약 학 석 사 학 위 논 문

Protective immunity induced by Respiratory Syncytial Virus Glycoprotein fragment in Neonatal Mice

RSV 신생아 단백질 백신 개발에 관한 연구

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Abbreviation

RSV : respiratory syncytial virus

Gcf : RSV Glyco protein core fragment

CT : cholera toxin

FI RSV : formalin inactivated RSV

i.n. : intranasal

Ig : immunoglobulin

BAL : bronchoalveolar lavage

Th : T helper

IFN-γ : interferon-gamma

Abstract

Respiratory syncytial virus (RSV) is the major cause of severe lower respiratory tract infection in infants and the elderly worldwide. The significant morbidity and mortality associated with this infection underscores the urgent need for an RSV vaccine development. In this study, we firstly showed that intranasal administration of RSV glycoprotein core fragment (Gcf) to neonatal mice was capable of inducing systemic humoral immune responses and had protective efficacy against RSV without lung eosinophilia, though antibody response was shifted to Th2. Next, we examined whether the presence of maternal anti-RSV antibodies affects the responsiveness and protection efficacy of Gcf in newborn mice, since infants can have RSV specific maternal antibodies due to frequent re-infection of RSV to adults. Intranasal

administration of Gcf induced antibody response, IFN-γ secretion and protected mice against RSV challenge without lung eosinophilia even in the presence of high level of RSV-specific maternal antibodies. Thus, our study suggests that Gcf can be applied as an effective and safe RSV vaccine during the neonatal period.

Key words: Respiratory syncytial virus, Glycoprotein, Neonate, Vaccine, Maternal effect, Mucosal

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1. Introduction

Human respiratory syncytial virus (RSV) is the most common cause of lower respiratory tract infection and bronchiolitis in infants and young children, causing significant morbidity and mortality worldwide [1]. While most adults except at high risk such as immunocompromised patients experience mild symptoms by RSV infection, it is reported by CDC that about 125,000 infant hospitalizations occur [2] and about 500 deaths break out every year due to RSV; over 80% of the deaths occur in babies younger than 1 year old [3] in the United States. It has been also reported that subsequent development of asthma and wheezing in childhood is related with severe lower respiratory infection by RSV in infancy [4, 5]. In the early 1960s, there was a vaccine trial using formalin-inactivated RSV (FI RSV), which failed in 2 deaths of children with

aggravated illness after natural RSV infection. Autopsy examination of these children reported that there were a number of neutrophils, peribronchiolar monocytic infiltration with significant tissue eosinophilia in their lungs [6]. In spite of exigent need for vaccine development, there is no licensed vaccine against RSV currently.

Immature myeloid dendritic cells of neonatal mice are incapable of driving the immune responses to Th1 against RSV [7]. RSV infection in neonatal period leads to substantial production of Th2 cytokines [8, 9] and subsequently results in lung eosinophil infiltration, the major characteristic of airway hypersensitive responses [10] and Th2-biased immune responses recur when mice were re-infected with RSV [11]. Since RSV infection does not assure full

protective immunity in humans, repetitive infections during life can happen [4, 12]. Importantly, maternal antibodies generated by one or more RSV infections before/during pregnancy are transmitted via placenta or breast milk and this passively transmitted antibody may influence the RSV vaccine-related immune responses in infants [13, 14]. Especially, the presences of high levels of maternal antibodies hinder immunogenicity of vaccine in the cases of measles, poliomyelitis, tetanus, diphtheria and pertussiss [15, 16].

Recently, Kim S *et al.* generated RSV G attachment glycoprotein core fragment (Gcf, a.a 131 to 230) as a RSV vaccine candidate and showed its substantial antiviral and chemotactic effects in adult mouse model [17]. Thus, we attempted to evaluate the vaccine efficacy of Gcf in neonatal mouse model

considering its importance in neonatal period and exhibited the immune protection of mice after immunizations. Further, we demonstrated that induction of immune responses in neonatal mice is not influenced by passively transmitted maternal antibodies.

2. Materials and Methods

2-1. Mice

Female BALB/c mice for maternal effect test, 6-8 week-old, were purchased from Charles River Laboratories (Orient Bio, Korea) or Samtako Bio Korea. Neonatal BALB/c mice (2-5 day-old) for immunization were also obtained from Samtako Bio Korea or bred under specific pathogen-free condition of International Vaccine Institute. All studies involving animals were performed under the regular guidelines of Institutional Animal Care and Use Committees (IACUC) of International Vaccine Institute (2011-014).

2-2. Preparation of antigen and RSV stocks

The recombinant RSV (RSV A2 strain) G protein fragment from 131 to 230

amino acids (Gcf) was expressed in *E.coli* and purified as described [17]. A stock of RSV A2 strain was propagated in HEp-2 cells (American Type Culture Collection, Manassas, VA) in 150mm cell culture dishes. After 4 days from inoculation, virus was harvested and titer was determined by plaque assay. HEp-2 cells were maintained in minimum essential media (MEM) containing Earle's salts, L-glutamine, 10% fetal bovine serum (FBS) (Hyclone, South Logan, Utah), and 1% penicilline-streptomycin (Gibco, Grand Island, NY).

2-3. Immunization and challenge

1 week-old neonatal BALB/c mice were immunized with five different substances via i.n. or footpad routes: live RSV ($5x10^4$ PFU), Gcf (20μg), Gcf

(20μg) with cholera toxin (CT, LIST BIOLOGICAL LBAS INC. Campbell, CA) adjuvant (1μg), and PBS were administered via intranasal (i.n.) route whereas FI RSV ($1x10^5$ PFU) was injected to each footpad of mice. Two weeks later, they were primed with the same antigen and adjuvant. After 4-5 weeks from the last immunization, the mice were challenged with live RSV ($1x10^6$ PFU).

To test the maternal effect of antigen exposure before pregnancy, female BALB/c mice were intranasally immunized twice with live RSV ($1x10^5$ PFU) or PBS at two-week intervals, and mated 4 weeks after the last immunization to obtain offspring. When their neonates were 1 week-old, the mice were divided into two subgroups and immunized: one group was immunized with

Gcf (20μg) plus CT adjuvant (1μg) via i.n. route twice at two-week intervals and the other was PBS control group. After 5 weeks of last immunization, the mice were challenged with live RSV ($1x10^6$ PFU). 4 days after challenge, eosinophil population in bronchoalveolar lavage (BAL) fluid was analyzed by flow cytometry and plaque assay was performed using lung homogenates.

2-4. Analysis of cells in BAL fluids

Four days after RSV challenge, mice from each group were sacrificed and their tracheas were washed two times with 700μl of PBS. The collected BAL fluid was centrifuged and BAL cells were resuspended in 1ml of PBS. Cells in the 50μl of the PBS were counted using heamacytometer to calculate the total BAL cell numbers and the rest of the total cells were incubated with violet

fluorescent reactive dye (Invitrogen, Eugene, OR) for 10 min in room temperature. After 10 min, cells were washed with 1ml PBS and blocked with purified CD16/CD32 Fc block (clone 2.4G2; BD Pharmingen) for 5 min. After blocking, 50μl of antibody cocktail containing anti-CD45-APC (clone 30-F11; BD Pharmingen), CD11c-FITC (clone HL3; BD Pharmingen), Siglec F-PE (clone E50-2440; BD Pharmingen) were added to cells and incubated at 4ºC for 30 min. Cells were subsequently washed two times with PBS (2% FBS) and fixed with 200μl of paraformaldehyde. Cells were analyzed using BD FACS LSR flow cytometer and data were analyzed by FlowJo software (version 7.2.5).

2-5. RSV detection and cytokine analysis in the lung

After BAL fluid collection from the mice, lungs were perfused with PBS containing heparin (10U/ml) until blood was removed thoroughly from the lung. After perfusion, lung tissues were washed with PBS and then homogenized with 70μm cell strainer (BD Labware, Franklin Lakes, NJ) with 2.5ml of MEM (10% FBS). The lung homogenates were centrifuged at 1800rpm for 5 min and 100μl of supernatants were inoculated into 90% confluent HEp-2 cells in 6-well plates. 4 days after incubation at 37ºC, the each well of plates were stained with neutral red and maintained additional incubation for 24h at 37ºC, after that PFU was determined by plaque counting. The leftover supernatants were stored at -80ºC until the cytokine analysis afterward. Cytokine levels in the supernatants were assessed as compared with mouse Th1/Th2/Th17 cytokines standards of BD Cytometric

Beads Assay (CBA) kit (BD Biosciences, San Jose, CA) according to manufacturer's recommendations.

2-6. ELISA

The RSV specific or Gcf specific antibody levels in blood sera were measured by ELISA. Mice were put under anesthesia using anesthetic solution composed of ketamine hydrochloride (Yuhan. Co.Ltd., Seoul, Korea) and xylazine hydrochloride (Rompun®, Bayer Korea, Seoul, Korea) through intraperitoneal route and blood was subsequently collected from tails or orbital sinus of mice. The blood was centrifuged at 13,000rpm for 10min and obtained sera were stored at -20ºC. 96-well ELISA plates were coated with 100μL of RSV A2 (5×10^3 PFU/well) or Gcf (200ng/well) diluted in PBS at 4

ºC overnight. After blocking with 5% skim milk for 1 hr and subsequent four times washing with PBS containing 0.05% Tween 20 (Sigma Aldrich Co., St. Louis, MO), serially diluted sera in blocking buffer were added to each well and incubated for 2 hr at 37 ºC. After washing, HRP conjugated goat anti-mouse IgG, IgG1, IgG2a or IgA (Southern Biotech, Birmingham, AL) were diluted in blocking buffer 1:3,000 and incubated in each well for 1 hr at RT. Peroxidase substrate (TMB) (Moss, Pasadena, MD) was added in each well after washing and the reaction was stopped by 0.5 N HCl. The plates were analyzed at 450nm by ELISA reader (Molecular Devices, Sunnyvale, CA). The antibody titer was expressed as reciprocal $\log_2$ titer of serum dilution showing 0.2 of absorbance at 450 nm.

2-7. Statistical analysis

Data were analyzed using Prism software program (version 5, GraphPad Prism) and expressed as the means±SEMs. Statistical significances were performed by using an unpaired, two-tailed Student's *t* test. *P* values less than 0.05 were considered statistically significant.

3. Results

3-1. Gcf vaccination in neonatal mice induces humoral immunity

Previous study has reported that mucosal vaccination with either Gcf or Gcf plus CT adjuvant (Gcf/CT) could induce Ag-specific B cell immune responses in adult BALB/c mice [17]. To determine whether Gcf vaccination is capable of effectively inducing humoral immune responses in neonatal mice, 7-day-old mice were immunized intranasally and boosted 14 days later (Fig. 1A). With blood sera collected on day 35, it was determined whether either Gcf or Gcf/CT immunization elicited antibody immune responses to RSV or Gcf protein (Fig. 1B and 1C, respectively). Notable RSV-specific serum IgG response in Gcf/CT immunized group was comparable to those of either FI RSV or RSV group and even the immunization of Gcf alone induced

considerable RSV-specific serum IgG response (Fig.1B). In terms of IgG2a and IgG1 antibody isotypes, representing Th1 and Th2 $CD4^+$ T cell responses respectively, Gcf/CT immunized mice showed dominant IgG1 response and a low level of IgG2a response to RSV. RSV-specific IgG2a was not detected in either FI RSV or Gcf alone-immunized group (Fig.1B). In terms of Gcf-specific antibody level in sera, all the isotypes included in the assay, IgG1, IgG2a and IgA isotype were detected in Gcf/CT immunized group. IgA in response to recombinant Gcf was not detected in the rest of groups. Gcf alone immunized group showed lower level of Gcf-specific antibodies than Gcf/CT group but similar levels of antibodies from FI RSV or RSV immunized groups (Fig 1C). RSV immunized group showed higher IgG1/IgG2a ratio (=2.31) in response to whole RSV compared to 6-8 weeks old adult mice (data not shown;

Figure 1. Systemic RSV and Gcf-specific antibody responses after Gcf immunization in neonatal mice. (A) Mice were immunized twice with PBS, Gcf, Gcf+CT, or RSV via intranasal route or with FI RSV via footpad when they were 7 and 21 days-old. After 2 weeks of the second immunization, blood sera were gathered from each mouse. (B) RSV (left) or Gcf (right)-specific antibody titers were determined by ELISA. Data are expressed as means± SEM of n=5-7 mice in each group. The results are representative of two independent experiments. *B.D.* means below detection level.

IgG1/IgG2a=0.95), while this RSV immunized neonate group showed 1.30 of IgG1/IgG2a suggesting higher Th1 response to Gcf itself compared to whole RSV particles. Collectively, these results suggest that Gcf or Gcf/CT could induce systemic humoral immune responses effectively *in neonatal mice, which is rather toward to Th2 response compared to adult mice*.

3-2. Effect of Gcf immunization on lung eosinophilia and protection against RSV

Next, we investigated whether vaccination with Gcf promotes the lung eosinophilia upon live RSV challenge, since exposure to live RSV after FI RSV vaccination leaded to vaccine-enhanced illness such as airway obstruction and excessive eosinophilia [18] and it was shown that

immunization with FI RSV developed Th2-skewed immune responses after live RSV infection in a mouse model [19]. As described in Fig.1A, mice were challenged with live RSV A2 and BAL fluids were obtained after 4 days. While FI RSV immunized mice had substantial increase of eosinophils in BAL (Fig.2A), Gcf/CT immunized group showed a low level of eosinophils in BAL like RSV immunized group. Mice immunized with Gcf alone showed few eosinophils like PBS negative control group. These data demonstrate that the mice immunized with Gcf or Gcf/CT does not develop vaccine-induced immunopathology after RSV infection.

Further studies were carried out to test if Gcf immunization would ameliorate viral load in the lung after challenge with RSV. Lungs were obtained from the

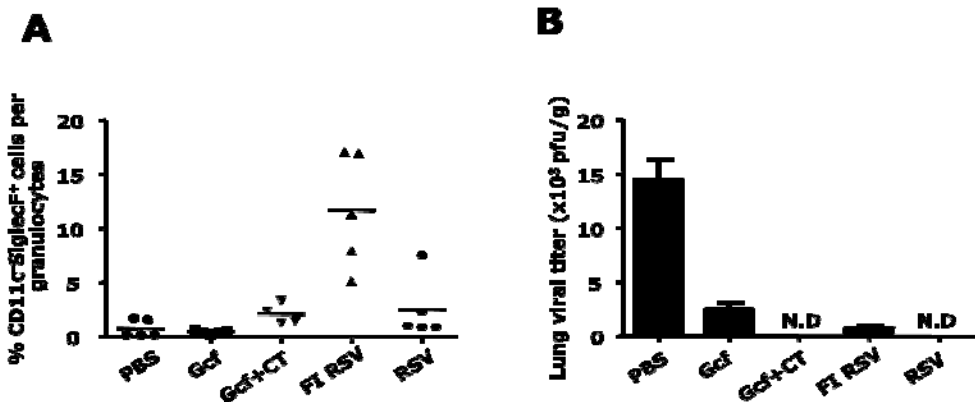

Figure 2. Low BAL eosinophilia and protective efficacy against RSV by intranasal Gcf immunization at neonatal period. (A) Mice were immunized twice and challenged by RSV as in Fig. 1A. 4 days after challenge, cells were isolated from BAL fluids and stained with fluorescence-labeled anti-CD45, CD11c, and Siglec-F antibodies. The percentages of eosinophils, namely $CD11c^{-}SiglecF^{+}$ cells, among granulocytes were shown. Data are shown as means±SEM with n=4-5 mice per group and representative of two independent experiments. (B) Lungs were harvested from the mice and viral mice sacrificed on day 56 and lung homogenates were prepared for viral titers were assessed by plaque assay on day 4 after challenge. *PFU*, plaque forming units.

titration. At day 4 post-infection to HEp-2 cell, viral loads in the lung homogenates were assessed by plaque counting. No virus was detected in lung homogenates of Gcf/CT-immune mice, whereas PBS control group showed significant lung viral titers (Fig.2B). In addition, Gcf protein alone also provided potent protection which was previously reported in adult mice [17]. These results indicate that intranasal immunizations of Gcf protein greatly improve viral clearance against RSV infection.

3-3. Gcf/CT vaccination induces immune response regardless of maternal antibody

We further examined whether the pre-existing RSV-specific maternal antibody have an effect on the immunogenicity of Gcf in neonates. Female

mice were infected with RSV twice intranasally at two weeks interval and then mated with male to get pups possessing RSV specific maternal antibodies. The pups were immunized with either Gcf/CT or PBS at the age of 1 week and 3 weeks followed by RSV challenge at the age of 8 weeks (Fig.3A). We investigated the antibody responses following the immunization of Gcf/CT in the presence of RSV-specific maternal antibodies.

In contrast to PBS control mother group, either RSV or Gcf-specific serum IgG from the RSV immunized mother mice showed long-term maintenance over 5 weeks in their progeny even without immunization (Fig.3B, C). Gcf/CT-immunized offspring from PBS control mothers showed increase of IgG titer after booster immunizations and kept gradual IgG titer increase until

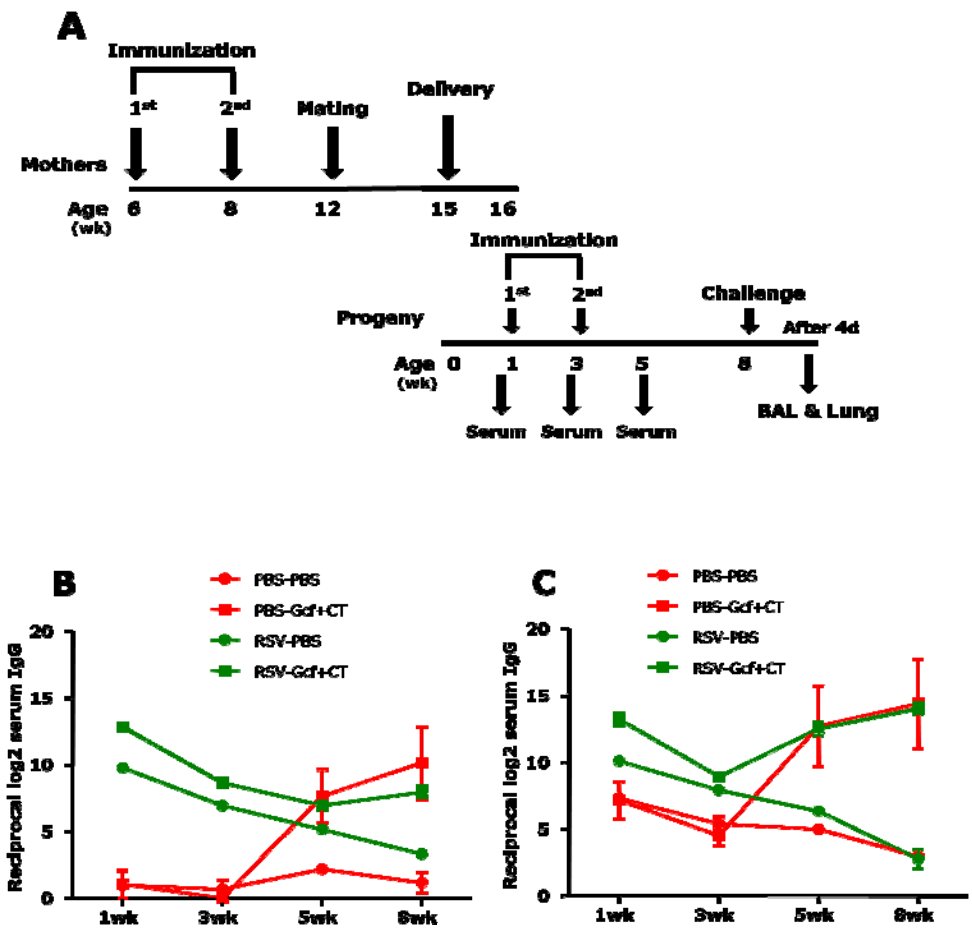

Figure 3. Anti-RSV and anti-Gcf IgG changes in sera of immunized offspring born from differently immunized mothers. (A) Schematic design of experimental protocol. 6-week-old Balb/c mother mice were administered twice intranasally with PBS or RSV before mating. After 7 weeks from the last immunization, pups were delivered from the immunized mice and subsequently immunized twice with either PBS or Gcf plus CT at 1 and 3-

week-old. Blood sera were collected four times from the pups: before performing two immunizations (1, 3 weeks of age), at 5 weeks of age, and on the day of sacrifice. When 8 weeks old, offspring were challenged with RSV and analyzed 4 days later. (B) Serum IgG level alterations that were derived from vertical antibody transfer from mothers or direct immunization to offspring, Kinetics of antibody levels in sera specific to RSV (B) as well as Gcf (C) is shown. Data are expressed as means± SEM of n=3-10 mice in each group.

8 weeks. In Gcf/CT- immunized offspring from RSV infected mothers, IgG titer specific to Gcf or RSV began to increase from either 3 or 5 weeks old. Taken together, administration of Gcf/CT is sufficiently immunogenic even in the presence of preexisting transmitted RSV-specific maternal antibodies.

3-4. Maternal antibodies have little effect on recruitment of eosinophil and protective efficacy against RSV challenge

We next compared the degree of airway hypersensitive responses and protection efficacy in pups possessing pre-existing maternal antibodies. As described in Fig.3A, the progeny born from either RSV-infected or naïve mothers were i.n. challenged with RSV A2 following i.n. immunization with Gcf/CT twice at the age of 1 week and 3 week, and then both BAL fluids and

lungs were obtained 4 days after challenge. The pups immunized with Gcf/CT showed significant increase of total BAL cells regardless of the presence of maternal antibodies (Fig.4A). However, despite the increase of total cell counts in BAL fluids, an eosinophil infiltration was minimal in Gcf/CT immunized pups.

Next, Th2 type cytokines, represented by IL-4, and IL-6, were measured using lung supernatants by cytometric beads assay (Fig.4B). There was no difference in IL-4 production between all the groups. Gcf/CT-immune offspring born from RSV immunized mothers or naïve mothers showed increase of IL-6 compared to no immunized neonate group born from same mothers, however, there was no statistical significant difference between these

Figure 4. Effect of maternal immune responses on neonatal Gcf immunization by analysis of immune responses in lungs (A) Neonatal mice were vaccinated and challenged with live RSV as described in Fig.3. BAL fluids were collected from their lungs at 4 days after challenge and analyzed.

The number of total cell count (TCC) and eosinophils (Eos) in BAL are shown.Lungs were harvested and cytokine levels in the lung supernatant were determined by cytometric beads assya (B) and viral titer was determined by plaque assay (C). Data are expressed as means± SEM of n=3-10 mice in each group.

two Gcf/CT immunized groups.

In addition, high levels of IFN-γ was detected in neonatally Gcf/CT immunized group and there was no statistically significant difference in Gcf/CT immunized offsprings born from either RSV immunized or naïve mothers (Fig.4B). Moreover, regardless of the presence of RSV-specific maternal antibody, Gcf/CT immunization leaded full protection against RSV replication in the lungs (Fig.4C).

Collectively, these results support that i.n. inoculations of Gcf/CT to neonatal mice could elicit protective immunity without vaccine-enhanced lung eosionophlia even with an increase in BAL cells in Gcf/CT immunized pups, regardless of the presence of maternal anti-RSV antibodies.

4. Discussions

Our study clearly shows that i.n. administrations of Gcf to neonatal mice induce not only prominent humoral immune responses but also protection against RSV infection without enhanced illness regardless of the presence of passively transmitted RSV-specific maternal antibodies from mothers as did in adult mice [17].

Since exacerbated morbidity and mortality that are related to RSV infections reach its peak between 2 to 4 months after birth in human, the most suitable RSV vaccine would be administered in early life and elicit effective immune responses in newborns [20, 21]. Although RSV vaccine development has been actively investigated over years using several animal models generating

numerous vaccine candidates, most studies were conducted focusing adult animal models [11]. Since there are some immunological differences between adults and neonates in terms of immaturity of immune system, Th2 shifted immune responses and effect of maternal antibodies in neonates [13], it is worth to use neonatal animal model to investigate the RSV vaccine efficacy. Our data indicated that neonatal immune responses are towards to Th2 based on that IgG1 isotype is more dominant than IgG2a even in response to live RSV vaccination (Fig. 1B), which is contrary to the isotype ratio in adults (data not shown). Although antibody responses to Gcf is Th2 dominant, eosinophils infiltration into the lung after Gcf with or without CT vaccination followed by RSV challenge is ignorable compared to that of FI RSV vaccination.

It is known that viral vaccine-related antibody responses in respect of rabies virus, canine parvovirus, pseudorabies virus and feline rhinotracheitis virus are suppressed in infants by maternally acquired antibodies [22-26]. There were several evidences even in adult mice that passive transfer of RSV antibodies suppresses the immune responses against RSV F and G protein immunizations [27, 28]. Previously, J.E. Crowe et al. reported that while primary systemic and mucosal Ab responses induced by their live RSV vaccine candidate were inhibited by passive transfer of RSV-specific serum Abs, cell-mediated immune responses was not inhibited [29]. Actually, due to nature of RSV re-infections through the whole life, RSV-specific antibodies of maternal origin can play an important role for a few weeks to months providing passive immunity to offspring [30-32], whereas they may interrupt the vaccine-

associated immune responses in infants. However, our results demonstrate that the history of RSV infection of mother mice does not affect neonatal vaccine efficacy of CT-adjuvanted Gcf. It could be resulted from that immunogenicity of nonglycosylated Gcf purified from *E.coli* is different from that glycosylated G protein from either live RSV or recombinant vaccinia virus. Therefore, humoral immune response to Gcf in offspring may not be affected by pre-existing maternal live RSV-specific antibodies. Indeed, Gcf-specific Ig titer increase was detected immediately after booster immunization. Similar result has been reported that neonatal immunization with BBG2Na developed antibody immune response even in the presence of RSV maternal antibodies [33], however, Gcf showed efficient vaccine efficacy even without any fusion proteins and exhibited safety with minimal lung eosinophilia followed by RSV

infection (Fig.3B).

In conclusion, our findings show that Gcf is a cost-effective and prospective vaccine candidate not inducing any deteriorated vaccine-induced illness caused by neonatal immunization. . Future studies are needed to define the mechanism of Gcf itself-related immune responses in neonates and more safe administration routes for the development of applicable human vaccine.

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한글 초록

Respiratory syncytial virus(RSV)는 전 세계적으로 영유아와 노인의 하기도 감염을 일으키는 주요 원인 인자로 알려져 있다. 또한 영유아기에 발생한 RSV 감염은 유병율과 사망율을 크게 증가시킨다는 점에서 그 중요성이 매우 크지만, 아직까지 개발된 RSV 백신은 존재하지 않는다.

본 연구에서 우리는 비강 루트를 통해 신생아 마우스에 Gcf를 접종시 폐의 호산구 증가 없이 면역 반응과 혈청 항체 반응이 유도 되며, Gcf 접종 후 RSV에 감염시 폐의 바이러스 농도가 효과적으로 줄어들어 숙주를 효과적으로 방어하는 것을 확인하였다. 다음으로 우리는 RSV에 감염된 적이 있는 성체 마우스로부터 태어난 신생아 마우스를 이용하여 모체 항체의 이행이 Gcf의 RSV에 대한 방어 작용에 영향을 미치는지 확인해 보았다. RSV는 한번 감염되었다고 해서 평생 면역이 지속 되지 않기 때문에, 일생 동안 빈번한 재감염이 일어나고, 이 때 생성된 항체는 임신시 태아에게 전달 되게 된다. 실험 결과, 모체 항체의 존재에도 불구하고 비강 루트를 통한 Gcf의 접종은 항체 반응과 IFN-γ 생성을 효과적으로 유도하였고, 호산구의 증가 없이 숙주를 효과적으로 방어하였다.

결론적으로, 이 연구는 Gcf가 신생아기에 사용될 수 있는 효과적이고 안전한 RSV vaccine 후보가 될 수 있음을 시사하고 있다.