

Efficient surface display of single-chain variable fragments against tumor necrosis factor α on engineered probiotic *Saccharomyces boulardii* and its application in alleviating intestinal inflammation *in vivo*

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ARTICLE INFO

Keywords:

Saccharomyces boulardii

Single-chain variable fragment

Yeast surface display

ABSTRACT

Saccharomyces boulardii is a probiotic that can alleviate inflammation in the gut. In this study, a novel surface display system was developed by employing two different single-chain variable fragments (scFvs) against human tumor necrosis factor α as model anti-inflammatory proteins. We first optimized the expression levels of the scFvs by selecting strong constitutive promoters, of which expression cassettes were integrated into the *S. boulardii* chromosome using the CRISPR/Cas9-based genome editing system. As the signal peptide and anchoring motif are key factors affecting the display efficiency of target proteins, we next sought to find their optimal combination for the development of an efficient surface display system in *S. boulardii*. Among various combinations of signal peptide and anchoring motif, the engineered *S. boulardii*, which displayed scFvs using the SED1 signal peptide and DAN4 glycosylphosphatidylinositol domain (derived from *Saccharomyces cerevisiae*) as the signal peptide and anchoring motif, respectively, exhibited the highest display efficiency. Finally, the anti-inflammatory effects of the engineered *S. boulardii* strains displaying a high yield of scFvs, including a low disease activity index score, prevention of colon shortening, and reduction in pro-inflammatory cytokines, were validated using a colitis mouse model. Therefore, we believe that our approach has potential applications in the development of engineered *S. boulardii* displaying other valuable proteins.

1. Introduction

Saccharomyces cerevisiae var. *boulardii* is the most commonly used probiotic yeast for the treatment of gastrointestinal tract disorders, such as diarrhea and inflammatory bowel disease (IBD) [1]. In particular, *S. boulardii* outperforms other probiotic microorganisms in terms of immunomodulation through secretory immunoglobulin type A and interleukin-10 stimulation [2]. A previous study showed that its anti-inflammatory effects are not limited to the gut but are also observed in distant organs through systemic circulation [3]. In addition, given its complete elimination from the gut within five days after discontinuing its administration [4], *S. boulardii* has enormous potential for the in-gut production of therapeutic agents.

Among several yeast expression systems, surface display systems, which express proteins of interest (POIs) on the cell surface, offer the advantage of improved activity and stability against changes in temperature, pH, and proteases [5]. Although *S. cerevisiae* and *S. boulardii* exhibit more than 99 % similarity in their genome sequences [6], some genetic tools developed in *S. cerevisiae* do not work well in *S. boulardii* [7, 8]. For this reason, surface display systems that have been extensively developed using *S. cerevisiae* as a host strain are not directly applicable to *S. boulardii*. To date, three studies have reported the successful display of POIs on the surface of *S. boulardii* using an endogenous glycosylphosphatidylinositol (GPI) domain of AGA1, AGA2, CWP2, and SED1 as an anchoring motif [9–11]. However, a systematic approach to improving the surface display efficiency of POIs in *S. boulardii* has not

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Table 1

Saccharomyces boulardii strains and plasmids used in this study.

Name	Description	Reference
Strains		
<i>S. boulardii</i>	ATCC MYA-796	ATCC
P _{PGK} Mα	<i>S. boulardii</i> containing the P _{PGK} -MFα _{SP} -scFv ^{TSA2} -His ₆ -T _{CYC1} expression cassette	This study
P _{PGK}	<i>S. boulardii</i> containing the P _{PGK} -scFv ^{TSA2} -His ₆ -T _{CYC1} expression cassette	This study
P _{GPD}	<i>S. boulardii</i> containing the P _{GPD} -scFv ^{TSA2} -His ₆ -T _{CYC1} expression cassette	This study
P _{TEF1}	<i>S. boulardii</i> containing the P _{TEF1} -scFv ^{TSA2} -His ₆ -T _{CYC1} expression cassette	This study
P _{HXT7}	<i>S. boulardii</i> containing the P _{HXT7} -scFv ^{TSA2} -His ₆ -T _{CYC1} expression cassette	This study
S1-A1	<i>S. boulardii</i> containing the P _{HXT7} -SED1 _{SP} -myc tag-scFv ^{TSA2} -linker-AGA1 _{GPI} -T _{CYC1} expression cassette	This study
Mα-A1	<i>S. boulardii</i> containing the P _{HXT7} -MFα _{SP} -myc tag-scFv ^{TSA2} -linker-AGA1 _{GPI} -T _{CYC1} expression cassette	This study
S1-D4	<i>S. boulardii</i> containing the P _{HXT7} -SED1 _{SP} -myc tag-scFv ^{TSA2} -linker-DAN4 _{GPI} -T _{CYC1} expression cassette	This study
Mα-D4	<i>S. boulardii</i> containing the P _{HXT7} -MFα _{SP} -myc tag-scFv ^{TSA2} -linker-DAN4 _{GPI} -T _{CYC1} expression cassette	This study
P ^{B_{MEL}} _{TEF1}	<i>S. boulardii</i> containing the P _{TEF1} -scFv ^{B_{MEL}} -His ₆ -T _{CYC1} expression cassette	This study
P ^{B_{MEL}} _{HXT7}	<i>S. boulardii</i> containing the P _{HXT7} -scFv ^{B_{MEL}} -His ₆ -T _{CYC1} expression cassette	This study
S1-A1 ^{B_{MEL}}	<i>S. boulardii</i> containing the P _{TEF1} -SED1 _{SP} -myc tag-scFv ^{B_{MEL}} -linker-AGA1 _{GPI} -T _{CYC1} expression cassette	This study
Mα-A1 ^{B_{MEL}}	<i>S. boulardii</i> containing the P _{TEF1} -MFα _{SP} -myc tag-scFv ^{B_{MEL}} -linker-AGA1 _{GPI} -T _{CYC1} expression cassette	This study
S1-D4 ^{B_{MEL}}	<i>S. boulardii</i> containing the P _{TEF1} -SED1 _{SP} -myc tag-scFv ^{B_{MEL}} -linker-DAN4 _{GPI} -T _{CYC1} expression cassette	This study
Plasmids		
pCas9_AUR	p414-TEF1p-Cas9-CYC1t, modified Cas9 expression plasmid, Aur ^R	[19]
pgRNA-CS6-HYB	SNR52p-gCS6-SUP4t, 2 μ origin, Hyg ^R	[8]

been attempted. The promoter, signal peptide, anchoring motif, and linker play a crucial role in the expression, secretion, and display of target proteins in *S. cerevisiae* [5]. In this study, we sought to efficiently display POIs on the surface of *S. boulardii* by determining the optimal combination of promoter, signal peptide, and anchoring motif derived from *S. cerevisiae*.

As model proteins, we chose two single-chain variable fragments (scFvs), scFv^{TSA2} and scFv^{B_{MEL}}, against human tumor necrosis factor α (TNFα). The scFvs consisting of the variable regions of the heavy and light chains of immunoglobulins have provided an alternative to full-length monoclonal antibodies in therapeutic applications owing to better pharmacokinetic properties and lower production costs [12]. While scFv^{TSA2} is a novel scFv constructed through computer-guided molecular design [13], scFv^{B_{MEL}} is derived from a hybridoma cell line 2-179-E11 [14]. TNFα is a representative pro-inflammatory cytokine that plays an important role in the pathogenesis of inflammatory bowel disease and tumor formation [15]; hence, anti-TNFα therapy is one of the most effective strategies for treating chronic inflammatory diseases [16]. Although TNFα inhibitors have clear benefits, anti-TNFα therapy nonetheless has certain drawbacks, such as immunogenicity and instability in terms of its physicochemical properties, leading to primary and secondary non-response to anti-TNFα therapy [17]. These limitations can be overcome with novel strategies designed to improve delivery of TNFα inhibitors to the inflamed colon. Therefore, the two scFvs against TNFα were included as model proteins not only because the development of an efficient surface display system has promising applications in various fields of medicine and biotechnology, but also because surface display of the scFvs can maximize the beneficial effects of *S. boulardii* on the gastrointestinal tract.

In this study, the SED1 signal peptide (SED1_{SP}) and DAN4 GPI domain (DAN4_{GPI}) were found to be the optimal signal peptide and anchoring motif, respectively. As a result, constitutive expression of

scFv^{TSA2} and scFv^{B_{MEL}} under the transcriptional control of strong promoters with the aid of SED1_{SP} and DAN4_{GPI} resulted in a significant increase in display efficiency on the surface of *S. boulardii*. Finally, the efficiency of the engineered *S. boulardii* strains capable of displaying anti-TNFα scFvs was evaluated using an IBD mouse model.

2. Materials and methods

2.1. Strains and plasmids

The *E. coli* TOP10 strain (Invitrogen, Carlsbad, CA, USA) was used for gene cloning. *S. boulardii* ATCC MYA-796 was used for the surface display of scFvs. All plasmids and *S. boulardii* strains used in this study are listed in Table 1.

2.2. Genetic manipulation

The genes coding for scFv^{TSA2} and scFv^{B_{MEL}} were synthesized by Bionics (Seoul, Republic of Korea). The primer sets used for amplification of the scFv^{TSA2} and scFv^{B_{MEL}} genes and construction of their expression cassettes are listed in Table S1. The resulting PCR products were combined using NEBuilder HiFi DNA Assembly Master Mix (New England Biolabs, Ipswich, MA, USA), as specified by the manufacturer.

The engineered *S. boulardii* strains expressing various scFv expression cassettes were constructed by introducing the expression cassettes into the CS6 locus [8], an intergenic region between the gene coding for tRNA and *HIP1* in chromosome VII of *S. boulardii*. To do so, the CRISPR/Cas9-based genome editing system was used, as previously described [18]. Briefly, the following primer sets were used for constructing repair DNA fragments containing the scFv expression cassettes: (1) SK35 and SK36 for the PGK promoter (P_{PGK})-based expression cassettes, (2) KH45 and SK36 for the GPD promoter (P_{GPD})-based expression cassettes, (3) KH47 and SK36 for the TEF1 promoter (P_{TEF1})-based expression cassettes, and (4) KH46 and SK36 for the truncated HXT7 promoter (P_{HXT7})-based expression cassettes. The resulting repair DNA fragments were introduced into the CS6 locus of *S. boulardii* ATCC MYA-796 harboring pCas9_AUR [19] with the aid of a pgRNA-CS6-HYB plasmid [8] expressing guide RNA specific for the CS6 locus. All primers used for genetic manipulation are listed in Table S1.

2.3. Media and culture conditions

Luria-Bertani medium (10 g/L tryptone, 5 g/L yeast extract, and 10 g/L NaCl) containing 50 μg/mL ampicillin was used for cultivation of *E. coli*. *S. boulardii* strains were pre-cultured at 30°C and 230 rpm for 48 h in yeast peptone (YP) medium (10 g/L of yeast extract and 20 g/L of Bacto™ Peptone) containing 20 g/L glucose. Cells at the stationary phase of the pre-culture were harvested and inoculated into main cultures with an initial optical density at 600 nm (OD₆₀₀) of 1.0. Batch fermentation experiments were performed at 30°C and 250 rpm for 72 h in a baffled flask containing 100 mL of YP medium (10 g/L of yeast extract and 20 g/L of Bacto™ Peptone) with 50 g/L of glucose.

2.4. Protein preparation and analysis of scFv expression

After cell cultivation, the culture broth was centrifuged at 15,000 rpm for 10 min to collect the extracellular protein fraction. To collect the intracellular protein fraction, cells were harvested at OD₆₀₀ × culture (mL) = 50 (i.e., if the OD₆₀₀ was 20, then 2.5 mL of culture was harvested). The harvested cells were resuspended in Y-PER™ (Thermo Fisher Scientific, Waltham, MA, USA) and lysed according to the manufacturer's instructions.

To visualize the proteins, the protein samples were fractionated by 12 % (w/v) sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The resulting fractionated proteins were transferred to polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA). For the

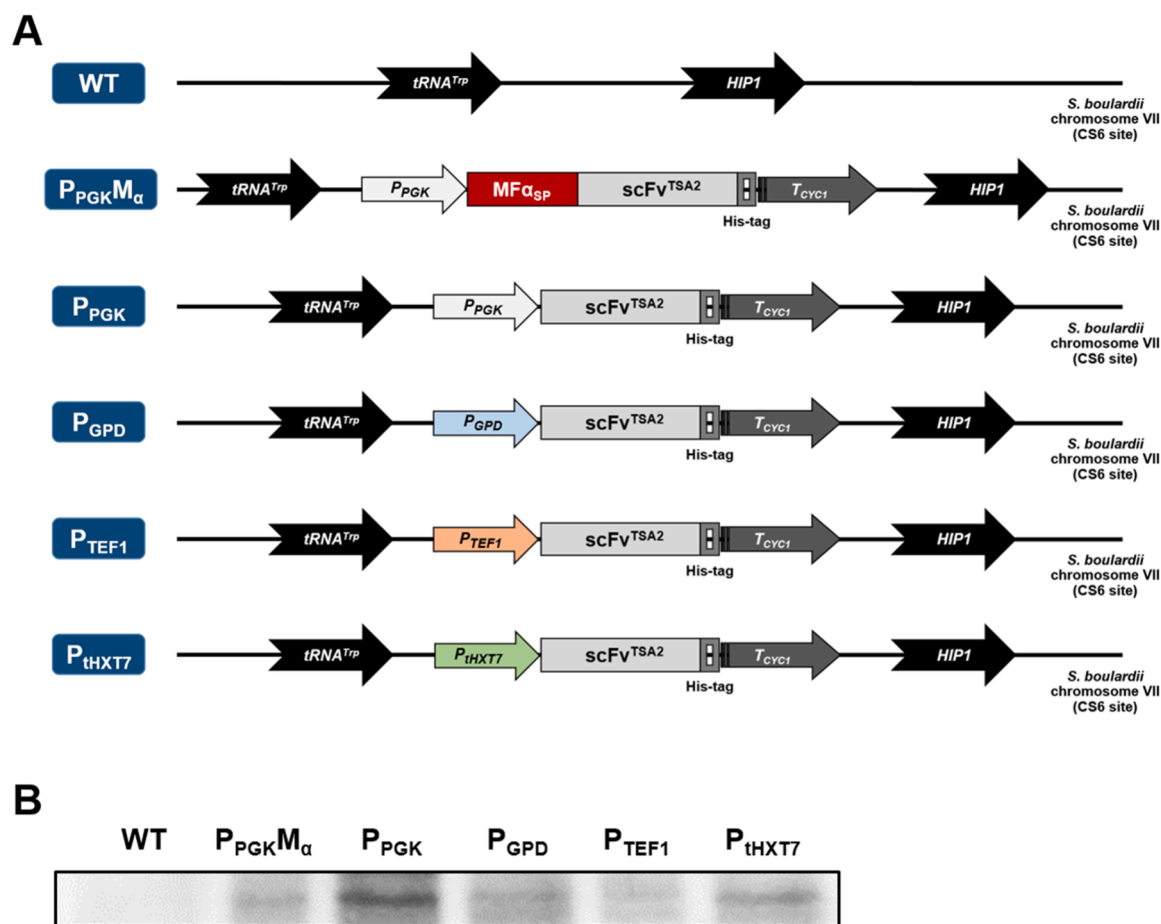


Fig. 1. Selection of the optimal promoter for scFv^{TSA2} expression. (A) Schematic diagrams of the scFv^{TSA2} protein and the structures of its expression cassettes. P_{PGK} , PGK promoter; P_{GPD} , GPD promoter; P_{TEF1} , TEF1 promoter; P_{tHXT7} , truncated HXT7 promoter; MF α _{SP}, mating factor α signal peptide; *T_{CYC1}*, *CYC1* terminator. (B) Western blot analysis to determine the optimal promoter for scFv^{TSA2} expression. Cell lysates of various engineered *S. boulardii* strains expressing scFv^{TSA2} were prepared and analyzed using an anti-polyhistidine antibody.

immunoblot assays, His₆-fusion proteins were detected using an anti-polyhistidine antibody (Abcam, ab18184) according to the manufacturer's instructions.

2.5. Immunofluorescence

Immunofluorescence generated by the engineered *S. boulardii* strains was detected according to a previous study [20]. Briefly, after treatment with anti-myc primary antibody (Santa Cruz, sc-42) and secondary anti-mouse Alexa Fluor 488-conjugated antibody (Invitrogen, A-11001), the cells were visualized using a Zeiss LSM 800 confocal laser scanning fluorescence microscope (Carl Zeiss, Oberkochen, Germany) with a 488 nm excitation filter and analyzed using Zen lite software (Carl Zeiss).

2.6. Animals

The animal experiments were approved by the Institutional Animal Care and Use Committee of Chung-Ang University (202301020090). The *in vivo* animal experiments were conducted in accordance with the National Research Council's Guide for the Care and Use of Laboratory Animals. Female Balb/c mice (6 weeks old) were purchased from DooYeol Biotech (Anyang, Republic of Korea). The mice were housed in ventilated cages (5 mice per cage) and kept for 1 week in our facility for acclimation.

2.7. Therapeutic effect against IBD model

An acute dextran sulfate sodium salt (DSS)-induced IBD mouse model was prepared following previously reported studies [8,21], with minor modifications. In brief, mice were randomly divided into the following five groups: (1) water without DSS (control [CON] group, $n = 3$), (2) water containing 4 % (w/v) DSS and not treated with any yeast ($n = 5$), (3) water containing 4 % DSS and treated with the wild-type (WT) *S. boulardii* ($n = 5$), (4) water containing 4 % DSS and treated with the S1-D4 strain (Table 1) ($n = 5$), and (5) water containing 4 % DSS and treated with the S1-D4^{B_{ME}L} strain (Table 1) ($n = 5$). For IBD induction, the mice were treated with drinking water containing 4 % DSS (molecular weight = 40,000, Alfa Aesar, Ward Hill, MA, USA) for 7 days, followed by normal water. In contrast, the control groups received normal water only. To evaluate the therapeutic effect of the engineered *S. boulardii* strains expressing scFv^{TSA2} and scFv^{B_{ME}L}, the mice were administered 1×10^9 CFU (100 μ L of 1×10^{10} CFU per mouse) of the yeast strains (WT, S1-D4, and S1-D4^{B_{ME}L}) via oral gavage daily during the entire experiment (from Day 0 to Day 7 for mice treated with DSS). During the treatment, symptoms of colitis were assessed daily according to the disease activity index (DAI) scoring system described previously [8,22], with some modifications. DAI was obtained as the mean score encompassing the three parameters weight loss, stool consistency, and presence or absence of blood in feces. The mice were sacrificed on Day 8 and their colons were collected to assess the therapeutic efficacy of the engineered *S. boulardii* strains. The length of collected colon tissues was measured, and the tissues were gently washed with Dulbecco's

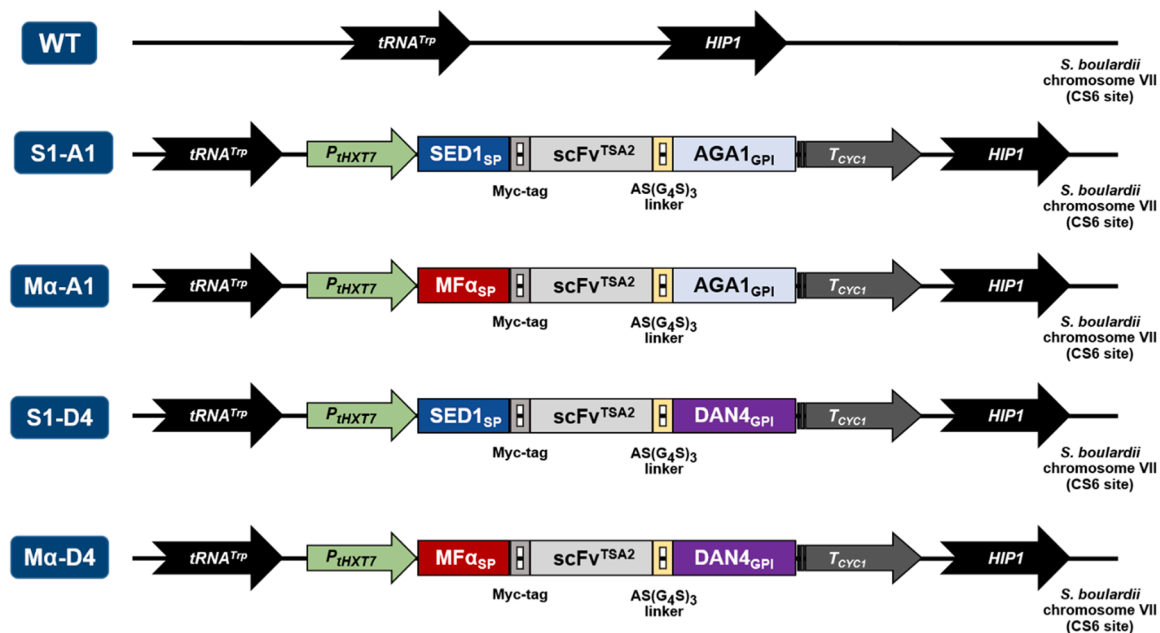


Fig. 2. Schematic representation of the expression cassettes constructed for scFv^{TSA2} display. *P*_{HXT7}, truncated *HXT7* promoter; SED1_{SP}, SED1 signal peptide; MFA_{SP}, mating factor α signal peptide; GPI: glycosylphosphatidylinositol domain; *T*_{CYC1}, *CYC1* terminator.

phosphate-buffered saline. Then, two 1-cm samples of the distal section were collected for histological assessment and inflammatory cytokine determination.

2.8. Histological assay

For histological observations, the distal portions of colon tissues of mice were fixed in 4 % paraformaldehyde. Then, the colon tissues were embedded in paraffin, cross-sectioned into 3-μm slices, and stained with hematoxylin and eosin (H&E). The sections were observed with a light microscope (BX50, Olympus, Tokyo, Japan). Images were acquired with an automated slide scanner (TW-SM01, TaeWoong Medical Co. Ltd, Goyang, Republic of Korea). The severity of acute IBD was blindly evaluated using histological sections according to previously reported criteria [23,24].

2.9. Cytokine assays

To determine the concentrations of pro-inflammatory cytokines in the colon, the segments of distal colon were mechanically homogenized in cold RIPA lysis buffer (Biomax, Guri, Republic of Korea) containing universal protease inhibitor cocktail (Biomax, Guri, Republic of Korea) using a homogenizer (Daihan Scientific Co., Wonju, Republic of Korea). The tissue lysates were centrifuged for 30 min at 15,000 rpm and 4°C, and then the supernatant was collected and stored at −80°C. TNF-α, interleukin (IL)-6, and IL-1 beta (IL-1β) levels in the supernatant were measured using the respective enzyme-linked immunosorbent assay (ELISA) kits (R&D systems, Minneapolis, MN, USA), according to the manufacturer's instructions.

2.10. Statistical analysis

The biological experiments were performed three times, and the data are reported as the mean ± standard deviation (S.D.). Statistical analyses were performed using the Systat Software (Chicago, IL, USA). For statistical comparison, we performed two-tailed Student's *t*-test and one-way ANOVA. Statistical significance was indicated as *p* < 0.05.

3. Results and discussion

3.1. Design of the scFv^{TSA2} expression system in *S. boulardii*

*P*_{PGK} and mating factor α signal peptide (MFA_{SP}) are a strong constitutive promoter and signal peptide pairing frequently used in *S. cerevisiae* [25]. According to a previous study that reported the successful expression of a luciferase from the firefly *Photinus pyralis* (FLuc) in *S. boulardii* under the transcriptional control of *P*_{PGK} [8], we initially attempted to produce scFv^{TSA2} in the extracellular fraction as a free form by attaching MFA_{SP} to the N-terminus of scFv^{TSA2} under the transcriptional control of *P*_{PGK} (Fig. 1A). However, scFv^{TSA2} expression was detected only in the intracellular fraction of the *P*_{PGK}Mα strain (Table 1) containing the *P*_{PGK}-MFA_{SP}-scFv^{TSA2}-His₆-*T*_{CYC1} expression cassette (Fig. 1A). Because finding the optimal conditions for scFv expression and secretion simultaneously proved challenging, we decided to optimize the surface display of scFv^{TSA2} after choosing the optimal promoter capable of moderately increasing the scFv expression level. To this end, we compared the expression levels of scFv^{TSA2} generated under the transcriptional control of four strong constitutive promoters (*P*_{PGK}, *P*_{GPD}, *P*_{TEF1}, and *P*_{HXT7}) derived from *S. cerevisiae* [26,27]. It is important to note that an inducible promoter system including the galactose-inducible promoter was not considered in this study because this system requires inducers that are not usually present in the gastrointestinal tract. Similarly, the scFv expression cassettes were integrated into chromosome VII of *S. cerevisiae* for stable expression of scFv without the requirement of selection pressure. While the *P*_{PGK} induced the strongest scFv^{TSA2} expression, moderate expression of scFv^{TSA2} was observed in the *P*_{HXT7}-based expression system (Fig. 1B). Of the two promoters, *P*_{HXT7} was chosen as the optimal promoter for future experiments on the surface display of scFv^{TSA2}. This was because overly strong expression of heterologous proteins in *S. cerevisiae* and *S. boulardii* often causes metabolic burden on the host and hence aggravates cell growth [8,28,29].

3.2. Surface display of scFv^{TSA2} on *S. boulardii*

Membrane proteins are typically synthesized with a cleavable signal peptide that targets them for cell wall localization [30]; hence, the

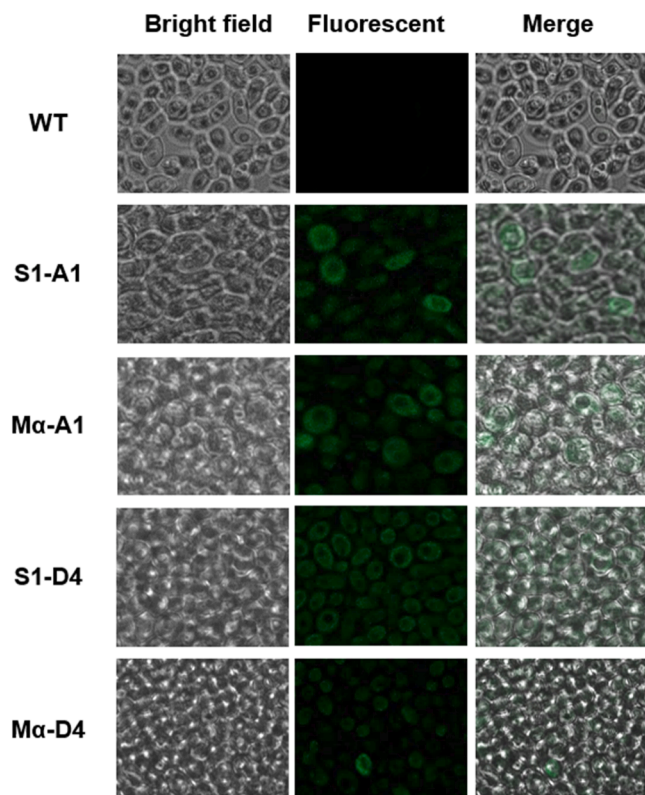


Fig. 3. Confirmation of cell surface display of scFv^{TSA2} by immunofluorescence. WT, *S. boulardii* ATCC MYA-796; S1-A1, *S. boulardii* ATCC MYA-796 containing the P_{HXT7}-SED1_{sp}-myc tag-scFv^{TSA2}-linker-AGA1_{GPI}-T_{CYC1} expression cassette; Mα-A1, *S. boulardii* ATCC MYA-796 containing the P_{HXT7}-MFα_{sp}-myc tag-scFv^{TSA2}-linker-AGA1_{GPI}-T_{CYC1} expression cassette; S1-D4, *S. boulardii* ATCC MYA-796 containing the P_{HXT7}-SED1_{sp}-myc tag-scFv^{TSA2}-linker-DAN4_{GPI}-T_{CYC1} expression cassette; Mα-D4, *S. boulardii* ATCC MYA-796 containing the P_{HXT7}-MFα_{sp}-myc tag-scFv^{TSA2}-linker-DAN4_{GPI}-T_{CYC1} expression cassette.

addition of an optimal signal peptide is important to maximize the display efficiency of POIs. Recently, it was reported that a novel signal peptide derived from SED1 (SED1_{sp}), a major GPI protein of *S. cerevisiae* in the stationary phase, improved secretory production and surface display of POIs in both *S. cerevisiae* [31] and *S. boulardii* [32]. Therefore, the scFv^{TSA2} expression cassettes with N-terminal SED1_{sp} in addition to the conventional signal peptide (MFα_{sp}) were constructed for the surface display of scFv^{TSA2} (Fig. 2).

The anchoring motif is another key factor that affects the display efficiency of POIs. The surface display system with a-agglutinin consisting of two components (AGA1 and AGA2) is widely used for the surface display of POIs in various yeast strains [33]. However, the two-component system has been reported to have lower display efficiency than a one-component display system due to the limited binding capacity between AGA1 and AGA2 and the instability of the disulfide bond, which is essential for POI anchoring in the two-component system under reducing conditions [5,34]. Therefore, we decided to exploit a one-component display system in which the GPI domain is directly attached at the C-terminus of the POI. To this end, GPI domains derived from AGA1 and DAN4 were fused with scFv^{TSA2}, and their display efficiency was compared. In addition, the AS(G₄S)₃ linker, consisting of a Ser and Gly repeat sequence [34], was placed between scFv^{TSA2} and the GPI domain to increase the flexibility of the fusion protein (Fig. 2). The scFv^{TSA2} expression in the four engineered strains was confirmed using western blot analysis (Fig. S1).

Next, we compared the display efficiency of scFv^{TSA2} using immunofluorescence microscopy to identify the best combination of signal

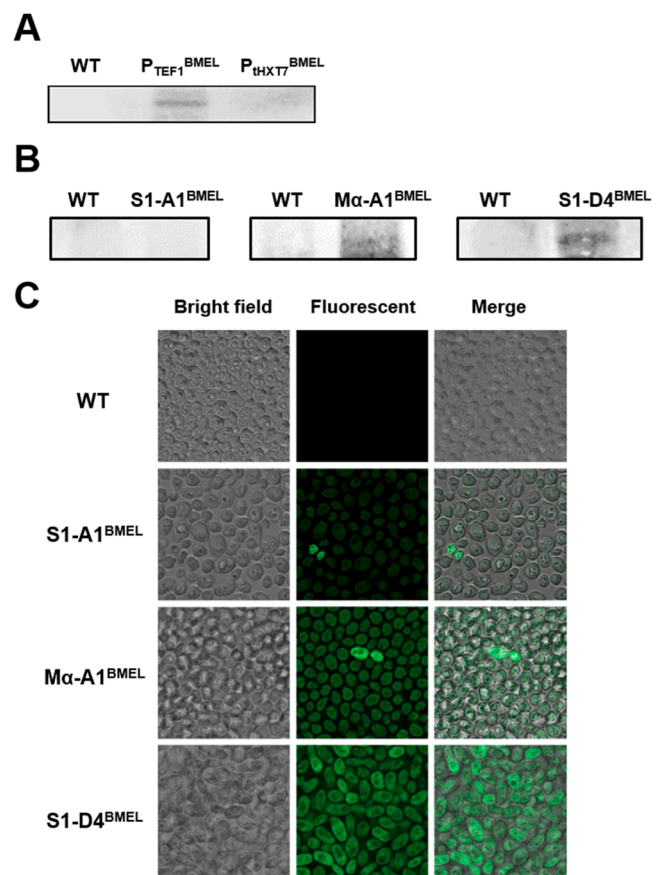


Fig. 4. Surface display of scFv^{BMEL} on *S. boulardii*. Western blot analysis to determine the optimal promoter (A) and combination of signal peptide and anchoring motif (B) for scFv^{BMEL} display. Cell lysates of various engineered *S. boulardii* strains expressing scFv^{BMEL} were prepared and analyzed using an anti-polyhistidine antibody. (C) Confirmation of cell surface display of scFv^{BMEL} by immunofluorescence. WT, *S. boulardii* ATCC MYA-796; P_{TEF1}-scFv^{BMEL}, *S. boulardii* ATCC MYA-796 containing the P_{TEF1}-scFv^{BMEL}-His₆-T_{CYC1} expression cassette; P_{HXT7}-scFv^{BMEL}, *S. boulardii* ATCC MYA-796 containing the P_{HXT7}-scFv^{BMEL}-His₆-T_{CYC1} expression cassette; S1-A1^{BMEL}, *S. boulardii* ATCC MYA-796 containing the P_{TEF1}-SED1_{sp}-myc tag-scFv^{BMEL}-linker-AGA1_{GPI}-T_{CYC1} expression cassette; Mα-A1^{BMEL}, *S. boulardii* ATCC MYA-796 containing the P_{TEF1}-MFα_{sp}-myc tag-scFv^{BMEL}-linker-AGA1_{GPI}-T_{CYC1} expression cassette; S1-D4^{BMEL}, *S. boulardii* ATCC MYA-796 containing the P_{TEF1}-SED1_{sp}-myc tag-scFv^{BMEL}-linker-DAN4_{GPI}-T_{CYC1} expression cassette.

peptide and the GPI domain. Among the engineered strains, only the S1-D4 strain produced a strong signal of evenly distributed green fluorescence, whereas heterogeneity of scFv^{TSA2} display was observed in the homogeneous populations of the S1-A1, Mα-A1, and Mα-D4 strains (Fig. 3). This finding indicates that virtually all scFv^{TSA2} expressed in the S1-D4 strain was displayed on the cell surface, and that the combination of signal peptide and GPI domain played a crucial role in the display efficiency of scFv^{TSA2}. Collectively, from these observations, we concluded that the SED1_{sp} and DAN4_{GPI} were the optimal signal peptide and anchoring motif, respectively, for the efficient display of scFv^{TSA2}.

In contrast to a previous study that reported the highest POI display efficiency using endogenous *S. boulardii* SED1_{GPI} as the anchoring motif [11], scFv^{TSA2} expression was not detected when *S. cerevisiae* SED1_{GPI} was attached to the C-terminus of scFv^{TSA2}, regardless of the type of signal peptide (data not shown). Additional experiments are underway to identify the key factors that led to the different results. For instance, the different amino acid sequences of SED1_{GPI} in *S. cerevisiae* and *S. boulardii* may play a role in the expression of heterologous proteins.

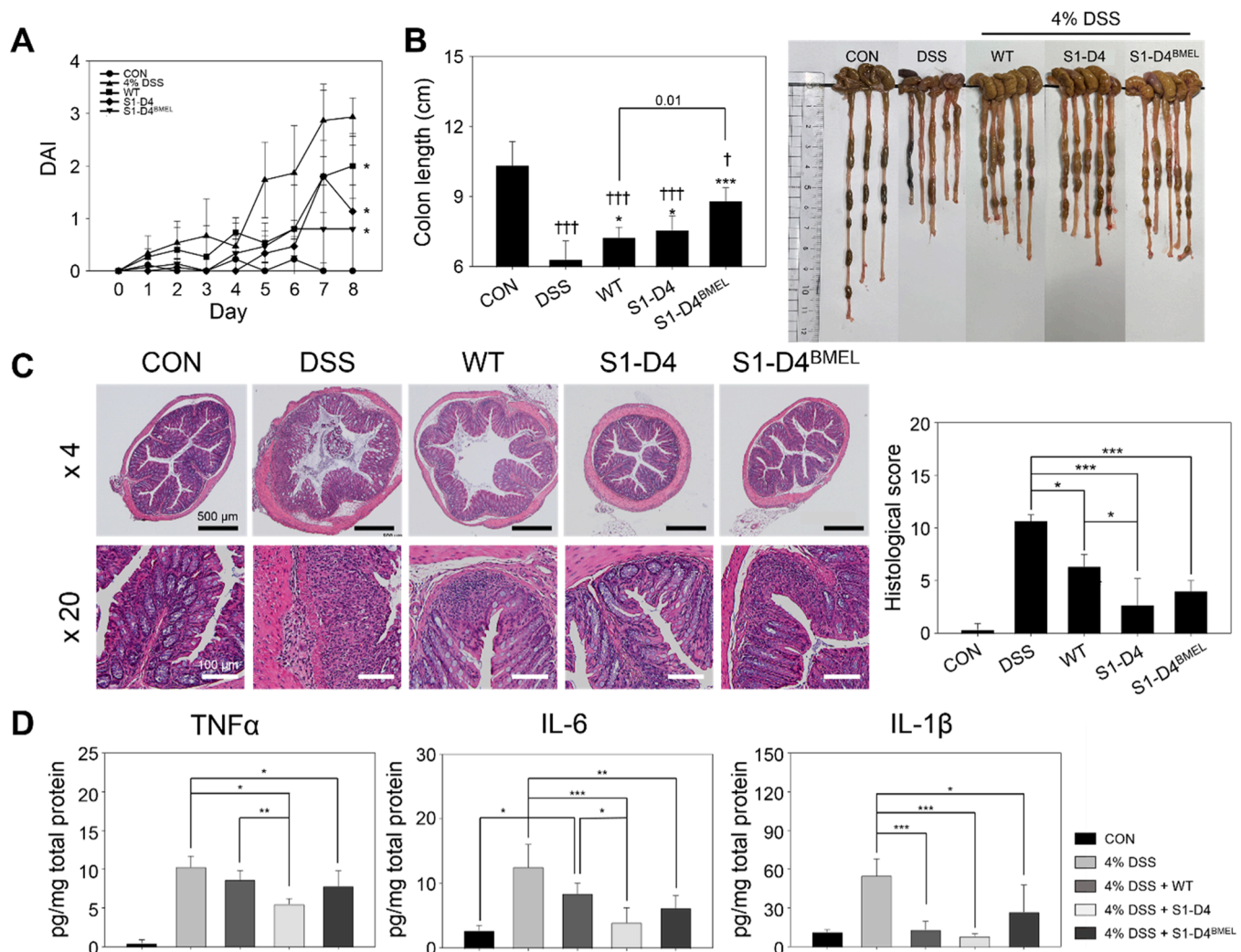


Fig. 5. Treatment efficacy of the engineered *S. boulardii* displaying anti-TNF α scFv in DSS-induced IBD mice. (A) Disease activity index (DAI) scores of each group from Day 0 to Day 8. * $p < 0.05$ vs the DSS group. (B) Colon lengths and photos of colon of mice in different groups. $\dagger p < 0.05$ and $\dagger\dagger p < 0.001$ vs the CON group; * $p < 0.05$ and *** $p < 0.001$ vs the DSS group. (C) Histological analysis (H&E staining) and histopathological scores of the colon tissues of mice in different groups. Scale bar = 500 μ m (upper) and 100 μ m (lower). * $p < 0.05$, and *** $p < 0.001$. (D) Levels of pro-inflammatory cytokines, including TNF α , IL-6, and IL-1 β , in colon tissues of mice determined using the respective ELISA kits on Day 8. Data are represented as the mean $\pm$ SD. CON: the normal group, DSS: 4 % DSS without any yeast, WT: 4 % DSS with wild-type *S. boulardii*, S1-D4: 4 % DSS with *S. boulardii* ATCC MYA-796 containing the P_{HXT7} -SED1_{SP}-myc tag-scFv^{TSA2}-linker-DAN4_{GPI}-TCYC1 expression cassette, S1-D4^{BMEL}: 4 % DSS with *S. boulardii* ATCC MYA-796 containing the P_{TEF1} -SED1_{SP}-myc tag-scFv^{BMEL}-linker-DAN4_{GPI}-TCYC1 expression cassette. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

3.3. Efficient display of scFv^{BMEL} on the cell surface via the combination of SED1_{SP} and DAN4_{GPI}

Before searching for the best combination of signal peptide and GPI domain for scFv^{BMEL} display, we compared the expression levels of scFv^{BMEL} generated from four different promoters (P_{PGK} , P_{GPD} , P_{TEF1} , and P_{HXT7}). Of the four expression cassettes constructed, attempts to introduce the scFv^{BMEL} expression cassettes containing P_{PGK} and P_{GPD} were unsuccessful (data not shown), which was likely due to the metabolic burden caused by the unbalanced expression of scFv^{BMEL} driven by P_{PGK} and P_{GPD} . This result is consistent with our previous finding that FLuc expression cassettes could be introduced into *S. boulardii* only when the FLuc expression levels were moderate [8]. Therefore, scFv^{BMEL} was designed to be under the transcriptional control of P_{TEF1} and P_{HXT7} (Fig. S2). Of these two promoters, P_{TEF1} was selected for subsequent experiments because only P_{TEF1} successfully induced scFv^{BMEL} expression (Fig. 4A).

As in the case of scFv^{TSA2}, expression cassettes were constructed for scFv^{BMEL} secretion by combining SED1_{SP} and MF α _{SP} with AGA1_{GPI} and

DAN4_{GPI}. Consistent with our findings, only certain expression cassettes could be inserted into the *S. boulardii* genome among the four expression cassettes expressing POIs. Specifically, we constructed engineered *S. boulardii* strains containing the scFv^{BMEL} expression cassette of the P_{TEF1} -SED1_{SP}-myc tag-scFv^{BMEL}-linker-AGA1_{GPI}-TCYC1 (S1-A1^{BMEL}), P_{TEF1} -MF α _{SP}-myc tag-scFv^{BMEL}-linker-AGA1_{GPI}-TCYC1 (M α -A1^{BMEL}), or P_{TEF1} -SED1_{SP}-myc tag-scFv^{BMEL}-linker-DAN4_{GPI}-TCYC1 (S1-D4^{BMEL}). We were unable to construct an engineered *S. boulardii* strain containing the MF α _{SP}-myc tag-scFv^{BMEL}-linker-DAN4_{GPI}-TCYC1 expression cassette despite multiple attempts (Fig. S2). More research is in progress to identify the key factors that determine the success of inserting gene expression cassettes into the *S. boulardii* genome. While significant levels of scFv^{TSA2} expression were observed in the M α -A1^{BMEL} and M α -D4^{BMEL} strains, scFv^{TSA2} expression in the S1-A1^{BMEL} strain was barely detectable in the western blot analysis (Fig. 4B). In the fluorescence microscopy results, as was the case with scFv^{TSA2} display, only the combination of SED1_{SP} and DAN4_{GPI} produced a strong and evenly distributed green fluorescent signal (Fig. 4C). In conclusion, the positive effects of the combination of SED1_{SP} and DAN4_{GPI} on display efficiency

were also confirmed for scFv^{BMEL}.

3.4. Therapeutic effect of the engineered *S. boulardii* strains displaying scFv^{TSA2} and scFv^{BMEL} in IBD

TNF α , the central pro-inflammatory mediator, participates in IBD pathogenesis [35,36]. Therefore, TNF α inhibitors, such as infliximab, have been considered as major therapeutic agents for IBD treatment [37, 38]. We examined the therapeutic effects of the S1-D4 and S1-D4^{BMEL} strains capable of displaying anti-TNF α scFvs with high yield using the DSS-induced IBD mouse model. To assess the therapeutic effect of the S1-D4 and S1-D4^{BMEL} strains, the mice were orally administered each strain (WT, S1-D4, and S1-D4^{BMEL}) from Day 0 to Day 7 during the treatment using water containing 4 % DSS, whereas the CON and DSS groups did not receive any strain. The DAI score of the DSS group increased steadily after DSS treatment unlike that in the CON group (Fig. 5A). In contrast, mice administered the WT, S1-D4, and S1-D4^{BMEL} strains showed a slower increase in DAI scores than those administered DSS. Notably, there were ameliorations of IBD symptoms in the groups administered the S1-D4 and S1-D4^{BMEL} strains. These results indicated that the engineered *S. boulardii* strains displaying scFv^{TSA2} and scFv^{BMEL} effectively reduced DSS-induced IBD in mice.

The length of harvested colons was also assessed (Fig. 5B). Compared with that in the CON group (10.33 $\pm$ 1.02 cm), the colon length of the DSS group was significantly reduced to 6.30 $\pm$ 0.79 cm. However, the colon length increased to 7.24 $\pm$ 0.44, 7.56 $\pm$ 0.61, and 8.80 $\pm$ 0.57 cm after treatment with the WT, S1-D4, and S1-D4^{BMEL} strains, respectively. Moreover, the colon length in the S1-D4 and S1-D4^{BMEL} treatment groups increased significantly by 20 and 40 %, respectively, compared to that in the DSS group. These results indicate that treatment with the engineered *S. boulardii* strains prevented colon shortening in the IBD mouse model.

In addition, H&E staining was performed to confirm the therapeutic effect in IBD. The colon tissue of the healthy CON group had evident intestinal crypts and intact mucosa without any signs of mucosal damage, whereas the colon tissue of the DSS group exhibited severe damages of mucosa with the disappearance of crypts and loss of Goblet cells. Mice treated with the WT, S1-D4, and S1-D4^{BMEL} strains showed marked improvements in the restoration of crypt structure and mucosal integrity. In concordance with the findings derived from colon tissue section imaging, the histological score for the DSS group was the highest among all groups, with notable decreases observed in all *S. boulardii*-treated groups compared with the DSS group. In particular, administration of the S1-D4 strain significantly attenuated the severity of the colon damage compared with the WT strain. These results demonstrate that *S. boulardii* strains displaying anti-TNF α scFvs have promising therapeutic effects in IBD.

To further investigate the therapeutic effects of the engineered yeasts displaying scFvs with high efficiency, the concentrations of pro-inflammatory cytokines in the colon tissue of IBD mice were determined using ELISA. Abnormal increase in pro-inflammatory cytokines in colon tissue plays a crucial role in IBD progression [39,40]. The DSS-mediated induction of inflammation significantly increased the expression of TNF α , IL-6, and IL-1 β (Fig. 5D). After treatment with WT *S. boulardii*, the TNF α , IL-6, and IL-1 β levels were reduced slightly, confirming that *S. boulardii* has an anti-inflammatory effect as a probiotic yeast [1]. Notably, the expression of pro-inflammatory cytokines in the groups administered engineered *S. boulardii* strains, especially the S1-D4 strain, were significantly lower than those in the DSS and WT groups. These results suggest that *S. boulardii* displaying anti-TNF α scFvs can attenuate DSS-induced IBD in mice by neutralizing TNF α via its probiotic and anti-inflammatory effects on IBD, providing a novel strategy for IBD treatment. A previous study demonstrated that mucosally administered *Lactococcus lactis* strain expressing an anti-TNF α scFv reduced colonic and systemic inflammatory symptoms in an IBD model [41]. Based on these findings, we expect that the engineered *S. boulardii*

strains constructed in this study can be used to treat IBD and other inflammatory diseases.

4. Conclusions

In this study, we demonstrated a novel strategy to display scFvs against human TNF α on the surface of *S. boulardii*. Although the optimal promoters for driving the expression of two scFvs (scFv^{TSA2} and scFv^{BMEL}) were different, the combination of the same signal peptide (SED1_{SP}) and anchoring motif (DAN4_{GPI}) significantly enhanced their display efficiency on the cell surface. Our findings demonstrated that the engineered *S. boulardii* strains displaying scFv^{TSA2} and scFv^{BMEL} alleviated intestinal inflammation in the DSS-induced colitis mouse model. The approach employed in this study can be applied to not only treat IBD but also construct engineered *S. boulardii* strains displaying various POIs with high efficiency.

CRedit authorship contribution statement

Kim Young-Woo: Visualization, Investigation. **Park Ji Sun:** Visualization, Methodology, Investigation. **Park Kyeongsoon:** Validation, Supervision. **Lee Woohyuk:** Validation, Methodology. **Han Kanghee:** Methodology, Investigation. **Kim Sun-Ki:** Writing – original draft, Formal analysis, Conceptualization.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

This study was financially supported by the National Research Foundation of Korea Grant (2021R1A4A1022206, 2022R1C1C1003154, and RS-2024-00440975), funded by the Korean Ministry of Science, ICT and Future Planning. This research was also supported by a grant (21153MFDS605) from the Ministry of Food and Drug Safety in 2025.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.nbt.2025.02.003.

Data availability

Data will be made available on request.

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