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Immune Responses to Plant-Derived Recombinant Colorectal Cancer Glycoprotein EpCAM-FcK Fusion Protein in Mice

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Abstract

Epidermal cell adhesion molecule (EpCAM) is a tumor-associated antigen (TAA), which has been considered as a cancer vaccine candidate. The EpCAM protein fused to the fragment crystallizable region of immunoglobulin G (IgG) tagged with KDEL endoplasmic reticulum (ER) retention signal (EpCAM-FcK) has been successfully expressed in transgenic tobacco (*Nicotiana tabacum* cv. Xanthi) and purified from the plant leaf. In this study, we investigated the ability of the plant-derived EpCAM-FcK (EpCAM-FcK^{P}) to elicit an immune response *in vivo*. The animal group injected with the EpCAM-FcK^{P} showed a higher differentiated germinal center (GC) B cell population (~9%) compared with the animal group injected with the recombinant rhEpCAM-Fc chimera (EpCAM-Fc^{M}). The animal group injected with EpCAM-FcK^{P} (~42%) had more differentiated T follicular helper cells (Tfh) than the animal group injected with EpCAM-Fc^{M} (~7%). This study demonstrated that the plant-derived EpCAM-FcK fusion antigenic protein induced a humoral immune response in mice.

Key Words: Colorectal cancer, EpCAM, Germinal center B cells, Transgenic plants

INTRODUCTION

The protein expression system of plants has been considered a potential alternative to produce immunotherapeutic proteins for various biomedical applications and has several advantages, such as the capability to produce large amounts of recombinant immunotherapeutic proteins economically and ensuring post-translational modifications (Lee and Ko, 2017; Song *et al*., 2019; Park *et al*., 2020; Song *et al*., 2021). In our previous study, we expressed the epidermal cell adhesion molecule (EpCAM) antigenic glycoprotein fused to the fragment crystallizable region of immunoglobulin G (IgG Fc), creating the fused protein EpCAM-Fc expressed in transgenic tobacco (*N. tabacum* cv. Xanthi) plants, and used this fused protein as a vaccine candidate for colorectal cancer (Lu *et al*., 2012; Lim *et al*., 2015; Kim *et al*., 2020). The plant-derived EpCAM-Fc recombinant protein have been confirmed to induce the production of IgGs against EpCAM in mice (Lu *et al*., 2012). Our previous studies only showed the endpoint results that immunization with the plant-derived EpCAM-Fc produced an anti-EpCAM IgG response. However, the molecular immune response at the cellular level by the plant-derived EpCAM-Fc recombinant protein has not been fully investigated. The immune system is made up of a population of diverse cells and molecules responsible for antibody production and protection from diseases via their collective, coordinated responses (Nicholson, 2016; Rich and Chaplin, 2019). Production of long-lived antibody-secreting plasma and memory B cells is essential for protective immunity against reinfection and diseases. The production of plasma cells and memory B cells occurs in the germinal centers (GCs) within follicles (B cell zone) of secondary lymphoid tissues (Stebegg *et al*., 2018). Follicular helper CD4^{+} T cells (TFH) are specialized B cell helper cells (Crotty, 2011). Distinct features of TFH cells include the expression of chemokine receptor 5 (CXCR5), programmed cell death protein 1 (PD-1), serum amyloid P

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(SAP), interleukin (IL)-21, and inducible T cell costimulator (ICOS), and the absence of B lymphocyte-induced maturation protein-1 (Blimp-1) (Ma *et al*., 2012). TFH cells are important for the formation of GCs. Once GCs are formed, TFH cells are required to maintain and regulate the germinal center for B cell differentiation into plasma and memory B cells (Haynes *et al*., 2007; King, 2011; Ma *et al*., 2012). In this study, we determined whether the plant-derived EpCAM-FcK induced activation of GCs and if plasma and memory B cells were generated by injecting the plant-derived EpCAM-FcK into mice and investigating the generation of cells related to the activation of GCs. This study is the first to demonstrate that plant-derived EpCAM-FcK fusion antigenic proteins induced activation of GCs *in vivo*.

MATERIALS AND METHODS

Expression and purification of EpCAM-FcK in transgenic tobacco plant

Transgenic tobacco plants expressing the EpCAM-FcK recombinant protein were generated using Agrobacterium-mediated transformation (Lu *et al*., 2012; Kim *et al*., 2020) (Fig. 1). The EpCAM-FcK recombinant protein was obtained from transgenic tobacco plants described in a previous study (Kim *et al*., 2020). The expression of the plant-derived EpCAM-FcK (EpCAM-FcK^{P}) was confirmed by western blot analyses conducted with human IgG Fc-specific and anti-EpCAM IgG antibodies (Jackson ImmunoResearch, West Grove, PA, USA) as described in a previous study (Lu *et al*., 2012).

Purification of recombinant fusion EpCAM-IgG FcK proteins in transgenic plant leaf

Recombinant EpCAM-FcK proteins were purified by affinity chromatography as described in a previous study (Park *et al*., 2015a; Kim *et al*., 2020). Transgenic tobacco leaves were harvested and homogenized using a blender in an extraction buffer (37.5 mM Tris-HCl, pH 7.5; 50 mM NaCl; 15 mM EDTA; 75 mM sodium citrate; and 0.2% sodium thiosulfate) (Hamilton Beach, Glen Allen, VA, USA) as described in our previous study (Park *et al*., 2015a). In brief, total soluble proteins were extracted by ammonium precipitation, and EpCAM-FcK^{P} was purified using equal volumes of total soluble proteins and binding buffer (20 mM sodium phosphate, pH 7.0). The mixture was centrifuged (15,000 g) at 4°C for 30 min. The supernatant was filtered using a 0.45 μm Millex filter (Merck Millipore, Burlington, MA, USA) and applied to a HiTrap Protein G HP column (GE Healthcare, Chicago, IL, USA). The proteins were eluted from the column with an elution buffer (0.1 M glycine-HCl, pH 2.7). The eluted protein samples were dialyzed overnight with PBS (pH 7.4) and were concentrated to 1 mg/ml using Amicon Ultra spin columns with a 10 kDa cut-off (PALL Co., Washington, NY, USA). The purified protein samples were resolved by a 12% SDS PAGE and stained

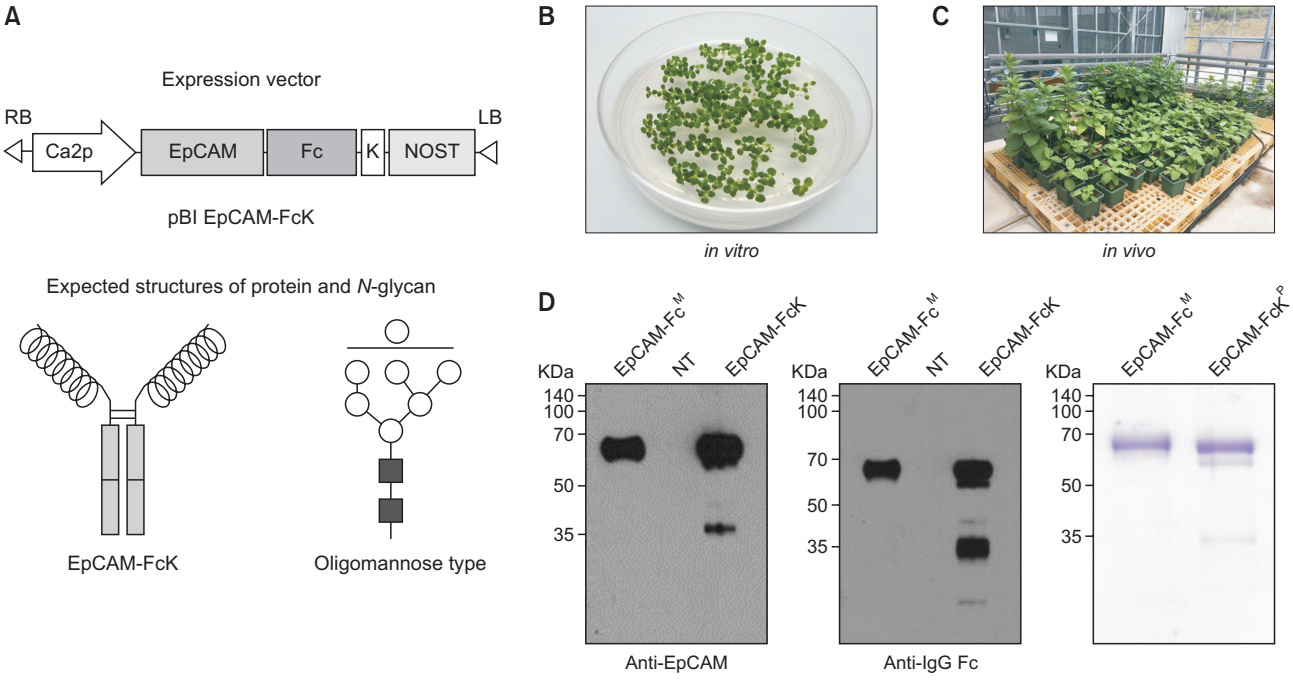

Fig. 1. Expression and purification of the EpCAM-FcK in transgenic tobacco plant. (A) EpCAM-FcK expression cassette in the plant expression vector for Agrobacterium-mediated tobacco plant. Ca2p, Cauliflower mosaic virus 35S promoter with duplicated enhancer region; EpCAM, Epidermal cell adhesion molecule; Fc, IgG constant region fragment; K, KDEL; ER, retention signal; NOS-T, terminator of Nopaline synthase gene. The KDEL ER retention signal was tagged to the C- terminus of the IgG Fc. Expected protein structure; Black spring and grey rectangular white bars indicate EpCAM and IgG Fc, respectively. *N*-glycan structure (Lim *et al*., 2015); N-acetylglucosamine, black square; mannose, white circle (Lim *et al*., 2015). (B) *In vitro* transgenic plant seeds carrying the EpCAM-FcK transgene. (C) *In vivo* soil-grown transgenic plants expressing the EpCAM-FcK transgene. (D) Western blots (left and middle) confirming the expression of the EpCAM-FcK recombinant protein in transgenic plants using anti-EpCAM and anti-human IgG_1 Fc antibodies. Coomassie blue stained SDS-PAGE (right) visualizing the purified EpCAM-FcK from transgenic plant leaf.

with Coomassie brilliant blue R250 (Thermo Fisher Scientific, Waltham, MA, USA). The purchased rhEpCAM-Fc chimera (EpCAM-Fc^{M}) (100 ng/μL) (R&D Systems, Minneapolis, MN, USA) was used as a positive control.

Animals

Six-week-old male BALB/c mice (Japan SLC, Inc. Hamamatsu, Shizuoka, Japan) were maintained in a pathogen-free environment. All mice experiments were approved by the Institutional Animal Care and Use Committee (IACUC) at Chung-Ang University (Seoul, Korea) (Approval ID: 14-0015) in accordance with the guidelines of the Korean Council on Animal Care.

Immunization of EpCAM-Fc^{P} in mice

Eight-week-old male BALB/c mice were intraperitoneally (i.p.) injected three times at two-week intervals with 10 μg of EpCAM-FcK^{P} or the positive control, EpCAM-Fc^{M} (R&D systems) in 100 μL and included the adjuvant aluminum hydroxide (Sigma Aldrich, St. Louis, MO, USA). Blood samples were collected before the experiment, ten days after the second immunization, and nine days after the third immunization by retro-orbital sinus bleeding (Yardeni *et al*., 2011). mice were bled individually and sacrificed. After sacrificing the mouse, the spleen was harvested (Fig. 2).

Surface plasmon resonance analysis

Steady-state equilibrium binding of the serum from mice vaccinated with EpCAM-Fc^{M} or EpCAM-FcK^{P} to EpCAM proteins was carried out at 25°C using a ProteOn XPR36 surface plasmon resonance biosensor (Bio-Rad Labs, Hercules, CA, USA). The EpCAM proteins were injected in the horizontal orientation of the ProteOn XPR36 fluidics system using a flow rate of 40 μL/min for 90 s (60 μL). Sera from the mice vaccinated with EpCAM-FcK^{P} or EpCAM-Fc^{M} were injected in the vertical orientation of the ProteOn XPR36 fluidics system for six min at 25 μL/min (150 μL). Running buffer was injected simultaneously in the channel to correct for the loss of captured supernatant antibodies from the chip sensor surface during the experiment as previously described (Nahshol *et al*., 2008). The binding kinetics data of the anti-EpCAM IgG to EpCAM protein were analyzed using Bio-Rad ProteON manager software. Affinity measurements were calculated using the Langmuir with Mass transfer algorithm (Khurana *et al*., 2009; Park *et al*., 2014).

Dendritic cell and CD4^{+} T cell isolation

Spleens were removed, and single-cell suspensions were

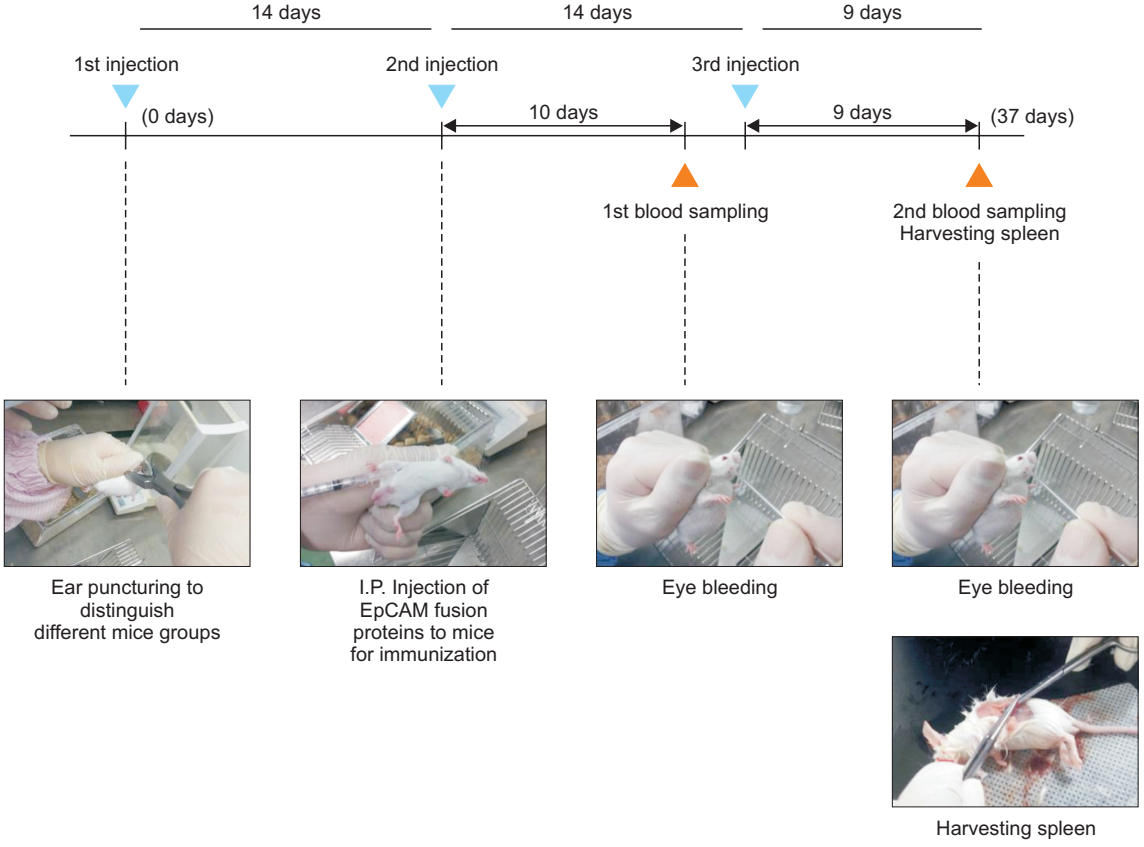

Fig. 2. Schematic diagram of the immunization plan for the plant-derived EpCAM-FcK fusion proteins in mice. Six-week male BALB/c mice were maintained in a pathogen-free environment. Eight-week-old male BALB/c mice were injected three times with 10 μg of EpCAM-FcK^{P} or EpCAM-Fc^{M} at two-week intervals. The first blood samples were collected by retro-orbital bleeding ten days after the second round of immunization. The second blood samples were collected by retro-orbital bleeding nine days after the third round of immunization. The spleens were harvested at the same time as the second blood samples.

prepared with 400 U/mL collagenase D (Roche, Basel, Switzerland). The CD11c$^+$ cells were enriched using magnetic-activated cell sorting (MACS) (Miltenyi Biotech, Bergisch Gladbach, Germany). OVA-specific CD8$^+$ or CD4$^+$ T cells were prepared from ovalbumin in the context of MHC class 1 (OT-1) or ovalbumin in the context of MHC class 2 (OT-2) mice. In brief, single-cell suspensions from spleens were prepared, and CD8$^+$ T cells or CD4$^+$ T cells were enriched by MACS sorting. All flow cytometry data were acquired by BD FACS LSR II and analyzed by FlowJo software (TreeStar, Stanford, CA, USA). In 96-well cell culture plates, naïve DC and CD4$^+$ T cells were co-cultured in the presence of EpCAM-FcK^P or EpCAM-Fc^M and then analyzed by flow cytometry.

Cytokine assay

An immune CD4$^+$ T cell cytokine production assay was conducted naive DCs cocultured with BALB/c T cells at a ratio of 1:10 (DC, 0.3×10^5: T cell, 0.3×10^6) in 96-well U-bottom plates at 37°C. After 72 h, supernatants were collected, and IL-4 and IL-10 production was analyzed by cytometric bead array (CBA) flex sets (BD Bioscience, San Jose, CA , USA) and flow cytometry. Data were analyzed with Flow Cytometric Analysis Program (FCAP) Array software (TreeStar, Ashland, OR, USA).

Statistical analysis

The compared values were the mean ± SD. IL-4 (pg/mL) and IL-10 (pg/mL) concentrations was compared using the unpaired t-test. Less than 0.01 (**) or 0.05 (*) of *p*-values means statistically significance. Statistical analysis was conducted using Excel (Microsoft Corporation, Redmond, WA, USA).

RESULTS

Generation of transgenic plants

The cDNA encoding the EpCAM fused to the Fc fragment of human IgG_1 tagged with a Lys-Asp-Glu-Leu (KDEL) endoplasmic reticulum (ER) retention signal (EpCAM-FcK) was cloned into a plant expression vector (pBI EpCAM-FcK) (Fig. 1A), where the expression cassette for of the EpCAM-FcK gene was under the control of the duplicated Cauliflower Mosaic Virus 35S promoter (Ca2p) and Nopaline synthase gene terminator (NosT) (Fig. 1A). The pBI EpCAM-FcK was transferred into *Agrobacterium tumefaciens* strain LBA4404 for Agrobacterium-mediated plant transformation (Lu *et al*., 2012; Kim *et al*., 2020). Transgenic tobacco was generated and grown *in vitro* (Kim *et al*., 2020) (Fig. 1B).

Growth of transgenic plants expressing EpCAM-FcK proteins and EpCAM-FcK purification

In vitro transgenic plant seeds carrying and expressing the EpCAM-FcK transgene (Fig. 1B) were transplanted to a soil pot for growth *in vivo*. Leaf biomass was collected for purification of EpCAM-FcK proteins (Fig. 1C). The expression of EpCAM-FcK protein was confirmed by Western blot using anti-EpCAM and anti-human IgG Fc antibodies (Fig. 1D left and middle, respectively). The purified EpCAM-FcK protein was visualized on an SDS-PAGE gel (Fig. 1D right). The western blot showed the expected EpCAM-FcK protein band of around 70 kDa in the leaf sample from the transgenic plant (Fig. 1D left and middle). No band was observed in the leaf sample from the non-transgenic plant (Fig. 1D). The Coomassie blue stained SDS-PAGE gel showed the EpCAM-FcK band at 68 kDa (Fig.1D right).

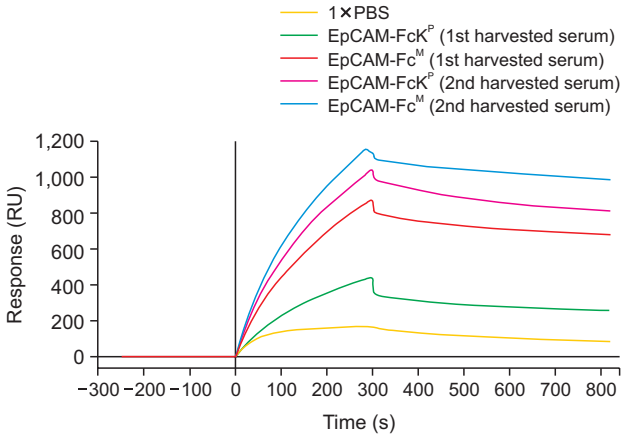

Fig. 3. Kinetic analysis confirming an immune response that induced anti-EpCAM IgGs in mouse vaccinated with EpCAM-FcK^P or EpCAM-Fc^M antigens using SPR. The response curves, blue, pink, red, and green, are from the second harvested serum from mice vaccinated with EpCAM-Fc^M, the second harvested serum from mice vaccinated with EpCAM-FcK^P, the first harvested serum from mice vaccinated with EpCAM-Fc^M and the first harvested serum from mouse vaccinated with EpCAM-FcK^P, respectively. The recombinant protein EpCAM-Fc was coated on a sensor chip. The first serum samples were harvested ten days after the second injection of the vaccines. The second serum samples were harvested nine days after the third injection of the vaccines. PBS was injected into a mouse as a negative control.

Kinetic analysis to confirm the immune response inducing anti-EpCAM IgGs in mouse vaccinated with EpCAM-FcK antigens using SPR

Kinetic analysis of antigen-antibody interactions using surface plasmon resonance (SPR) showed that the first harvested serum from mice vaccinated with EpCAM-FcK^P (~420 RU) was lower than the first harvested serum from mice vaccinated with EpCAM-Fc^M (~810 RU). In mice vaccinated with EpCAM-FcK^P, the RU value (~1,020) of the second harvested serum increased almost 2.5 times from the RU value (~420) of the first harvested serum (Fig. 3). In mice vaccinated with EpCAM-Fc^M, the RU value (~1,150) of the second harvested serum increased only 1.4 times from the RU value of the first harvested serum (Fig. 3). The difference in RU values between the second and first harvest in the EpCAM-FcK^P group was significantly larger than the difference between the second and first harvest in the EpCAM-Fc^M group indicating that EpCAM-FcK^P had a relatively higher immunization boosting effect (Fig. 3).

Flow cytometry analysis of germinal center (GC) B cell

FACS was conducted to compare the percentage of differentiated B cells in the GCs from spleens of mice injected with PBS, EpCAM-FcK^P, or EpCAM-Fc^M. Representative FACS plots of the GC B cells (GL7$^+$ FAS$^+$) were obtained from mice nine days after injections and were generated with a gating strategy of CD19$^+$ IgD$^-$. GL7 and FAS are B cell activation markers (Naito *et al*., 2007; Hao *et al*., 2008). The GC is a

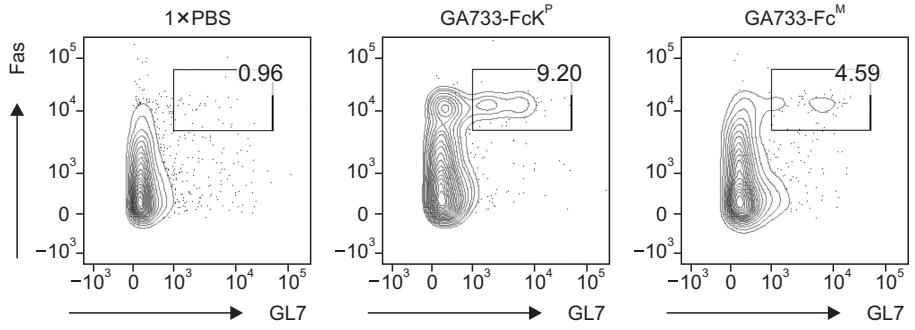

Fig. 4. Flow cytometry analysis of germinal center (GC) B cells obtained from mice vaccinated with EpCAM-FcK^P or EpCAM-Fc^M. Flow cytometry of GC B cells nine day after injection with the vaccine (gated on CD19$^+$ IgD$^-$ GL7$^+$ FAS$^+$). Numbers represent the percentage of differentiated B cells in the GCs.

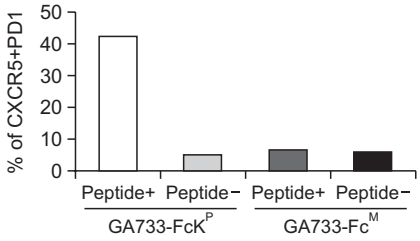

Fig. 5. T follicular helper (TFH) cell activation from mice spleens in the response to immunization with EpCAM-FcK^P or EpCAM-Fc^M. In 96-well cell culture plates, naïve dendritic cells (0.03 M) were cocultured with CD4$^+$ T cells (0.3 M) isolated from the mice injected with EpCAM-FcK^P or EpCAM-Fc^M and with or without proteins in the presence of a LPS (100 ng/mL) adjuvant for three days. Percentage of CXCR5+PD1 was calculated from [(CD4$^+$ T cell expressing CXCR5 and PD1/total CD4$^+$ T cells)×100].

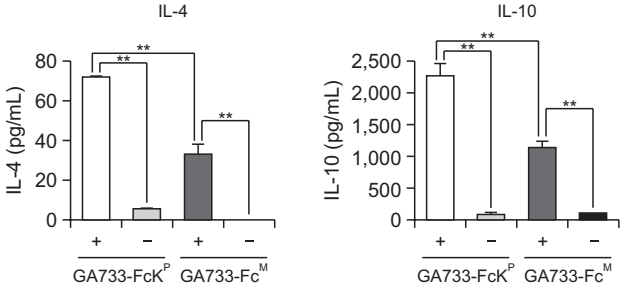

Fig. 6. Analysis of IL-4 and IL-10 in CD4$^+$ Th2 cells obtained from mice injected with EpCAM-FcK^P or EpCAM-Fc^M. EpCAM antigens induced the differentiation of functional effector T cells. CD4$^+$ T cell cytotoxicity was measured by flow cytometry. Nine days after the mice were injected with splenocytes pulsed with EpCAM-FcK or without EpCAM-FcK proteins. Naive DCs were cocultured with BALB/c T cells at a ratio of 1:10 (DC, 0.3×10^5: T cell, 0.3×10^6) in a 96-well U-bottom plate at 37°C. After 72 h, the supernatants were collected, and a CBA assay measured IL-4 and IL-10 levels. The *p*-values<0.01 (**) means a statistically significant difference (unpaired t-test).

specialized microstructure that occurs in secondary lymphoid organs, mainly in response to T cell-dependent antigen immune response (Hao *et al*., 2008). Mature B cells entering the GC undergo editing of their immunoglobulin gene through somatic hypermutation and class-switching recombination and differentiate into memory or plasma cells (Laidlaw and cyster, 2021). The EpCAM-FcK^P injected mice (9.2%) had almost a two times higher percentage of dual positive (GL7$^+$, FAS$^+$) GC B cells compared with the EpCAM-Fc^M injected mice (4.9%) (Fig. 4), suggesting that in the EpCAM-FcK^P injected mice, the EpCAM-FcK^P activated and proliferate B cells becoming a blasting B cell, eventually forming germinal center (Nojima *et al*., 2011). The PBS injected mice (0.96%) had the lowest percentage of differentiated GC B cell population.

T follicular helper (Tfh) cell activation from mouse spleens in response to antigen immunization

CXCR5 and PD1 are major markers of T follicular helper cells (Tfh) (Haynes *et al*., 2007; Ma *et al*., 2012; Jang and Youn, 2013). This result showed that the injections of EpCAM-FcK^P (~42%) resulted in more cells to differentiate to Tfh cells than EpCAM-Fc^M injections (~7%) (Fig. 5) indicating that antigens purified from plants had a higher effect on immunization than antigens derived from mammalian cells.

Analysis of IL-4 and IL-10 cytokine factors in the CD4$^+$ subset of Th2 cells

The levels of IL-4 and IL-10 corresponded with the number of Th2 cells (Ma *et al*., 2012) and were analyzed using supernatant from the cocultured CD4$^+$ Th2 cells isolated from mice injected with EpCAM-FcK^P or EpCAM-Fc^M (Fig. 6). The concentrations of IL-4 and IL-10 were higher in the CD4$^+$ Th2 cells from the mice injected with the EpCAM-FcK^P compared with the mice injected with EpCAM-Fc^M. These results indicate that EpCAM-FcK^P is more effective at inducing IL-4 and IL-10 expression in Th2 cell compared with EpCAM-Fc^M and indicates that antigens purified from plants could produce a more effective immunization than mammalian-derived antigens.

DISCUSSION

This study demonstrated that plant-derived EpCAM-FcK recombinant proteins induced the immune response to produce anti-EpCAM IgGs, the GC activation, and Tfh cell enhancement. The EpCAM-FcK^P protein has been successfully expressed in transgenic tobacco plant leaves (Kim *et al*., 2020). The EpCAM-FcK^P protein was injected into mice in order to

investigate the effect on the protective immune response. The EpCAM has been considered a useful vaccine candidate for colorectal cancer (Verch *et al*., 2004). One of the essential functions of a vaccine is stimulating the molecular immune response. In our previous study, EpCAM-FcK^{P} induced an immune response and produced anti-EpCAM-Fc IgGs in a mouse model (Lu *et al*., 2012). In addition, anti-EpCAM-Fc IgGs inhibited the growth of human colorectal cancer cells that were xenografted on nude mice (Kim *et al*., 2020). However, the effect of EpCAM-FcK^{P} on the immune response at the cell level has not been studied, mainly related to the GC. Thus, we investigated whether EpCAM-FcK^{P} could induce GC activation, which is essential for protective immune response.

In this study, transgenic plants expressing the transgene EpCAM-FcK were grown *in vivo*, and EpCAM-FcK was successfully purified from transgenic plants. The purified EpCAM-FcK^{P} was injected into mice to determine the effects of EpCAM-FcK^{P} on the immune response and activation of the GC. The activation of the GC is an essential process for initiating and modulating an immune response (Stebegg *et al*., 2018; Choi and Morel, 2020). In a kinetic analysis of antigen-antibody interactions using SPR, the EpCAM-FcK^{P} injected mice had a higher RU value than the EpCAM-Fc^{M} injected mice. These results suggest that the EpCAM-FcK^{P} can induce an immune response and produce anti-EpCAM antibodies.

The GC activation is one of the essential cellular responses that contribute to immune responses (Mesin *et al*., 2016; Stebegg *et al*., 2018). The GC is a specialized microstructure within B cell follicles found in secondary lymphoid organs where mature B cells are activated and differentiate into memory B and plasma cells that secrete long-lived antibodies (Hamel *et al*., 2012; Mesin *et al*., 2016; Stebegg *et al*., 2018). In this study, we confirmed that EpCAM-FcK^{P} induced B cell activation and we investigated the B cells highly expressing both GL7^{+} and FAS^{+} B cell activation markers. The mice injected with EpCAM-FcK^{P} had a higher percentage of GL7^{+} FAS^{+} B cells than those mice injected with EpCAM-Fc^{M}, suggesting that EpCAM-FcK^{P} induces B cell activation in the GC.

Tfh cells play a significant role in providing help to B cells to produce antibodies against foreign pathogens and activating the GC (Park *et al*., 2013). GC B cells positively correlate with Tfh cells (Kerfoot *et al*., 2011; Mintz and Cyster, 2020). Furthermore, without the help of Tfh cells, GCs would not form, and there would be no protective immune responses or production of long-lived antibodies secreted by plasma and memory B cells (Kerfoot *et al*., 2011; Stebegg *et al*., 2018; Palm and Henry, 2019). In this study, the expression of CXCR5 and PD1 in spleens from mice injected with EpCAM-FcK^{P} confirmed that EpCAM-FcK^{P} induced Tfh cells in the GC. CXCR5 is required to recruit Tfh cells into the GC, and the increased expression of CXCR5 results in increased Tfh cells entering the follicles and activating B cells. PD1 is found on the surface of Tfh cells and forms synapses with B cells, eventually generating antibody-producing plasma cells (Perreau *et al*., 2013; Barnett *et al*., 2014).

The immunomodulatory cytokine IL-4 upregulates IL-10 in effector CD4^{+} T cells. Tfh cells are a subset of effector CD4^{+} T cells. IL-10 secretion in Tfh cells regulates the emerging GC response within the follicle (Guthmiller *et al*., 2017; Laidlaw *et al*., 2017). In addition, the Tfh regulates the GC response through IL-4 secretion consequently, promoting GC B cell maturation (Tangye *et al*., 2013). In this study, IL-4 and IL-10 expressions in CD4^{+} Th2 cells were higher in the EpCAM-FcK^{P} injected mice than in the EpCAM-Fc^{M} injected mice, indicating that EpCAM-FcK^{P} increased the GC related immune response compared with EpCAM-Fc^{M}. These results were not unexpected since mice injected with EpCAM-FcK^{P} had a stronger immune response than mice injected with EpCAM-Fc^{M} (Lu *et al*., 2012).

This study demonstrated that EpCAM-FcK^{P} is a potential vaccine candidate for colorectal cancer and induced an immune response activating the GC and B cells through Tfh cells. Furthermore, our study suggests that the plant expression system can be a reliable producer of recombinant cancer antigens to be used as vaccines and activate GC B cells for an immune response.

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REFERENCES

Barnett, L. G., Simkins, H. M., Barnett, B. E., Korn, L. L., Johnson, A. L., Wherry, E. J., Wu, G. F. and Laufer, T. M. (2014) B cell antigen presentation in the initiation of follicular helper T cell and germinal center differentiation. *J. Immunol.* **192**, 3607-3617.

Choi, S.-C. and Morel, L. (2020) Immune metabolism regulation of the germinal center response. *Exp. Mol. Med.* **52**, 348-355.

Crotty, S. (2011) Follicular helper CD4 T cells (Tfh). *Annu. Rev. Immunol.* **29**, 621-663.

Guthmiller, J. J., Graham, A. C., Zander, R. A., Pope, R. L. and Butler, N. S. (2017) Cutting edge: IL-10 is essential for the generation of germinal center B cell responses and anti-plasmodium humoral immunity. *J. Immunol.* **198**, 617-622.

Hamel, K. M., Liarski, V. M. and Clark, M. R. (2012) Germinal center B-cells. *Autoimmunity* **45**, 333-347.

Hao, Z., Duncan, G. S., Seagal, J., Su, Y.-W., Hong, C., Haight, J., Chen, N.-J., Elia, A., Wakeham, A. and Li, W. Y. (2008) Fas receptor expression in germinal-center B cells is essential for T and B lymphocyte homeostasis. *Immunity* **29**, 615-627.

Haynes, N. M., Allen, C. D., Lesley, R., Ansel, K. M., Killeen, N. and Cyster, J. G. (2007) Role of CXCR5 and CCR7 in follicular Th cell positioning and appearance of a programmed cell death gene-1high germinal center-associated subpopulation. *J. Immunol.* **179**, 5099-5108.

Jang, E. and Youn, J. (2013) The niche of follicular helper T cells in systemic autoimmune diseases. *Hanyang Med. Rev.* **33**, 1-9.

Kerfoot, S. M., Yaari, G., Patel, J. R., Johnson, K. L., Gonzalez, D. G., Kleinstein, S. H. and Haberman, A. M. (2011) Germinal center B cell and T follicular helper cell development initiates in the interfollicular zone. *Immunity* **34**, 947-960.

Khurana, P., Boeckx, L., Lauriks, W., Leclaire, P., Dazel, O. and Allard, J. F. (2009) A description of transversely isotropic sound absorbing porous materials by transfer matrices. *J. Acoust. Soc. Am.* **125**, 915-921.

Kim, D. S., Kang ,Y. J., Lee, K. J., Qiao, L., Ko, K., Kim, D. H., Myeung, S. C. and Ko, K. (2020) A plant-derived antigen-antibody complex induces anti-cancer immune responses by forming a large quaternary structure. *Int. J. Mol. Sci.* **21**, 5603.

King, C. (2011) A fine romance: T follicular helper cells and B cells. *Immunity* **34**, 827-829.
Laidlaw, B. J. and Cyster, J. G. (2021) Transcriptional regulation of memory B cell differentiation. *Nat. Rev. Immunol.* **21**, 209-220.
Laidlaw, B. J., Lu, Y., Amezquita, R. A., Weinstein, J. S., Vander Heiden, J. A., Gupta, N.,T., Kleinstein, S. H., Kaech, S. M. and Craft, J. (2017) Interleukin-10 from CD4+ follicular regulatory T cells promotes the germinal center response. *Sci. Immunol.* **2**, eaan4767.
Lee, J. H. and Ko, K. (2017) Production of recombinant anti-cancer vaccines in plants. *Biomol. Ther. (Seoul)* **25**, 345-353.
Lim, C.-Y., Lee, K. J., Oh, D.-B. and Ko, K. (2015) Effect of the developmental stage and tissue position on the expression and glycosylation of recombinant glycoprotein GA733-FcK in transgenic plants. *Front. Plant Sci.* **5**, 778.
Lu, Z., Lee, K.-J., Shao, Y., Lee, J.-H., So, Y., Choo, Y.-K., Oh, D.-B., Hwang, K.-A., Oh, S. H., Han, Y. S. and Ko, K. (2012) Expression of GA733-Fc fusion protein as a vaccine candidate for colorectal cancer in transgenic plants. *J. Biomed. Biotechnol.* **2012**, 364240.
Ma, C. S., Deenick, E. K., Batten, M. and Tangye, S. G. (2012) The origins, function, and regulation of T follicular helper cells. *J. Exp. Med.* **209**, 1241-1253.
Mesin, L., Ersching, J. and Victora, G. D. (2016) Germinal center B cell dynamics. *Immunity* **45**, 471-482.
Mintz, M. A. and Cyster, J. G. (2020) T follicular helper cells in germinal center B cell selection and lymphomagenesis. *Immunol. Rev.* **296**, 48-61.
Nahshol, O., Bronner, V., Notcovich, A., Rubrecht, L., Laune, D. and Bravman, T. (2008) Parallel kinetic analysis and affinity determination of hundreds of monoclonal antibodies using the ProteOn XPR36. *Anal. Biochem.* **383**, 52-60.
Naito, Y., Takematsu, H., Koyama, S., Miyake, S., Yamamoto, H., Fujinawa, R., Sugai, M., Okuno, Y., Tsujimoto, G., Yamaji, T., Hashimoto, Y., Itohara, S., Kawasaki, T., Suzuki, A. and Kozutsumi, Y. (2007) Germinal center marker GL7 probes activation-dependent repression of N-glycolylneuraminic acid, a sialic acid species involved in the negative modulation of B-cell activation. *Mol. Cell. Biol.* **27**, 3008-3022.
Nicholson, L. B. (2016) The immune system. *Essays Biochem.* **60**, 275-301.
Nojima, T., Haniuda, K., Moutai, T., Matsudaira, M., Mizokawa, S., Shiratori, I., Azuma, T. and Kitamura, D. (2011) *In-vitro* derived germinal centre B cells differentially generate memory B or plasma cells *in vivo*. *Nat. Commun.* **2**, 465.
Palm, A.-K. E. and Henry, C. (2019) Remembrance of things past: long-term B cell memory after infection and vaccination. *Front. Immunol.* **10**, 1787.
Park, H.-J., Kim, D.-H. and Choi, J.-M. (2013) Germinal center formation controlled by balancing between follicular helper T cells and follicular regulatory T cells. *Hanyang Med. Rev.* **33**, 10-16.
Park, S.-R., Lim, C.-Y., Kim, D.-S. and Ko, K. (2015a) Optimization of ammonium sulfate concentration for purification of colorectal cancer vaccine candidate recombinant protein GA733-FcK isolated from plants. *Front. Plant Sci.* **6**, 1040.
Park, S. R., Lee, J.-H., Kim, K., Kim, T. M., Lee, S. H., Choo, Y.-K., Kim, K. S. and Ko, K. (2020) Expression and *in vitro* function of anti-breast cancer llama-based single domain antibody VHH expressed in tobacco plants. *Int. J. Mol. Sci.* **21**, 1354.
Park, S. R., Shin, Y. K., Lee, K. J., Lee, J. H., Hedin, D., Mulvania, T., Lee, S. H. and Ko, K. (2014) Expression, glycosylation and function of recombinant anti-colorectal cancer mAb CO17-1A in SfSWT4 insect cells. *Entomol. Res.* **44**, 39-46.
Perreau, M., Savoye, A.-L., De Crignis, E., Corpataux, J.-M., Cubas, R., Haddad, E. K., De Leval, L., Graziosi, C. and Pantaleo, G. (2013) Follicular helper T cells serve as the major CD4 T cell compartment for HIV-1 infection, replication, and production. *J. Exp. Med.* **210**, 143-156.
Rich, R. R. and Chaplin, D. D. (2019) The human immune response. In Clinical Immunology, pp. 3-17.e1. Elsevier.
Song, I., Kang, Y. J., Choi, S. L., Han, D., Kim, D. S., Lee, H. K., Lee, J. C., Park, J., Kim, D. S. and Ko, K. (2019) Purification of plant-derived anti-virus mAb through optimized pH conditions for coupling between protein A and epoxy-activated beads. *PeerJ* **7**, e6828.
Song, I., Lee, Y. K., Kim, J. W., Lee, S.-W., Park, S. R., Lee, H. K, Oh, S., Ko, K., Kim, M. K., Park, S. J., Kim, D. H., Kim, M.-S., Kim, D. S. and Ko, K. (2021) Effect of an endoplasmic reticulum retention signal tagged to human anti-rabies mAb SO57 on its expression in *Arabidopsis* and plant growth. *Mol. Cells* **44**, 770-779.
Stebegg, M., Kumar, S. D., Silva-Cayetano, A., Fonseca, V. R., Linterman, M. A. and Graca, L. (2018) Regulation of the germinal center response. *Front. Immunol.* **9**, 2469.
Tangye, S. G., Ma, C. S., Brink, R. and Deenick, E. K. (2013) The good, the bad and the ugly—TFH cells in human health and disease. *Nat. Rev. Immunol.* **13**, 412-426.
Verch, T., Hooper, D. C., Kiyatkin, A., Steplewski, Z. and Koprowski, H. (2004) Immunization with a plant-produced colorectal cancer antigen. *Cancer Immunol. Immunother.* **53**, 92-99.
Yardeni, T., Eckhaus, M., Morris, H. D., Huizing, M. and Hoogstraten-Miller, S. (2011) Retro-orbital injections in mice. *Lab. Anim.* **40**, 155-160.