A rice-based soluble form of a murine TNF-specific llama variable domain of heavy-chain antibody suppresses collagen-induced arthritis in mice

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Abbreviations: Ab, Antibody; CIA, collagen-induced arthritis; CII, bovine type II collagen; CTB, cholera toxin B subunit; ELISA, enzyme-linked immunosorbent assay; GST, glutathione *S*-transferase; mTNF, mouse tumor necrosis factor alpha; PBS, phosphate buffered saline; RNAi, RNA interference; SDS-PAGE, SDS–polyacrylamide gel electrophoresis; TNF, tumor necrosis factor alpha; TNFR, TNF receptor; T-DNA, transfer DNA; VHH, llama variable domain of heavy-chain antibody fragment; WT, wild-type.

Summary

Tumor necrosis factor alpha (TNF) plays a pivotal role in chronic inflammatory diseases such as rheumatoid arthritis and Crohn's disease. Although anti-TNF antibody therapy is now commonly used to treat patients suffering from these inflammatory conditions, the cost of treatment continues to be a concern. Here, we developed a rice transgenic system for the production of a llama variable domain of a heavy-chain antibody fragment (VHH) specific for mouse TNF in rice seeds (MucoRice-mTNF-VHH). MucoRice-mTNF-VHH was produced at high levels in the rice seeds when we used our most recent transgene-overexpression system with RNA interference technology that suppresses the production of major rice endogenous storage proteins while enhancing the expression of the transgene-derived protein. Production levels of mTNF-VHH in rice seeds reached an average of 1.45% (w/w). Further, approximately 91% of mTNF-VHH was released easily when the powder form of MucoRice-mTNF-VHH was mixed with PBS. mTNF-VHH purified by means of single-step gel filtration from rice PBS extract showed high neutralizing activity in an *in vitro* mTNF cytotoxicity assay using WEHI164 cells. In addition, purified mTNF-VHH suppressed progression of collagen-induced arthritis in mice. These results show that this rice-expression system is useful for the production of neutralizing VHH antibody specific for mTNF.

1. Introduction

Tumor necrosis factor alpha (TNF) is considered to be responsible for chronic inflammatory conditions such as rheumatoid arthritis (Maini et al., 1995) and Crohn's disease (van Dullemen et al., 1995). It binds cell-surface receptor 1 (TNFR1, p55) and receptor 2 (TNFR2, p75). TNFR1 is expressed by all human tissues and is the major signaling receptor for TNF. TNFR2 is expressed in most immune cells and mediates limited biological responses. Both TNFR1 and TNFR2 signal through nuclear factor kappa B and MAP kinase to initiate pro-inflammatory gene transcription (Tracey et al., 2008). Currently, five TNF inhibitors (adalimumab, golimumab, infliximab, certolizumab, and etanercept) are available for clinical use (Thalayasingam and Isaacs, 2011). Adalimumab, golimumab, and infliximab are complete antibodies, whereas certolizumab is a Fab fragment conjugated to polyethylene glycol (PEG) and etanercept is a fusion protein of two TNFR2 receptor extracellular domains and an Fc fragment. However, these antibodies as pharmaceuticals are expensive at a cost of \$12,000–15,000 annually per patient in the case of infliximab or etanercept (Feldmann, 2002). A more cost-effective production method or alternative anti-TNF therapy is, therefore, needed.

The variable domain of llama heavy-chain antibodies (VHH) consists of a single domain and constitutes the smallest, naturally occurring antigen-binding domain (Hamers-Casterman et al., 1993). Because of its small and simple form, VHH is easy to produce as a recombinant protein (Arbabi Ghahroudi et al., 1997; Frenken et al., 2000). VHHs against murine and human TNF have already been reported, and C-terminal hexahistidine-tagged TNF-VHHs have been expressed in *Escherichia coli* and purified. Moreover, anti-murine TNF-VHH has been shown to block the development of collagen-induced arthritis in mice (Coppieters et al., 2006). Expression of TNF-VHH in plants (*Arabidopsis thaliana*) has also been reported, but the expression level was very low (Winichayakul et al., 2009). The use of a transient expression system (Teh and Kavanagh, 2010)

and expression as a fusion protein in *Nicotiana benthamiana* (Conrad et al., 2011) improved the expression level, but purification of VHH or the VHH-fusion protein in *N. benthamiana* resulted in low yields.

Rice seed has been shown to provide a suitable environment for the long-term stable expression and accumulation of a foreign protein at room temperature without loss of immunogenicity. Accordingly, a rice-based vaccine expressing the cholera toxin B subunit (CTB), MucoRice-CTB, has attracted interest as a vaccine production, storage, and delivery system for immunization (Nochi et al., 2007; Tokuhara et al., 2010). Recently, we advanced the MucoRice system by using RNA interference (RNAi) technology to suppress the production of the two major endogenous storage proteins, 13 kDa prolamin and glutelin A, to increase CTB expression; CTB was expressed in MucoRice-CTB with RNAi at a level six times that in MucoRice-CTB without the RNAi trigger (Yuki et al., 2013). More recently, we expressed a soluble form of anti-rotavirus VHH (which we termed 'MucoRice-ARP1) in the MucoRice system with or without RNAi (Tokuhara et al., 2013). MucoRice-ARP1 with RNAi led to 10 times higher expression of ARP1 compared with that in the absence of RNAi.

Here, we show that MucoRice with RNAi is a useful production system for the soluble form of mTNF-VHH (1.32% of the seed weight). In addition, MucoRice-mTNF-VHH could be purified, with a high yield, by single-step gel filtration after PBS extraction, and it suppressed progression of collagen-induced arthritis in mice.

2. Materials and methods

2.1. DNA constructs and transformation into rice plants

mTNF-VHH **MT1** was derived from llama immunized with mouse TNF (Coppieters et al., 2006; Plagmann et al., 2009). The mTNF-VHH gene was synthesized with optimized codon usage for rice as shown in Supplementary Table 1 (Nochi et al., 2007) and was subcloned into pMD19 (TaKaRa Bio Inc., Tokyo, Japan). The resultant plasmid, pMD-19 mTNF-VHH, was digested with XbaI and SacI, and the mTNF-VHH fragment was subcloned into the XbaI and SacI restriction sites of pUC198AM (Kuroda et al., 2010) and inserted into a binary transfer-DNA (T-DNA) vector (pZ2028) containing an overexpression cassette for mTNF-VHH and a combination cassette for RNAi suppression of the major rice endogenous storage proteins 13-kDa prolamin and glutelin A (pZH2Bik45G1B) (Kuroda et al., 2010; Yuki et al., 2013). For the RNAi suppression of rice storage protein expression, we used a 45-bp fragment of the rice 13-kDa prolamin **signal sequence gene** and a 129-bp fragment of the rice glutelin gene (see Supplementary Table 2). Because the trigger sequence of 13k-Da prolamin also contains its signal sequence, we used the 10k-Da prolamin signal sequence for the mTNF-VHH expression cassette to avoid down-regulation of mTNF-VHH expression. The Acs I–Mlu I fragment of the mTNF-VHH expression cassette was subcloned into pZH2Bik45-G1B. The resultant expression vector for the expression of mTNF-VHH together with the combination RNAi cassette was named pZH2B-mTNF-VHH-ik45-G1B. Next, we transformed a japonica rice plant cultivar, Nipponbare, **with** the plasmid by using an Agrobacterium-mediated method, as described previously (Nochi et al., 2007a). **Several independent transgenic rice lines were generated for MucoRice-mTNF-VHH, and the mTNF-VHH accumulation levels in the seeds were determined by use of SDS-PAGE followed by Western blotting. The plant line that accumulated the highest levels of mTNF-VHH in its seed was selected and advanced to the T4 generation by self-crossing to obtain homozygous lines.**

2.2. Protein expression and purification of TNF-VHH

For the production of recombinant mTNF-VHH (rTNF-VHH), the expression vector (pGEX-6P-3; GE Healthcare Biosciences, Pittsburgh, PA) containing the mTNF-VHH (pGEX6P3-mTNFVHH) gene was transformed into *Escherichia coli* competent cells [Rosetta 2(DE3) pLysS; Merck, Darmstadt, Germany] by using a standard method (Yuki et al., 2010). To purify the glutathione *S*-transferase (GST)-fused TNF-VHH protein, the sonicated cell sample was purified on a column packed with glutathione sepharose 4 FF (GE Healthcare) in phosphate buffered saline (PBS). The fusion protein on the column was treated with PreScission Protease (GE Healthcare) overnight at 4°C to cleave the GST from the TNF-VHH, and the digested fraction containing the TNF-VHH was collected. Finally, purification by gel filtration on a Sephadex G-100 (GE Healthcare) column was performed.

Total seed proteins were extracted from transgenic rice seeds as described previously (Nochi et al., 2007a). Briefly, rice seeds were ground into powder by using a Multi-beads shocker (Yasui Kikai, Osaka, Japan) and extracted in sample buffer (2% [w/v] sodium dodecyl sulfate [SDS], 5% [w/v] β -mercaptoethanol, 50 mM Tris-HCl (pH 6.8), and 20% [w/v] glycerol). The extracted proteins were analyzed by means of SDS-polyacrylamide gel electrophoresis (SDS-PAGE) with a 12% polyacrylamide gel (12% NuPAGE Bis-Tris Gel; Life Technologies, Carlsbad, CA) and Western blotting with a polyclonal rabbit anti-rTNF-VHH antibody that was prepared in our laboratory by using purified rTNF-VHH. The level of mTNF-VHH in MucoRice-mTNF-VHH was determined by means of densitometric analysis (GS-800; Bio-Rad Laboratories, Hercules, CA) of the western blot as a standard of purified rTNF-VHH.

For the preparation of mTNF-VHH from MucoRice-mTNF-VHH, mTNF-VHH was extracted with PBS from MucoRice-mTNF-VHH powder by incubation for 30 min at room temperature (rice

water). After centrifugation, the supernatant and the pellet were analyzed by SDS-PAGE and Western blotting. Purified TNF-VHH proteins from MucoRice-TNF-VHH were prepared from the rice PBA extract containing the mTNF-VHH by using gel filtration on a Sephadex G-100 column (GE Healthcare). Silver staining was performed by using a SilverQuest staining kit (Invitrogen) according to the manufacturer's protocol.

To analyze the purified mTNF-VHH, 15 μ L was injected into a Superose 12 column (GE Healthcare) equilibrated with PBS. Carbonic anhydrase (29 kDa), ribonuclease A (13.7 kDa), and aprotinin (6.5 kDa) were used as molecular weight marker proteins (GE Healthcare). The analytical conditions were as follows: flow rate, 0.5 mL/min; detection, absorbance at 280 nm; HPLC system, LC-20A (Shimadzu, Kyoto, Japan). The concentrations of total proteins were measured with a 2-D Quant kit (GE Healthcare Biosciences) with BSA as the standard. The total protein extracted by the sample buffer containing SDS was purified by using the 2-D Clean-Up Kit (GE Healthcare Biosciences) and was then measured by using the 2-D Quant kit according to the manufacturer's protocol.

For 2D-PAGE, 16.25 μ g of purified mTNF-VHH was dissolved in 250 μ L of lysis buffer containing 1% IPG buffer pH 3–10 non-linear (GE Healthcare Biosciences) and then applied to Immobiline Drystrips (pH 3–10 NL, 13cm, GE Healthcare), which were rehydrated for 12 h at 20 °C. First-dimensional IEF was then carried out using an Ettan IPGphor III (GE Healthcare Biosciences) under the following conditions: 500 V for 4 h; 1000 V for 1 h; 8000 V for 2.5 h; and 8000 V for 1.5 h. After being focused, the strips were equilibrated for 15 min in equilibration buffer (100 mM Tris-HCl [pH 8.0], 6 M Urea, 30% [v/v] glycerol, 2% [w/v] SDS, and a small amount of BPB) containing 0.5% (w/v) dithiothreitol, and were then incubated for 15 min only this time in equilibration buffer containing 4.5% (w/v) iodoacetamide. Second-dimensional SDS-PAGE was performed at 220 V for 3 h on 10%–20% gradient acrylamide gels (14 $\times$ 14 cm gel length, DRC Co.,

Ltd., Tokyo, Japan). After electrophoresis, the gels were stained with Coomassie Brilliant Blue R-250 (Invitrogen). For immunoblot analysis, the separated proteins were transferred to PVDF membranes (Merk Millipore), which were then incubated overnight at 4 °C with 5% (w/v) skim milk-TBS containing 0.05% (w/v) Tween 20 (TBS-T). The membranes were then incubated for 90 min at room temperature with each antibody (1 µg/mL of anti-recombinant RAG2 rabbit IgG or of anti-recombinant rTNF-VHH rabbit IgG diluted with TBS-T). After three 10-min washes with TBS-T, the membranes were incubated for 1 h at room temperature with peroxidase-labeled anti-mouse IgG (Jackson) or peroxidase-labeled anti-rabbit IgG (Jackson) that was diluted 20,000 times with TBS-T. After another three 10-min washes with TBS-T, peroxidase activity was measured by using SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific) according to the manufacturer's protocol. The signal intensity of the bands was detected by using the ImageQuant LAS 4000 mini (GE Healthcare Biosciences).

2.3. Mass spectrometry

To identify the mTNF-VHH expressed in transgenic rice, we performed a mass spectrometry analysis as previously described (Oyama et al., 2009). MucoRice-mTNF-VHH powder (20 mg) was lysed in 1 mL of sample buffer (2% SDS, 5% β-mercaptoethanol, 50 mM Tris-Cl (pH 6.8), and 20% glycerol) and separated by using SDS-PAGE on a 12% NuPAGE Bis-Tris Gel (Life Technologies). The mTNF-VHH band was cut out of the gel, digested at 37 °C overnight with 0.2 µg of trypsin (Sequence grade; Promega, Madison, WI), desalted, and then concentrated to a volume of 20 µL. The sample was then injected into a direct nanoflow liquid chromatography system (Dina; KYA Thecknologies, Tokyo, Japan) and sprayed into a quadropole time-of-flight tandem mass spectrometer (QSTAR Elite; Applied Biosystems, Carlsbad, CA) or a linear ion trap Orbitrap mass spectrometer (LTQ Orbitrap; Thermo Fisher Scientific, Suwanee, GA). Analysis of the data using the

Mascot search server confirmed the complete amino acid sequence of the mTNF-VHH band expressed in transgenic rice.

2.4. Immunohistochemical and immuno-electronmicroscopic analyses of MucoRice-mTNF-VHH

To evaluate the localization of mTNF-VHH in MucoRice-mTNF-VHH seeds, rice seeds at day 14 after flowering were fixed with 4% paraformaldehyde (Nacalai Tesque, Kyoto, Japan). Frozen sections of seed were reacted with a polyclonal rabbit anti-rTNF-VHH antibody and visualized with 3, 3'-diaminobenzidine. The distribution of mTNF-VHH in MucoRice seed was also analyzed by immuno-electronmicroscopy as previously described (Saito et al., 2012). Rice seeds (at 14 day after flowering) were fixed with 4% paraformaldehyde and 0.1% glutaraldehyde (Nacalai Tesque) and embedded in LR White resin (London Resin, Hampshire, U.K.). Ultrathin sections (100 nm) were stained with the polyclonal rabbit anti-TNF-VHH antibody, followed by gold particle-conjugated anti-rabbit IgG (18 nm; Jackson ImmunoResearch Laboratories, PA, USA). Then, the sections were stained with 2% uranyl acetate followed by Reynold's lead and examined by use of a transmission electron microscope (HITACHI, Tokyo, Japan).

2.5. In vitro neutralization assay

An *in vitro* neutralization assay was carried out by using the WEHI cytotoxicity assay (Espevik and Nissen-Meyer, 1986). WEHI164 cells were grown to 90% confluence in RPMI1640 medium supplemented with 10% fetal calf serum, 50 units/mL penicillin, and 50 µg/mL streptomycin sulfate. The cells were plated at 2×10^4 cells/well (0.1 mL) in a 96-well plate and incubated for 24 h. The cells were then incubated with a cytotoxic concentration (2 ng/mL) of mouse TNF (R&D Systems, Minneapolis, MN) for 24 h. Cell viability was determined by using a cell counting kit (Dojindo, Kumamoto, Japan). The ID₅₀ was defined as the amount that inhibited 1 ng of mouse TNF.

2.6. Animals

Male DBA/1J mice were purchased from Japan SLC, Inc. (Shizuoka, Japan). All mice were housed at 22°C on a 12-h/12-h light/dark cycle. Food and tap water were provided *ad libitum*. All experiments were performed according to the Guidelines for Use and Care of Experimental Animals and approved by the Animal Committee of the Institute of Medical Science of the University of Tokyo.

2.7. Induction and treatment of collagen-induced arthritis (CIA)

Male DBA/1J mice (8-weeks-old) were immunized intradermally at the base of the tail with 200 µg of bovine type II collagen (CII; Cosmo-bio, Tokyo, Japan) emulsified in an equal volume of Freund's complete adjuvant (H37Ra; Difco, Detroit, MI). On day 21 post-immunization, mice were given a CII booster emulsified in an equal volume of Freund's incomplete adjuvant (Difco) by using the same procedure. Treatment of arthritis was initiated daily from day 21. Mice were intraperitoneally injected daily with PBS as a control or with 200 µL (100 mg/kg; n = 6) of purified mTNF-VHH from MucoRice-mTNF-VHH until day 42. **As an additional control without CII immunization, mice were intraperitoneally injected daily with PBS or with 200 µL (100 mg/kg; n = 3) of purified mTNF-VHH from MucoRice-mTNF-VHH until day 20.**

Mice were monitored daily for clinical signs and symptoms of arthritis from day 28. The clinical severity of arthritis for each paw was graded as follows: 0 = normal, 1 = redness and swelling in only 1 digit, 2 = moderate redness and swelling in more than one digit or entire paw, 3 = severe redness and swelling in entire paw, 4 = maximum inflammation. The clinical score for clinical severity was graded for each paw, resulting in a maximum clinical score of 16 per mouse.

2.8. Histopathology

Mice were euthanized at the end of the treatment (day 42 post-immunization). Knees were removed, fixed with 4% paraformaldehyde (Nacalai Tesque), decalcified in 10% [w/v] EDTA solution, and then embedded in paraffin. Tissues were cut into 5- μ m thick sections and stained with hematoxylin and eosin.

2.9. Detection of antigen-specific Ab production by ELISA

Antigen-specific Ab responses were determined as described previously (Nochi et al., 2007). Briefly, the wells of 96-well microtiter plates (BD Falcon, Franklin Lakes, NJ) were coated with 100 μ L of purified rice-based mTNF-VHH at 5 μ g/mL and incubated overnight at 4 °C. After the mice received their final injection (i.e., on day 21), two-fold serial dilutions of serum from the control mice (injected with PBS or purified mTNF-VHH without CII immunization) were added to the wells for 2 h at room temperature (RT) after being blocked with 1% BSA. After the plates were washed, HRP-conjugated goat anti-mouse IgG or HRP-conjugated goat anti-mouse IgE, each diluted 1:4000 (SouthernBiotech, Birmingham, AL), was added and the plates were kept for 1 h at RT. The reaction was finally developed with a TMB Microwell Peroxidase Substrate System (XPL, Caithersburg, MD). Endpoint titers were expressed as the reciprocal $\log_2$ of the last dilution, which gave an OD₄₅₀ of 0.1 greater than the negative control.

2.10. Data analysis

The Wilcoxon's rank test was used for statistical analysis of the arthritis clinical score in CIA mice (Fig. 5A). The Kaplan-Meier method was used for statistical analysis of progression of arthritis in CIA mice (Fig. 5B). * P < 0.05 vs. PBS. ** P < 0.01 vs. PBS.

3. Results

3.1. Expression of a soluble form of MucoRice-mTNF-VHH

To develop MucoRice with a high mTNF-VHH expression level, we inserted the mTNF-VHH gene between an endosperm-specific promoter (13-kDa prolamin promoter) that was combined with the prolamin signal peptide gene and the prolamin terminator. In addition, we introduced a combination cassette for RNAi suppression of the 13-kDa prolamin and glutelin A rice storage proteins into the T-DNA vector to increase the expression level of mTNF-VHH in rice seed (Fig. 1). Then, we transformed the plasmid into the japonica rice plants (Nipponbare) by using *Agrobacterium tumefaciens*-mediated transformation and selected the line with the highest expression of mTNF-VHH from 48 MucoRice-mTNF-VHH lines. We used this line as MucoRice-mTNF-VHH for all further assessments.

As expected, RNAi decreased the levels of prolamin and glutelin (as acid and basic chains of gutelin) in MucoRice-mTNF-VHH. SDS-PAGE analysis showed that MucoRice-mTNF-VHH was produced as a single protein band with a molecular weight of 13.8 kDa (Fig. 2A). Because all of the peptides from the trypsin digestion of the MucoRice-mTNF-VHH band were obtained by MS/MS analysis (Fig. 2B, Supplemental Table 3), we were able to confirm the complete sequence of MucoRice-TNF-VHH and show that the mouse TNF-VHH sequence was authentic, with the exception of the addition of an N-terminal Ser-Arg (SR) dipeptide that resulted from the introduction of the XbaI site between the signal peptide and the mTNF-VHH gene (Figs. 1, 2B). Using densitometry analysis of western blot with rTNF-VHH as a standard, we observed that the expression level of TNF-VHH reached an average of 14.5 mg per gram rice seed. The TNF-VHH was released easily from the MucoRice-TNF-VHH powder with PBS at room temperature, as shown in Fig. 2B. The yield of soluble TNF-VHH was, on average, 13.2 mg per gram of rice powder. The

purification yield and purity of the rice PBS extract were 91% and 27.4%, respectively.

3.2. Localization of TNF-VHH in rice-seed

Immuno-histochemical analysis showed that TNF-VHH was mainly expressed in the sub-aleurone layer in MucoRice-TNF-VHH seeds, but not in wild-type (WT) seeds (Fig. 3A). Rice seed storage proteins accumulated in two different types of protein bodies (PB-I and PB-II); PB-I formed spherical structures with ring-shaped layers and PB-II formed electron-dense, irregularly shaped granules in WT seeds. Immuno-electronmicroscopic analysis revealed that PB-II formation was strongly disturbed, and PB-I formed smaller rough-shaped structures in MucoRice-TNF-VHH compared with WT (Fig. 3B). In addition, MucoRice-TNF-VHH formed a PB-like structure that was not found in WT seeds (Fig. 3B). TNF-VHH predominantly localized to the cytoplasm and PB-II (Fig. 3B), which was consistent with the high solubility of MucoRice-TNF-VHH in PBS, but it did not localize to the PB-like structure.

3.3. Single-step purification and neutralization of TNF with purified TNF-VHH in vitro

To assess the inhibitory activity of TNF-VHH *in vitro*, we extracted TNF-VHH from MucoRice-TNF-VHH rice powder in PBS and then purified it by using gel filtration on a Sephadex G-100 column (Fig. 4A). When 25 g of MucoRice-TNF-VHH powder was used, we obtained 300 mg of TNF-VHH by gel filtration. The purification yield and purity from rice seeds were 83% and 90.4%, respectively. When purified TNF-VHH was analyzed by means of HPLC with a Superose 12 column (Fig. 4B), the TNF-VHH that produced roughly a single band on SDS-PAGE showed a single peak with a molecular weight of 13.6 kDa.

Next, we assessed the inhibitory activity of TNF-VHH against TNF by using TNF-sensitive WEHI 164 cells (Fig. 4C). Purified rice-based TNF-VHH and *E.coli*-based TNF-VHH both inhibited TNF-induced cell death in almost the same dose-dependent manner with ID₅₀ values of 16 and 22

nM, respectively. Taken together these results demonstrate that rice-based TNF-VHH can be purified with high quality and a high yield by single-step gel filtration and that rice-based TNF-VHH has high biological activity that suppresses TNF *in vitro*.

3.4. Purified MucoRice-TNF-VHH suppresses collagen-induced arthritis in mice

The therapeutic effect of purified rice-based TNF-VHH was investigated in CIA-induced mice. Mice were given intraperitoneally TNF-VHH daily beginning on day 21 (total 20 doses, 2 mg in PBS per dose) after CII immunization. Control mice were injected with PBS after CII immunization. Additional control mice that did not undergo CII immunization were injected with 20 doses of 2 mg of purified rice-based TNF-VHH alone or with PBS. The clinical score was assessed on the basis of the effect on paw swelling. The TNF-VHH group had significantly lower clinical scores and the disease was prevented from spreading to unaffected joints in the DBA/1 mice compared with the control group (Fig. 5A). Injection of TNF-VHH significantly decreased the incidence of arthritis compared with that of the control group. In addition, TNF-VHH-treated mice showed a delay in disease onset (Fig. 5B). To further evaluate arthritis, hematoxylin and eosin-stained sections of knee joints from the TNF-VHH and control groups were compared. Histological analysis showed synovial proliferation and inflammatory cells infiltration was significantly decreased in the knee joints of mice treated with TNF-VHH compared with those of control mice (Fig. 5C). The results showed that treatment with mTNF-VHH significantly inhibited the infiltration of inflammatory cells into the synovium. However, the control mice that did not undergo CII immunization showed no clinical changes and no mTNF-VHH-specific IgG or IgE immune responses to TNF-VHH or PBS alone (Supplementary Fig. 3). These results show that rice-based TNF-VHH can suppress arthritis symptoms in mice.

4. Discussion

We previously reported that vaccine antigens, including a heavy chain fragment of botulinum neurotoxin (Yuki et al., 2012) and the cholera toxin B-subunit (Yuki et al., 2013), were produced at high levels in rice seed (or MucoRice) by using an antigen overexpression system together with RNAi technology to suppress the production of the main endogenous storage proteins prolamin and glutelin. More recently, we successfully expressed the variable domain of a rotavirus-specific llama heavy-chain antibody (VHH) as a soluble protein at high levels in rice seeds (Tokuhara et al., 2013). In this study, we used mTNF-VHH to demonstrate the universal applicability of the MucoRice system for soluble antibody expression. MucoRice-mTNF-VHH was purified in one step, after PBS extraction, with high yield. In addition, purified mTNF-VHH showed significant suppression of mTNF both in an *in vitro* cell assay and in an *in vivo* collagen-induced arthritis model in mice.

Because we used RNAi suppression of the rice major endogenous storage proteins to increase the production of mTNF-VHH in rice seed, the expression of 13-kDa prolamin and glutelin A decreased compared with WT rice (Fig. 2A). The mature rice seed of MucoRice-mTNF-VHH was downsized by about 90% compared with WT rice-seed; this may have been caused by stress due to the introduction of VHH and/or RNAi genes. (Fig. 3A). In fact, the suppression of prolamin and glutelin caused PB-II formation to be strongly disturbed and PB-I to form smaller rough-shaped structures in MucoRice-mTNF-VHH compared with in WT (Fig. 3B). mTNF-VHH accumulated inside the partially destroyed PB-II, in the cytoplasm, and at the surface of the smaller PB-I, which are the soluble components of rice. When mTNF-VHH is expressed as a secretory protein and trafficked into seed endosperm tissue, its subcellular destination may be affected by its interaction with seed storage proteins in the ER lumen. Suppression of storage proteins by use of RNAi led to the partial destruction of PB-II and to the formation of smaller PB-I as well as PB-like structures, which may

have caused the unexpected localization of mTNF-VHH at intercellular locations such as the plasma membrane/cell wall in MucoRice-mTNF-VHH. Although we expressed the cholera toxin B subunit (CTB) as a cholera vaccine in the MucoRice system with RNAi in a separate study, CTB localizes to a PB-like structure in addition to PB-I, the plasma membrane/cell wall, and the cytoplasm in rice seeds (Kurokawa et al., 2013). This localization of CTB to a PB-like structure prevents the CTB from being fully PBS soluble. In this regard, MucoRice-mTNF-VHH also formed PB-like structures (Fig. 3B) but mTNF-VHH did not localize there, which may explain the full water solubility of mTNF-VHH. ARP1 (rotavirus-VHH) was also fully water soluble and accumulated inside the partially destroyed PB-II and in the cytoplasm of rice seed in the MucoRice expression system with RNAi (Tokuhara et al., 2013). Because ARP1 sequences, including three ARP1-specific complementary-determining region sequences (#31–34, #49–58, and #98–112 residues in ARP1) (Pant et al., 2006) share 68% homology with mTNF-VHH sequences, the common structure of these VHHs appears to be highly conserved and may promote a preference to accumulate in PB-II/cytoplasm but not PB-I/PB-like structures in rice seed.

Although there are many of reports on protein expression in plants for medical use, few of these reports confirm the full amino acid sequence of the expressed protein. Because unexpected plant-specific GlcNAc-Fuc and Man-Xyl carbohydrates have been found in patients allergic to pollen allergens (van Ree, 2002), it is important to analyze the post-translational modification of protein-based pharmaceuticals such as antibodies from plants. In this study, we identified the full amino acid sequence of MucoRice-mTNF-VHH by means of MS/MS analysis. Although there are no putative N-link sugar-attaching sites such as N-X-S/T or N-X-C sequences in mTNF-VHH (Matsui et al., 2011), we confirmed the existence of MucoRice-mTNF-VHH without any sugar chain post-translational modification, including O-linked carbohydrate attachment (Moriguchi et al., 2011).

In addition to high protein expression and water solubility, we could purify mTNF-VHH from the MucoRice system by simple protein purification. Three hundred milligrams of TNF-VHH could be purified from 25 g of rice seeds with an 83% yield. Unlike expression systems in *E.coli* and *N.benthamiana* (Coppieters et al., 2006; Teh and Kavanagh, 2010), MucoRice-mTNF-VHH could be expressed without an affinity-tag such as a His-tag and can be purified by single-step gel filtration with 90.4% purity, although this may not be applicable as a first step for large-scale purification. To confirm the identities of the contaminating proteins, we performed 2-DE of the purified mTNF-VHH. As shown in Supplementary Figure 2, the purified mTNF-VHH contained a few endogenous rice seed proteins. Western blot analysis with an Ab to RAG2 indicated that the contaminating proteins of 14–16 kDa represented amylase/trypsin inhibitor-like family proteins (Adachi et al., 2003; Kurokawa et al., 2013). The inhibition potency of TNF-VHH against TNF in an *in vitro* cell assay was almost the same as that of rTNF-VHH derived from *E.coli*. These results demonstrate MucoRice-TNF-VHH is a high-yield, water-soluble, bioactive rice-based antibody fragment against TNF.

To assess whether MucoRice-TNF-VHH could suppress rheumatoid arthritis, we used a mouse collagen-induced arthritis model. In an earlier study, a bivalent form of mouse TNF-VHH, named MT1-MT1, was expressed in an *E.coli* system together with the monomeric mouse TNF-VHH (Coppieters et al., 2006), and it was reported that the neutralizing activity of MT1-MT1 increased that of the monomeric form by about 500-fold. Because administration of MT1-MT1 at 15 mg/kg was effective in suppressing the expression of arthritis in an *in vivo* mouse model, we used purified MucoRice-mTNF-VHH at 100 mg/kg (2mg/mouse) to assess the efficacy of MucoRice-mTNF-VHH. Purified MucoRice-TNF-VHH suppressed the development of arthritis in terms of both clinical progression (Fig. 5A, B) and inflammatory symptoms in histological sections (Fig. 5C). Although purified mTNF-VHH contained a few rice amylase/trypsin inhibitor-like family proteins, the control

group mice that were given purified mTNF-VHH and did not undergo CII immunization showed no serum antigen-specific IgG or IgE immune-responses, as determined by use of an ELISA containing purified mTNF-VHH as the coating antigen. These results demonstrate that MucoRice-TNF-VHH successfully suppressed the development of arthritis in a collagen-induced mouse model.

In conclusion, we have developed a high-yield, water-soluble, rice-based antibody fragment that has inhibitory activity against TNF *in vitro* and reduces the development of arthritis in a collagen-induced mouse model. The MucoRice-TNF-VHH system provides a novel approach to obtain VHH against TNF to prevent and treat TNF-induced inflammation at low cost. This system may also be able to provide alternative, bio-similar anti-TNFs as follow-on versions of original drugs. This technology could be extended to the production of antibody fragments against other cytokines and pathogens.

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Figure Legends

Fig. 1. T-DNA vector for overexpression of mTNF-VHH in rice seeds.

We constructed a tandem T-DNA vector containing an overexpression cassette for mTNF-VHH, controlled by the 13-kDa prolamin promoter, and an RNAi cassette for **suppression of the rice endogenous 13-kDa prolamin and glutelin A storage proteins, controlled by the mUbi promoter.** CaMV35S-P, cauliflower mosaic virus 35S promoter; mHPT, mutant hygromycin phosphotransferase; Nos-T, nos terminator; 13P-P, 13-kDa prolamin promoter; 10P-S, signal sequence of 10-kDa prolamin; 13P-T, 13-kDa prolamin terminator; mUbi-P, ubiquitin promoter; LB, T-DNA left border; RB, T-DNA right border.

Fig. 2. Expression of TNF-VHH in MucoRice-mTNF-VHH and PBS solubility of MucoRice-mTNF-VHH.

(A) SDS-PAGE and western blot analysis of mTNF-VHH expressed in MucoRice-mTNF-VHH seeds. (B) PBS solubility of MucoRice-mTNF-VHH. Rice powder (**100 µg**) was incubated with PBS for 30 min at room temperature, and the supernatant (S) and pellet (P) were then separated. A rabbit anti-rTNF-VHH antibody was used for the western blot analysis of mTNF-VHH. Purified recombinant TNF-VHH derived from *E. coli* (rTNF-VHH, **1 µg**) was used as a positive control.

Fig. 3. Localization of mTNF-VHH in MucoRice-mTNF-VHH seed.

(A) The expression of mTNF-VHH proteins in MucoRice-mTNF-VHH or WT seed was analyzed by immunohistochemistry using an anti-TNF-VHH antibody. (B) The localization of mTNF-VHH proteins in MucoRice-mTNF-VHH or WT seed was analyzed by immuno-electronmicroscopy using

a gold particle-conjugated anti-rTNF-VHH antibody. PB: Protein Body; CW: Cell Wall; Asterisks: PB-like structure.

Fig. 4. Purification of mTNF-VHH from MucoRice-mTNF-VHH and the inhibitory activity of mTNF-VHH against TNF.

(A) SDS-PAGE analysis of purified mTNF-VHH from MucoRice-mTNF-VHH rice powder. (B) Assessment of purified mTNF-VHH. The purity of rice-based mTNF-VHH was assessed by using HPLC with a Superose 12 column in PBS. (C) *In vitro* neutralization assay against TNF. A neutralization assay against TNF was used in which WEHI 164 cells were incubated with mTNF-VHH for 24 h in the presence of TNF (2 ng/mL).

Fig. 5. Effects of mTNF-VHH on the clinical progression of collagen-induced arthritis in mice.

DBA/1J mice were immunized with type II collagen (CII). From day 21 post-immunization, the mice were treated with PBS (n = 8) or 2 mg of purified MucoRice-mTNF-VHH (n = 6) daily until day 42. (A) Clinical scores in mTNF-VHH-treated CIA mice. $^{**}P < 0.01$ vs. PBS. (B) Decreased incidence of arthritis in mTNF-VHH-treated CIA mice. After immunization with CII, mice were observed for clinical signs and symptoms of disease. $^{*}P < 0.05$ vs. PBS. (C) Histopathology of knee joints in mTNF-VHH-treated mice on day 42. Treatment with mTNF-VHH significantly inhibited the infiltration of inflammatory cells into the synovium compared with PBS treatment. Arrows, inflammatory cells

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Figure(s)

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(A) Immunohistochemistry

(B) Immune-electronmicroscopy

Bar = 500 nm

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Molecular weight maker proteins
 1 : Carbonic anhydrase (29 KDa)
 2 : Ribonuclease A (13.7kDa)
 3 : Aprotinin (6.5 kDa)

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