농학박사학위논문

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천 인 수

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In Su Cheon

(Supervised by Prof. Cheol-Heui Yun)

Abstract

Respiratory syncytial virus (RSV) is one of the major pathogens causing respiratory tract infection and disease in infants, young children and immunocompromised patients worldwide. In addition, almost all children have experienced RSV infection by 2 to 3 years of age. However, currently there is no vaccine available for human use.

RSV G glycoprotein (RSVG) plays a role in attachment on the host cell and induces neutralizing antibody response which is involved in protective response against RSV infection in the host. However, RSVG, especially $CD4^+$ T cell epitope region within RSV G protein, is known to induce immunopathology.

Mucosal vaccination could induce humoral and cellular immune responses at systemic compartment and mucosal site. However, intranasal route is known to redirect antigen to central nerve system that leads to serious side effect like Bell's palsy although it is rare. In contrast, sublingual route is seemingly safer than intranasal route despite the

fact that its immunogenicity is low. The purpose of this study is to examine RSV vaccine candidate without immunopathology using RSV G protein core fragment (Gcf) administered through sublingual route in mouse.

In the first study, the effect of Gcf immunization routes via sublingual or intranasal routes in mouse was compared when RSV was infected subsequently. Unexpectedly, Gcf sublingual vaccination induced vaccine-enhanced disease, mediated by Th17 response, but intranasal vaccination of Gcf did not vaccine-enhanced disease. The vaccine-enhanced disease by formalin inactivated-RSV (FI-RSV) was mediated through Th2 response as it has been already reported.

In the second study, since $CD4^+$ T cell epitope within RSV G protein was reported to be responsible for the immune response and immunopathology, it was modified for safe and effective RSV vaccine when administered by sublingual route. The amino acid (a.a.) positions 185 and 188, critical sites to activate T cells, were mutated to generate mGcf in order to vanish vaccine-enhanced disease. Then, to recover the function of CD4 helper T cell activity, mGcf was fused with $CD4^+$ T cell epitope from RSV F (F_{51-66}) protein, and generated Th-mGcf. As a result, Th-mGcf induced humoral immune response and provided protection against RSV without immunopathology.

Taken together, vaccine-enhanced disease induced in mice sublingually immunized with Gcf following RSV challenge was mediated by Th17 response. The mutation of $CD4^+$ T cell epitope in Gcf (mGcf) resulted in lower immunogenicity without immunopathology. However, lower immunogenicity by mutation of $CD4^+$ T cell epitope was recovered by conjugation of another $CD4^+$ T cell epitope from RSV F protein (Th-mGcf). Finally, this Th-mGcf immunization via sublingual route after RSV challenge induced protective immune response without vaccine enhanced disease and it might be

potential candidate for RSV vaccine.

Key words: $CD4^+$ T cell epitope, G glycoprotein (RSVG), Immunopathology, Mucosal vaccination route, Respiratory syncytial virus (RSV), Sublingual (s.l.), Vaccine,

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List of Abbreviations

a.a.: amino acid

Ab: antibody

Ag: antigen

AGcf/BCD4: recombinant RSV A2 Gcf with its $CD4^+$ T cell epitope replaced with the corresponding a.a. sequence from RSV B Gcf

AHR: airway hyperresponsiveness

BAL: bronchoalveolar lavage

BB: albumin-binding domain of the streptococcal protein G

BBG2Na: a.a. residues 130-230 of RSV G protein fused with albumin-binding domain of the streptococcal protein G

BGcf/ACD4: recombinant RSV B1 Gcf with its $CD4^+$ T cell epitope replaced with the corresponding a.a. sequence from RSV A2 Gcf

CBA: cytometric bead array

***cp*:** cold-passage

CT: cholera toxin

CTL: cytotoxic T lymphocyte

DCs: dendritic cells

dsRNA: double-stranded RNA

ELISA: enzyme-linked immunosorbent assay

F: fusion protein of RSV

FI-RSV: formalin inactivated RSV

FI-RSVB: formalin inactivated RSV derived from B subtype

f.p.: foot-pad

G: G protein of RSV

GAGs: glycosaminoglycans

Gcf: G protein core fragment

H&E: hematoxylin and eosin

IFN: interferon

IL: interleukin

ILCs: innate lymphoid cells

i.n.: intranasal

IRF: interferon regulatory factor

L: large protein of RSV

LAL: limulus amebocyte lysate

LB medium: Luria-Bertani medium

M: matrix protein of RSV

MdLN: mediastinal lymph node

MHC: major histocompatibility complex

mGcf: recombinantly modified Gcf in which two amino acids corresponsding to a.a. position 185 and 188 in Gcf

N: nucleocapsid protein of RSV

NALT: nasal-associated lymphoid tissue

NK cells: natural killer cells

NS1: non-structural proteins 1 of RSV

NS2: non-structural proteins 2 of RSV

P: phosphoprotein of RSV

PAS: periodic acid-Schiff

PFP: purified F protein

PIV: parainfluenza virus

PRRs: pattern recognition receptors

RIG-I: retinoic acid-inducible gene 1

RNPs: ribonucleoproteins

RSV: respiratory syncytial virus

RSVG: respiratory syncytial virus G protein

s.c.: subcutaneous

SH: small hydrophobic protein

s.l.: sublingual

Th-mGcf: recombinantly modified Gcf which $CD4^+$ T cell epitope from RSV A2 F protein is fused to mGcf

TLR: toll-like receptor

TMB: tetramethylbenzidine

TNF: tumor necrosis factor

***ts*:** temperature-sensitive

TSLP: thymic stromal lymphopoietin

vvF: vaccinia virus expressing the F protein of RSV

vvG: vaccinia virus expressing the G protein of RSV

vvM2: vaccinia virus expressing the M2 protein of RSV

wtGcf (wtAGcf): Gcf derived from RSV A2

wtBGcf: Gcf derived from RSV B1

Review of Literature

1. RSV

Respiratory syncytial virus (RSV) was first discovered in 1956 from chimpanzee exhibiting cold-like illness [1]. The humans who worked with the chimpanzees were infected and showed the similar, yet mild, symptoms like respiratory tract illness and coryza. Subsequently, virus was isolated from patients with respiratory tract illness [2, 3]. Both viruses from chimpanzee and human were similar cytopathology in tissue culture forming of syncytia [2, 3].

1.1 Characteristics

RSV belongs to the family Paramyxoviridae, the subfamily *Pneumovirinae* and the genus *Pneumovirus* and is a negative sense, non-segmented RNA virus [4]. There are two distinct subtypes of virus, A and B depending on the amino acid (a.a.) sequence in its G protein [5]. RSV has 10 genes encoding 11 proteins, three envelope proteins including glycoprotein (G), fusion (F) proteins and small hydrophobic (SH) protein, five other structural proteins, the large (L) protein, nucleocapsid (N), phosphoprotein (P), matrix (M), and M2-1, and two non-structural proteins (NS1 and NS2), and M2-2 [6]. Fig. 1 shows the schematic diagram for the composition of genetic structure and proteins of RSV.

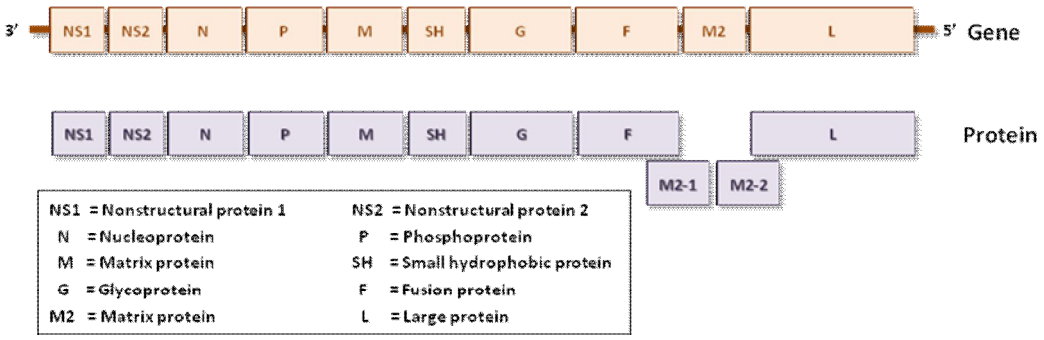

Figure 1. Schematic diagram for functional domains of RSV gene and protein.

G protein is involved in the attachment of virion on host cell surface and F protein helps the fusion between virion envelope and host cell plasma membrane leading to the penetration of virion. G and F proteins are the major targets of neutralizing antibodies [7]. Another membrane protein, SH is not crucial for the efficient viral growth [4]. The role of SH protein is unclear although some evidence suggested that it enhances cell to cell fusion or makes pore-like structures acting like cation channel which influences on membrane permeability [8-10]. L, N and P proteins constitute structural ribonucleoproteins (RNPs), which are the major functional units for the viral transcription and replication [11, 12]. P and L proteins function as polymerase and N protein is essential for the encapsidation of RNA [12, 13]. M protein is important in forming virus-like particles [14] and M2-1 protein is known to be associated with RNA transcription [15]. The function of M2-2 is balancing RNA synthesis between transcription and RNA replication [16]. NS1 and NS2 proteins antagonize type I

interferon (IFN)-mediated antiviral effects through the inhibition of interferon regulatory factor 3 (IRF3) [17, 18]. Summary of RSV protein functions is shown in Table 1.

Table 1. RSV proteins and their function.

RSV Protein	Function
Nonstructural-1 (NS-1)	Inhibition of type I IFN production
Nonstructural-2 (NS-2)	Inhibition of type I IFN production
Nucleocapsid (N)	Major nucleocapsid protein
Phosphoprotein (P)	Nucleocapsid associated, cofactor in RNA synthesis
Matrix(M)	Virion assembly
Small Hydrophobic (SH)	Pentameric ion channel
Glycoprotein (G)	Attachment to the plasma membrane of host cell
Fusion (F)	Fusion and cell entry
M2-1	Supportive of mRNA transcription
M2-2	The balance between RNA replication and transcription
Large (L)	Major polymerase

If RSV infects host, the virus particles hold on to the host cell membrane by binding of viral G protein to cellular glycosaminoglycans (GAGs), particularly heparin sulfate and chondroitin sulfate B, leading to the fusion of host plasma cell membrane and viral envelop by RSV F protein [19, 20]. After the fusion, viral components enter into the host cell cytoplasm. RNA synthesis takes place using L, P and N proteins in the cytoplasm. The mRNA accumulation leads to M2-2 protein accumulation, and the balance of RNA synthesis shift from transcription to RNA replication [16]. Then, virus

assembly and budding occur at the cell surface and progeny viruses are released from the host cell [4, 21].

1.2 Epidemiology

RSV is one of the major pathogens which cause respiratory tract disease and hospitalization in infants or children [4]. It caused 73,400 to 126,300 hospitalizations of children younger than 1 year in USA by bronchiolitis and pneumonia annually [22]. Also, in another study, infection rate of RSV showed 68.8 % and 82.6 % in the children in USA during the first year of life and the second year of life, respectively, suggesting that most of children were infected at least once against RSV by 24 months and half of them were re-infected [23]. The infection rate of RSV showed over 40% in Korea [24]. RSV infection is also risk at elderly or immunodeficient patients including bone marrow transplant recipients or cardiopulmonary patients [25-27]. But there was no difference of duration of illness, temperature, respiratory rate between HIV- and non HIV- infected children [28].

There are two subtypes of RSV, A and B, often co-existing during an epidemic although there is a predominant subtype in each outbreak [29-31]. RSV infection depends on the season in temperate zone. Generally, RSV epidemic lasts from late fall to spring but not during summer [32, 33]. However, RSV infection occurs during the rainy season in tropical or subtropical climate zone [34].

Several risk factors including male gender, concurrent heart or lung disease, high sibling number, day care attendance, exposure to smoke, lower family income, family

history of atopy or asthma and lack of breast feeding have been suggested for the infection and the severe RSV disease [35, 36].

1.3 Immune responses and pathogenesis in RSV infection

1.3.1 Immune responses

The transmission of RSV seems to be though large droplets or fomite contamination, but not small particle aerosols, as a major mode of spread [37]. The symptoms for RSV infection appear about 4 to 5 days after RSV infection [38].

RSV is known to infect the airway and lung tissues of the host. At first, the virus primarily invades airway epithelial cells via pattern recognition receptors (PRRs) (Fig. 2). RSV activates host cells through F protein - Toll-like receptor 4 (TLR4) - MyD88 signaling [39], double-stranded RNA (dsRNA) generated during the RSV replication

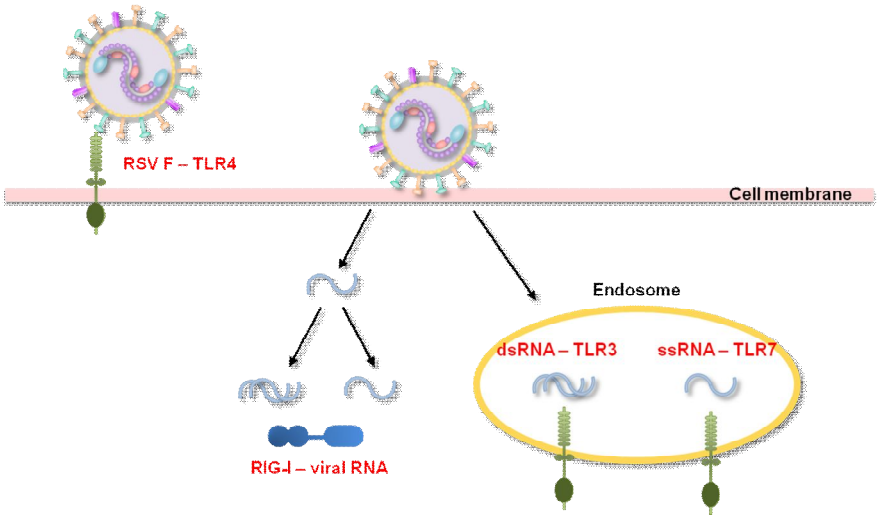

Figure 2. Interaction of RSV with host cell.

cycle - TLR3 [40] and 5' triphosphate structure of viral RNA - retinoic acid-inducible gene 1 (RIG-I) [41]. The activated cells produce chemokines (CCL2, CCL5, interleukin-8 (IL-8), CXCL10) and cytokines (IFN-γ, IL-2, IL-5, IL-9, IL-12, thymic stromal lymphopoietin (TSLP), Type I IFNs) through nuclear factor-kappa B (NF-κB) and IRF-3 [42] that are associated with the recruitment of various leukocytes including neutrophils, eosinophils, macrophages, dendritic cells (DCs), and natural killer (NK) cells from the peripheral blood into infected tissues [43]. Infiltrated cells produce cytokines such as IFN-γ, IL-4, IL-5, IL-12 and IL-13, and antigen presenting cells move to draining lymph node to activate T cells. Cytokines released by innate cells affect the recruitment of eosinophils or differentiation of $CD4^+$ T cells generating and enhancing of the for B and $CD8^+$ T cell responses [44]. NK cells infiltrated at early time of viral infection play a role in killing infected cells whereas $CD8^+$ T cells function at later time (Fig. 3).

There is an increasing number of investigation regarding innate lymphoid cells (ILCs). It is reported that ILC-2 is involved in respiratory tract disease [45, 46]. They are activated by IL-25, IL-33 or TSLP that released mostly from epithelial cells. Also, ILC-2 produces Th2 type cytokines and growth factors including IL-5 and IL-13, but not IL-4 and is involved in the restoration phase after lung damage [47]. However, during RSV infection, exact function of ILCs is yet to be further defined.

It is well known that cytokines as a signal 3, together with major histocompatibility complex (MHC)-antigenic determinant as a signal 1 and interaction of co-stimulatory molecules as signal 2, affects the differentiation of naïve T cells. IFN-γ secreted by NK cells, initially recruited into infection site, not only activates $CD8^+$ T

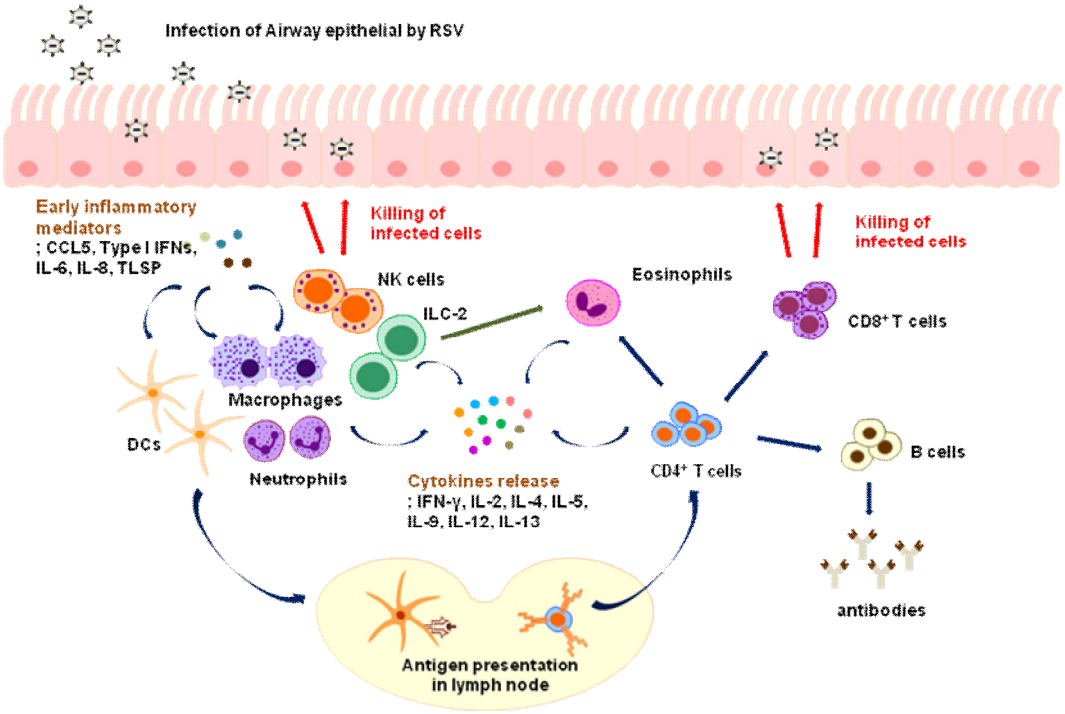

Figure 3. Host immune responses induced by RSV infection.

cells but also helps the differentiation of $CD4^+$ naïve T cells to Th1 $CD4^+$ T cells. Also, IL-12 produced by mainly DCs drives naïve T cells toward to IFN-γ secreting Th1 differentiation and consequently enhances cytotoxic $CD8^+$ T cells [48]. These $CD8^+$ T cells secreting IFN-γ are involved in the clearance of virus by killing infected cells or by restricting inflammation in the lungs [49]. It is also supported by the results from RSV study that depletion of $CD4^+$ or $CD8^+$ T cells extended the period of RSV shedding [50].

While the cellular responses appear to play in the clearance of virus infected cells and prevention of RSV shedding after the infection, humoral response has a

responsibility for the protection by preventing RSV infection [51]. RSV infection indeed induces specific anti-RSV antibodies, IgM, IgG, IgA and even IgE, in the host [52, 53]. Studies using palivizumab which is RSV specific antibodies suggested that RSV-specific antibodies are responsible to prevent or inhibit the severity of infection [51, 54]. F or G proteins induce RSV-specific neutralizing antibodies critical for the prevention of RSV infection where both secretory IgA and serum-derived IgG are responsible for the neutralization [55]. IgE, however, is reported that it is related to the pathogenesis [56].

1.3.2 Immunopathogenesis

The disease symptoms during primary infection of RSV vary among individuals including upper respiratory infections, fever, lower respiratory infection from mild to life-threatening bronchiolitis and pneumonia. Furthermore, immunopathogenesis is caused by various reasons such as personal genetic status and postnatal ages [21, 57]. When RSV infects the host, who are susceptible to RSV infection, host showed more severe disease such as increased inflammations in the lung, detrimental polarization of $CD4^+$ T cell responses or impaired $CD8^+$ T cell responses [58]. Table 2 shows the factors associated with immunopathology during RSV infection.

After RSV infection, proinflammatory cytokines and chemokines released are involved in the expression of adhesion molecules and recruitment of immune cells into the airway [59]. Neutrophils are one of the major cell types infiltrated into the airway as it occurs typically in case of acute inflammation [60] and closely related to severe RSV disease [61]. Pulmonary eosinophilia is also well known in severe RSV disease and the

Table 2. Effector molecules and responses of immunopathology during RSV infection.

Effector molecules	Proinflammatory or immunoregulatory cytokines; IL-4, IL-6, IL-8, TNF-α, TSLP Antibody; IgE
Responses	Shift to or persistence of Th2 $CD4^+$ T cell responses Recruitment and activation of other immune cells into the airways and lungs Activation of mast cells and eosinophils

levels of eosinophil degranulation protein are increased in respiratory tract in patients with severe RSV disease [62, 63]. Eosinophils are known to be infiltrated by chemokines including eotaxin/CCL11 and regulated on activation, normal T cell expressed and secreted (RANTES) and Th2 cytokines like IL-5 and IL-13 [64].

T cell responses are important for the clearance of viral infection and, at the same time, are also associated with pathology during RSV infection. It is well known that the balance between Th1 and Th2 cells is important for the switching between protective and pathogenic responses to RSV infection [4]. Especially, Th2 response is related more to the significant pulmonary pathogenesis including eosinophilia and production of mucus [51, 65, 66]. Neonate and young infants can be more sensitive for RSV infection since the $CD4^+$ T cell response is more biased toward Th2 response [67]. RSV infection is known to induce Th17 response resulting in upregulation of mucus production and inhibition of $CD8^+$ T cell activity [68].

$CD8^+$ T cell responses seem to be involved in both pathogenesis and viral

clearance. As shown by depletion of $CD8^+$ T cell study during RSV infection, $CD8^+$ T cell responses are related to RSV associated disease [50, 69]. It is proposed that robust bystander $CD8^+$ T cell responses in addition to detrimental virus specific $CD8^+$ T cell responses may produce excessive proinflammatory cytokines unnecessarily causing lung damage [70].

It has been reported that RSV specific IgE was present in nasopharyngeal washes and sera from children with severe lung disease by RSV infection [71]. RSV specific IgE is shown to induce degranulation of activated mast cells [72]. Subsequently, RSV infection resulted in exaggerated airway responsiveness (AHR) dependent on the interaction between IgE and FcεRI on mast cells in IgE sensitized mice [72] and these results were similar to lung disease including bronchoalveolar lavage (BAL) eosinophilia and mucus hyperproduction in neonatal mice having RSV specific IgE following secondary RSV infection [73].

1.3.3 Viral evasion

Even though specific immune responses are induced against primary RSV infection, many people have gone through reinfections throughout life. This implies that immunity against RSV remain incomplete [6] as RSV has specific mechanisms to escape host immune responses [74]. For example, NS-1 and -2 inhibit induction of type I and II IFNs through blocking of IRF3 [18, 75]. Also, heavy glycosylation of G protein is obstacle factor for neutralizing antibodies [76]. It is known that soluble G proteins bind to neutralizing antibodies leading to prevent the recognition of virus particle by

antibodies [74].

1.4 Animal models and limitation

One of the major difficulties for RSV studies is no proper animal model exist at present. However, RSV has been investigated in various animal models due to several advantages; study of complex biological system, controlled intervention experiments, genetic influences, test pathophysiological hypotheses, easy handling and ethical issues [57, 77]. Table 3 shows the advantages and disadvantages of RSV animal models.

Table 3. Advantages and disadvantages of RSV animal models.

Animal	Advantages	Disadvantages
Mouse	Easy handling Immunological reagent Extensive experience (genetic manipulation) Similar genome to that of human	Semi-permissive
Cotton rat	100-fole more susceptible than mice Replicate to relatively high titers	Semi-permissive Immunological reagent Weak-easily agitate
Non-human primate	Isolated first from chimpanzee Similar disease symptoms to human Replicate	Expensive Ethical issues Skilled expert

1.4.1 Mouse

Mouse is not appropriate animal model for the RSV study because RSV replication is semi-permissive [78]. Despite the disadvantage of susceptibility, mouse is actively used in RSV study because of strengths as following; easy handling, easy to get immunological reagents, extensive experience with gene targeting such as transgenic or gene deficient mouse and similar genomes, relative non-animal models, to that of human [57, 77]. Among mouse strains, Balb/c was more susceptible than C57BL/6 when viral replications were examined in lungs and nose after viral inoculation [78].

1.4.2 Cotton rat

Cotton rat, *Sigmodon hispidus,* is the animal frequently used to study RSV infection. Like in mouse, they are also semi-permissive for RSV replication [79], but about 100-fold more susceptible than in mouse [80]. RSV can replicate to relatively high titers in the nasal and lungs of naïve cotton rats [81-83]. Furthermore, RSV can infect and are detected in upper and lower respiratory tract of cotton rat, unlike mouse [81]. Nonetheless, there are several weaknesses in cotton rat model. First, it is not easy to get immunological reagents specific to cotton rat when compared to those of mice [84]. Also, special training is required for handling of cotton rats because they are sensitive and easily agitated [80, 84].

1.4.3 Non-human primate

RSV was isolated originally from chimpanzee [1] and disease symptoms such as rhinorrhea or sneezing also were monitored in chimpanzees [85]. RSV replicates in

chimpanzee so that it is a good animal model for RSV study [86]. However, there are limitations to study RSV in chimpanzee. Most of all, it is very expensive to maintain and perform the experiment. And ethical issues together with facilities and skilled expert for handling chimpanzees are another hurdle [77, 87].

2. RSV vaccine

A number of investigations are attempted to develop effective RSV vaccines. There are various processes to develop RSV vaccine and some of them had or have been under clinical test (Table 4).

2.1 Types of RSV vaccine

2.1.1 FI-RSV

Formalin-inactivated RSV (FI-RSV) was the first RSV vaccine in clinical trials during the 1960s [89]. FI-RSV absorbed to alum adjuvant was vaccinated through intramuscular route. However, the results were disappointed. Children vaccinated with FI-RSV did not have protective efficacy and some showed exacerbated pulmonary disease following natural RSV infection. The rate of hospitalization was more than 80% and two even died [89]. On the other hand, children who had not been vaccinated with FI-RSV induced mild symptoms upon subsequent RSV infection and only 5% of patients were hospitalized [88, 89].

It turned out that FI-RSV vaccine did not elicit neutralizing antibodies [101]. The level

of RSV in the lungs of died children were high and antibodies which induced by vaccination with FI-RSV did not have neutralizing activity against the virus [89, 102]. It has been suggested that protein structures on the surface of FI-RSV were disrupted during the formalin treatment leading to the lack of neutralizing antibody responses [83, 101, 103]. RSV activates host cell through TLRs (e.g. F protein of RSV – TLR4, RSV single-stranded RNA genome – TLR7) [39, 104], but FI-RSV cannot activate the

Table 4. RSV vaccine candidates in clinical trials.

Experimental approach	Comments	Status	Ref.
Inactivated			
FI-RSV	Potentiation of RSV disease among RSV-naïve infants and children	Discontinued (exacerated pathology)	[88, 89]
Live attenuated			
Cold-passage/temperature sensitive	Immunogenic in RSV-naïve and -seropositive subjects	Phase I/IIa	[90, 91]
Genetically engineered by reverse genetics	Challenges of phenotype (over- or under-attenuation in clinical studies) and genotype (genetic instability/variability during host replication)	Phase I/IIa	[92, 93]

(Table 4 continued on the next page)

(Table 4 continued from the previous page)

Experimental approach	Comments	Status	Ref.
Vectored			
PIV3 (F protein)	Acceptable safe and immunogenic	Phase I/IIa	[94, 95]
Subunit			
Purified F protein (PFP-1, 2, 3)	Elicits anti-RSV antibodies Inverse correlation between Homogeniety of RSV F protein preparation and immunogenicity	Phase III-discontinued (no preventive efficacy)	[96, 97]
BBG2Na	Phase II - immunogenic	Phase III-discontinued (type III hypersensitivity reactions)	[10, 98]
F/G/M mixed formulation	Immunogenic in healthy and elderly high-risk adults	Phase II	[99, 100]

Modified from *Clin Lab Med 2009; 29* and *BMB reports 2011; 4*

host cells via TLRs because the disrupted membrane by formalin treatment cannot be recognized by TLRs. Thus, FI-RSV cannot stimulate B cells at sufficient level by TLR signaling and subsequently resulted in low avidity antibodies [102, 105]. That is, FI-RSV vaccine induces antibodies but they do not have neutralizing activity and the avidity of antibody is low.

Also, peribronchiolar mononuclear cell infiltration including excessive eosinophilia was observed in the analysis of lung tissue from died children [89]. It has been demonstrated that FI-RSV vaccine induced not only virus-specific polarized Th2 responses but also lack of $CD8^+$ T cell responses against the virus [103, 106]. Th1 cells secrete IFN-γ and induce cell-mediated immunity for protection against intracellular pathogens whilst Th2 cells secrete IL-4, IL-5 and IL-13, and are associated with eosinophilia, IgE production and airway hypersensitivity so that the balance between Th1 and Th2 responses is the key to the immunopathology [50, 107]. Thus, T cell responses by FI-RSV lead to inefficient IFN-γ secreting $CD8^+$ T cells [108] as well as to recruitment of cells like eosinophils [109, 110] and neutrophils [111] into the lower respiratory tract. Also, it appears that vaccine-enhanced disease induced by FI-RSV immunization is not dependent on the immunization route because intramuscular as well as footpad route of FI-RSV immunization induced vaccine-enhanced disease [105, 112].

2.1.2 Live attenuated RSV vaccine

One of the strategies with strong potential for the development of RSV vaccine is producing live attenuated RSV vaccine. There are several mechanisms to generate live attenuated RSV vaccine candidates (Table 5); cold-passage (*cp*) strains which have had serial passages at suboptimal temperatures [113], chemical mutagenesis for temperature-sensitive (*ts*) phenotypes [90], and reverse genetic [114].

In order to attenuate more and, at the same time, augment host response against these RSV vaccines, some of the aforementioned mechanisms have been combined to

generate a new live attenuated RSV vaccine. One of them, *cp* strains are combined with chemical mutagenesis and cold-passaged *ts* (*cpts*) viruses have been generated and tested in clinical trials [92]. Another generation is rA2cp248/40/1030ΔSH which *cpts* 248/404 was engineered the mutation of L protein (a.a. 1030) and the deletion of SH gene. [115]. Above all, when live attenuated RSV vaccine candidates are under consideration, the most critical point is the appropriate balance between over-attenuation and under-attenuation because it can lead to safe but inefficient vaccine efficacy or effective vaccine but with disease, respectively [116].

Table 5. Strategies of live attenuated/genetically engineered RSV derivatives.

Strategy	Methods	Example
Cold-passage (*cp*)	Serial passage at suboptimal temperature	*cp*RSV; 52 serial passages at progressively lower temperatures (final temperature 26℃)
Temperature-sensitive (*ts*)	Mutation by chemical	*ts*1; infected to BK cells at 28℃ in the presence of 10^{-4}M 5-fluorouracil (5FU) for 8 days
Reverse genetics	Engineer recombinant RSV strains bearing attenuated phenotype while maintaining genetic stability	rA2*cp*ΔNS2; *cp* derivative bearing deletions in the NS2 gene

2.1.3 Vector-based RSV vaccines

There are several virus vectors for RSV vaccine development to express RSV protein. For example, vaccinia virus or adenovirus has been engineered to express G or F proteins [117-119]. However, it has not induced sufficient immune responses and protective effect [120, 121]. Immunization with vaccinia virus recombinants that express the F or G glycoprotein of RSV strain A2 showed a half protective effect in the lung of chimpanzees challenged with RSV where all immunized chimpanzees were infected in the upper respiratory tract.

RSV vaccine has been engineered using parainfluenza (PIV), Newcastle disease virus, or Sendai virus expressing RSV protein [122-124]. When these viruses are delivered through the respiratory tract, they do not have tropism to human. Furthermore, the effect of maternal antibodies can be avoided because there are very low levels of pre-existing immunity against these viruses in humans [125]. In particular, PIV vectored RSV vaccine showed protective efficacy in African green monkeys and it is in clinical trials [126].

Bacteria including *Salmonella*, *Streptococcus*, and *Staphylococcus* expressing RSV recombinant protein have been also considered as the delivery tool [127-129]. *Staphylococcus* expressing RSV G peptide on the surface was administrated in mice via intranasal (i.n.) route. It induced antigen specific serum IgG with a balanced IgG1 and IgG2a, and some of mice were partially protected against RSV challenge [127]. Also, *Streptococcus gordonii*, commensal bacterium, was engineered to express RSV G protein and immunized subcutaneously (s.c.) or intranasally in mice. Both elicited

antigen-specific antibodies, especially, i.n. immunized mice induced antigen-specific IgA in BAL although the protective responses were not complete [128]. Additionally, attenuated *Salmonella* typhimurium was used as delivery system. Plasmid DNA encoding RSV F gene was transformed into a live attenuated *Salmonella* typhimurium and it was administrated orally in mice. This vaccine showed increased antigen-specific antibodies and cytotoxic T lymphocyte (CTL) response compared to control DNA vaccine. However, it led to partial protective response, like aforementioned two other vaccine candidates [129].

2.1.4 Subunit vaccines

RSV F and G proteins are known to be the antigens for neutralization antibody response so that a major target for subunit vaccine. Several subunit vaccine candidates have been developed. For example, RSV F and G proteins purified from RSV infected cells and F/G chimeric proteins produced from baculovirus-expression system had been tested [130, 131]. However, these subunit vaccines in rodents induced similar results to those of FI-RSV such as poor neutralizing antibodies and lung pathology [132]. However, it has been reported that various adjuvants like ligands to TLRs can reduce side effects and enhance immunogenicity of subunit vaccine [133, 134]. Co-immunization of G protein with TLR ligand, such as CpG ODN, led to reduce pulmonary eosinophilia and Th2 response evidenced by decreased IL-5^+ T cells and increase of IFN-γ^+ T cells.

There are some subunit vaccine candidates in pre- or clinical strategies in

practice: purified F proteins (PFP), FG chimeric proteins, combinations of RSV F, G and M proteins, or bacterially derived RSV G derivative (BBG2Na). PFP derivatives are subunit vaccines purified from Vero cells infected with RSV [96, 135]. It has been administrated with alum to human [136] and the results showed that PFP immunization induced more than 4-fold in RSV-neutralizing antibody titers [97, 137]. However, RSV infectivity to vaccinees of PFP was not significantly different from its control recipients [100, 137, 138]. Another subunit vaccine candidate, BBG2Na has been generated by fusion of amino acids 130-230 of RSV G protein and the albumin-binding domain of streptococcal protein and it has been tested in pre- and clinical evaluation [98, 139]. Although BBG2Na has protective efficacy, it also caused unanticipated adverse effect like purpura in some vaccinees and therefore clinical trials were stopped [140].

2.2 Vaccine-enhanced pathogenesis

As mentioned above, FI-RSV did not protect children completely from natural RSV infection and exacerbated lung disease like the infiltration of eosinophils into airway and bronchopneumonia [89, 141]. Mice primed with FI-RSV or recombinant vaccinia virus expressing G glycoprotein of RSV (vvG) followed by intranasal challenge with RSV showed vaccine-enhanced disease [142, 143]. Therefore, resolution for the vaccine-enhanced disease inducing immunopathology is a critical issue to make effective RSV vaccine.

What makes effective vaccine is induction of proper immune responses and quick activation following infection (of course without side-effect) so that it leads to

limit virus replication, antigen load, and magnitude of the immune response [141]. There are two possibilities to enhance illness after the RSV challenge or infection in the RSV vaccinated animals and humans. First, vaccine induces ineffective immune responses that cannot clear the virus. Thus, it results in higher replication of virus since the protection efficacy is not present causing high magnitude but ineffective immune response. As a result, it leads to severe inflammation and disease because of ineffective immune response. Second, appropriate immune responses induced by vaccination can clear the virus however, if the composition or magnitude of the response is inappropriate, then it may cause the disease although it attenuates the viral replication [141].

Antibody is one of the prime defense tactics of adaptive immune response to prevent RSV infection that is associated with protection of airway epithelial cells and reduction of antigen accumulation [125]. However, immunization with FI-RSV induced ineffective humoral immune response with low neutralizing and low fusion-inhibiting activity [101, 144]. That is, FI-RSV vaccination induces high quantities of antibody having low functional (neutralizing) activity and this antibody binds virus and leads to immune complex-mediated inflammation after RSV infection.

It is well known that, under the certain circumstances, T cells could contribute to induce vaccine-enhanced disease. There are several studies suggested that the balance between Th1 versus Th2 response is crucial for the outcome and severity of the disease. For instance, Th1 responses are associated with viral clearance after the viral infection. In contrast, Th2 responses tend to prolong virus replication, and sometimes even enhance immunopathology [66, 145, 146]. In many RSV vaccine studies, $CD4^+$ T cell

response affects on vaccine-enhanced disease leading to immunopathology [147]. Indeed, it is demonstrated that immunization with FI-RSV or vvG induced Th2 cytokine response and it is associated with vaccine-enhanced disease including pulmonary eosinophilia or weight loss [109, 143, 148]. Also, $CD4^+$ T cells expressing Vβ14 TCR is responsible for the eosinophilia and increased Th2 cytokine production in vvG vaccinated mice and it is proved that Th2-like pulmonary injury can be decreased by depletion of $CD4^+$ $V\beta14^+$ T cells [107, 112].

Type 2 cytokines, IL-4 and IL-13, are associated with decreased $CD8^+$ T cell response [149, 150]. The roles of Th1 and Th2 $CD4^+$ T cells in immunopathology are depicted in Fig.4. The cytokine milieu which occurs at the initial phase of immune response is important for the differentiation of naïve $CD4^+$ T cells into Th1 or Th2 cells.

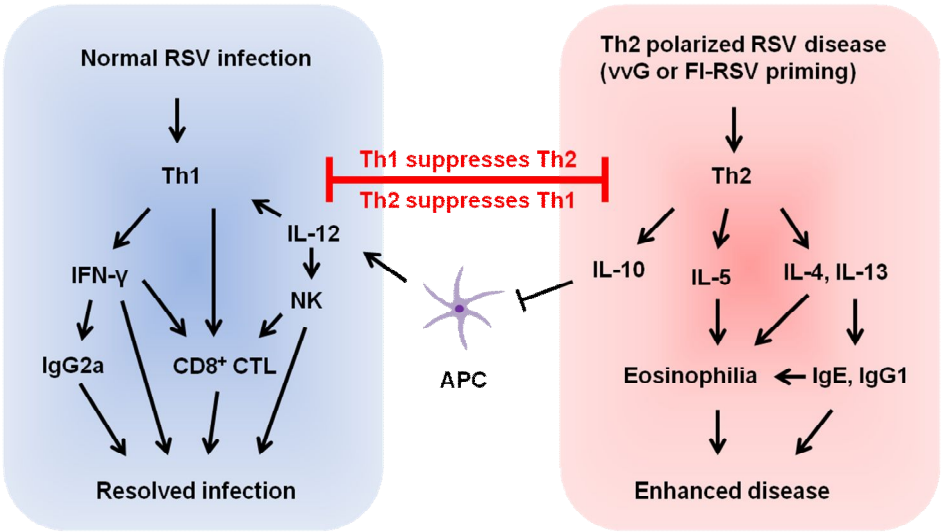

Figure 4. Postulated mechanism of vaccine-enhanced disease in the host with RSV infection.

In general, when virus including RSV is infected the host, Th1 response is mainly induced by antigen presenting cells producing IL-12. This cytokine derive from native T cells to Th1 and activate NK cells. Subsequently, IFN-γ is released by Th1 and NK cells and activates $CD8^+$ T cells which have cytotoxicity and kill the infected cell. Moreover, IFN-γ helps the isotype switching of B cells to produce IgG2a and it is involved in enhancement of Th1 response or resolution of infection site.

In contrast, when there is milieu to induce Th2 response by priming of vvG or FI-RSV, Th2 response is promoted after the RSV infection. At the same time, Th2 cytokines including IL-5, IL-4 and IL-13 suppress Th1 response, subsequently, Th2 response is enhanced. Th2 cytokines then induce the influx of immune cells, especially eosinophils, into lung or airway and the isotype switching of B cell antibody to IgG1 or IgE which further enhance lung disease.

In most studies, RSV-specific $CD8^+$ T cells are related to reduction of vaccine-enhanced disease by producing IFN-γ [151]. But, $CD8^+$ cytotoxic T cell response is also associated with the induction of illness. When RSV specific cytotoxic T cells were adoptively transferred into immunodeficient mice infected with RSV, the hemorrhagic pneumonia and illness were increased [152]. It is also reported that RSV-specific $CD8^+$ T cell response inhibits pulmonary eosinophilia induced by $CD4^+$ T cell response in vvG or FI-RSV immunized mice [108].

There are several parameters to measure the vaccine-enhanced disease in mouse model. Pulmonary eosinophilia, Th2 response, body weight loss and cytokine responses toward Th1 response were typical parameters for vaccine-enhanced disease in FI-RSV

clinical trials and animal experiments [153, 154]. Airway hyperreactivity, mucus production and inflammation can be also used as indicators for vaccine-enhanced disease [155]. However, it has been reported that eosinophils do not and associated with RSV vaccine-enhanced disease like body weight loss, clinical illness, enhanced Penh [156]. Furthermore, the mechanisms of vaccine-enhanced disease appear to be dependent on the type of vaccine. For example, Th1 and Th2 responses are contributed to induce vaccine-enhanced disease in mice immunized with vvG whereas only Th1 response is related to vaccine-enhanced disease in mice immunized with vvG and recombinant vaccinia virus expressing M2 protein (vvM2) or fusion protein (vvF) of RSV [153]. Therefore, symptoms and thereof the parameters to measure vaccine-enhanced disease are various.

2.3 Factors for effective RSV vaccine

In general, RSV infection occurs during the first year after the birth so that there are several factors including host factor, virus factor and safety to be considered for an effective RSV vaccine [51, 116, 157].

In terms of host factor, the age of vaccinees is important for RSV vaccine development. Since infants and young children are the primary target population for the RSV vaccination, the immune immaturity of vaccinees should be considered when design the vaccine. Maternal antibodies present in the infants may have a negative impact due to impediment of the immune response by the vaccine. It is known that reinfection of RSV is common occurrence because RSV infection does not induce

proper memory response. Thus, vaccine is also needed for whom have been infected with RSV in the past and have pre-existing immunity against RSV. For these populations, strategy for the vaccination should focus on boosting protective immunity [10].

As virus factors, RSV vaccine should induce protective response against both A and B subtype despite the genetic variability of RSV genome including the glycosylation of F and G protein [51]. Viral evasion system is also important factor in order to augment protective immune response against RSV infection after vaccination [10].

It is never too much to emphasize safety issue. Because of the failure of FI-RSV, the safety and vaccine-enhanced disease should be carefully and seriously considered for the development of effective RSV vaccine. Furthermore, vaccination route is one of critical factors to be considered for effective vaccine development. In most RSV vaccine studies, vaccination route has not been significantly considered and it has been vaccinated by systemic route such as intramuscular or subcutaneously, or sometimes mucosal route like intranasal. However, it is known that mucosal vaccination route can induce both systemic and local mucosal immune response [158, 159]. In contrast, systemic vaccination route induce effective systemic immune response but not mucosal immune response [160]. Since RSV infects respiratory tract, mucosal route which acts on respiratory tract might be more effective [161, 162]. Among mucosal routes, immunization via sublingual and intranasal routes induce certain immune response in respiratory tract [163]. The advantages and disadvantages of mucosal routes for the

vaccination are shown in the Table 6.

Table 6. Advantages and disadvantages of intranasal and sublingual administration.

	Intranasal	Sublingual
Advantages	Systemic antibodies Mucosal antibodies CTL responses	Systemic antibodies Mucosal antibodies CTL responses No neurological adverse effect to CNS Easy of administration
Disadvantages	Neurological adverse effect to CNS	Small volume Unpleasant taste of some drugs

Intranasal vaccination is effective route for the induction of systemic and mucosal immunity in the gastric mucosa and the respiratory tract. Particularly, it has been investigated for the vaccine against pathogens which infect respiratory tract. For example, FluMist which is live attenuated vaccine for influenza virus is administrated by intranasal route and it provides effective protection against seasonal infection [164]. In the RSV vaccine studies, there are some cases tried to administrate by intranasal route [165]. Unfortunately, intranasal route shows sometimes unfavorable effect where the vaccine can reached the brain and it caused side effect like Bell's palsy [166].

Sublingual route has recently been considered for the attractive novel strategy for mucosal vaccination against some pathogens. Sublingual route induces mucosal and systemic immune responses which are similar effect to intranasal route. In contrast to

intranasal route, s.l. route has no side effect for redirecting to central nerve system [163]. And s.l. immunization showed the induction of immune response more effectively in the upper respiratory tract than in i.n. immunization [167]. However, in the most of studies to date, s.l. immunization does not induce strong enough immune response although it requires small amount of antigen for the vaccination [168].

3. RSV G glycoprotein

RSV G glycoprotein is a type II transmembrane glycoprotein and is involved in attachment on the surface of host cells. It is significantly good target for RSV vaccine because G protein can induce neutralizing antibodies and have function of chemotatic activity despite it is antigenically variable between strains.

3.1 Characteristics

The G and F proteins can induce neutralizing antibodies and protective immunity against RSV infection in animal model [55, 169]. Whereas F protein is conserved considerably [170], G protein has antigenic diversity between RSV A and B subtype with 53% homology [171-173]. It is reported that virus deleted G protein showed reduction in infectivity *in vivo* [114]. G protein is expressed on the surface of virion, *i.e.* anchored on the membrane or secreted into the extracellular environment [174, 175]. Soluble form of G protein can be detected as early as 6 hr after the infection, faster than membrane-anchored G protein [175].

Despite the diversity of G protein between A and B subtypes, there is a central

conserved region compose of 13 amino acids residue (Fig. 5). This region is completely conserved among RSV strains and overlaps with cysteine residues (at a.a. 173, 176, 182 and 186). This cysteine residue forms secondary structure to make cysteine noose by disulfide bonds between Cys-173 and Cys-186, and Cys-176 and Cys-182 [176]. Furthermore, a CX3C motif is located within this cysteine residue. This CX3C motif involves Cys-182 and Cys-186 and acts like fractalkine for leukocyte chemotaxis [177].

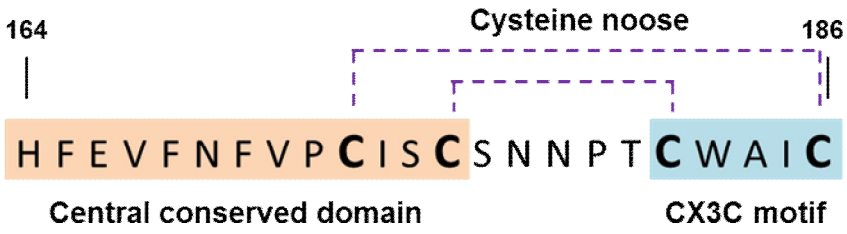

Figure 5. Conserved domain, cysteine noose and CX3C motif within G glycoprotein.

It has been suggested that G protein is involved in viral evasion. G protein is highly glycosylated, which interferes with the recognition by antibodies. Thus, it may act to evade the humoral response [178]. Also, secreted G protein appears to act as a decoy. Previous study found that infection with recombinant RSV lacking soluble form of G protein resulted in milder disease than that of wild type RSV [179]. Thus, secreted G proteins help virus to evade from host immune system as it impedes RSV-specific antibody binding to the virus [180].

3.2 T cell epitope and its modification

$CD4^+$ T cells are important for the regulation of humoral and cellular immune responses. $CD4^+$ T cell response specific to RSV G protein is related to immunopathology and mice immunized with RSV G protein showed immunopathology polarizing Th2 response which is similar result to that induced by FI-RSV vaccination upon RSV infection [107, 143, 145]. For example, vvG vaccination induced memory $CD4^+$ T cells producing Th2 cytokines and these cells were responsible for vaccine-enhanced disease [143, 181]. Moreover, when RSV G specific Th2 $CD4^+$ T cells are transferred into naïve recipient mouse, lung eosinophilia was induced after RSV challenge [145]. Therefore, studies using $CD4^+$ T cell response become important to attenuate vaccine-enhanced disease for safer and more effective vaccine development.

By using several RSV G protein frame shift mutants, a region spanning a.a. 193-205 within the G protein was reported to induce infiltration of eosinophils into the airway depending on $CD4^+$ T cell recognition of G protein fragment [182]. Additionally, a.a. residues 184 to 198 of RSV G protein, known as $CD4^+$ T cell epitope, are responsible for the induction of eosinophilia and inflammatory cytokines in mice immunized with vvG and then challenged with RSV [183]. Another study showed that immunization with RSV G peptide 184-198 conjugated to KLH as a carrier protein induce pulmonary eosinophilia following challenge with RSV suggesting that this portion of G protein is associated with pulmonary eosinophilia [184].

RSV G protein induced simultaneously both Th1 and Th2 response and RSV G residues 183 to 197 was a single dominant site recognized by both Th1 and Th2 effector

cells [183]. In addition, it is reported that Ile at position 185 or Arg at position 188 plays a crucial role in T cell recognition [185], and Arg at position 188 and Lys at position 192 play a role in protective immunity and eosinophilia against RSV [186].

3.3 RSV G glycoprotein as an RSV vaccine candidate

RSV G glycoprotein can be a good RSV vaccine candidate. First, G protein induces neutralizing antibodies and is one of the principle RSV immunogen [187]. Also, it is expressed at early time after the infection. Therefore, G protein has been investigated for the development of RSV vaccine [112].

It is reported that vvG prevents the replication of RSV in mice [169]. However, vvG vaccination also induced pulmonary pathology followed by RSV infection [143, 169]. According to the studies for vaccine-enhanced disease in mice primed with vvG upon RSV challenge, it is found that IL-13 is important for pulmonary eosinophilia whereas IL-4 is critical cytokine for pulmonary eosinophilia in mice immunized with FI-RSV following RSV challenge [173, 188]. Interestingly, RSV $G_{183\text{-}195}$-specific $CD4^+$ T cells expressing Vβ14 chain is associated with the enhanced disease observed in vvG-primed mice, but it does not contribute to vaccine-enhanced disease in FI-RSV-primed mice [112].

BBG2Na is designed by fusion of a.a. residues 130-230 of RSV G protein with the albumin-binding domain (BB) of the streptococcal protein G [189]. To note, there are several advantages of BB fusion strategy. First, BB provides extended exposure of the immunogen because the *in vivo* half-life of proteins is increased [190]. BB also

served as a carrier protein by significantly enhancing antibody response [191]. Moreover, it has been reported that BB binds to albumin in human, mouse, and rat serum with high affinity, therefore, the efficiency of purification for BB fusion protein can be increased by affinity chromatography on albumin-Sepharose [192]. BBG2Na induced protective response without immunopathology including adverse Th2 type response in adult and neonatal mice [193-196]. In the early studies of BBG2Na in humans, the results were positive in general but it did not progress to further clinical trials because of rare unexpected development of purpura in some trials [98, 140, 197].

Adenoviral vector expressing G protein has been also studied for RSV vaccine development. A recombinant replication-deficient adenovirus-based vaccine expressing the soluble core domain of G protein (a.a. residues 130 to 230), rAd/3xG, was designed and evaluated for the RSV vaccine candidate. Mice immunized with rAd/3xG induced mucosal IgA and serum IgG responses, and showed long-term protection after RSV challenge without vaccine-enhanced disease [118]. However, there is a pre-existing immunity to rAd vectors in the normal population [198] and it may cause to decrease the immune response to the vaccine in terms of magnitude and frequency of T cell response and vector-induced antibody responses [199].

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General Introduction

One of the major causes for the hospitalization in respiratory viral disease is respiratory syncytial virus (RSV), which infects almost all children, especially infants worldwide. However, no vaccines are available at the moment. In the 1960's, formalin inactivated-RSV (FI-RSV) was tested in clinical trial. However, it failed because children vaccinated with FI-RSV showed exacerbated lung disease following RSV infection and two died. Through further studies to investigate immunopathology, the biased Th2 response and pulmonary eosinophilia were shown in children immunized with FI-RSV upon RSV infection. Because the failure of FI-RSV as RSV vaccine has been a trauma, the safety concern is strictly considered as a top priority for the development of the vaccine.

Mucosal vaccination has been considered for the administration of RSV vaccine because of several advantages. (1) It could induce immune responses systemically and at mucosal site where pathogen invades and infects at the first place in the host. (2) It does not need needle for the vaccination. (3) For respiratory pathogen, the optimal mucosal routes could be either intranasal or sublingual route. Unfortunately, it has been reported the side effect of intranasal route which is the redirection of vaccine into central nerve system. It is to note that investigation on RSV vaccine has been focused more on immunopathology and mucosal route of administration for the vaccine tried the most so far was intranasal route only.

RSV G protein (RSVG), for the attachment to target cell, is known to induce neutralizing antibody, thus it can be a good target antigen for RSV vaccine. Indeed, it has been studied for the development of RSV vaccine using vaccinia vector expressing G protein (vvG). However, it is reported that G protein is involved in immunopathology and $CD4^+$ T cell epitope in the G protein appears to be mainly responsible for the immunopathology after RSV infection. The immunization of RSV G protein core fragment (Gcf), amino acid (a.a.) 131 to 230 and relatively conserved region between RSV A and B subtype, through mucosal route elicited antigen-specific antibody in serum and bronchoalveolar lavage (BAL) in the previous study. Aims of the present studies were to investigate proper mucosal route for RSV vaccination and to develop RSV vaccine without immunopathology using G protein core fragment.

In the chapter 1, to study the effect of mucosal immunization with Gcf as an RSV vaccine candidate, immune responses leading to the protection against RSV infection were investigated after the immunization with Gcf via sublingual or intranasal route and then challenged with RSV. Also, immunopathology was examined including pulmonary eosinophilia and body weight loss. Furthermore, the mechanism of vaccine-enhanced disease by Gcf immunization was investigated.

In the chapter 2, to develop safer and more effective RSV vaccine using Gcf via sublingual route, $CD4^+$ T cell epitope was modified. To reduce immunopathology, $CD4^+$ T cell epitope within RSV A2 G protein was modified (mGcf). And to recover the function of $CD4^+$ T cells, another $CD4^+$ T cell epitope from RSV A2 F protein was fused

to mGf (Th-mGcf) and then examined for the efficacy of the vaccine candidate in mice.

Chapter 1

Comparison of vaccine-enhanced disease by sublingual and intranasal routes in mice immunized with G protein fragment derived from Respiratory Syncytial Virus A2 following viral infection

1. Introduction

Respiratory syncytial virus (RSV) causes severe respiratory infection in infants, the elderly and immunocompromised individuals, however its vaccine is not yet available [1]. In the 1960s, clinical trial with formalin-inactivated RSV (FI-RSV) vaccine showed severe disease symptoms including bronchiolitis and pneumonia in children following RSV natural infection [2, 3]. Further studies have demonstrated that pulmonary eosinophilia and predominant Th2 responses were evident coincide with vaccine-enhanced disease by FI-RSV immunization upon RSV infection [3].

The RSV G protein, the major RSV attachment protein, is considered to have important role in protection against RSV infection [4]. Therefore, RSV G protein has been studied as a target for RSV vaccine for instance, by using recombinant vaccinia virus expression system (vvG). However, mice immunized with vvG showed vaccine enhanced disease including pulmonary eosinophilia upon RSV challenge [5, 6]. Moreover, pulmonary eosinophilia caused by RSV G protein seemed to be closely related with the induction of RSV G protein-specific $CD4^+$ T cell response [7].

It has been demonstrated that not only Th2 response but also Th17 response is involved in RSV-associated pathology [8]. Th17 cells, producing IL-17, are known to induce pathogenesis of several inflammatory and autoimmune diseases [9]. IL-17 increased in infants infected with RSV [10] is associated with pathology during the infection [11]. Further study suggested the possibility that Th17 response is related with vaccine enhanced disease [12].

It is clear that vaccination route is crucial for the induction of proper immune response at infection site [13]. RSV infects and replicates in respiratory tract suggesting that effective vaccination might be induced through intranasal (i.n.) [14, 15] and sublingual (s.l.) [16] routes. Both vaccination routes could induce systemic and mucosal immune responses under the certain circumstances. Indeed, it has been previously showed that immunization with purified G protein fragment (Gcf), amino acids (a.a) 131-230, via intranasal or sublingual route induced antigen-specific antibodies in the sera and BAL fluid, providing protective immunity against RSV challenge [17]. However, intranasal route has a side effect where the vaccination sometimes redirects antigenic molecules to central nerve system causing Bell′s palsy [18]. No studies so far had looked into the safety and efficacy issues with sublingual route in RSV vaccination scheme.

In the present study, the administration route of s.l. was compared to that of i.n. using Gcf as a RSV vaccine candidate, and investigated the efficacy and the precise mechanism of immunopathology induced by Gcf immunization.

2. Materials and Methods

1) Materials

For cell culture or virus preparation, MEM and FBS was purchased from Life Technologies (Grand Island, NY, USA) and Lonza (Basel, Switzerland), respectively. To measure the levels of cytokines, inflammatory cytokine cytometric bead array (CBA) kit, Th cytokine CBA kit and Flex set (IL-5, IL-13) were purchased from BD Biosciences (San Diego, CA, USA) and for TGF-β, enzyme-linked immunosorbent assay (ELISA) Duo set were purchased from R&D Systems (Minneapolis, MN, USA). Also, all reagents for flow cytometry staining, Golgi Plug, Cytofix/Cytoperm solution, anti-mouse CD4-APC-Cy7, CD44-FITC, anti-mouse IFN-γ-APC, anti-mouse IL-17-PE, anti-mouse IL-5-APC, CD11c-FITC, CD45-APC, Siglec-F-PE or Ly6G-V450, were purchased from BD Bioscience. For determine of antibody levels, HRP-conjugated goat anti-mouse IgG was purchased from Southern Biotechnology (Birmingham, AL, USA).

2) Virus preparation

RSV A2 strain was amplified as previously described [17]. Briefly, virus was propagated in HEp-2 cells (ATCC, Manassas, VA, USA) grown in MEM containing 3 % of FBS and harvested at day 3 or 4 post challenge when extensive syncytia were observed by microscope. Virus titer was determined by standard RSV plaque assay.

3) Construction of plasmids and purification of RSV G protein fragment

The plasmid containing Gcf derived from RSV A2 G protein gene was prepared

as previously described [17]. Gcf plasmid was transformed into *E. coli* BL21 (DE3) strain (Novagen, Madison, WI, USA) and *E. coli* was grown overnight at 37°C in Luria-Bertani (LB) medium supplemented with 100 μg/ml ampicillin. The culture was transferred into fresh LB medium and cultured at 37°C while shaking at 180 rpm until OD_{600} of 0.6~0.8. Protein expression was induced by the addition of 0.5 M IPTG for 4 hrs and the cells were harvested by centrifugation at 6,000 rpm for 10 min. The cell pellets were suspended in binding buffer (20 mM Tris, 0.5 M Nacl, pH 7.9) and disrupted by sonication on ice. After sonication, the soluble and insoluble fractions were separated by centrifugation for 40 min at 20,000 rpm. To harvest Gcf, the soluble fractions were applied to a Talon metal affinity column, washed with binding buffer containing 20 mM imidazole, and then the proteins were eluted by using an elution buffer (300 mM imidazole, 20 mM Tris, 0.5 M NaCl, pH 7.4). The purified proteins were dialyzed against 1 x PBS. The endotoxin in each purified protein was removed by using Triton X-114 as previously described [19]. The endotoxin level of each protein was measured by the limulus amebocyte lysate (LAL) assay kit according to the instructions (Lonza). The purified proteins were electrophoresed on 15% SDS-PAGE and the protein bands were visualized by staining with Coomassie Brilliant Blue (Biorad, Hercules, CA, USA). The protein concentration was determined by Bradford protein assay kit (Biorad). The purified proteins were stored at -80°C until use.

4) Mice and immunization

Female BALB/c mice, 6-8 weeks old, were purchased from Orient Bio Inc.

(Seoul, Korea). IL-17 knockout mice were provided from Yoon-Keun Kim (POSTECH, Korea). All mice were maintained under specific pathogen-free conditions and all studies were approved by Institutional Animal Care and Use Committee (IACUC) at International Vaccine Institute (Approval No. 2012-018). Each mouse was immunized with 20 μg of purified Gcf protein together with 2 μg of cholera toxin (CT) (List Biological Lab. Inc. Campbell, CA, USA) via the s.l. or i.n. route on day 0 and day 14. For s.l. immunization, the anesthetized mice were immunized with 15 μl of prepared vaccines underneath the tongue using a pipette. Following s.l. immunization, mice were maintained with heads placed in ante flexion for 30 min. For i.n. immunization, total 20 μl of vaccines were administered into each nostril of the anesthetized mice. Three weeks after the last immunization, the mice were challenged i.n. with 2 x 10^6 or 2 x 10^5 PFU of live RSV A2. As control groups, mice were immunized each with 2 μg of CT sublingually, or 1x10^5 PFU of FI-RSV via foot-pad (f.p.).

5) Cytokines

Mice were immunized on days 0 and 14 and challenged with RSV A2 as described above. On expected days (1 - 9 days) post challenge, BAL fluids were collected by centrifugation and inflammatory CBA kit for IL-6, TNF-α, MCP-1 and IFN-γ and Th cytokine CBA kit for IL-2, IL-4, and IL-17 were used, according to the protocol. IL-5 and IL-13 were measured using Flex set. TGF-β was measured by ELISA Duo set. Briefly, each BAL fluid, went through the oxidation-reduction procedure to activate TGF-β, was added into the plate coated with TGF-β antibody. After incubation

for 2 hrs, samples were reacted with detection antibody conjugated with biotin and streptavidin-HRP and developed by substrate tetramethylbenzidine (TMB) (Millipore, Bedford, MA, USA). To stop the reaction, 2 M H_2SO_4 was added and the value of each well at OD_{450nm} was read by microplate reader (Molecular Devices, Sunnyvale, CA, USA).

6) T cell response

Mice were immunized on days 0 and 14 and challenged with RSV A2 as described above. On day 5 post-challenge, lungs or mediastinal lymph node (MdLN) was harvested and single cells were collected through 70 μm cell strainer (BD Biosciences) using serum-free RPMI. To examine the cytokine-producing cells, cells were stimulated with 10 μg/ml of a.a. 183-195 G peptide (WAICKRIPNKKPG) and incubated for 18hr for IFN-γ and IL-17 or 36hr for IL-5. For accumulation of cytokines, Golgi Plug was treated before intracellular staining for 12hr. The cells were stained with anti-mouse CD4-APC-Cy7 and CD44-FITC. Then, the cells were fixed, permeabilized using Cytofix/Cytoperm solution, and stained with anti-mouse IFN-γ-APC and anti-mouse IL-17-PE or anti-mouse IL-5-APC. The cells were analyzed using flow cytometry (FACS LSR II; BD Biosciences).

7) Measurement of eosinophils or neutrophils in the BAL or Lung

BAL samples were collected as described previously [19]. Briefly, mice were sacrificed and BAL samples were collected using PBS via tracheotomy and the cells

were separated from the BAL fluid by centrifugation. To measure percentage of eosinophils or neutrophils, lungs from RSV-infected mice were isolated on the expected day post-RSV challenge and processed through 70 μm cell strainer using serum-free MEM. The cells were stained with CD11c-FITC, CD45-APC and Siglec-F-PE for eosinophils or CD11c-FITC, CD45-APC, Ly6G-V450 for neutrophils. The percentages of Eosinophil or neutrophil were acquired using a FACS LSR II and flow cytometric data were analyzed by using FlowJo software (Tree Star, San Carlos, CA, USA). The absolute numbers of eosinophils or neutrophils in BAL and lung were calculated based on the percentage of $CD45^+SiglecF^+CD11c^-$ cells for eosinophils or $CD45^+Ly6G^+CD11c^-$ for neutrophils by flow cytometry and total cell numbers of BAL or lung.

8) ELISA for detection of antibodies

Levels of antigen-specific antibodies in the immune sera were measured by ELISA. In brief, 96-well plates (Nunc, Roskilde, Denmark) were pre-coated with 2 X 10^3 PFU of purified RSV A2 in 100 μl of PBS/well overnight at 4°C. After blocking with PBS containing 5% skim milk for 1 hr at room temperature, sera were individually serially diluted in blocking buffer to each well and incubated for 1 hr at 37°C, followed by the addition of 1:3,000 diluted HRP-conjugated goat anti-mouse IgG. After incubation for 1 hr at room temperature, the color reaction was developed with peroxidase substrate, TMB followed by cease the reaction by the addition of 2 M H_2SO_4. The absorbance at wavelength 450 nm was measured by a microplate reader. The

endpoint titer was determined by O.D. cut-off values of 0.2.

9) Virus titration in mouse lung

To determine the viral titers after the challenge, lungs from RSV-infected mice were isolated on day 4 post-RSV challenge. The lungs were then minced through 70 μm cell strainer using serum-free MEM. Supernatant was collected and RSV titers were determined by plaque assay using HEp-2 cells. The virus titers in the whole lung were indicated as PFU.

10) Histology

For histology studies, mice were immunized with 20 μg of Gcf via s.l. or i.n. route in the present of 2 μg of CT in a prime-boost regimen. For control, mice immunized with 2 μg of CT through s.l. route or with $1x10^5$ PFU of FI-RSV in the same scheme with Gcf. After three weeks, immunized and naïve mice were challenged with 2 x 10^6 PFU live RSV A2. After 4 days post challenge, whole lungs were fixed with 4% formalin and embedded in paraffin. Lung sections were stained with hematoxylin and eosin (H&E) or periodic acid-Schiff (PAS) to show inflammation or mucus production, respectively.

11) Statistical analysis

Statistical differences were analyzed by an ANOVA using GraphPad software. The difference was considered statistically significant when the *P* value was less than

0.05.

3. Results

1) Effect of administration route on the immune response and pathogenesis following RSV infection.

Vaccination route is critical factor for effective vaccine. Since RSV invades through respiratory tract, it has been shown previously that Gcf induced humoral responses when mice were vaccinated through s.l. or i.n. route [17]. In the present study, I compared the immune responses and immunopathology induced by these two different routes of Gcf vaccination. Mice were immunized mice with 20 μg of Gcf in the present of CT via sublingually or intranasally. As the positive control for immunopathology, FI-RSV was immunized into the foot-pad and CT adjuvant alone was injected as a negative control. Two weeks after the second immunization, the levels of RSV A-specific serum IgG were measured. As expected, all immunization regimens induced significantly (P<0.05) higher level of RSV-specific serum IgG than that of CT immunization (Fig. 1-1A). For the protective efficacy of mucosal Gcf vaccination, viral titers were analyzed in the lung from immunized mice following RSV A2 challenge. After 4 days, very low viral titers were found in all mice immunized while it was significantly higher in mice treated with CT alone (Fig. 1-1B).

Next, body weight loss and pulmonary eosinophilia were examined after the challenge with RSV in immunized mice to test vaccine enhanced disease. Interestingly, mice immunized with Gcf by s.l. route experienced the most severe weight loss and delayed its recover following RSV challenge compared to other groups (Fig. 1-1C). Also,

when mice were challenge with high dose of RSV, body weight showed more decreased (data not shown). At day 4 post-challenge, the percentage of eosinophils in BAL collected from mice immunized with FI-RSV or Gcf via s.l. route showed significantly ($P<0.05$) high when compared to that from mice immunized with Gcf via i.n. route. There were no eosinophils in BAL of mice immunized with CT (Fig. 1-1D).

These results indicated that immunization with Gcf induce RSV-specific IgG responses and viral clearance regardless of vaccination route. Furthermore, immunization with Gcf through s.l. route showed more severe vaccine-enhanced disease including body weight loss and eosinophilia in BAL compared to immunization with Gcf via i.n. route.

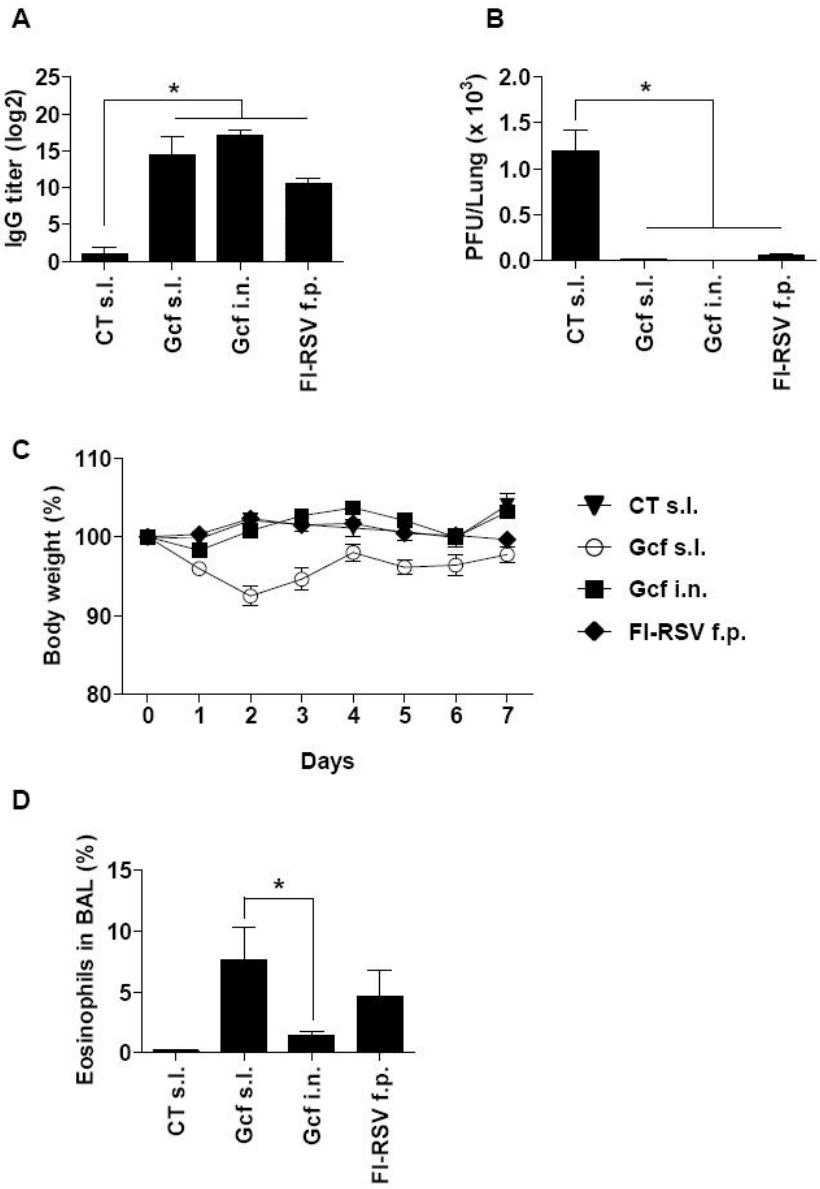
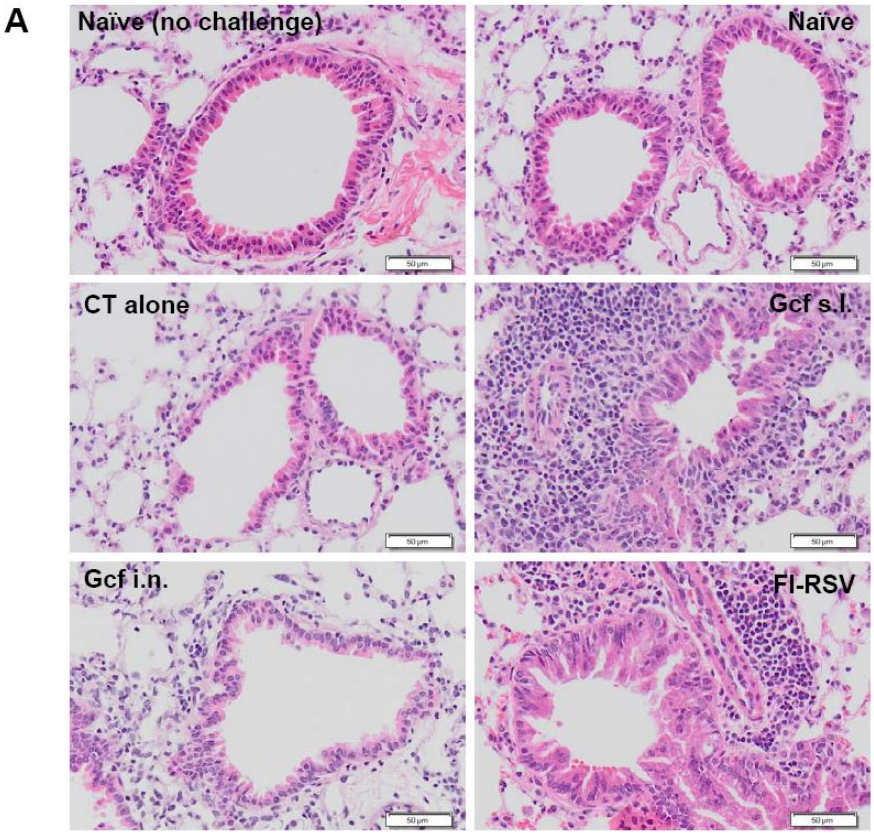


Figure 1-1. Antibody production, protective response, and vaccine-enhanced disease in mice vaccinated with Gcf through different routes. Mice were immunized twice with 20 μg of Gcf via s.l. or i.n. route in the presence of 2 μg of CT or CT alone

sublingually or with 1 x 10^5 PFU of FI-RSV via foot-pad route. (A) RSV A2 specific serum IgGs were determined by ELISA on day 13 after the second immunization. (B) Three weeks after the last immunization, mice were challenged with 2 x 10^6 PFU live RSV A2. The viral titer in the lungs was checked by plaque on day 4 post-challenge. (C) Body weight was monitored daily after the challenge. (D) On day 4 post-challenge, the percentage of eosinophils in BAL was examined by $CD45^+SiglecF^+CD11c^-$ cell using flow cytometry. The results are expressed as mean ± S.E.M for the group (n = 5). * denotes that the values are significantly different at $P < 0.05$.

2) Histological effect by G protein fragment administration route.

To confirm whether immunization with Gcf via s.l. route induce more severe pathology following RSV challenge, mice were immunized intranasally or sublingually with Gcf and then challenged with RSV. At day 4 post challenge, histological sections of the lung were stained with H&E (Fig. 1-2A) or PAS (Fig. 1-2B). Mice immunized sublingually with Gcf or immunized with FI-RSV following RSV challenge resulted in severe peribronchial and perivascular inflammatory infiltrate and mucus production in the lungs. Mice immunized with Gcf i.n. showed less cells infiltrated than that via s.l. route. It is to note that eosinophilic infiltration was shown in the lung from mice immunized with Gcf through s.l. route while few eosinophils in mice immunized via i.n. were found. These results suggest that immunization with Gcf s.l., but not with i.n., induced vaccine-enhanced disease following RSV infection.


A
Naïve (no challenge)
Naïve
CT alone
Gcf s.l.
Gcf i.n.
FI-RSV

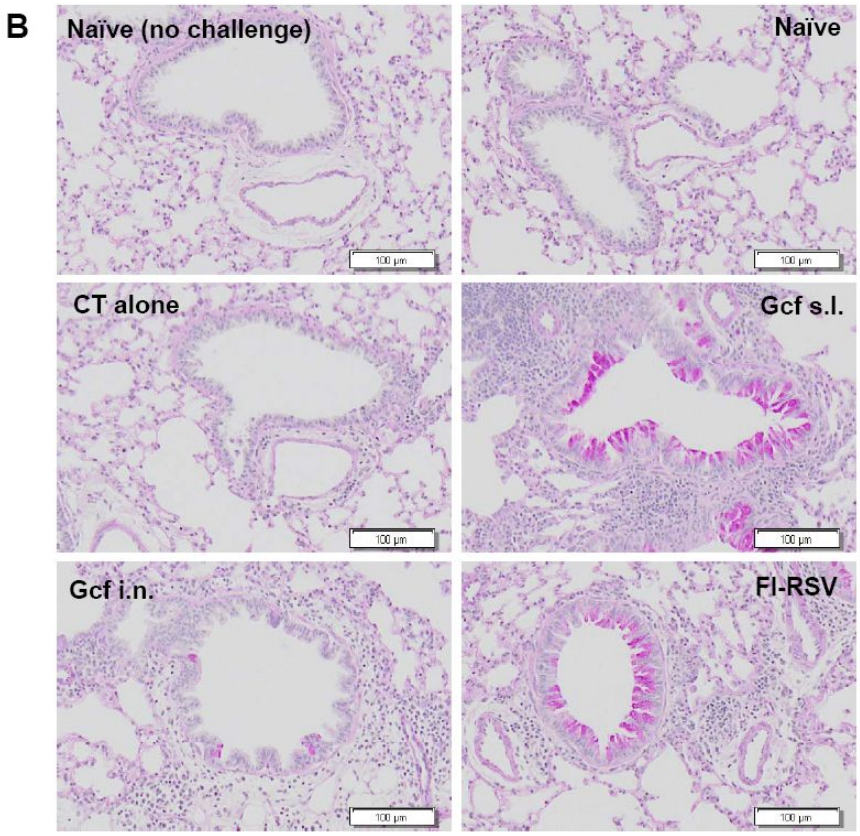


Figure 1-2. Histological examination of the lungs in mice vaccinated with Gcf through different routes. Mice were immunized twice with 20 μg of Gcf via s.l. or i.n. route in the presence of 2 μg of CT or CT alone sublingually or with 1 x 10^5 PFU of FI-RSV via foot-pad route. Three weeks after the last immunization, mice were challenged with 2 x 10^6 PFU live RSV A2. Lungs were harvested on day 4 post-challenge and stained with (A) H&E or (B) PAS. Scale bar for H&E: 50 μm; scale bar for PAS: 100 μm.

3) Inflammatory cytokines induced by G protein fragment vaccination route following RSV infection

To examine whether different administration routes are associated with different inflammatory responses, cytokines associated with inflammation were measured in BAL from mice immunized with Gcf through different routes or FI-RSV as positive control of vaccine-enhanced disease after the challenge. At day 2 post-challenge, regarding as an early time point, mice immunized with Gcf s.l. induced the release of substantial amount of cytokines compared to those in mice immunized with Gcf i.n. or CT alone. And the levels of cytokines in mice immunized with CT alone following RSV challenge were similar to that in naïve mice infected with RSV (data not shown). As shown in Fig. 1-3A, IL-6 was significantly produced in mice immunized Gcf s.l. than in i.n. or CT alone, which was even higher than that from mice immunized with FI-RSV. Both TNF (Fig. 1-3B) and MCP-1 (Fig. 1-3C) showed the highest levels in BAL at day 2. Moreover, immunization of Gcf via s.l. route or FI-RSV increased significantly than other immunization, as expected. Collectively, in the present study, results demonstrate that immunization with Gcf s.l. induces inflammatory response upon RSV infection that is similar to the mice immunized with FI-RSV at early time point following virus challenge.

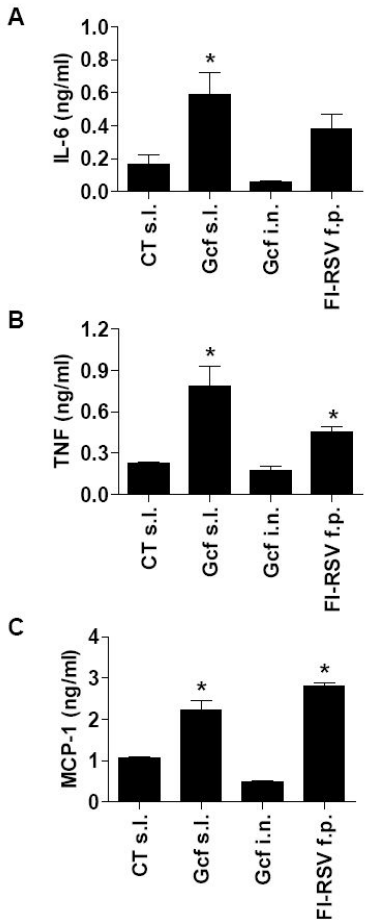


Figure 1-3. Changes of inflammatory cytokines in mice vaccinated with Gcf through different routes. Mice were immunized and challenged as described above. The BAL fluid was collected before RSV challenge or at day 2 post-RSV challenge and performed CBA assay for the analysis of cytokines, (A) IL-6, (B) TNF, (C) MCP-1. The results are expressed as means ± S.E.M. for the group (n = 3). The data are representative of three separate experiments. Significant differences at $P < 0.05$ from results compared with the CT are denoted as *.

4) T cell response by G protein fragment vaccination route.

It is well known that vaccine-enhanced disease mediated by FI-RSV or vvG is caused by Th2 biased responses [6, 20]. To compare Th2 response in mice immunized through different route, mice immunized in a prime-boost regimen with Gcf by different routes were challenged with RSV. At day 5 post-challenge, mononuclear cells were isolated from the lung or MdLN, stimulated with $G_{183\text{-}195}$, $CD4^+$ T cell epitope within G protein, and examined by intracellular staining for IFN-γ, IL-5 and IL-17, representing Th1, Th2 and Th17 $CD4^+$ T cells, respectively. RSV G-specific IFN-γ-producing $CD4^+$ T cells (Fig. 1-4A) in the MdLN were significantly increased in mice immunized with Gcf through s.l. routes than any other groups. The G-specific $IL\text{-}5^+$ $CD4^+$ T cells (Fig. 1-4B) increased significantly in the lung of mice immunized with FI-RSV. However, immunization sublingually with Gcf did not induced G-specific-$IL\text{-}5^+CD4^+$ T cell despite it induced eosinophilia or body weight loss after RSV challenge (Fig. 1-1C and D). The G-specific $IL\text{-}17^+CD4^+$ T cells (Fig. 1-4C) increased significantly in the lung from mice immunized with Gcf regardless of administration routes. Because the cell numbers in lung of mice immunized with Gcf through sublingual route were significantly higher than that in mice immunized with Gcf via intranasal route (Fig. 1-4E), immunization sublingually with Gcf might induce more total G-specific $IL\text{-}17^+CD4^+$ T cells response than immunization intranasally with Gcf. Particularly, immunization with Gcf sublingually increased notably the G-specific $IL\text{-}17^+CD4^+$ T cells in MdLN similar to result of $IFN\text{-}\gamma^+$ $CD4^+$ T cells. However, the G-specific $IL\text{-}17^+CD4^+$ T cells showed very low levels in mice immunized with FI-RSV. Since it is

reported that Th17 cells producing IFN-γ is involved in the pathology in autoimmune experimental inflammation [21], T cells were investigated whether they are expressing both IFN-γ and IL-17. Surprisingly, IFN-γ and IL-17 double positive $CD4^+$ T cells were increased in MdLN from only mice immunized with Gcf through s.l. route, but not i.n. route (Fig. 1-4D) although the total cell numbers of MdLN were not different among groups (Fig. 1-4F).These results mean that immunization sublingually with Gcf shows the possibility of T cell differentiation into Th17 response than any other groups.

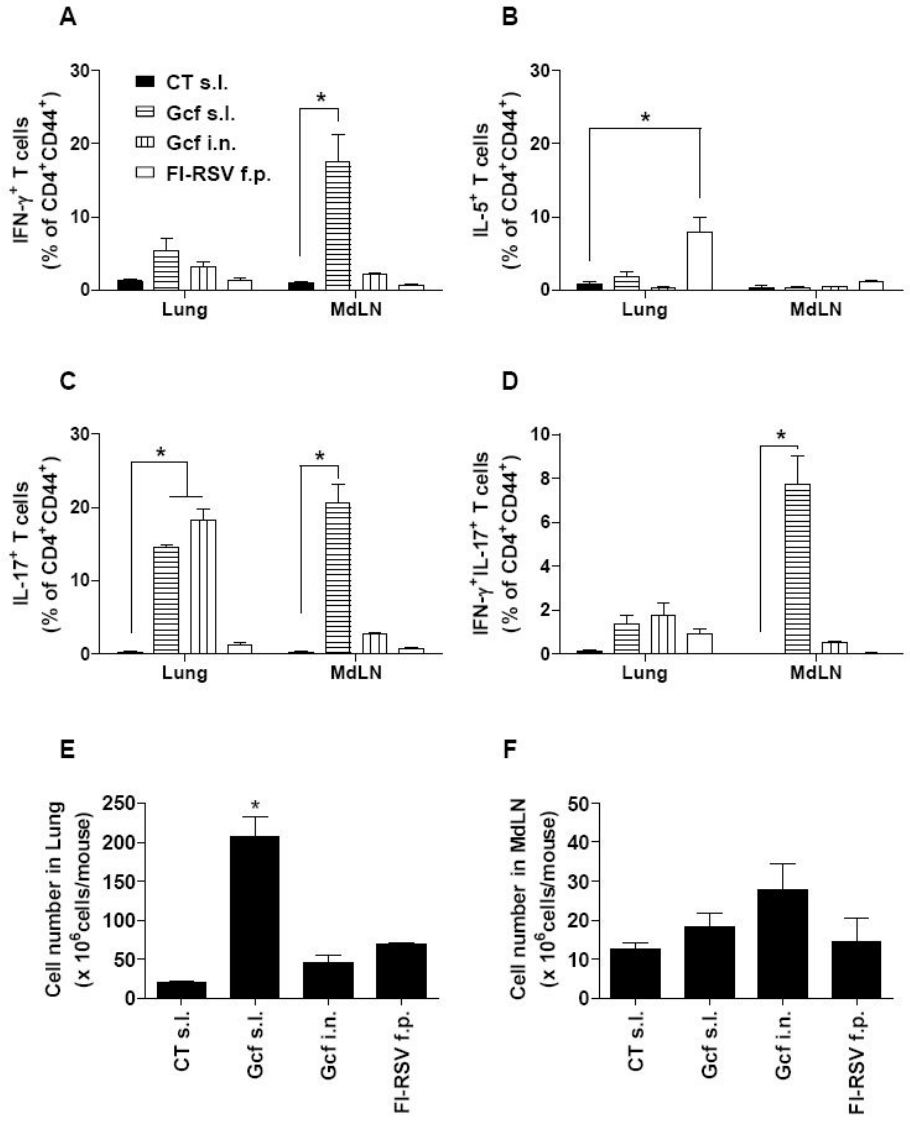


Figure 1-4. T cell responses in mice vaccinated through different routes. Mice were immunized and challenged as described above. Mononuclear cells from lung or MdLN were prepared at day 5 post-challenge and re-stimulated with G peptide (a.a. 183-195). (A) IFN-γ, (B) IL-5, (C) IL-17 or (D) both IFN-γ and IL-17 producing $CD4^+CD44^+$ T

cells were determined by intracellular staining assay using flow cytometry. Total cell numbers of (E) lung and (F) MdLN were counted. The results are expressed as means ± S.E.M. for the group (n = 3). The data are representative of three separate experiments. Significant differences at $P < 0.05$ from results compared with the CT are denoted as *.

These results suggest that major T cell response induced by FI-RSV is mainly Th2 T cell response, as it is already demonstrated in many studies, whilst vaccine-enhanced disease by Gcf via s.l. route following RSV infection seemed to be caused by Th1 and/or Th17 T cell response.

Next, cytokines were checked which are related with specific Th response in BAL fluid from mice immunized with Gcf via both routes, CT or FI-RSV upon RSV challenge. IFN-γ, representative Th1 cytokine, was released from day 1 and peaked at day 3 post challenge and then decreased from mice immunized with Gcf sublingually upon RSV challenge (Fig. 1-5A). But IFN-γ was not detected during all checking days in mice immunized with Gcf intranasally post RSV challenge. After 5 days post challenge, IFN- γ was produced in mice immunized with CT. Also, mice immunized with FI-RSV produced IFN-γ at day 5, but no significant. As expected, IL-4 was secreted only in mice immunized with FI-RSV (Fig. 1-5B). Interestingly, IL-17 was detected only in mice immunized with Gcf sublingually at day 3 post challenge, but there were no IL-17 production in other groups (Fig. 1-5C).

To confirm whether immunization with Gcf through s.l. route drive $CD4^+$ T cells to Th17 response, the cytokines associated with Th17 differentiation were checked. IL-6 was produced in all groups at first day after challenge. However, mice immunized with Gcf through s.l. route produced relatively higher amount of IL-6 than that via i.n. route. Additionally, IL-6 was secreted in only mice immunized sublingually with Gcf, but not other groups at day 3 post challenge (Fig. 1-6A). TGF-β, another important cytokine for Th17 differentiation, was also more induced in mice immunized with Gcf sublingually

following RSV challenge than that immunized with Gcf via i.n. route (Fig. 1-6B).

Taken together, Th1 or Th17 seem to mediate inflammatory response by Gcf immunization sublingually following RSV infection and Th17 response may have a major cause for the vaccine enhanced disease.

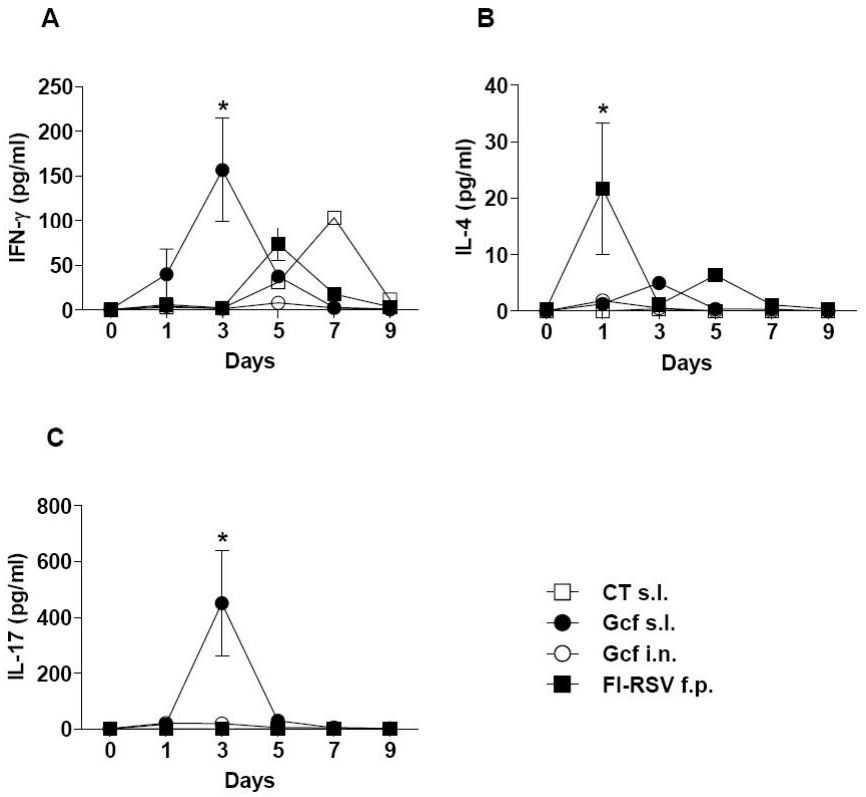


Figure 1-5. Cytokine production based on $CD4^+$ Th cell subsets in mice vaccinated with Gcf through different routes. Mice were immunized as described above. Three weeks after the last immunization, mice were challenge with 2 x 10^5 PFU live RSV A2. The BAL fluid was collected at indicated days after the challenge. Levels of (A) IFN-γ, (B) IL-4 and (C) IL-17 were determined using CBA mouse Th1/Th2/Th17 cytokine kit. The results are expressed as means ± S.E.M. for the group (n = 4). The data are representative of two separate experiments. Significant differences at $P < 0.05$ from results compared with the CT are denoted as *.

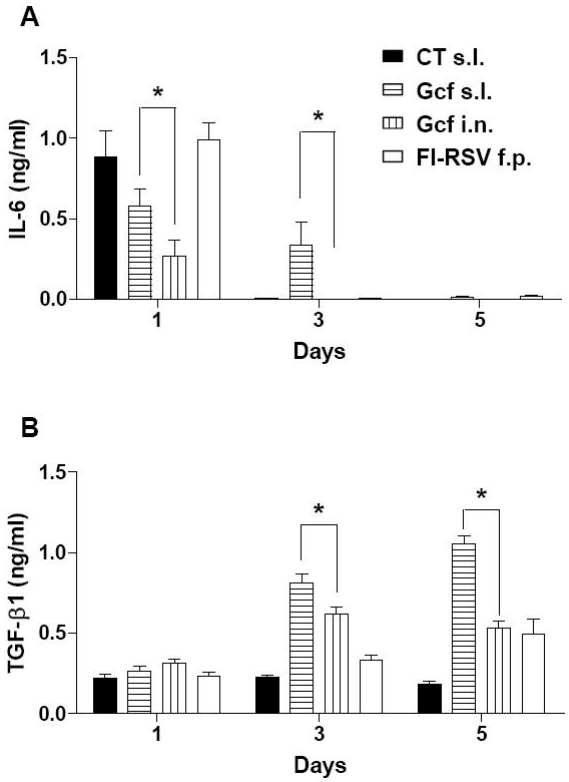


Figure 1-6. Changes of cytokines associated with Th17 differentiation. Mice were immunized as described above. After three weeks last immunization, mice were challenge with 2 x 10^5 PFU live RSV A2. The BAL fluid was collected at indicated days after the challenge. (A) IL-6 levels were determined using CBA mouse inflammatory cytokine kit. (B) TGF-β levels were determined by ELISA. The results are expressed as means ± S.E.M. for the group (n = 4). The data are representative of two separate experiments. * denotes that the values are significantly different at $P < 0.05$.

5) Infiltration of granulocytes.

Mice immunized with Gcf sublingually produced substantial amount of IL-17 (Fig. 1-5), and it is well known that IL-17 is related to neutrophil infiltration [22]. Thus, the recruitments of neutrophil into BAL and lung were checked in mice immunized with Gcf through both routes, CT or FI-RSV following RSV challenge. The absolute number of neutrophils in BAL was significantly higher in mice immunized with Gcf via s.l. route than any other groups from 3 days post challenge and sustained until day 7 (Fig. 1-7A). Gcf immunization in mice via i.n. route also induced the infiltration of neutrophils into BAL, but the absolute number of neutrophils was dramatically lower than that by s.l. route. In the lung, the number of neutrophils was increased in mice immunized with Gcf through s.l. route at days 3 and 5 post-challenge whilst no neutrophilia was observed in any other group. Fig. 1-7B shows that neutrophils in mice immunized with Gcf via s.l. route are still present at days 3 post challenge.

As shown in Fig. 1-1D, eosinophils were infiltrated into BAL in mice immunized with Gcf sublingually upon RSV challenge. Since it is reported that eosinophilia in mice immunized RSV G protein upon RSV infection depends on IL-5 and IL-13 [23-25], these cytokines were checked in BAL fluid. IL-5 was produced in mice immunized with Gcf sublingually at early time after the RSV challenge whereas the mice immunized with FI-RSV following RSV challenge secreted IL-5 at not only early but also late time points (Fig. 1-8A). Also, Gcf immunization via s.l. route dramatically induced IL-13 when compared to other groups (Fig. 1-8B). These results suggest that IL-5 seems to be associated with eosinophilia infiltration in both Gcf via s.l.

route and FI-RSV immunization. Furthermore, eosinophilia in mice immunized with Gcf via s.l. upon RSV challenge might be dependent more on IL-13 than that in mice immunized with FI-RSV.

Timing to secret IL-5 was different between Gcf immunization through s.l., route and FI-RSV immunization. Thus, the time kinetics of eosinophilia was examined in the airway in both groups. The number of eosinophils in mice immunized with Gcf sublingually increased at early time, 3 days post challenge, and peaked at day 5 while that in BAL from mice immunized with FI-RSV showed from day 5, later time, and peaked at day 7 (Fig. 1-8C). Interestingly, the number of eosinophils in lung increased in mice immunized with Gcf via s.l. route, but not in FI-RSV immunized mice (Fig. 1-8D). However, the percentage of eosinophils in lung of mice immunized with FI-RSV showed biphasic increased at days 1 and 7 post challenges which are similar pattern to IL-5 production (Fig. 1-9). Collectively, these data imply that Gcf sublingual and FI-RSV immunization induce vaccine enhanced disease by different mechanisms bringing about eosinophil infiltration at different time point.

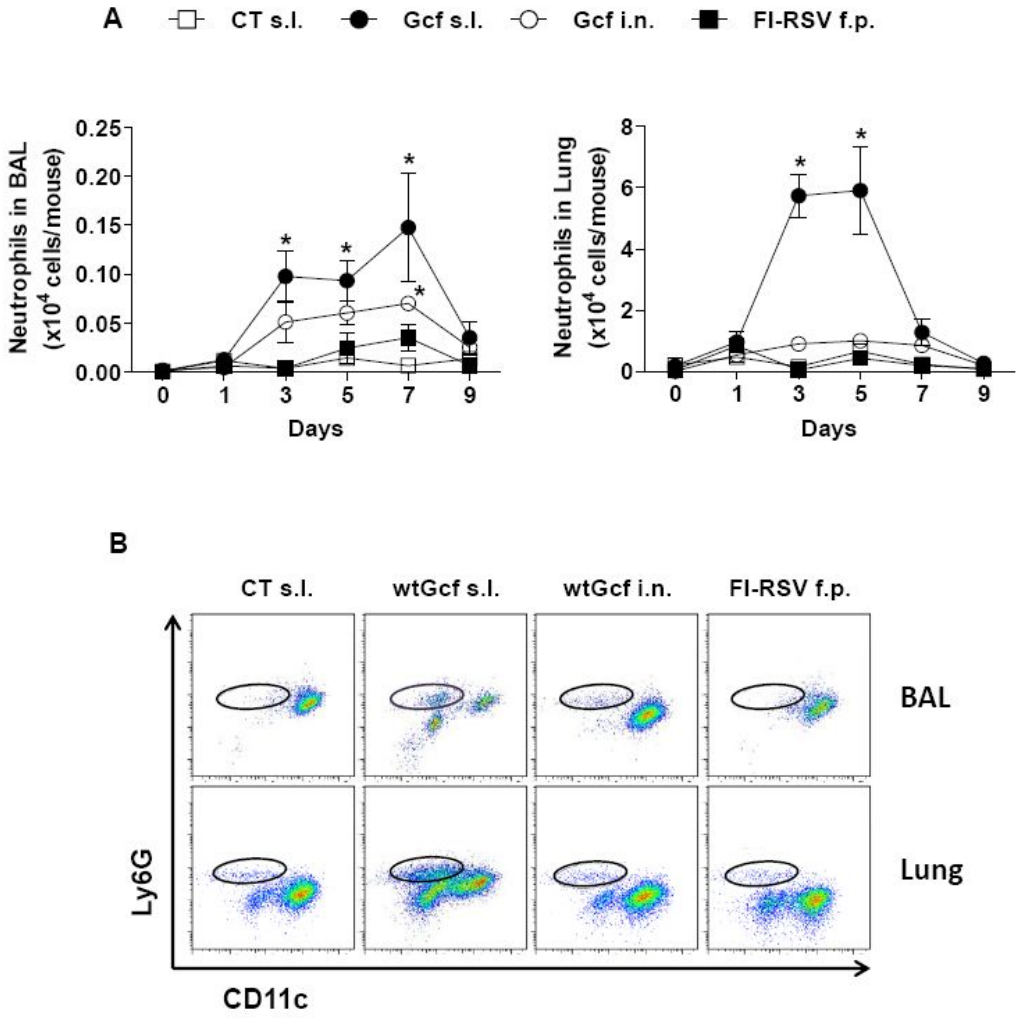


Figure 1-7. Infiltration of neutrophils in mice vaccinated with Gcf through different routes. Mice were immunized as described above. After three weeks last immunization, mice were challenge with 2 x 10^5 PFU live RSV A2. Infiltration of pulmonary neutrophils was monitored after the RSV challenge. (A) The numbers of neutrophils in BAL and lung were calculated based on (B) the percentage of

$CD45^{+}Ly6G^{+}CD11c^{-}$ by flow cytometry and total cell numbers of BAL or lung. The results are expressed as means ± S.E.M. for the group (n = 4). The data are representative of two separate experiments. Significant differences at $P < 0.05$ from results compared with the CT are denoted as *.

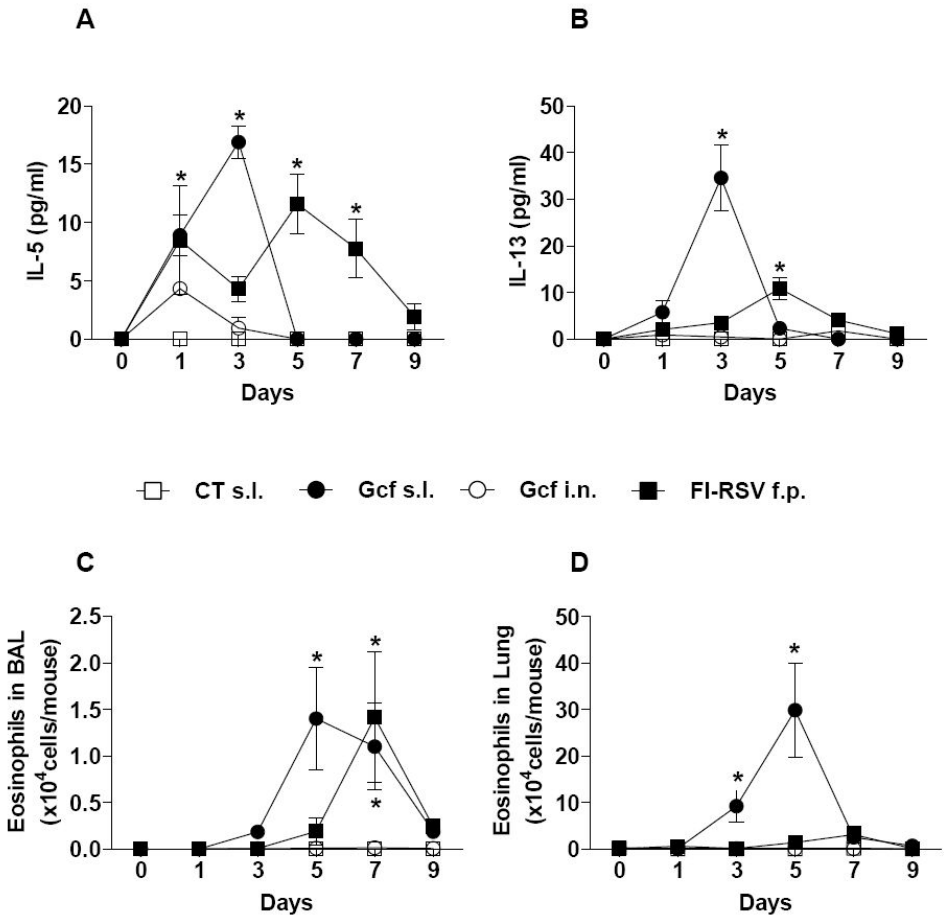


Figure 1-8. Cytokines related to the increase of eosinophils in mice vaccinated with Gcf through different routes. Mice were immunized as described above. After three weeks last immunization, mice were challenge with 2 x 10^5 PFU live RSV A2. (A) IL-5 and (B) IL-13 were determined by mouse Flex Set. The infiltration of pulmonary eosinophils was monitored after the RSV challenge. The numbers of eosinophils in (C) BAL and (D) lung were calculated based on the percentage of $CD45^+SiglecF^+CD11c^-$ cells by flow cytometry and total cell numbers of BAL or lung. The results are expressed

as means ± S.E.M. for the group (n = 4). The data are representative of two separate experiments. Significant differences at $P < 0.05$ from results compared with the CT are denoted as *.

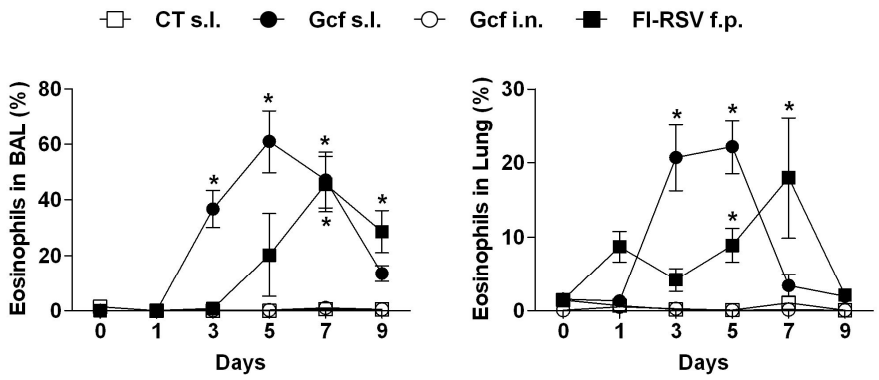


Figure 1-9. Infiltration of eosinophils in mice vaccinated with Gcf through different routes. Mice were immunized as described above. After three weeks last immunization, mice were challenge with 2 x 10^5 PFU live RSV A2. The infiltration of pulmonary eosinophils was monitored after the RSV challenge. The percentage of eosinophils in BAL and lung were examined by $CD45^+SiglecF^+CD11c^-$ cells using flow cytometry. The results are expressed as means ± S.E.M. for the group (n = 4). The data are representative of two separate experiments. * denotes that the values are significantly different at $P < 0.05$.

6) Effect of IL-17 on vaccine-enhanced immunopathology.

To confirm whether IL-17 is important for the induction of vaccine-enhanced immunopathology, wild type or IL-17 KO mice were immunized with Gcf through s.l. or i.n. route and challenged with live RSV A2. Body weigh was monitored daily after the challenge. IL-17 KO mice immunized with Gcf via s.l. route showed minimal body weight loss until day 2, and then completely recovered, which was similar to that of mice with i.n. (Fig. 1-10A). In addition, there was no eosinophilia in the airway of IL-17 KO mice immunized either sublingually or intranasally with Gcf following viral challenge (Fig. 1-10B). These results determine that IL-17 is crucial factor to induce vaccine-enhanced disease in sublingual immunization of Gcf following RSV infection.

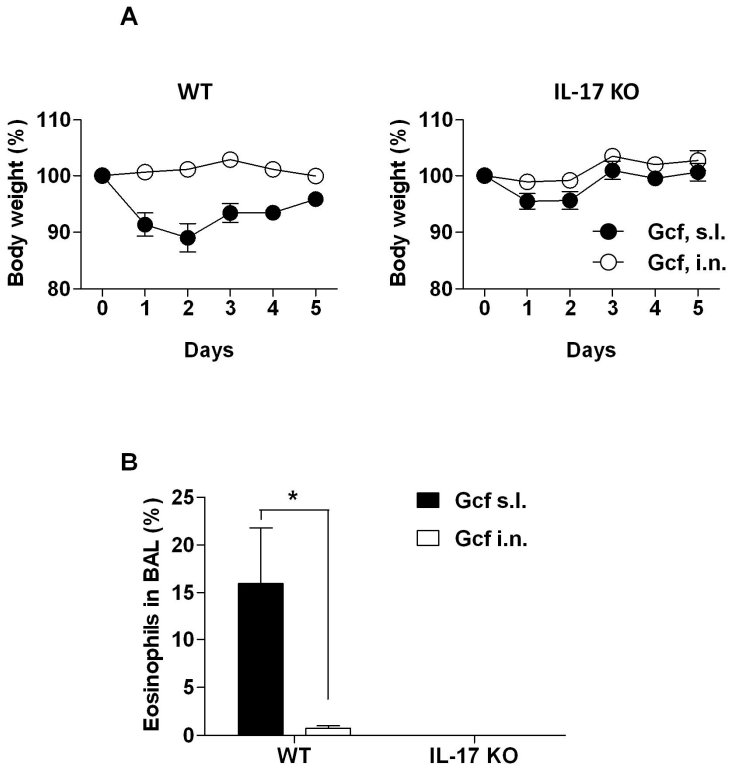


Figure 1-10. Effect of IL-17 on vaccine-enhanced disease following RSV challenge in mice immunized with Gcf. Wild type or IL-17 KO mice were immunized twice with 20 μg of Gcf via s.l. or i.n. route in the presence of 2 μg of CT. Three weeks after the last immunization, mice were challenge with 2 x 10^6 PFU live RSV A2. (A) Body weight loss was monitored daily after the challenge. (B) On day 5 post challenge, the percentage of eosinophils in BAL was tested for $CD45^+SiglecF^+CD11c^-$ by flow cytometry. The results are expressed as mean ± S.E.M for the group (n = 5). * denotes that the values are significantly different at $P < 0.05$.

4. Discussion

In the present study, I demonstrated followings; 1) both mucosal vaccination, s.l. and i.n., with purified Gcf together with CT induce antigen-specific antibody that provide protection against RSV infection. 2) s.l. immunization with Gcf in the presence of CT exacerbates disease following RSV infection, but not i.n. immunization. 3) s.l. immunization with Gcf in the presence of CT induces vaccine-enhanced disease through Th17 $CD4^+$ T cell response while 4) immunization with FI-RSV induces the disease through Th2 cell response.

Mucosal vaccination could induce immune responses systemically and, at the same time, at mucosal site [26, 27]. Since RSV infects through respiratory tract, intranasal or sublingual route might be a proper route for RSV vaccination. Indeed, it has been shown previously that Gcf immunization through mucosal route, i.n. or s.l., induced Ag-specific Abs [17]. Generally, it is known that sublingual route has low immunogenicity compared to intranasal route, but sublingual route has record for the risk for the redirection of vaccine antigen to brain, unlike intranasal route. Thus, there has been an attempt to augment immunogenicity via s.l. administration. Indeed, several studies reported s.l. vaccination against respiratory pathogen. Influenza vaccine was effective when immunized with adjuvant via s.l. route although the immunogenicity was lower than that by i.n. route [19, 28]. Furthermore, sublingual administration of other antigen induced stronger systemic and mucosal immune response than that by transdermal [29], subcutaneous [30] and oro-gastirc route [31, 32]. However, in the

results for RSV vaccine, immunization with Gcf through s.l. route showed severe diseased, unexpectedly, compared to that by i.n. route following RSV challenge. Not only FI-RSV but also Gcf s.l. immunization induced high levels of inflammatory cytokines at early time upon RSV challenge. In contrast, Gcf i.n. immunization produced similar or lower levels of inflammatory cytokines when compared to control mice. These results are shown that Gcf s.l. immunization resulted to enhance inflammatory responses against viral infection but not Gcf i.n. immunization.

Vaccine-enhanced disease by FI-RSV is mediated by pulmonary eosinophilia and Th2 response [20, 33]. In the results, FI-RSV immunization showed the induction of $CD4^+$ T cells producing IL-5 after the infection whereas Gcf s.l. immunization induced increase of $CD4^+$ T cells producing both IFN-γ and IL-17. Furthermore, IL-17 was produced only in mice immunized with Gcf through s.l. route. Interestingly, IL-4 was increased in mice immunized with FI-RSV but not with s.l. Gcf immunization suggesting that vaccine-enhanced disease by FI-RSV immunization following RSV infection is mediated by Th2 cell response while the responses of Gcf s.l. was mediated by Th1 and/or Th17 responses. It is known that IL-12 and IFN-γ are important for the differentiation to Th1 cells, and IL-6 together with TGF-β are prominent for the differentiation of Th17 cells [34, 35]. Gcf s.l. immunization did not induced IL-12p70 (data not shown), but induced high levels of IL-6 in BAL fluid, which maintained until 3 ~ 4 day after the RSV challenge indicating that Gcf s.l. immunization may stimulate antigen presenting cells such as dendritic cells to induce Th17 response.

IL-17 is mainly produced by Th17 cells and it has been known that IL-17 cells

are responsible for host defense against bacteria, fungi or protozoa [36]. However, it is also increased in autoimmune disease or inflammation [37] and related with asthma or airway inflammation in the lung [38, 39]. In the results of present study, mice immunized with Gcf via s.l. route produced significant levels of IL-17 in BAL fluid and lung (data not shown) following RSV challenge but not in mice immunized with FI-RSV. It has been reported that both IL-17 and IFN-γ are produced by $CD4^+$ effector T cells in autoimmune disease including multiple sclerosis and rheumatoid arthritis [21, 40] and $IL\text{-}17^+IFN\text{-}\gamma^+CD4^+$ T cells are responsible for pathogenicity in EAE [41]. Gcf immunization through s.l. route upon RSV infection increased $CD4^+$ T cells producing both IL-17 and IFN-γ, which was coincide with high levels of IL-17 and IFN-γ in BAL fluid and lung (data not shown) when compared to those in mice immunized with Gcf i.n. In addition, asthma is known to be associated with airway neutrophilia in an IL-17-dependent manner [42]. The results also showed that Gcf immunization via s.l. route following RSV challenge increased infiltration of neutrophils to BAL and lung which appeared to be mediated by IL-17 produced after the Gcf s.l. immunization.

Eosinophilia has been an important marker for vaccine-enhanced disease of RSV vaccine in BAL and lung. Eosinophilia in vaccine-enhanced disease by FI-RSV depends on IL-4 [23, 43] whilst IL-5 and IL-13 are involved in immunopathology after the G protein immunization [24, 44]. Herein, the results also demonstrated that Gcf s.l. immunization induced eosinophilia coincident with IL-5 and IL-13, but not IL-4, production. Furthermore, when IL-17 knockout mice were immunized with Gcf through s.l. route, minimal changes on the body weight and no eosinophil infiltration were found.

Taken together, this implies that Gcf s.l. immunization, unlike FI-RSV immunization, induce IL-17^{+}IFN-γ^{+} CD4^{+} T cells responsible for immunopathogenesis after RSV infection.

Collectively, these results describe that Gcf s.l. immunization induced vaccine-enhanced disease following RSV infection, which is mediated by Th17 response. Therefore, when RSV vaccine is administrated via sublingual route, it should be cautiously considered to attenuate immunopathology.

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Chapter 2

Development of safe and effective RSV vaccine by modified CD4 epitope in G protein Core Fragment (Gcf)

1. Introduction

Respiratory syncytial virus (RSV), consisting of A and B subtype, is a major causative agent of severe lower respiratory tract disease in infants, young children, and the elderly worldwide. Nevertheless, there is no RSV vaccine licensed for human use. Although considerable efforts have been invested for the development of safe and effective RSV vaccines, none has been successful owing to the difficulties in achieving the proper balance of safety and efficacy. Formalin-inactivated RSV (FI-RSV) was the first RSV vaccine candidate introduced in the 1960s. However, FI-RSV caused enhanced respiratory disease hallmarked by pulmonary eosinophilia and predominant Th2 type cytokine response following subsequent RSV infection in individuals who received this vaccine [146, 233].

The G glycoprotein of RSV, a major attachment protein, is a potentially important target for the induction of neutralizing antibodies and protective antiviral immune response [171, 234]. For example, BBG2Na, a subunit vaccine candidate, has shown to elicit immune response in small and large animals and been evaluated in human clinical trials [141, 235, 236]. Moreover, studies evaluating BBG2Na in combination with different adjuvants and by different routes of administration have further confirmed the role for the RSV G protein in protection against RSV infection [141, 235, 236]. Further, a study on the serum reactivity to various RSVG epitopes using sera harvested from RSV A- and B-infected human subjects reported a significant increase in cross-subtype IgG response against the central conserved region of the

RSVG [237], suggesting that the Abs specific to the central conserved region of the RSVG may be able to neutralize both A and B subtypes of RSV and provide heterosubtypic protection. As such, various RSVG-derived vaccine candidates including recombinant vaccinia virus expressing RSV G protein (vvG) have been evaluated. However, mice vaccinated with vvG developed enhanced lung disease accompanied by pulmonary eosinophilia following intranasal RSV infection [145, 153, 238]. Further studies have suggested the induction of Th2-biased $CD4^+$ T cell response concomitant with the secretion of excess Th2 cytokines as the cause of vvG immunization leading to the enhanced lung disease [145, 147, 183].

It is worth noting that symptoms of immunopathology caused by vvG immunization, such as eosinophilia and Th2-biased responses, interestingly, were similar to those caused by FI-RSV immunization [90, 147, 239]. Importantly, studies have further identified RSVG-specific subset of $CD4^+$ T cells expressing Vβ14 TCR as the culprit behind the pulmonary eosinophilia and exaggerated Th2 cytokine production [109, 114]. Furthermore, mice immunized with the KLH-conjugated peptide corresponding to RSV $G_{184\text{-}198}$, which is the $CD4^+$ T cell epitope within RSV G protein, induces severe pulmonary eosinophilia upon live RSV challenge strongly suggesting the involvement of amino acid (a.a.) residues 184-198 of RSVG to the RSVG-mediated immunopathology [186].

It has been shown in the previous study that RSV G protein fragment of residues 131 to 230 (Gcf) elicits Ag-specific immune response *in vivo* after mucosal administration [167]. Gcf immunization with CT, as a mucosal adjuvant, induced higher

level of antibody than those in mice immunized with Gcf alone. However, Gcf vaccine elicited significant lung eosinophilia when it was co-immunized with CT meaning that it still has the potential to cause immunopathological symptoms when it is combined with an adjuvant such as CT [167].

Most studies of RSV G protein were performed with RSV A subtype, and there has been only a limited amount of studies on RSV B subtype and its G protein, despite both A and B subtype are co-circulated. Therefore, in the present study, various modified recombinant RSV Gcfs were engineered to circumvent the potential RSVG mediated immunopathology against RSV A and B subtype. These modified Gcfs were evaluated for vaccine-induced immune response and vaccine-enhanced disease such as eosinophilia in the airway and body weight loss. These various Gcf-derivatives demonstrated a promising potential as novel RSV vaccine candidates.

2. Materials and Methods

1) Ethics statement

All animal studies were approved by Institutional Animal Care and Use Committee (IACUC) at International Vaccine Institute (Approval No. 2011-008).

2) Virus preparation

RSV A2 strain and B strain (CH18537 or HRSV-B isolate, KR/B/10-12) were amplified as previously described [167, 240]. Briefly, virus was propagated in HEp-2 cells (ATCC, Manassas, VA) grown in MEM (Life Technologies, Grand Island, NY, USA) and harvested at day 3 or 4 post-infection when extensive syncytia were observed in infected cells. Virus titer was determined by standard RSV plaque assay.

3) Construction of plasmids expressing various Gcf proteins

Illustrative schemes of plasmid expressing each Gcf protein are depicted in Fig. 2-1. The plasmid containing wtAGcf gene was prepared as previously described [167]. The mGcf gene, which was designed to produce mutations at a.a. 185 and 188 inside the $CD4^+$ T cell epitope region within wtAGcf, was generated with the forward primer (5'-TGG GCT GCC TGC AAA GCA ATA CCA AAC AAA AAA CCA GGA-3') and the reverse primer (5'-GGG CCC AAG CTT GGG CTT GGT GGT GGG TAC TTC-3') by site-directed mutagenesis using wtAGcf gene as the template. The Th-mGcf gene possessing RSV F protein $CD4^+$ T cell epitope at N terminus of mGcf was generated with the primers 5'-CCC GAA TTC CC GGT TGG TAT ACC AGT GTT ATA ACT ATA

GAA TTA AGT AAT ATT AAG GAA GTC AAG ACC AAA AAC ACA ACA-3' and 5'-GGG CCC AAG CTT GGG CTT GGT GGT GGG TAC TTC-3' by polymerase chain reaction (PCR) using plasmid pET21d-mGcf as the template. The wtBGcf (a.a. 131 to 230), AGcf/BCD4 and BGcf/ACD4 genes were synthesized by Bioneer (Korea). The $CD4^+$ T cell epitope inside wtAGcf or wtBGcf (a.a. 183 to 195) was replaced with the corresponding region within wtBGcf (derived from RSV CH18537) or wtAGcf (derived from RSV A2), respectively, to create AGcf/BCD4 or BGcf/ACD4. The DNA sequences were confirmed by Macrogen (Seoul, Korea).

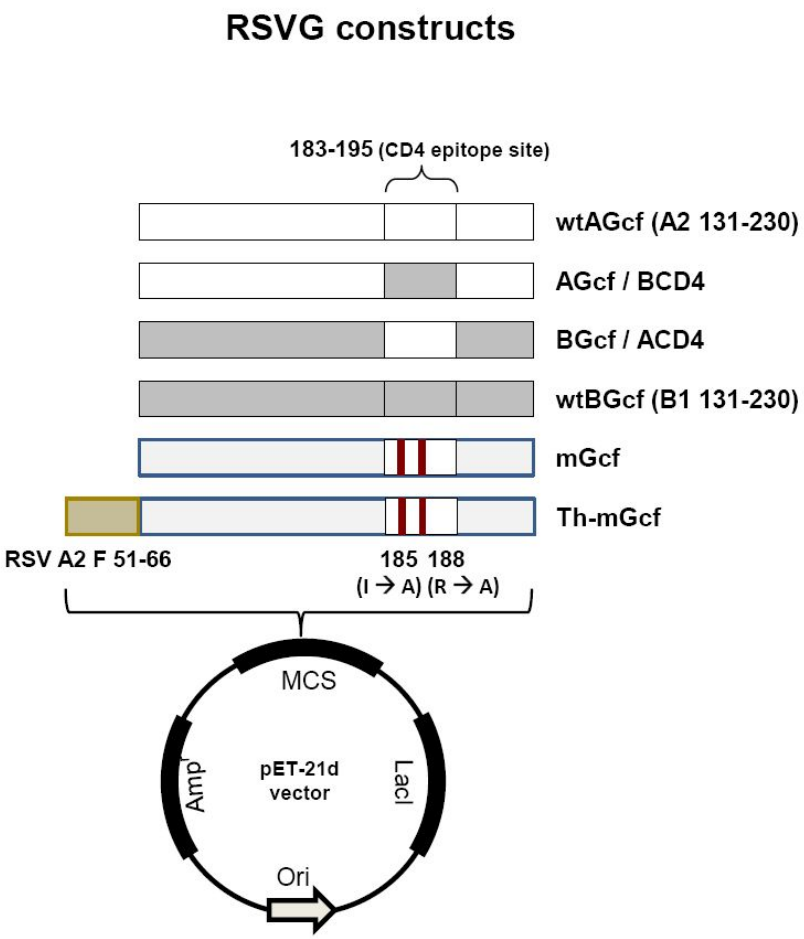

Figure 2-1. Construction of plasmids expressing various Gcf proteins. wtAGcf, AGcf/BCD4, BGcf/ACD4, wtBGcf, mGcf or Th-mGcf genes were cloned into pET21d vector to express recombinant Gcf proteins in *E. coli*.

4) Expression and purification of various Gcf proteins

E. coli BL21 (DE3) strain (Novagen) transformed with each plasmid was grown overnight at 37°C in Luria-Bertani (LB) medium supplemented with 100 μg/ml of ampicillin. The overnight culture was transferred into fresh LB medium and cultured at 37°C while shaking at 180 rpm until OD_{600} of 0.6~0.8. Each protein expression was induced by adding IPTG of 0.5 M for 4 hrs and the cells were harvested by centrifugation at 6,000 rpm for 10 min. The cell pellets were suspended in binding buffer (20 mM Tris, 0.5 M Nacl, pH 7.9) and disrupted by sonication on ice. After sonication, the soluble and insoluble fractions were separated by centrifugation for 40 min at 20,000 rpm. For the Th-mGcf protein, the insoluble fraction was dissolved in binding buffer containing 6 M urea. After centrifugation for 30 min at 18,000 rpm, the supernatant was applied to a Talon metal affinity column (Clontech, Palo Alto, CA). For the wtAGcf, wtBGcf, AGcf/BCD4, BGcf/ACD4 and mGcf, the soluble fractions were applied to a Talon metal affinity column. The columns were washed with binding buffer containing 20 mM imidazole, and then the proteins were eluted using an elution buffer (300 mM imidazole, 20 mM Tris, 0.5 M NaCl, pH 7.4). The purified proteins were dialyzed against 1 x PBS. The endotxoin in each purified protein was removed by using Triton X-114 as previously described [213]. The endotoxin level of each protein was measured by the limulus amebocyte lysate (LAL) assay kit according to the instructions (Lonza, Switzerland). To note, endotoxin levels of the proteins were less than 5 EU/mg. The purified proteins were electrophoresed on 15% SDS-PAGE and the bands were visualized by staining with Coomassie Brilliant Blue. The protein concentration was

determined by Bradford protein assay kit (Biorad, CA, USA). The purified proteins were stored at -80°C until use.

5) Mice and immunization

Specific pathogen free, female BALB/c mice aged 6 weeks were purchased from Orient Bio Inc. (Korea) and all mice were maintained under specific pathogen-free conditions. Mice were immunized with 20 μg of each purified Gcf proteins with 2 μg of CT (List Biological Lab. Inc. Campbell, CA) via the sublingual (s.l.) route on day 0 and day 14. As control groups, mice were immunized with CT sublingually, $1x10^5$ PFU of FI-RSV via foot-pad, or $1x10^5$ PFU of live RSV through intranasal (i.n.) route. For s.l. immunization, the anesthetized mice were immunized with 15 μl of prepared vaccines underneath the tongue using a pipette. Following s.l. immunization, mice were maintained with heads placed in ante flexion for 30 min. For i.n. immunization, total 20 μl of prepared vaccines were administered into each nostril of the anesthetized mice. Three weeks after the last immunization, the mice were challenged i.n. with 2×10^6 PFU of live RSV A2 or 2×10^6 or 4×10^6 PFU of live CH18537 or KR/B/10-12 for B subtype RSV.

6) T cell response

Mice were immunized twice on days 0 and 14, and challenged with RSV A2 as described above. On day 4 post-challenge, the lungs were harvested and strained through 70 μm cell strainer (BD Biosciences, San Diego, CA, USA) using serum-free

RPMI. To examine the cytokine-producing cells, lung mononuclear cells were stimulated with 10 μg/ml a.a. 183-195 G peptide (WAICKRIPNKKPG from wtAGcf, KSICKTIPSNKPK from wtBGcf) or 1 μg/ml of anti-CD3 and anti-CD28 antibodies (BD Biosciences) and incubated for 5 hr in the presence of Golgi Plug™ (BD Biosciences). The cells were then stained with anti-mouse CD4 (BD Biosciences) for surface markers. The cells were fixed, permeabilized using Cytofix/Cytoperm solution (BD Biosciences), and stained with anti-mouse IFN-γ. Stained cells were acquired using flow cytometry (FACS LSR II; BD Biosciences) and analyzed by FlowJo (Tree Star, San Carlos, CA, USA). For ELISPOT, the plates (Millipore, Billerica, MA, USA) were coated with 100 μl of coating anti-IFN-γ (e-Bioscience, San Diego, CA, USA) at a concentration of 10 μg/ml overnight at 4°C and blocked with RPMI-1640 (Life Technologies, Grand Island, NY, USA) containing 10% FBS (Lonza, Switzerland) for 30 min at 37°C. Then 2 x 10^5 or 5 x 10^4 lung cells were transferred into each well and stimulated with 10 μg/ml of G (183-195) peptide (WAICKRIPNKKPG) or F (51-66) peptide (MGWYTSVITIELSNIK) for 24 hr at 37°C. Following intensive washing with PBS, biotinylated anti-IFN-γ antibodies (e-Bioscience) were added into each well, and incubated overnight at 4°C. Next day, the plates were incubated with streptavidin-HRP (BD Biosciences) for 1hr at room temperature. Spots were developed by adding AEC-H_2O_2 chromogenic substrate (Sigma-Aldrich) and counted.

7) Measurement of eosinophils in the BAL

BAL samples were collected as described previously [213] on day 4 post-

challenge. Briefly, mice were sacrificed and BAL samples were collected using PBS via tracheotomy. Cells were separated from the BAL fluid by centrifugation and stained with CD11c-FITC, CD45-APC and Siglec-F-PE (BD Biosciences). BAL eosinophil levels were evaluated using a FACS Calibur (BD Biosciences) and flow cytometric data were analyzed by using FlowJo software.

8) ELISA

Levels of Abs in the immune sera were measured by enzyme-linked immunosorbent assay (ELISA). In brief, 96-well plates (Nunc, Roskilde, Denmark) were pre-coated with 100 μl/well of 2 x 10^3 PFU of purified RSV A2 virus in PBS overnight at 4°C. After blocking with PBS containing 5% skim milk for 1 hr at room temperature, each serum sample was serially diluted in blocking buffer to each well and incubated for 1 hr at 37°C, followed by addition of 1:3,000 diluted HRP-conjugated goat anti-mouse IgG (Southern Biotechnology, Birmingham, AL, USA). After incubation for 1 hr at room temperature, tetramethylbenzidine (TMB, Millipore) was added to develop the color and then the reaction was stopped by adding 2M H_2SO_4. The absorbance at wavelength 450 nm was measured by an ELISA reader (Molecular Devices, Sunnyvale, CA, USA). The endpoint titer was determined by O.D. cut-off values of 0.2.

9) Virus titration in mouse lung

To determine the viral titers after challenge, lungs from RSV-infected mice were isolated on day 4 post-RSV challenge. The lungs were then strained through 70 μm cell

strainer (BD Biosciences) using serum-free MEM. Supernatant was collected and RSV titers were determined by plaque assay using HEp-2 cells. The virus titer in the lung data are expressed as PFU per lung.

10) Statistical analysis

Statistical tests were performed by using Student's *t* test. The difference was considered statistically significant when the *P* value was less than 0.05.

3. Results

1) Effect of CD4+ T cell epitope in wtAGcf on the immune response and pathogenesis following RSV A subtype infection

Neutralizing Ab is a major correlate of protection against RSV infection, and RSV G protein is known to induce neutralizing Ab responses in numerous studies [171]. Interestingly, however, previous study also demonstrated that adoptive transfer of RSVG-specific Type 2 $CD4^+$ helper T cells from RSVG-sensitized mice causes severe pulmonary disease in recipient mice following RSV challenge [147]. Therefore, it was examined whether immunization of mice with recombinantly modified Gcf, deficient in $CD4^+$ T cell epitope, could offer protection against RSV infection while eliminating RSVG-mediated lung immunopathology. Mice were immunized with the recombinant wild type Gcf derived from RSV A2 (wtAGcf), recombinant RSV A2 Gcf with its $CD4^+$ T cell epitope replaced with the corresponding a.a. sequence from RSV B Gcf (AGcf/BCD4), recombinant RSV B Gcf with its $CD4^+$ T cell epitope replaced with the corresponding a.a. sequence from RSV A2 Gcf (BGcf/ACD4), recombinant wild type Gcf derived from RSV B1 (wtBGcf), FI-RSV, or CT adjuvant alone. Subsequently, the levels of RSV A2-specific serum IgGs were measured 13 days after the second immunization. These results showed that wtAGcf, BGcf/ACD4, and FI-RSV immunization induced significant levels of RSV-specific serum IgGs compared to CT immunization (Fig. 2-2A). However, AGcf/BCD4 or wtBGcf immunization failed to induce significant levels of RSV A2-specific serum IgGs.

Next, virus titers were examined in the lung, eosinophil infiltration in BAL, and body weight changes in immunized mice following RSV A2 challenge. At day 4 post-challenge, there was detected no RSV in the lungs of mice immunized with wtAGcf or BGcf/ACD4. RSV titer in the lung was significantly decreased in mice immunized with wtBGcf or FI-RSV compared to control group immunized with CT alone. However, although a moderate reduction of viral titers in the lung was observed in mice immunized with AGcf/BCD4, this reduction was not significant (Fig. 2-2B). Interestingly, eosinophils in BAL collected from mice immunized with wtAGcf, BGcf/ACD4, or FI-RSV were significantly increased compared to mice immunized with CT alone. However, AGcf/BCD4 or wtBGcf immunization did not cause such increase (Fig. 2-2C). Moreover, mice immunized with wtAGcf or BGcf/ACD4 experienced more severe weight loss and slower weight recovery following RSV challenge compared to other immunization groups (Fig. 2-2D).

These results indicate that $CD4^+$ T cell epitope within RSV AGcf is essential for the induction RSV-specific Ab responses and viral clearance. However, these results also suggest that the same $CD4^+$ T cell epitope is functionally linked to the enhancement of RSV disease as shown by significant increase in the eosinophilic recruitment to the airway mucosa and severe body weight loss only when the mice were immunized with Gcf containing this $CD4^+$ T cell epitope. Taken together, these data suggest that $CD4^+$ T cell epitope within RSV A2 Gcf possesses a dual function in mediating the development of protective immunity and vaccine-enhanced disease.

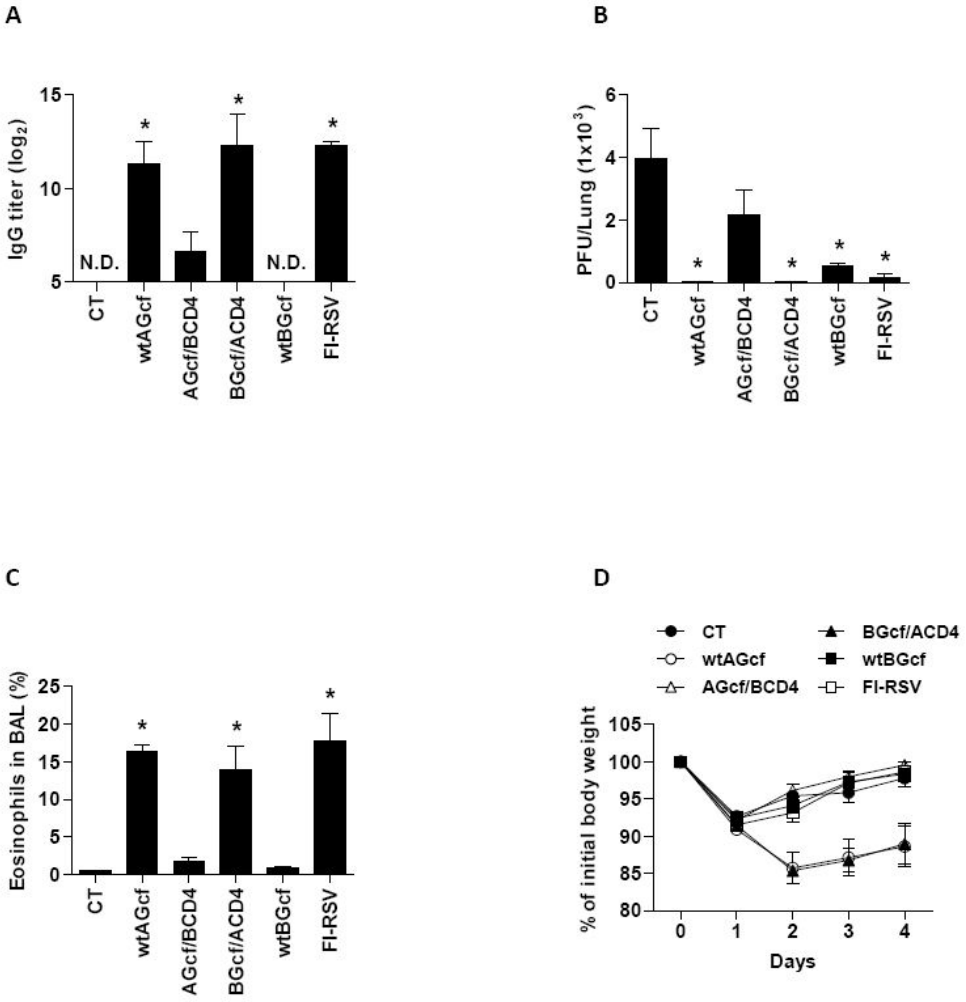

Figure 2-2. Effect of CD4$^+$ T cell epitope on immune response and pathogenesis induced by wtAGcf. Mice were immunized twice with 20 μg of wtAGcf, AGcf/BCD4, BGcf/ACD4, or wtBGcf in the presence of 2 μg of CT or CT alone sublingually or with 1 x 10^5 PFU of FI-RSV through foot-pad route. (A) RSV A2 specific serum IgGs were measured by ELISA 13 days after the second immunization. At day 4 post-challenge with 2 x 10^6 PFU of RSV A2, (B) the viral replication in the lungs was determined by

plaque assay and (C) the pulmonary eosinophils were measured and by flow cytometry. (D) Body weight loss was monitored daily after the viral challenge. The results are expressed as mean ± S.E.M. for the group (n = 5). Significant differences at $P < 0.05$ from results compared with the CT are denoted as *.

2) Immune response and eosinophilia by wtBGcf after RSV B subtype infection

To examine whether RSV B Gcf shares the same region a.a. as a $CD4^+$ T cell epitope, mice were immunized with wtAGcf, AGcf/BCD4, BGcf/ACD4, wtBGcf, FI-RSV derived from B subtype (FI-RSVB), or CT alone using the same immunization regiment used above and the levels of RSV B-specific serum IgG were measured 13 days after the second immunization. The results indicate that mice immunized with BGcf/ACD4, wtBGcf, or FI-RSVB produced significantly higher levels of RSV B-specific serum IgG than those of mice immunized with CT alone, albeit the levels of RSV B-specific serum IgG generated by BGcf/ACD4 immunization was considerably low compared to those by wtBGcf or FI-RSVB immunization (Fig. 3A). It was noting that no or very low RSV B-specific serum IgG were detected in mice immunized with wtAGcf, AGcf/BCD4, or CT alone (Fig 2-3A).

Next, to examine whether immunization with wtBGcf could induce pulmonary eosinophilia following subsequent infection with RSV B subtype, mice were immunized twice with wtBGcf or FI-RSVB and challenged with RSV B subtype (CH18537) 14 days after the second immunization. At day 4 post-challenge, in the percentage of eosinophils in BAL harvested from mice immunized with FI-RSVB as expected was considerable increase, but not from wtBGcf-immunized mice (Fig. 2-3B), indicating that immunization with wtBGcf, unlike wtAGcf, does not cause pulmonary eosinophilia upon subsequent infection with the homologous RSV subtype.

Taken together, these results demonstrate that immunization with RSV B Gcf

induces RSV B–specific Ab responses without promoting excessive eosinophilic recruitment into the airway upon RSV B subtype challenge. Furthermore, a.a. 183-195 within RSV B Gcf is neither essential in the induction of RSV B-specific Ab responses and nor function as a $CD4^+$ T cell epitope.

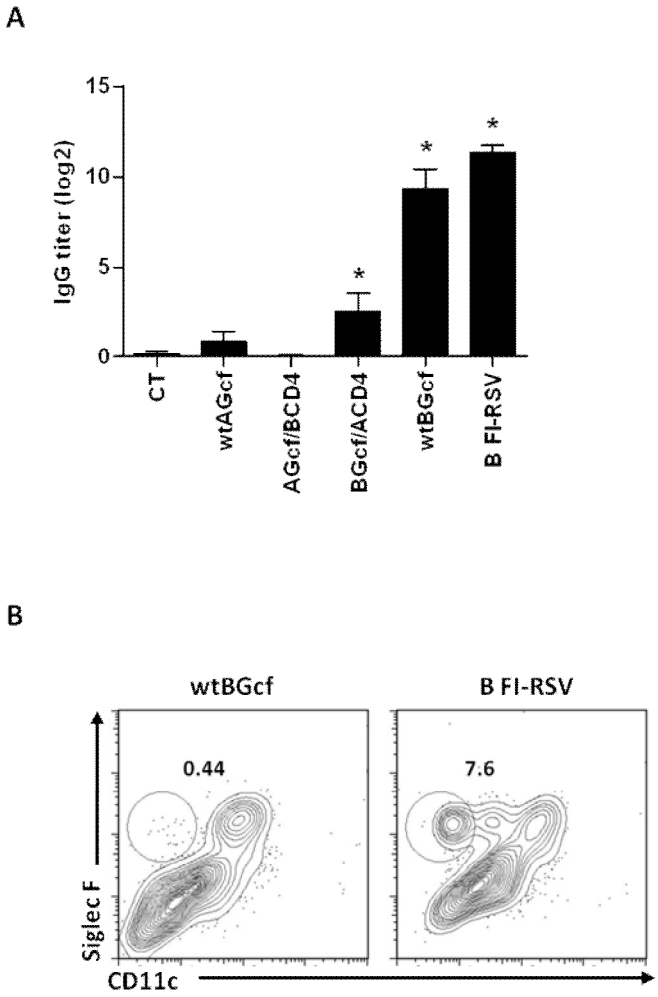

Figure 2-3. Antibody production and eosinophilia in mice immunized with wtBGcf. Mice were immunized twice with 20 μg of wtAGcf, AGcf/BCD4, BGcf/ACD4, or wtBGcf in the presence of 2 μg of CT or CT alone sublingually or with $1x10^5$ PFU of B type FI-RSV through foot-pad route. (A) RSV B type specific serum IgGs were measured by ELISA 13 days after the second immunization. * denotes significant differences at $P < 0.05$ from results compared with the CT. (B) For the pulmonary

eosinophilia, mice were immunized twice with 20 μg of wtBGcf in the presence of 2 μg of CT sublingually or with 1 x 10^5 PFU of B type FI-RSV through foot-pad route. At day 4 post challenge with 2 x 10^6 PFU of CH18537, the pulmonary eosinophils were detected by flow cytometry. The results are expressed as mean ± S.E.M. for the group (n = 3).

3) Amino acid residues 183-195 within RSV BGcf lack functionality as $CD4^+$ T cell epitope

I further validated the possibility that a.a. residues 183-195 within RSV BGcf contain $CD4^+$ T cell epitope functionality. To this end, mice were immunized with either wtAGcf or wtBGcf in a prime-boost regimen and challenged them with RSV A or B subtype, respectively. At 4 day post-challenge, lung mononuclear cells were isolated and stimulated with $wtBG_{183\text{-}195}$, $wtAG_{183\text{-}195}$, or a mixture of anti-CD3 and -CD28 antibodies as positive control, and RSVG-specific IFN-γ response in the $CD4^+$ T cells was evaluated. The results showed a robust induction of RSVG-specific IFN-γ response in $CD4^+$ T cells in the lung mononuclear cells harvested from mice immunized with wtAGcf, but not from mice immunized with wtBGcf (Fig. 2-4). Overall, these results confirm my hypothesis that a.a. residues 183-195 within RSV BGcf do not function as a $CD4^+$ T cell epitope.

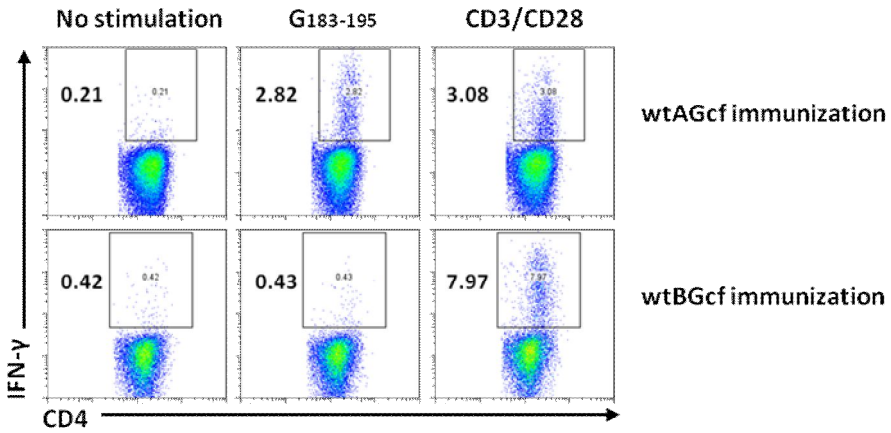

Figure 2-4. CD4 epitope in wtGcf. Mice were immunized twice with 20 μg of wtAGcf, or wtBGcf in the presence of 2 μg of CT sublingually in a prime-boost regimen. At day 4 post challenge with 2 x 10^6 PFU of RSV A or B subtype, respectively, lung mononuclear cells were prepared from the lung tissue and re-stimulated with G peptides (a.a. 183-195) from wtAGcf or wtBGcf or 1 μg/ml of anti-CD3 and anti-CD28 antibodies as positive control. T cell responses were measured IFN-γ secreting $CD4^+$ T cells by intracellular staining.

4) T cell response by Gcfs

A previous study by Varga *et al.* has shown that alanine-substitution at a.a. position 185 or 188 within RSVG can inhibit $CD4^+$ T cell activation by RSVG [187]. Therefore, a recombinantly modified Gcf (mGcf) were generated in which two amino acids corresponding to a.a. positions 185 and 188 in Gcf were substituted with alanine by point-mutation. Furthermore, since eliminating the $CD4^+$ T cell epitope would negate the induction of RSVG-specific immunity, another modified Gcf (Th-mGcf) was generated by fusing a $CD4^+$ T cell epitope from RSV F protein (F_{51-66}) to the N-terminus of mGcf to maintain $CD4^+$ T cell-mediated RSVG-specific immune responses.

In order to examine the ability of modified Gcf to stimulate $CD4^+$ T cells, mice were immunized sublingually in a prime-boost regimen with wtAGcf, mGcf, Th-mGcf, or CT and challenged with RSV A2. At day 4 post-challenge, lung mononuclear cells were isolated, stimulated with $G_{183-195}$-(Fig. 2-5A) or F_{51-66}-(Fig. 2-5B) and IFN-γ^+ response was evaluated by ELISPOT assay. As expected, the numbers of IFN-γ-secreting cells were significantly ($P < 0.05$) increased with $G_{183-195}$ stimulation in cells from mice immunized with wtAGcf and with F_{51-66} stimulation in cells from mice immunized with Th-mGcf, respectively. The ability of modified Gcf to stimulate CD4+ T cells was further confirmed by intracellular cytokine staining using flow cytometry (data not shown). Collectively, the results of present study demonstrate that recombinantly fusing $CD4^+$ T cell epitope from RSV F protein to mGcf, which lost its original G protein $CD4^+$ T cell-stimulating functionality due to mutations within its T cell epitope, effectively restores mGcf's ability to stimulated $CD4^+$ T cells.

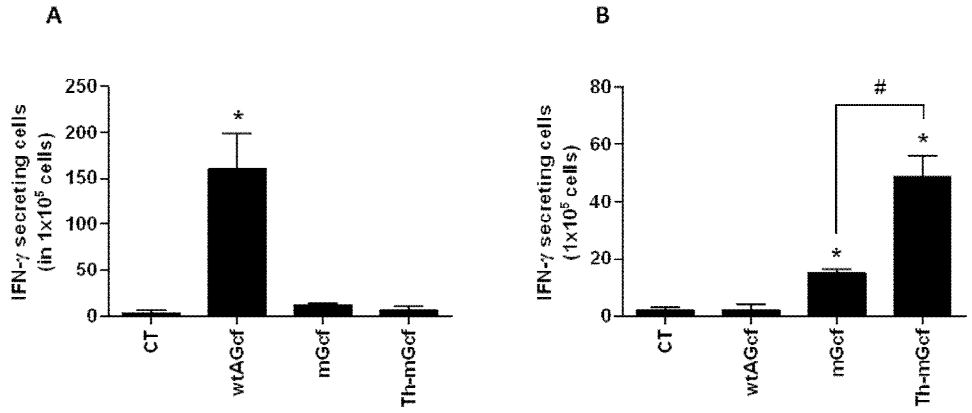

Figure 2-5. T cell response by modified Gcf. Mice was immunized twice with Gcfs sublingually or FI-RSV through foot-pad route and then challenged with RSV A2 three weeks after the second immunization. At day 4 post-challenge, lung mononuclear cells were prepared from the lung tissue and re-stimulated with (A) G (a.a. 183-195) or (B) F (a.a. 51-66) peptide. T cell responses were detected as IFN-γ secreting cells by ELISPOT. The results are expressed as means ± S.E.M. for the group (n = 3). Significant differences from results with the CT are marked with asterisks (*), while significant differences between mGcf and Th-mGcf are marked with sharp (#), respectively *(* or #, $P < 0.05$)*.

5) Immunogenicity and protective efficacy of recombinant Gcfs against homologous RSV A type infection

Next, various aspects of RSV-specific immune responses after wtAGcf, mGcf, or Th-mGcf immunization were examined and compared with live RSV and FI-RSV immunization followed by RSV challenge. Mice were immunized with the respective antigen in a prime-boost regimen and, at 13 days after the second immunization, RSV A2-specific serum IgG levels were determined by ELISA. The results demonstrate that mice immunized with wtAGcf, Th-mGcf, FI-RSV, and live RSV produced significantly ($P < 0.05$) higher RSV-specific serum IgGs compared to CT-immunized negative control group (Fig. 2-6A). Importantly, the level of RSV A2-specific serum IgGs in Th-mGcf immunized mice was greater than that in mGcf-immunized group. However, RSV-specific serum IgGs induced by Th-mGcf immunization were lower than those induced by wtAGcf, FI-RSV, or live-RSV immunization (Fig. 2-6A)

In order to examine the efficacy of Th-mGcf immunization in facilitating viral clearance, immunized mice were challenged intranasally with RSV A2. At day 4 post-challenge, lungs were harvested from RSV-challenged mice, and viral plaque assays were performed with the lung homogenates to detect RSV titers. The results indicate that wtAGcf, Th-mGcf, and FI-RSV immunization significantly reduced lung viral titers compared to CT immunization, whereas mGcf immunization did not lead to a significant reduction of viral titers (Fig. 2-6B). Notably, Th-mGcf immunization showed significantly enhanced lung viral clearance compared to mGcf immunization.

Furthermore, eosinophil recruitment to BAL was examined following RSV A2

challenge in Th-mGcf immunized mice as previous studies have shown that mice primed with RSVG experience exacerbated disease and pulmonary eosinophilia when subsequently challenged with RSV [241]. As expected, RSV challenge in FI-RSV immunized mice caused substantial recruitment of eosinophils, composing ~25% of the BAL cells (Fig. 2-6C). Mice that received wtAGcf immunization, although in lesser degree than FI-RSV immunization, also showed significant eosinophil recruitment following RSV challenge, composing ~10% of the BAL cells. It is important to note that Th-mGcf immunization, along with mGcf or live RSV immunization, did not induce significant eosinophil recruitment. In addition, weight loss in immunized mice was measured following RSV challenge in order to assess the protection from morbidity conferred by Th-mGcf immunization. The results show that, while mice immunized with wtAGcf experienced ~15% reduction in their body weight by day 2 post-challenge, weight loss in mice immunized with CT, mGcf, Th-mGcf, FI-RSV, or live RSV was less severe and began to recover their body weight at day 1 post-challenge (Fig. 2-6D).

Taken together, these results indicate that fusing F_{51-66} to mGcf restores the ability of mGcf to induce RSV-specific Ab response and viral clearance. Furthermore, Th-mGcf did not induce excessive pulmonary eosinophil recruitment and body weight loss upon Gcf immunization followed by RSV challenge.

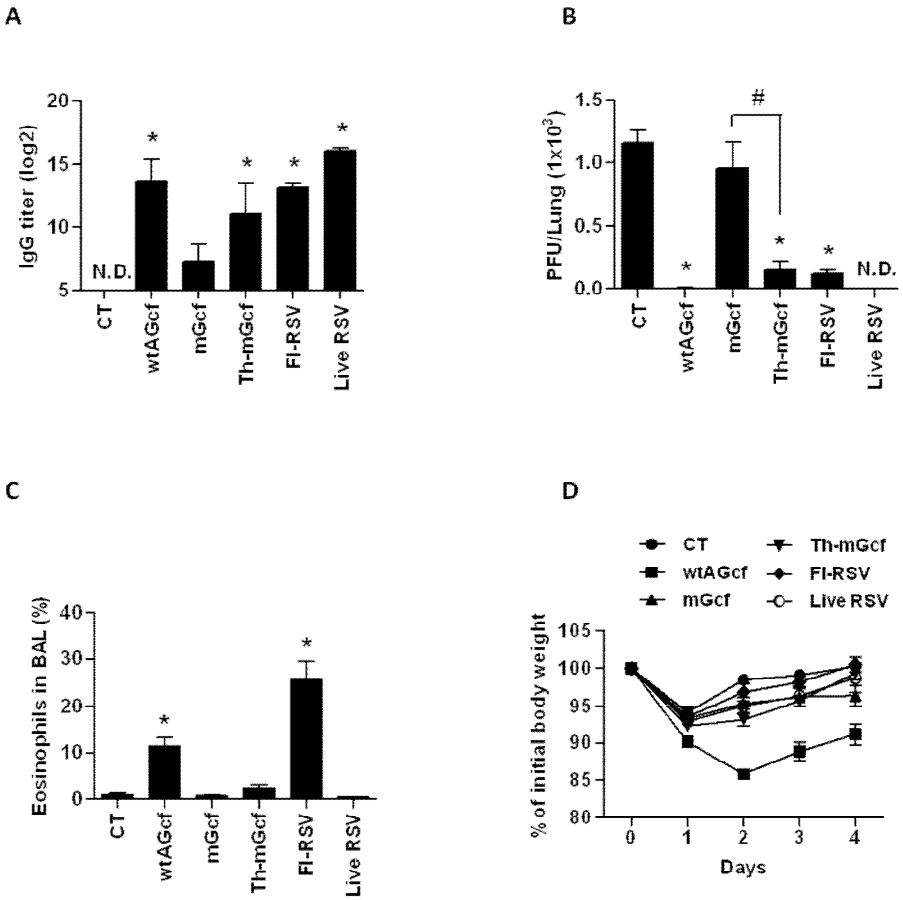

Figure 2-6. Effect of modified Gcf on the infection of homologous RSV A type. Mice (n=5) were immunized with 20 μg of wtAGcf, mGcf, or Th-mGcf sublingually in the presence of 2 μg of CT, 1 x 10^5 PFU of FI-RSV through foot-pad or 1x10^5 PFU of live RSV A2 intranasally on days 0 and 14 and (A) RSV A2 specific serum IgG was measured by ELISA two weeks after the second immunization. The mice were challenged with 2 x 10^6 PFU of RSV A2 three weeks after the last immunization. (B) The viral replication in the lungs was determined by plaque assay at day 4 post-

challenge and (C) the pulmonary eosinophils were examined by flow cytometry. Significant differences from results with the CT are marked with asterisks (*), while significant differences between mGcf and Th-mGcf are marked with sharp (#) respectively *(* or #, $P < 0.05$)*. (D) The body weight loss was monitored daily after the viral challenge. N.D., not detected. The results are expressed as means ± S.E.M. for the group (n = 5). The data are representative of three separate experiments.

6) Immunogenicity and protective efficacy of recombinant Gcfs against heterologous RSV B type infection

RSV is classified into two different subtypes: A and B, depending on the a.a. sequence in its G protein [5]. In order to examine whether immunization with Th-mGcf, derived from RSV A subtype, can induce specific immune responses against the B subtype, mice were immunized with wtAGcf, mGcf, Th-mGcf, FI-RSV, live RSV A2, or CT alone, and RSV B-specific serum IgGs were measured 13 days after the last immunization (Fig. 2-7A). In the present study, the results show that mice immunized with FI-RSV or live RSV A2 produced RSV B subtype-specific IgGs despite the use of RSV A subtype origin FI-RSV and live RSV for the immunization. However, mice immunized with wtAGcfs, mGcf, or Th-mGcf did not produce RSV B subtype-specific serum IgGs.

Next, immunized mice were challenged with RSV B (KR/B/10-12) subtype, and virus titer in the lung and eosinophil infiltration in BAL were compared at day 4 post-challenge. The results indicate that, compared to CT immunization, immunization with wtAGcf, Th-mGcf, FI-RSV, or live RSV A2 led to a significant reduction in virus titer in the lung following RSV B (KR/B/10-12) subtype challenge (Fig. 2-7B). The mGcf immunization also caused a slight decrease in the virus titer with no statistical significant. Moreover, significant increase in the percentage of eosinophils in BAL was observed in FI-RSV immunized mice as expected. However, immunization with wtAGcf, mGcf, Th-mGcf, or live RSV A2 showed few or no eosinophils in BAL (Fig. 2-7C). Furthermore, no significant differences in weight loss were found among the

immunization groups following RSV B subtype challenge (Fig. 2-7D). Collectively, these results demonstrate that RSV A2-derived Th-mGcf can confer cross-protective immunity against both subtypes of RSV without causing vaccine-mediated enhancement of disease upon RSV infection.

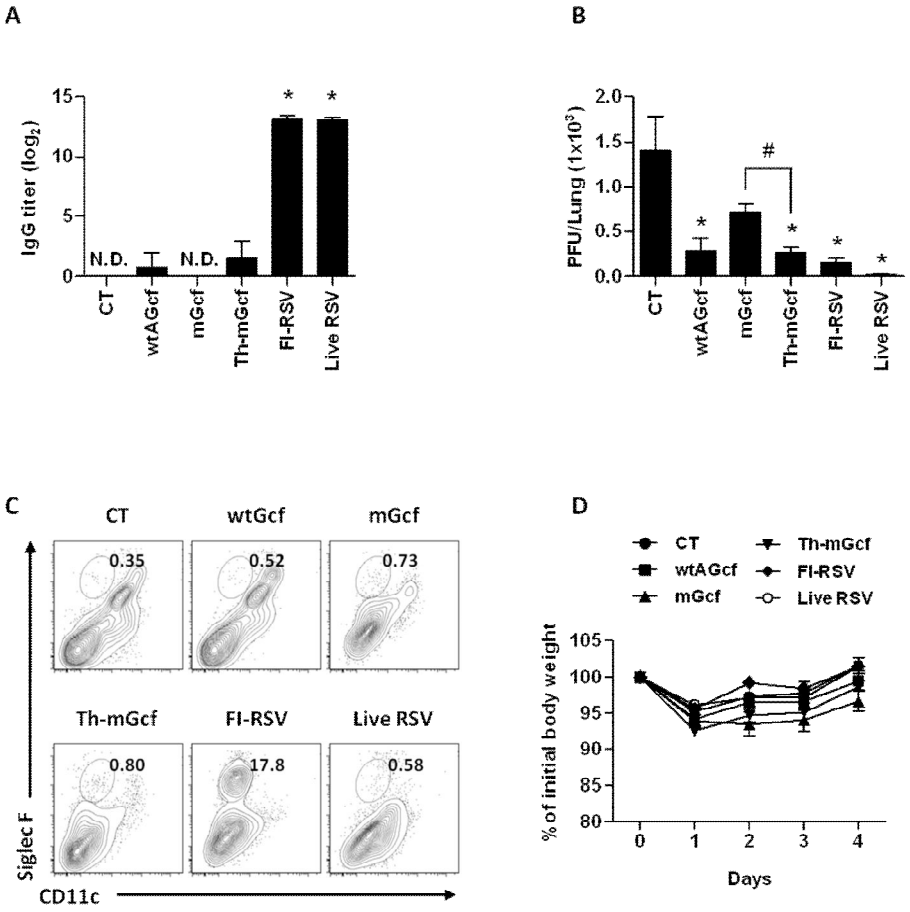

Figure 2-7. Effect of modified Gcf on the infection of heterologous RSV B type. (A) The mice (n = 5) were immunized with 20 μg of Gcf, mGcf or Th-mGcf sublingually in the presence of 2 μg of CT, 1 x 10^5 PFU of FI-RSV through foot-pad or 1 x 10^5 PFU of live RSV intranasally on days 0 and 14 and the level of RSV B type specific serum IgG was measured by ELISA 13 days after the second immunization. (B-D) The mice were challenged with 4 x 10^6 PFU of RSV B type (KR/B/10-12) three weeks after the last immunization. (B) The level of viral replication in the lungs was determined by plaque

assay at day 4 post-challenge and (C) the pulmonary eosinophils were examined by flow cytometry. Significant differences from results with the CT are marked with asterisks (*), while significant differences between mGcf and Th-mGcf are marked with sharp (#) respectively *(* or # $P < 0.05$)*. (D) The body weight loss was monitored daily after the viral challenge. N.D., not detected. The results are expressed as means ± S.E.M. for the group (n = 5). The data are representative of three separate experiments.

4. Discussion

RSV is a major respiratory pathogen in infants, elderly, and immunocompromised, often causing a severe lower respiratory tract infection. Currently, however, there is no licensed vaccine available for human use. RSV G protein can be a promising RSV vaccine candidate because it induces RSV-specific antibody responses, although $CD4^+$ T cell epitope within G protein has been shown to be related to immunopathology. In this regard, various recombinant RSV Gcfs which is RSV G protein fragment (a.a. 131-230) were engineered, expecting protective immune response without immunopathology.

The results from the present study highlights: (1) unlike RSV A subtype, a.a. residues 183-195 in Gcf of RSV B subtype do not function as $CD4^+$ T cell epitope, (2) Gcfs from RSV B subtype, unlike RSV A subtype, do not induce eosinophilia after RSV B subtype challenge, and (3) Gcf derived from RSV A subtype containing modified $CD4^+$ T cell epitope can protect mice against both RSV A and B subtypes without immunopathology.

Studies have indicated that RSV A subtype infection occurs with higher frequency and may exhibit more severe disease symptoms than those of RSV B subtype infection, but both strains often circulate together during RSV epidemics and contribute to the RSV antigenic heterogeneity and reinfection [173, 242]. Moreover, shifting in the predominance of circulating RSV subtypes occurs in 1-2 year cycle [243]. Therefore, an effective RSV vaccine should confer protective immunity against both subtypes of RSV.

However, there has been about very limited studies on the vaccine against RSV B subtype infection.

It is well known that G protein is antigenically variable and the sequence homology of G proteins between A and B subtype is approximately 53% [174]. Nevertheless, G protein of RSV B subtype also contain 13 a.a. peptide which is conserved in both subtypes of RSV [244], 4 conserved Cys residues [175], and a CX3C chemokine motif [245]. It was investigated whether wtBGcf containing these regions can induce protection against RSV infection. Both wtAGcf and wtBGcf elicit RSV-specific serum IgG against the homologous subtype, but not against the heterologous subtype. Importantly, wtBGcf did not induce eosinophilia and a.a. 183-195 in wtBGcf was identified to not possess $CD4^+$ T cell epitope functionality in contrast to wtAGcf, which has a dual function of eliciting RSVG-specific $CD4^+$ T cell immunity and promoting immunopathology. This study indicates that wtBGcf does not induce vaccine-enhanced disease. However, these results suggest that $CD4^+$ T cell epitope in wtBGcf may be present at a different region other than a.a. 183-195, because the mice immunized with wtBGcfs produced RSV-specific serum IgGs, suggesting helper T cell activity must have fostered B cell class switching.

Numerous studies demonstrated that G protein is critically associated with immunopathology in mice immunized with G protein followed by RSV infection. It has been shown that the mice immunized with conserved region of RSV G protein reduced the various parameters of RSV disease, including weight loss, pulmonary inflammation, and lung viral titer [246, 247]. Importantly, however, RSV G protein appears to be

involved in the enhancement of RSV disease pathogenesis in RSVG-primed animals by eliciting aberrant T helper cell responses [114, 147, 233, 248]. It has been reported that a.a. residue 184-193 of RSV G protein is associated with pulmonary eosinophilia [186]. Accordingly, it has also shown in this study that $CD4^+$ T cell epitope within RSV A2 Gcf is directly correlated, not only with the induction of RSVG-specific antibody responses and viral clearance, but also with the vaccine-mediated eosinophilia and severe body weight loss.

In the previous study, it was demonstrated that a region within the RSV Gcf containing four cysteine residues (CX3C motif) is responsible for the induction of RSVG-specific immune response [167]. In order to maintain the immunogenicity while eliminate $CD4^+$ T cell epitope-mediated eosinophilia, mGcf which lost the function of $CD4^+$ T cell epitope [187] and Th-mGcf was generated. This new Th-mGcf induced RSV-specific IgG response and viral clearance effect, but not pathogenesis. In addition, mice immunized with Th-mGcf had increased number of F_{51-66}–specific IFN-γ-secreting cells in the lung, suggesting that Th-mGcf immunization may also boost the aspects of cell-mediated immunity that are important for viral clearance (e.g. Th1 or CTL response).

Interestingly, mice immunized with Th-mGcf did not exhibit strong RSV B subtype specific serum IgG response, but significantly enhanced viral clearance without causing severe weight loss or exaggerated recruitment of eosinophil to the airway mucosa. Given the light of the fact that mice immunized with mGcf, without the F_{51-66} peptide fusion, failed to effectively clear the virus, it is probably that the protection from

RSV B subtype was rendered by Th-mGcf's ability to generate F_{51-66} –specific IFN-γ response.

In conclusion, in the present study, the findings describe that the vaccination approach using the modified Gcf engineered to eliminate its original T cell epitope may be a promising strategy in developing a novel RSV vaccine for cross-protection against the both subtypes of RSV while preventing RSVG-mediated lung immunopathology.

5. References

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General Conclusion

RSV G protein could be a good candidate for the development of RSV vaccine because it induces neutralizing antibodies for the protective immunity against RSV infection although it is also associated with immunopathology. Therefore, it is necessary to attenuate immunopathology but to sustain or increase the immune responses when RSV G protein is considered for the immunization. On the other hand, administration route is an important factor for safe and effective vaccine. Because RSV infect through respiratory tract, the vaccine candidate should actively induce the immune responses in the nasal-associated lymphoid tissue (NALT).

In the present study, administration route inducing proper protective immunity in the respiratory tract and its mechanism in mouse immunized with RSV vaccine candidate was investigated. Although it is known that s.l. administration does not induce side effects shown in intra nasal including redirection of antigen into central nerve system, s.l. immunization with Gcf induced symptoms of vaccine enhanced disease such as pulmonary eosinophilia and body weight loss following RSV challenge. In addition, vaccine enhanced disease induced by immunization with Gcf through s.l. route was mediated by Th17 response after the RSV infection whereas by FI-RSV induced Th2 response suggesting that immunopathology by Gcf and FI-RSV was mediated through a different mechanism.

Next, to overcome immunopathology induced by Gcf sublingual immunization following RSV infection, $CD4^+$ T cell epitope portion within Gcf was modified. The mutation at a.a. position 185 and 188 (mGcf) resulted a loss of function that induces

immune response, but it recovered by fusion with $CD4^+$ T cell epitope from RSV F protein, Th-mGcf. It was noting that immunization with Th-mGcf sublingual route induced humoral immune response and did not lead to recruitment of eosinophils in the airway after the RSV infection. Moreover, immunization with Th-mGcf s.l. showed partial cross-protective against RSV B subtype.

In conclusion, these results show that vaccine enhanced disease induced by Gcf sublingual immunization following RSV infection is mediated by Th17 response and the immunopathology can be overcome by modifying $CD4^+$ T cell epitope within Gcf.

Summary in Korean

호흡기성 융합 바이러스 (RSV)는 영유아나 면역력이 약화된 환자에게 호흡기관에 감염되어 질병을 유도하는주요 원인균이다. 또한 생 후 2~3세에 이르면 대부분의 아이들이 호흡기성 융합 바이러스에 한번 이상 감염되었던 경험이 있을 정도로 감염빈도는 매우 높다. 1960년대 호흡기성 융합 바이러스 백신으로 FI-RSV가 임상시험 되었다. 그러나 백신 접종 후 자연적으로 호흡기성 융합 바이러스에 감염되었을 때, 오히려 FI-RSV로 백신 접종 하였던 아이들에서 호흡기성 병리적 증상을 더욱 악화되었다. 이러한 백신 접종 후 바이러스 감염 시에, 백신을 접종한 경우 더욱 증가되는 병리적 현상은 호흡기성 융합 바이러스 백신개발에 장애가 되었으며, 현재 사람에게 사용될 수 있는 호흡기성 융합 바이러스 백신은 없는 실정이다.

호흡기성 융합 바이러스 G 당단백질은 바이러스가 숙주 세포 표면에 부착하는 역할을 한다. 또한 G 당단백질은 숙주에서 호흡기성 융합 바이러스 감염 방어에 관여하는 중화 항체를 유도할 수 있다. 그러나, 호흡기성 융합 바이러스 G 단백질은 면역 병리현상에도 관계가 있다고 밝혀져 있으며 특히, G 단백질 내에 있는 $CD4^+$ T 세포 에피톱이 깊은 연관이 있다.

점막을 통해 주입되는 백신은 접종되는 부위의 조직이나 장기뿐만 아니라 신체 전체적으로 체액성 및 세포성 면역 반응을 유도할 수 있다. 그러나 비강 투여를 통한 백신은 항원이 중추신경계로 들어가 벨의

마비(Bell's palsy)와 같은 심각한 부작용을 초래한다는 보고가 있다. 반면 설하 접종을 통한 백신은 비강 투여에 비해 면역성은 좀 낮을지라도 부작용이 없이 좀 더 안전한 것으로 알려졌다. 따라서 본 연구의 목적은 호흡기성 융합 바이러스 G 당단백질 중 모든 호흡기성 융합 바이러스에 비교적 높게 보존되어 있는 중심 단백질 조각인 Gcf를 이용하여 설하 접종을 통한 면역 병리현상이 없는 안전한 호흡기성 융합 바이러스 백신을 개발하는 것이다.

첫째, 점막 백신 경로인 설하 또는 비강 투여를 통한 Gcf 백신 효과를 비교하였다. 두 가지 점막 백신 경로 모두 호흡기성 융합 바이러스에 특이적인 항체를 생성하고, 바이러스에 감염될 때 바이러스가 폐에서 증식하지 못하였다. 하지만, Gcf를 설하 경로를 이용하여 접종한 그룹에서는 호흡기성 융합 바이러스 감염 후 유도되는 백신에 의해 증가되는 병리적 현상이 유도되었다. 이와는 달리, Gcf의 비강 경로를 통해 접종한 그룹에서는 호흡기성 융합 바이러스 감염 후 백신에 의한 병리적 현상이 유도되지 않았다. 이러한 현상은 Th17 반응에 의해 매개되었으며, 이는 이미 잘 알려진 FI-RSV가Th2 반응을 유도함으로써 바이러스 감염 후 병리적 현상을 더욱 증가시키는 반응과는 다른 기전을 보였다.

두번째 주제로 Gcf 내에 존재하는 $CD4^+$ T 세포에 대한 에피톱이 면역 반응과 면역병리 현상에 중요한 역할을 하기 때문에 이 부분을 변형시켰다. 변형된 Gcf는 설하 경로를 통해 접종되었음에도, 바이러스 감염 시 병리적 질병이 유도되지 않으면서 바이러스에 특이적인 면역반응을 유도하여, 안전하고 효과적인 호흡기성 융합 바이러스 백신을 개발하였다. 특히,

185번과 188번에 위치한 아미노산은 T 세포의 활성 및 질병 유도에 중요한 역할을 하는 부분으로 보고되었기 때문에 이 부분을 변형 시킴으로써 (mGcf) T 세포의 활성 및 질병 유도를 억제하였다. 또한 B 세포를 자극하여 항체의 생성을 돕는 $CD4^+$ T 세포의 기능을 회복하기 위해 RSV F 단백질에 존재하는 $CD4^+$ T 세포의 에피톱 부분을 mGcf와 결합시켜 Th-mGcf를 생산하였다. 이를 마우스에 실험한 결과 Th-mGcf는 병리적 현상 없이 항체 생성 및 RSV 감염에 대해 방어할 수 있는 반응을 유도하였다.

본 결과들을 정리하면, Gcf의 설하 경로를 통해 접종된 후 호흡기성 융합 바이러스에 감염되면 백신에 접종된 그룹에서 오히려질병이 더욱유도되었다. 이러한 면역 병리학현상은 Th17 반응에 의해 매개되며, FI-RSV 백신이 Th2 반응에 의해 질병을 유도하는 것과는 다른 기전으로 백신에 의해 증가되는 질병을 유도하였다. 또한 Gcf 내의 $CD4^+$ T 세포의 에피톱을 변형시켜 만든 Th-mGcf는 설하 경로를 통한 접종 후 RSV 감염 시에 면역 병리현상 없이 면역 반응 및 방어 반응을 유도하여 호흡기성 융합 바이러스 백신으로써 효과를 보였다.

주용어: 호흡기성 융합 바이러스, 백신, 점막백신경로, 설하, G 당단백질, 면역병리, $CD4^+$ T 세포 에피톱