timeline is shown in Figure 4.

The Chinese PEDV strain reported in 2012 was genetically related to the PEDV strain isolated in the United States (Huang et al., 2013). Interestingly, the PEDV strains isolated from Korea and Taiwan after the US outbreak were genetically related to the US PEDV strain (Cho et al., 2014).

The US outbreak was a notable turning point in PED research because many

veterinary scientists including the US scientists have put efforts on the development of effective PED vaccines which reflect the genetic characteristics of the PEDV strain isolated during the US outbreak.

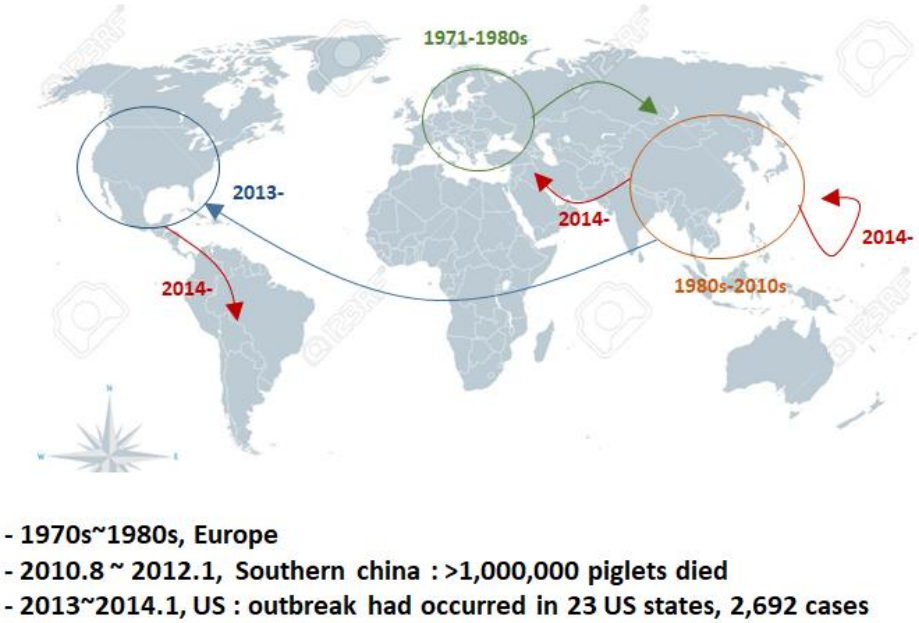


Figure 4. PED progression upon timeline.

PEDV spread throughout Europe during the 1970s and 1980s, subsequently spread to Asia and became endemic in swine in Asian countries. A severe PED outbreak occurred in Southern China in 2010, which was characterized by high mortality in neonatal piglets. PED outbreaks were reported in the USA in 2013 and the responsible emerging strains of PEDV were spread throughout the world Asian and European countries.

(3) Outbreaks in South Korea

The first PED case in South Korea was confirmed in 1992 (Kweon et al., 1993) though some researchers insisted that PEDV already existed since 1987. PED outbreaks have occurred every year since 1992 and became endemic, resulting substantial economic losses to domestic swine industry until 2010. The majority of farms seemed to be affected with endemic PEDV infection as 91.8 % of tested farms in broad periods (30–150 days of age) had sero-positive pigs through a serological study in 2007 (Park and Pak, 2009). Since early 2000s, the vaccination program for PED prevention using inactivated and modified attenuated vaccines based on SM98-1 or DR-1 has been introduced. Though these measurements resulted in a decline of PEDV-associated diarrheal disease outbreaks compared to non-vaccinated periods, incessant PED epidemics in vaccinated farms have occurred. In 2010-2011, after South Korea was hit by severe outbreaks of the foot-and-mouth disease (FMD), more than 3 million pigs have been slaughtered and the prevalence of PEDV infections was declined with only intermittent outbreaks from 2010 to 2013. After November 2013, however, the severe PED epidemics occurred significantly again and affected more than 40 % of pig farms (Lee and Lee, 2014). In 2018, the highest number of died pigs from PEDV infection was recorded, providing a necessity for the development of an effective PED vaccine. The number of died pigs and farms as a result of PEDV infection in South Korea is listed in Table 1.

Table 1. Current outbreak of PED in South Korea.

Year	2010	2011	2012	2013	2014	2015	2016	2017	2018
Head	3092	289	10	4703	33646	17657	8963	8004	34966
Farm	12	5	1	12	169	95	82	101	221

The information in table were supplied by Korea Animal Health Integrated System

3) Diagnosis and control measurements of PED

(1) Diagnosis

As signs of the PEDV infection were not unique and were indistinguishable from other viruses such as Transmissible gastroenteritis virus (TGEV) and porcine deltacoronavirus, different detection methods to confirm the presence of PEDV or its antigens other than diagnosis based on clinical signs or histopathological lesions are necessary. These methods include immunohistochemistry (IHC) tests, immunofluorescence (IF), enzyme-linked immunosorbent assays (ELISA) and diverse reverse-transcription polymerase chain reaction (RT-PCR) techniques. Diagnosis based on nucleotide detection confers rapidness and sensitivity. S gene sequencing as well as conventional and real-time RT-PCR systems support monitoring of genetic diversity of PEDV among diverse isolates. Detection of PEDV antibodies through a number of serological assays are essential not only for monitoring PEDV infection in affected farms under endemic situation, but also for monitoring the immunity level after sow immunization. When estimating titers of protective antibodies in serum and colostrum which piglets would receive from sows, virus neutralization (VN) test could be useful. However, time-consuming aspect together with complexity of this assay makes another approaches attractive. IFA and indirect ELISA methods based on S antigens are equally specific but easier to perform and less time-consuming than VN test. IFA and ELISA approaches based on whole virus particles are useful to determine infection status in weaner

to finisher pigs in affected farms.

(2) Control measurement

Strict biosecurity, blocking the entrance of virion into swine farms, is one of the best measurements for control and prevention of acute PED outbreaks (Lee, 2015). It can be accomplished by minimizing the entrance of any material, which could be in contact with the virion. As most disinfectants can inactivate the PEDV, they should be utilized to all personnel, fomites, and external visitors that could be contact with PEDV. Other biosecurity measurements other than disinfection include restricting contact between the farm interior and trailers or drivers during the transporting process at the pig farm or between the slaughter facilities and drivers when unloading (Park and Lee, 2009). All newly arriving animals should be isolated to monitor their health status for a certain period.

In countries where PED is considered an epidemic or endemic, vaccination of sows is the best and fundamental strategy in order to control and prevent any additional damage caused by the disease. As piglets, the most vulnerable targets for PEDV infection, are so young that could not evoke adequate immune response after vaccination, protective immunity conferred by maternal antibodies via colostrum and milk is necessary.

The majority of commercial PED vaccines fall into the category of inactivated or live attenuated vaccines (LAVs) (Song et al., 2015). PV-5, a Vero cell-attenuated vaccine, was developed first in Japan and subsequently became available in South Korea. Additionally, the method of cell-culture adaptation was

also utilized to attenuate South Korean virulent PEDV strains, DR-13and SM98-1 (Kweon et al., 1999;Song et al., 2007). While these types of vaccines have staying power due to positive results in experimental conditions, their overall effectiveness in the field and concerns with the well-being and safety issues are still being debated. In addition, the effectiveness of some of those commercial PEDV vaccines used in Korea has become controversial after an outbreak of PED in the United States (Song et al., 2015). The low to moderate effectiveness of current commercial PEDV vaccines seems to be due to genetic differences between field strains and vaccine (Ayudhya et al., 2012) which are the reason for the failure of cross-protection to recent PEDV strains after vaccination. These disadvantages of vaccines manipulating viral particles lead researchers to develop other kinds of vaccines.

Currently, other types of vaccines have been developed as new candidates of PED. These include RNA/DNA vaccines as well as subunit vaccines manufactured from mammalian cells. In 2014, an RNA particle vaccines based on alphavirus vector for PED was developed by US company, Harris Vaccine Inc. (Kim et al., 2016). They manipulated Venezuelan equine encephalitis (VEE) virus, one of Alphavirus members, as a vaccine vector to produce heterologous PED antigens. Although this vaccine can make it possible for vaccinations in emergency situations as it can be produced faster than traditional one, it is still expensive to use as an alternative. Recombinant PED subunit vaccines also have been vigorously studied among researchers. Molecular fusion adjuvant

conjugated PED subunit vaccines have been developed and tested in pig which are produced from mammalian cells(Chang et al., 2018;Li et al., 2018). Commercially available PED vaccines and their disadvantages are listed in Table 2.

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Table 2. Available PED vaccines and their limitation.

***Strain*/Name**	**Type**	**Disadvantages**
KPEDV-9 *P-5V* *PEDV DR13* *SM-98* *SM-98*	LAV Inactivated vaccine	▪ Expensive, time-consuming, labor intensive ▪ Low efficacy ▪ Safety issues ▪ Strain evolutions problem
Porcine Epidemic Diarrhea, RNA	RNA vaccine	▪ Costly to produce ▪ Lower immunogenicity than LAV
PED-CUP-B2014/ IMMUNIS-PED-M *G2b*/Zoetis	Inactivated vaccine Inactivated vaccine	▪ Risk of being infectious

2. Development of recombinant subunit vaccine for PED

1) Subunit vaccine

(1) Subunit vaccine

A vaccine is a biological preparation that activates adaptive immunity to a particular disease, mostly infectious disease as well as cancer, in host. A vaccine generally contains an agent that resembles a disease-causing pathogen and is often made from attenuated or killed forms of the pathogen, its toxins, or a component of it. The agent stimulates host immune system to recognize the agent as a non-self, and subsequently to recognize and destroy any of the microorganisms related to that agent that host may encounter in the future. Vaccines can be classified as prophylactic and therapeutic vaccine. Prophylactic vaccine is the vaccine for the purpose of prevention against future infection, while therapeutic vaccine is the vaccine for the purpose of cure after infection (Plotkin, 2003).

The administration of vaccines is the most effective method of preventing infectious diseases (Gellin, 2000). Worldwide eradication of smallpox and the restriction of diseases including polio, measles, and tetanus are the result of vaccine development. Vaccinations of influenza vaccine, the HPV vaccine, and the chicken pox vaccine also have been proven as an effective measurement.

There are many types of vaccines as described in Table 3. Vaccines are

macroscopically classified as whole body vaccine and recombinant vaccine. Whole body vaccines are divided into the live attenuated vaccine and inactivated (or killed) vaccine. The attenuated vaccines contain living microbe that has been weakened by approximately hundred rounds of cultivation under conditions that reduce their virulent properties while inactivated vaccines contain inactive micro-organisms that have been killed by chemical, radiation, or heat. Though whole body vaccines have a capacity to induce high immune responses as they contain all components of pathogen in addition to replication in live attenuated vaccine case, they have several disadvantages such as safety, time-consuming and labor intensive aspect of production (Jeong and Seong, 2017). Recombinant vaccines use part instead of entire whole microbe and are classified as subunit vaccine, DNA vaccine and recombinant vector vaccine depending on the form it conveys. Subunit vaccines contain specific parts of the antigen as a protein form produced by host cells such as bacteria, yeast, or mammalian cell while as a DNA form for DNA vaccine case. Recombinant vector vaccines deliver DNA encoding the antigen as DNA vaccine, however, use an attenuated virus or bacterium as a vector. Subunit vaccines also can be produced by gathering antigens from cultivated microbes after purification which make them to be excluded from the classification of recombinant vaccines in some cases. Recombinant vaccines can overcome the disadvantages of whole body vaccines as they do not utilize live micro-organisms nor time-consuming attenuation process, however, they tend to elicit immune responses lower than whole body

vaccines because of confined component they have.

Table 3. Pros and cons of several types of vaccines.

	Whole body vaccines		Recombinant vaccines		
	Live attenuated vaccine	Inactivated vaccine	Subunit vaccine	Nucleic acid-based vaccine	Recombinant viral vectors
Pros	Good efficacy	Better safety than LAV	Good safety	Good productivity Good stability	Good efficacy
Cons	Safety issue	Safety issue	Poor efficacy	Poor efficacy	Vector immunity

Instead of using the entire microbe, subunit vaccines include only the part of microbe that stimulate the immune system of the host (Moyle and Toth, 2013). These vaccines use not only protein subunit but also even epitopes, specific parts of the antigen that B cells or T cells recognize and bind to, in some cases. Because subunit vaccines include only the specific antigens excluding all the other components that make up the pathogen, the chances of adverse effects such as viral reversion are low. By virtue of safe aspects, many subunit vaccines are preferentially used in children and elders (Tristram et al., 1993;Falsey and Walsh, 1997). In addition, subunit vaccines have a high productive efficiency as their design can be quickly done which make it possible for vaccinations in emergency situations to and as large-scale production process of them is

relatively simple and easy. However, the immunogenicity they stimulate is low *per se* which necessitate the use of additional measurements such as adjuvant. A recombinant subunit vaccine has been made and commercialized for the hepatitis B virus, influenza virus, human papillomavirus etc. (Moyle and Toth, 2013)

Though subunit vaccines can be obtained from the microbe after cultivation, they can be manufactured in host cells such as bacteria, yeast, or mammalian cells by using recombinant DNA technology and especially called as "recombinant subunit vaccine". Simplified schematic view of recombinant subunit vaccine production is listed in Figure 5.

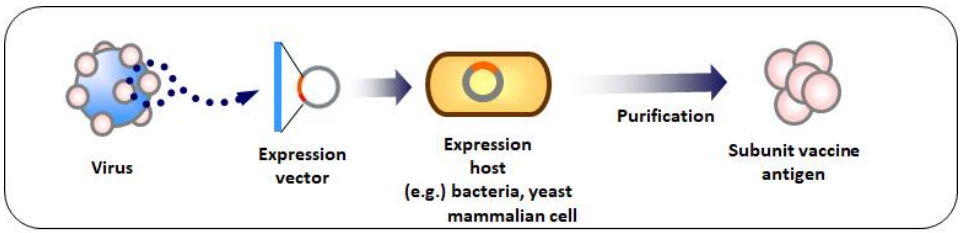

Figure 5. Schematic view of recombinant subunit vaccine production.

The DNA sequence encoding the antigen from the virus can be inserted into expression vector. This vector can be transformed into expression host such as bacteria which produce target antigen as a subunit vaccine component.

(2) PED subunit vaccine

In spite of their advantages, recombinant PED subunit vaccines have been studied with a relative slow pace which may be attributed to the difficulties of antigen production in bacteria. In fact, the spike protein of PED virus need special measurements to produce in bacteria (Piao et al., 2016b). Instead, many researchers have tried to develop recombinant PED subunit vaccine through another host including yeast, insect cell and mammalian cells.

N Makadiya and colleagues produced S1 domain of PEDV S protein as a target antigen by using yeast, insect cell and mammalian cell (Makadiya et al., 2016). Although S1 antigen expressed by mammalian cell has the highest yield with N-glycosylation, it failed to provide complete protection against PEDV challenge. To enhance the efficacy of PED subunit vaccine, Q Li and colleagues have adapted flagellin fusion adjuvant to COE region of PEDV spike protein (rSF-COE-3D) and tested its efficacy through challenge experiment (Li et al., 2018). They suggested that rSF-COE-3D could support a better protection through the improved production of mucosal IgA in feces, serum IgA compared to COE alone. YC Chang and colleagues produced trimeric spike subunit vaccine from HEK 293 cells and immunized through intramuscular (IM) route together with heat-labile enterotoxin B (LTB) to test protective effect in pig after challenge (Chang, Chang et al. 2018). They suggested that the development of a vaccine regimen for inducing mucosal immunity is necessary because the IM

immunization with LTB as the adjuvant supported insufficient protection. Plant cells have also been utilized to produce PED antigen as a subunit vaccine. Z Khamis produced elastin-like polypeptide fusion PED membrane protein through *Nicotiana benthamiana* (Khamis, 2016). Though he did not evaluate the efficacy to enhance the immune response in animal, however, the production of virus-like particles (VLPs) were confirmed for the first time for PED subunit vaccine.

2) Production of recombinant protein

(1) Host systems for heterologous protein expression

Expression of numerous genes in various systems have been explored with the advent of recombinant DNA technology to produce many therapeutics and vaccines as a form of recombinant proteins (Gomes et al., 2016). Accumulated researches suggest that as each expression system has unique characteristics for the production of recombinant protein, it is necessary to consider suitable expression system for the protein of interest. Bacterial expression system is widely used because of its several advantages such as simple media requirement, or high level of recombinant protein expression yield with rapidness. However, there are some limitations such as improper folding of protein which subsequently produce insoluble aggregates rather than soluble protein, lack of post-translational modifications, or the accumulation of endotoxins. Expression systems utilizing mammalian cells can overcome these problems. Proper protein folding, post-translational modifications including glycosylation with patterns the same as human, and product assembly, which are important for fully functional activity are possible in this system, however, complicated aspect of technology together with high cost is major disadvantages of it. Yeast systems is considered the hybrid of bacteria and mammalian cell system as they adapt advantages of each system. As they are eukaryotes, they confer post-translational modifications despite the glycosylation patterns are not similar to human. Rapid

growth in low cost medium is guaranteed as they seem to be the simplest eukaryotes. Though the production yield of this system is better than mammalian system, however, the cost-effectiveness is markedly lower than bacteria system. The merits and demerits of each system are listed in Table 4. For the development recombinant subunit vaccine for livestock industry, cost is considered the most important factor which make it an attractive choice to select bacteria as an expression system. *Escherichia coli* (*E. coli*) is the representative host of bacteria system.

Table 4. Merits and demerits of different expression host systems.

Host system	Merits	Demerits
Escherichia coli	Easy Quick (rapid growth rate) Economical (capacity for continuous fermentation)	Absence of machinery for removing introns Codon bias Lack of post-translational modifications Production of proteins in the insoluble form or in the form of inclusion bodies Degradation of proteins Accumulation of endotoxins
Yeast system	Rapid growth in low cost medium Appropriate post-translational modifications No endotoxins production	Hyperglycosylation of proteins Codon bias
Mammalian cells/systems	Proper protein folding Appropriate post-translational modifications and product assembly Proper glycosylation	High cost Complicated technology Potential contamination with animal viruses

(2) Protein solubility

Recombinant expression of proteins in *E. coli*, despite of significant benefits in terms of ease-of-use, cost, often results in the misfolding of the protein into inclusion bodies (IBs), biologically inactive aggregates (Fahnert et al., 2004). The high expression levels of *E. coli* along with the nature of the recombinant protein which is not originated from the expression host and considered "non-native" appears to be the main reason for the aggregation of the protein in *E. coli* (Lilie et al., 1998).

To deal with this problem, many researchers have tried to utilize IBs because they have some positive attributes such as very high levels of protein expression, ease of isolation, resistance against cellular protease (Singh et al., 2005). In this regard, many refolding methods have been explored to recover purified IBs into soluble proteins. (Singh et al., 2015). However, it is typically difficult to optimize the refolding condition which mostly results in refolding of small fraction of the input protein. Moreover, purification of the refolded fraction is also hard to optimize, making it as a best choice to enhance soluble expression and prevent IBs formation.

To prevent IBs production, various approaches have been suggested including lowering physiological rate by modulating induction temperature or inducer concentration, and utilization of solubility enhancing tags (Swartz, 2001). Strategies to get soluble proteins in the course of recombinant protein production

in *E. coli* are listed in Figure 6.

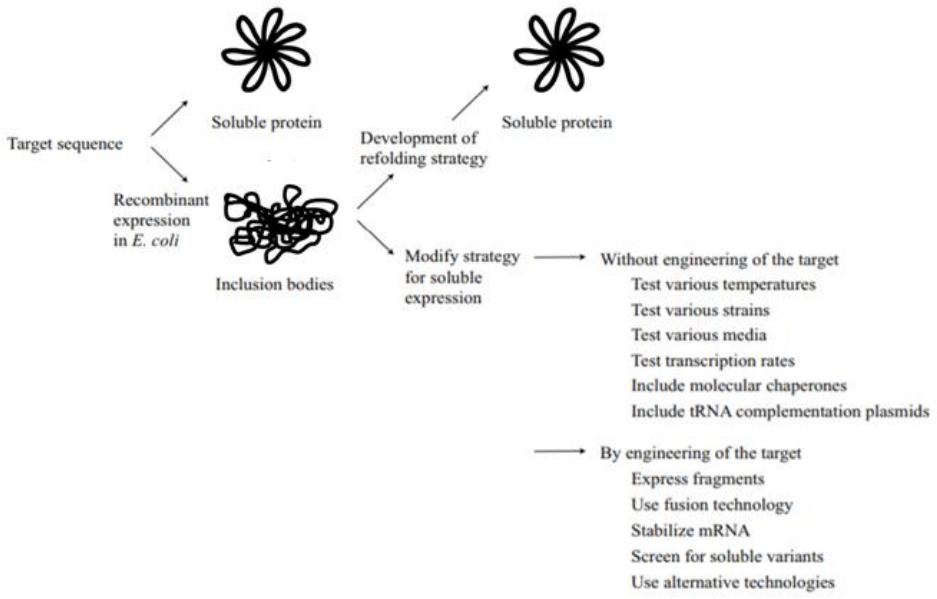

Figure 6. Strategies to get soluble proteins in *E. coli.*

To get soluble target protein, soluble expression can be enhanced through the modulation of physiological rate or solubility enhancing tag utilization. In addition, soluble proteins can be directly recovered from IBs as well.

(3) Chaperone system

Chaperone co-expression strategy is another approach to facilitate soluble expression in *E. coli*. Chaperones are molecular machinery complex that bind to newly synthesized polypeptide chains, prevent inappropriate interactions or aggregations and help them fold correctly into their native structure. Molecular chaperone teams such as the GroEL-GroES and the DanK-DnaJ-GrpE and a ribosome-associated trigger factor chaperone which work as a folding catalyst are found in *E. coli* cytosol and profoundly engaged in the folding process.

Amongst multitude chaperone plasmids which express their respective chaperone protein, pTf16, which expresses trigger factor *tig*, was selected for this study as it enhanced the solubility of both PED antigens, S1D and COE, in our previous study (Piao et al., 2016b).

Trigger factor *tig* is the only ribosome-associated chaperone in bacteria. It constitutively expressed in the cytosol and outnumbers ribosomes 5:2 (Crooke et al., 1988). It binds directly to the ribosome transiently and hijack the growing polypeptide chains (Hoffmann et al., 2010). The unique shape of *tig* prevents inappropriate interactions along with delaying the folding of the protein (Figure 7) (Hoffmann et al., 2010).

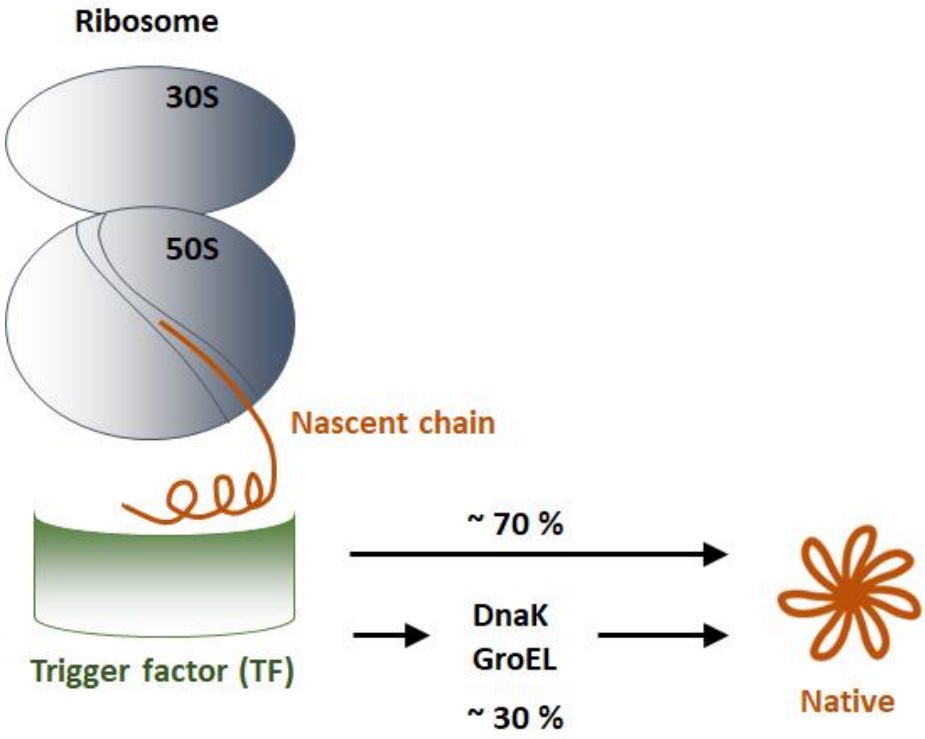

Figure 7. The role of trigger factor *tig* chaperone in folding process.

Trigger factor *tig* can binds directly to ribosome and catch nascent polypeptides. It helps the initial folding steps and growing polypeptide chains are protected from misfolding as well as aggregation.

3) Target antigen for recombinant PED subunit vaccine

To develop recombinant subunit vaccine for PED, specific part of PEDV should be selected as a target antigen. A successful antigen should satisfy two requirements to confer solid protection against broad range of PEDV strains: the inclusion of neutralizing epitopes which is responsible for the induction of actual protective neutralizing antibodies and the conserved aspects of these neutralizing epitopes to confer cross-protection among various strains of PEDV.

To characterize an adequate antigen, many researchers have explored various regions in PEDV spike protein which is expressed on the surface of the virus and responsible for the entry of the virion into host cell by binding to pAPN. In 2002, SH Chang and colleagues selected COE region as a potent antigen based on the sequence information for the neutralizing epitope of the TGEV, a virus closely related to PEDV and also belong to *Coronaviridae*. Polyclonal antisera generated using the recombinant protein of this region significantly inhibited plaque formation by PEDV, demonstrating that this region of spike protein possesses neutralizing epitopes like TGEV (Chang et al., 2002). Later, D Sun and colleagues have tried to identify the most important antigenic region of S1 by examining the ability of truncated S1-GST fusion proteins to react with immune serum against PEDV and to elicit neutralization antibodies in immunized animals (Sun et al., 2007). They suggested that S1D (636-789 aa) was able to react with PEDV antiserum and neutralization antibodies were induced in mice. Above mentioned researches, however, have limitations for the

application of present situation because they use prototype strains Brl/87, CV777, respectively which are largely different from emerging non-S INDEL strains.

Epitope screening studies for the PEDV S protein of emerging strains have not researched until 2017. C Li and colleagues produced PED spike protein from mammalian cell and isolated 11 monoclonal antibodies (mAbs) which recognize diverse region of the spike protein through mouse immunization (Li et al., 2017). Among them, 4 mAbs recognize S0 region of the spike protein. The S0 region of PEDV spike protein interacts with a sugar co-receptor 5-N-acetylneuraminic acid which is expressed on epithelial cells and supports PEDV infection (Liu et al., 2015). They revealed that all of the S0-specific mAbs can recognize the S0 region of other non-S INDEL PEDV strains including PEDV GDU (GenBank accession no. KU985230), PEDV USA, a contemporary US non-S INDEL isolate through ELISA and IFA. They also found out that mAbs neutralize above mentioned strains of the PEDV through neutralization assay using Vero cell, which indicates that S0 could be one of the good subunit vaccine candidates with neutralization epitopes and cross reactivity. mAbs recognizing the SB region also neutralized broad strains of PEDV including S INDEL as well as non-S INDEL, suggesting this region also could be a potent target antigen for recombinant PED subunit vaccine. Integrated summary of epitope information for PEDV spike protein from previous studies is listed in Table 5.

In this study, *E. coli* was selected as a host cell for PEDV antigen production. Although S0 and SB contain potent neutralization epitopes in their intra-

structures, they could not stimulate neutralizing antibodies after vaccination when they are produced in *E. coli* which does not support the post translational modification *per se*. Interestingly, in an indirect ELISA based on the S0 region of PEDV spike protein produced from *E. coli* showed positive correlations between optical density (OD) values of pig sera and virus neutralization titers (Li et al., 2015). Therefore, the S0 region of PEDV spike protein was selected as a target antigen for recombinant PED subunit vaccine in this study.

Table 5. Integrated summary of epitope information of PEDV spike protein.

Reference	Specific region of PEDV S							Note			
	S0	SA		SB (COE)	SCD	hinge	S2	Immunized strain	tag	Host	Target strain
Chang SH, 2002				√ (NAb)				Brl/87		*E. coli*	CV777
Dongbo Sun, 2007	√		√	√	√ (NAb)			CV777	GST	*E. coli*	CV777
Dongbo Sun, 2008						Ab		CV777	GST	*E. coli*	CV777
Chunhua Li, 2017	√ (NAb)	√	√	√ (NAb)	√			GDU	mFc	293T	various
Faten A Okda, 2017				√ (NAb)	√	√ (NAb)	√ (NAb)	various		various	various

Epitope information of PEDV spike protein in previous researches are integrated and listed. √ indicates the tested region in each reference and NAb indicates the region that contains neutralization epitopes revealed in that study. “Various” indicates the PEDV strains utilized in those researches which include both S and non-S INDEL strains.

3. Adjuvant system

1) Adjuvant

(1) Adjuvant

An adjuvant is a pharmacological or immunological agent which modifies the effect of other agents such as subunit vaccine antigen (Coffman et al., 2010). Adjuvants could be applied not only as a “scala” which is responsible for the magnitude of immune response, but also as a “vector” as for the type of immune response. Generally, inclusion of adjuvants to a vaccine boosts the magnitude of immune response by producing more immunological weapons such as antibodies and long-lasting immunity, thus minimizing the needed dose of antigen. In some cases, adjuvants may also be used to enhance the efficacy of a vaccine by guiding the immune response to particular type: for example, adjuvants consist of oligonucleotides that contain unmethylated CpG motifs could be used to induce Th1-type immune response which is responsible for cellular immune response rather than humoral immune response and utilized as a cancer vaccine or vaccines for intracellular pathogen (Scheiermann and Klinman, 2014).

Various types of adjuvants have been explored for the past seven decades. Though the exact mechanism of action of them are not fully defined yet, they are considered to exert their respective adjuvant effect through enforcing a particular part of the induction pathway of host adaptive immune response against antigen Figure 8 (De Haes et al., 2012). In this regard, adjuvants have been grouped in

classes to provide a clearer description of how they work. These classes include ‘delivery systems’, ‘immunostimulants’ and ‘T cell epitope donor’ although many adjuvants are not classified in these groups. Delivery systems refer to adjuvants whose main purpose is to sustain or maximize the amount of antigens to immune cells, thus maintaining the amount input antigens in Figure 8. Commonly used adjuvants including the insoluble aluminum salts adjuvant and the water in oil emulsions are the representative examples of delivery systems. Both adjuvants are designed to extend the duration of antigen persistence at the injection site (O’Hagan and De Gregorio, 2009). Immunostimulants are substances that stimulate the immune system by inducing activation of any of its components. Stimulating the innate immune response is the common strategy to enhance the efficacy of vaccine as a immunostimulant. T cell epitope donors are the proteins that contain T cell epitopes which can be loaded to MHC class II and make the antigen up-taking B cells to be assisted by helper T cells specific for the epitopes they donate.

Adjuvants have been generally used to enhance the immunogenicity of subunit vaccines to supplement its low immunogenicity (Perrie et al., 2008). In this regard, adjuvants were applied in this study.

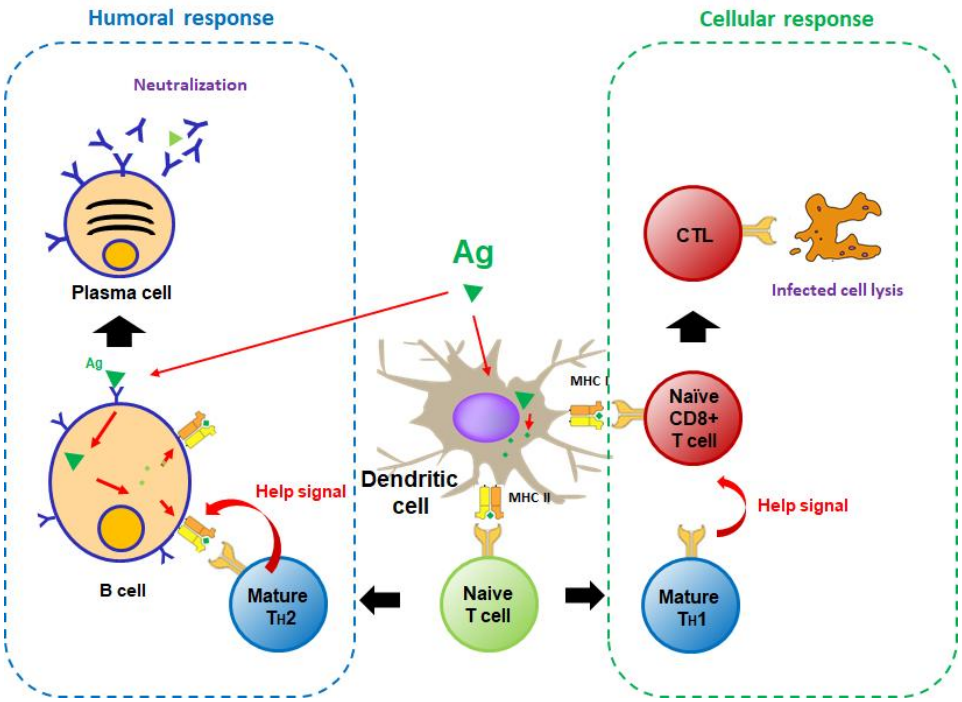

Figure 8. Induction of host adaptive immune response against antigen.

Detailed induction mechanism of host adaptive immune response against antigen is described. TH, helper T cell; CTL, cytotoxic T lymphocyte; Ag, antigen; MHC, major histocompatibility complex

(2) Immunostimulants

Immunostimulants are the group that has direct effects on the innate immune system, providing an immunological driving force to adaptive immune system (O'Hagan and De Gregorio, 2009) as innate and adaptive immune response are closely linked (Paul, 2011). Pathogen associated molecular pattern (PAMP) is the representative example of immunostimulants.

To induce adaptive immune responses which are the final mean of the vaccine, innate immune responses should be activated as a result of pathogen recognition. As pathogens have PAMPs in their own bodies, host can recognize them as an alarm through pattern recognition receptors (PRRs) and prepare the adaptive immune responses to combat those pathogens. This phenomenon could be utilized by adjuvants such as immunostimulators.

The PAMPs used as an adjuvant include Toll-like receptor (TLR), Nod-like receptors (NLRs), and RIG-I-like receptors (RLRs) agonists. This diverse group of ligands stimulates innate PRRs, inducing intracellular signaling cascades that ended up cytokine secretion and immune cell activation. TLR9 agonist CpG is one example of the PAMP adjuvant. These oligodeoxynucleotide sequences promote strong Th1 responses and have been applied in many vaccine studies (Shirota et al., 2001;Scheiermann and Klinman, 2014). Christian and colleagues demonstrates that CpG oligodeoxynucleotides (ODNs) directly stimulate human B cells and plasmacytoid dendritic cells through TLR-9 and also the

proliferation and maturation of natural killer cells, monocytes/macrophages and T cells indirectly, eventually resulting in the production of Th1-type and proinflammatory cytokines, chemokines and polyreactive IgM. CpG ODNs improve and activate the development of vaccine-induced responses. They also improve the formation of memory, thereby supporting the duration of adaptive immunity.

Flagellin is also a representative example of TLR agonist (Mizel and Bates, 2010). Flagellin can activate host immune responses via TLR5 as well as NLRC4/NAIP5. Flagellin has a number of advantages that make it an attractive adjuvant for us in human vaccines. It is effective at very low doses and does not promote IgE responses. It can also easily be made in large amounts under GMP conditions.

(3) T cell epitope donor

For some vaccines such as PED vaccines or addiction vaccines, the induction of humoral immune response is utmost important. As the signal 2 in B cells supported by helper T cells (Th2-cell in Figure 8) through T cell epitope complexed with major histocompatibility complex (MHC) class II molecule is the most important part on the induction of humoral immune response, conjugation of carrier proteins that contain potent T cell epitopes in their intra-structures with the target antigen could confer or maximize signal 2, subsequently the titers of antibodies that specific for target antigen in these cases.

This kind of adjuvant could show great value when it fused to non-protein antigens such as polysaccharides derived from pathogens. The first case used in human was the Haemophilus influenzae type b (Hib) conjugate vaccine which was combined with one of several different carrier proteins that support T cell epitopes, such as the diphtheria toxoid or the tetanus toxoid (Ahmad and Chapnick 1999). The conjugation of polysaccharide antigens to diverse proteins can result in improved immunogenicity of vaccines. Soon after such vaccine was made available, the rates of Hib infection reduced markedly, with a reduction of approximately 90 % between 1987 and 1991. The clear advantages of conjugate vaccines in enhancing the protective responses of young infants and the senescent immune systems of the elderly who have immature immune systems have been made clear.

Complement fragment C3d is also an example of T cell epitope donor. Originally, C3d was believed to stimulate B-cell complement receptor 2 (CR2), which is responsible for the stimulation of B cells. New evidence suggests that C3d instead donates T cell epitopes to antigen up-taking cells and produces an adjuvant effect through C3d-specific autoreactive T cells, which exist circulating as either naïve or memory helper T cells (De Groot, Ross et al. 2015).

2) Adjuvant fusion strategy

(1) Fusion adjuvant

Some of adjuvants could be utilized in conjugation with the target antigen (Moyle, 2017). Adjuvants that utilized as this strategy are called 'fusion adjuvant'. Generally, adjuvants that act as an immunopotentiator have frequently been applied as a fusion adjuvant which enable them to generate significantly higher immune responses in comparison to mixtures of antigen and adjuvant (Zeng et al., 2002;Mizel and Bates, 2010;Zom et al., 2012). Meanwhile, T cell epitope donors should be utilized as a fusion adjuvant as they have to engulfed by antigen uptaking cells.

This strategy has advantages over using mixtures of each in terms of production and immunological perspectives. From the production perspectives, the method of fusion is very simple. Protein based antigen-adjuvant fusion is one-step process with the help of recombinant DNA technology which does not require the use of any complex chemistry (Figure 9). In addition, conjugation of highly soluble fusion adjuvant to many hard-to-express antigens enhances the overall solubility of them (Rhee et al., 2012). Finally, making one good manufacturing practice (GMP) is enough for fusion product to get FDA permission, while mixture regimen should make GMPs for each protein separately. From immunological perspectives, conjugation of targeting adjuvant could reduce the amount of antigen needed. In addition, the conjugation of

immunopotentiators to antigens has been demonstrated to elicit higher immune responses than mixtures of them in many previous researches (Huleatt et al., 2007;Kreutz et al., 2012;Huang et al., 2016;Moyle, 2017). This stronger response occurs because conjugation ensures the delivery and uptake of both antigen and adjuvant by the same antigen presenting cell (Blander and Medzhitov, 2006;Zom et al., 2012;Kastenmüller et al., 2014). In this regard, bacterial flagellin, bacterial lipoproteins, Type III repeat extra domain A of fibronectin, and heat shock proteins have been frequently utilized as a fusion adjuvant (Zeng et al., 2002;Huang et al., 2007;Mizel and Bates, 2010;Julier et al., 2015).

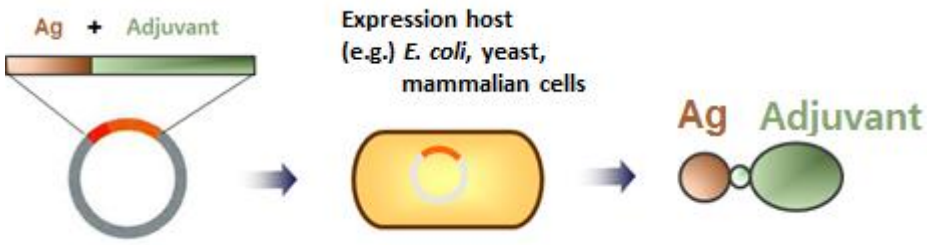

Figure 9. Schematic view of antigen-adjuvant fusion protein production.

Fusion protein consists of antigen and adjuvant could be easily produced by using recombinant DNA technology.

(2) Fusion adjuvant candidate: Flagellin

Flagellin, a monomeric subunit of bacterial flagella. To detect flagellin present on flagellated bacteria, the cells of the innate immune system are equipped with two germline-encoded receptors. The transmembrane receptor TLR5 detects extracellular flagellin, thus activating a number of innate and adaptive immune cells via methods such as secretion of proinflammatory cytokines. Injection of virulence factors containing flagellin from invading microbes results in flagellin being found within the cytosol of macrophages and being recognized by the cytosolic receptor NLRC4/NAIP5, which supports a stronger pathogen killing environment through IL-18, IL-1β and pyroptosis (Miao et al., 2007).

As described before, the PRR stimulating ability of flagellin leads researchers to utilize it as an adjuvant. The role of these two receptors on, in particular, flagellin's adjuvant effect is suggested to be complementary, as a single knockout of either pathway did not interfere with the adjuvancy of flagellin, while a double-knockout resulted in a significant reduction of the adjuvancy of flagellin (Vijay□Kumar et al., 2010;López-Yglesias et al., 2014).

In view of the potency of flagellin as a fusion adjuvant, considerable effort has focused on its adjuvant mechanism. Flagellin could induce immune responses through above mentioned two PRRs. Especially for humoral immune response, flagellin has an ability to activate a number of innate immune

processes that are crucial for the development of humoral immune response, which include the recruitment of T and B lymphocytes to secondary lymphoid sites, the induction of pro-inflammatory cytokines and chemokines by host cells, DC activation, and direct activation of T cells. In addition, efficient and rapid accumulation of flagellin and its fusion proteins at draining lymph node after intramuscular administration results in their high concentration at that place.

Generally, the physical conjugation of flagellin with antigens has been proven to exert stronger immune responses over non-conjugated mixtures in many previous researches (Mizel and Bates, 2010). It also has been suggested that flagellin enhances the solubility of many hard-to-express proteins after conjugation (Rhee et al., 2012). In this regard, it has been frequently used as a molecular fusion adjuvant for the past few decades (Ben-Yedidia and Arnon, 1998;Ben-Yedidia et al., 1999;Lee et al., 2006;Mizel et al., 2009;Song et al., 2009;Mizel and Bates, 2010;Nguyen et al., 2011;Moyle, 2017;Puth et al., 2017) (Table 6).

Table 6. List of researches utilizing flagellin as a fusion adjuvant.

Reference	Recombinant vaccine	Route of administration	Response
Ben-Yedidia and Arnon, 1998	Flagellin-influenza virus hemagglutinin epitopes	s.c.	Ab production
Song, Zhang et al., 2009	Flagellin-influenza virus hemagglutinin globular head domain	s.c.	Ab production Protection against *in vivo* challenge
Huleatt, Nakaar et al., 2008	Flagellin-influenza virus M2e ectodomain	s.c.	Ab production Protection against *in vivo* challenge
Ben-Yedidia, Tarrab-Hazdai et al., 1999	Flagellin-Schistosoma mansoni epitope	i.n.	Ab production Protection against *in vivo* challenge

s.c., subcutaneous; i.n., intranasal; Ab, antibody

(3) Fusion adjuvant candidate: Complement based adjuvant

The complement system is a part of the innate immune system consists of a lot of blood small proteins synthesized in liver which circulate as inactive precursors. Proteases in the system cut specific proteins when stimulated by several triggers to release cytokines and initiate an amplifying cascade of further cleavages. They eventually lead to clear pathogens and damaged cells from an organism via opsonization, promotion of inflammation, and direct attack the pathogen's membrane (Noris and Remuzzi, 2013). Recently, it has become apparent that the complement system is not operative only in the extracellular space but also intracellular space. Activation of C3 and C5, for example, has been revealed to exert multiple effects such as direct antimicrobial defense or cell differentiation and metabolism.

One consequence of the complement activation is the physical attachment of fragments of C3, to the membrane of the pathogen. Further cleavage process of C3 generates C3d. In 1996, Dempsey and colleagues utilized C3d as a fusion adjuvant for the first time and suggested that C3d exerted adjuvant effect by stimulating B-cell complement receptor 2 (CR2), which is responsible for the stimulation of B cells (Dempsey et al., 1996;Zhang et al., 2007). Then, C3d has been widely applied to many subunit vaccine antigens as a fusion adjuvant due to its effectiveness of enhancing the humoral immune responses to target antigens (Dempsey et al., 1996;Green et al., 2003;Li et al., 2003;Watanabe et al., 2003;Wang et al., 2004a;Wang et al., 2004b;Koch et al., 2005;Zhang et al., 2011)

(Table 7).

However, sustained adjuvant effect of C3d fusion in CR2 knock-out mice leads researchers to think that other mechanisms would be exist (Haas et al., 2004). AS De Groot and colleagues suggest that C3d instead donates T cell epitopes to antigen up-taking cells and produces an adjuvant effect through C3d-specific autoreactive T cells (Knopf et al., 2008;De Groot et al., 2015). They confirmed that C3d has many T cell epitopes unusually and the autoreactive T cells responsible for many C3d epitopes existed circulating as either naïve or memory helper T cells in adult human (De Groot et al., 2015). Decisively, they found that the mutation of T cell epitopes in P28, the main region of C3d for fusion adjuvant effect, which is fused to antigen resulted in the depletion of the adjuvant effect of P28 in mice (De Groot et al., 2015). The schematic view of suggested mechanism of the C3d fusion adjuvant is described in Figure 10.

Table 7. List of researches utilizing flagellin as a fusion adjuvant.

Reference	Vaccine type	Antigen-adjuvant	Route of administration	Response
Dempsey et al., 1996	Subunit vaccine (L cell)	HEL-(C3d3)	s.c. x2	1. vs. HEL alone: same serum IgG with 1/10000 dose 2. vs. CFA, HEL alone: same serum IgG with 1/100 dose
Wang et al., 2004	DNA vaccine	hCGβ-(C3d3)	i.m. x2	vs. hCGβ alone: same IgG with 1/243 dose
Watanabe et al., 2003	Subunit vaccine (293T cell)	[vs. Influenza] HA-(C3d3)	i.n. x2	vs. sHA alone: nasal igA 100 fold, serum IgG induced protection to challenge (100 % vs. 0 %)
Wang et al., 2004	Subunit vaccine (293T cell)	[vs. BVDV] E2s-(C3d3)	s.c. x2	vs. E2s alone: 100 fold serum IgG with 1/100 dose
Koch et al., 2005	Subunit vaccine (S2 cell; Insect cell)	[vs. HIV] Gp120-(C3d2)	s.c. x4	vs. gp120 alone: serum IgG 2 fold (O.D.)

S.C., subcutaneous; i.m., intramuscular; i.n., intranasal; HEL, Hen egg

lysozyme; CFA, complete freund's adjuvant; hCGβ, human chorionic gonadotropin; HA, hemagglutinin; BVDV, Bovine viral diarrhea virus; HIV, human immunodeficiency virus.

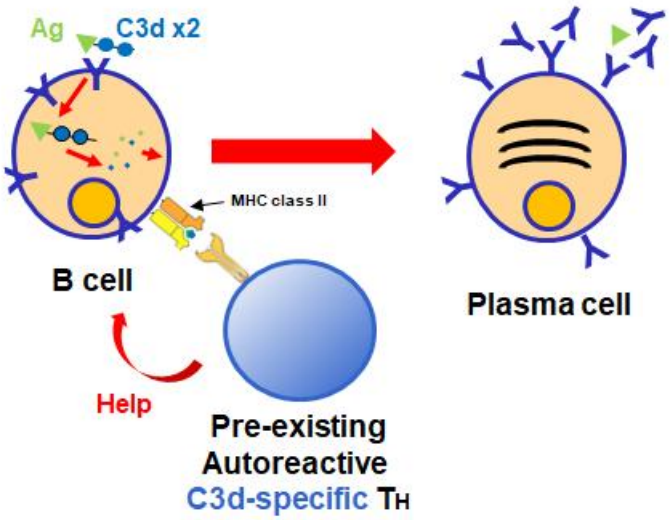

Figure 10. Schematic view of proposed mechanism of C3d as a fusion adjuvant.

The fused C3d with the target antigen is expected to exert its adjuvant effect by donating T cell epitopes in its intra-structure to MHC class II molecules in antigen-specific B cells and enable them to be assisted by C3d-specific autoreactive helper T cells to become plasma cells. T_H, helper T cell; Ag, antigen.

Study I. Effects of Flagellin Conjugation to S0 as a Fusion Adjuvant

1. Introduction

Flagellin is one of PAMP proteins that are recognized by two PRRs, TLR5 and NLRC4/NAIP5, which are expressed on a number of immune cells. For several decades, flagellin has been vigorously utilized as a fusion against many antigens (Lee et al., 2006;Mizel et al., 2009;Mizel and Bates, 2010;Nguyen et al., 2011;Moyle, 2017;Puth et al., 2017), and both receptors are involved in its adjuvancy. Flagellin has also been utilized as a fusion partner to enhance the solubility of many aggregation-prone proteins (Rhee et al., 2012). Therefore, the conjugation of flagellin to different terminals of S0 could influence not only solubility but also immunogenicity. However, the fusion effect of flagellin varies depending on the target antigen or its conjugation direction (Mizel and Bates, 2010;Lin et al., 2016b).

In study I, the flagellin from *Vibrio vulnificus,* FlaB, was conjugated to the C- or N-terminus of S0 to generate S0-F and F-S0, respectively. Firstly, the effect of flagellin conjugation to S0 on the enhancement of solubility was tested. To further ameliorate the solubility of each recombinant protein, trigger factor chaperone *tig* was introduced, and the solubility of each recombinant protein was evaluated in the presence or absence of *tig*. The ability of the conjugated

flagellin to enhance the S0-specific humoral immune response was also evaluated in Balb/c mice and different outcomes depending on the direction of fusion were identified. The underlying mechanisms of the results were investigated through *in vitro* and *in vivo* experiments. These findings provide important information for the development of a novel PED vaccine as well as flagellin-based immune therapeutics.

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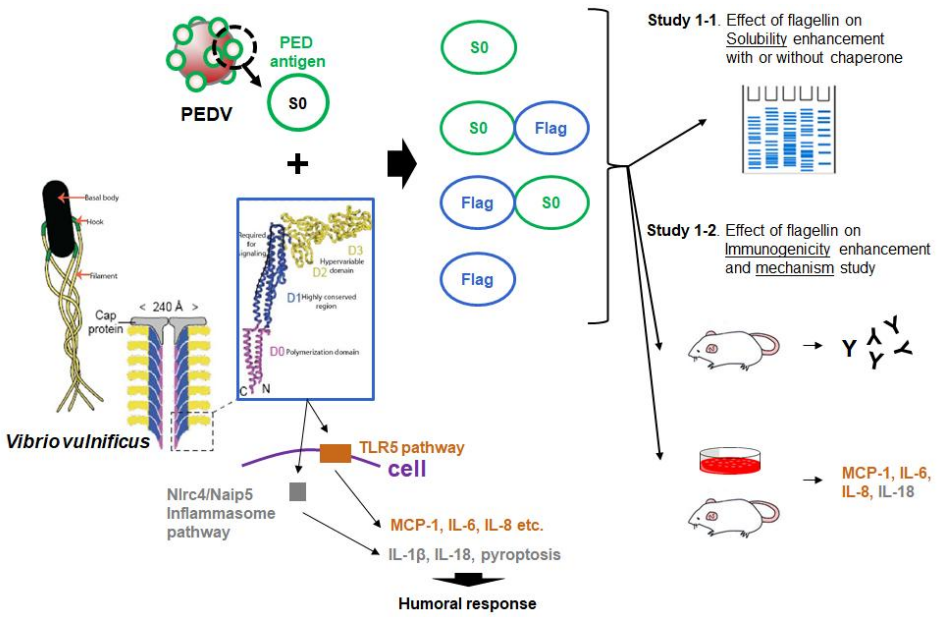


Figure 11. Graphical abstract of study I.

Bacterial flagellin from *Vibrio vulnificus,* FlaB, was used as a fusion adjuvant candidate. FlaB was fused to the C- or N-terminus of S0 to generate S0-F and F-S0, respectively. The effect of flagellin conjuation to S0 on solubility was evaluated in the presence or absence of chaperone *tig*. The effect of flagellin conjugation to S0 on immunogenicity was also evaluated in mice and the underlying mechanism was deduced through *in vitro* and *in vivo* experiments.

2. Materials and methods

1) Expression and purification of recombinant proteins

(1) Vector construction for recombinant proteins

The nucleotide sequence of the spike protein from the PEDV K14JB01 strain (GenBank accession No. KJ623926) was used as a reference to synthesize a partial protein from the S0 (amino acids 25-229) gene. The whole genome sequence from *Vibrio vulnificus* YJ016 (GenBank accession No. BA000037) was used as a reference to synthesize FlaB. Each sequence was codon-optimized using the table of codon usage in *E. coli* before synthesis (Genescript, USA and Bioneer, Korea) and PCR-amplified using various primer pairs provided in Table 8.

The nucleotide sequence of S0 was amplified by PCR using the primers NS0-F/XS0-R and cloned into the vector pET28a(+) (Novagen, USA) using *NheI* and *XhoI* restriction enzyme sites (pS0). The FlaB DNA fragment was amplified by PCR using BFlaB-F/XFlaB-R primers and was cloned into pET28a(+). The resulting construct was digested using *NheI* and *BamHI* restriction enzymes and ligated with the S0 DNA fragment that was previously PCR-amplified using NS0-F/BS0-R primers (pS0-F). The FlaB DNA fragment, which was PCR-amplified using SFlaB-F/NFlaB-R primers, was cloned into pET28a(+) pretreated with rSAP. The resulting construct, which contained the correct FlaB direction, was digested using *NheI* restriction enzymes and ligated with the

PCR-amplified S0 DNA fragment using NS0-F/XS0-R primers (pF-S0). To express FlaB alone, the FlaB DNA fragment PCR-amplified using NFlaB-F/XFlaB-R primers was cloned into pET28a(+) using *NheI* and *XhoI* restriction enzyme sites (pF). As pET28a(+) supports 6His-tag nucleotide sequences on both ends of the multiple cloning site, the resulting recombinant proteins contained an additional 6His-tag on both the C- and N-terminal regions. DNA sequences of the resulting expression vectors were verified by the dideoxy-chain termination method.

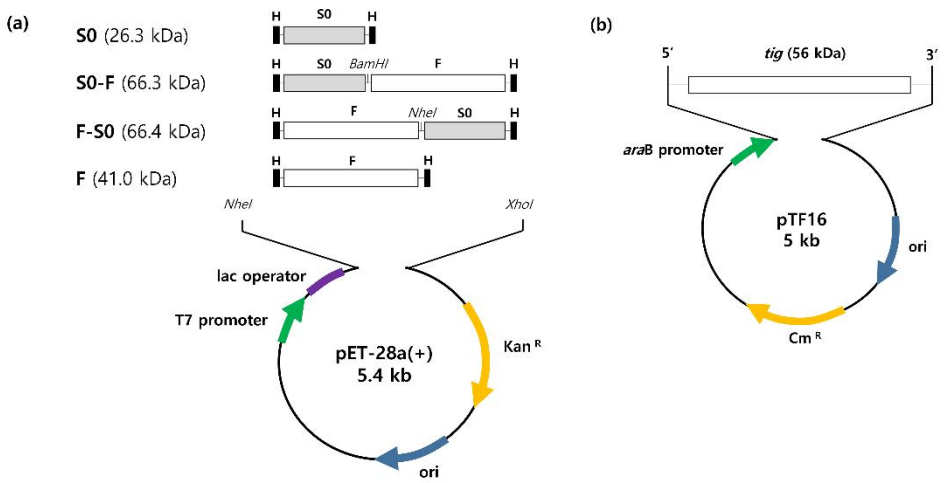


Figure 12. Schematic view of the expression vector system.

(a) Construction of expression vectors for recombinant S0, S0-F, F-S0 and F. (b) Vector map of tig expression plasmid pTF16 purchased from TAKARA BIO INC. S0, truncated region of PEDV spike protein; F, FlaB from *Vibrio vulnificus*;

H, 6X His-tag.

Table 8. PCR primer sequences for study I.

Names	Sequences
NS0-F	5'-GCTAGC CGTTGCAGCGCGAACAC
XS0-R	5'-CTCGAG CGGTTGGTAGCTAATGCC
BS0-R	5'-GGATCC CGGTTGGTAGCTAATGCC
SFlaB-F	5'-ACTAGT ATGGCAGTGAATGTAAA
NFlaB-R	5'-GCTAGC GCCAAGCAGGCTTAACG
BFlaB-F	5'-GGATCC ATGGCAGTGAATGTAAA
XFlaB-R	5'-CTCGAG GCCAAGCAGGCTTAACG
NFlaB-F	5'-GCTAGC GCAGTGAATGTAAA

(2) Protein expression in the presence or absence of *tig*

Each expression plasmid (pS0, pS0-F, pF-S0 and pF) was transformed into *E. coli* BL21(DE3) (Invitrogen, USA). A seed culture was prepared with a single colony of recombinant *E. coli* and grown overnight in LB broth containing 50 μg/ml kanamycin. One percent v and grown until the optical density at 600 nm (O.D. 600) reached 0.5. Cultures were then treated with 0.3 mM isopropyl-β-D-thiogalactopyranoside (IPTG) to induce expression of the protein of interest and grown at 25 ℃ for 5 h with shaking set to 200 rpm.

To express each recombinant protein in the presence of the chaperone *tig*, each expression plasmid (pS0, pS0-F and pF-S0) was transformed into *E. coli*

BL21(DE3)/pTf16 (Takara, Japan), which can induce the expression of trigger factor chaperone *tig*. A seed culture was prepared by inoculating a single colony in LB broth containing 50 μg/ml kanamycin and 20 μg/ml chloramphenicol overnight at 37 ℃. *tig* was expressed before the induction of each recombinant protein. One percent of the seed culture was inoculated into LB broth supplemented with 0.5 mg/ml L-arabinose, 20 μg/ml chloramphenicol and 50 μg/ml kanamycin until the O.D. 600 reached 0.35. At this point, cells were cooled to 4 ℃ for 15 min and subsequently induced with 0.1 mM IPTG at 15 ℃ for 24 h.

(3) Analysis of protein solubility with SDS-PAGE and densitometer

Cells were harvested, resuspended in GNβ buffer (50 mM Tris-Cl, 300 mM NaCl, 10 % glycerol, 2 mM β-mercaptoethanol, 0.5 % NP-40, 5 mM imidazole, pH 7.9) and reacted with 1 mg/ml lysozyme for 30 min at 37 ℃. Subsequently, cells were disrupted on ice by sonication (VCX750, SONICS, Newtown, CT, USA) using a preset program (45 cycles of 2 sec On/5 sec Off, amp 40 %), followed by centrifugation at 20,000 × g for 15 min at 4 ℃. After centrifugation, supernatants were collected as a soluble fraction, while pellets were solubilized using 50 mM Tris-Cl, 2 M urea, pH 12.5 buffer and collected as an insoluble fraction. Equivalent volumes of solubilization buffer versus supernatants were used to prepare the insoluble fraction. The 20 μl supernatants of insoluble and

soluble fractions of each recombinant protein were analyzed by 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The gels were stained with Coomassie Brilliant Blue by 3 times of heating in a microwave oven for 70 seconds, cooled down on a rocker for 5 min and destained with 25% methanol and 7.5% acetic acid solution overnight. The band densities of each recombinant protein were measured using Image Lab software (Bio-Rad Laboratories). The insoluble fraction of S0 was subsequently refolded as described previously (Piao et al., 2016a). Overall scheme of protein expression followed by solubility analysis is described in Figure 13.

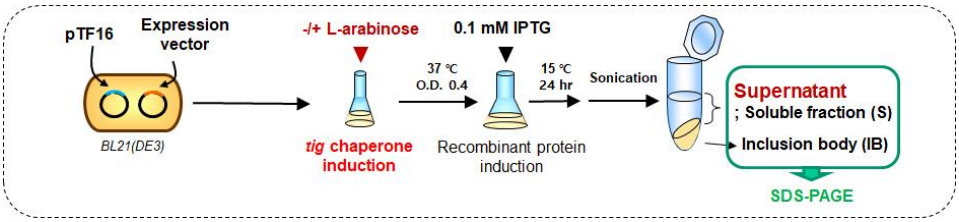


Figure 13. Overall scheme of recombinant protein expression and solubility analysis.

Expression vector for each recombinant protein was transformed into *E. coli* BL21 (DE3) together with pTF16. Recombinant cells were grown and induced *tig* expression by adding L-arabinose, subsequently induced by adding IPTG. Then, cells were disrupted and centrifuged to generate soluble (S) and inclusion body (IB) fractions. Each fraction was subsequently analyzed by SDS-PAGE.

(4) Purification, dialysis and endotoxin removal of recombinant

proteins

The soluble fractions of S0-F and F-S0, which were expressed in the presence of *tig*, were filtered with a 0.45 μm syringe filter (Sigma, USA) and purified using affinity chromatography on a Ni^{2+}-NTA column. His-bound resin (Thermo Scientific, USA) was packed into a column, equilibrated with 2 volumes of binding buffer and charged with 3 volumes of charging buffer. After washing with 5 volumes to remove uncharged nickel ions, the column was loaded with filtered soluble fraction of recombinant protein. Then, the column was washed with washing buffer containing different imidazole (IMD) concentrations to remove non-specific proteins. The 6x His-tag bearing recombinant protein was eluted with elution buffer. The composition of the various buffers used for Ni^{2+}-NTA affinity chromatography is listed in Table 9. Each fraction was analyzed by SDS-PAGE.

Purified samples were dialyzed against phosphate buffered saline (PBS) for at 4 ℃ three times and subsequently concentrated using Amicon ultra-15 centrifugal filters (Merck, Germany). Contaminating lipopolysaccharide (LPS) was further removed by using an endotoxin removal spin column (Thermo Scientific, USA), and the residual LPS content of each recombinant protein was determined using the ToxinSensor Chromogenic LAL Endotoxin Assay Kit (Genescript, USA). The LPS levels of each recombinant protein were below 0.03 EU/ml and 0.0075 EU per dose.

Table 9. Buffer compositions for Ni^{2+}-NTA affinity chromatography.

Buffer		Imidazole	Tris-Cl	NaCl	$NiSO_4$	pH	Volume
Charging buffer		-	-	-	50 mM	-	5
GNβ buffer		5 mM	50 mM	300 mM	-	7.9	5
Washing buffer	1	5 mM					5
	2	10 mM					5
	3	20 mM					5
Elution buffer		300 mM					5

2) *in vivo* immunization

(1) Animals

Six-week-old female naïve BALB/c mice (17-19 g) were purchased from Samtako (Osan, Korea). The mice were maintained under standard pathogen-free conditions on a 12:12 h light/dark cycle (5 mice per cage), monitored three times weekly and provided with free access to food and water during experimentation. All mice were divided randomly into different groups before immunization and adapted for 1 week before any treatments. Animal experiments were performed in accordance with the guidelines for the care and use of laboratory animals under the approval of the animal ethics committee at Seoul National University (No. SNU-161114-4). After the final serum collection of each experiment, the mice were euthanized with CO_2 inhalation.

(2) Mouse immunization experiment

Six-week-old BALB/c mice (n=5) were immunized intramuscularly with 30 pmol of each recombinant protein (0.76 μg of S0, 2 μg of S0-F or F-S0 and 0.76+1.24 μg of S0/F per mouse, a total of 5 groups, total n=25) three times at 2-week intervals after adaptation for one week. Complete freund's adjuvant (CFA) and Incomplete freund's adjuvant (IFA) were mixed with antigens for the priming and boosting of mice, respectively. Treatment group information and regimens are listed in Table 10. Blood samples were collected on days 13, 27,

and 42 (sacrifice) from intrapetrosal veins with a disposable syringe and delivered into sterilized tubes. Serum was separated by centrifugation at 12,000 rpm for 3 min using a serum separator tube (BD microtainer, NJ, USA). Overall scheme is described in Figure 14.

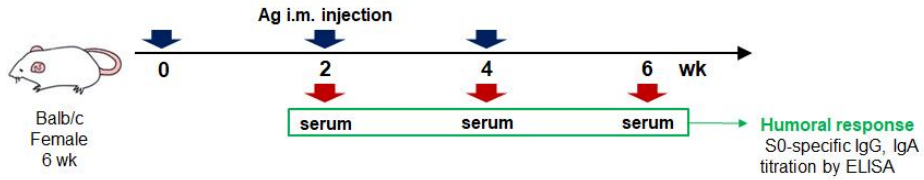


Figure 14. Overall scheme of mouse immunization experiment.

Each mouse was immunized with regimens listed in Table 10 three times at 2-week intervals. Blood samples were collected on days 13, 27, and 42 and analyzed by ELISA to evaluate S0-specific IgG and IgA titers.

Table 10. Treatment group information and regimens for mouse immunization experiment in study I.

	Antigen	Note	Amount		Oil adjuvant	Solution	Volume	Route	n
			pmol/mouse	μg/mouse					
1	None	PBS only	---	---	CFA (0 wk) + IFA (2, 4 wk)	PBS	50 μL	I.M.	5
2	S0	PED Ag	30	0.76					
3	S0-Flagellin (S0-F)	Flagellin fused to C' terminus of S0		2.0					
4	Flagellin-S0 (F-S0)	Flagellin fused to N' terminus of S0							
5	S0 + Flagellin	S0 mixed with 1 x Flagellin	30 + 30	0.76 + 1.24					

I.M., intramuscular administration; Ag, antigen; wk, week

(3) ELISA

Antibody production was examined by ELISA in serum samples collected at days 0, 13, 27, and 42. Briefly, 96-well immunoplates (SPL, Korea) were coated with 10 μg/ml of purified recombinant S0 (100 μl/well) in carbonate-bicarbonate buffer (pH 9.6) at 37 ℃ for 2 h. Coated plates were blocked with 0.5 % skim milk in PBS (100 μl/well) for 1 h at room temperature. Then, serially diluted serum from a 1:100 dilutions was added to the wells (100 μl/well) and incubated at 37 °C for 2 h. After incubation, horseradish peroxidase-conjugated goat anti-mouse IgG, IgG1, IgG2a (1:2000 dilutions, 100 μl/well) or IgA (1:5000 dilutions, 100 μl/well) was added to the designated wells for 1 h at room temperature. Appropriate washing of each well was performed between each step with PBS containing 0.05 % Tween 20. Tetramethylbenzidine (TMB) solution (Sigma-Aldrich, USA) was added to the wells (100 μl/well) as a horseradish peroxidase (HRP) substrate and incubated for 30 min without light interference, followed by the addition of stop solution (0.16 M H_2SO_4; 100 μl/well) to stop the enzymatic reaction. Finally, the absorbance was measured at 450 nm using a microplate reader (Infinite® 200 PRO, USA). ELISA results were calculated by Softmax Pro version 5.4.1. and expressed as the endpoint titer.

3) Mechanism studies

(1) Analysis of IL-8 and MCP-1 release by Caco-2 cells

Caco-2 cells (KCTC, Korea) were seeded at 1×10^5 per well in 48-well plates (SPL, Korea) and incubated at 37 ℃ for 5 days in Dulbecco's modified Eagle's medium (Invitrogen, USA) supplemented with 10 % fetal bovine serum (Gibco, USA) in an atmosphere containing 5 % CO_2. Cells were then cultivated overnight without fetal bovine serum (FBS) and subsequently stimulated with different concentrations of each recombinant protein for 6 h. The supernatants were harvested, and cytokine levels were quantified using enzyme-linked immunosorbent assay (ELISA) kits (Cusabio, China) according to manufacturer's instruction.

(2) Analysis of IL-6 and IL-18 *in vivo*

Six-week-old BALB/c mice (n=3) were injected intraperitoneally with 150 pmol of each recombinant protein in 100 μl PBS (3.8 μg of S0, 10 μg of S0-F or F-S0 and 6.2 μg of F per mouse, a total of 4 groups, total n=12). Sera were collected at 1 and 4 h postinjection from intrapetrosal veins with a disposable syringe and delivered into sterilized tubes. Serum was separated by centrifugation at 12,000 rpm for 3 min using a serum separator tube (BD microtainer, NJ, USA). Cytokine levels were analyzed using IL-6 and IL-18 ELISA kits (Invitrogen, USA, and Cusabio, China, respectively) according to manufacturer's instruction.

3. Results and discussion

1) Effect of flagellin conjugation to S0 on solubility enhancement

(1) Solubility enhancement in the absence of *tig*

To evaluate the effect of flagellin conjugation on solubility enhancement, the solubility of each recombinant protein expressed in generic condition where chaperone *tig* were not co-expressed was checked firstly. Each expression vector was transformed into *E. coli* BL21(DE3) followed by expression induction with IPTG. After cell disruption by sonication, soluble lysates were separated from insoluble lysates. To evaluate soluble expression, soluble and insoluble fractions of each recombinant protein were confirmed through SDS-PAGE followed by Coomassie Blue staining. 99 % of flagellin was expressed as a soluble protein, while the majority of S0 was expressed as an insoluble protein. The majority of F-S0 was expressed as an insoluble protein, however, 59 % of S0-F was expressed as a soluble protein (Figure 15). These data suggest that flagellin conjugation to the C-terminus of S0 improved solubility in the *E. coli* expression system without *tig* expression.

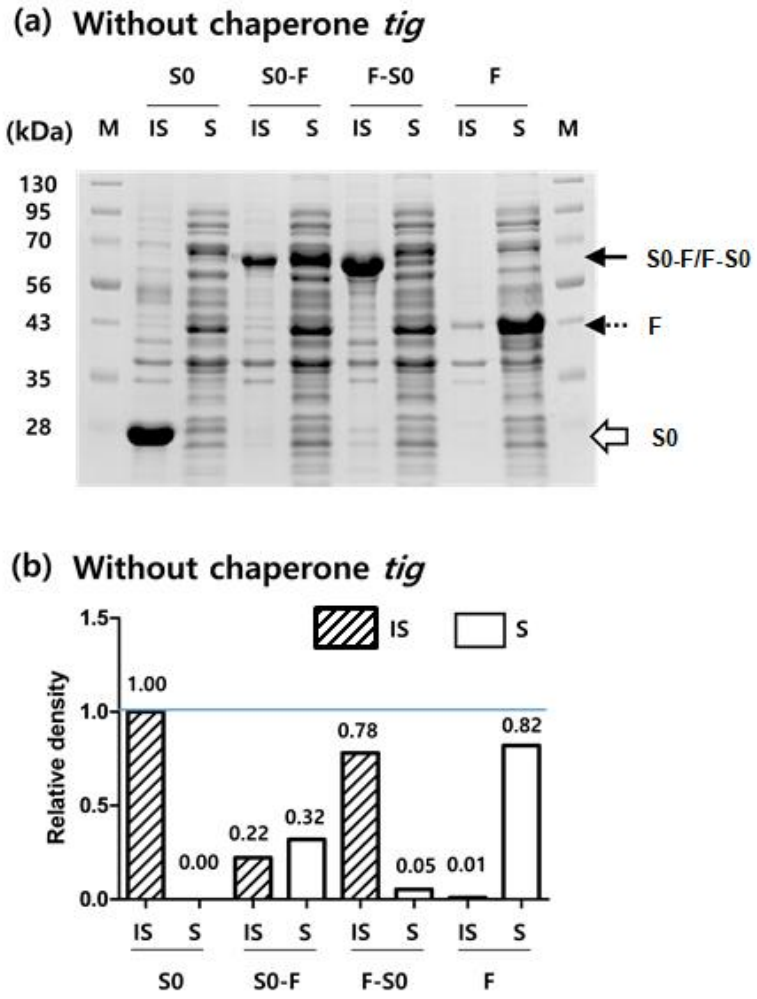


Figure 15. Analysis of solubility of recombinant proteins in the absence of the chaperone *tig*.

(a) Insoluble and soluble fractions of each recombinant protein were analyzed by SDS-PAGE followed by Coomassie Blue staining in the absence of *tig* **(b)** Band densities of target recombinant protein from (a) were analyzed using ImageJ. Densities were calculated using the relative ratio of the S0 insoluble fraction

without the chaperone *tig*. Respective values presented for each corresponding bar. IS, Insoluble fraction; S, Soluble fraction; M, Marker; Black arrow, Location of S0-F or F-S0; Dotted arrow, Location of F, Empty arrow, Location of S0

(2) Solubility enhancement in the presence of *tig*

To improve the solubility of the recombinant proteins, trigger factor chaperone *tig*, which has been shown to assist protein folding during synthesis and the initial folding process, was introduced and coexpressed with the respective protein of interest. The *tig* expression vector pTF16 [Figure 12 (b)] was co-transformed into *E. coli* BL21(DE3) together with expression vectors for each target recombinant protein (pS0, pS0-F and pF-S0). Each target protein was induced by adding IPTG approximately 3 hours after L-arabinose addition. In contrast to S0, which had 66 % solubility, 99 % of S0-F and F-S0 were expressed as soluble proteins (Figure 16), suggesting that flagellin conjugation also improved the solubility of recombinant proteins in the presence of *tig*. Furthermore, coexpression of *tig* ameliorated the solubility of S0, S0-F and F-S0 from 0 % to 66 %, 59 % to 99 %, and 6 % to 99 %, respectively. Taking this result into account, not only flagellin conjugation but also the coexpression of chaperone *tig* improved the final solubility of S0-related recombinant proteins.

Interestingly, the total amount of F-S0 expression was approximately 6.2- and 1.5-times higher than S0-F in the presence or absence of *tig*, respectively, despite their similar molecular weights (Figure 15, Figure 16). This difference in protein levels indicates that the N-terminal flagellin sequence may confer efficient translation initiation, resulting in improved production of F-S0. In fact, it is generally accepted that conjugation of a solubility enhancer to the N-terminus of a target protein can confer efficient translation initiation, which makes it a more

favorable choice for soluble expression than the C-terminal fusion (Costa et al., 2014). The fusion of flagellin to the C-terminus of S0 (S0-F) improved soluble expression in comparison to the N-terminal conjugation (F-S0) or S0 alone in the absence of *tig* (Figure 15). *tig* enhances the solubility of recombinant protein through by assisting with the protein folding process. Aggregation-prone proteins, including the S1D and COE region of PEDV's spike protein, which are otherwise totally insoluble, have been expressed as soluble proteins with the assistance of *tig* (Piao et al., 2016b). In contrast, the condition lacking *tig* had a relatively lower capacity to fold recombinant protein correctly and make soluble protein. Therefore, fast translation of F-S0 in the absence of *tig* is thought to produce IBs rather than soluble protein despite of its total protein expression level being 1.5-times higher than the S0-F protein level.

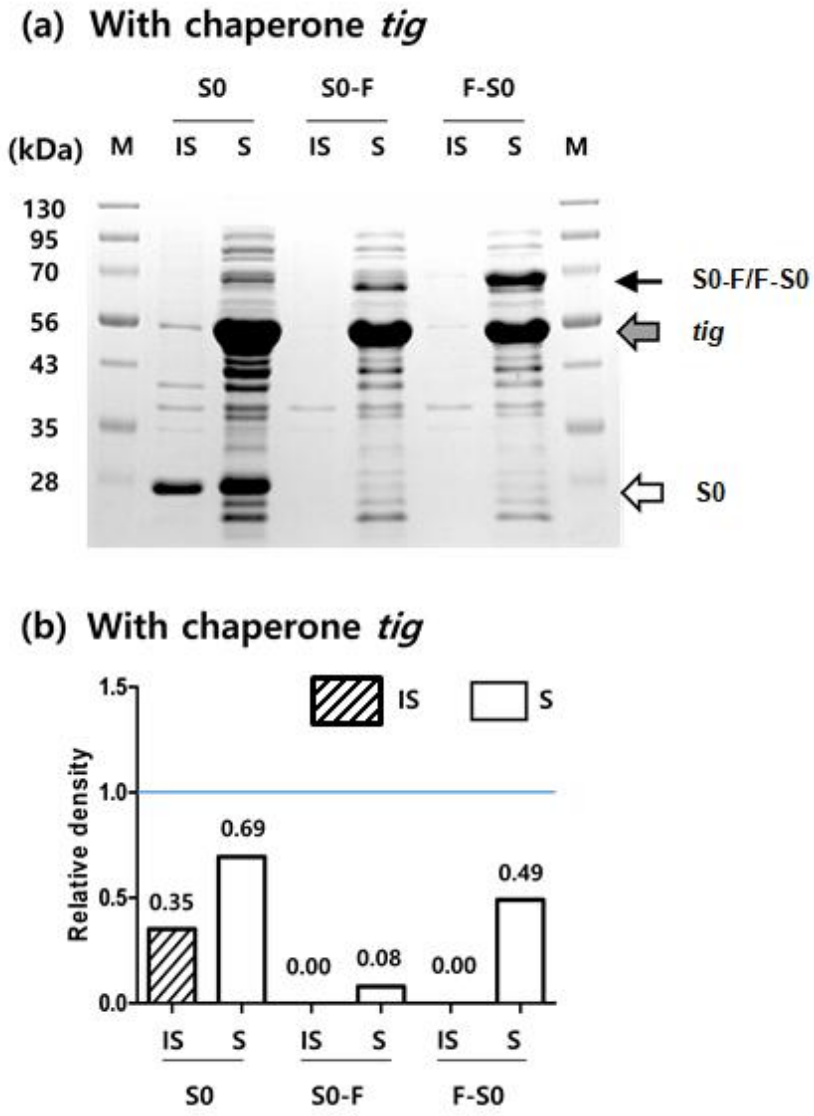


Figure 16. Analysis of solubility of recombinant proteins in the absence of the chaperone *tig*.

(a) Insoluble and soluble fractions of each recombinant protein were analyzed by SDS-PAGE followed by Coomassie Blue staining in the presence of the coexpressed chaperone *tig* **(b)** Band densities of target recombinant protein from

(a) were analyzed using ImageJ. Densities were calculated using the relative ratio of the S0 insoluble fraction without the chaperone *tig* in Figure 15. Respective values presented for each corresponding bar. IS, Insoluble fraction; S, Soluble fraction; M, Marker; Black arrow, Location of S0-F or F-S0; Grey arrow, Location of *tig*, Empty arrow, Location of S0.

(3) Final isolation of recombinant proteins

For further experiments, soluble lysate of each recombinant protein using Ni^{2+}-NTA affinity chromatography was purified, subsequently removed endotoxins and verified through SDS-PAGE followed by Coomassie Blue staining (Figure 17). The representative band of each recombinant protein appeared at its right molecular weight location in the IPTG-induced whole cell lysate lane compared to the lane of IPTG non-induced whole cell lysate (lane 1 of Figure 17). The purities of S0-F, F-S0 and F were 90.3 %, 92.6 % and 99 %, respectively (Table 11). Due to the difficulties of purifying S0 as a soluble lysate, S0 insoluble aggregates were solubilized, refolded and subsequently purified as described previously (Piao et al., 2016a). Purified S0-F and F-S0 had markedly lower expression yields than purified F, in contrast to high purity of each recombinant protein. There was a significant loss of target protein during the purification process judging by the differences between the band intensities of the soluble and purified fractions (lanes 3 and 4 of Figure 17). It is inferred that both 6His-tags bound to the N- and C-termini of S0-F and F-S0 were difficult to access by the Ni^{2+} metal ions during the purification process. In this regard, long flexible linkers could reduce steric hindrance and enable S0-F or F-S0 to be purified well.

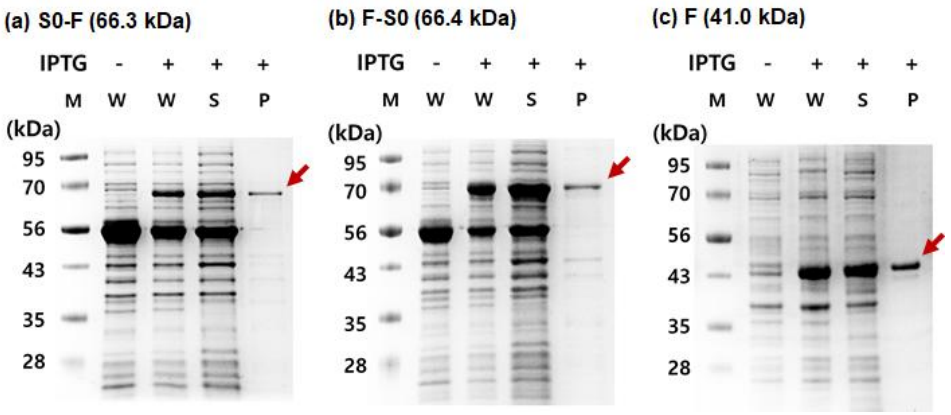


Figure 17. Purification of S0-F, F-S0 and F.

(a) S0-F, (b) F-S0 and (c) F were expressed in *E. coli* BL21 (DE3). After sonication, soluble fractions were purified. Normalized amounts of each sample were analyzed using SDS-PAGE followed by Coomassie Blue staining. M, Marker; W, Whole cell lysates; S, Soluble fraction; P, Purified fraction; Red arrows, the location of each recombinant protein.

Table 11. Purity and yield of S0-F, F-S0 and F.

Recombinant protein	Purity (%)	Yield (mg/L)
S0-F	90.4	0.64
F-S0	92.6	1.17
F	99	5.88

2) Effect of flagellin conjugation to S0 on humoral immune response

(1) Evaluation of adjuvant effect of flagellin conjugation *in vivo*

In past decades, flagellin has been uitilized as a fusion adjuvant for a broad range of antigens (Lee et al., 2006;Mizel et al., 2009;Mizel and Bates, 2010;Nguyen et al., 2011;Moyle, 2017;Puth et al., 2017). However, there have been few researches evaluating the effect of the flagellin's fusion position on its adjuvant activity. Therefore, the ability of S0-F and F-S0 to induce S0-specific humoral immune responses was tested in mice. Mice were distributed to five groups and immunized intramuscularly three times with PBS, 30 pmol of S0 (0.76 μg), S0-F (2 μg), F-S0 (2 μg) and a mixture of S0/F (0.76+1.24 μg) at 2-week intervals. Sera were collected at the 2, 4 and 6-week postinjection, and S0-specific IgG levels were assessed by indirect ELISA. Interestingly, the group immunizing F-S0 had significantly higher titers of S0-specific IgG and IgA compared to all other groups at 6-week postinejction. Sera collected at the 2 and 4-week postinjection had a similar tendency or S0-specific titers as the 6-week postinjection data (Figure 18). A the PEDV neutralization titers have positive correlations to indirect ELISA results which utilized S0 produced from *E. coli* as a coating protein, the S0-specific antibodies in Figure 18 is thought to be the neutralizing antibodies (Li et al., 2015).

The serum S0-specific IgG2a and IgG1 titers as indicators of Th1 and Th2

type immune responses (Stevens et al., 1988), respectively, was also evaluated at 6-week postinjection. Data were expressed as a ratio of IgG2a/IgG1. Figure 19 indicates that the S0 immunization induced stronger S0-specific IgG1 response than IgG2a response, except from one mouse, demonstrating that S0 inherently induce Th2-type immune response against itself. While S0-F and F-S0 indcued IgG1 more than IgG2a, flagellin alone induced IgG2a more than IgG1. These data demonstrate that flagellin skewed the type of immune response from Th2 to Th1. S0-F or F-S0 is expected to have an additional advantage if they are used as a mucosal vaccine because Th2 cell-derived cytokines including IL-5 and IL-6 are crucial for the induction of IgA at mucosal site (Yamamoto et al., 1996).

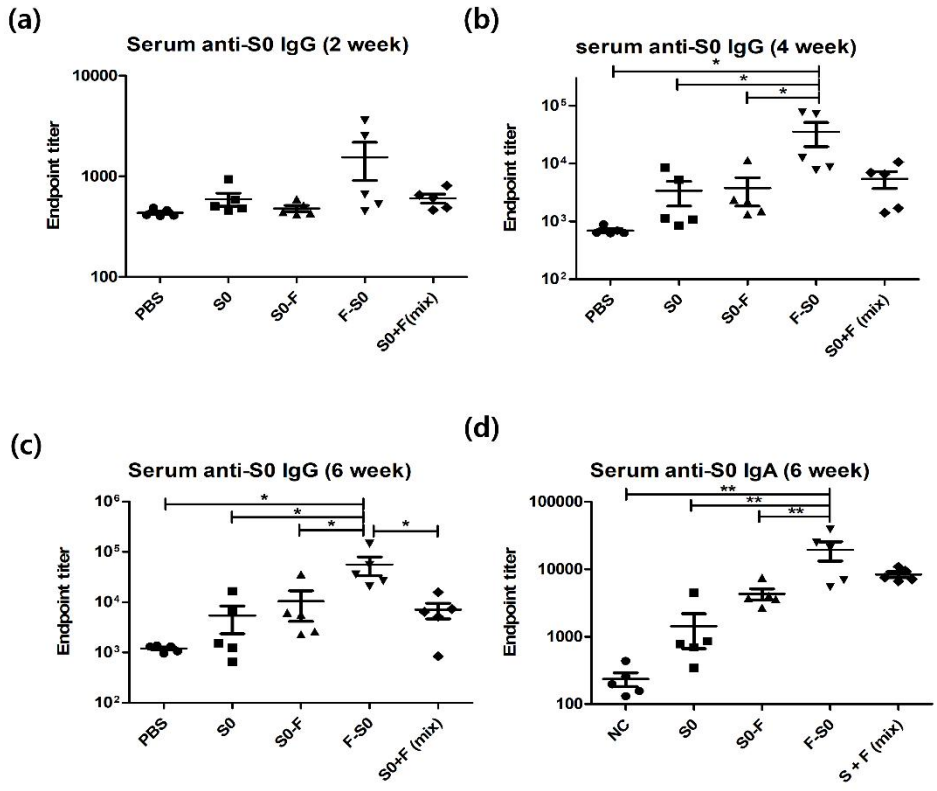


Figure 18. S0-specific humoral immune response in mice after immunization of each recombinant protein.

Mice were immunized with the indicated recombinant proteins. Blood samples from **(a)** 2, **(b)** 4, and **(c, d)** 6-week postinjection were analyzed with an S0-coated sandwich ELISA. Data represented as the mean of five individual mice ± standard error of the mean and plotted in log 10 scale. *, $P < 0.05$, **, $P < 0.01$.

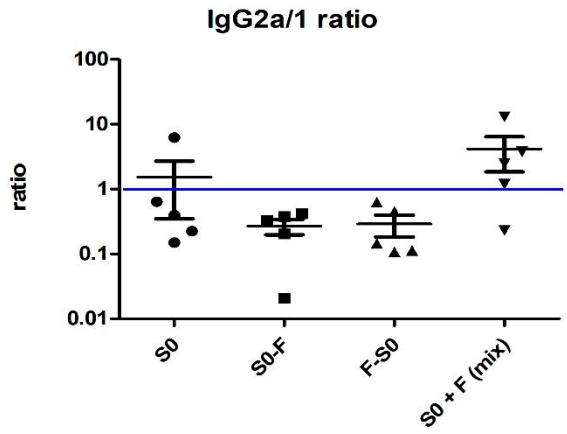


Figure 19. IgG2a and IgG1 subtypes of S0-specific IgG at 6 weeks postinjection.

Blood samples from 6 weeks postinjection were analyzed by ELISA, and the IgG2a/IgG1 ratio was calculated. All data were plotted on a log 10 scale.

(2) Evaluation of recombinant proteins on PRR *in vitro*

Flagellin has been shown to be a highly potent adjuvant through TLR5 in a number of immune cells (Hajam et al., 2017). To interpret the differences of S0-specific IgG outcomes among various groups, the epithelial cell line Caco-2, which expresses TLR5 constitutively, was used to evalute the TLR5 stimulating ability of each recombinant protein. Each recombinant protein was treated to Caco-2 cells at 1, 10, or 100 nM. Representative cytokines for the TLR5 pathway (IL-8 and MCP-1) were measured in the conditioned medium by ELISA 72 h after treatment. As shown in Figure 20, S0-F, F-S0 and F significantly induced IL-8 than S0 alone control at 10 and 100 nM. F treatment induced IL-8 more significantly than S0-F or F-S0 treatment at 1 nM treatment condition. Similar patterns were confirmed for MCP-1 (Figure 20b). There was no clear difference in the ability of S0-F and F-S0 to induce IL-8 or MCP-1.

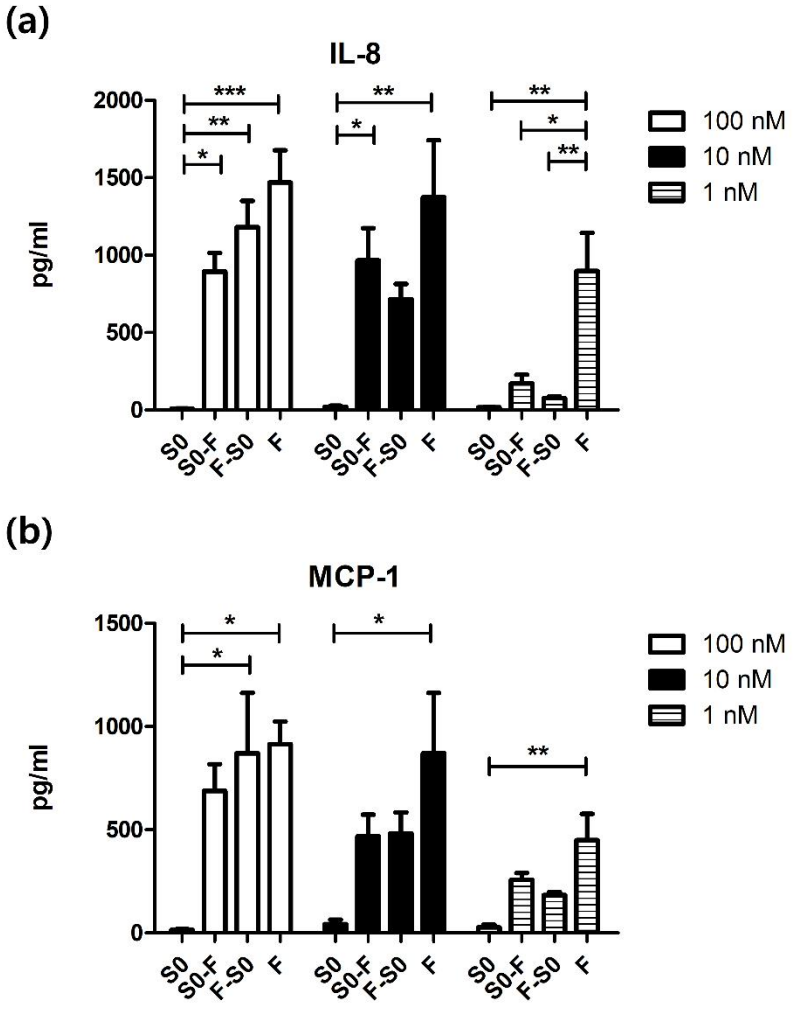


Figure 20. The TLR5 activation properties of S0, S0-F, F-S0 and F in the Caco-2 model.

(a) IL-8 and **(b)** MCP-1, downstream cytokines of TLR5, were secreted from Caco-2 cells 72 h after treatment with 1, 10, 100 nM of S0, S0-F, F-S0 and F. Supernatants were analyzed by ELISA. *, ** and *** indicate $p < 0.05$, 0.01 and 0.001, respectively.

(3) Evaluation of recombinant proteins on PRR *in vivo*

For more in-depth analysis, each recombinant protein's ability to activate the TLR5 pathway was investigated *in vivo*. Equivalent molar values of each recombinant protein were injected intraperitoneally into each mouse and sera were analyzed by ELISA to detect IL-6, a marker of TLR5 activation. The data revealed that the F treatment induced IL-6 levels stronger than S0-F or F-S0 treatments at 4 h (Figure 21a), consistent with the *in vitro* data (Figure 20). S0-F and F-S0 had a tendency to have superior ability in IL-6 stimulation than S0, however, it was lower than F.

Flagellin can be recognized not only by the TLR5 on the cell surface but also by the cytosolic receptor NLRC4/NAIP5 (Miao et al., 2007). Flagellin's adjuvant effect on the humoral immune response can be varied by activation extent of both receptors (Vijay□Kumar et al., 2010;Li et al., 2016). The ability of each recombinant protein to activate the cytosolic receptor NLRC4/NAIP5, therefore, was also confirmed by measuring IL-18, its responsive cytokine, from sera. Interestingly, the S0-F induced serum IL-18 levels significantly higher than all other groups at 1 h (Figure 21b). Similar patterns were also confirmed at 4 h.

The genetic conjugation of F-S0 may be the reason of its superior ability to induce S0-specific IgG relative to the mixture of S0/F. In fact, it is generally accepted that the physical fusion of immunopotentiators to antigens has been proved to induce stronger immune responses than separated treatments in many

previous researches (Huleatt et al., 2007;Kreutz et al., 2012;Huang et al., 2016;Moyle, 2017). The conjugation ensures the delivery and uptake of both antigen and immunopotentiator by the same antigen presenting cell, making the potentiation of antigen-specific immune responses, ended up with strong immune response induction (Blander and Medzhitov, 2006;Zom et al., 2012;Kastenmüller et al., 2014). Indeed, the conjugation of flagellin to various antigens has been shown to support stronger antigen-specific humoral immune responses than mixture regimen (Huleatt et al., 2007;Delavari et al., 2015;Moyle, 2017).

The role of two receptors for flagellin on its adjuvant effect is suggested to be complementary (Vijay□Kumar et al., 2010;López-Yglesias et al., 2014), however, the NLRC4/NAIP5 pathway is thought to interfere flagellin's adjuvancy in the presence of intact TLR5 activation. It was demonstrated that FliC-L3A flagellin which has point mutations and lacks the ability to activate NLRC4/NAIP5 induced a significantly higher antigen-specific serum IgG titer than wild-type flagellin when utilized as an adjuvant with p24, a model antigen, in mice (Li et al., 2016). A/J mouse, which is naturally partially deficient in *Naip5*, showed higher IgG responses to ovalbumin (OVA) than C57BL/6 mice after immunization with a mixture of flagellin and OVA (López-Yglesias et al., 2014). Our data revealed that S0-F treatment induced IL-18, a representative cytokine of the NLRC4/NAIP5 pathway, stronger than F-S0 or F treatment in mice (Figure 21b), demonstrating that the superior ability of S0-F to activate the

NLRC4/NAIP5 pathway is thought to interfere flagellin's adjuvant effect and reduce serum S0-specific IgG in spite of its conjugation advantage.

Flagellin showed various abilities to activate its cognate receptors depending on its status of conjugation. In fact, there have been several researches evaluating the effect of the directionality of the flagellin conjugation to its bioactivity. For example, flagellin conjugation to the C-terminus of HPV-16 E7 protein activated the TLR5 pathway 10-fold more stronger than its counterpart, while flagellin conjugation to the N-terminus of E7 stimulated the NLRC4/NAIP5 pathway significantly stronger than its counterpart or even flagellin alone form (Lin et al., 2016b). Wenzhi Tan *et al*. also showed that FlaB conjugated to the C-terminus of the recombinant norovirus P domain activated TLR5 stronger than FlaB alone through an NF-kB luciferase reporter assay (unpublished data). It was speculated that the receptor binding domains of flagellin may be influenced by the presence of the S0 protein, causing the NLRC4/NAIP5 binding domain of S0-F to be more exposed than the binding domain F-S0 or F. Based on the inference, the structure model for S0-F, F-S0 and F was suggested in Figure 22.

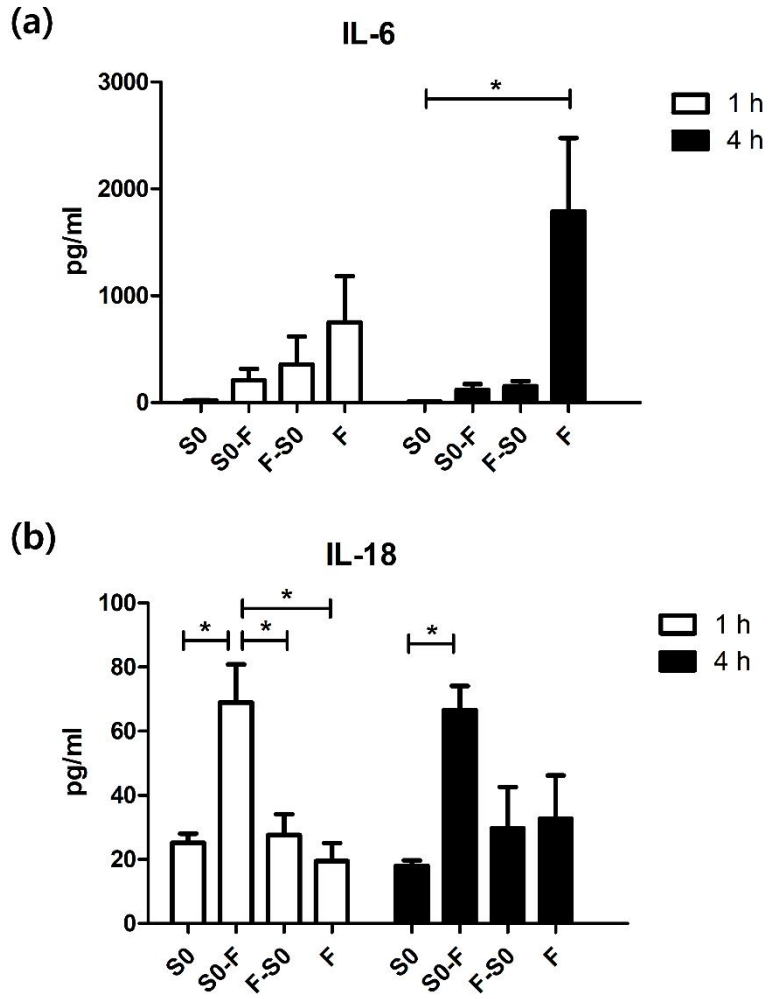


Figure 21. The TLR5 and NLRC4/Naip5 activation properties of S0, S0-F, F-S0 and F in a mouse model.

Mice were intraperitoneal injected with equivalent moles of S0, S0-F, F-S0 and F. Blood was collected 1 and 4 h after injection. From blood samples, **(a)** IL-6 and **(b)** IL-18, downstream cytokines of TLR5 and Nlrc4/Naip5, respectively, were analyzed by ELISA. * and ** indicate $p < 0.05$ and 0.01, respectively.

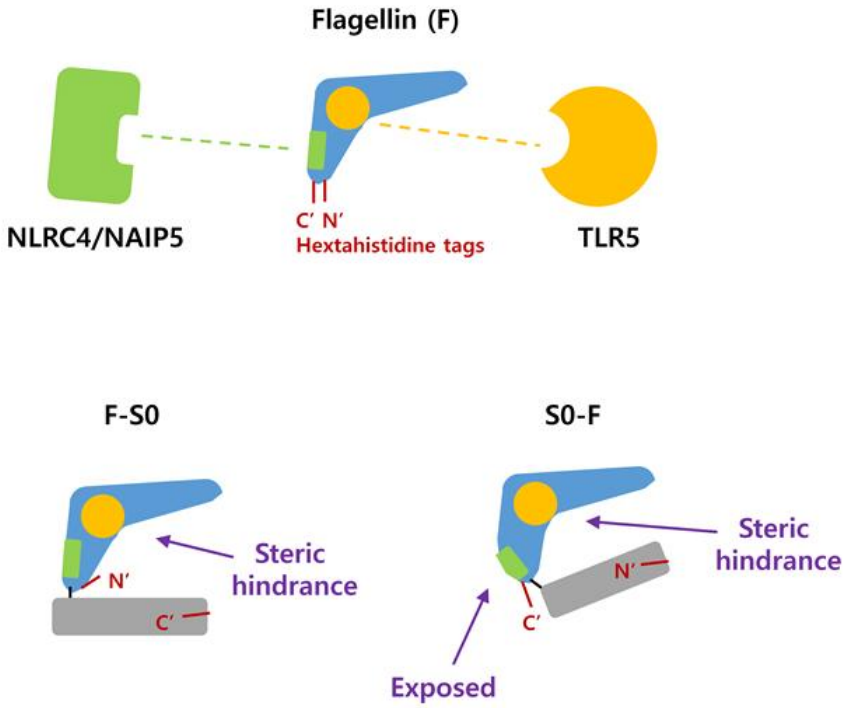


Figure 22. Proposed diagram of structural model for S0-F, F-S0 and F.

The TLR5 binding site of each F-S0 and S0-F could be interfered by conjugated S0. In contrast, the NLRC4/NAIP5 binding site of S0-F is inferred to be exposed after S0 conjugation, while that of F-S0 might not be exposed

4. Conclusion

In this study, flagellin, *Vibrio vulnificus* FlaB, was conjugated to the N- or C-terminus of PEDV antigen S0 and evaluated its ability to improve protein solubility and an antigen-specific humoral immune response (Figure 23). The ability of immunopotentiator conjugation with an antigen to enhance the solubility and immunogenicity of the vaccine was confirmed once again. Conjugation of flagellin to S0 enhanced the solubility of the recombinant proteins when combined with molecular chaperone *tig*. Though conjugation of flagellin did not ameliorate the solubility of all other proteins, however, flagellin has been shown to be a valuable candidate for enhancing antigen solubility in proteins that are prone to form aggreagation such as S0. Conjugation of flagellin to the N-terminus of S0 enhanced the S0-specific humoral immune response more than any other regimen using flagellin. However, flagellin conjugation to the N-terminus of other antigens (e.g. HIV gp120, HPV E7) is not guaranteed to ameliorate the antigen-specific humoral response because flagellin's ability to activate TLR5 or NLRC4/NAIP5 in its position differs depending on the conjugated antigens. Rather, it is recommended that flagellin-conjugated antigens having inferior ability to activate NLRC4/NAIP5 is thought to be good candidates for increased antigen-specific humoral immune response induction. Meanwhile, S0-specific IgG1 was the dominant form of antibodies compared to IgG2a when S0 was injected. S0 seems to inherently induce Th2-type immune response against itself as the induction of IgG2a and IgG1 represent Th1- and

Th2-type immune responses, respectively (Stevens et al., 1988) (Figure 19. Flagellin alone induced IgG2a more than IgG1 in contrast to S0-F and F-S0 case, which indicates that flagellin retrogresses the type of immune response from Th2 to Th1. As other flagellins generally induce a mixed Th1/Th2 type of immune response, the use of flagellin from *Vibrio vulnificus* could offer good opportunities for cancer vaccine development.

Here, the adjuvant effect of flagellin was evaluated by the induction of systemic humoral immune response (Figure 18). Induction of the virus-specific IgA at colostrum/milk after mucosal immunization is an important factor in terms of lactogenic immunity though many researches vaccinated the sow through intramuscular route (Subramaniam et al., 2018) (Langel et al., 2016). Further works using target animals, however, is necessary for the development of this antigen as an effective vaccine. These findings provide important informations for the rational design of not only a PED vaccine but also flagellin-based immunotherapeutics.

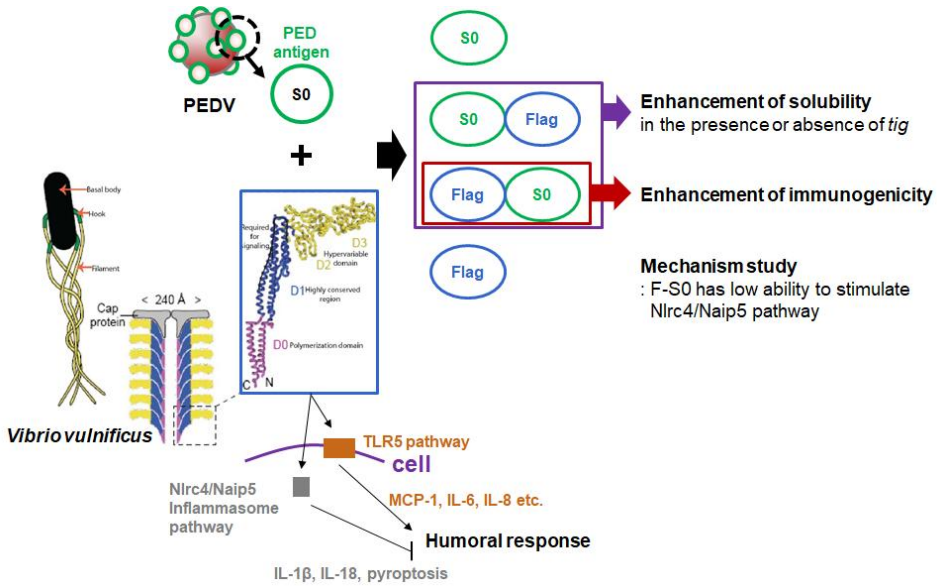


Figure 23. Conclusion of study I.

Conjugation of flagellin derived from *Vibrio vulnificus* to S0 enhanced overall protein solubility in the presence or absence of chaperone *tig*. Only F-S0, however, enhanced antigen-specific immune response against S0. Both genetic conjugation form and low ability to stimulate NLRC4/Naip5 pathway were inferred to be the reason of F-S0's superior adjuvancy

Study II. Effects of Complement Fragment C3d, C4d Conjugation to S0 as a Fusion Adjuvant

1. Introduction

C3d is one of a small split byproduct fragments originated from the complement C3, which covalently bind to broad range of pathogens (Noris and Remuzzi, 2013). C3d has been frequently applied to many subunit vaccine antigens as a fusion adjuvant due to its effectiveness of enhancing the humoral immune responses to target antigens (Dempsey et al., 1996;Green et al., 2003;Li et al., 2003;Watanabe et al., 2003;Wang et al., 2004a;Wang et al., 2004b;Koch et al., 2005;Zhang et al., 2011). The C3d possesses an unnaturally high frequency of T cell epitopes responsible for many types of host MHC class II molecules (Knopf et al., 2008;De Groot et al., 2015). Thus, when the B cells uptake the subunit vaccine constructed by fusion of the target antigen with C3d, they could present both T cell epitopes not only from the target antigen but also from the C3d, then the humoral immune response could be facilitated through C3d-specific autoreactive T cells which could recognize the T epitopes from the C3d, in addition to target antigen-specific T cells via MHC class II molecules (De Groot et al., 2015). In fact, C3d-specific autoreactive memory T cells are circulating in the periphery of healthy humans (Knopf et al., 2008;De Groot et al., 2015).

In study II, another endogenous candidate protein which possess a high T cell epitope frequency was explored with a hypothesis that they could be utilized as efficient fusion adjuvants to construct subunit vaccine if they also had an activation mechanism of the autoreactive T helper cells like the C3d. A split fragment derived from the complement C4, C4d, was selected as a novel endogenous candidate protein for the fusion adjuvant through *in silico* analysis among various serum proteins by evaluation of T cell epitope frequency in their intra-structures. The biological function of the C4d is still unknown in major part although there have been some reports that C4d might be associated with the control of the complement cascade (Platt 2002, Murata and Baldwin III 2009).

After selection of C4d, the conjugation effects of C3d and C4d to the target antigen S0 were evaluated. First, the solubility enhancement of C3d and C4d conjugation to S0 was confirmed in the presence of *tig* expression. Next, the effects of C3d and C4d conjugation to S0 on humoral immune response enhancement were evaluated. To validate the potential of the C4d as a novel fusion adjuvant through the mechanism of T cell epitope donation, IL-4 secreting T cells specific for C4d were also enumerated by ELIspot analysis. These observations provide information that the C3d and C4d could be utilized as an adjuvant regardless of its fusion to target antigen on the development of PED subunit vaccine.

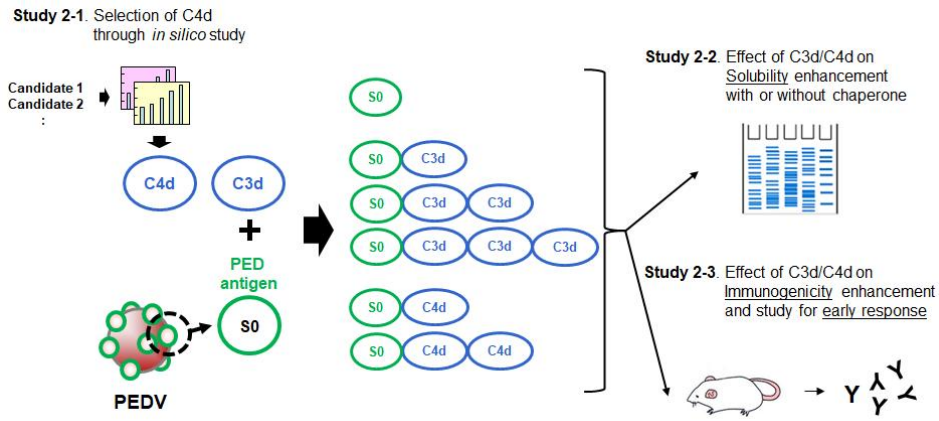


Figure 24. Graphical abstract of study II.

C3d and C4d were used as a fusion adjuvant candidate on the development of PED subunit vaccine. C4d was selected as a novel fusion adjuvant through *in silico* study. The effect of C3d and C4d conjugation to S0 on solubility was evaluated in the presence of chaperone *tig*. The effect of their conjugation to S0 on immunogenicity was also evaluated in mice.

2. Materials and methods

1) *in silico* analysis

(1) IEDB analysis tool

The purpose of IEBD analysis tool usage in this study is the selection of protein with high T cell epitope frequencies among various protein candidates (Figure 25). The frequency of T-cell epitopes of various protein candidates reported to bind pathogens broadly was identified using the IEDB analysis tool (http://tools.immuneepitope.org/main/tcell/). This tool cleaves the input protein sequence into peptide fragments appropriately sized for binding with MHC class II. Each peptide fragment was analyzed against six common MHC class II alleles that cover about 90% of the human population (DRB1*01:01, DRB1*03:01, DRB1*07:01, DRB1*09:01, DRB1*11:01, DRB1*15:01) (Southwood et al., 1998) and assigned a Z-score with lower Z-scores denoting a high binding ability to MHC class II. Z-scores below 5 or 10 were designated as the thresholds indicating relative high binding ability. The total number of fragments with Z-scores either below 5 or below 10 were aggregated and divided by the length of each input protein to create “the frequency of T cell epitopes”. The principles of IEDB analysis tool including the process of T cell epitope frequency calculation are described in Figure 26. The entry accession numbers of each protein candidate are listed in Table 12.

The T cell epitope frequencies of proteins from mouse origin including C3d,

C4d, and serum albumin were also analyzed through IEDB analysis tool against H-2 I A^d and H-2 I E^d.

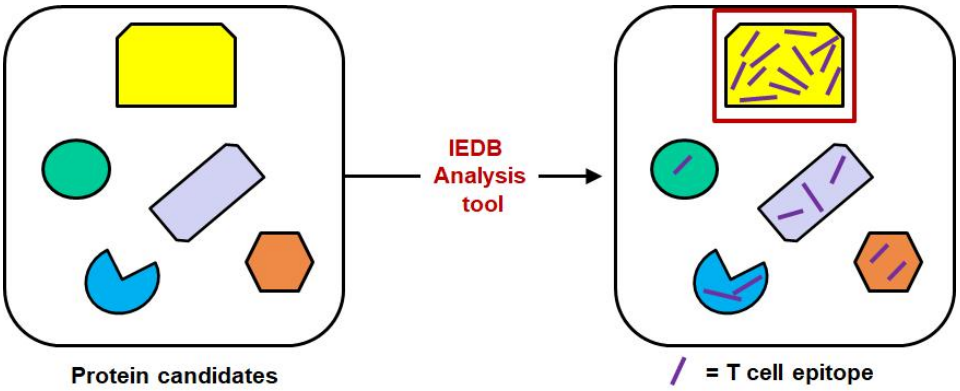


Figure 25. Proposed diagram of structural model for S0-F, F-S0 and F.

IEDB analysis tool was used to select protein with high T cell epitope frequency among various protein candidates in study II.

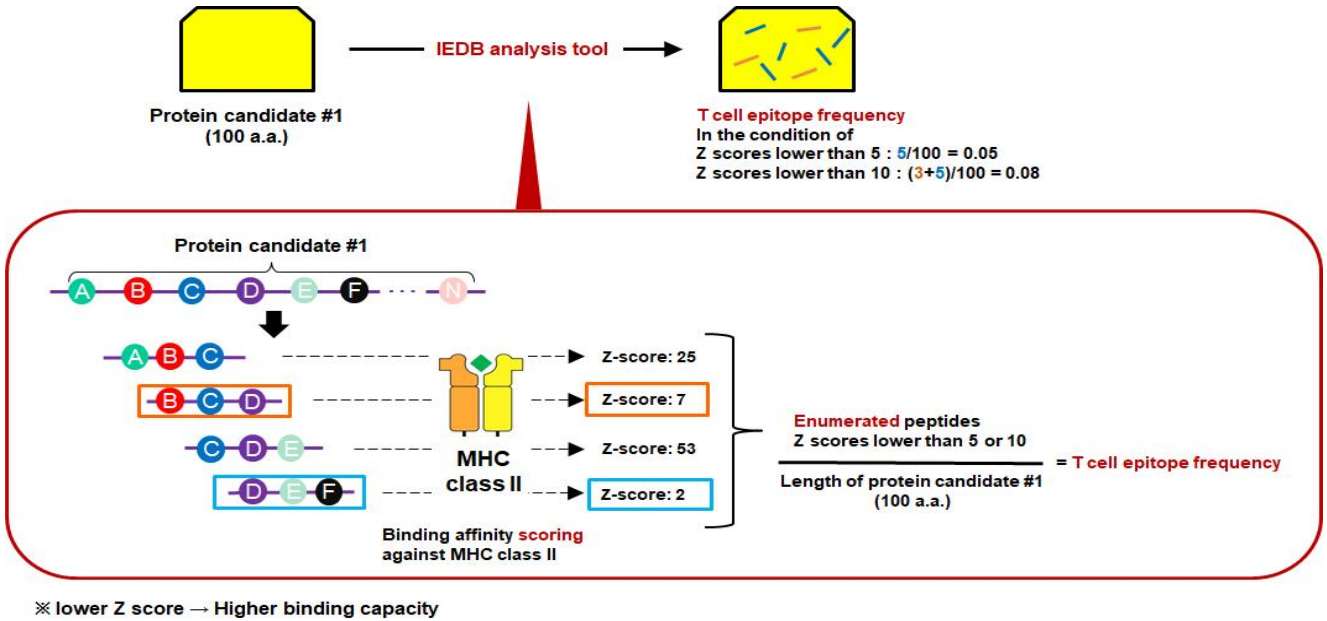


Figure 26. The principles of IEDB analysis tool.

Possible peptide fragments binding to MHC class II from protein candidate #1 are evaluated their affinity to bind MHC class II and get Z scores. Enumerated peptides in the condition of Z scores lower than 5 or 10 are divided by length of protein of interest, generating T cell epitope frequency.

Table 12. Information of protein candidates.

Name	Entry accession number	Position (a.a.)	Remark
C3d	P01024	1002-1303	C3
C4d	P0C0L4	957-1336	C4
Factor Bb	P00751	260-764	Factor B
MBL	P11226	21-248	PRRs for lectin pathway
Ficolin-1	O00602	30-326	
Ficolin-2	Q15485	26-313	
Ficolin-3	O75636	24-299	
Serum albumin	A0A0C4DGB6	19-604	Negative control

MBL, Mannose binding lectin 2; PRR, pattern recognition receptor

(2) BLAST

The sequence similarity of proteins was analyzed by standard protein BLAST at NCBI with an algorithm of blastp (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The results are expressed as the percentage of "identicals" or "positives". "identicals" refers to the number of identical amino acids, while "positives" refers to the number and fraction of residues for which the alignment scores have positive values. Entry accession numbers of analyzed sequence are listed in Table 13.

Table 13. Entry accession numbers of C3d and C4d from various origins.

	C3d		C4d	
	Entry accession number	**Position (a.a.)**	**Entry accession number**	**Position (a.a.)**
Mouse	P01027	1002-1303	P01029	953-1332
Rat	P01026	1002-1303	P08649	952-1331
Pig	P01025	1001-1302	B0LFE9	955-1333
Human	P01024	1002-1303	P0C0L4	957-1336

2) Expression and purification of recombinant proteins

(1) Vector construction for recombinant proteins

The nucleotide sequence of spike protein from PEDV K14JB01 strain (GenBank accession No. KJ623926) was used as a reference to synthesize a partial protein, S0 (25-229 amino acid) gene. The whole genome sequence from mouse C3d (1002-1303 amino acid from Entry accession number P01027) and mouse C4d (953-1332 amino acid from Entry accession number P01029) were used as a reference to synthesize the mC3d unit and mC4d unit which have *BglII* restriction enzyme site at 5'-end and *BamHI*, *XhoI* restriction enzyme sites at 3'-end. The final mC3d unit, mC4d unit and S0 sequence were synthesized (Bioneer, Korea) after codon-optimization using the table of codon usage in *E. coli*.

The nucleotide sequence of S0 was also amplified by PCR using NS0-F/BS0-R primers and cloned into pET28a(+) vector using *NheI* and *BamHI* restriction enzyme sites (pS0B) to clone pS0-mC3d1, pS0-mC3d2, and pS0-mC3d3 as a precursor vector. The nucleotides of mC3d unit after restriction by *BglII* and *XhoI* was cloned into a pS0B using *BamHI* and *XhoI* restriction enzyme sites (pS0-mC3d1). *BamHI* and *BglII* are isocaudomers and generate nucleotide sequence GGATCT after ligation which is translated to produce Gly-Ser linker and are not cut by both restriction enzyme *BamHI* and *BglII*. The mC3d unit with *BglII* and *XhoI* sticky ends was ligated again into pS0-mC3d1 which was

digested using *BamHI* and *XhoI* restriction enzyme sites (pS0-mC3d2). The mC3d unit with *BglII* and *XhoI* sticky ends was ligated again into pS0-mC3d2 which was digested using *BamHI* and *XhoI* restriction enzyme sites (pS0-mC3d3). To express mC3d alone, mC3d unit sequence was PCR-amplified using NmC3d-F/XmC3d-R primers and cloned into pET28a(+) vector using *NheI* and *XhoI* restriction enzyme sites (pmC3d). For expression vectors utilizing mC4d (pS0-mC4d1, pS0-mC4d2, and pmC4d), the same cloning strategy was applied as expression vectors utilizing mC3d (pS0-mC3d1, pS0-mC3d2, and pmC3d).

Additionally, pET28a(+) vectors support hexahistidine tag (6X His) nucleotide sequences flanking both ends of the multiple cloning site, resulting recombinant proteins would containing 6X His on both C- and N-terminus of expressed recombinant proteins. DNA sequences of the resulting expression vectors were verified through Sanger sequencing. The nucleotide sequence of various primers for PCR-amplification are listed in Table 14

Table 14. PCR primer sequences for study II.

Names	Sequences
NS0-F	5'-GCTAGC CGTTGCAGCGCGAACAC
XS0-R	5'-CTCGAG CGGTTGGTAGCTAATGCC
BS0-R	5'-GGATCC CGGTTGGTAGCTAATGCC
NmC4d-F	5'-GCTAGC ACTCTTGAAATTCC
XmC4d-R	5'-CTCGAG ACCTCTACCCATACT
NmC3d-F	5'-GCTAGC CACCTGATTGTTACCC
XmC3d-R	5'-CTCGAG ACGGCTCGGCAGG

(2) Protein expression in the presence or absence of *tig*

Each of recombinant plasmid (pS0, pS0-mC3d1, pS0-mC3d2, pS0-mC3d3, pmC3d, pS0-mC4d1, pS0-mC4d2, and pmC4d) was transformed into the expression host *E. coli* BL21(DE3) together with pTf16 (Takara, Japan) which could express chaperone *tig* after L-arabinose induction. A seed culture was prepared by inoculating with a single colony of recombinant *E. coli* and grown overnight in LB broth containing 50 μg/mL of kanamycin and 20 μg/mL of chloramphenicol. 1% of the seed culture was inoculated to LB broth supplemented with 0.5mg/mL of L-arabinose, 20 μg/mL of chloramphenicol and 50 μg/mL kanamycin and grown until optical density at 600 nm reached 0.4 at which time cells were cooled to 4 °C on ice for 10 min. For the expression of recombinant proteins in the absence of *tig*, the addition of L-arabinose was omitted. Cultures were then stimulated using 0.1 mM IPTG to induce target protein expression and grown at 15 °C, 150 rpm for 24 hours.

(3) Analysis of protein solubility with SDS-PAGE and densitometer

Following processes including cell harvest, lysozyme treatment, disruption, collection of soluble and insoluble fraction, and analysis of protein solubility through SDS-PAGE followed by densitometer are the same as described above. Overall scheme of protein expression and solubility analysis are described in Figure 13.

(4) Purification, dialysis and concentration of recombinant proteins

Soluble fractions of each recombinant protein were purified using affinity chromatography on a Ni^{2+}-NTA column. Purified samples were dialyzed against phosphate buffered saline (PBS) changed three times at 4 °C and concentrated down using Amicon ultra-15 centrifugal filter tubes (Merck, Germany). Fractions of each recombinant protein were analyzed through SDS-PAGE followed by Coomassie Blue staining. Band densities of each recombinant protein were measured to calculate the purity using ImageJ software. The S0 in insoluble fractions was subsequently refolded as described previously (Piao et al., 2016a).

3) *In vivo* immunization

(1) Mouse immunization experiment for C3d adjuvant

6-week-old BALB/c mice (n=4) were immunized intramuscularly with 40 pmol of each recombinant protein (1.00 μg of S0, 2.35 μg of S0-mC3d1, 3.67 μg of S0-mC3d2, and 5.00 μg of S0-mC3d3) three times at 2-week intervals after adaptation for one week. Mice were also immunized with 1.0+4.00 μg of S0+mC3d per mouse as a non-conjugated mixture control for S0-mC3d3. CFA and IFA were mixed with antigens for the priming and boosting of mice, respectively. Treatment group information and regimens are listed in Table 15. Blood samples were collected on days 13, 27, and 42 (sacrifice) from intrapetrosal veins with a disposable syringe and delivered into sterilized tubes. Serum was separated by centrifugation at 12,000 rpm for 3 min using a serum separator tube (BD microtainer, NJ, USA). Overall scheme is described in Figure 14.

Table 15. Treatment group information and regimens for mouse immunization experiment with C3d conjugated recombinant proteins.

	Antigen	Note	Amount		Oil adjuvant	Solution	Volume	Route	n
			nmol/mouse	µg/mouse					
1	None	PBS only	---	---	CFA (0 wk) + IFA (2, 4 wk)	PBS	50 µL	I.M.	5
2	S0	PED Ag	40	1.0					
3	S0-mC3d1	S0 fused to one repeat of mC3d		2.35					
4	S0-mC3d2	S0 fused to two repeats of mC3d		3.67					
5	S0-mC3d3	S0 fused to three repeats of mC3d		5.00					
6	S0 + mC3d (x3)	S0 mixed with 3 x mC3d	40 + 120	1.0 + 4.00					

(2) Mouse immunization experiment for C4d adjuvant

6-week-old Balb/c mice (n=4) were immunized intramuscularly with 0.12 nmol of each recombinant protein (3.0 µg of S0, 8.0 µg of S0-mC4d1, 12.9 µg of S0-mC4d2) three times at 2-week intervals after adaptation. Mice were also immunized with 3.0+5.2 µg of S0+mC4d and 3.0+10.4 µg of S0+mC4d per mouse as a non-conjugated mixture control for S0-mC4d1 and S0-mC4d2, respectively. CFA and IFA were mixed with antigens for the priming and boosting of mice, respectively. Treatment group information and regimens are listed in Table 16. Blood samples were collected on days 13, 27, and 42 (sacrifice) from intrapetrosal veins with a disposable syringe and delivered into sterilized tubes. Serum was separated by centrifugation at 12,000 rpm for 3 min using a serum separator tube (BD microtainer, NJ, USA). Splenocytes of each

mouse were collected on day 42 postinjection and analyzed by ELISPOT assay. Overall scheme is described in Figure 27.

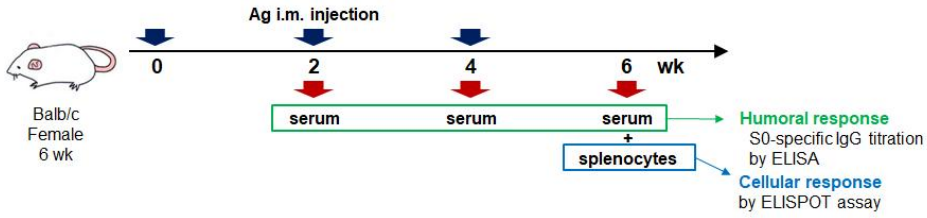


Figure 27. Overall scheme of mouse immunization experiment in study II (C4d).

Each mouse was immunized with regimens listed in Table 16 three times at 2-week intervals. Blood samples were collected on days 13, 27, and 42 and analyzed by ELISA to evaluate S0-specific IgG titers. Splenocytes were collected on day 42 and analyzed by ELISPOT assay to evaluate IL-4 secreting T cells.

Table 16. Treatment group information and regimens for mouse immunization experiment with C4d conjugated recombinant proteins.

	Antigen	Note	Amount		Oil adjuvant	Solution	Volume	Route	n
			nmol/mouse	μg/mouse					
1	None	PBS only	---	---	CFA (0 wk) + IFA (2, 4 wk)	PBS	50 μL	I.M.	4
2	S0	PED Ag	0.12	3.0					
3	S0-mC4d1	S0 fused to one repeat of C4d		8.0					
4	S0-mC4d2	S0 fused to two repeats of C4d		12.9					
5	S0-mC4d	S0 mixed with 1 x mC4d	0.12 + 0.12	3.0 + 5.2					
6	S0 + mC4d (x3)	S0 mixed with 3 x mC4d	0.12 + 0.24	3.0 + 10.4					

(3) ELISA

To detect S0-specific IgG levels, sera were analyzed by indirect ELISA. 96-well immunoplate (SPL, Korea) were coated with 1 μg/ml of purified recombinant S0 (100 μl/well) in carbonate-bicarbonate buffer (pH 9.6) at 37 ℃ for 2 h. Coated plates were blocked with 0.5 % skim milk in PBS containing 0.05% of Tween 20 (100 μl/well) for 1h at room temperature. Then, 5-fold serially diluted sera starting from 1:100 dilution fold were added into the wells (100 μl/well) and incubated at 37 °C for 2 h. After incubation, horse radish peroxidase-conjugated goat anti-mouse IgG (1:2000 dilutions, 100 μl/well) were added to the designated wells for 1 h at room temperature. 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 (100 μl/well) as a HRP substrate and incubated for 30 min without light interference, followed by the addition of stop solution (0.16 M H2SO4; 100 μl/well) to stop the enzymatic reaction. Finally, the absorbance was measured at 450 nm using microplate reader (Infinite® 200 PRO, USA). ELISA results are expressed as the endpoint titer.

(4) ELISPOT assay

The number of IL-4 secreting T cells specific for each protein including S0, mC4d, and mC3d was determined by ELISPOT assay. At 6-week postinjection,

mice were sacrificed by cervical dislocation and spleens removed using aseptic technique. Spleens were crushed and filtered through a 40 μm single-cell nylon mesh (Falcon, Becton Dickson, Paramus, N.J.). Cells were pelleted and erythrocytes were lysed using ACK lysis buffer (Gibco, USA). Cells were subsequently washed with PBS and resuspended in RPMI 1640 medium with 10% fecal bovine serum (FBS) (Gibco, USA). Splenocytes were added to the wells (106 cells/well) of IL-4+ ELISpot plate purchased from RnD SYSTEMS® kit (R&D Systems, USA) after coating with anti-mouse IL-4 antibody and stimulated with each protein (final concentration 5 μg/mL) or media as a control. After incubating the plate for 72 hours at 37°C with 5% CO_2, spots were developed according to the manufacturer instructions. The spots were visualized by the A.EL.VIS ELISPOT software Eli Analyze (A.El.VIS, USA) and exact spot counts were determined using the naked eye. Numbers refer to spot-forming cells (SFC) per 10^6 splenocytes.

3. Results and discussion

1) *In silico* selection of C4d as a fusion adjuvant

C3d have many T cell epitopes responsible for many types of host MHC class II molecule and work as a potent fusion adjuvant by donating those T cell epitopes (Knopf et al., 2008;De Groot et al., 2015). To find a novel endogenous protein candidate for the effective fusion adjuvant such as C3d, several serum proteins reported having a pathogen binding ability like C3d had been chosen and analyzed to determine the frequency of T cell epitopes in their intra-structures using the IEDB analysis tool. The candidates of serum proteins are listed below; Factor Bb which is a cleavage fragment of the plasma protein factor B that form a C3-convertase by binding to C3b on the pathogen surface (Noris and Remuzzi, 2013); Four PRRs including mannose-binding lectin (MBL), ficolin-1, ficolin-2, and ficolin-3 that circulate in blood or extracellular fluids and recognize carbohydrates on microbial surfaces, subsequently trigger the lectin pathway (Fujita et al., 2004;Noris and Remuzzi, 2013).

In IEDB tool, the amino acid sequence of each protein were fragmented into the small size of peptides appropriate for the binding to the MHC class II molecule and their binding affinity against the set MHC class II molecules were assessed to generate Z-scores. As the lower Z-score represents the higher binding affinity, the number of T cell epitopes of each protein could be deduced by enumerating the fragmented peptides having lower Z-scores; lower than 10 or

5 in this case (Figure 28). The frequency of T cell epitopes could also be calculated by subsequent normalization with their respective protein sizes. Six common MHC class II alleles (DRB1*01:01, DRB1*03:01, DRB1*07:01, DRB1*09:01, DRB1*11:01, DRB1*15:01) were set in this experiment to cover about 90 % of the human population (Southwood et al., 1998).

Data revealed that C3d had 0.68 and 1.41 frequency value of T cell epitopes in the condition of Z-scores lower than 5 and 10, respectively, while serum albumin, which was set as a negative control, had 0.33 and 0.77 (Figure 28). Among the serum proteins analyzed for selection of novel fusion adjuvant candidate, C4d had 0.54 and 1.12, which presented a similarly high frequency of T cell epitopes compared to C3d in the condition of Z-scores lower than 5 and 10, respectively.

In addition, the T cell epitope frequencies of mouse C3d and C4d sequence were also evaluated against mouse H-2 I A^d and E^d through IEDB analysis tool. Data revealed that mouse sequence of C3d and C4d have 0.162 and 0.171 value of T cell frequencies in the condition of Z scores lower than 10 compare to 0.098 value of serum albumin sequence set as a control (Figure 29). The T cell frequency value of mouse C4d in the condition of Z scores lower than 5, was even higher than that of C3d or serum albumin (0.092 in C4d case versus to 0.056 and 0.050 of C3d and serum albumin, respectively). C4d sequence has 0.092 and 0.171 value of T cell epitope frequency compare to 0.045 and 0.119 in C4 sequence except for C4d region in the condition of Z scores lower than 5 and 10, respectively, indicating that C4 has enriched T cell epitope in C4d region

(Figure 30). In this regard, C4d was selected as the endogenous protein candidate for a fusion adjuvant to construct model subunit vaccine. The sequence similarities among mouse, rat, pig and human C3d or C4d are listed in Table 17 and Table 18. The sequence similarities between C3d and C4d in mouse, rat, pig and human are listed in Table 19.

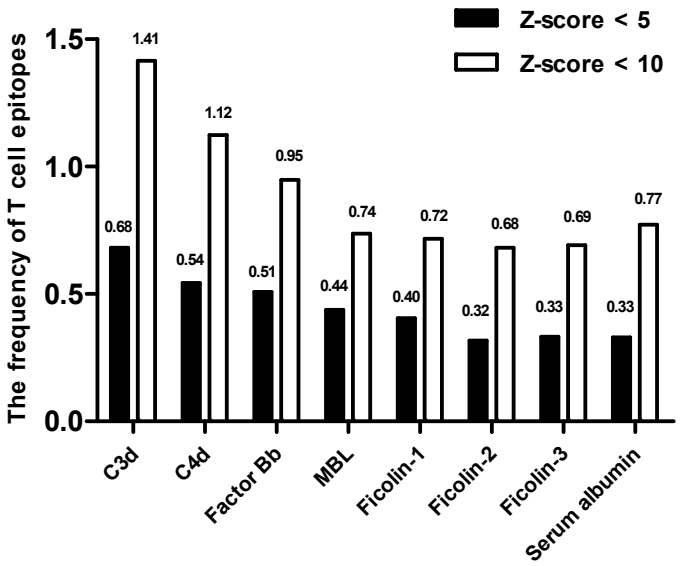


Figure 28. Analysis of the frequency of T cell epitopes on various serum protein candidates through IEDB analysis tool.

The frequency of T cell epitopes of each protein were analyzed against six MHC class II alleles including DRB1*01:01, DRB1*03:01, DRB1*07:01, DRB1*09:01, DRB1*11:01 and DRB1*15:01 through IEDB analysis tool under the condition of Z-scores below 5 (black bars) and below 10 (white bars).

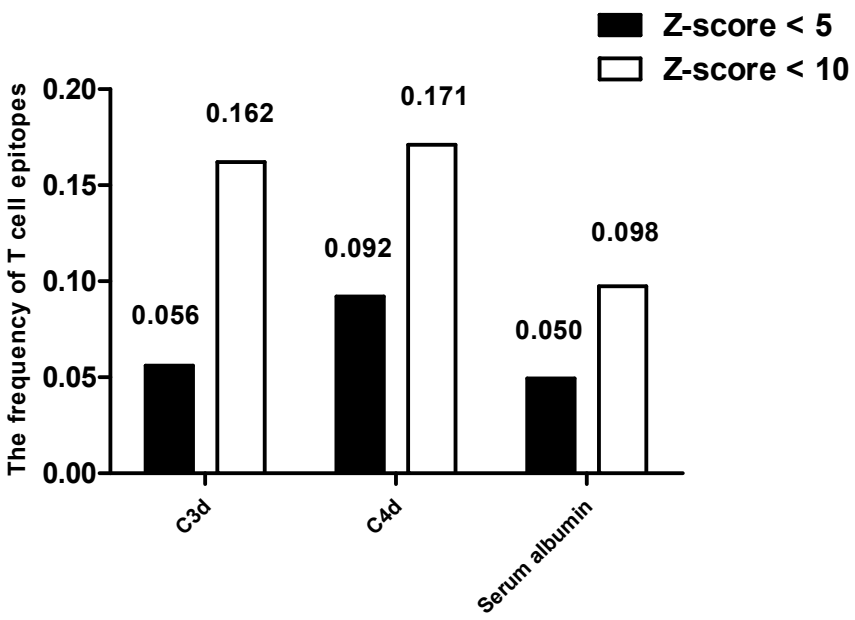


Figure 29. Analysis of T cell epitope frequency of mouse C3d and C4d against mouse H-2 I through IEDB analysis tool.

The frequency of T cell epitopes of each protein were analyzed against mouse H-2 I including H-2 I A^d and H-2 I E^d through IEDB analysis tool under the condition of Z-scores below 5 (black bars) and below 10 (white bars).

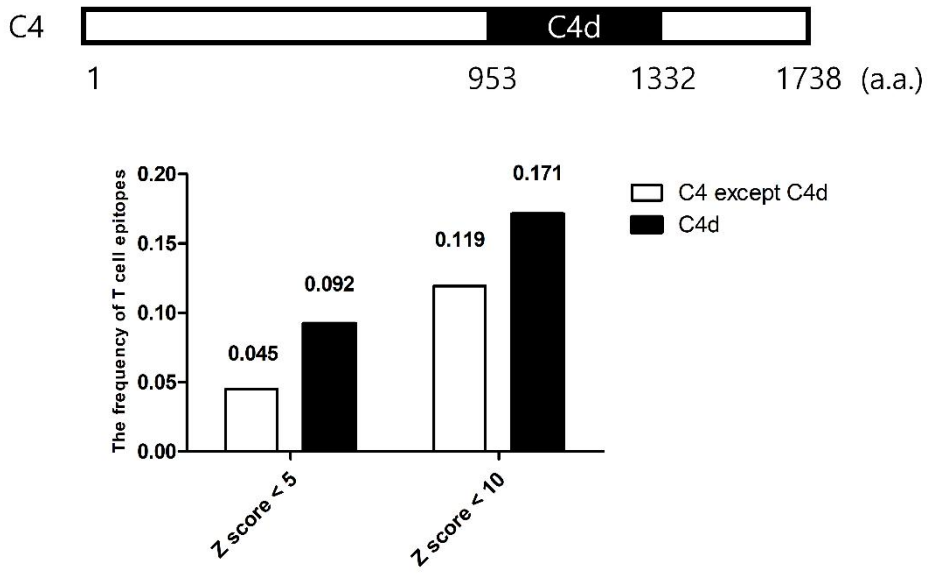


Figure 30. Analysis of T cell epitope frequency in C4 sequence.

The frequency of T cell epitopes of C4d sequence and C4 sequence except for C4d region were analyzed against mouse H-2 I including H-2 I A^d and H-2 I E^d through IEDB analysis tool under the condition of Z-scores below 5 and 10.

Table 17. C3d sequence similarities among mouse, rat, pig and human.

	mouse	rat	pig	human
mouse		94 (96)	82 (92)	84 (91)
rat	94 (96)		82 (91)	83 (90)
pig	82 (92)	82 (91)		80 (88)
human	84 (91)	83 (90)	80 (88)	

Values listed in table represent amino acid sequence percentage of identities

(positives) between each host.

Table 18. C4d sequence similarities among mouse, rat, pig and human.

	mouse	rat	pig	human
mouse		89 (94)	76 (87)	80 (89)
rat	89 (94)		78 (89)	83 (90)
pig	76 (87)	78 (89)		82 (89)
human	80 (89)	83 (90)	82 (89)	

Values listed in table represent amino acid sequence percentage of identities (positives) between each host.

Table 19. Sequence similarities between C3d and C4d of mouse, rat, pig and human.

		C3d			
		mouse	**rat**	**pig**	**human**
C4d	**mouse**	33 (46)			
	rat		33 (46)		
	pig			34 (50)	
	human				33 (48)

Values listed in table represent amino acid sequence percentage of identities (positives) between C3d and C4d.

2) Effect of C3d and C4d conjugation to S0 on solubility enhancement

(1) Solubility enhancement ability of C3d conjugation against S0

To evaluate the effect of C3d conjugation on solubility enhancement, the solubility of each recombinant protein including S0, S0-mC3d1, S0-mC3d2, and S0-mC3d3 was checked in the presence or absence of *tig*. Each vector was transformed into *E. coli* BL21(DE3) together with the *tig* expression vector pTF16 [Figure 12 (b)] and the *tig* expression was regulated by adding L-arabinose or not to make the *tig* presence or absence condition, respectively. Subsequently, IPTG were added to induce each protein of interest. Soluble lysates were separated from insoluble lysates after disruption by sonication. Soluble and insoluble fractions of each recombinant protein were confirmed through SDS-PAGE followed by Coomassie Blue staining to assess the solubility of each recombinant protein as described in materials and methods.

Data revealed that not only S0 alone, but also other recombinant proteins including S0-mC3d1, S0-mC3d2, and S0-mC3d3 were expressed almost entirely as an insoluble form in the absence of *tig*. In contrast, all of recombinant proteins were expressed as a soluble form in the presence of *tig* (Figure 31): coexpression of *tig* improved the solubility of S0, S0-mC3d2, and S0-mC3d3 from 0 % to 62 %, 0 % to 42 %, and 3 % to 35 %, respectively. For S0-mC3d1, though it was hard to quantitate the soluble expression accurately as the band of

it was overlaps with *tig* band, the enhanced band densities of S0-mC3d1 in this position compare to other recombinant proteins suggest that the solubility of S0-mC3d1 was improved in part. Improvement of S0 solubility in the presence of *tig* was confirmed once again [Figure 31 (b), (d)]. Though the coexpression of *tig* improved the solubility of each recombinant protein, the conjugation of C3d could not further ameliorate their solubility.

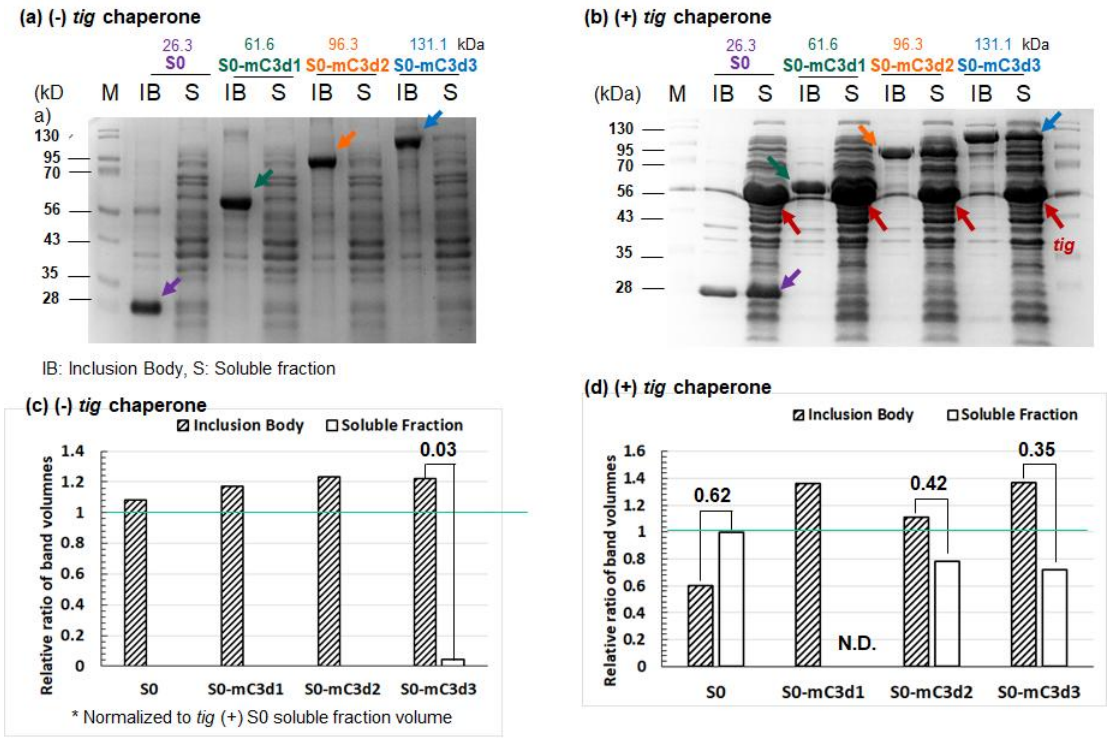


Figure 31. Soluble and insoluble expression of S0, S0-mC3d1, S0-mC3d2, and S0-mC3d3 in the presence or absence of *tig*.

Soluble and insoluble expression of each recombinant protein were evaluated by SDS-PAGE and densitometer analysis

(2) Solubility enhancement ability of C4d conjugation against S0

The effect of C4d conjugation on solubility enhancement was also evaluated. The solubility of each recombinant protein including S0, S0-mC4d1, S0-mC4d2, and mC4d was evaluated in the presence or absence of *tig*. Each vector was transformed into *E. coli* BL21(DE3) together with the *tig* expression vector pTF16 [Figure 16 (b)] and the *tig* expression was regulated by L-arabinose addition. Further processes are identical with C3d conjugation case.

S0 was expressed almost entirely as an insoluble protein, while 85 % of mC4d was expressed as a soluble protein. In contrast to S0, which was expressed mostly as an insoluble protein, the conjugation of mC4d to S0 greatly improved their solubility to 41 % and 62 % for S0-mC4d1 and S0-mC4d2, respectively, indicating that mC4d conjugation to S0 improved solubility in the *E. coli* expression system without *tig* expression [Figure 32 (a), (c)]. Besides, all of recombinant proteins except mC4d alone were expressed with improved solubility in the presence of *tig* [Figure 32 (b), (d)] : coexpression of *tig* improved the solubility of S0, S0-mC4d1, and S0-mC4d2 from 0 % to 60 %, 41 % to 75 %, and 62 % to 98 %, respectively. For mC4d alone, it is inferred that already high solubility (~85%) make it insensitive to the *tig* assistance. The ability of mC4d conjugation to improve the solubility is also confirmed in the condition of *tig* expression. The conjugation of mC4d to S0 improved the solubility of the recombinant protein from 60 % to 75 % and 60% to 96 % in S0-mC4d1, S0-mC4d2 case compare to S0 alone. Taking these results into account,

both mC4d conjugation and chaperone *tig* coexpression increased the solubility of S0-related recombinant proteins.

In addition, mC4d alone have a high expression amount as well as high solubility. As the fusion of a solubility enhancer to the N-terminus of a target protein can enable efficient translation initiation, the conjugation of mC4d to the N-terminus of S0 is inferred to have not only a high solubility but also a high amount of expression. Further works are needed to confirm this expectation.

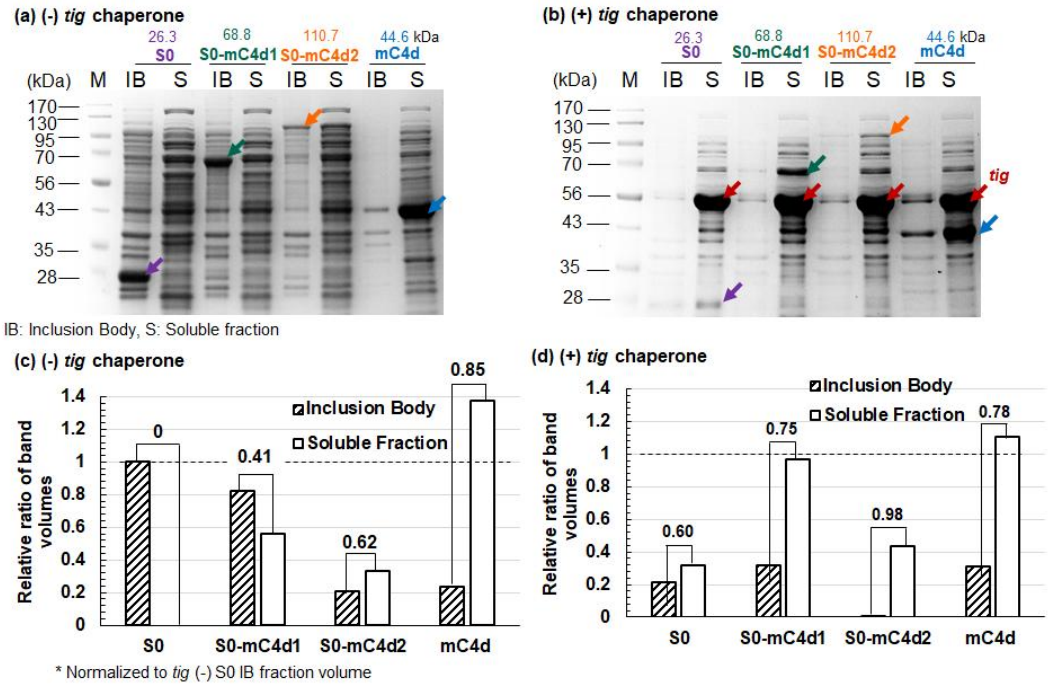


Figure 32. Soluble and insoluble expression of S0, S0-mC4d1, S0-mC4d2, and mC4d in the presence or absence of *tig*.

Soluble and insoluble expression of each recombinant protein were evaluated by SDS-PAGE and densitometer analysis. This figure is obtained from the thesis of YS Kim Cho.

(3) Final isolation of recombinant proteins

For further experiments, soluble lysates of each recombinant protein was purified using Ni^{2+}-NTA affinity chromatography and confirmed through SDS-PAGE followed by Coomassie Blue staining (Figure 33). The representative band of each recombinant protein appeared at the appropriate molecular weight location. The purities of S0-mC3d1, S0-mC3d2, S0-mC3d3, S0-mC4d1, and S0-mC4d2 were 95 %, 90%, 92 %, 95 %, and 89 %, respectively (Table 20). In contrast to high purity of each recombinant protein, purified S0 conjugated series remarkably lower expression yields than purified mC3d or mC4d the same as flagellin case. Differences between the band intensities of the soluble and purified fractions and the comparable high level of band densities between soluble and sample flow through fractions suggested that there was a significant amount of target protein loss during the purification process (Figure 33). As mentioned in study I, both 6His-tags bound to the N- and C-termini of each protein were difficult to access by the Ni^{2+} metal ions during the purification process.

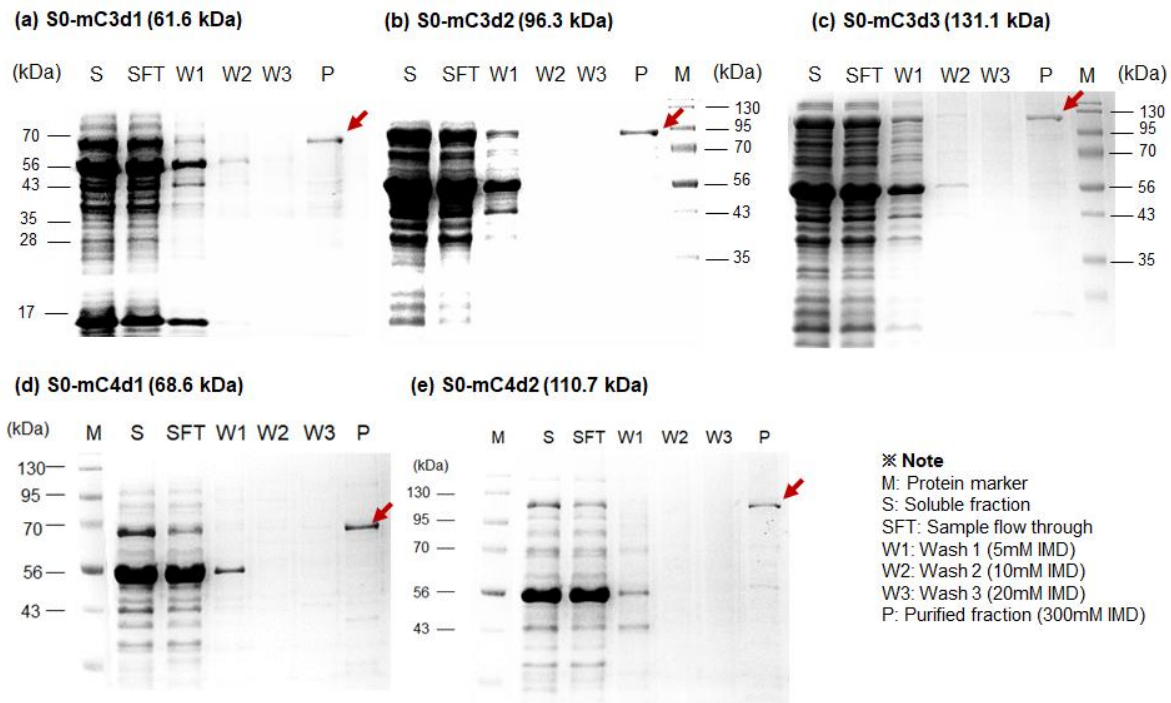


Figure 33. Large scale production and purification of S0-mC3d and S0-mC4d series by Ni^{2+}-NTA affinity chromatography.

Each recombinant protein was purified by using Ni^{2+}-NTA affinity chromatography as described in materials and methods. Figure (d) and (e) are obtained from the thesis of YS Kim Cho.

Table 20. Purity and yield of S0-mC3d and S0-mC4d series.

Recombinant proteins	Purity	Yield (mg/L)
S0-mC3d1	95	3.35
S0-mC3d2	90	2.58
S0-mC3d3	92	1.30
S0-mC4d1	95	2.40
S0-mC4d2	89	1.66
mC3d	97	16.3
mC4d	98	20.0

3) Effect of C3d and C4d conjugation to S0 on humoral immune response

(1) Evaluation of adjuvant effect of C3d conjugation *in vivo*

Complement fragment C3d has been widely utilized as a fusion adjuvant for a broad range of recombinant antigens in past two decades (Lee et al., 2006;Mizel et al., 2009;Mizel and Bates, 2010;Nguyen et al., 2011;Moyle, 2017;Puth et al., 2017); however, it has not been applied to antigen derived from PEDV yet. Therefore, to validate the potential of the C3d as a fusion adjuvant, mice immunization studies have been conducted using a representative target antigen, S0, derived from the surface protein of porcine epidemic diarrhea virus, after the construction of model subunit vaccine conjugating with C3d. Six groups of mice were immunized three times intramuscularly with PBS, 40 pmol of 40 pmol of each recombinant protein (1.0 μg of S0, 2.35 μg of S0-mC3d1, 3.67 μg of S0-mC3d2 and 5.00 μg of S0-mC3d3) and 1.0+4.0 μg of S0+mC3d as a non-conjugated mixture control for S0-mC3d3 at 14-day intervals. Sera were collected at the 2, 4 and 6-week postinjection, and S0-specific IgG titers were evaluated by indirect ELISA.

At 2-week postinjection, every groups showed no significant changes in their serum S0-specific IgG titers [Figure 34 (a)]. However, the IgG levels increased in the groups treated with C3d adjuvants [S0-mC3d1, S0-mC3d2, and S0-mC3d3] compared with the controls including untreated and S0 treated group at

4-week postinjection [Figure 34 (b)]. Interestingly, the group immunized with S0 + mC3d (x3) also showed significantly increased titers of serum S0-specific IgG together with the group immunized with S0-mC3d3 at this time point. Sera collected at the 6-week postinjection had a similar tendency as the 4-week postinjection sera, though the group immunized with S0-mC3d3 had reduced anti-S0 IgG titers compare to other groups at this time point (Figure 34).

The higher level of IgG titers specific for S0 clearly demonstrated that C3d could also be utilized to PED subunit vaccine as did in various other target antigens. Furthermore, C3d alone also could be applied as an adjuvant as it has greatly high yield (16.3 mg/L) compare to fusion forms including S0-mC3d1, S0-mC3d2, and S0-mC3d3 (3.35 mg/L, 2.58 mg/L, and 1.30 mg/L, respectively) as listed in Table 20. Meanwhile, the adjuvant effect of mC3d alone has raised the mechanism of its action, because C3d has been suggested to utilize as a conjugated form as it influence antigen uptaking cells directly (Dempsey et al., 1996;Knopf et al., 2008;De Groot et al., 2015). As all of the previous studies utilizing C3d as a fusion adjuvant have not included non-conjugated mixture control, the adjuvant effect of C3d alone in this study has raised an important question in terms of mechanism.

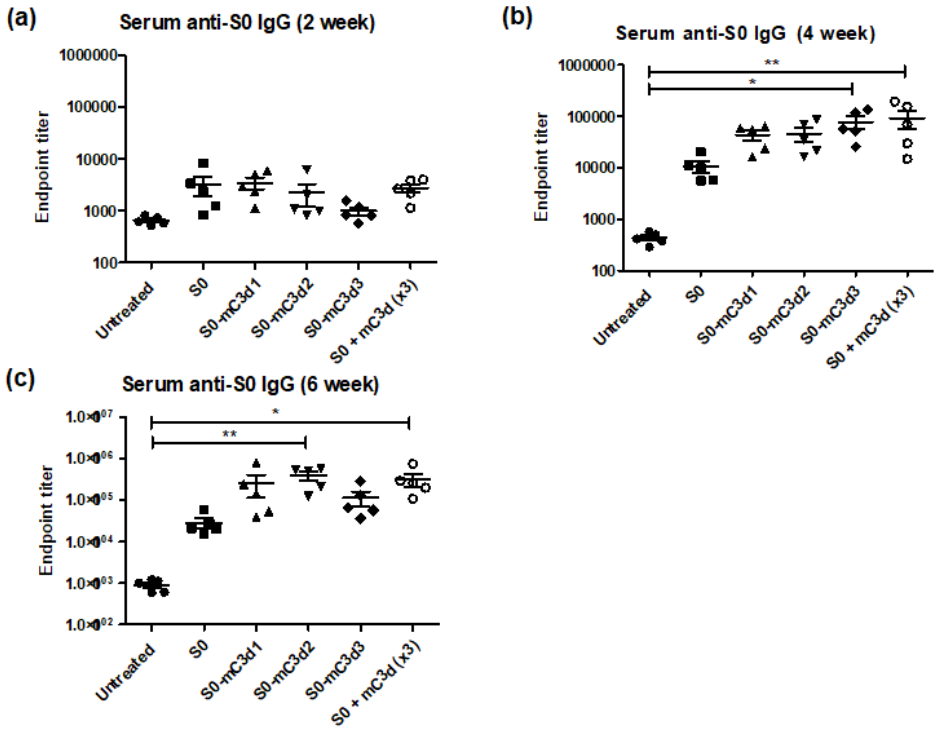


Figure 34. S0-specific IgG titers after immunization with mC3d conjugated recombinant proteins.

Mice were immunized with the indicated recombinant proteins. Blood samples from **(a)** 2, **(b)** 4, and **(c)** 6-week postinjection were analyzed to detect anti-S0 IgG titers with an S0-coated sandwich ELISA. Data represented as the mean of five individual mice ± standard error of the mean and plotted in log 10 scale. *, $P < 0.05$, **, $P < 0.01$.

(2) Evaluation of adjuvant effect of C4d conjugation *in vivo*

As the complement fragment C3d, the split product that covalently bound to broad range of pathogens, has been widely utilized as a fusion adjuvant against various antigens mainly through T cell epitope donation, another complement fragment C4d which has high frequency of T cell epitopes was selected as a novel fusion adjuvant candidate in the hope that it supports T cell epitopes to antigen uptaking cells as C3d did. The fused C4d with the target antigen is expected to exert its adjuvant effect by donating T cell epitopes in its intra-structure to MHC class II molecules in antigen-specific B cells and enable them to be assisted by C4d-specific IL-4 secreting autoreactive helper T cells in addition to antigen-specific one to become plasma cells like C3d case (Figure 35). To validate the potential of the C4d as a novel fusion adjuvant through the mechanism of T cell epitope donation, mice immunization studies have been conducted using S0 as a target antigen after the construction of model subunit vaccine conjugating with C4d.

The systemic humoral immune response against S0 was analyzed through measuring IgG titers in murine serum to elucidate the adjuvanticity of fused mC4d. S0, S0 fused mC4d (S0-mC4d1 and S0-mC4d2), or mixtures of S0 and mC4d [S0 + mC4d and S0 + mC4d (x2)] were administered intramuscularly into each mouse on day 0, 14, and 28. Anti-S0 IgG titers were measured from serum collected on day 13, 27, and 42 by indirect ELISA (Figure 27). At 2 week-post injection, every groups showed no significant changes in their serum S0-specific

IgG titers [Figure 36 (a)]. However, the IgG levels increased in the groups treated with C4d adjuvants [S0-mC4d1, S0-mC4d2, S0 + mC4d, and S0 + mC4d(x2)] compared with the controls including untreated and S0 treated group at 4 week-post injection [Figure 36 (b)], then the gap in IgG levels between the C4d adjuvant-treated groups and the control groups was differed significantly at 6 week-post injection [Figure 36 (c)]. Interestingly, mC4d alone exerted a potent adjuvant effect by increasing the titers of serum S0-specific IgG significantly higher than S0 alone or PBS, the level of which is similarly high with the groups immunized with S0-mC4d1 and S0-mC4d2 at 4 and 6 week-post injection as mC3d did in Figure 34.

As mC4d is expected to act as a T cell epitope donor, the number of mC4d-specific IL-4 secreting T cells was enumerated in addition to S0-specific one by ELISPOT assay at 6-week postinjection. Splenocytes extracted from the spleen of each immunized mouse were stimulated with blank media, or 10 μg of S0, mC4d, or mC3d, respectively, then population of the activated T cells secreting IL-4 in each treated group was enumerated (Figure 37). The number of spots after S0, mC4d or mC3d treatment was designated as the populations of S0-, mC4d- and mC3d-specific IL-4 secreting T cells. All the analyzed groups, except the untreated group, included the high populations of S0-specific IL-4 secreting T cells. S0-mC4d2 and S0 + mC4d groups had significantly higher number of mC4d-specific IL-4 secreting T cells than media control. S0-mC4d1 group had higher number of mC4d-specific IL-4 secreting T cells than media

control, though there's no statistical significance. Interestingly, in contrast to AS Groot's report that C3d-specific autoreactive memory T cells are circulating in adult human, mouse has no pre-existed T cells specific for mC3d or mC4d. In contrast, some of repeated experiments showed early immune response against S0 in C4d conjugated groups at 7d post-injection. Accurate and sophisticated experiments are necessary to unravel the true effect of complement fragment conjugation for the induction of early immune response and a potential harmful consequences of complement fragment specific autoreactive T cells such as host autoimmune response against complement fragment producing cells.

Throughout the study II, the free complement fragment including C3d and C4d induced high anti-S0 IgG titers similar to its conjugation from (Figure 34, Figure 36). It was inferred that C4d would be free to exert its function regardless of its fusion to S0. However, mC4d-specific T cells were activated in S0-mC4d1, S0-mC4d2, and S0 + mC4d groups, suggesting that mC4d-specific T cells could be involved in the course of immune response in part. Though the overall populations of them were lower than S0-specific one (Figure 37), the direct comparison of each T cell populations, however, is not relevant because the *in vitro* activation condition for each T cell populations may not be same. Therefore, the mechanism of complement fragment adjuvant is not determined yet and should not be predicated. Further works are needed to unravel the genuine mechanism of their adjuvant effect.

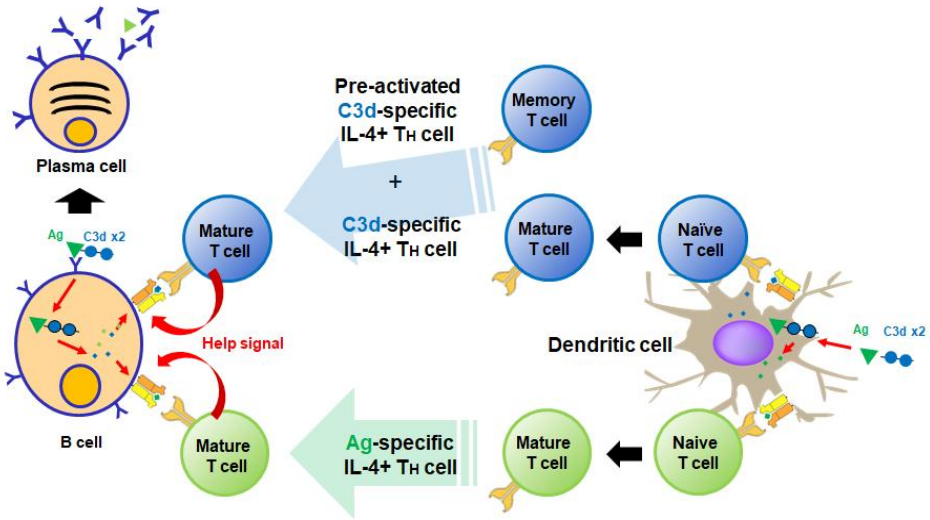


Figure 35. Proposed mechanism of C3d fusion adjuvant.

The fused C3d with the target antigen is expected to exert its adjuvant effect by donating T cell epitopes in its intra-structure to MHC class II molecules in antigen-specific B cells and enable them to be assisted by C3d-specific IL-4 secreting autoreactive helper T cells in addition to antigen-specific one to become plasma cells. T_H, helper T cell; Ag, antigen.

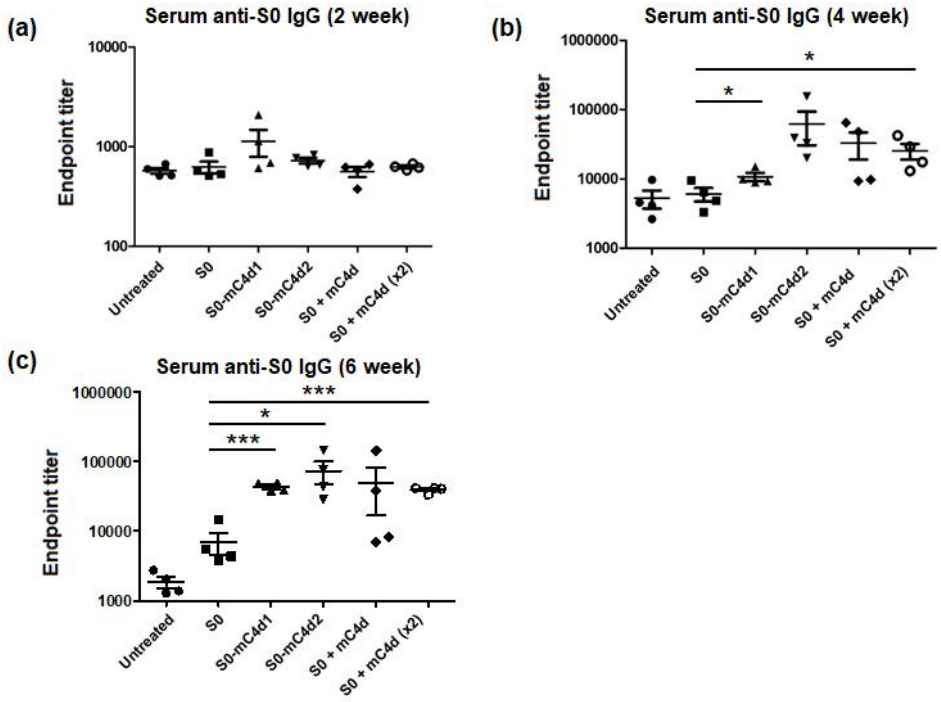


Figure 36. S0-specific IgG titers after immunization with mC4d conjugated recombinant proteins.

Mice were immunized with the indicated recombinant proteins. Blood samples from **(a)** 2, **(b)** 4, and **(c)** 6-week postinjection were analyzed to detect anti-S0 IgG titers with an S0-coated sandwich ELISA. Data represented as the mean of five individual mice $\pm$ standard error of the mean and plotted in log 10 scale. *, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$. This figure is obtained from the thesis of YS Kim Cho.

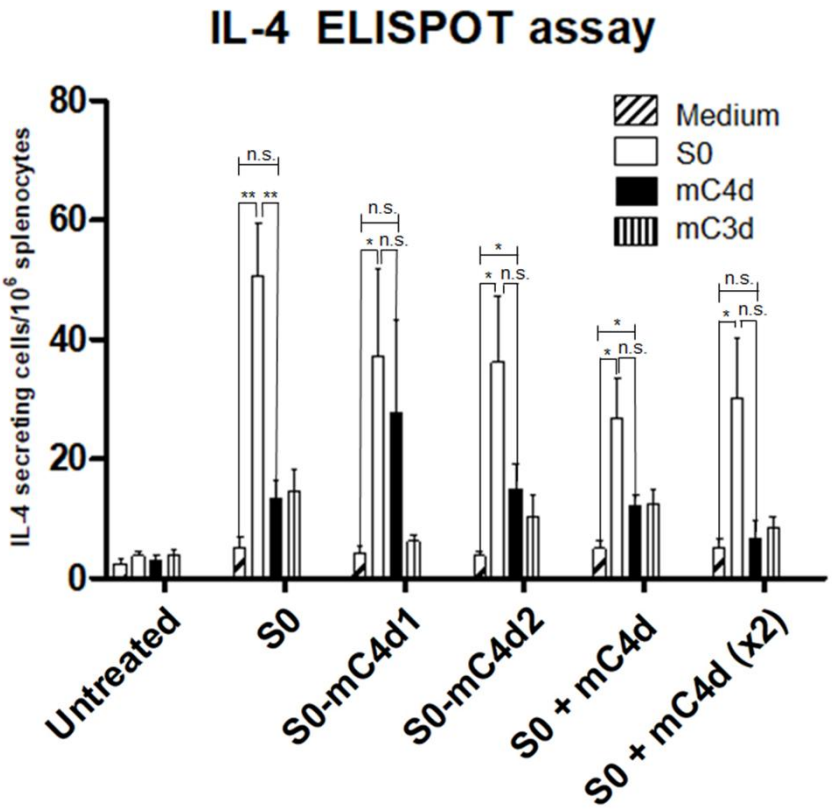


Figure 37. Analysis of IL-4 secreting T cells by ELISPOT assay.

Mice were immunized intramuscularly with recombinant proteins. Six weeks later, spleen cells were stimulated with 10μg of blank media, S0, mC4d, or mC3d. Single-color IL-4 ELISpot assays were used to determine the frequencies of cells secreting IL-4. Frequencies (spots per million spleen cells) were calculated as described in Materials and Methods. Frequencies of spots that reacted from no stimulation, or S0, mC4d, or mC3d stimulation for each treatment group. *, $P < 0.05$. This figure is obtained from the thesis of YS Kim

Cho.

4. Conclusion

In this study, the complement fragments mC3d and mC4d were conjugated to PEDV antigen S0 and its ability to improve protein solubility and an antigen-specific humoral immune response was evaluated in mice (Figure 38).

Conjugation of mC4d to S0 improved the solubility of the recombinant proteins regardless of the existence of chaperone *tig*. In this regard, mC4d could be a good candidate for enhancing antigen solubility in other proteins that are prone to form IBs. Further works should be done to test its ability to enhance protein solubility when it fused to N-terminus of target protein as it is translated high rate with soluble form.

From the perspective of immunogenicity, the adjuvant effect of C3d conjugation was once again confirmed. Moreover, the conjugation of complement fragment C4d which was selected as a novel fusion adjuvant candidate through *in silico* study, also elicited adjuvant effect against S0 in mice. Unexpectedly, both C3d and C4d exerts adjuvant effect on humoral responses not only in the status of conjugation to target antigen but also in the form of non-conjugated mixtures. Therefore, C3d and C4d could be utilized as a potent adjuvant regardless of their fusion status.

Though the mechanism of their action is not determined in this study, the possibility of the involvement of IL-4 secreting mC4d-specific T cell was confirmed. Further works are urgently needed to unravel the genuine mechanism

of complement based adjuvant.

These findings provide crucial information for the rational design of a PED vaccine and complement fragment-based immunotherapeutics.

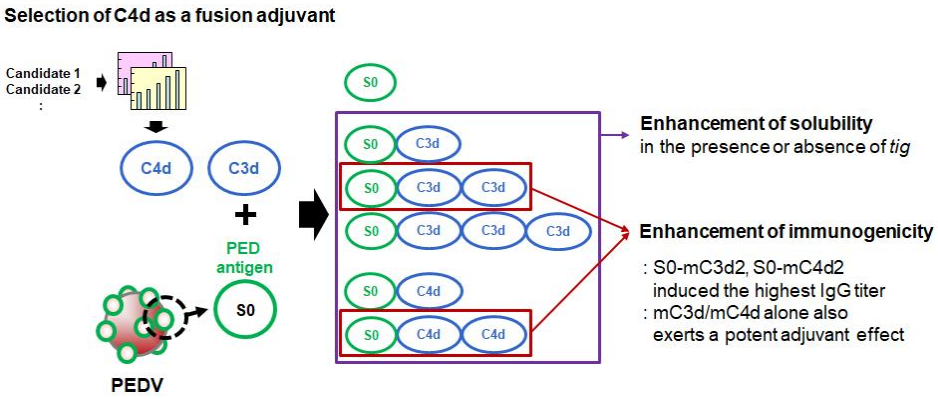


Figure 38. Conclusion of study II.

Complement fragment C4d was selected as a novel fusion adjuvant through *in silico* study. Each complement fragement adjuvants was conjugated to S0 in tendem, generating S0-C3d1, S0-C3d2, S0-C3d3, S0-C4d1 and S0-C4d2. C3d conjugated recombinant proteins did not ameliorate the solubility of S0, however, C4d conjugated recombinant proteins improve the solubility of S0 in the presence or absence of *tig*. Both adjuvants enhanced the immunogenicity of S0 regardless of their conjugation. Each complement fragement specific T cells were inferred to be involved in their adjuvant effect.

Study III. Selection of a Fusion Adjuvant

1. Introduction

To develop a successful PED subunit vaccine, the recombinant protein candidates have been researched should be compared at the same time (Figure 39). For flagellin based recombinant proteins, F-S0 was selected for study III as it induced significantly higher anti-S0 IgG titers than any other regimens (Figure 18). For complement based adjuvants, S0-mC3d2 and S0-mC4d2 were selected as fusion adjuvant candidates for study III because they enhanced higher anti-S0 IgG titers than S0-mC3d1 and S0-mC4d1 in average, respectively (Figure 34 and Figure 36). The mC3d and mC4d which was used in non-conjugated mixture control were also selected as adjuvant candidates as they have tremendous high yield compare to other fusion proteins.

To protect piglets, the main contributor to substantial economic loss, it is imperative to immunize sows to establish the passive immunity of piglets via colostrum/milk. Therefore, enhancement of the PEDV-specific antibodies at mucosal area is a prerequisite for PED vaccine development. Not only for PED case, other diseases require mucosal immune response as most of the infectious agents make an infection through mucosal route. In this regard, the ability to induce mucosal immune response after mucosal administration was also considered together with systemic immune response after intramuscular immunization.

In this study, the recombinant protein candidates were evaluated to induce S0-specific antibody responses after systemic and mucosal administration in mice. The results were interpreted together with yield information of each recombinant protein candidate to select the most immunogenic PED subunit vaccine protein.

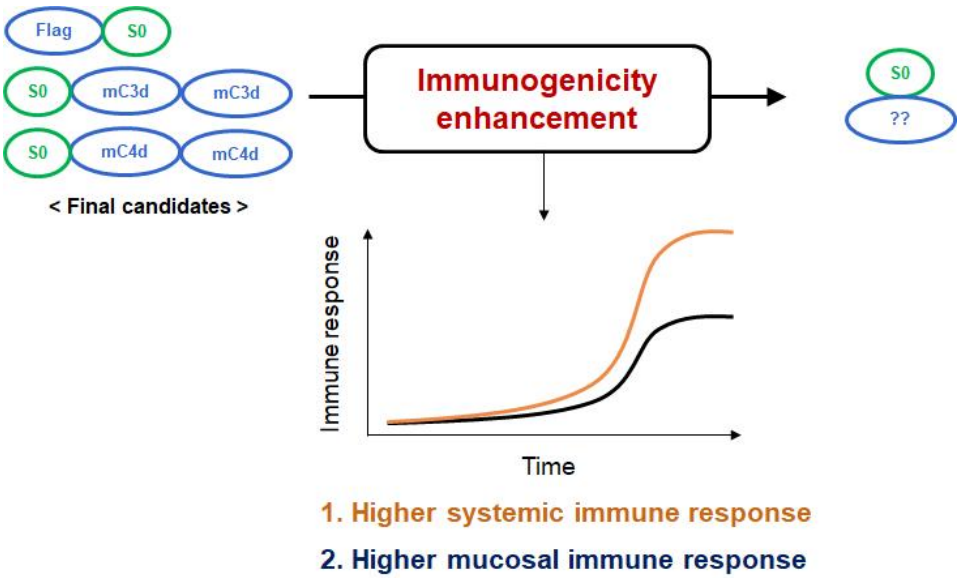


Figure 39. Graphical abstract of study III.

Recombinant protein candidates were compared in terms of immunogenicity enhancement after systemic and mucosal administration to select the most immunogenic PEDV subunit vaccine antigen

2. Materials and methods

1) *In vivo* immunization

(1) Intramuscular administration in mouse

6-week-old Balb/c mice (n=4) were immunized intramuscularly with 80 pmol of each recombinant protein (1.9 μg of S0, 4.81 μg of F-S0, 6.97 μg of S0-mC3d2, and 8.01 μg of S0-mC4d2) three times at 2-week intervals after adaptation for one week. Mice were also immunized with 1.9+5.41 μg of S0+mC3d and 1.9+6.46 μg of S0+mC4d per mouse as a non-conjugated mixture control for S0-mC3d2 and S0-mC4d2, respectively. CFA and IFA were mixed with antigens for the priming and boosting of mice, respectively. Treatment group information and regimens are listed in Table 21. Blood samples were collected on days 13, 27, and 42 (sacrifice) from intrapetrosal veins with a disposable syringe and delivered into sterilized tubes. Serum was separated by centrifugation at 12,000 rpm for 3 min using a serum separator tube. Overall scheme is described in Figure 17.

Table 21. Treatment group information and regimens for intramuscular mouse immunization experiment.

	Antigen	Note	Amount		Oil adjuvant	Solution	Volume	Route	n
			pmol/mouse	μg/mouse					
1	None	PBS only	---	---	CFA (0 wk) + IFA (2, 4 wk)	PBS	50 μL	I.M.	4
2	S0	PED Ag	80	1.9					
3	Flagellin-S0	Flagellin fused to N' terminus of S0		4.81					
4	S0-mC3d2	S0 fused to two repeats of mC3d		6.97					
5	S0-mC4d2	S0 fused to two repeats of mC4d		8.01					
6	S0 + mC3d (x2)	S0 mixed with 2 x mC3d	80 + 160	1.9 + 5.41					
7	S0 + mC4d (x2)	S0 mixed with 3 x mC4d		1.9 + 6.46					

(2) Intranasal administration in mouse

6-week-old BALB/c mice (n=4) were immunized through intranasal route by dropping 21 μl of PBS containing 58 pmol of each recombinant protein (1.37 μg of S0, 1.45 μg of F-S0, 5.00 μg of S0-mC3d2, and 5.75 μg of S0-mC4d2) into the nostrils under anesthesia three times at 2-week intervals after adaptation for one week. Mice were also immunized with 1.37+3.88 μg of S0+mC3d and 1.37+4.63 μg of S0+mC4d per mouse as a non-conjugated mixture control for S0-mC3d2 and S0-mC4d2, respectively. 1 μg of cholera toxin (CT, LIST BIOLOGICAL LABORATORIES, Inc.) per mouse were mixed with each recombinant protein regimen for the priming and boosting of mice, respectively. Treatment group information and regimens are listed in Table 22. Blood samples were collected on days 13, 27, and 42 (sacrifice) from intrapetrosal veins with a disposable syringe and delivered into sterilized tubes. Mucosal fluids from nasal,

bronchoalveolar lavage (BAL), and vagina were collected on day 42 (sacrifice) and delivered into sterilized tubes. Sera and mucosal fluids were analyzed by ELISA to detect anti-S0 antibodies. Overall scheme is described in Figure 40.

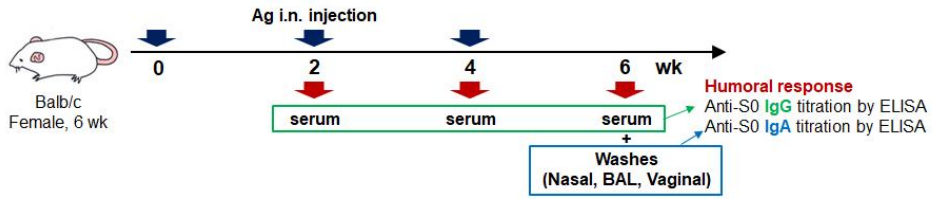


Figure 40. Overall scheme of intranasal mouse immunization experiment.

Each mouse was immunized with regimens listed in Table 22 three times at 2-week intervals. Blood samples were collected on days 13, 27, and 42 and analyzed by ELISA to evaluate S0-specific IgG titers. Mucosal fluids were collected on day 42 and analyzed by ELISA to evaluate S0-specific IgA titers.

Table 22. Treatment group information and regimens for intranasal mouse immunization experiment.

	Antigen	Note	Amount		Additional adjuvant	Solution	Volume	Route	n
			pmol/mouse	μg/mouse					
1	None	PBS only	---	---	CT (1 μg/mouse)	PBS	21 μL	I.N.	4
2	S0	PED Ag	58	1.37					
3	Flagellin-S0	Flagellin fused to N' terminus of S0		1.45					
4	S0-mC3d2	S0 fused to two repeats of mC3d		5.00					
5	S0-mC4d2	S0 fused to two repeats of mC4d		5.75					
6	S0 + mC3d (x2)	S0 mixed with 2 x mC3d	58 + 116	1.37 + 3.88					
7	S0 + mC4d (x2)	S0 mixed with 3 x mC4d		1.37 +4.63					

I.N., Intranasal administration; CT, cholera toxin

(3) Collection of mucosal fluid

Mucosal fluids from nasal, BAL, and vagina from each mouse were collected on day 42 (sacrifice) and delivered into sterilized tubes. The bronchi were exposed from the neck, a truncated needle with a syringe containing 300 μl PBS was inserted through a small pore on each bronchus. BAL wash specimens were obtained by two consecutive tracheal and lung infusions and aspirations. The nasal wash specimens were obtained by a single 400 μl infusion of PBS in the laryngeal region and then collection from the nostrils. For vaginal wash specimens, the vagina was washed 3 times with 50 μl of PBS. The harvested supernatants were collected by centrifugation (1000g-force and 10 min at 4 °C) to remove cellular debris. All of the samples were stored at−70 °C until analysis.

2) Sample analysis

(1) ELISA for serum samples

The anti-S0 IgG titers in each serum were analyzed by indirect ELISA. Detailed methods are the same as ELISA above mentioned in study I and II. Briefly, 96-well immunoplate (SPL, Korea) were coated with purified recombinant S0 (0.1 ㎍/well) at 37 ℃ for 2 h. Coated plates were blocked with 0.5 % skim milk in PBS containing 0.05% of Tween 20 for 1h at room temperature. Then, 5-fold serially diluted sera starting from 1:100 dilution fold were added into the wells and incubated at 37 °C for 2 h. The HRP-conjugated goat anti-mouse IgG (1:2000 dilutions) were added to each well for 1 h at room temperature. 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 30 min without light interference, followed by the addition of stop solution. Finally, the absorbance was measured at 450 nm using microplate reader (Infinite® 200 PRO, USA). ELISA results are expressed as the endpoint titer.

(2) ELISA for mucosal fluids

To detect S0-specific IgA levels, each mucosal wash specimen was analyzed by indirect ELISA. The coasting and blocking steps are the same as above mentioned methods. After blocking process, samples from nasal wash, BAL

wash (1:10) and vaginal wash (1:10) were added into the wells (100 µl/well) and incubated at 37 °C for 2 h. After incubation, HRP-conjugated goat anti-mouse IgA (1:4000 dilutions, 100 µl/well) were added to the designated wells for 1 h at room temperature. 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 (100 µl/well) as a HRP substrate and incubated for 30 min without light interference, followed by the addition of stop solution (0.16 M H2SO4; 100 µl/well) to stop the enzymatic reaction. Finally, the absorbance was measured at 450 nm using microplate reader (Infinite® 200 PRO, USA). ELISA results are expressed as the O.D. value measured at 450 nm. The results were finally calibrated by dilution fold (dilutions of 1:10 for BAL IgA, 1: 1 for nasal wash IgA, 1:10 for vaginal IgA).

3. Results and discussion

1) Comparison of the systemic adjuvanticity of fusion adjuvants

To select the most immunogenic final recombinant proteins, systemic adjuvanticity of each recombinant proteins after intramuscular administration was firstly compared at the same time.

The immunogenicity of each recombinant proteins after intramuscular administration was analyzed first. At 2-week postinjection, every groups showed no significant changes in their serum S0-specific IgG titers which are correspond to study I and II though one mouse in F-S0 group had relative high level of antibody response [Figure 41 (a)]. At 4-week postinjection, the anti-S0 IgG titers of F-S0 group were induced significantly higher than untreated control group, though other adjuvanted groups had a tendency to have high level of anti-S0 IgG titers than control groups including untreated and S0. The adjuvant effect of non-conjugated mixture mC3d and mC4d is confirmed once again as shown in Figure 34 and Figure 36, respectively. All of the groups using adjuvant [F-S0, S0-mC3d2, S0-mC4d2, S0 + mC3d (x2), and S0 + mC4d (x2)] had a similarly high titers of anti-S0 IgG than control groups including untreated and S0 at 4 and 6-week postinjection [Figure 41 (b), (c)]. At 6-week postinjection, only S0 + mC3d (x2) group had a statistical significance compare to S0 control group. Take into consideration that S0 + mC3d (x3) had a similar level of anti-S0 IgG titers compare to S0-mC3d2 in study II (Figure 34), all of adjuvanted groups are

inferred to have similarly high levels of anti-S0 IgG titers at 6-week postinjection. The similar level of immune response may be derived from the plateu of immune response. To differentiate this problem, further works are needed to evaluated immune responses using lower amount of recombinant proteins than 80 pmol per mouse.

As the similar adjuvanticity among various recombinant protein candidates was confirmed in the condition of systemic immunization, other factors are needed to determine their effectiveness such as the ability of enhancing mucosal immune responses.

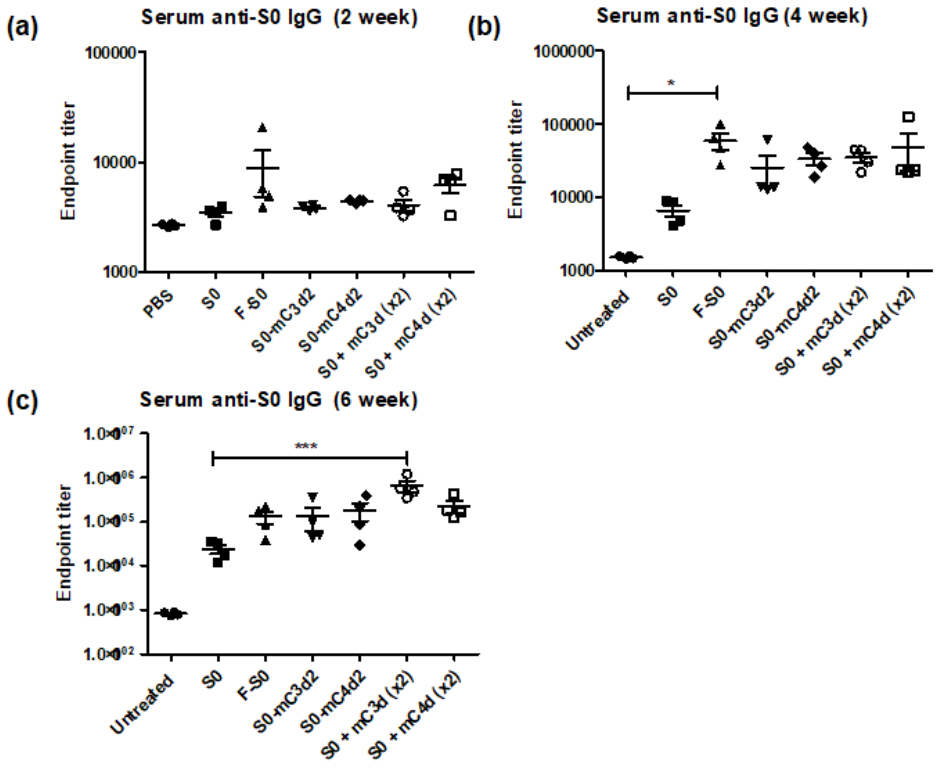


Figure 41. S0-specific IgG titers in serum after intramuscular immunization with various recombinant proteins.

Mice were immunized with the indicated recombinant proteins through intranasal administration. Blood samples from **(a)** 2, **(b)** 4, and **(c)** 6-week postinjection were analyzed to detect anti-S0 IgG titers with an S0-coated sandwich ELISA. Data represented as the mean of four individual mice $\pm$ standard error of the mean and plotted in log 10 scale. *, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$.

2) Comparison of the mucosal adjuvanticity of fusion adjuvants

Researchers have been extensively explored a potent mucosal adjuvant because many infectious diseases invade the host through this route. As mucosal administration of vaccine confers adaptive immune responses to various mucosal areas including oral, intestinal, nasal, or vaginal area via common mucosal immune system, it is an attractive strategy to combat diverse infectious diseases including PED. In this regard, the ability of each recombinant proteins to enhance mucosal immune response was analyzed through measuring IgA titers in mucosal fluids after intranasal immunization.

Mice were immunized intranasally with each recombinant proteins mixed with cholera toxin as listed in Table 22. After 6 weeks, IgA antibody titers were measured from mucosal specimen of nasal wash, BAL wash and vaginal wash of each mouse. In addition, anti-S0 IgG titers were also measured in blood samples taken from 2, 4, and 6-week postinjection.

Data revealed that the flagellin, mC4d, and mC3d fusion groups (F-S0, S0-mC3d2 and S0-mC4d2) induced more IgA antibody production than in mice injected with solitary S0 in all mucosal wash specimens (Figure 42). The tendency of anti-S0 IgA titers among groups is similar, suggesting that S0-sepcific IgA was induced via common mucosal immune system. In contrast to systemic immunization, the mC4d fusion groups produced a significantly higher antibody titer than in the mC3d fusion groups in respect to mucosal immunity.

Though flagellin conjugated group (F-S0) induced higher anti-S0 IgA titers than S0-mC3d2 in average, the standard deviation of this group is high and the average of anti-S0 IgA titers is lower than S0-mC4d2. Interestingly, the mC4d or mC3d mixture regimens did not have an adjuvant effect on antibody induction, contrary to systemic injection with mixture regimens (Figure 42 and Figure 43), suggesting that conjugation of mC3d or mC4d was the main factor to confer the mucosal adjuvant activity for them. This data also prove once again that mC3d- or mC4d-specific autoreactive T cells could be the reason, in part, for the high level of mC4d conjugated groups in study II (Figure 36). In this regard, the higher level of anti-S0 IgA titers in S0-mC4d2 group compare to S0-mC3d2 group is inferred to be the difference of the activity between mC3d- and mC4d specific autoreactive T cells. As AD Groot and colleagues suggested that the memory type of mC3d-specific autoreactive T cells are circulating in blood of adult human, this type of T cells could be deduced to exist in mice. As mucosal area is vulnerable to meet various pathogens, complement fragments such as C3d or C4d could be exposed to host immune systems as a result of complement pathway activation, subsequently the mC3d- and mC4d-specific autoreactive T cells could be arranged in this area.

The serum S0-specific IgG titers after intranasal administration appear to have similar tendency compared to IgA titers amongst the various treatment groups (Figure 43), demonstrating the adjuvant effect of S0-mC4d2.

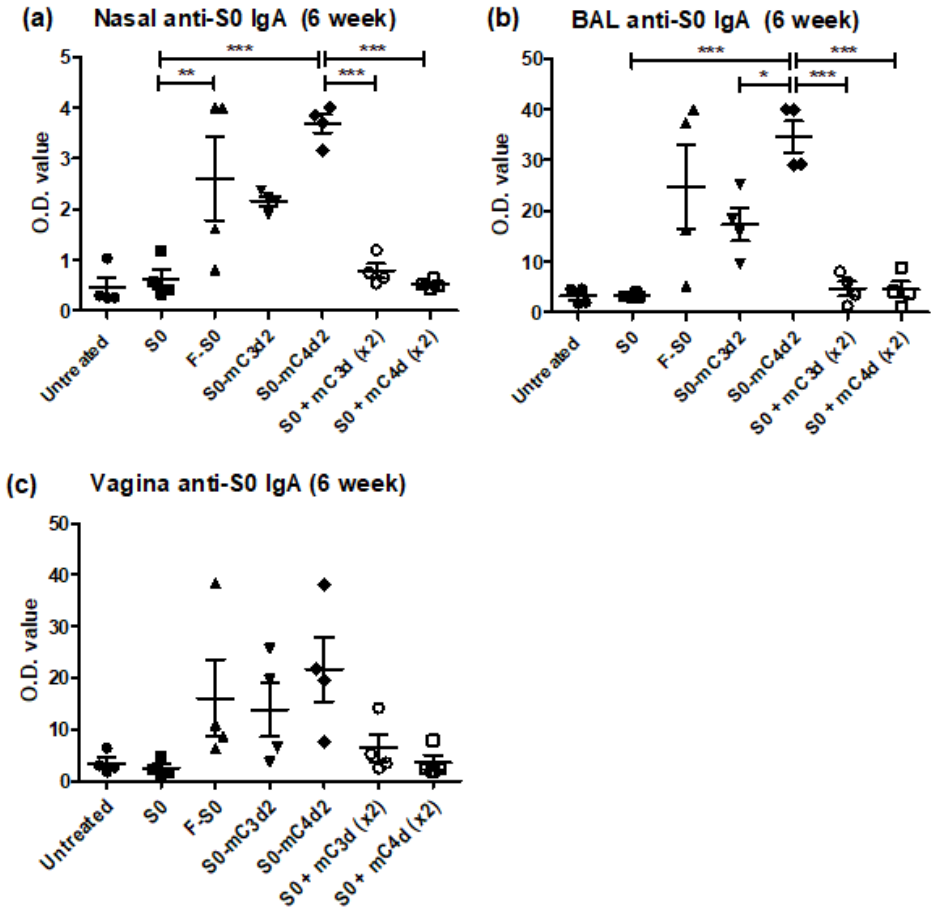


Figure 42. S0-specific IgA titers in mucosal fluids after intranasal immunization with various recombinant proteins.

Mice were immunized with the indicated recombinant proteins through intranasal administration. Mucosal specimens from **(a)** nasal wash, **(b)** BAL wash, and **(c)** vaginal wash at 6-week postinjection were analyzed to detect anti-S0 IgA titers with an S0-coated ELISA. Data represented as the mean of four individual mice $\pm$ standard error of the mean. *, $P < 0.05$, **, $P < 0.01$, ***, $P <$

0.001.

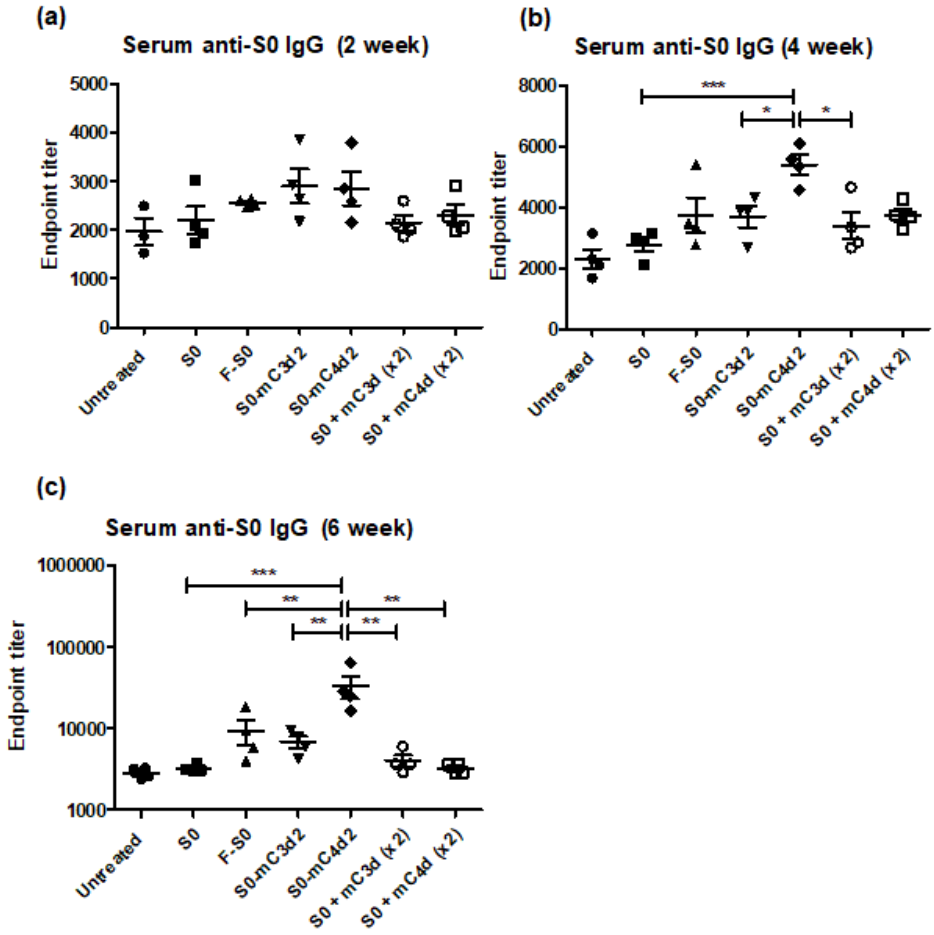


Figure 43. S0-specific IgG titers in serum after intranasal immunization with various recombinant proteins.

Mice were immunized with the indicated recombinant proteins through intranasal administration. Blood samples from **(a)** 2, **(b)** 4, and **(c)** 6-week postinjection were analyzed to detect anti-S0 IgG titers with an S0-coated sandwich ELISA. Data represented as the mean of four individual mice $\pm$ standard error of the mean and plotted in log 10 scale. *, $P < 0.05$, **, $P < 0.01$,

***, $P < 0.001$.

3) Selection of a PED subunit vaccine protein

Selected PED subunit vaccine protein candidates were F-S0, S0-mC3d2, S-mC4d2, S0 + mC3d (x2) and S0 + mC4d (x2). Of these, final PED subunit vaccine protein was tried to be selected in terms of immunogenicity enhancement. As the production time and materials are same for all the candidates, "yield" was chosen as a factor for "cost". For systemic regimen, all of the PED subunit vaccine protein candidates induced similarly high S0-specific IgG titers (Figure 41). Therefore, mC3d or mC4d alone was finally selected as the most immunogenic adjuvant for systemic PED subunit vaccine in terms of their high yield (Table 23). In spite of their high yield, however, mC3d or mC4d could not ameliorate the mucosal immune response. Therefore, S0-mC4d2 was finally selected as the best mucosal PED subunit vaccine as this recombinant protein could induce significantly higher anti-S0 IgA titers than any other regimen in mice (Figure 42).

Table 23. Profiles of final adjuvant candidates.

	Yield (mg/L)	Immunogenicity enhancement	
		I.M.	I.N.
F-S0	5.2	↑	↑
S0-mC3d2	2.58	↑	↑
S0-mC4d2	1.66	↑	↑↑↑
mC3d	16.3	↑↑	-
mC4d	20	↑	-

I.M., intramuscular administration; I.N., intranasal administration

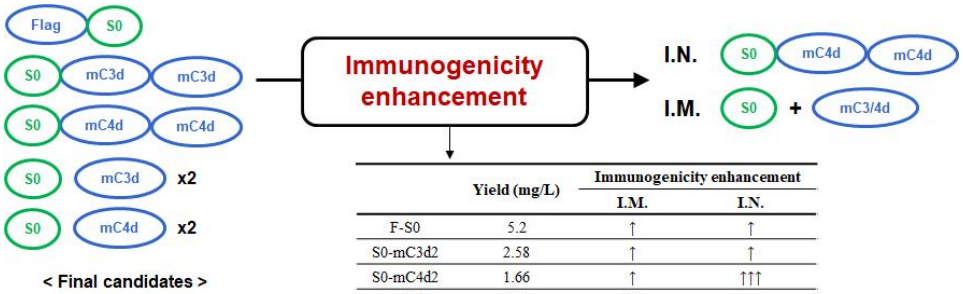
4. Conclusion

In this study, the recombinant protein candidates that have been researched throughout the study I and II were compared at the same time to select the best PED subunit vaccine protein in terms of immunogenicity enhancement.

All of the recombinant protein candidates induced similarly high level of anti-S0 antibody titers in serum compare to control group after systemic administration. In this regard, taking the high production yield of mC3d and mC4d into consideration, they were finally selected as an adjuvant of PED subunit vaccine for intramuscular administration. Meanwhile, S0-mC4d2 enhanced the highest anti-S0 IgA titers at various mucosal areas compare to any other recombinant proteins after intranasal administration. Therefore, S0-mC4d2 was finally selected as the most immunogenic mucosal PED subunit vaccine.

In addition, the results from intranasal mouse immunization suggested that mC3d or mC4d may exerted their adjuvant effect through autoreactive T cells specific for themselves at mucosal area. This observation also gave a clue to the interpretation of systemic immune response that the adjuvant effect of mC4d was in part attributed to the assistance of mC4d-specific T cells.

The selected adjuvant and the regimen could be applied not only to PED subunit vaccine, but also other vaccines.



	Yield (mg/L)	Immunogenicity enhancement	
		I.M.	I.N.
F-S0	5.2	↑	↑
S0-mC3d2	2.58	↑	↑
S0-mC4d2	1.66	↑	↑↑↑
mC3d	16.3	↑↑	-
mC4d	20	↑	-

Figure 44. Conclusion of study III.

S0-mC4d2 was selected as the most immunogenic PEDV subunit vaccine antigen after intranasal administration based on its superior ability to enhance mucosal immune response against S0. mC3d and mC4d were selected as the most immunogenic adjuvant after intramuscular administration complexed with S0 based on its superior ability to enhance systemic immune response against S0 together with its overwhelming production efficiency

Perspectives for Further Study

In this study, the effects of fusion adjuvants to enhance immunogenicity of PEDV subunit vaccine were investigated. Though this study has provided immunological insights for using flagellin and complement adjuvants when conjugated with PEDV S0 antigen, it is necessary to discuss about the application of fusion adjuvants for other antigens. Therefore, in this section, further considerations about using fusion adjuvants on the development of subunit vaccine would be suggested based on the improvement points of this study.

For the perspective of protein production, the genetic fusion of highly soluble adjuvants with hard-to-express antigens seems to recover the insoluble expression of antigen. Though mC3d did not improve the solubility of S0, however, it still could be utilized as a solubility enhancer because it could enhance the solubility of S1D, another truncated region of PEDV surface protein other than S0, from 0 % to 95 % (data not shown). Although fusion adjuvants could be a solubility enhancer, the purification efficiency of each recombinant protein should be improved. In fact, all conjugated proteins in this study became highly soluble than S0 alone, however, most of the soluble protein were not purified well, resulting the purification efficiency lower than 10 % on average. Take flagellin adjuvant into consideration, purified S0-F, F-S0 had significantly lower yield than flagellin alone. Differences between band intensities of soluble

and purified fraction revealed that there's big loss in purification process (lane 3 and 4 of Figure 17). We expect that both hexahistidine tags binding to N- and C-terminal of S0-F, F-S0 are exist hard to be approached by Ni metal ions during purification process. As soluble lysates of S0 were not purified at all, both hexahistidine tags binding to N- and C-terminal of S0 might be hidden, suggesting hexahistidine tags attached to S0 part of S0-F or F-S0 were not worked. Despite hexahistidine tags attached to flagellin worked well during purification process (Figure 17), they might be hindered by conjugated S0 in S0-F or F-S0, because N- and C-terminus position of flagellin is very close. According to this insistence, long flexible linkers could reduce steric hindrance and enable S0-F or F-S0 to be purified well. Purified S0 conjugated with C3d or C4d had also significantly lower yield than their alone forms similar to flagellin case. It is inferred that hexahistidine tag on N-terminus of C3d or C4d can be attributed to the superior purification of C3d or C4d alone, as the hexahistidine tag on N-terminus of S0 was revealed not well operated on purification. Therefore, for complement fragment case, conjugation of them with N-terminus of S0 is recommended.

For the immunological perspectives, more informative indicators could be helpful to determine the immunogenicity. Of antigen-specific antibodies, neutralizing antibody is utmost important for the protection of pathogen infection. In fact, S0 produced through E. coli system in this study is different from S0 of original PEDV, because the S0 protein originally have an N-

glycosylation site at 213. However, according to Yufeng's research (Li Yufeng et al., 2015) there were positive correlations between virus neutralization titers and indirect ELISA results in which they used S0 antigen produced from bacteria as a coating protein. Thus, it is inferred that the induced serum IgG in this study may be the neutralizing antibodies, however, more direct assays to evaluate antigen-specific neutralizing antibodies should be included for informative results. It is also important that the immunization experiment should be tested on target animal as different animals have distinct immunological features. This is especially relevant for complement fragment adjuvant case because mouse C3d sequence tested in mouse cannot be utilized as a swine vaccine adjuvant. Thus, target animal should be used as an experimental model when complement based adjuvants are tested.

For the perspective of complement fragment adjuvants, the mechanism of their action should be unraveled for its further application. Though AS Groot and colleagues suggested that C3d-specific autoreactive T cell could be one of the reasons of its adjuvant effect, data revealed that other mechanisms might be existed as C3d mixture regimen also enhanced antigen-specific immune response similarly high to conjugated form in this study. This ability of C3d or C4d may be worked only in systemic immune system, not in mucosal system. Together, the activation of C4d-specific T cell should be evaluated sophisticatedly by using peptide stimulation followed by FACS analysis to clarify the mechanism of action of C4d's adjuvancy. In addition, pre-existed C3d

and C4d-specific memory T cells should also be determined not only in mouse but also in target animals including human and pig for further application.

Overall Conclusion

The PED outbreaks in the South Korea, occurred every year since 1992 and became endemic, resulting substantial economic losses to domestic swine industry. Since, early 2000, the vaccination program for PED prevention using inactivated and modified attenuated vaccines has been introduced and resulted in a decline of PEDV associated diarrheal disease outbreaks. Despite of the decline the PED outbreaks, the highest number of death pigs from PEDV infection was recorded in the year 2018, providing an immediate resolution to develop an effective PED vaccine.

The most currently used PED vaccines are live attenuated (LAV) or inactivated vaccine which have inherent issues related to safety and eventually cost-effectiveness of its use. Particularly, the effectiveness of some of those vaccines have become controversial after the appearance of virulent non-S INDEL strains, which consequently stressed the need for development of a novel PED vaccine.

Despite of low immunogenicity, subunit vaccines have many advantages over traditional LAVs including safety, simplicity and mass production through E. coli systems. However, candidate antigens of PED subunit vaccine, such as S0, have been expressed as insoluble aggregates in Escherichia coli (E. coli) which are not suitable to use be as a vaccine. In order to overcome the low solubility and low immunogenicity of the PED subunit vaccine, fusion adjuvants including

flagellin and complement fragment C3d, C4d were introduced and investigated to enhance the solubility and immunogenicity of the PED subunit vaccine antigen, S0.

In the study I, flagellin, *Vibrio vulnificus FlaB*, was conjugated to the N- or C-terminus of PEDV antigen S0 to generate S0-F and F-S0, respectively. The ability of recombinant proteins to improve protein solubility and an antigen-specific humoral immune response was investigated. In contrast to S0, which expressed almost entirely as an insoluble protein, 99 % of flagellin was expressed as a soluble protein and 59 % of S0-F was also expressed as a soluble protein. This results suggests S0 improved solubility in the E. coli expression system in generic expression condition when flagellin is conjugated to the C-terminus of S0. In order to improve the solubility of the recombinant proteins, trigger factor chaperone tig, was introduced and co-expressed with the respective protein of interest. In this condition, compare to S0, which had 66 % solubility, 99 % of S0-F and F-S0 were expressed as soluble proteins, suggesting that flagellin conjugation also improved the solubility of recombinant proteins in the presence of *tig*. Thus, these results demonstrate that both flagellin conjugation and chaperone *tig* coexpression increased the solubility of S0-related recombinant proteins.

In contrast to S0-F, which 59 % was expressed as a soluble protein, F-S0 was expressed as an IB form. By fusion, a solubility enhancer to the N-terminus of a target protein can enable efficient translation initiation. The rapid translation of

F-S0 in the absence of *tig* produces IBs rather than the soluble protein despite of its total protein expression level being 1.5-times higher compare to the S0-F protein level.

The ability of S0-F and F-S0 to induce S0-specific humoral immune responses was tested in mice. Interestingly, the F-S0 immunized group had significantly high levels of S0-specific IgG and IgA compared to all other groups at the 6-week post-injection. The results from the effects of the conjugation suggests the superior ability of F-S0 to induce S0-specific IgG compared to the mixture of S0/F. Data results demonstrates that S0-F treatment induced significantly higher levels of IL-18, a representative cytokine of the NLRC4/NAIP5 pathway, compared to F-S0 or F treatment in mice, suggesting that the superior ability of S0-F to activate the NLRC4/NAIP5 pathway may interfere with flagellin's adjuvancy and reduce serum S0-specific IgG. Meanwhile, except for one mouse, S0-immunized group displayed higher dominance of S0-specific IgG1 response compared to IgG2a response, suggesting that S0 carries an inherent ability to induce Th2-type immune response to itself. In contrast to S0-F and F-S0, flagellin alone induced IgG2a higher compared to IgG1, suggesting that flagellin may distort the type of immune response from Th2 to Th1. The cytokines derived to TH2 cells, such as IL-5 and IL-6, are important for the generation of IgA at mucosal site, and when S0-F or F-S0 is utilized as a mucosal vaccine, additional advantages are expected.

In the study II, through *in silico* study, the complement fragment C4d was

selected as a novel fusion adjuvant candidate, and the conjugation effect of C4d as well as C3d on the enhancement of solubility and immunogenicity was investigated. In order to identify a novel endogenous protein candidate for the effective fusion adjuvant such as C3d, several serum proteins known to have a pathogen binding ability like C3d has been chosen and analyzed to determine the frequency of T cell epitopes in their intra-structures using the IEDB analysis tool. Date results demonstrates that the human C4d had 0.54 and 1.12, which presented a similarly high frequency of T cell epitopes among human serum protein candidates in the condition of Z-scores lower than 5 and 10, respectively. The T cell frequency value of mouse C4d in the condition of Z scores lower than 5, was greater than C3d or serum albumin (0.092 in C4d case versus to 0.056 and 0.050 of C3d and serum albumin, respectively). In this regard, C4d was chosen as the endogenous protein candidate for a fusion adjuvant to construct model subunit vaccine.

In addition, conjugation of mC4d to S0 improved the solubility of the recombinant proteins regardless of the existence of chaperone *tig*, suggesting that C4d could be a good candidate for enhancing antigen solubility in other proteins that are prone to form IBs, while conjugation of C3d had no effect on solubility enhancement. The conjugation of mC3d and mC4d to S0 also enhanced S0-specific IgG titers in serum against S0 in mice. Unexpectedly, both C3d and C4d carried adjuvant effect on humoral responses not only in the status of conjugation to target antigen, but also in the form of non-conjugated mixtures.

Therefore, regardless of the fusion status, C3d and C4d could be utilized as adjuvant. As mC4d is expected to act as a T cell epitope donor, we enumerated the mC4d-specific IL-4 secreting T cells in addition to S0-specific one by ELISPOT assay at 6-week post-injection. As mC4d is expected to act as a T cell epitope donor, the number of mC4d-specific IL-4 secreting T cells was enumerated in addition to S0-specific one by ELISPOT assay at 6-week postinjection. S0-mC4d2 and S0 + mC4d groups had significantly higher number of mC4d-specific IL-4 secreting T cells than media control. S0-mC4d1 group had higher number of mC4d-specific IL-4 secreting T cells than media control, though there's no statistical significance. These data suggest that mC4d-specific T cells could be involved in the course of immune response in part.

In study III, the recombinant protein candidates, selected from the study I and II, were compared at the same time to select the best PED subunit vaccine protein in terms of immunogenicity enhancement. The immunogenicity of each recombinant proteins after intramuscular administration was analyzed first. Data revealed that all of the groups using adjuvant [F-S0, S0-mC3d2, S0-mC4d2, S0 + mC3d (x2), and S0 + mC4d (x2)] had a similar high level titers of anti-S0 IgG compared to control groups including untreated and S0 at 4 and 6-week post-injection. As mC3d and mC4d had high production yield, they were finally selected as the best adjuvant of PED subunit vaccine for intramuscular administration. The ability of each recombinant proteins to enhance mucosal immune response was also analyzed by measuring IgA titers in mucosal fluids

after intranasal immunization. The result shows that S0-mC4d2 enhanced the highest anti-S0 IgA titers at various mucosal areas compare to any other recombinant proteins after intranasal administration. Therefore, S0-mC4d2 was finally selected as the best immunogenic mucosal PED subunit vaccine. Interestingly, the mC4d or mC3d mixture regimens did not have an adjuvant effect on antibody induction, contrary to systemic injection with mixture regimens, suggesting that conjugation of mC3d or mC4d was the main factor to confer the mucosal adjuvant activity.

In conclusion, the most immunogenic recombinant proteins for intramuscular and intranasal PED subunit vaccine were selected. These recombinant proteins cab be used not only as a sole subunit vaccine, but also as a starting material for enhanced vaccine by applying other adjuvants. In particular, the novel complement fragment adjuvant C4d could be applied to PEDV antigen, in addition to various antigens for other vaccines. Thus, following investigation provide critical insights for the development of a novel PED vaccine and the immunotherapeutics based on flagellin or complement fragments.

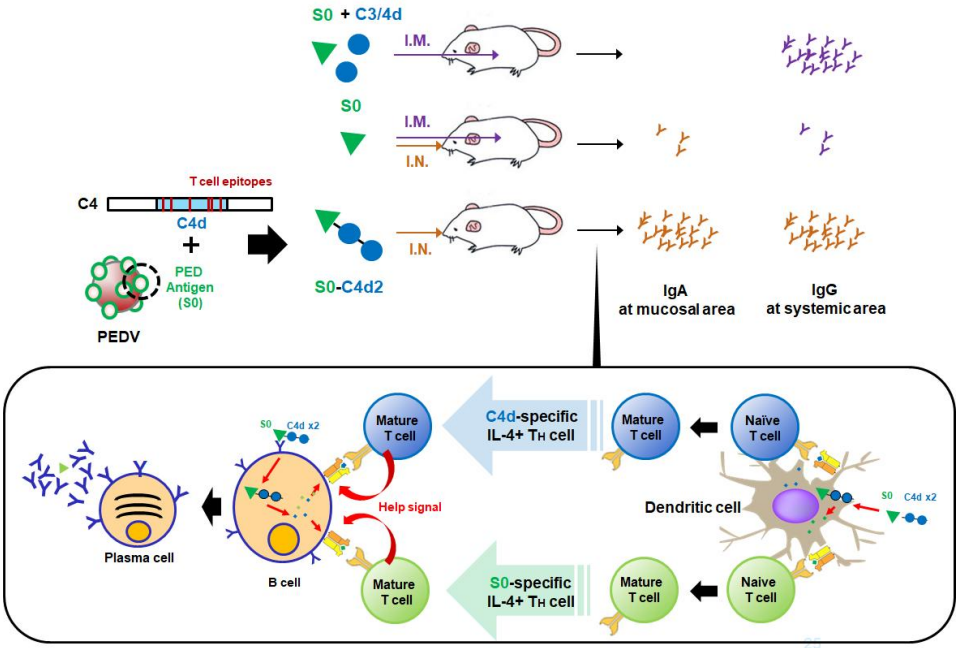


Figure 45. Schematic view of overall conclusion.

S0-mC4d2 has a potent adjuvant effect in relation to IgA induction at mucosal area after intranasal immunization (I.N.). C4d-specific T cells are inferred to be involved in this process. C3d and C4d have a potent adjuvant effect after intramuscular administration (I.M.) when they are immunized with S0 separately.

Literature Cited

Ayudhya, S.N.N., Assavacheep, P., Thanawongnuwech, R.J.T., and Diseases, E. (2012). One World–One Health: the threat of emerging swine diseases. an Asian perspective. 59**,** 9-17.

Ben-Yedidia, T., and Arnon, R. (1998). Effect of pre-existing carrier immunity on the efficacy of synthetic influenza vaccine. *Immunology letters* 64**,** 9-15.

Ben-Yedidia, T., Tarrab-Hazdai, R., Schechtman, D., and Arnon, R. (1999). Intranasal administration of synthetic recombinant peptide-based vaccine protects mice from infection by Schistosoma mansoni. *Infection and immunity* 67**,** 4360-4366.

Blander, J.M., and Medzhitov, R. (2006). Toll-dependent selection of microbial antigens for presentation by dendritic cells. *Nature* 440**,** 808.

Chang, S.-H., Bae, J.-L., Kang, T.-J., Kim, J., Chung, G.-H., Lim, C.-W., Laude, H., Yang, M.-S., and Jang, Y.-S. (2002). Identification of the epitope region capable of inducing neutralizing antibodies against the porcine epidemic diarrhea virus. *Molecules and cells* 14**,** 295-299.

Chang, Y.-C., Chang, C.-Y., Tsai, P.-S., Chiou, H.-Y., Jeng, C.-R., Pang, V.F., and Chang, H.-W. (2018). Efficacy of heat-labile enterotoxin B subunit-adjuvanted parenteral porcine epidemic diarrhea virus trimeric spike subunit vaccine in piglets. *Applied microbiology and biotechnology* 102**,** 7499-7507.

Chen, J., Wang, C., Shi, H., Qiu, H., Liu, S., Chen, X., Zhang, Z., and Feng, L. (2010). Molecular epidemiology of porcine epidemic diarrhea virus in China. *Archives of virology* 155**,** 1471-1476.

Cho, Y.-Y., Lim, S.-I., Kim, Y.K., Song, J.-Y., Lee, J.-B., and An, D.-J. (2014). Complete genome sequence of K14JB01, a novel variant strain of porcine epidemic diarrhea virus in South Korea. *Genome Announc.* 2**,** e00505-00514.

Coffman, R.L., Sher, A., and Seder, R.A. (2010). Vaccine adjuvants: putting innate immunity to work. *Immunity* 33**,** 492-503.

Costa, S., Almeida, A., Castro, A., and Domingues, L. (2014). Fusion tags for protein solubility, purification and immunogenicity in Escherichia coli: the novel Fh8 system. *Frontiers in microbiology* 5**,** 63.

Crooke, E., Guthrie, B., Lecker, S., Lill, R., and Wickner, W.J.C. (1988). ProOmpA is stabilized for membrane translocation by either purified E. coli trigger factor or canine signal recognition particle. 54**,** 1003-1011.

De Groot, A.S., Ross, T.M., Levitz, L., Messitt, T.J., Tassone, R., Boyle, C.M., Vincelli, A.J., Moise, L., Martin, W., and Knopf, P.M. (2015). C3d adjuvant effects are mediated through the activation of C3d□specific autoreactive T cells. *Immunology and cell biology* 93**,** 189-197.

De Haes, W., Pollard, C., Vanham, G., and Rejman, J. (2012). "“Wrapped Up”

Vaccines in the Context of HIV-1 Immunotherapy," in *Immunodeficiency*. IntechOpen).

Delavari, S., Sohrabi, M., Ardestani, M.S., Faezi, S., Tebianian, M., Taghizadeh, M., Shajiei, A., Hosseini, S.Y., Moghaddampour, M., and Mahdavi, M. (2015). Pseudomonas aeruginosa flagellin as an adjuvant: superiority of a conjugated form of flagellin versus a mixture with a human immunodeficiency virus type 1 vaccine candidate in the induction of immune responses. *Journal of medical microbiology* 64**,** 1361-1368.

Dempsey, P.W., Allison, M.E., Akkaraju, S., Goodnow, C.C., and Fearon, D.T. (1996). C3d of complement as a molecular adjuvant: bridging innate and acquired immunity. *Science* 271**,** 348-350.

Ding, Z., Fang, L., Jing, H., Zeng, S., Wang, D., Liu, L., Zhang, H., Luo, R., Chen, H., and Xiao, S. (2014). Porcine epidemic diarrhea virus nucleocapsid protein antagonizes beta interferon production by sequestering the interaction between IRF3 and TBK1. *Journal of virology* 88**,** 8936-8945.

Fahnert, B., Lilie, H., and Neubauer, P. (2004). "Inclusion bodies: formation and utilisation," in *Physiological Stress Responses in Bioprocesses*. Springer), 93-142.

Falsey, A.R., and Walsh, E.E.J.V. (1997). Safety and immunogenicity of a respiratory syncytial virus subunit vaccine (PFP-2) in the institutionalized elderly. 15**,** 1130-1132.

Fujita, T., Matsushita, M., and Endo, Y. (2004). The lectin□complement pathway–its role in innate immunity and evolution. *Immunological reviews* 198**,** 185-202.

Gellin, B. (2000). Vaccines and infectious diseases: Putting risk into perspective. *American Medical Association Briefing on Microbial Threats, National Press Club, Washington, DC*.

Gomes, A.R., Byregowda, S.M., Veeregowda, B.M., and Balamurugan, V. (2016). An overview of heterologous expression host systems for the production of recombinant proteins.

Green, T.D., Montefiori, D.C., and Ross, T.M.J.J.O.V. (2003). Enhancement of antibodies to the human immunodeficiency virus type 1 envelope by using the molecular adjuvant C3d. 77**,** 2046-2055.

Haas, K.M., Toapanta, F.R., Oliver, J.A., Poe, J.C., Weis, J.H., Karp, D.R., Bower, J.F., Ross, T.M., and Tedder, T.F. (2004). Cutting edge: C3d functions as a molecular adjuvant in the absence of CD21/35 expression. *The Journal of Immunology* 172**,** 5833-5837.

Hajam, I.A., Dar, P.A., Shahnawaz, I., Jaume, J.C., and Lee, J.H. (2017). Bacterial flagellin—a potent immunomodulatory agent. *Experimental & molecular medicine* 49**,** e373.

Hoffmann, A., Bukau, B., and Kramer, G.J.B.E.B.a.-M.C.R. (2010). Structure and function of the molecular chaperone Trigger Factor. 1803**,** 650-661.

Horzinek, M., Egberink, H., Ederveen, J., and Callebaut, P. (1988).

Characterization of the structural proteins of porcine epizootoc diarrhea virus, strain CV777. *American journal of veterinary research* 49**,** 1320-1324.

Huang, C.-Y., Chen, C.-A., Lee, C.-N., Chang, M.-C., Su, Y.-N., Lin, Y.-C., Hsieh, C.-Y., and Cheng, W.-F. (2007). DNA vaccine encoding heat shock protein 60 co-linked to HPV16 E6 and E7 tumor antigens generates more potent immunotherapeutic effects than respective E6 or E7 tumor antigens. *Gynecologic oncology* 107**,** 404-412.

Huang, Q., Yu, W., and Hu, T. (2016). Potent antigen-adjuvant delivery system by conjugation of Mycobacterium tuberculosis Ag85B-HspX fusion protein with arabinogalactan-Poly (I: C) conjugate. *Bioconjugate chemistry* 27**,** 1165-1174.

Huang, Y.-W., Dickerman, A.W., Piñeyro, P., Li, L., Fang, L., Kiehne, R., Opriessnig, T., and Meng, X.-J. (2013). Origin, evolution, and genotyping of emergent porcine epidemic diarrhea virus strains in the United States. *MBio* 4**,** e00737-00713.

Huleatt, J.W., Jacobs, A.R., Tang, J., Desai, P., Kopp, E.B., Huang, Y., Song, L., Nakaar, V., and Powell, T. (2007). Vaccination with recombinant fusion proteins incorporating Toll-like receptor ligands induces rapid cellular and humoral immunity. *Vaccine* 25**,** 763-775.

Jarvis, M.C., Lam, H.C., Zhang, Y., Wang, L., Hesse, R.A., Hause, B.M., Vlasova, A., Wang, Q., Zhang, J., and Nelson, M.I.J.P.V.M. (2016). Genomic and evolutionary inferences between American and global strains of porcine epidemic diarrhea virus. 123**,** 175-184.

Jeong, H., and Seong, B.L. (2017). Exploiting virus-like particles as innovative vaccines against emerging viral infections. *Journal of Microbiology* 55**,** 220-230.

Julier, Z., Martino, M.M., De Titta, A., Jeanbart, L., and Hubbell, J.A. (2015). The TLR4 agonist fibronectin extra domain A is cryptic, exposed by elastase-2; use in a fibrin matrix cancer vaccine. *Scientific reports* 5**,** 8569.

Kastenmüller, W., Kastenmüller, K., Kurts, C., and Seder, R.A. (2014). Dendritic cell-targeted vaccines—hope or hype? *Nature Reviews Immunology* 14**,** 705.

Khamis, Z. (2016). Producing a subunit vaccine for porcine epidemic diarrhea virus.

Kim, H., Lee, Y.-K., Kang, S.C., Han, B.K., Choi, K.M.J.C., and Research, E.V. (2016). Recent vaccine technology in industrial animals. 5**,** 12-18.

Kim, Y., and Lee, C. (2014). Porcine epidemic diarrhea virus induces caspase-independent apoptosis through activation of mitochondrial apoptosis-inducing factor. *Virology* 460**,** 180-193.

Knopf, P.M., Rivera, D.S., Hai, S.H., Mcmurry, J., Martin, W., and De Groot, A.S. (2008). Novel function of complement C3d as an autologous helper T□cell target. *Immunology and cell biology* 86**,** 221-225.

Koch, M., Frazier, J., Sodroski, J., and Wyatt, R.J.V. (2005). Characterization of antibody responses to purified HIV-1 gp120 glycoproteins fused with the molecular adjuvant C3d. 340**,** 277-284.

Kocherhans, R., Bridgen, A., Ackermann, M., and Tobler, K. (2001). Completion of the porcine epidemic diarrhoea coronavirus (PEDV) genome sequence. *Virus genes* 23**,** 137-144.

Kreutz, M., Giquel, B., Hu, Q., Abuknesha, R., Uematsu, S., Akira, S., Nestle, F.O., and Diebold, S.S. (2012). Antibody-antigen-adjuvant conjugates enable co-delivery of antigen and adjuvant to dendritic cells in cis but only have partial targeting specificity. *PloS one* 7**,** e40208.

Kweon, C.-H., Kwon, B.-J., Jung, T.-S., Kee, Y.-J., Hur, D.-H., Hwang, E.-K., Rhee, J.-C., and An, S.-H. (1993). Isolation of porcine epidemic diarrhea virus (PEDV) in Korea. *Korean Journal of Veterinary Research* 33**,** 249-254.

Kweon, C.-H., Kwon, B.-J., Lee, J.-G., Kwon, G.-O., and Kang, Y.-B. (1999). Derivation of attenuated porcine epidemic diarrhea virus (PEDV) as vaccine candidate. *Vaccine* 17**,** 2546-2553.

Langel, S.N., Paim, F.C., Lager, K.M., Vlasova, A.N., and Saif, L.J. (2016). Lactogenic immunity and vaccines for porcine epidemic diarrhea virus (PEDV): historical and current concepts. *Virus research* 226**,** 93-107.

Lee, C. (2015). Porcine epidemic diarrhea virus: an emerging and re-emerging epizootic swine virus. *Virology journal* 12**,** 193.

Lee, D.-K., Cha, S.-Y., and Lee, C. (2011). The N-terminal region of the porcine epidemic diarrhea virus spike protein is important for the receptor binding. *Korean J Microbiol Biotechnol* 39**,** 140-145.

Lee, S., and Lee, C. (2014). Outbreak-related porcine epidemic diarrhea virus strains similar to US strains, South Korea, 2013. *Emerging infectious diseases* 20**,** 1223.

Lee, S.E., Kim, S.Y., Jeong, B.C., Kim, Y.R., Bae, S.J., Ahn, O.S., Lee, J.-J., Song, H.-C., Kim, J.M., and Choy, H.E. (2006). A bacterial flagellin, Vibrio vulnificus FlaB, has a strong mucosal adjuvant activity to induce protective immunity. *Infection and immunity* 74**,** 694-702.

Li, B., Ge, J., and Li, Y. (2007). Porcine aminopeptidase N is a functional receptor for the PEDV coronavirus. *Virology* 365**,** 166-172.

Li, C., Li, W., De Esesarte, E.L., Guo, H., Van Den Elzen, P., Aarts, E., Van Den Born, E., Rottier, P.J., and Bosch, B.-J. (2017). Cell attachment domains of the PEDV spike protein are key targets of neutralizing antibodies. *Journal of virology***,** JVI. 00273-00217.

Li, D.-J., Wang, H.-M., Li, L., Zhao, X.-R., Wang, M.-Y., Zhu, Y., Meng, Y., and Yuan, M.-M.J.J.O.R.I. (2003). Gene fusion of molecular adjuvant C3d to hCGβ enhances the anti-hCGβ antibody response in DNA immunization. 60**,** 129-141.

Li, Q., Xu, Z., Wu, T., Peng, O., Huang, L., Zhang, Y., Xue, C., Wen, Z., Zhou, Q., and Cao, Y. (2018). A flagellin-adjuvanted PED subunit vaccine

improved protective efficiency against PEDV variant challenge in pigs. *Vaccine* 36**,** 4228-4235.

Li, W., Yang, J., Zhang, E., Zhong, M., Xiao, Y., Yu, J., Zhou, D., Cao, Y., Yang, Y., and Li, Y. (2016). Activation of NLRC4 downregulates TLR5-mediated antibody immune responses against flagellin. *Cellular & molecular immunology* 13**,** 514.

Li, Y., Zheng, F., Fan, B., Muhammad, H.M., Zou, Y., and Jiang, P. (2015). Development of an indirect ELISA based on a truncated S protein of the porcine epidemic diarrhea virus. *Canadian journal of microbiology* 61**,** 811-817.

Lilie, H., Schwarz, E., and Rudolph, R.J.C.O.I.B. (1998). Advances in refolding of proteins produced in E. coli. 9**,** 497-501.

Lin, C.-M., Saif, L.J., Marthaler, D., and Wang, Q. (2016a). Evolution, antigenicity and pathogenicity of global porcine epidemic diarrhea virus strains. *Virus research* 226**,** 20-39.

Lin, C.-N., Chung, W.-B., Chang, S.-W., Wen, C.-C., Liu, H., Chien, C.-H., and Chiou, M.-T. (2014). US-like strain of porcine epidemic diarrhea virus outbreaks in Taiwan, 2013–2014. *Journal of Veterinary Medical Science***,** 14-0098.

Lin, K.-H., Chang, L.-S., Tian, C.-Y., Yeh, Y.-C., Chen, Y.-J., Chuang, T.-H., Liu, S.-J., and Leng, C.-H. (2016b). Carboxyl-terminal fusion of E7 into Flagellin shifts TLR5 activation to NLRC4/NAIP5 activation and induces TLR5-independent anti-tumor immunity. *Scientific reports* 6**,** 24199.

Liu, C., Tang, J., Ma, Y., Liang, X., Yang, Y., Peng, G., Qi, Q., Jiang, S., Li, J., and Du, L. (2015). Receptor usage and cell entry of porcine epidemic diarrhea coronavirus. *Journal of virology* 89**,** 6121-6125.

López-Yglesias, A.H., Zhao, X., Quarles, E.K., Lai, M.A., Vandenbos, T., Strong, R.K., and Smith, K.D. (2014). Flagellin induces antibody responses through a TLR5-and inflammasome-independent pathway. *The Journal of Immunology***,** 1301893.

Makadiya, N., Brownlie, R., Van Den Hurk, J., Berube, N., Allan, B., Gerdts, V., and Zakhartchouk, A. (2016). S1 domain of the porcine epidemic diarrhea virus spike protein as a vaccine antigen. *Virology journal* 13**,** 57.

Miao, E.A., Andersen-Nissen, E., Warren, S.E., and Aderem, A. (Year). "TLR5 and Ipaf: dual sensors of bacterial flagellin in the innate immune system", in: *Seminars in immunopathology*: Springer), 275-288.

Mizel, S.B., and Bates, J.T. (2010). Flagellin as an adjuvant: cellular mechanisms and potential. *The Journal of Immunology* 185**,** 5677-5682.

Mizel, S.B., Graff, A.H., Sriranganathan, N., Ervin, S., Lees, C.J., Lively, M.O., Hantgan, R.R., Thomas, M.J., Wood, J., and Bell, B. (2009). Flagellin-F1-V fusion protein is an effective plague vaccine in mice and two species of nonhuman primates. *Clinical and Vaccine Immunology* 16**,** 21-28.

Moyle, P.M. (2017). Biotechnology approaches to produce potent, self-adjuvanting antigen-adjuvant fusion protein subunit vaccines. *Biotechnology advances* 35**,** 375-389.

Moyle, P.M., and Toth, I. (2013). Modern subunit vaccines: development, components, and research opportunities. *ChemMedChem* 8**,** 360-376.

Nguyen, C.T., Kim, S.Y., Kim, M.S., Lee, S.E., and Rhee, J.H. (2011). Intranasal immunization with recombinant PspA fused with a flagellin enhances cross-protective immunity against Streptococcus pneumoniae infection in mice. *Vaccine* 29**,** 5731-5739.

Noris, M., and Remuzzi, G. (Year). "Overview of complement activation and regulation", in: *Seminars in nephrology*: Elsevier), 479-492.

O'hagan, D.T., and De Gregorio, E.J.D.D.T. (2009). The path to a successful vaccine adjuvant–'the long and winding road'. 14**,** 541-551.

Oldham, J. (1972). Letter to the editor. *Pig Farming* 10**,** 2.

Park, C.-K., and Pak, S.-I. (2009). Infection patterns of porcine epidemic diarrhea virus (PEDV) by sera-epidemiological analysis in Korean pig farms. *Journal of Life Science* 19**,** 1304-1308.

Park, C., and Lee, C. (2009). Clinical examination and control measures in a commercial pig farm persistently infected with porcine epidemic diarrhea (PED) virus. *Journal of Veterinary Clinics* 26**,** 463-466.

Paul, W.E. (2011). Bridging innate and adaptive immunity. *Cell* 147**,** 1212-1215.

Pensaert, M., and De Bouck, P. (1978). A new coronavirus-like particle associated with diarrhea in swine. *Archives of virology* 58**,** 243-247.

Perrie, Y., Mohammed, A.R., Kirby, D.J., Mcneil, S.E., and Bramwell, V.W. (2008). Vaccine adjuvant systems: enhancing the efficacy of sub-unit protein antigens. *International journal of pharmaceutics* 364**,** 272-280.

Piao, D.-C., Lee, Y.-S., Bok, J.-D., Cho, C.-S., Hong, Z.-S., Kang, S.-K., and Choi, Y.-J. (2016a). Production of soluble truncated spike protein of porcine epidemic diarrhea virus from inclusion bodies of Escherichia coli through refolding. *Protein expression and purification* 126**,** 77-83.

Piao, D.-C., Shin, D.-W., Kim, I.-S., Li, H.-S., Oh, S.-H., Singh, B., Maharjan, S., Lee, Y.-S., Bok, J.-D., and Cho, C.-S. (2016b). Trigger factor assisted soluble expression of recombinant spike protein of porcine epidemic diarrhea virus in Escherichia coli. *BMC biotechnology* 16**,** 39.

Plotkin, S.A. (2003). Vaccines, vaccination, and vaccinology. *The Journal of infectious diseases* 187**,** 1349-1359.

Puth, S., Hong, S.H., Park, M.J., Lee, H.H., Lee, Y.S., Jeong, K., Kang, I.-C., Koh, J.T., Moon, B., and Park, S.C. (2017). Mucosal immunization with a flagellin-adjuvanted Hgp44 vaccine enhances protective immune responses in a murine Porphyromonas gingivalis infection model. *Human vaccines & immunotherapeutics* 13**,** 2794-2803.

Rhee, J.H., Lee, S.E., and Kim, S.Y. (2012). Mucosal vaccine adjuvants update. *Clinical and experimental vaccine research* 1**,** 50-63.

Scheiermann, J., and Klinman, D.M. (2014). Clinical evaluation of CpG

oligonucleotides as adjuvants for vaccines targeting infectious diseases and cancer. *Vaccine* 32**,** 6377-6389.

Sekhon, S.S., Nguyen, P.-L., Ahn, J.-Y., Lee, K.-A., Lee, L., Kim, S.Y., Yoon, H., Park, J., Ko, J.H., and Kim, Y.-H. (2016). Porcine epidemic diarrhea (PED) infection, diagnosis and vaccination: A mini review. *Toxicology and Environmental Health Sciences* 8**,** 277-289.

Shibata, I., Tsuda, T., Mori, M., Ono, M., Sueyoshi, M., and Uruno, K. (2000). Isolation of porcine epidemic diarrhea virus in porcine cell cultures and experimental infection of pigs of different ages. *Veterinary microbiology* 72**,** 173-182.

Shirota, H., Sano, K., Hirasawa, N., Terui, T., Ohuchi, K., Hattori, T., Shirato, K., and Tamura, G. (2001). Novel roles of CpG oligodeoxynucleotides as a leader for the sampling and presentation of CpG-tagged antigen by dendritic cells. *The Journal of Immunology* 167**,** 66-74.

Singh, A., Upadhyay, V., Upadhyay, A.K., Singh, S.M., and Panda, A.K. (2015). Protein recovery from inclusion bodies of Escherichia coli using mild solubilization process. *Microbial cell factories* 14**,** 41.

Singh, S.M., Panda, A.K.J.J.O.B., and Bioengineering (2005). Solubilization and refolding of bacterial inclusion body proteins. 99**,** 303-310.

Song, D., Moon, H., and Kang, B. (2015). Porcine epidemic diarrhea: a review of current epidemiology and available vaccines. *Clinical and experimental vaccine research* 4**,** 166-176.

Song, D., Oh, J., Kang, B., Yang, J.S., Moon, H., Yoo, H.S., Jang, Y., and Park, B. (2007). Oral efficacy of Vero cell attenuated porcine epidemic diarrhea virus DR13 strain. *Research in veterinary science* 82**,** 134-140.

Song, L., Zhang, Y., Yun, N.E., Poussard, A.L., Smith, J.N., Smith, J.K., Borisevich, V., Linde, J.J., Zacks, M.A., and Li, H. (2009). Superior efficacy of a recombinant flagellin: H5N1 HA globular head vaccine is determined by the placement of the globular head within flagellin. *Vaccine* 27**,** 5875-5884.

Southwood, S., Sidney, J., Kondo, A., Del Guercio, M.-F., Appella, E., Hoffman, S., Kubo, R.T., Chesnut, R.W., Grey, H.M., and Sette, A. (1998). Several common HLA-DR types share largely overlapping peptide binding repertoires. *The Journal of Immunology* 160**,** 3363-3373.

Stevens, T.L., Bossie, A., Sanders, V.M., Fernandez-Botran, R., Coffman, R.L., Mosmann, T.R., and Vitetta, E.S. (1988). Regulation of antibody isotype secretion by subsets of antigen-specific helper T cells. *Nature* 334**,** 255.

Subramaniam, S., Yugo, D.M., Heffron, C.L., Rogers, A.J., Sooryanarain, H., Leroith, T., Overend, C., Cao, D., and Meng, X.-J. (2018). Vaccination of sows with a dendritic cell-targeted porcine epidemic diarrhea virus S1 protein-based candidate vaccine reduced viral shedding but exacerbated gross pathological lesions in suckling neonatal piglets. *Journal of General Virology* 99**,** 230-239.

Sun, D., Feng, L., Shi, H., Chen, J., Liu, S., Chen, H., and Wang, Y. (2007).

Spike protein region (aa 636789) of porcine epidemic diarrhea virus is essential for induction of neutralizing antibodies. *Acta virologica* 51**,** 149-156.

Sun, R.-Q., Cai, R.-J., Chen, Y.-Q., Liang, P.-S., Chen, D.-K., and Song, C.-X. (2012). Outbreak of porcine epidemic diarrhea in suckling piglets, China. *Emerging infectious diseases* 18**,** 161.

Swartz, J.R. (2001). Advances in Escherichia coli production of therapeutic proteins. *Current opinion in biotechnology* 12**,** 195-201.

Tristram, D., Welliver, R., Mohar, C., Hogerman, D., Hildreth, S., and Paradiso, P.J.J.O.I.D. (1993). Immunogenicity and safety of respiratory syncytial virus subunit vaccine in seropositive children 18-36 months old. 167**,** 191-195.

Vijay□Kumar, M., Carvalho, F.A., Aitken, J.D., Fifadara, N.H., and Gewirtz, A.T. (2010). TLR5 or NLRC4 is necessary and sufficient for promotion of humoral immunity by flagellin. *European journal of immunology* 40**,** 3528-3534.

Vlasova, A.N., Marthaler, D., Wang, Q., Culhane, M.R., Rossow, K.D., Rovira, A., Collins, J., and Saif, L.J. (2014). Distinct characteristics and complex evolution of PEDV strains, North America, May 2013–February 2014. *Emerging infectious diseases* 20**,** 1620.

Wang, E., Guo, D., Li, C., Wei, S., Wang, Z., Liu, Q., Zhang, B., Kong, F., Feng, L., and Sun, D.J.P.O. (2016). Molecular characterization of the ORF3 and S1 Genes of porcine epidemic diarrhea virus non S-INDEL strains in seven regions of China, 2015. 11**,** e0160561.

Wang, K., Lu, W., Chen, J., Xie, S., Shi, H., Hsu, H., Yu, W., Xu, K., Bian, C., and Fischer, W.B. (2012). PEDV ORF3 encodes an ion channel protein and regulates virus production. *FEBS letters* 586**,** 384-391.

Wang, L., Sunyer, J.O., and Bello, L.J. (2004a). Fusion to C3d enhances the immunogenicity of the E2 glycoprotein of type 2 bovine viral diarrhea virus. *Journal of virology* 78**,** 1616-1622.

Wang, X.-L., Li, D.-J., Yuan, M.-M., Yu, M., and Yao, X.-Y.J.J.O.R.I. (2004b). Enhancement of humoral immunity to the hCGβ protein antigen by fusing a molecular adjuvant C3d3. 63**,** 97-110.

Watanabe, I., Ross, T.M., Tamura, S.-I., Ichinohe, T., Ito, S., Takahashi, H., Sawa, H., Chiba, J., Kurata, T., and Sata, T. (2003). Protection against influenza virus infection by intranasal administration of C3d-fused hemagglutinin. *Vaccine* 21**,** 4532-4538.

Yamamoto, M., Vancott, J.L., Okahashi, N., Marinaro, M., Kiyono, H., Fujihashi, K., Jackson, R.J., Chatfield, S.N., Bluethmann, H., and Mcghee, J.R. (1996). The Role of Th1 and Th2 Cells for Mucosal IgA Responsesa. *Annals of the New York Academy of Sciences* 778**,** 64-71.

Zeng, W., Ghosh, S., Lau, Y.F., Brown, L.E., and Jackson, D.C. (2002). Highly immunogenic and totally synthetic lipopeptides as self-adjuvanting immunocontraceptive vaccines. *The Journal of Immunology* 169**,** 4905-

4912.
Zhang, D., Xia, Q., Wu, J., Liu, D., Wang, X., and Niu, Z. (2011). Construction and immunogenicity of DNA vaccines encoding fusion protein of murine complement C3d-p28 and GP5 gene of porcine reproductive and respiratory syndrome virus. *Vaccine* 29**,** 629-635.
Zhang, L., Mallik, B., and Morikis, D. (2007). Immunophysical exploration of C3d–CR2 (CCP1-2) interaction using molecular dynamics and electrostatics. *Journal of molecular biology* 369**,** 567-583.
Zom, G.G., Khan, S., Filippov, D.V., and Ossendorp, F. (2012). "TLR ligand–peptide conjugate vaccines: toward clinical application," in *Advances in immunology*. Elsevier), 177-201.

Summary in Korean

돼지유행성설사병은 구토와 수양성 설사가 특징인 양돈질병으로, 모든 연령대의 돼지를 감염하지만, 특히 포유자돈에게 높은 치사율을 야기하여 양돈 농가에 큰 경제적 피해를 입히는 감염성 질병이다. 우리나라 역시 1992년부터 발발하여 풍토병 (endemic)이 되었다. 한 때 불활화 백신과 약독화 생백신의 도입으로 발병이 소폭 감소하였으나, 2013년 새로운 돼지유행성설사병 바이러스 변형체가 등장하면서 피해가 지속적으로 증가하여 2018년에는 사상 최고치의 발생 두수를 기록했다. 현재 상용화된 백신은 대부분 돼지유행성설사병 바이러스를 직접 배양하고, 약독화 과정을 거치기 때문에 노동 집약적이며, 안전성 문제, 빠른 대응의 문제가 있을 뿐 아니라 그 방어 효과 또한 기대에 미치지 못하는 상황이다. 따라서 상용화된 백신의 단점을 극복할 수 있는 아단위 백신의 개발이 절실한 상황이다. 본 연구에서는 아단위 백신의 큰 단점 중 하나인 낮은 면역원성을 극복하기 위해서 다양한 결합형 면역증강제 (fusion adjuvant)를 도입하여 그것의 생산성과 면역원성을 증진시키고자 하였다.

Study I 에서는 *Vibro vulnificus*로부터 유래한 박테리아 플라젤린을 항원인 돼지유행성설사병 바이러스 외피 단백질의 S0 부분에 도입하여 생산성 및 면역원성 증진을 확인하고, 그 메커니즘을 규명하였다.

플라젤린은 결합형 면역증강제로서 다른 항원에 많이 이용되어왔으며, 항원에 결합하는 방향에 따라 다른 효과를 내는 것으로 알려져 있다. 따라서 플라젤린을 S0의 C 말단에 도입한 S0-F와 N 말단에 도입한 F-S0를 대장균 BL21 (DE3)에서 생산하였고, 각각이 S0의 용해도 (solubility) 및 면역원성에 미치는 영향을 평가하였다.

플라젤린을 S0의 C말단에 결합할 경우, S0의 용해도를 크게 증가시킴을 확인했으며, 특히 샤페론 *tig*가 공통 발현된 조건에서는 S0 단독에 비해 S0-F, F-S0 모두 대부분의 단백질이 용해성을 띄는 것으로 나타나 생산성을 크게 증가시키는 것을 확인했다. 한편, 마우스 면역 *in vivo* 실험 결과 F-S0는 S0 혹은 S0와 F의 혼합물에 비해 유의적으로 높은 S0 특이적 혈중 IgG, IgA 항체를 유도하는 것으로 나타났다. 특히, F-S0는 S0-F에 비해서 유의적으로 높은 면역반응을 유도하는 것으로 확인되었다. 이는 플라젤린이 S0의 N 말단에 결합할 경우 구조 변화가 생기고, C말단에 결합하여 구조 변화가 생긴 단백질에 비해 NLRC4/NAIP5 신호 전달 경로를 덜 자극하기 때문인 것으로 추측된다.

Study II 에서는 in silico study를 통해 새로운 보체 절편 형태의 결합형 면역증강제 C4d를 발굴하고, C3d와 C4d를 항원 S0에 도입하여 생산성 및 면역원성 증진을 확인하였다. 보체 절편 C3d는 결합형

면역증강제로서 다른 항원에 많이 이용되어왔다. 이것은 자체적으로 높은 T 세포 항원결정부 (epitope) 밀도를 가지고 있고, 이것을 제공하는 것이 면역증강의 주요 기작으로 작용하는 것이 밝혀져 있다. 따라서 C3d와 같이 병원체에 물리적으로 붙는 내생 단백질 후보들의 서열 정보를 미국 국립생물정보센터의 데이터베이스에서 얻은 후, “IEDB analysis tool”을 통해 그들의 T 세포 항원결정부 밀도를 조사하였고, 후보군 중 가장 높은 밀도를 가진 C4d를 새로운 결합형 면역증강제로 선정하였다. 따라서 C3d를 S0에 결합한 S0-mC3d1, S0-mC3d2, S0-mC3d3와 C4d를 S0에 결합한 S0-mC4d1, S0-mC4d2를 대장균 BL21 (DE3)에서 생산하였고, 각각이 S0의 용해도 (solubility) 및 면역원성에 미치는 영향을 평가하였다.

C4d를 S0에 결합할 경우, S0의 용해도를 크게 증가시킴을 확인했으며, 특히 샤페론 *tig*가 공통 발현된 조건에서는 S0 단독에 비해 S0-mC4d1, S0-mC4d2 모두 대부분의 단백질이 용해성을 띄는 것으로 나타나 생산성을 크게 증가시키는 것을 확인했다. 하지만 C3d를 S0에 결합할 경우, S0의 용해도 측면에서 큰 효과가 없음을 확인하였다. 한편, 마우스 면역 *in vivo* 실험 결과 C3d와 C4d를 결합한 S0는 S0에 비해 유의적으로 높은 S0 특이적 혈중 IgG 항체를 유도하는 것으로 나타났다. S0-mC4d1과 S0-mC4d2의 경우 숙주의 mC4d 특

이적 T 세포에 의해 면역증강 효과가 나타날 수 있음을 확인하였다. 재미있게도, C3d, C4d를 S0와 혼합하여 처리한 경우 역시 S0에 대한 높은 혈중 IgG 항체를 유도함을 확인하였다.

Study III 에서는 앞서 연구한 다양한 면역증강제 결합형 돼지유행성설사병 아단위 백신 조성 중 가장 효과적인 것을 선정하였다. 후보 아단위 백신 조성은 F-S0, S0-mC3d2, S0-mC4d2와 혼합물인 S0 + mC3d (x2), S0-mC4d (x2)이다. 마우스 면역 *in vivo* 실험을 통해 이들이 전신면역반응 및 점막면역반응에 미치는 효과를 직접 비교하였고, 최종 아단위 백신 조성을 선정하였다.

근육 접종 백신의 경우 높은 전신면역반응을 유도하며, 매우 높은 생산 수율을 보이는 S0, C3d (혹은 C4d) 혼합물을 가장 효과적인 아단위 백신 조성으로 선정하였다. 점막 면역 백신의 경우 가장 높은 점막면역반응을 유도하는 S0-mC4d2를 아단위 백신 조성물로 선정하였다. 한편, 이 과정에서 S0, C3d (혹은 C4d) 혼합물은 점막 접종할 경우, 근육 접종의 경우와 달리 S0 특이적 면역반응을 전혀 일으키지 않음을 확인하였다. 이는 study II에서 C4d의 면역증강기작이 C4d 특이적 T 세포를 통해서 이루어질 수 있는 근거로 생각될 수 있다.

본 연구를 통해 근육 접종 및 점막 접종 측면에서 돼지유행성설사병 예방을 위한 비용 효과적인 아단위 백신 조성물을 선정하였다. 특

히 새로 개발한 면역증강제 C4d의 경우 다른 백신의 개발에 있어서 활용이 가능할 것이다. 이 연구에서 보여준 결과들은 돼지유행성설사병 백신 개발뿐만 아니라 플라젤린, C3d, C4d 결합형 면역증강제에 대한 통찰력을 제공한다.

주요어: 돼지유행성설사병, 돼지유행성설사병 바이러스, 돼지유행성설사병 백신, 재조합 단백질, 결합형 면역증강제, 아단위 백신, 플라젤린, 보체 절편, C3d, C4d

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