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). 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
(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-FcKP) 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 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 with Coomassie blue.
with Coomassie brilliant blue R250 (Thermo Fisher Scientific, Waltham, MA, USA). The purchased rhEpCAM-Fc chimera (EpCAM-Fc\textsuperscript{p}) (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\textsuperscript{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-Fc\textsuperscript{p} or the positive control, EpCAM-Fc\textsuperscript{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 \textit{et al.}, 2011). mice were killed 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\textsuperscript{p} or EpCAM-Fc\textsuperscript{M} 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 fluids system using a flow rate of 40 µL/min for 90 s (60 µL). Sera from the mice vaccinated with EpCAM-Fc\textsuperscript{p} or EpCAM-Fc\textsuperscript{M} were injected in the vertical orientation of the ProteOn XPR36 fluids 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 \textit{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 \textit{et al.}, 2009; Park \textit{et al.}, 2014).

**Dendritic cell and CD4\textsuperscript{+} T cell isolation**

Spleens were removed, and single-cell suspensions were
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 generated 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-FcKP or EpCAM-FcM 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, 0.3×10^6; T cell, 0.3×10^6) in 96-well U-bottom plates at 37°C. After 72 hours, 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: 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 expression cassette of for 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 tumefaciens-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).

**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 (~420 RU) was lower than the first harvested serum from mice vaccinated with EpCAM-FcM (~810 RU). In mice vaccinated with EpCAM-FcM, 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-FcK, 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 group was significantly larger than the difference between the second and first harvest in the EpCAM-FcM group indicating that EpCAM-FcK 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, or EpCAM-FcM. 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 + FAS+ are B cell activation markers (Naito et al., 2007; Hao et al., 2008). The GC is a...
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 oyster, 2021). The EpCAM-FcK injected mice (9.2%) had almost two times higher percentage of dual positive (GL7+, FAS+) GC B cells compared with the mice injected with EpCAM-FcM (0.96%) (Fig. 4), suggesting that in the EpCAM-FcK injected mice, the EpCAM-FcK 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 (~42%) resulted in more cells to differentiate to Tfh cells than EpCAM-FcM injections (~7%) (Fig. 5) indicating that antigens purified from plants had a higher effect on immunization than antigens derived from mammalian cells.

**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 protein has been successfully expressed in transgenic tobacco plant leaves (Kim et al., 2020). The EpCAM-FcK 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° 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° on the immune response at the cell level has not been studied, mainly related to the GC. Thus, we investigated whether EpCAM-FcK° could induce GC activation, which is essential for protective immune response.

In this study, transgenic plants expressing the transgene EpCAM-FcK was grown in vivo, and EpCAM-FcK was successfully purified from transgenic plants. The purified EpCAM-FcK° was injected into mice to determine the effects of EpCAM-FcK° 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° injected mice had a higher RU value than the EpCAM-FcM° injected mice. These results suggest that the EpCAM-FcK° 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; Steberg 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 differentiated into memory B and plasma cells that secrete long-lived antibodies (Hamel et al., 2012; Mesin et al., 2016; Steberg et al., 2018). In this study, we confirmed that EpCAM-FcK° 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° had a higher percentage of GL7° FAS° B cells than those mice injected with EpCAM-FcM°, suggesting that EpCAM-FcK° 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; Steberg et al., 2018; Palm and Henry, 2019). In this study, the expression of CXCR5 and PD1 in spleens from mice injected with EpCAM-FcK° confirmed that EpCAM-FcK° 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 (Perreaux 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° injected mice than in the EpCAM-FcM° injected mice, indicating that EpCAM-FcK° increased the GC related immune response compared with EpCAM-FcM°. These results were not unexpected since mice injected with EpCAM-FcK° had a stronger immune response than mice injected with EpCAM-FcM° (Lu et al., 2012).

This study demonstrated that EpCAM-FcK° 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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