

Modification of Fc-fusion protein structures to enhance efficacy of cancer vaccine in plant expression system

Sohee Lim¹ , Hyun Joo Chung², Yoo Jin Oh³, Peter Hinterdorfer³, Soon Chul Myung², Young-Jin Seo⁴ and Kisung Ko^{1,*} 

¹BioSystems Design Lab, Department of Medicine, College of Medicine, Chung-Ang University, Seoul, Korea

²Department of Urology, College of Medicine, Chung-Ang University, Seoul, Korea

³Department of Applied Experimental Biophysics, Johannes Kepler University, Linz, Austria

⁴Department of Life Science, Chung-Ang University, Seoul, Korea

Received 29 October 2024;

accepted 3 December 2024.

*Correspondence (Tel +82-2-820-5666; fax +82-2-813-5387; email ksko@cau.ac.kr)

Summary

Epithelial cell adhesion molecule (EpCAM) fused to IgG, IgA and IgM Fc domains was expressed to create IgG, IgA and IgM-like structures as anti-cancer vaccines in *Nicotiana tabacum*. High-mannose glycan structures were generated by adding a C-terminal endoplasmic reticulum (ER) retention motif (KDEL) to the Fc domain (FcK) to produce EpCAM-Fc and EpCAM-FcK proteins in transgenic plants via *Agrobacterium*-mediated transformation. Cross-fertilization of EpCAM-Fc (FcK) transgenic plants with Joining chain (J-chain, J and JK) transgenic plants led to stable expression of large quaternary EpCAM-IgA Fc (EpCAM-A) and IgM-like (EpCAM-M) proteins. Immunoblotting, SDS–PAGE and ELISA analyses demonstrated that proteins with KDEL had higher expression levels and binding activity to anti-EpCAM IgGs. IgM showed the strongest binding among the fusion proteins, followed by IgA and IgG. Sera from BALB/c mice immunized with these vaccines produced anti-EpCAM IgGs. Flow cytometry indicated that the EpCAM-Fc fusion proteins significantly activated CD8⁺ cytotoxic T cells, CD4⁺ helper T cells and B cells, particularly with EpCAM-FcK^P and EpCAM-Fc^P (FcK^P) × J^P (JK^P). The induced anti-EpCAM IgGs captured human prostate cancer PC-3 and colorectal cancer SW620 cells. Sera from immunized mice inhibited cancer cell proliferation, migration and invasion; down-regulated proliferation markers (PCNA, Ki-67) and epithelial–mesenchymal transition markers (Vimentin); and up-regulated E-cadherin. These findings suggest that *N. tabacum* can produce effective vaccine candidates to induce anti-cancer immune responses.

Keywords: cancer vaccine, EpCAM, colorectal cancer, prostate cancer, Fc-fusion protein, plant crossing.

Introduction

The epithelial cell adhesion molecule (EpCAM) is a transmembrane glycoprotein recognized as a tumour-associated antigen, initially identified in colon carcinomas (Herlyn *et al.*, 1979; Went *et al.*, 2004). While EpCAM is expressed at low levels in normal human tissues, its expression is significantly up-regulated in various human carcinomas, including colorectal and prostate cancers (Van der Gun *et al.*, 2010; Went *et al.*, 2004). EpCAM is a promising prognostic and diagnostic marker and an immunotherapeutic antibody and vaccine target for various human cancers (Van der Gun *et al.*, 2010).

Fc-fusion proteins consist of an immunoglobulin (Ig) Fc region directly linked to an antigenic protein, serving as a vaccine platform. Most Fc-fusion proteins are based on the IgG Fc region, forming homodimers that enhance immunogenicity through Fc receptor (FcR) interactions on immune cells, such as antigen-presenting cells (APCs) (Chomczynski and Mackey, 1995; Nimmerjahn and Ravetch, 2008). These fusion proteins improve antigen stability and solubility, facilitate cost-effective purification via Fc-specific affinity chromatography and extend the therapeutic proteins' half-life (Carter, 2011; Kang *et al.*, 2023; Lu *et al.*, 2012; Oh *et al.*, 2023).

Plant expression systems do not require cold storage, are scalable for large-scale production and lack pathogenic animal

contaminants (Ko, 2014; Laere *et al.*, 2016; Lu *et al.*, 2012; Rigano and Walmsley, 2005; Takeyama *et al.*, 2015). Additionally, plant systems facilitate post-translational modifications, such as *N*-glycosylation, which are crucial for biological activity. These systems can be engineered to avoid allergenic plant-specific glycans, such as $\beta(1,2)$ -xylose and $\alpha(1,3)$ -fucose, using the Lys-Asp-Glu-Leu (KDEL) endoplasmic reticulum (ER) retention motif (Fischer *et al.*, 2004; Lu *et al.*, 2012; Munro and Pelham, 1987). Several plant-derived vaccines have been produced, with some advancing to clinical trials (Laere *et al.*, 2016; Takeyama *et al.*, 2015).

This study aims to produce a large quaternary structural cancer vaccine by modifying plant protein and glycan structures. Specifically, we fused EpCAM to the Fc regions of IgG, IgA or IgM, which naturally exist in monomeric (IgG), dimeric (IgA) and pentameric or hexameric (IgM) forms, respectively. The resulting vaccine features high-mannose glycan structures devoid of plant-specific residues, achieved by tagging the C terminus of the Fc region with an ER retention motif.

In this study, we produced the plant-derived EpCAM-Fc (FcK) and EpCAM-Fc (FcK) × J (JK) [referred to as EpCAM-Fc^P (FcK^P) and EpCAM-Fc^P (FcK^P) × J^P (JK^P)] as effective cancer vaccine candidates using the Fc regions of IgA or IgM combined with the KDEL ER retention motif in *Nicotiana tabacum*. These polymeric immunogens may enhance the immune response by delivering

multiple copies of EpCAM proteins to APCs (Kang *et al.*, 2023). The characterization and functionality of the EpCAM-Fc^P (FcK^P) and EpCAM-Fc^P (FcK^P) × J^P (JK^P) proteins were investigated both *in vitro* and *in vivo*.

Results

Generation of transgenic *N. tabacum* plants expressing EpCAM-Fc fusion proteins and J-chain

Transgenic lines expressing various EpCAM-Fc fusion proteins [EpCAM-IgG Fc (EpCAM-G), EpCAM-IgG FcK (EpCAM-GK), EpCAM-IgA Fc (EpCAM-A), EpCAM-IgA FcK (EpCAM-AK), EpCAM-IgM Fc (EpCAM-M) and EpCAM-IgM FcK (EpCAM-MK)] and Joining chain (J-chain, J and JK) were generated through *Agrobacterium*-mediated transformation (Figure 1). The EpCAM-Fc proteins were designed by fusing the Fc regions of IgG, IgA and IgM to EpCAM, incorporating an ER retention motif (KDEL) to ensure retention within the ER (Figure 1). The insertion, transcription and expression of EpCAM-Fc (FcK) and J (JK) genes in transgenic plants were confirmed using polymerase chain reaction (PCR) (Figure S1), RT-PCR (Figure S1) and immunoblot analyses (Figure 2). PCR was performed on three randomly selected transgenic lines to validate transgene insertion (Figure S1, left panels). Semi-quantitative RT-PCR confirmed mRNA expression in these plants (Figure S1, right panels). Immunoblot analysis demonstrated protein expression across the tested EpCAM-Fc (FcK) and J (JK) transgenic lines. Notably, the EpCAM proteins tagged with KDEL exhibited higher expression levels compared to those without KDEL, although EpCAM-MK and EpCAM-M showed similar expression levels.

Generation of F₁ transgenic plants through cross-fertilization

Following confirmation of protein expression in the EpCAM-Fc and EpCAM-FcK transgenic plants, lines exhibiting high protein levels were selected and transferred them to soil pots for greenhouse cultivation. Transgenic plants expressing EpCAM-A (Figure 3a), EpCAM-AK (Figure 3b), EpCAM-M (Figure 3c) and EpCAM-MK (Figure 3d) were cross-fertilized with J or JK transgenic plants (Figure 3), yielding F₁ lines, including EpCAM-AJ, EpCAM-AJK, EpCAM-AKJ, EpCAM-AKJK, EpCAM-MJ, EpCAM-MJK, EpCAM-MKJ and EpCAM-MKJK, corresponding to dimeric and pentameric structures (Figure 3). The F₁ seeds were germinated on a Murashige and Skoog (MS) medium containing kanamycin, and F₁ seedlings were used for subsequent experiments.

Expression and purification of F₁ transgenic plants from cross-fertilization

The EpCAM-Fc (FcK) × J (JK) gene transcription and expression were confirmed in the F₁ plants through RT-PCR and immunoblot analyses (Figure 4). RT-PCR analysis revealed EpCAM mRNA in the F₁ plants (Figure 4a). Specifically, the IgA Fc band was detected only in the EpCAM-A and EpCAM-AK F₁ plants, while the IgM Fc band was observed exclusively in the EpCAM-M and EpCAM-MK F₁ plants (Figure 4a). The J-chain band was present in all F₁ plants, indicating successful construct generation. Protein expression was confirmed for the EpCAM-Fc (FcK) × J (JK) in the F₁ lines. Notably, unlike the EpCAM-Fc (FcK) transgenic lines, the F₁ lines did not significantly differ in protein expression levels, regardless of the presence of the KDEL tag (Figure 4b).

Following confirmation of protein expression levels in F₁ transgenic plants expressing EpCAM-Fc (FcK) × J (JK), high-expression lines were selected for further analysis. These selected *in vitro* F₁ transgenic plants were propagated *in vitro* and subsequently transferred them to soil pots for leaf biomass production. The EpCAM-Fc^P (FcK^P) × J^P (JK^P) proteins were purified from *N. tabacum* leaves (400 g). This yielded EpCAM-G^P (1.6 mg), EpCAM-GK^P (19.2 mg), EpCAM-A^P (3.3 mg), EpCAM-AK^P (5.1 mg), EpCAM-M^P (4.96 mg) and EpCAM-MK^P (6.14 mg) per kilogram of harvested leaves. Purified proteins from the eluted fractions were analysed using SDS-PAGE stained with Coomassie Brilliant Blue (Figure S2), showing that greater quantities of EpCAM-FcK^P proteins were obtained than those of EpCAM-Fc^P proteins. The yields of purified EpCAM-AJ^P, EpCAM-AJK^P, EpCAM-AKJ^P, EpCAM-AKJK^P, EpCAM-MJ^P, EpCAM-MJK^P, EpCAM-MKJ^P and EpCAM-MKJK^P were 3.42, 2.12, 2, 4.32, 4.28, 2.6, 5.1 and 3.24 mg per kilogram of leaves, respectively (Figure S3).

ELISA to confirm binding of anti-human EpCAM IgG to EpCAM-Fc^P (FcK^P) or EpCAM-Fc^P (FcK^P) × J^P (JK^P)

ELISA analysis was used to assess anti-human EpCAM IgG binding to EpCAM-Fc^P (FcK^P) and EpCAM-Fc^P (FcK^P) × J^P (JK^P) (Figure 5). Proteins, including EpCAM-Fc^P (FcK^P), EpCAM-Fc^P (FcK^P) × J^P (JK^P), mammalian-derived EpCAM-IgG Fc (EpCAM-G^M) and recombinant 6× His tag-EpCAMK (H6-EpCAMK^P) (Figure S4), were coated at 100 ng per well on 96-well Maxisorp Nunc-immuno plates. The plates were subsequently treated with an anti-human EpCAM antibody. The overall binding activity was ranked as follows: EpCAM-G^P < H6-EpCAMK^P = EpCAM-GK^P < EpCAM-A^P < EpCAM-AKJK^P < EpCAM-M^P < control (EpCAM-G^M) < EpCAM-AK^P < EpCAM-AJ^P < EpCAM-MK^P < EpCAM-MJ^P < EpCAM-MKJK^P. Comparing EpCAM-Fc^P and EpCAM-FcK^P, the strongest binding was M type > A type > G type. For the EpCAM-Fc^P (FcK^P) × J^P (JK^P), the binding activity was EpCAM-AKJK^P < EpCAM-AJ^P < EpCAM-MJ^P < EpCAM-MKJK^P. Overall, EpCAM-Fc^P (FcK^P) and EpCAM-Fc^P (FcK^P) × J^P (JK^P) exhibited stronger binding than H6-EpCAMK^P, except EpCAM-G^P. M-type proteins demonstrated the highest binding activity, followed by A-type proteins and then G-type proteins.

Induction of anti-EpCAM IgGs in BALB/c mice immunized with EpCAM-Fc^P (FcK^P) and EpCAM-Fc^P (FcK^P) × J^P (JK^P) proteins

To evaluate the immunological efficacy of EpCAM-Fc^P (FcK^P) and EpCAM-Fc^P (FcK^P) × J^P (JK^P) proteins, BALB/c mice were immunized with purified recombinant proteins (5 µg) and aluminium hydroxide adjuvant via intraperitoneally (IP) injection, administered four times at 2-week intervals (Figure 6a). Blood samples were collected via retro-orbital bleeding 10 days after the second immunization to assess anti-EpCAM antibody induction. A second set of blood samples was collected 10 days after the fourth injection, and the spleens were harvested (Figure 6a). ELISA analysis was conducted to measure the levels of anti-EpCAM IgGs in the sera collected after the second immunization (Figure 6b). 96-well plates were coated with H6-EpCAMK^P proteins, incubated with sera from immunized mice as the primary antibody and subsequently treated the solution with an anti-murine IgG heavy chain (HC)-conjugated horseradish peroxidase (HRP) antibody for detection. The order of antibody induction in the blood samples after the second injection was EpCAM-G^M = EpCAM-G^P < EpCAM-GK^P < EpCAM-A^P < EpCAM-

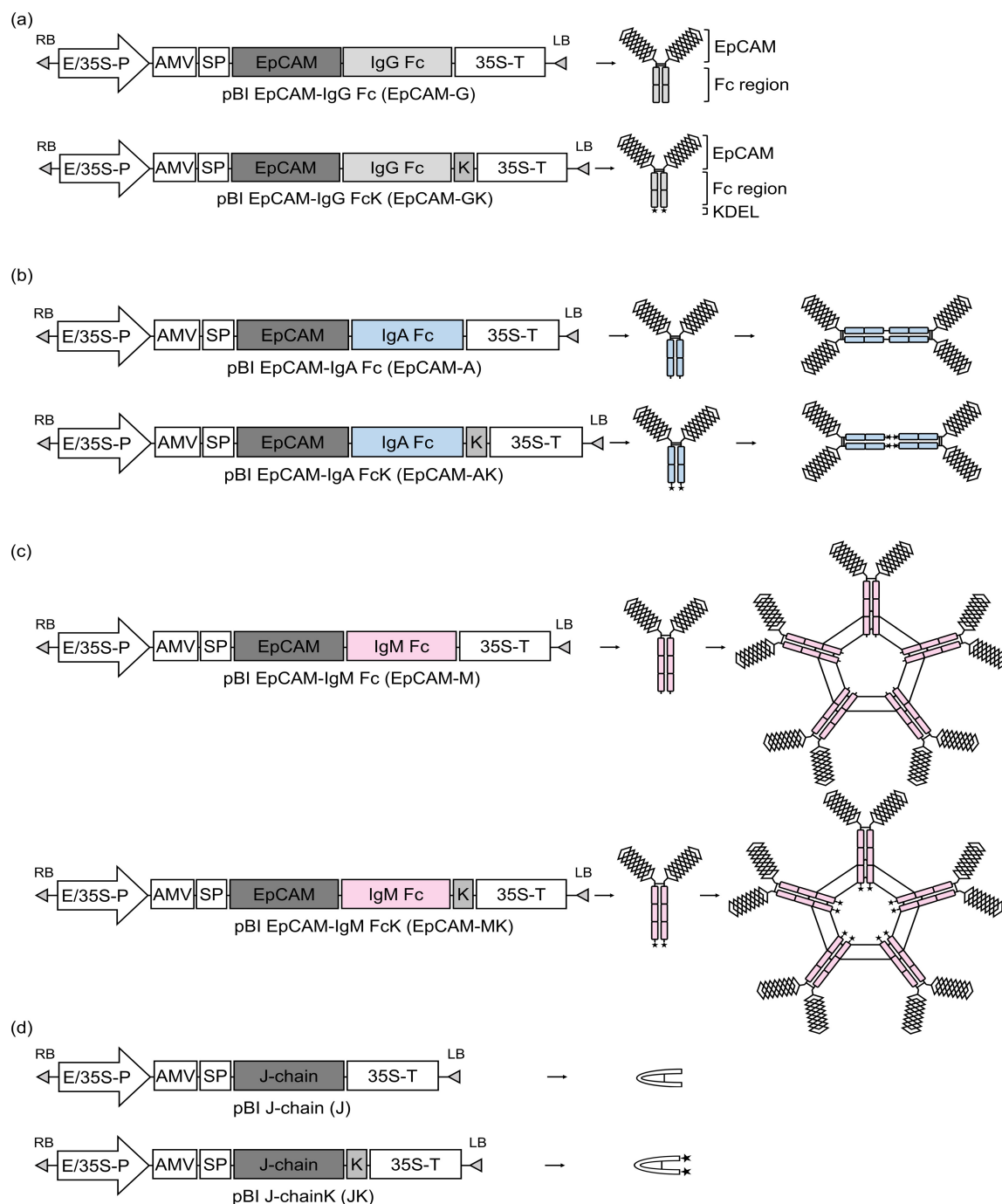


Figure 1 Schematic diagram of the gene expression cassettes in plant expression vectors and the protein structures of EpCAM-Fc (FcK) and J (JK) fusion proteins. This figure illustrates the gene expression cassettes for various EpCAM fusion proteins designed for *Agrobacterium*-mediated plant transformation. (a) EpCAM-IgG Fc (EpCAM-G) and EpCAM-IgG FcK (EpCAM-GK). (b) EpCAM-IgA Fc (EpCAM-A) and EpCAM-IgA FcK (EpCAM-AK). (c) EpCAM-IgM Fc (EpCAM-M) and EpCAM-IgM FcK (EpCAM-MK). (d) J and JK fusion proteins. Each construct was incorporated into the plant expression vector pBI 121. The components include E/35S-P, enhanced duplicated Cauliflower Mosaic Virus 35S promoter; AMV, untranslated leader sequence from RNA4 of Alfalfa Mosaic Virus; SP, signal peptide for the ER; K, KDEL ER retention motif; 35S-T, 35S gene terminator from Cauliflower Mosaic Virus. We depicted the expected structures of the recombinant EpCAM-Fc fusion proteins to highlight their configurations and functionalities.

$AJ^P < EpCAM-AK^P < EpCAM-MK^P = EpCAM-MJ^P < EpCAM-AKJK^P = EpCAM-M^P < EpCAM-MKJK^P$ (Figure 6b). Generally, proteins with the KDEL tag showed higher anti-EpCAM

IgGs induction compared to those without. M-type proteins exhibited the strongest induction. A-type proteins were more effective than G-type proteins.

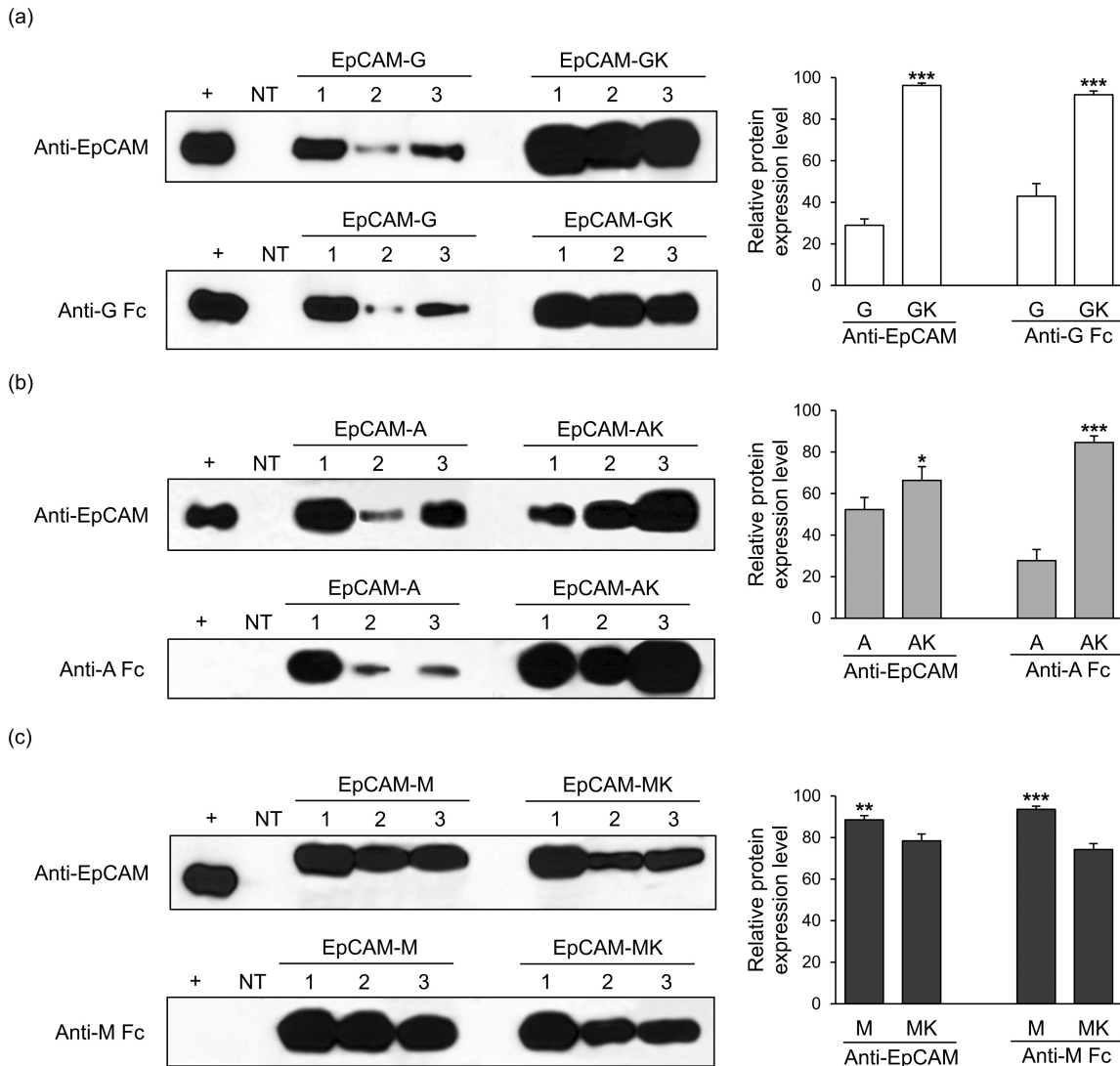


Figure 2 Immunoblot analysis of recombinant EpCAM-G, EpCAM-GK, EpCAM-A, EpCAM-AK, EpCAM-M and EpCAM-MK proteins in transgenic *N. tabacum* plants. This figure presents the immunoblot analysis that confirms the expression of EpCAM proteins fused with various immunoglobulin Fc regions in transgenic *N. tabacum* plants. Leaf samples from each transgenic plant were homogenized in 1 × PBS for analysis. The recombinant EpCAM-Fc and EpCAM-FcK fusion proteins using (a) murine anti-human EpCAM antibody (upper panel) and goat anti-human IgG Fc antibody (lower panel), (b) murine anti-human EpCAM antibody (upper panel) and goat anti-human IgG Fc antibody (lower panel) and (c) murine anti-human EpCAM antibody (upper panel) and goat anti-human IgM μ chain antibody (lower panel). Relative protein expression levels of EpCAM and each Fc region are shown in graphs (a, b, c) as mean $\pm$ standard deviation. +, positive control (commercial EpCAM-IgG Fc); NT, negative control (non-transgenic *N. tabacum* plant); 1–3, transgenic lines expressing the respective proteins; G, A, M, EpCAM-G, EpCAM-A and EpCAM-M (without KDEL motif), respectively; GK, AK, MK, EpCAM-GK, EpCAM-AK and EpCAM-MK (with KDEL motif), respectively. Statistical significance was determined using a Student's *t*-test to compare two groups. Asterisks indicate significance levels: **P* < 0.05, ***P* < 0.01 and ****P* < 0.001.

Moreover, proteins that underwent cross-fertilization with J demonstrated superior immunogenicity compared to J-free proteins. ELISA analysis was performed on sera collected 10 days post-immunization to confirm the enhanced induction of anti-EpCAM IgGs following the fourth immunization (Figure 6c). The order of IgG induction from the immunized mice was EpCAM-G^M < EpCAM-G^P < EpCAM-GK^P < EpCAM-AJ^P < EpCAM-A^P < EpCAM-AK^P < EpCAM-AKJK^P < EpCAM-M^P < EpCAM-MK^P < EpCAM-MKJK^P < EpCAM-MJ^P (Figure 6c). Proteins with the KDEL tag generally had better induction effects than those without. Consistent with earlier results, M-type proteins led in induction,

followed by A-type proteins, then G-type proteins. Additionally, proteins cross-fertilized with J exhibited superior immunogenicity compared to J-free proteins.

CD4⁺ and CD8⁺ T-cell responses in immunized mice

Flow cytometry analysis was performed to assess the activation of CD8⁺ cytotoxic T lymphocytes (gated on CD3⁺ CD69⁺ CD4[−] CD8⁺) and CD4⁺ helper T lymphocytes (gated on CD3⁺ CD69⁺ CD8[−] CD4⁺) isolated from the blood and spleens of mice immunized with EpCAM-Fc^P (Fck^P) or EpCAM-Fc^P (Fck^P) × J^P (JK^P) 10 days following the second and fourth immunizations

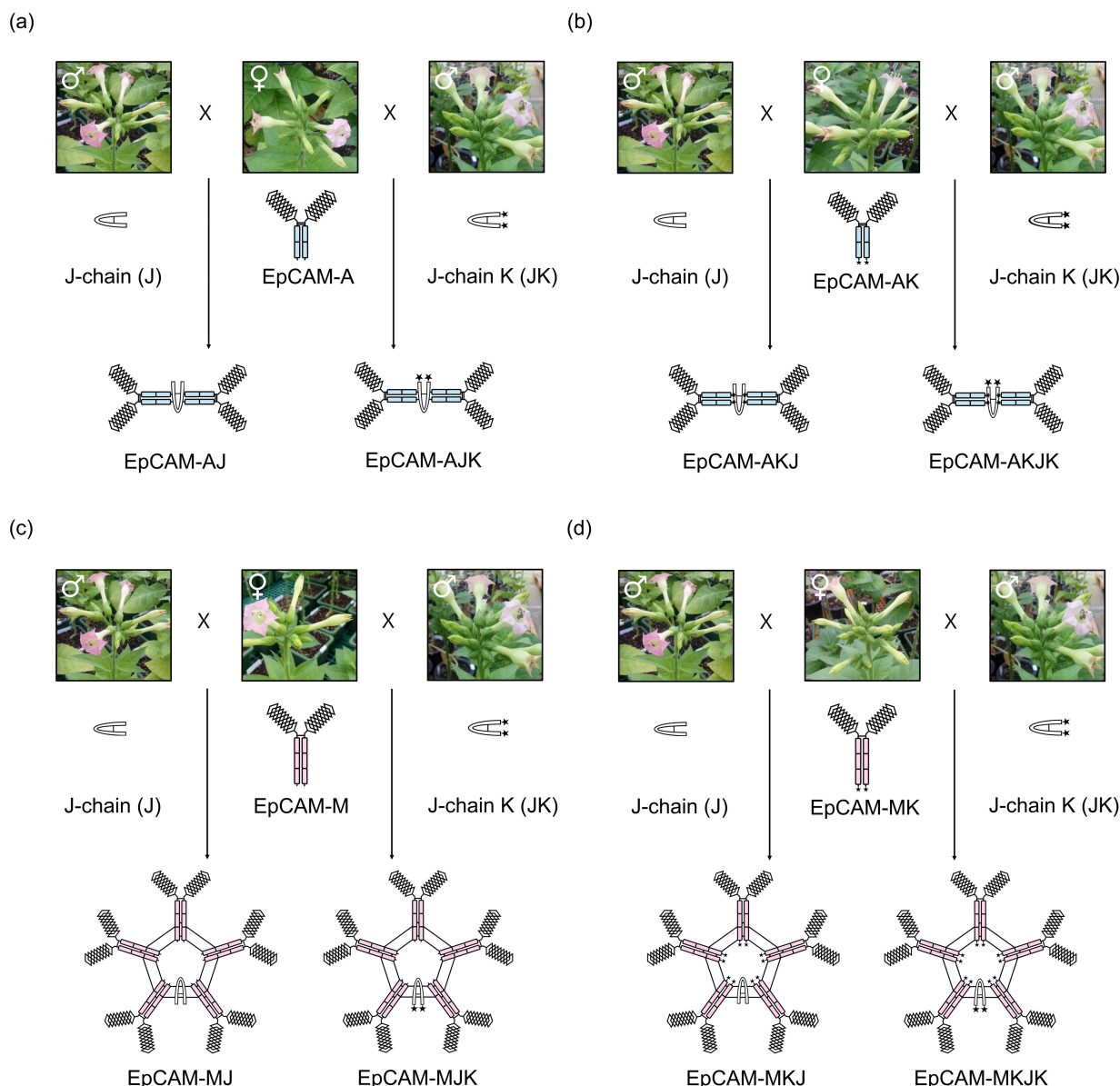


Figure 3 Cross-fertilization of transgenic plants expressing EpCAM-Fc proteins. This figure illustrates the cross-fertilization of transgenic plants expressing EpCAM-Fc variants (EpCAM-A, EpCAM-AK, EpCAM-M and EpCAM-MK) with J and JK transgenic plants. The resulting F₁ seedlings expressed various combinations: EpCAM-AJ, EpCAM-AJK, EpCAM-AKJ, EpCAM-AKJK, EpCAM-MJ, EpCAM-MJK, EpCAM-MKJ and EpCAM-MKJK. (a) Transgenic plants expressing EpCAM-A. (b) Transgenic plants expressing EpCAM-AK. (c) Transgenic plants expressing EpCAM-M. (d) Transgenic plants expressing EpCAM-MK. The aim of each cross-fertilization was to generate seedlings with the desired combinations of EpCAM-Fc fusion proteins.

(Figure 7a–d). The lymphocytes were stained with anti-murine CD4, CD8, CD3 and CD69 antibodies to identify T cells, with CD69 as an early marker of activation (Cibrian and Sanchez-Madrid, 2017). CD8⁺ cytotoxic T cells in mice immunized with EpCAM-Fc^P (FcK^P) were slightly activated (Figure 7b, left). In contrast, CD8⁺ cytotoxic T cells in mice immunized with EpCAM-Fc^P (FcK^P) × J^P (JK^P) were significantly activated. CD4⁺ helper T cells in the group immunized twice with EpCAM-Fc^P (FcK^P) were not activated, whereas those immunized with EpCAM-Fc^P (FcK^P) × J^P (JK^P) were significantly activated, similar to CD8⁺ T cells (Figure 7b, right). Flow cytometry was conducted on splenocytes from the fourth immunization to confirm CD4⁺ and CD8⁺ T-cell responses (Figure 7c,d). Isolated splenocytes were stained and analysed, revealing that

CD8⁺ and CD4⁺ T cells from mice immunized with EpCAM-Fc^P (FcK^P) and EpCAM-Fc^P (FcK^P) × J^P (JK^P) were significantly activated (Figure 7d). CD4⁺ helper T cells from mice immunized with M-type proteins (excluding EpCAM-M) demonstrated significant activation compared to other proteins (Figure 7d, right).

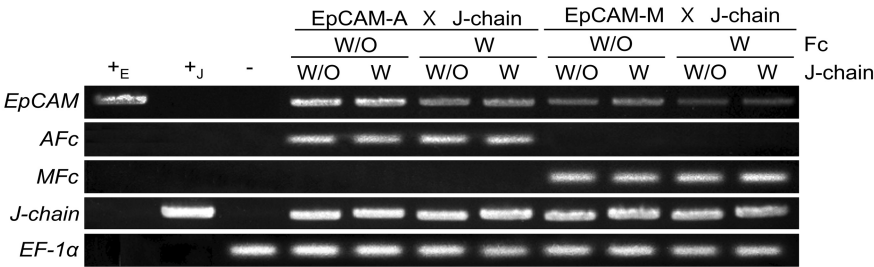
CD80⁺ and CD86⁺ B-cell responses in immunized mice

Flow cytometry analysis was also utilized to evaluate B-cell (gated on CD3[−] CD19⁺) activation in the blood and splenocytes of immunized mice 10 days after the second and fourth immunizations with EpCAM-Fc^P (FcK^P) and EpCAM-Fc^P (FcK^P) × J^P (JK^P) (Figure 8a–d). Lymphocytes were isolated from the blood of immunized mice 10 days post-second immunization (Figures 6a

and 8a,b). The isolated lymphocytes were stained with anti-murine CD3, CD19, CD80 and CD86 antibodies to detect B cells. B cells from mice immunized with EpCAM-G^P, EpCAM-A^P and EpCAM-M^P proteins exhibited significant activation of CD80 and CD86. B cells from those immunized with all EpCAM-Fc^P and EpCAM-Fc^P (FcK^P) × J^P (JK^P) variants were inactivated

(Figure 8b). Notably, B cells from mice immunized with EpCAM-Fc^P (FcK^P) × J^P (JK^P) showed significant inactivation. These findings indicate a correlation between T-cell and B-cell activation. Mice with activated T cells exhibited inactivated B cells, suggesting that signals generated by T cells had not yet been transmitted to B cells.

(a)



(b)

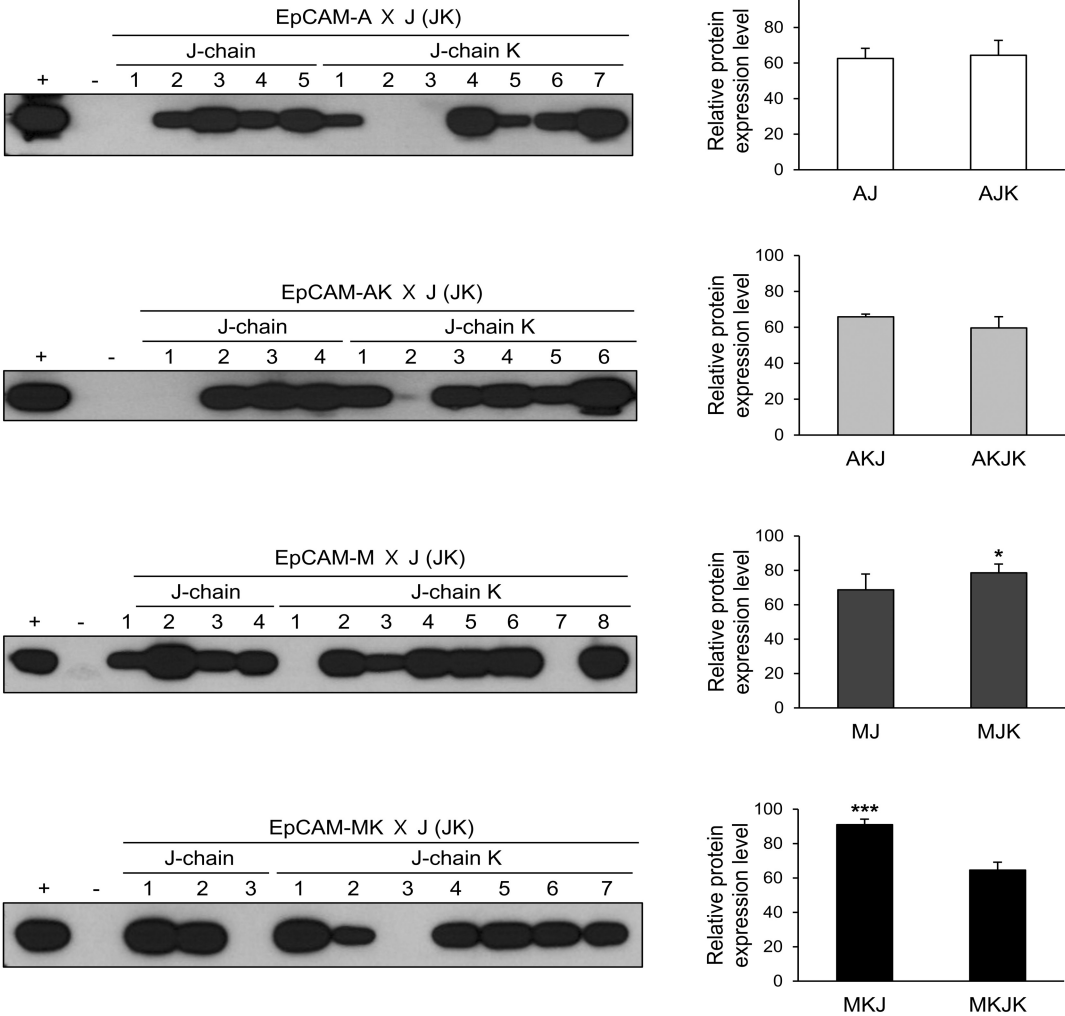


Figure 4 RT-PCR and immunoblot analyses to confirm transcription and protein expression of EpCAM-AJ, EpCAM-AJK, EpCAM-AKJ, EpCAM-AKJK, EpCAM-MJ, EpCAM-MJK, EpCAM-MKJ and EpCAM-MKJK in F₁ plants. (a) RT-PCR analysis to confirm mRNA transcription of transgenes in selected F₁ plants. RT-PCR was performed to confirm the mRNA transcription of the *EpCAM* gene and different immunoglobulin Fc regions and *J* genes in F₁ transgenic plants. Each gene's relative transcription level was normalized to the geometric mean of the internal control gene *EF-1α*. In EpCAM-A × J-chain, W/O (W/O): *EpCAM-AJ*, W/O (W): *EpCAM-AJK*, W (W/O): *EpCAM-AKJ*, W (W): *EpCAM-AKJK*. In EpCAM-M × J-chain, W/O (W/O): *EpCAM-MJ*, W/O (W): *EpCAM-MJK*, W (W/O): *EpCAM-MKJ*, W (W): *EpCAM-MKJK* transgene; +_E and +_J: positive control, pBI EpCAM-G and pBI JK in *DH5α* each; −: negative control, non-transgenic *N. tabacum* plant. 5 μL of each sample was loaded. (b) Immunoblot analysis was performed to confirm the expression of recombinant proteins in F₁ plants. Leaf samples from each F₁ transgenic plant were ground with 1 × PBS. The recombinant EpCAM-Fc (FcK) × J (JK) fusion proteins were detected using murine anti-human EpCAM antibody. +, positive control, commercial EpCAM-IgG Fc; −, negative control, non-transgenic *N. tabacum* plant; Number, the number of F₁ line.

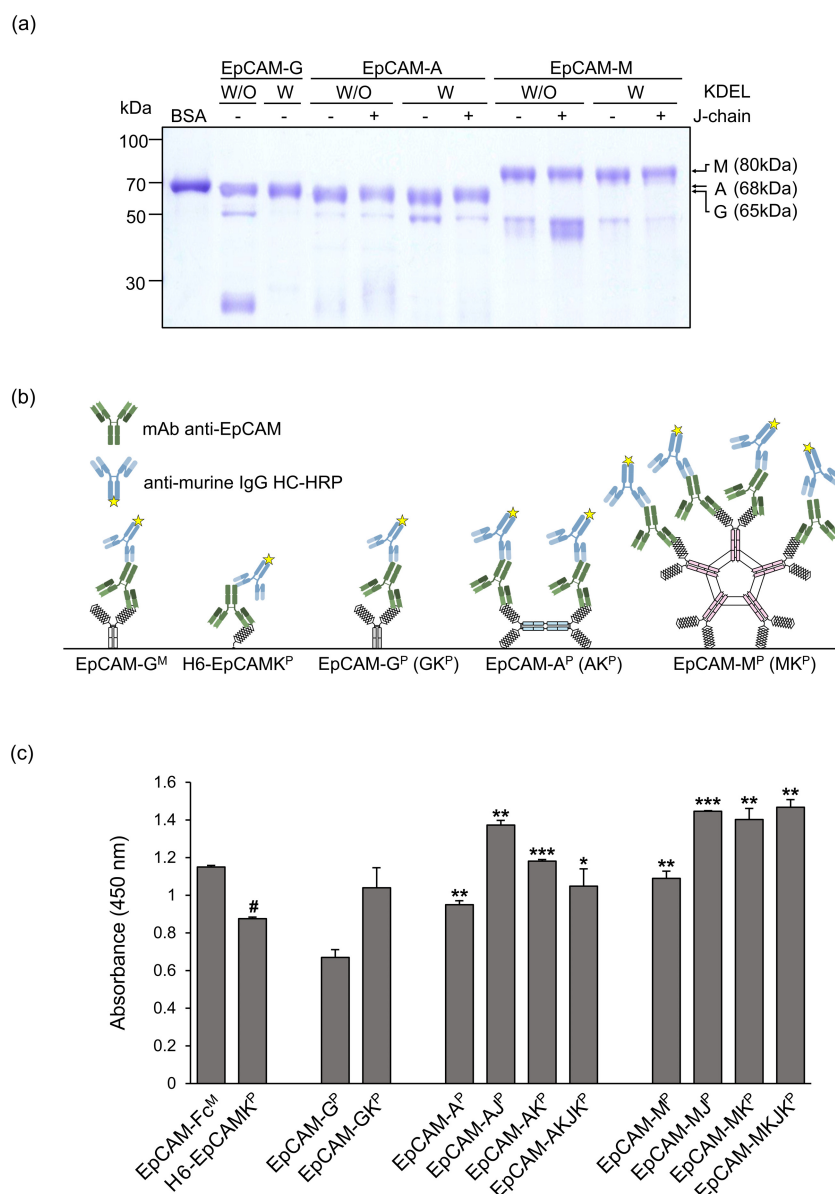


Figure 5 ELISA analysis to measure the binding affinity of EpCAM-Fc^P (FcK^P) or EpCAM-Fc^P (FcK^P) × J^P (JK^P) to anti-human EpCAM antibody. (a) SDS-PAGE analysis to compare relative quantity and purity of EpCAM-Fc^P (FcK^P) or EpCAM-Fc^P (FcK^P) × J^P (JK^P) from plant leaf biomass. All protein samples (2 μg) were loaded and stained with Coomassie Brilliant Blue. (b) A schematic diagram of indirect ELISA is provided to confirm the binding activity of EpCAM-Fc^P (FcK^P) or EpCAM-Fc^P (FcK^P) × J^P (JK^P) to anti-human EpCAM antibody. Each purified EpCAM-Fc^P (FcK^P) or EpCAM-Fc^P (FcK^P) × J^P (JK^P) recombinant protein (100 ng/well) was coated on the 96-well immuno plates. The primary antibody used was murine anti-EpCAM antibody, and the secondary antibody was HRP-conjugated goat anti-murine IgG HC. (c) Optical density levels for binding affinity are presented as the mean ± standard deviation from three independent experiments. Statistical significance was assessed using a Student's *t*-test, comparing each group to the control group (#). Asterisks indicate the following levels of significance: **P* < 0.05, ***P* < 0.01 and ****P* < 0.001.

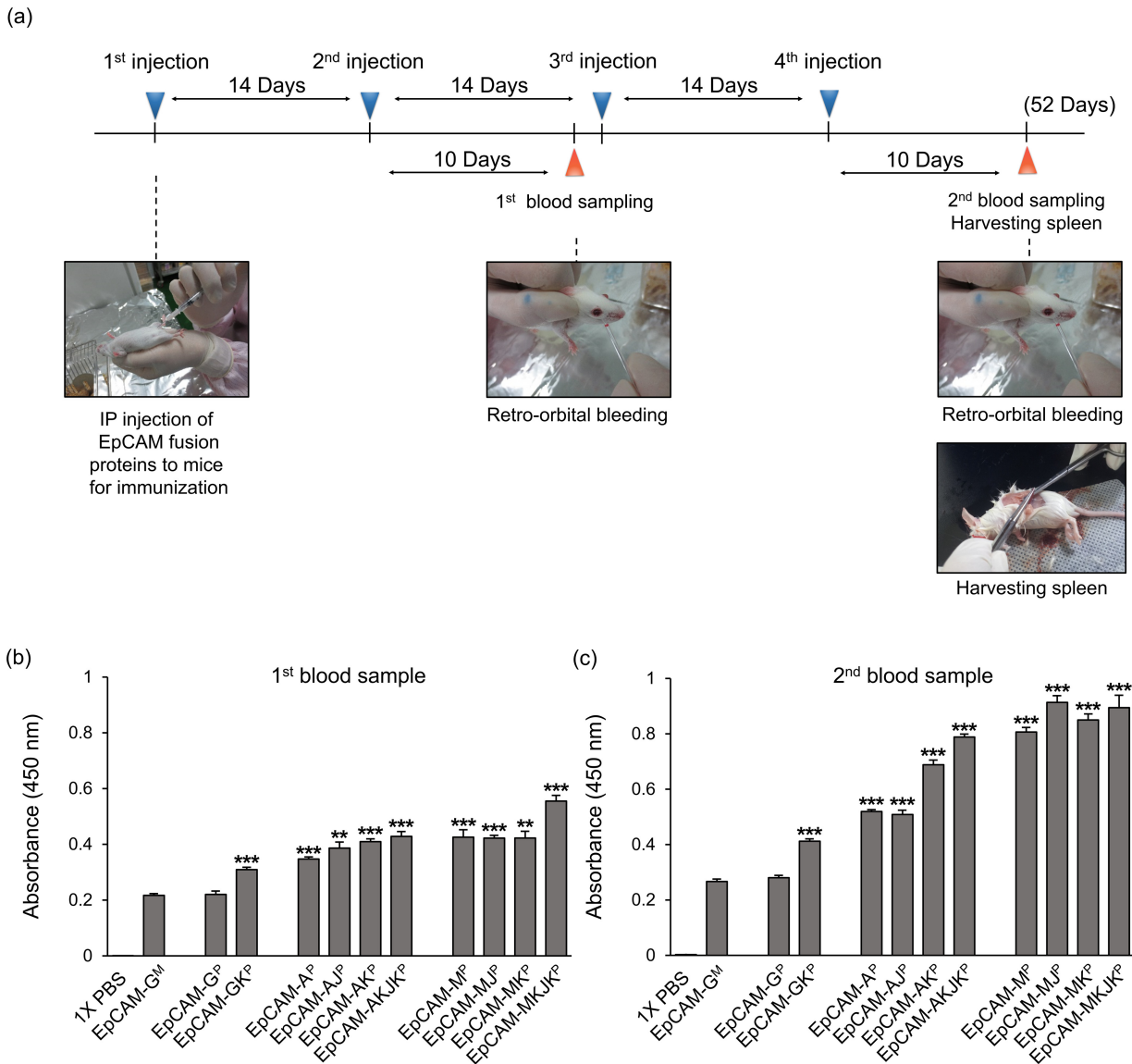


Figure 6 ELISA analysis to confirm induction of anti-EpCAM IgGs in sera of BALB/c mice with the second and fourth immunizations. (a) Experimental design for murine immunization of EpCAM-Fc^P (FcK^P) or EpCAM-Fc^P (FcK^P) × J^P (JK^P). Six-week-old female BALB/c mice were maintained in a pathogen-free environment. Seven-week-old female BALB/c mice were immunized IP four times at 2-week intervals with 5 µg of the EpCAM-Fc^P (FcK^P) or EpCAM-Fc^P (FcK^P) × J^P (JK^P) with aluminium hydroxide adjuvant. (b) The first blood samples were collected 10 days after the second immunization via retro-orbital bleeding. (c) The second blood samples were collected 10 days after the fourth injection via retro-orbital bleeding, and the spleens were harvested simultaneously. The existence of anti-EpCAM antibodies in the sera from the second (b) and fourth (c) immunized mice was confirmed by ELISA. H6-EpCAMK proteins were used as solid-phase antigens on 96-well ELISA plates, and incubations were conducted with positive and negative controls and sera harvested 10 days after the second (b) and fourth (c) immunizations. Optical density levels of 1:2000 serum dilutions are presented as the mean ± standard deviation from three independent experiments, including five mice per group. Statistical significance was assessed using a Student's *t*-test to compare each group to the control group. Asterisks indicate the following levels of significance: **P* < 0.05, ***P* < 0.01 and ****P* < 0.001.

Conversely, T cells were inactivated in mice with activated B cells, indicating successful signal transmission from T cells to B cells. To further investigate the B-cell immune response in BALB/c mice spleens, flow cytometry analysis was conducted 10 days after the fourth immunization with either EpCAM-Fc^P (FcK^P) or EpCAM-Fc^P (FcK^P) × J^P (JK^P) (Figure 8c,d). Splenocytes were isolated and stained for B-cell analysis (gated on CD3⁺ CD19⁺) (Figure 8c). All B cells from the immunized mice receiving EpCAM-Fc^P (FcK^P) and EpCAM-Fc^P (FcK^P) × J^P (JK^P) proteins were activated (Figure 8d). Notably, B cells from mice immunized with EpCAM-Fc^P (FcK^P) × J^P (JK^P) exhibited significant activation

(Figure 8d). Unlike the second immunization, T and B cells were activated following the fourth immunization. T-cell and B-cell responses were significantly enhanced with the fourth immunization, demonstrating the immunogenic potential of the tested vaccine candidates.

***In vitro* activation of murine bone marrow-derived dendritic cells (BMDCs) treated with EpCAM-Fc^P (FcK^P) or EpCAM-Fc^P (FcK^P) × J^P (JK^P)**

Dendritic cells (DCs), potent APCs, play a critical role in initiating immune responses by activating naïve T cells (Wang *et al.*, 2016).

Flow cytometry was performed to confirm the *in vitro* activation of BMDCs obtained from the bone marrow (BM) of mice treated with EpCAM-Fc^P (FcK^P) or EpCAM-Fc^P (FcK^P) × J^P (JK^P) (Figure 9). For activation, murine BMDCs were incubated in a medium containing either EpCAM-Fc^P (FcK^P) or EpCAM-Fc^P (FcK^P) × J^P (JK^P) proteins for 2 days. The activated BMDCs (gated on CD11c⁺ MHC II⁺) were stained with anti-murine CD11c, anti-murine MHC II and anti-murine CD86 antibodies for flow cytometric analysis. The results indicated that all incubated BMDCs treated with EpCAM-Fc^P (FcK^P) and EpCAM-Fc^P (FcK^P) × J^P (JK^P) proteins were activated. Notably, BMDCs treated with EpCAM-MKJK^P exhibited the highest activation levels, as evidenced by increased MHC II and CD86 expression.

***In vivo* immune response following activation of murine BMDCs with EpCAM-MK^P and EpCAM-MKJK^P**

Among the plant-derived EpCAM recombinant proteins, EpCAM-MKJK^P demonstrated the most effective *in vitro* DC activation (Figure 9). Therefore, an *in vivo* experiment was conducted using EpCAM-MKJK^P to assess its effects on immune response (Figure 10). We intravenously (IV) injected 7-week-old female mice with activated BMDCs (10⁶ cells) treated with negative control (1 × PBS), positive control (R848), EpCAM-MK^P, EpCAM-MK^P + R848, EpCAM-MKJK^P and EpCAM-MKJK^P + R848. Then, CD86 and MHC II expression were measured to evaluate the activation of each treated BMDC via flow cytometry. BMDCs (gated on CD11c⁺) were stained with anti-murine CD11c, anti-murine MHC II and anti-murine CD86 antibodies. BMDCs treated with EpCAM-MKJK^P exhibited significantly higher activation levels in CD86 expression compared to those treated with EpCAM-MK^P and the negative control (Figure S5). To confirm the *in vivo* immune response, the populations of B and T cells in the spleens of mice injected with activated BMDCs were measured via flow cytometry (Figure 10bc).

Splenocytes were stained with anti-murine CD3 and CD19 antibodies to detect B cells (gated on CD3⁺ CD19⁺). We observed negative control (46.3%), positive control (59%), EpCAM-MK^P (53.9%), EpCAM-MK^P + R848 (61.2%), EpCAM-MKJK^P (61.6%) and EpCAM-MKJK^P + R848 (64.7%). These data indicate a significant increase in the B-cell population in mice treated with EpCAM-MKJK^P. Additionally, splenocytes were stained with anti-murine CD3, CD4 and CD8 antibodies to detect CD8⁺ cytotoxic (gated on CD3⁺ CD8⁺) and CD4⁺ helper T lymphocytes (gated on CD3⁺ CD4⁺). Results showed an increase in the CD8⁺ T-cell population following treatment with EpCAM-MK^P and EpCAM-MKJK^P, while the CD4⁺ T cells remained unchanged. These findings suggest that plant expression systems can effectively produce functional recombinant EpCAM proteins, which may be vaccine candidates capable of inducing immune responses. These responses can generate antibodies targeting proteins highly expressed in cancer cells through DC activation.

Inhibition effect of sera from mice immunized with EpCAM-MK^P and EpCAM-MKJK^P on EpCAM-positive cancer cell migration

RT-PCR analysis confirmed the EpCAM expression in PC-3 and SW620 cell lines (Figure S6a). Additionally, immunocytochemistry (ICC) was conducted to assess the binding affinity between EpCAM positive cancer cells and anti-EpCAM IgGs derived from sera of immunized mice with EpCAM-MK^P and EpCAM-MKJK^P proteins. ICC analysis revealed distinct binding of anti-EpCAM antibodies to PC-3 (Figure S6c) and SW620 (Figure S6d) cells.

Thus, both PC-3 and SW620 cell lines were applied to the anti-cancer activity studies.

A wound healing analysis was conducted to assess the inhibitory effects of sera from mice immunized with EpCAM-MK^P and EpCAM-MKJK^P on cell migration in both PC-3 and SW620 cancer cell lines (Figure 11a,b). In the PC-3 cell line, sera from both EpCAM-MK^P and EpCAM-MKJK^P significantly inhibited cell migration at both 1:500 and 1:100 serum dilutions compared to the negative control (Figure 11a). The EpCAM-MKJK^P serum exhibited a pronounced suppressive effect on PC-3 cell migration. The number of migrated cells was as follows: negative control (101 ± 3.8 cells), positive control (37 ± 1.5 cells), EpCAM-MK^P 1:500 serum dilution (74 ± 2.6 cells), EpCAM-MK^P 1:100 serum dilution (89 ± 1.3 cells), EpCAM-MKJK^P 1:500 serum dilution (40 ± 3.4 cells) and EpCAM-MKJK^P 1:100 serum dilution (49 ± 0.6 cells). In the SW620 cell line, both EpCAM-MK^P and EpCAM-MKJK^P sera similarly inhibited migration at 1:8000 and 1:2000 serum dilutions compared to the negative control (Figure 11b). Again, the EpCAM-MKJK^P serum significantly suppressed SW620 cell migration: negative control (780 ± 14 cells), positive control (386 ± 4 cells), EpCAM-MK^P 1:8000 serum dilution (564 ± 6.4 cells), EpCAM-MK^P 1:2000 serum dilution (491 ± 11.7 cells), EpCAM-MKJK^P 1:8000 serum dilution (364 ± 3.8 cells) and EpCAM-MKJK^P 1:2000 serum dilution (278 ± 5.6 cells). Cell migration was markedly reduced in PC-3 and SW620 cells treated with EpCAM-MKJK^P serum.

Transwell invasion assay to confirm inhibition activity of sera from immunized mice by EpCAM-MK^P and EpCAM-MKJK^P on PC-3 and SW620 cell movement

To further investigate the inhibition of cell invasion, a transwell invasion assay was conducted with sera from mice immunized with EpCAM-MK^P and EpCAM-MKJK^P (Figure 11c,d). In the PC-3 cell line, both EpCAM-MK^P and EpCAM-MKJK^P sera significantly inhibited invasion at 1:500 and 1:100 serum dilutions compared to the negative control (Figure 11c). The EpCAM-MKJK^P serum reduced PC-3 cell invasion: negative control (83 ± 1.1 cells), positive control (33.4 ± 1.2 cells), EpCAM-MK^P serum 1:500 dilution (59.3 ± 2.5 cells), EpCAM-MK^P serum 1:100 dilution (64.8 ± 1.5 cells), EpCAM-MKJK^P serum 1:500 dilution (27.7 ± 0.3 cells) and EpCAM-MKJK^P serum 1:100 dilution (32.5 ± 0.5 cells). In the SW620 cell line, both EpCAM-MK^P and EpCAM-MKJK^P sera also inhibited invasion at 1:8000 and 1:2000 serum dilutions compared to the negative control (Figure 11d). The EpCAM-MKJK^P serum significantly reduced SW620 cell invasion, with the following cell counts: negative control (35.5 ± 0.9 cells), positive control (7.5 ± 0.4 cells), EpCAM-MK^P serum 1:8000 dilution (27 ± 0.2 cells), EpCAM-MK^P serum 1:2000 dilution (25 ± 0.5 cells), EpCAM-MKJK^P serum 1:8000 dilution (14.3 ± 0.3 cells) and EpCAM-MKJK^P serum 1:2000 dilution (12.6 ± 0.4 cells). Overall, invasion was significantly reduced in both PC-3 and SW620 cells treated with the EpCAM-MKJK^P serum, indicating its potent inhibitory effects on cancer cell migration and invasion.

Expression of EMT-related genes, *E-cadherin* and *Vimentin*, in PC-3 and SW620 cells treated with sera from mice immunized with EpCAM-MK^P and EpCAM-MKJK^P

Epithelial-mesenchymal transition (EMT) is a critical process associated with the loss of cell-to-cell contact and polarity in epithelial cancer cells (Juan *et al.*, 2018). EMT, a key early event

in the progression from early stage to invasive malignancies, is characterized by decreased *E-cadherin* (Kang and Massague, 2004) expression. Given the reduction in the migration and invasion of PC-3 and SW620 cells following treatment with sera

from EpCAM-MK^P and EpCAM-MKJK^P immunized mice, we investigated the expression levels of EMT marker genes, *E-cadherin* and *Vimentin*, using real-time PCR (Figure 11e,f). EpCAM-MK^P and EpCAM-MKJK^P sera significantly increased *E-*

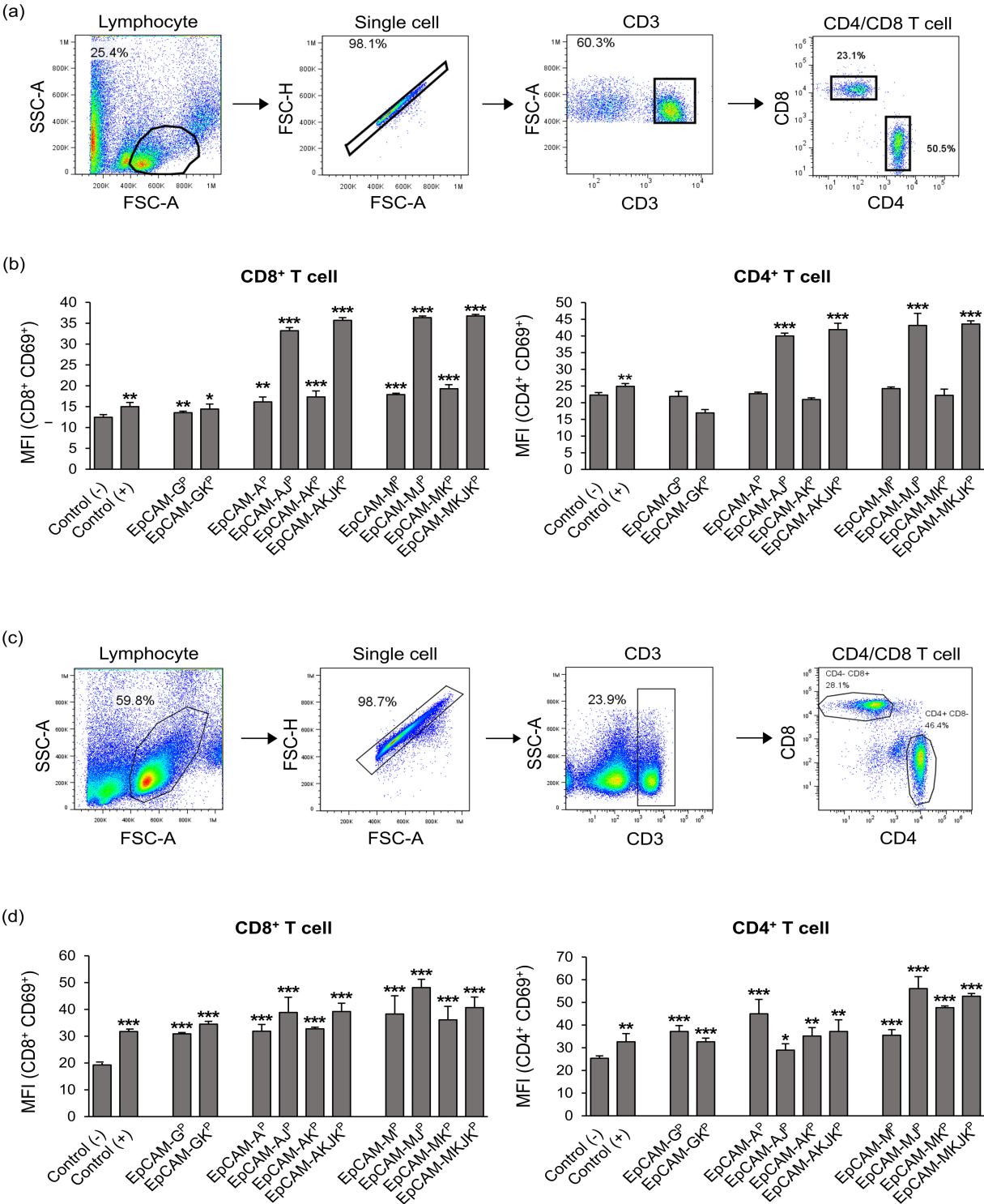


Figure 7 Flow cytometry analysis of antigen-specific CD8⁺ and CD4⁺ T cells to confirm the cellular immune response induced by EpCAM-Fc^P (FcK^P) or EpCAM-Fc^P (FcK^P) × J^P (JK^P) recombinant proteins in the blood and spleen of mice following the second and fourth immunizations. (a) Gating strategy for identifying CD8⁺ cytotoxic and CD4⁺ helper T lymphocytes from mice immunized with EpCAM-Fc^P (FcK^P) or EpCAM-Fc^P (FcK^P) × J^P (JK^P). Blood samples were collected 10 days after the second immunization via retro-orbital bleeding. (b) Differences in CD8⁺ cytotoxic T cells and CD4⁺ helper T cells 10 days post-second immunization. (b left) Representative flow cytometry plots show T-cell activation marker CD69 expression in gated CD8⁺ T cells. The bar graphs represent the mean fluorescence intensity (MFI) of activated CD8⁺ CD69⁺ cytotoxic T cells (*n* = 5). (b right) Representative flow cytometry plots show the expression of CD69 in gated CD4⁺ T cells, with bar graphs indicating the MFI of activated CD4⁺ CD69⁺ helper T cells (*n* = 5). (c) Gating strategy for identifying CD8⁺ and CD4⁺ T lymphocytes from the spleens of immunized mice using flow cytometry. Splenocytes were isolated 10 days after the fourth immunization. (d) Flow cytometry results confirming CD8⁺ and CD4⁺ T-cell immune responses 10 days after the fourth immunization. (d left) Bar graphs show the MFI of activated CD8⁺ CD69⁺ cytotoxic T cells (*n* = 5). (d right) Bar graphs show the MFI of activated CD4⁺ CD69⁺ helper T cells (*n* = 5). Control (+): positive control, blood from mice injected with commercial EpCAM-G^M; Control (−): negative control, blood from mice injected with 1 × PBS. Data are presented as means ± standard deviation from independent experiments, each evaluating five mice per group. Statistical significance using a Student's *t*-test comparing to the negative control group, with * indicating differences at **P* < 0.05, ***P* < 0.01 and ****P* < 0.001.

cadherin expression in both PC-3 and SW620 cells. These sera reduced the mesenchymal marker gene *Vimentin* expression in both cell lines. The EpCAM-MKJK^P serum significantly increased *E-cadherin* expression and effectively lowered *Vimentin* expression levels. Sera containing anti-EpCAM IgGs induced by plant-derived recombinant proteins effectively inhibits PC-3 and SW620 cell invasion, promoting a more epithelial-like phenotype by enhancing *E-cadherin* expression and suppressing *Vimentin* expression.

Discussion

EpCAM, highly expressed in most human epithelial tumours, circulating tumour cells and cancer stem cells, is associated with adhesiveness, tissue stabilization, proliferation, metastasis and tumour growth promotion (Baeuerle and Gires, 2007; Balzar et al., 1999; Patriarca et al., 2012). It is also stably expressed and up-regulated during cancer progression (Patriarca et al., 2012). The EpCAM protein has been considered a vaccine to induce specific B- and T-cell immune responses for immunotherapeutic applications in cancer therapy (Mosolits et al., 2004; Went et al., 2004, 2006). Cancer vaccine triggers adaptive immune responses through an antigen to control and prevent cancer (Menaria et al., 2013; Wong et al., 2016; Wong-Arce et al., 2017). Clinical trials regarding long-term anti-cancer response with specific immune memory cells will contribute to the effective elimination of malignant cancers (Menaria et al., 2013).

In a previous size-dependent immunogenicity study, an antigen-conjugated bead (~0.04–0.05 µm in viral range) induced combined antibody and CD8 T-cell immunity comparable to leading adjuvants (Fifis et al., 2004). Several previous studies have shown that immune complexes are potent activators of DCs and can prime stronger immune responses than antigens alone (Mekhaieil et al., 2011; Rafiq et al., 2002; Schuurhuis et al., 2006). In research by Johannes et al., IgM Fc + J-chain increased the binding of a PD-L1 to PD-1 test interaction up to 1000-fold compared to standard IgG Fc fusion proteins (Ammann and Trowsdale, 2014).

In this study, to construct the large quaternary structure with a modified glycan structure, EpCAM proteins were fused to the IgG, IgA and IgM Fc regions for EpCAM-G, EpCAM-A (dimeric) and EpCAM-M (pentameric or hexameric), respectively. The fusion proteins were produced in transgenic *N. tabacum* plants (Johansen et al., 2000; Sorensen et al., 2000). Furthermore, the EpCAM-A and EpCAM-M expressing plants were cross-fertilized

with J-chain expressing plants to generate EpCAM-A × J and EpCAM-M × J with a large quaternary structure. These polymeric Fc fusion antigenic proteins might improve the immune response by delivering multiple copies of the EpCAM protein to APCs. The primary purpose of this study was to investigate the potential of plant-derived EpCAM-Fc^P (FcK^P) and EpCAM-Fc^P (FcK^P) × J^P (JK^P) proteins in inducing *in vitro* and *in vivo* immune responses.

Plant expression systems have several advantages over conventional production systems, such as low upstream process cost, scalability and a lack of zoonotic pathogen contamination (Ko, 2014; Laere et al., 2016; Rigano and Walmsley, 2005). Additionally, a plant expression system is suitable for post-translational protein modifications, including the *N*-glycosylation required for biological activity and co-expression of multiple proteins using plant cross-fertilization (Fischer et al., 2004; Jin et al., 2023). In this study, *N. tabacum* was used to produce the EpCAM-Fc fusions with relatively high amounts of recombinant proteins (Conley et al., 2011; Sheen, 1983).

The *N. tabacum* plant is a non-food/non-feed crop that eliminates the risk of plant-derived recombinant proteins entering the food chain (Rymerson et al., 2002; Twyman et al., 2003) and is an effective plant host for recombinant protein production (Conley et al., 2011). *Agrobacterium*-mediated transformations were successfully conducted to generate transgenic plants expressing EpCAM-G, EpCAM-GK, EpCAM-A, EpCAM-AK, EpCAM-M and EpCAM-MK including J and JK proteins. Through crossing those transgenic plants expressing EpCAM-A, EpCAM-AK, EpCAM-M, EpCAM-MK with J and JK transgenic plants, EpCAM-AJ, EpCAM-AJK, EpCAM-AKJ, EpCAM-AKJK, EpCAM-MJ, EpCAM-MJK, EpCAM-MKJ and EpCAM-MKJK transgenic plants were generated. The insertion and expression of EpCAM-Fc (FcK) and J (JK) genes were confirmed by PCR analysis at the DNA level, RT-PCR analysis at the RNA level and immunoblot analysis at the protein level. When attached to the C-terminal end, the ER retention motif KDEL retains or returns glycoproteins in the ER (Ko et al., 2003). The KDEL-tagged proteins in transgenic plants typically improve production levels (Fiedler et al., 1997; Sharp and Doran, 2001).

An immunoblot analysis showed that the proteins with KDEL had better expression than those without KDEL (Fiedler et al., 1997; Ko et al., 2003; Munro and Pelham, 1987). To form stable large quaternary structures, we cross-fertilized each plant expressing the EpCAM-A, EpCAM-AK, EpCAM-M and EpCAM-MK with a plant expressing J-chain. J-chain is required for the dimerization of EpCAM-A/EpCAM-AK and pentamerization of EpCAM-M/EpCAM-MK to increase stability (Ammann and

Trowsdale, 2014; Johansen *et al.*, 2000; Redwan *et al.*, 2006). EpCAM-Fc (FcK) × J (JK) expression was confirmed by RT-PCR analysis at the RNA level and an immunoblot assay at the protein

level from cross-fertilized plants. Unlike the transgenic lines of EpCAM-Fc (FcK), the cross-fertilized lines showed no significant difference in protein expression by the KDEL effect. The proteins

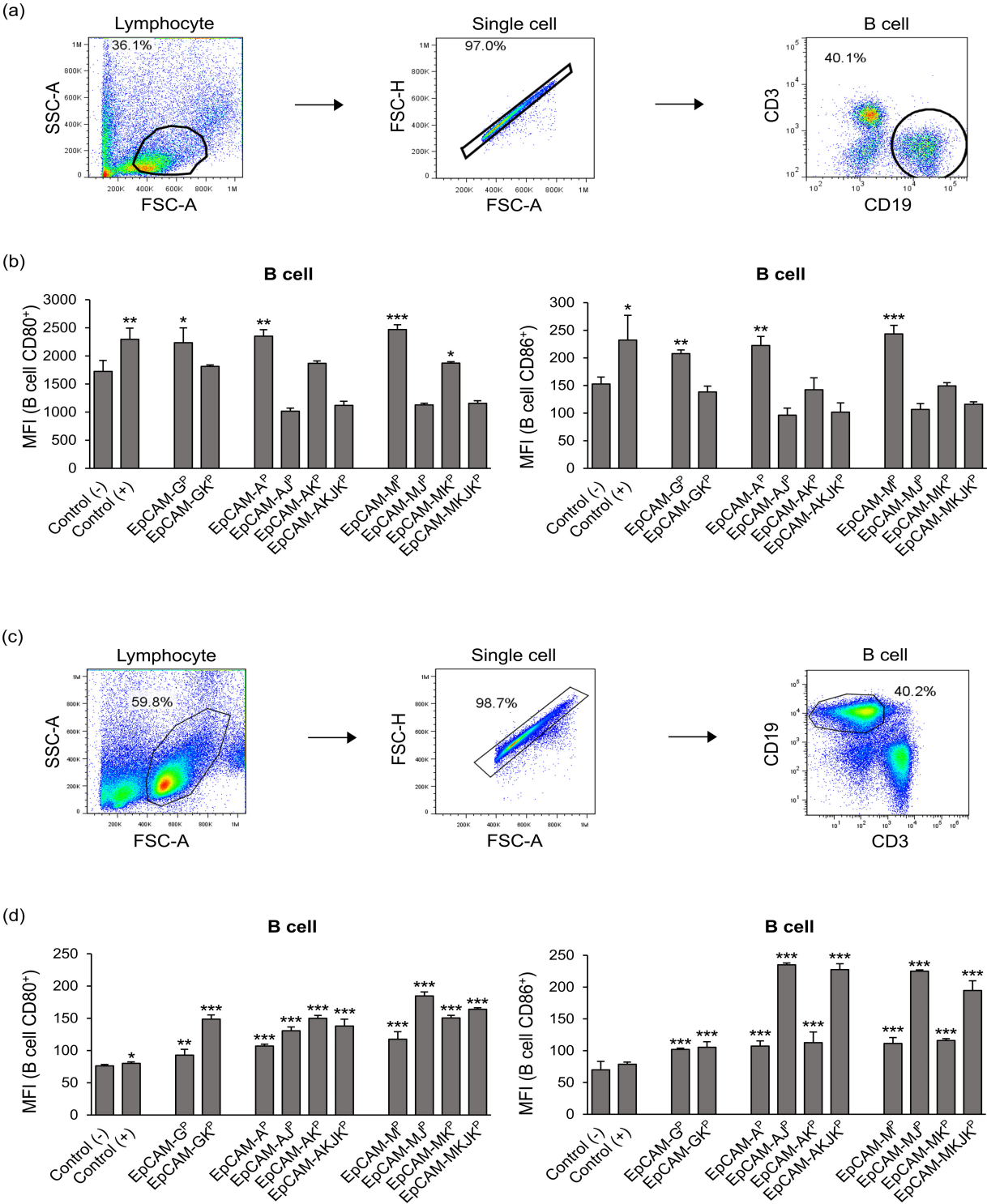


Figure 8 Flow cytometry analysis to evaluate CD80⁺ and CD86⁺ B-cell immune responses induced by EpCAM-Fc^P (FcK^P) or EpCAM-Fc^P (FcK^P) × J^P (JK^P) recombinant proteins in the blood and spleen of mice. (a) Gating strategy for identifying B cells from the blood of mice immunized with EpCAM-Fc^P (FcK^P) or EpCAM-Fc^P (FcK^P) × J^P (JK^P) by flow cytometry. (b) Differences in B-cell populations were observed 10 days after the second immunization. (b left) Bar graphs display the mean fluorescence intensity (MFI) of activated CD19⁺ CD80⁺ B cells (*n* = 5). (b right) Bar graphs show the MFI of activated CD19⁺ CD86⁺ B cells (*n* = 5). (c) Gating strategy for identifying B cells from the spleens of mice immunized with EpCAM-Fc^P (FcK^P) or EpCAM-Fc^P (FcK^P) × J^P (JK^P) by flow cytometry. (d) Differences in B-cell populations were observed 10 days after the fourth immunization. (d left) Bar graphs indicate the MFI of activated CD19⁺ CD80⁺ B cells (*n* = 5). (d right) Bar graphs present the MFI of activated CD19⁺ CD86⁺ B cells (*n* = 5). Control (+): positive control, splenocytes from mice injected with commercial EpCAM-G^M; Control (−): negative control, splenocytes from mice injected with 1 × PBS. Data are presented as means ± standard deviation from independent experiments, each evaluating five mice per group (**P* < 0.05, ***P* < 0.01 and ****P* < 0.001).

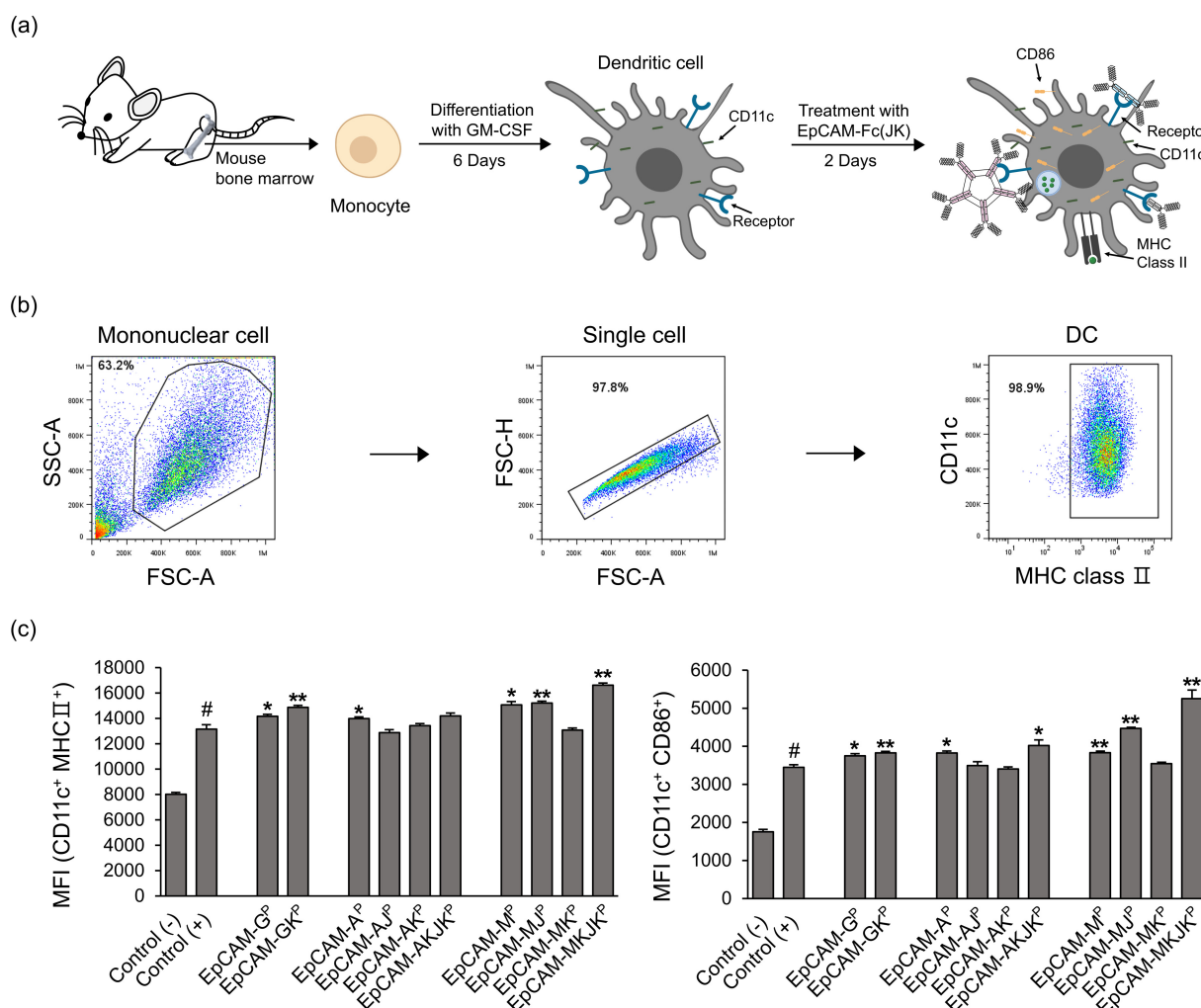


Figure 9 *In vitro* activation of murine dendritic cells (DCs) to present EpCAM-Fc^P (FcK^P) and EpCAM-Fc^P (FcK^P) × J^P (JK^P) antigens using bone marrow-derived DCs (BMDCs) from mice. (a) Schematic diagram illustrating the antigen presentation process of EpCAM-Fc^P (FcK^P) and EpCAM-Fc^P (FcK^P) × J^P (JK^P) in murine DCs *in vitro*. Monocytes were isolated from mouse bone marrow (BM) cells. BM cells were cultured with mouse GM-CSF for 6 days to differentiate BMDCs. Subsequently, BMDCs were incubated with a medium containing either EpCAM-Fc^P (FcK^P) or EpCAM-Fc^P (FcK^P) × J^P (JK^P) proteins for 2 days to activate the BMDCs. (b) Gating strategy for identifying DCs treated with EpCAM-Fc^P (FcK^P) or EpCAM-Fc^P (FcK^P) × J^P (JK^P) using flow cytometry. DCs were isolated from mouse BM. (c) Flow cytometry analysis to confirm the cellular immune response induced by EpCAM-Fc^P (FcK^P) or EpCAM-Fc^P (FcK^P) × J^P (JK^P) recombinant proteins in DCs *in vitro*. Differences in DC activation were assessed 2 days after incubation with the antigens. (c left) Bar graphs show the mean fluorescence intensity (MFI) of activated CD11c⁺ MHC II⁺ DCs. (c right) Bar graphs present the MFI of activated CD11c⁺ CD86⁺ DCs. Data are presented as means ± standard deviation from three independent experiments (**P* < 0.05, ***P* < 0.01 and ****P* < 0.001). Control (+): positive control, BMDCs activated with R848; Control (−): negative control, BMDCs activated with 1 × PBS.

were successfully purified for the animal immunization experiments.

Mice were immunized to investigate the immune effectiveness of the purified EpCAM-Fc recombinant proteins. ELISA and flow

cytometry using the first harvested blood samples from immunized mice revealed that plant-derived EpCAM-Fc proteins effectively induced an immune response to produce anti-EpCAM IgGs in the serum. ELISA results showed that the M-type proteins had the best

vaccination effect, and the A-type proteins had a better immunization effect than the G-type proteins. The proteins with KDEL had a better immune response effect than proteins without KDEL, and the proteins with J were superior to J-free proteins. Flow cytometry analysis was conducted to confirm the immune response of T cells and B cells from blood harvested 10 days after the second immunization. After antigen processing and presentation by APCs, humoral and cellular immunity are induced,

activating CD8⁺ T and CD4⁺ T helper 1 and helper 2 types (Itoh *et al.*, 2009; Xiang *et al.*, 2006). The expression of CD69, an early lymphocyte activation marker, in CD8⁺ cytotoxic and CD4⁺ helper T lymphocytes confirmed the effect of vaccine candidates on adaptive immunity (Cibrian and Sanchez-Madrid, 2017).

In this study, flow cytometry analysis revealed that both CD8⁺ cytotoxic T cells and CD4⁺ helper T cells from the mice who received a second immunization of plant-derived EpCAM-Fc^P

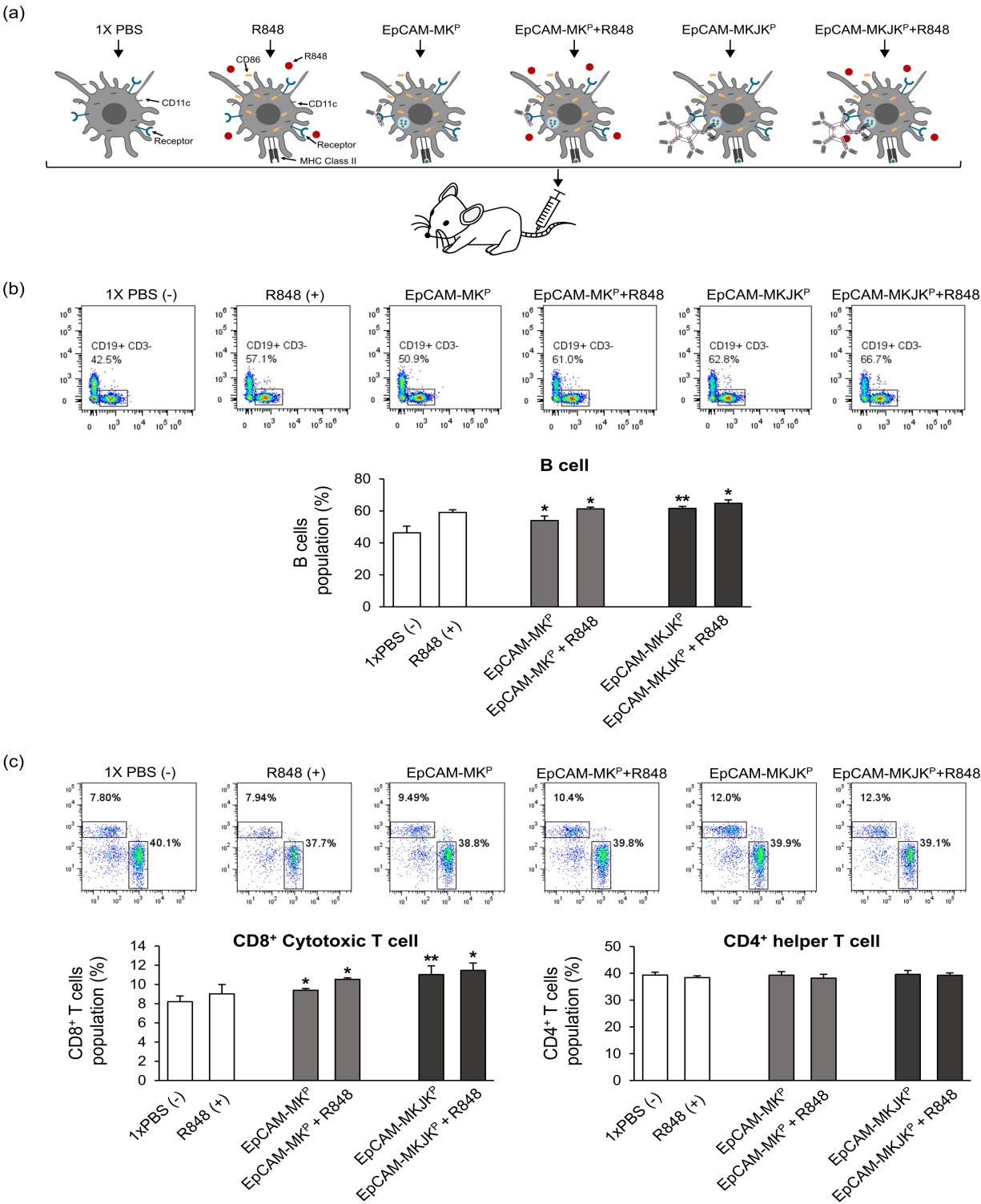


Figure 10 Flow cytometry analysis confirming B- and T-cell responses from the spleens of mice injected with dendritic cells (DCs) activated by EpCAM-MK^P or EpCAM-MKJK^P. (a) Experimental design to assess the effect of DCs activated by EpCAM-MK^P or EpCAM-MKJK^P *in vivo*. DCs were isolated from mouse bone marrow (BM) and cultured with 20 ng/mL GM-CSF for 6 days. On day 6, the culture medium was refreshed, and the following treatments were added for 1 day: negative control (1 × PBS), positive control (R848), EpCAM-MK^P, EpCAM-MK^P + R848, EpCAM-MKJK^P and EpCAM-MKJK^P + R848. Seven-week-old female mice were intravenously injected with 10⁶ DCs activated by the respective treatments. Spleen samples were harvested 10 days post-injection. (b) B-cell response in the spleens of mice injected with DCs activated by 1 × PBS, R848, EpCAM-MK^P, EpCAM-MK^P + R848, EpCAM-MKJK^P and EpCAM-MKJK^P + R848. Differences in B-cell populations were assessed 10 days after injection. Representative flow cytometry plots show the expression of B-cell populations in gated CD19⁺ CD3[−] B cells from independent experiments, each evaluating three mice per group. Bar graphs depict the percentage of activated CD19⁺ CD3[−] B cells. (c) CD8⁺ and CD4⁺ T-cell responses in the spleens of mice injected with DCs activated by the aforementioned treatments. Differences in CD8⁺ and CD4⁺ T-cell populations were evaluated 10 days post-injection. Representative flow cytometry plots illustrate the expression of CD8⁺ and CD4⁺ T-cell populations in gated CD3⁺ CD8⁺ or CD3⁺ CD4⁺ T cells from independent experiments, each evaluating three mice per group. Bar graphs display the percentage of activated CD3⁺ CD8⁺ cytotoxic T cells and CD3⁺ CD4⁺ helper T cells. Data are presented as means ± standard deviation from independent experiments (**P* < 0.05, ***P* < 0.01 and ****P* < 0.001).

(FcK^P) proteins were slightly activated. Both T cells of mice immunized by EpCAM-Fc^P (FcK^P) × J^P (JK^P) proteins were significantly activated. Antigen-specific B and T lymphocytes are vital for humoral immune responses to various antigens (Garside *et al.*, 1998; Mitchison, 1971). After antigen presentation by APCs, exogenous antigens are displayed on MHC II molecules, which activate CD4⁺ helper T cells.

To produce antigen-specific antibodies, the B cell encounters its specific antigen, receives additional signals from a CD4⁺ helper T cells and differentiates into plasma cell-secreting antibodies (Garside *et al.*, 1998; Harwood and Batista, 2010; Wong *et al.*, 2016). CD80⁺ and CD86⁺ B-cell responses have been investigated to confirm the effect of vaccine candidates on adaptive immunity (Nakajima *et al.*, 1998; Suvas *et al.*, 2002). Each B cell of the mice immunized with EpCAM-G^P, EpCAM-A^P and EpCAM-M^P proteins was significantly activated. The B cells of the mice immunized with EpCAM-FcK^P and EpCAM-Fc (FcK^P) × J^P (JK^P) were inactivated, EpCAM-Fc^P (FcK^P) × J^P (JK^P). B cells were inactivated in mice with activated T cells, suggesting that the signal generated by T cells was not transmitted to B cells. T cells were inactivated in mice with activated B cells, indicating that the signal generated by T cells was already transmitted to B cells. The close relationship between T and B cells is mediated by antigen presentation, and signals from T cells induce B cells to produce antibodies (Suvas *et al.*, 2002). T cells are always activated by an antigen-presenting DC first, and the activated T cell interacts with B cells (Eynon and Parker, 1992; Garside *et al.*, 1998).

To confirm the boost induction of anti-EpCAM IgGs by EpCAM-Fc^P recombinant proteins from mice immunized with BALB/c four times, we performed an ELISA analysis to measure the level of anti-EpCAM IgGs in the sera collected 10 days after the fourth immunization. More antibodies were produced in the mice after the fourth injection compared to the second injection. EpCAM-M or EpCAM-Fc fusion proteins with KDEL tagging showed higher anti-EpCAM IgG levels among the treatments. In flow cytometry analysis to confirm the immune response with spleens harvested 10 days after the fourth immunization, all of the CD8⁺ cytotoxic T cells and CD4⁺ helper T cells of the mice immunized by EpCAM-Fc^P (FcK^P) proteins were significantly activated, unlike the second injection-derived immune response. CD4⁺ helper T cells of the mice immunized with M-type proteins, except for EpCAM-M, were significantly activated. A flow cytometry analysis with the B cells from the spleens of immunized BALB/c-immunized mice showed that all of the B cells of the immunized mice by EpCAM-Fc^P (FcK^P) and EpCAM-Fc^P (FcK^P) × J^P (JK^P) proteins were activated. B cells of the mice immunized with

EpCAM-Fc^P (FcK^P) × J^P (JK^P) proteins were significantly activated. Unlike the second immunization, T and B cells were significantly activated after the fourth immunization.

These ELISA and flow cytometry data demonstrated that the plant-derived EpCAM-Fc recombinant proteins can induce immune responses to produce anti-EpCAM IgGs. The effective immune response orders were ranked: 1. Protein without KDEL < Protein with KDEL, 2. G-type protein < A-type protein << M-type protein, 3. Protein without J < Protein with J. These results suggest that the higher immune response of the vaccine with the KDEL motif may be due to the effective mannose receptor-mediated antigen presentation with high-mannose *N*-glycan by DCs (Apostolopoulos and McKenzie, 2001). The M-type proteins induced the best immune responses since it is the largest protein structure that DCs preferentially uptake like the virus-sized particles (20–200 nm) (Xiang *et al.*, 2006; Zhao *et al.*, 2014). The J-chain in the EpCAM-Fc fusion proteins resulted in a better immune response because more polymer-type proteins were assembled from the protein present with the J-chain for the polymerization of IgA (dimer) and IgM (pentamer) to increase stability (Johansen *et al.*, 2000).

DCs, the most important specialized APCs, efficiently uptake and process antigens as the initiator of immune responses. They are often targeted in cancer vaccine studies (Wang *et al.*, 2016; Zhao *et al.*, 2014). The most common method for preparing DC-based vaccines, used in about 97% of clinical trials, involves reinfusion of *ex vivo* manipulated DCs (Constantino *et al.*, 2016; Lee *et al.*, 2023). To date, sipuleucel-T (Provenge) is the FDA-approved autologous *ex vivo* DC vaccine that has demonstrated significant efficacy in phase III trials for metastatic castration-resistant prostate cancer (Cheever and Higano, 2011; Higano *et al.*, 2009; Kantoff *et al.*, 2010). In this study, DC activation assay was conducted to confirm the mechanism of the EpCAM-Fc^P (FcK^P) or EpCAM-Fc^P (FcK^P) × J^P (JK^P) and the antigen presentation effect by BMDCs, the initial immune response by MHC II and CD86, which are DC activation markers (Al-Ashmawy, 2018). Most BMDCs incubated with the EpCAM-Fc^P (FcK^P) and EpCAM-Fc^P (FcK^P) × J^P (JK^P) proteins were activated, with the EpCAM-MKJK^P protein the most effective. DCs prefer to uptake virus-size particles (20–200 nm) to induce an immune response (Xiang *et al.*, 2006; Zhao *et al.*, 2014).

In the *in vitro* DC activation assay, the EpCAM-MKJK^P had the best activation effect. Thus, an *in vivo* DC activation experiment was conducted with EpCAM-MKJK^P to confirm the *in vitro* results. We found that BMDCs treated with EpCAM-MKJK^P were significantly activated, which increased the population of B cells

and CD8⁺ cytotoxic T cells. There was no difference in the population of CD4⁺ helper T cells in the six groups. These results demonstrate that a plant expression system can produce a functional vaccine candidate to produce antibodies that

recognize the target proteins highly expressed on cancer cells via DC activation.

Furthermore, cell experiments were performed to determine whether the anti-EpCAM IgGs produced from immune responses

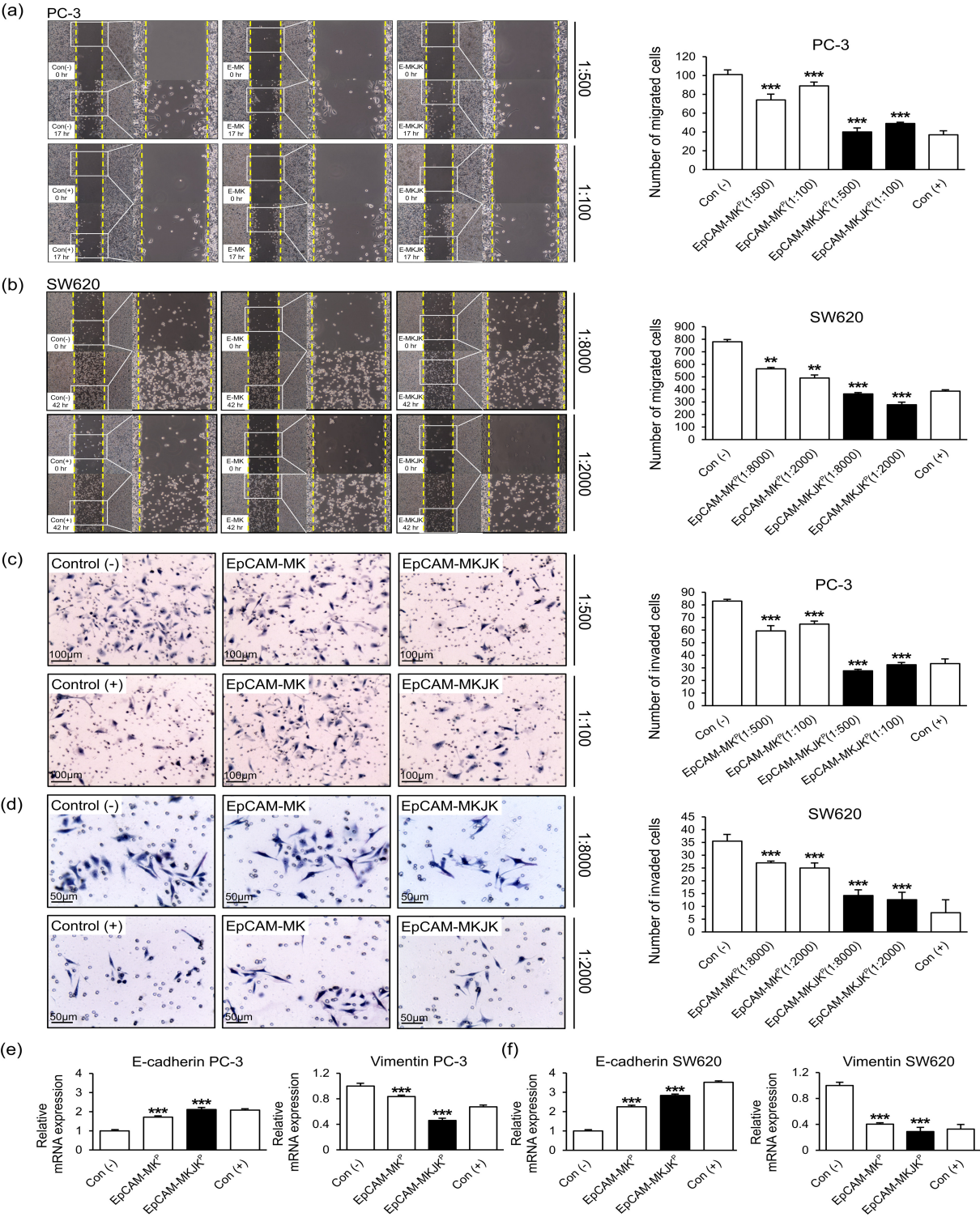


Figure 11 Inhibition activity of sera from immunized BALB/c mice with EpCAM-MK^P and EpCAM-MKJK^P proteins on the migration and invasion of PC-3 and SW620 cells. (a) Migration assay of PC-3 cells treated with sera from immunized BALB/c mice using EpCAM-MK^P and EpCAM-MKJK^P proteins. Sera were collected 10 days after the fourth immunization. PC-3 cell migration was assessed using different serum dilutions (1:500 and 1:100) for 17 h. (a, right) Bar graphs show the number of migrated cells. (b) Migration assay of SW620 cells treated with sera from immunized BALB/c mice with EpCAM-MK^P and EpCAM-MKJK^P proteins. Sera were collected 10 days post-immunization. SW620 cell migration was evaluated using serum dilutions (1:8000 and 1:2000) for 42 h. (b, right) Bar graphs display the number of migrated cells. Control (–): negative control (cells treated with sera from mice injected with 1 × PBS); Control (+): positive control (cells treated with commercial anti-EpCAM antibody, 10 µg); E-MK and E-MKJK: cells treated with sera from mice injected with EpCAM-MK^P and EpCAM-MKJK^P, respectively. (c) Invasion assay of PC-3 cells treated with sera from immunized BALB/c mice with EpCAM-MK^P and EpCAM-MKJK^P proteins. Sera were collected 10 days after the fourth immunization. Transwell invasion assays were conducted using different serum dilutions (1:500 and 1:100) for 48 h. Control (–): negative control (cells treated with sera from mice injected with 1 × PBS); Control (+): positive control (cells treated with commercial anti-EpCAM antibody, 10 µg); EpCAM-MK^P and EpCAM-MKJK^P: cells treated with sera from mice injected with EpCAM-MK^P and EpCAM-MKJK^P. (c, right) Bar graphs illustrate the number of invaded cells. (d) Invasion assay of SW620 cells treated with sera from immunized BALB/c mice with EpCAM-MK^P and EpCAM-MKJK^P proteins. Sera were collected 10 days after the fourth immunization. Transwell invasion assays were performed using serum dilutions (1:8000 and 1:2000) for 96 h. Control (–): negative control (cells treated with sera from mice injected with 1 × PBS); Control (+): positive control (cells treated with commercial anti-EpCAM antibody, 1 µg); EpCAM-MK^P and EpCAM-MKJK^P: cells treated with sera from mice injected with EpCAM-MK^P and EpCAM-MKJK^P. Bar graphs show the number of invaded cells. (e) Real-time PCR analysis of EMT-related genes *E-cadherin* and *Vimentin* expression in mRNA from PC-3 cells treated with sera from immunized BALB/c mice with EpCAM-MK^P and EpCAM-MKJK^P proteins. Sera were collected 10 days after the fourth immunization. Relative expression levels were normalized to 18S rRNA as the internal control. Control (–): negative control (mRNA from cells treated with sera from mice injected with 1 × PBS); Control (+): positive control (mRNA from cells treated with commercial anti-EpCAM antibody); EpCAM-MK^P and EpCAM-MKJK^P: mRNA from cells treated with sera from mice injected EpCAM-MK^P and EpCAM-MKJK^P. Data are presented as means ± standard deviation from three (A, B, C, D) or four (e, f) independent experiments (**P* < 0.05, ***P* < 0.01 and ****P* < 0.001).

induced by plant-derived vaccines recognized human cancer cells. EpCAM is more intensely expressed in tumour cells than the normal tissue in colorectal, prostate, pancreas, ovarian, breast and bladder carcinomas (Patriarca et al., 2012; Winter et al., 2003). Strong EpCAM expression has been reported in colon adenocarcinoma (close to 100%), while lower expression (80%) was detected in the very rare medullary colon carcinomas (Patriarca et al., 2012; Went et al., 2006). Strong EpCAM expression has also been reported in about 90% of prostate adenocarcinomas, whereas significantly higher expression of EpCAM is detected in hormone-refractory adenocarcinoma of the prostate than in untreated adenocarcinoma (Rao et al., 2005; Went et al., 2004).

Proliferation is integral to cancer progression and development and is manifested by altering the activity and expression of cell cycle-related proteins (Feitelson et al., 2015). Proliferation assay was performed to confirm the inhibition effect of anti-EpCAM IgGs in the sera of mice immunized by EpCAM-MK^P and EpCAM-MKJK^P on cancer cell proliferation in both PC-3 and SW620 cells and to determine the concentrations and time zones affecting cell proliferation (Figure S7). PC-3 and SW620 cell proliferation was, at certain times and doses, reduced by the EpCAM-MK^P and EpCAM-MKJK^P sera. In the real-time PCR, the EpCAM-MK^P and EpCAM-MKJK^P sera reduced *PCNA* and *Ki-67* expression levels in both PC-3 and SW620 cells; EpCAM-MKJK^P serum did so significantly. These data demonstrate that IgGs produced by plant-derived recombinant proteins effectively inhibited the proliferation of both PC-3 and SW620 cells.

The wound healing and transwell invasion assays were performed to investigate the effect of anti-EpCAM IgGs from the sera of mice immunized with EpCAM-MK^P and EpCAM-MKJK^P in cancer cell movements. In each migration and invasion assay of PC-3 and SW620 cells, both sera from the mice immunized with EpCAM-MK^P and EpCAM-MKJK^P inhibited migration and invasion compared to the negative control. The EpCAM-MKJK^P serum significantly suppressed migration and invasion in PC-3 and SW620 cells. PC-3 and SW620 inhibition on cell migration was positively correlated to cell invasion inhibition.

Epithelial cancer cell migration and invasion are involved in tumour metastasis. Tumour metastasis is associated with numerous factors, such as EMT (Juan et al., 2018; Kang and Massague, 2004). EMT is vital in the polarity loss of epithelial cancer cell-to-cell contacts and remodelling of the cytoskeleton during early-stage tumours and invasive malignancies (Kang and Massague, 2004; Thiery, 2002). *E-cadherin* expression is repressed by several key inducers of EMT (Kang and Massague, 2004; Thiery, 2002). *E-cadherin* is a central component of cell-to-cell adhesion junctions required for epithelial homeostasis. *Vimentin* is a mesenchymal cell marker (Kang and Massague, 2004; Thiery, 2002). Since EpCAM-MK^P and EpCAM-MKJK^P reduced the cell migration and invasion of PC-3 and SW620 cells, the expression of the EMT markers *E-cadherin* and *Vimentin* was investigated. The sera of mice immunized with EpCAM-MK^P and EpCAM-MKJK^P showed increased *E-cadherin* expression and decreased *Vimentin* expression in both PC-3 and SW620 cells.

EpCAM plays a vital role in cell-to-cell adhesion and cell signalling, proliferation, migration and differentiation (Ni et al., 2013; Patriarca et al., 2012). The knockdown of EpCAM suppressed prostate cancer proliferation and invasion and down-regulated *E-cadherin*, p-mTOR, p-Akt, p-4EBP1 and p-S6K expression (Ni et al., 2013). EpCAM plays a vital role in prostate cancer proliferation, metastasis and invasion via activation of the PI3K/Akt/mTOR signalling pathway. Knockdown of EpCAM in breast cancer was associated with decreased invasiveness and inhibition of the Ras/Raf/ERK signalling pathway and down-regulation of MMP-9, enhancing tumour progression by regulating migration, proliferation, angiogenesis and invasion (Dubrovskaya et al., 2009; Gao et al., 2015; Li et al., 2014; Osta et al., 2004). However, the mechanisms between EpCAM and cell signalling remain largely unknown.

This study demonstrates that plant expression systems can express and assemble large quaternary EpCAM-Fc fusion proteins via plant cross-fertilization to form functional and effective vaccines. These proteins can induce an immune response that produces anti-EpCAM antibodies to recognize the target antigenic proteins with anti-cancer activity.

Experimental procedures

Construction of plant expression vectors for EpCAM-Fc, EpCAM-FcK, J-chain and J-chain K for *Agrobacterium*-mediated transformation

A 30-amino acid N-terminal signal peptide derived from *Nicotiana tabacum* calreticulin was added to the synthetic DNA sequence encoding EpCAM (Thr17-Lys265; GenBank accession no. BC014785) (Lu *et al.*, 2012). ER retention motif (KDEL: Lys-Asp-Glu-Leu) was tagged to the C terminus (Mekhaieel *et al.*, 2011) to the synthetic DNA sequence for the human J-chain (Met1-Asp175; GenBank accession no. DQ88439) (So *et al.*, 2012). EpCAM was then fused to the human IgG Fc fragment (Val245-Gly476; GenBank accession no. Y14737), the human IgA Fc fragment (Pro104-Ala353; GenBank accession no. AJ294729) and the human IgM Fc fragment (Leu103-Tyr453; GenBank accession no. X57086) to generate the constructs EpCAM-G, EpCAM-A and EpCAM-M, respectively. The C termini of EpCAM-G, EpCAM-A and EpCAM-M were further tagged with the ER retention motif, resulting in EpCAM-GK, EpCAM-AK and EpCAM-MK, respectively (Figure 1). These genes were subcloned into the plant expression vector pBI121, utilizing the untranslated leader sequence of RNA4 from Alfalfa Mosaic Virus (AMV) under the control of the enhanced duplicated 35S promoter and the 35S gene terminator from Cauliflower Mosaic Virus (Figure 1).

Generation of transgenic plants expressing EpCAM-G (GK), EpCAM-A (AK) and EpCAM-M (MK)

The plant binary vectors pBI EpCAM-G, pBI EpCAM-GK, pBI EpCAM-A, pBI EpCAM-AK, pBI EpCAM-M, pBI EpCAM-MK, pBI J and pBI JK were transformed into *Agrobacterium tumefaciens* strain LBA4404. *Agrobacterium* was then inoculated onto wounded non-transgenic (NT) leaf tissue. The *Agrobacterium*-inoculated leaf slices underwent co-cultivation and were subsequently transferred to regeneration media until small shoots emerged (Kang *et al.*, 2023). These small shoots were then moved to MS medium supplemented with B5 vitamins (Duchefa Biochemie, Haarlem, Netherlands), containing cefotaxime (250 µg/mL) and kanamycin (100 µg/mL) *in vitro*, using magenta boxes (Sigma-Aldrich, St. Louis, MO, USA). To harvest biomass, the *in vitro* transgenic plants were transplanted into soil pots in a greenhouse.

Generation of F₁ plant seedlings from the cross-fertilization of transgenic plants expressing EpCAM-Fc (FcK) fusion proteins and J (JK)

For cross-fertilization, immature stamens were removed from the unopened flower buds of female plants. Anthers were collected from a male plant and dried them overnight to release pollen. The pollen from the male plant was paced onto the stigmas of the female plants (Figure 3). F₁ seeds from the cross-fertilized plants were germinated on MS medium containing 100 µg/mL kanamycin.

PCR analysis

Plant genomic DNA (gDNA) was isolated from 100 mg of leaf tissue using a Genomic DNA Extraction Kit Mini (Plant) (RBC Bioscience, Taipei, Taiwan). PCR amplification of plant gDNA was performed to confirm EpCAM-G (GK), EpCAM-A (AK), EpCAM-M (MK) and J (JK) insertion using specific primer pairs (Table S1). The PCR conditions included 30 cycles of 94 °C for 20 s, 57–61 °C for 30 s and 72 °C for 30 s using the Maxime PCR Premix Kit

(Intron Biotechnology, Seoul, Korea). Positive controls included pBI EpCAM-G (GK), pBI EpCAM-A (AK), pBI EpCAM-M (MK) and pBI J (JK) vectors, while gDNA from non-transgenic plants was the negative control.

Semi-quantitative RT-PCR analysis

Total RNA was isolated from 100 mg of leaf tissue using the TRIzol RNA isolation protocol (Chomczynski and Mackey, 1995). gDNA elimination and cDNA synthesis were performed using the QuantiTect Reverse Transcription Kit (Qiagen, Hilden, Germany). RT-PCR analysis was conducted to confirm EpCAM-Fc (FcK), JK and EpCAM-Fc (FcK) × J (JK) expression using the specified primer pairs (Table S1). The RT-PCR conditions involved 30 cycles of 94 °C for 20 s, 57–61 °C for 30 s and 72 °C for 30 s using the Maxime PCR Premix Kit (Intron Biotechnology, Seoul, Korea). The relative expression levels of each gene were normalized against EF-1α as an internal control. Positive controls included pBI EpCAM-Fc (FcK) and pBI JK vectors, while RNA from non-transgenic plants served as the negative control.

SDS-PAGE and immunoblot analyses

An immunoblot analysis was performed to confirm the expression of EpCAM and Fc regions of IgG, IgA and IgM in transgenic plants. Leaf samples (100 mg fresh weight) from each transgenic plant were homogenized with three volumes of 1× PBS to extract total soluble proteins. The homogenized samples were mixed with 5× loading buffer (1 M Tris-HCl, 50% glycerol, 10% SDS, 5% 2-mercaptoethanol, 1% bromophenol blue), boiled it for 5 min and then cooled the solution on ice. 20 µL of each sample was loaded onto a 10% SDS-PAGE gel. To compare the purity and quantity of EpCAM-Fc^P (FcK^P) and EpCAM-Fc^P (FcK^P) × J^P (JK^P) proteins, purified samples (2 µg) were loaded onto a 10% SDS-PAGE gel. After electrophoresis, the gel was stained with Coomassie Brilliant Blue for 40 min. For immunoblot analysis, the proteins were transferred to a nitrocellulose membrane (Merck Millipore, Burlington, NY, USA), and the membrane was blocked for 1 h at room temperature (RT) with 5% skim milk (Sigma-Aldrich, St. Louis, MO, USA) in 1× TBS with 0.5% (v/v) Tween 20 (1× TBS-T). The membranes were then incubated for 2 h at RT with murine anti-human EpCAM antibody (R&D Systems, Minneapolis, MN, USA) and goat anti-murine IgG HC conjugated to HRP (Abcam, Cambridge, UK) to detect human EpCAM protein. Subsequently, membranes were incubated with rabbit anti-human IgG Fcγ antibody conjugated to HRP (Jackson ImmunoResearch, West Grove, PA, USA), goat anti-human IgA Fc antibody conjugated to HRP (Thermo Fisher Scientific, Waltham, MA, USA) and goat anti-human IgM Fc antibody conjugated to HRP (Abcam, Cambridge, UK) for 2 h at RT to recognize human IgG Fc, IgA Fc and IgM Fc proteins, respectively. Following antibody incubation, the membranes were washed with 1× TBS-T for 1 h at RT. Protein bands were visualized by exposing the membranes to X-ray film (Feitelson *et al.*, 2015) using Clarity Western ECL Substrate (Bio-Rad, Hercules, CA, USA). Non-transgenic plant and recombinant human EpCAM/TROP-1 Fc chimera (R&D Systems, Minneapolis, MN, USA) were used as negative and positive controls, respectively.

Purification of EpCAM-Fc^P (FcK^P) and EpCAM-Fc^P (FcK^P) × J^P (JK^P) proteins from plant leaf biomass

Recombinant EpCAM-Fc^P (FcK^P) and EpCAM-Fc^P (FcK^P) × J^P (JK^P) proteins were purified from fresh leaves following established

protocols (Kang *et al.*, 2023; Lim *et al.*, 2022). The leaves (400 g) were homogenized in 1200 mL of pre-chilled extraction buffer (37.5 mM Tris-HCl, 50 mM NaCl, 15 mM EDTA, 75 mM sodium citrate and 0.2% sodium thiosulfate, pH 7.4) and centrifuged the solution at 9000 *g* for 30 min at 4 °C. The supernatant was filtered through Miracloth (Biosciences, La Jolla, CA, USA), the pH was adjusted to 5.1 by adding acetic acid (pH 2.4), and it was centrifuged at 10 000 *g* for 30 min at 4 °C. The supernatant was filtered again through Miracloth and adjusted the pH to 7.0 by adding 3 M Tris-HCl. Ammonium sulphate was added to achieve 8% saturation, and it was incubated for 2 h at 4 °C. The mixture was centrifuged at 9000 *g* for 30 min at 4 °C, and the supernatant was filtered through Miracloth.

Next, ammonium sulphate was added to achieve 22.6% saturation, and the solution was incubated overnight at 4 °C. The final solution was centrifuged at 9000 *g* for 30 min at 4 °C, and the pellet was resuspended in extraction buffer to 1/10 of the original volume. The resuspended solution was centrifuged at 10 000 *g* for 30 min at 4 °C and then filtered through Miracloth and a 0.45-μm filter (Merck Millipore, Burlington, NY, USA). Protein purification was carried out using protein A Sepharose 4 Fast Flow (GE Healthcare, Chicago, IL, USA) for EpCAM-G and EpCAM-GK, CaptureSelect IgA Affinity Matrix (Thermo Fisher Scientific, Waltham, MA, USA) for EpCAM-A and EpCAM-AK, and POROS CaptureSelect IgM Affinity Matrix (Thermo Fisher Scientific, Waltham, MA, USA) for EpCAM-M and EpCAM-MK. The purified proteins were dialysed against 1× PBS (pH 7.4) using Por 2 RC Dialysis Membrane Tubing with a molecular weight cutoff of 12 000–14 000 Dalton (Spectrum Chemical). The protein concentration was determined by NanoDrop and SDS-PAGE analyses, and the purified proteins were stored at –80 °C.

ELISA analysis

96-well Maxisorp Nunc-immuno plates (Sigma-Aldrich, St. Louis, MO, USA) were coated with 100 ng per well of either EpCAM-Fc^P (FcK^P) or EpCAM-Fc^P (FcK^P) × J^P (JK^P) diluted in 0.05 M Carbonate-Bicarbonate Buffer (pH 9.6, Sigma-Aldrich, St. Louis, MO, USA) and incubated overnight at 4 °C. Following incubation, the plates were washed four times with 200 μL of 1× PBS-T per well and blocked them with 5% skim milk (Sigma-Aldrich, St. Louis, MO, USA) in 1× PBS-T for 2 h at RT. After blocking, we added 100 ng and 25 ng of murine anti-human EpCAM antibody (R&D Systems, Minneapolis, MN, USA), diluted in 1× PBS-T, to the wells and incubated them for 2 h at 37 °C. The plates were washed again, and goat anti-murine IgG HC conjugated to HRP (Abcam, Cambridge, UK) was added at a dilution of 1:5000. The solution was incubated for 2 h at RT. After additional washing with 1× PBS-T, the plates were developed using 100 μL of SureBlue TMB Microwell Peroxidase Substrate and TMB Stop Solution (SeraCare, Milford, CT, USA). Absorbance at 450 nm was measured using an Epoch Microplate Spectrophotometer (BioTek, Winooski, VT, USA).

Immunization of BALB/c mice with EpCAM-G^P, EpCAM-GK^P, EpCAM-A^P, EpCAM-AJ^P, EpCAM-AK^P, EpCAM-AKJK^P, EpCAM-M^P, EpCAM-MJ^P, EpCAM-MK^P and EpCAM-MKJK^P proteins

Six-week-old female BALB/c mice were maintained in a pathogen-free environment. All animal experiments were conducted in accordance with protocols approved by the Chung-Ang University Animal Ethics Committee and complied with Korean Council

on Animal Care guidelines (Approval No: 2017-00050). Mice were IP immunized with EpCAM-G^P, EpCAM-GK^P, EpCAM-A^P, EpCAM-AJ^P, EpCAM-AK^P, EpCAM-AKJK^P, EpCAM-M^P, EpCAM-MJ^P, EpCAM-MK^P and EpCAM-MKJK^P proteins using an aluminium hydroxide adjuvant (Alhydrogel, 2%, InvivoGen, San Diego, CA, USA). At 7 weeks of age, mice (five per group) received four immunizations at 2-week intervals, with a total of 5 μg of protein in 150 μL per injection. Blood samples were collected via retro-orbital bleeding 10 days after the second immunization and 10 days after the fourth injection. Spleens were concurrently harvested during the second blood sample collection.

ELISA to confirm induction of anti-EpCAM IgGs

To confirm the induction of anti-EpCAM IgGs in the immunized BALB/c mice, serum samples were analysed using an ELISA. 96-well Maxisorp Nunc-Immuno plates (Sigma-Aldrich, St. Louis, MO, USA) were coated overnight at 4 °C with 100 ng per well of 6H-EpCAMK proteins diluted in 0.05 M carbonate-bicarbonate buffer (pH 9.6, Sigma-Aldrich, St. Louis, MO, USA). Following incubation, the plates were washed four times with 200 μL of 1× PBS-T. The solution was blocked with 5% skim milk in 1× PBS-T for 2 h at RT. After washing, serum samples were diluted 1:2000 in 1× PBS-T and incubated for 2 h at 37 °C. The plates were washed again and treated with goat anti-murine IgG HC antibodies conjugated to HRP (Jackson ImmunoResearch, West Grove, PA, USA) at a dilution of 1:5000. The plates were then incubated for 2 h at RT. After washing, 100 μL of SureBlue TMB Microwell Peroxidase Substrate was added to each well. The reaction was stopped with TMB Stop Solution (SeraCare, Milford, CT, USA). Absorbance at 450 nm was measured using an Epoch Microplate Spectrophotometer (BioTek, Winooski, VT, USA).

Isolation of lymphocytes and flow cytometry

Blood was collected 10 days after the second immunization via retro-orbital bleeding. To remove red blood cells (RBCs), blood samples were treated with ACK lysis buffer (Gibco, Waltham, MA, USA), vortexed and incubated for 5 min at RT. The samples were then centrifuged at 7800 *g* for 5 min at RT. The supernatant was discarded, and the cells were resuspended in 500 μL of ACK lysis buffer. After a second round of lysis, the cells were washed twice with 1× PBS. To detect T cells, blood cells were stained with the following antibodies: FITC-conjugated anti-murine CD4, APC-conjugated anti-murine CD8, PerCP-conjugated anti-murine CD3 and PE-Cy7A-conjugated anti-murine CD69 (Tonbo, San Diego, CA, USA). The cells were prepared as described, washed twice in flow cytometry buffer (2 mM EDTA, 0.1% sodium azide, 0.2% BSA in PBS) and centrifuged them at 7800 *g* for 5 min.

After removing the supernatant, the cells were resuspended in a flow cytometry buffer. Flow cytometric analysis was performed using an Attune™ NxT Acoustic Focusing Cytometer (Thermo Fisher Scientific, Waltham, MA, USA), and post-sort analysis was conducted with FlowJo software (Tree Star, Ashland, OR, USA). For B-cell detection, blood cells were stained with PerCP-conjugated anti-murine CD3, PE-conjugated anti-murine CD19, FITC-conjugated anti-murine CD80 and APC-conjugated anti-murine CD86 antibodies (Tonbo, San Diego, CA, USA) and analysed via flow cytometry. Each mouse's spleen was harvested 10 days after the last immunization. Spleens were passed through a cell strainer (SPL, Pocheon, Korea) using the plunger end of a syringe and ACK lysis buffer to create a single-cell suspension, followed by a 5-min incubation at RT. The cells were

washed with 1× PBS and centrifuged them at 310 **g** for 5 min at RT. After discarding the supernatant, splenocytes were stained for T-cell detection using FITC-conjugated anti-murine CD4, APC-conjugated anti-murine CD8, PerCP-conjugated anti-murine CD3 and PE-Cy7A-conjugated anti-murine CD69 antibodies (Tonbo, San Diego, CA, USA) and analysed them by flow cytometry. B cells were identified using PerCP-conjugated anti-murine CD3, PE-conjugated anti-murine CD19, FITC-conjugated anti-murine CD80 and APC-conjugated anti-murine CD86 antibodies (Tonbo, San Diego, CA, USA).

Isolation of murine BM and generation and activation of DCs

BMDCs were obtained by extracting the femur and tibia from the mice's hind legs, which we cut and dissected using sterile scissors and forceps to remove excess tissue and muscle. The intact bones were sterilized by dipping them in 70% ethanol (EtOH) for 3 min. Both ends of the bones were cut, and 1× PBS was injected into them. The BM cells were flushed until the bones were clear, and the suspension was collected in a tube through a cell strainer. The collected BM cells were centrifuged at 310 **g** for 5 min at RT, and the supernatant was discarded. The pellet was resuspended in ACK lysis buffer (Gibco, Waltham, MA, USA), incubated and then resuspended in 1× PBS. Another centrifugation was performed at 310 **g** for 5 min at RT. BM cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 1% penicillin–streptomycin (Welgene, Gyeongsan, Korea) and 20 ng/mL mouse Granulocyte-macrophage colony-stimulating factor (GM-CSF) (PeproTech, Rocky Hill, CT, USA) in a humidified incubator with 5% CO₂ at 37 °C for 6 days to facilitate BMDC differentiation. To activate BMDC activation, 4 × 10⁴ cells per well were incubated with a medium containing either EpCAM-Fc^P (FcK^P) or EpCAM-Fc^P (FcK^P) × J^P (JK^P) proteins for 2 days. The activated BMDCs were then stained with PerCP-conjugated anti-murine CD11c, PE-conjugated anti-murine MHC II and APC-conjugated anti-murine CD86 antibodies (Tonbo, San Diego, CA, USA) and analysed via flow cytometry.

In vivo DC activation by EpCAM-MK^P and EpCAM-MKJK^P

To confirm *in vivo* DC activation, 5 × 10⁶ BMDCs per 100 mm cell culture dish (SPL, Pocheon, Korea) were prepared. They were incubated them for 24 h with medium containing 1× PBS as a negative control, EpCAM-MK^P or EpCAM-MKJK^P proteins. The activation status of the BMDCs treated with EpCAM-MK^P, EpCAM-MKJK^P and 1× PBS was assessed by flow cytometry before injection. The activated BMDCs were stained with PerCP-conjugated anti-murine CD11c, PE-conjugated anti-murine MHC II and APC-conjugated anti-murine CD86 antibodies (Tonbo, San Diego, CA, USA) and analysed by flow cytometry. Seven-week-old female mice (three mice per group) were IV injected with 1 × 10⁶ activated BMDCs treated with either EpCAM-MK^P, EpCAM-MKJK^P or 1× PBS. Blood samples were collected 10 days post-injection via retro-orbital bleeding, and spleens were simultaneously harvested. The harvested splenocytes were prepared as described above and stained with PerCP-conjugated anti-murine CD3 and PE-conjugated anti-murine CD19 antibodies (Tonbo, San Diego, CA, USA) for B-cell detection by flow cytometry. For T-cell detection, splenocytes were stained with PerCP-conjugated anti-murine CD3, PE-conjugated anti-murine CD4 and APC-conjugated anti-murine CD8 antibodies (Tonbo, San Diego, CA, USA) and analysed by flow cytometry.

ICC

Cancer cell-based ICC was performed to confirm the binding affinity between human cancer cells and anti-EpCAM IgGs obtained from the sera of immunized mice treated with 1× PBS, EpCAM-MK^P and EpCAM-MKJK^P. Human cancer cell lines, HeLa, PC-3 and SW620, were grown on poly-L-lysine (Sigma-Aldrich, St. Louis, MO, USA) coated coverslips in 6-well plates (SPL, Pocheon, Korea) and fixed them in 4% paraformaldehyde solution (Biosesang, Seongnam, Korea) at RT for 30 min. Cells were washed twice with 1× PBS and permeabilized with 0.1% Triton X-100 (Sigma-Aldrich, St. Louis, MO, USA) in 1× PBS at RT for 10 min. After washing, cells were blocked with a 5% bovine serum albumin (BSA) solution (RMBio, Missoula, MT, USA) at RT for 1 h. Subsequently, the cells were incubated overnight at 4 °C with a 1:2000 serum dilution from immunized mice as the primary antibody. The anti-murine IgG HC antibody conjugated to FITC (Invitrogen, San Diego, CA, USA) was utilized as a secondary antibody at a dilution of 1:500. The mixture was then incubated at RT for 1 h to detect the induced anti-EpCAM IgGs. The cells were mounted on glass slides with ProlongTM Gold Antifade solution containing DAPI (Invitrogen, San Diego, CA, USA). The negative control (NC) was treated with serum from immunized mice using 1× PBS, and the positive control (PC) was treated with a commercial anti-EpCAM antibody. Fluorescent images were captured using an LSM 700 confocal microscope (Carl Zeiss, Jena, Germany).

Real-time PCR and semi-quantitative RT-PCR analyses to confirm EpCAM transcription in various cancer cells

Cervical carcinoma HeLa cells were maintained as a monolayer in Minimum Essential Medium, prostatic carcinoma PC-3 cells were cultured in Ham's F-12 K medium, and colorectal carcinoma SW620 cells were cultured in Leibovitz's L-15 medium (Gibco, Waltham, MA, USA). All media were supplemented with 10% FBS and 0.1% penicillin–streptomycin antibiotics, and the media were changed every 2 days.

Total RNA was isolated from HeLa, PC-3 and SW620 cells using the FavorPrep Blood/Cultured Cell Total RNA Purification Mini Kit (Favorgen Biotech, Ping-Tung, Taiwan). cDNA was synthesized using the QuantiTect Reverse Transcription Kit (Qiagen, Hilden, Germany). RT-PCR analysis was utilized to confirm EpCAM expression in each cancer cell line, with relative expression levels normalized to β -Actin as the internal control. The expression of EMT marker genes in the cancer cells was assessed using real-time PCR with the primer pairs detailed in Table S2. RT-PCR was conducted with 30 cycles of 94 °C for 20 s, 61 °C for 30 s and 72 °C for 30 s using the Maxime PCR Premix Kit (Intron Biotechnology, Seoul, Korea). Real-time PCR was performed using a two-step cycling protocol: denaturation at 95 °C for 5 s and annealing/extension at 60 °C for 10 s, requiring 40 cycles with the Rotor-Gene SYBR Green PCR Kit (Qiagen, Hilden, Germany) on a Rotor-Gene Q PCR machine (Chung *et al.*, 2018). Cycle threshold (Ct) values varied by no more than 0.1 among triplicates. Relative expression values for each gene were calculated using the 2^{−ΔΔCt} method normalized to 18S rRNA as the internal control. All experiments were performed in triplicate.

Cell proliferation assay

To assess the inhibitory effect of anti-EpCAM IgGs from the sera of immunized mice (using 1× PBS, EpCAM-MK^P and EpCAM-MKJK^P) on PC-3 and SW620 cell proliferation, an MTT cytotoxicity

assay was conducted using the Cell Counting Kit-8 (Dojindo Lab, Kumamoto, Japan). Human cancer cell lines PC-3 and SW620 (5×10^3 cells/well) were seeded in 96-well plates (SPL, Pocheon, Korea) and cultured until they reached 80% confluency. Cells were washed with $1 \times$ PBS and treated with serum dilutions of 0, 1:4000, 1:2000, 1:1000, 1:500, 1:200, 1:100 and 1:50, along with a commercial anti-EpCAM antibody as a positive control (0, 0.5, 1, 2, 4, 10 and 20 $\mu\text{g/mL}$) for 12, 24 and 36 h in PC-3 cells. For SW620 cells, the same procedure was followed with serum dilutions of 0, 1:16 000, 1:8000, 1:4000, 1:2000, 1:1000, 1:500 and 1:100 and the positive control (0, 0.25, 0.5, 1, 2, 4, 10 and 20 $\mu\text{g/mL}$) for 12, 24, and 48 h. Absorbance at 450 nm was measured using an Epoch Microplate Spectrophotometer (BioTek, Winooski, VT, USA). All experiments were performed in triplicate.

Wound healing migration assay

For the wound healing migration assay, 3×10^6 PC-3 cells and 5×10^6 SW620 cells per well were seeded in 6-well cell culture plates (SPL, Pocheon, Korea) with Culture-Inserts 2 Well (Ibidi, Verona, WI, USA) and culture until the cells reached confluence. After establishing a monolayer, the Culture-Inserts were removed using sterile forceps, and the plates were washed with $1 \times$ PBS to eliminate cell debris and non-adherent cells. The cells were treated with serum dilutions of 1:500 and 1:100 for PC-3 and 1:8000 and 1:2000 for SW620 and incubated them for 17 or 42 h at 37 °C, respectively. Cell migration was quantified, and images were captured using an inverted microscope (Olympus IMT-2, Olympus, Melville, NY, USA), a digital camera (OM-4ti, Olympus, Melville, NY, USA) and DMC Advanced image analysis software (INS Industry, Seoul, Korea).

Transwell invasion assay

A transwell invasion assay was conducted to evaluate the inhibitory effect of anti-EpCAM IgGs from the sera of immunized mice using Corning Matrigel Invasion Chamber 24-well plates with 8.0-micron pores (Corning, Corning, NY, USA). PC-3 and SW620 cells (2×10^4 cells) were seeded in 500 μL of serum-free medium in the upper chamber compartment. The lower compartment was filled with 800 μL of medium containing 20% FBS with serum dilutions of 1:500 and 1:1000 for PC-3 cells or 1:8000 and 1:2000 for SW620 cells, derived from mice immunized with $1 \times$ PBS, EpCAM-MK^P or EpCAM-MKJK^P, respectively. After incubation at 37 °C for 48 h for PC-3 cells and 96 h for SW620 cells, the invaded cells that migrated to the lower surface of the chamber were fixed and stained using the Diff-Quick Kit (Sysmex, Kobe, Japan). The cells were then dried and counted. Images were obtained using an inverted microscope (Olympus IMT-2, Olympus, Melville, NY, USA), a digital camera (OM-4ti, Olympus, Melville, NY, USA) and DMC Advanced image analysis software (INS Industry, Seoul, Korea).

Data statistics

All experiments were performed at least three times independently. Data are presented as means $\pm$ standard deviation (SD) from independent experiments. Statistical significance was determined using Student's *t*-test, with $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$.

Author contributions

Conceptualization, S. L., Y. J. S., K. K.; Data curation, S. L., H. J. C., Y. J. O., P. H., Y. J. S.; Formal analysis, S. L., H. J. C., K. K.;

Investigation, S. L., H. J. C., S. C. M., Y. J. S.; Methodology, S. L., H. J. C., Y. J. S.; Validation, S. L., H. J. C., Y. J. S.; Resource, S. C. M., Y. J. S., K. K.; Visualization, S. L., H. J. C.; Writing – original draft, S. L., K. K.; Supervision, S. C. M., Y. J. S., K. K.; Funding acquisition, K. K.; Project administration, K. K.; Writing – review and editing, Y. J. O., P. H., S. C. M., Y. J. S., K. K.

Acknowledgements

This research was supported by the National Research Foundation of Korea (NRF) grant funded by the Korea government (MSIT) (No. RS-2024-00409554)

Conflict of interest

The authors declare that they have no conflict of interest.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1 PCR and RT-PCR analyses to confirm transgene insertion and mRNA expression in transgenic *Nicotiana tabacum* plants expressing EpCAM-Fc (FcK) and JK proteins.

Figure S2 SDS-PAGE analysis of purified EpCAM-Fc and EpCAM-FcK recombinant proteins from transgenic *N. tabacum*.

Figure S3 SDS-PAGE analysis of purified EpCAM-Fc^P (FcK^P) × J^P (JK^P) recombinant proteins from transgenic plants.

Figure S4 Schematic diagram of the 6x His tag-EpCAMK gene expression cassette, its expression, and purification.

Figure S5 Flow cytometry analysis confirms activation of dendritic cells (DCs) by EpCAM-MK^P or EpCAM-MKJK^P recombinant proteins before injection.

Figure S6 RT-PCR and immunocytochemistry (ICC) analysis to confirm EpCAM expression in various cancer cells.

Figure S7 Proliferation assay of PC-3 and SW620 cells using sera from immunized BALB/c mice with EpCAM-MK^P and EpCAM-MKJK^P proteins. The sera were collected 10 days after the fourth immunization with EpCAM-MK^P and EpCAM-MKJK^P proteins.

Table S1 The sequences of gene-specific primers used for PCR and RT-PCR analysis.

Table S2 The sequence of gene-specific primers used for real-time PCR and qRT-PCR analysis.