

Kitagaki M., Isoda K., Kamada H., Kobayashi T., Tsunoda S., Tsutsumi Y., Niida T., Kujiraoka T., Ishigami N., Ishihara M., Matsubara O., Ohsuzu F., Kikuchi M.	Novel TNF- $\alpha$ receptor-1 antagonist treatment attenuates arterial inflammation and intimal hyperplasia in mice.	J. Atheroscler. Thromb	19(1)	36-46	2012
Yamashita T., Okamura T., Nagano K., Imai S., Abe Y., Nabeshi H., Yoshikawa T., Yoshioka Y., Kamada H., Tsutsumi Y., Tsunoda S.	Rho GDP-dissociation inhibitor alpha is associated with cancer metastasis in colon and prostate cancer.	Pharmazie	67(3)	253-5	2012
Yamashita T., Nagano K., Kanasaki S., Maeda Y., Furuya T., Inoue M., Nabeshi H., Yoshikawa T., Yoshioka Y., Itoh N., Abe Y., Kamada H., Tsutsumi Y., Tsunoda S.	Annexin A4 is a possible biomarker for cisplatin susceptibility of malignant mesothelioma cells	Biochem. Biophys. Res. Commun.	421(1)	140-4	2012
Morishige T., Yoshioka Y., Narimatsu S., Ikemizu S., Tsunoda S., Tsutsumi Y., Mukai Y., Okada N., Nakagawa S.	Mutants of lymphotoxin- $\alpha$ with augmented cytotoxic activity via TNFR1 for use in cancer therapy	Cytokine	61(2)	578-84	2012
Yamagishi Y., Watari A., Hayata Y., Li X., Kondoh M., Tsutsumi Y., Yagi K.	Hepatotoxicity of sub-nanosized platinum particles in mice.	Pharmazie,	68(3)	178-82	2013

Yoshikawa M., Mukai Y., Okada Y., Tsumori Y., Tsunoda S., Tsutsumi Y., Aird WC., Yoshioka Y., Okada N., Doi T., Nakagawa S.	Robo4 is an effective tumor endothelial marker for antibody-drug conjugates based on the rapid isolation of the anti-Robo4 cell-internalizing antibody.	Blood	121(14)	2804-13	2013
Iida M., Yoshida T., Watari A., Yagi K., Hamakubo T., Kondoh M.	A baculoviral display system to assay viral entry.	<i>Biol. Pharm. Bull.</i>	36(11)	1867-9	2013
Nagano K., Kanasaki S., Yamashita T., Maeda Y., Inoue M., Higashisaka K., Yoshioka Y., Abe Y., Mukai Y., Kamada H., Tsutsumi Y., Tsunoda S.	Expression of Eph receptor A10 is correlated with lymph node metastasis and stage progression in breast cancer patients.	<i>Cancer Medicine</i>	2	72-977	2013
Morishige T., Yoshioka Y., Narimatsu S., Ikemizu S., Tsunoda S., Tsutsumi Y., Mukai Y., Okada N., Nakagawa S.	Mutants of lymphotoxin- $\alpha$ with augmented cytotoxic activity via TNFR1 for use in cancer therapy.	<i>Cytokine</i>	61(2)	578-84	2013
Takano M., Yamashita T., Nagano K., Otani M., Maekura K., Kamada H., Tsunoda S., Tsutsumi Y., Tomiyama T., Mori H., Matsuura K., Matsuyama S.	Proteomic analysis of the hippocampus in Alzheimer's disease model mice by using two-dimensional fluorescence difference in gel electrophoresis.	<i>Neuroscience Letters</i>	534	85-9	2013

Yoshikawa M., Mukai Y., Okada Y., Tsumori Y., Tsunoda S., Tsutsumi Y., Aird WC., Yoshioka Y., Okada N., Doi T., Nakagawa S.	Robo4 is an effective tumor endothelial marker for antibody–drug conjugates based on the rapid isolation of the anti–Robo4 cell–internalizing antibody.	<i>Blood</i>	121(14):	2804–13,	2013
Yamashita T., Kamada H., Kanasaki S., Maeda Y., Nagano K., Abe Y., Inoue M., Yoshioka Y., Tsutsumi Y., Katayama S., Inoue M., Tsunoda S.	Epidermal growth factor receptor localized to exosome membranes as a possible biomarker for lung cancer diagnosis.	<i>Pharmazie</i>	68:	969–973,	2013
Nagano T., Higashisaka K., Kunieda A., Iwahara Y., Tanaka K., Nagano K., Abe Y., Kamada H., Tsunoda S., Nabeshi H., Yoshikawa T., Yoshioka Y., Tsutsumi Y.	Liver–specific microRNAs as biomarkers of nanomaterial–induced liver damage.	<i>Nanotechnology</i>	24	405102	2013
Li X., Saeki R., Watari A., Yagi K., Kondoh M.	Tissue distribution and safety evaluation of a claudin–targeting molecule, the C–terminal fragment of <i>Clostridium perfringens</i> enterotoxin.	<i>Eur. J. Pharm. Sci.</i>	14(52)	132–7	2014

Laboratory of Bio-Functional Molecular Chemistry<sup>1</sup>, Laboratory of Toxicology<sup>2</sup>, Graduate School of Pharmaceutical Sciences, Osaka University, Osaka, Japan

## Effect of 70-nm silica particles on the toxicity of acetaminophen, tetracycline, trazodone, and 5-aminosalicylic acid in mice

X. LI<sup>1</sup>, M. KONDOH<sup>1</sup>, A. WATARI<sup>1</sup>, T. HASEZAKI<sup>1</sup>, K. ISODA<sup>1</sup>, Y. TSUTSUMI<sup>2</sup>, K. YAGI<sup>1</sup>

Received September 13, 2010, accepted October 15, 2010

Masuo Kondoh, Ph. D., Laboratory of Bio-Functional Molecular Chemistry  
masuo@phs.osaka-u.ac.jp

Kiyohito Yagi, Ph. D., Laboratory of Bio-Functional Molecular Chemistry, Graduate School of Pharmaceutical Sciences, Osaka University, Suita, Osaka 565-0871, Japan  
yagi@phs.osaka-u.ac.jp

Pharmazie 66: 282–286 (2011)

doi: 10.1691/ph.2011.0778

Exposure to nano-sized particles is increasing because they are used in a wide variety of industrial products, cosmetics, and pharmaceuticals. Some animal studies indicate that such nanomaterials may have some toxicity, but their synergistic actions on the adverse effects of drugs are not well understood. In this study, we investigated whether 70-nm silica particles (nSP70), which are widely used in cosmetics and drug delivery, affect the toxicity of a drug for inflammatory bowel disease (5-aminosalicylic acid), an antibiotic drug (tetracycline), an antidepressant drug (trazodone), and an antipyretic drug (acetaminophen) in mice. Co-administration of nSP70 with trazodone did not increase a biochemical marker of liver injury. In contrast, co-administration increased the hepatotoxicity of the other drugs. Co-administration of nSP70 and tetracycline was lethal. These findings indicate that evaluation of synergistic adverse effects is important for the application of nano-sized materials.

### 1. Introduction

Nano-sized particles, which have a diameter of less than 100 nm, are widely used in medicine, food, and machinery. With their smaller size, the physical and chemical properties of their constituents change, so that they may be toxic, for example to the lungs or liver, even though macro-particles of the same materials are not (Byrne and Baugh 2008; Nishimori et al. 2009b). Some nano-sized particles show long-term accumulation or a wide distribution in the body (Byrne and Baugh 2008; Nishimori et al. 2009b; Xie et al. 2009; Yang et al. 2008).

Recent reports indicate that some nano-sized particles can generate reactive oxygen species (ROS) on their surfaces, leading to cellular injury (Jin et al., 2008; Sharma et al. 2007; Ye et al. 2010). There are also many drugs that cause adverse effects through the generation of ROS (Ali et al. 2002; Kovacic 2005; Xu et al. 2008). Thus, nano-sized particles might enhance the side-effects of some pharmaceutical drugs. Indeed, we have shown that 70-nm silica particles (nSP70) cause liver injury but that macro-sized silica particles with a diameter of 300 and 1000 nm do not (Nishimori et al. 2009b). Also, when co-administered to mice, nSP70 but not the macro-sized silica particles enhance the toxicity of cisplatin and paraquat (Nishimori et al. 2009a). Surprisingly, co-administration of cisplatin and nSP70 was lethal, suggesting that each chemical may have different synergistic effects in the presence of nano-sized materials. In the current study, to clarify the influence of nano-sized materials on the adverse effects of chemicals, we assessed the toxicity in mice of 5-aminosalicylic acid (an agent for treating inflammatory bowel disease), tetracycline (a broad-spectrum antibiotic), trazodone (an antidepressant), and acetaminophen (a common antipyretic analogue) in the presence or absence of nSP70.

### 2. Investigations and results

Several reports indicate that 5-aminosalicylic acid, which is used to treat inflammatory bowel disease, causes liver injury and interstitial nephritis (Deltenre et al. 1999; Margetts et al. 2001). Administration of 5-aminosalicylic acid caused an increase in ALT, AST and BUN levels (Fig. 1). Also, nSP70 dose-dependently elevated ALT and AST levels. Co-treatment with 5-aminosalicylic acid and nSP70 resulted in higher levels of ALT and AST than nSP70 alone. In contrast, changes in BUN levels in response to 5-aminosalicylic acid were not affected by nSP70.

Next, we investigated effect of nSP70 on tetracycline, a broad-spectrum antibiotic. As shown in Fig. 2A and 2B, administration of tetracycline did not elevate biochemical markers for liver injury. In contrast, co-administration with nSP70 resulted in the synergistic induction of liver injury. However, nSP70 alone did not cause kidney injury. Importantly, co-administration of 30 and 50 mg/kg nSP70 with tetracycline resulted in the death of 1 of 4 and 2 of 4 mice, respectively.

Finally, we investigated effect of nSP70 on toxicity of the antidepressant trazodone and the antipyretic analgesic acetaminophen. We found that nSP70 did not have a synergistic effect on the toxicity of trazodone (Fig. 3). In contrast, co-administration of acetaminophen with nSP70 caused synergistic liver injury (Fig. 4).

### 3. Discussion

In this study, we showed that nSP70 synergistically enhance the toxicity of 5-aminosalicylic acid, tetracycline, and acetaminophen but not trazodone. To avoid direct interac-

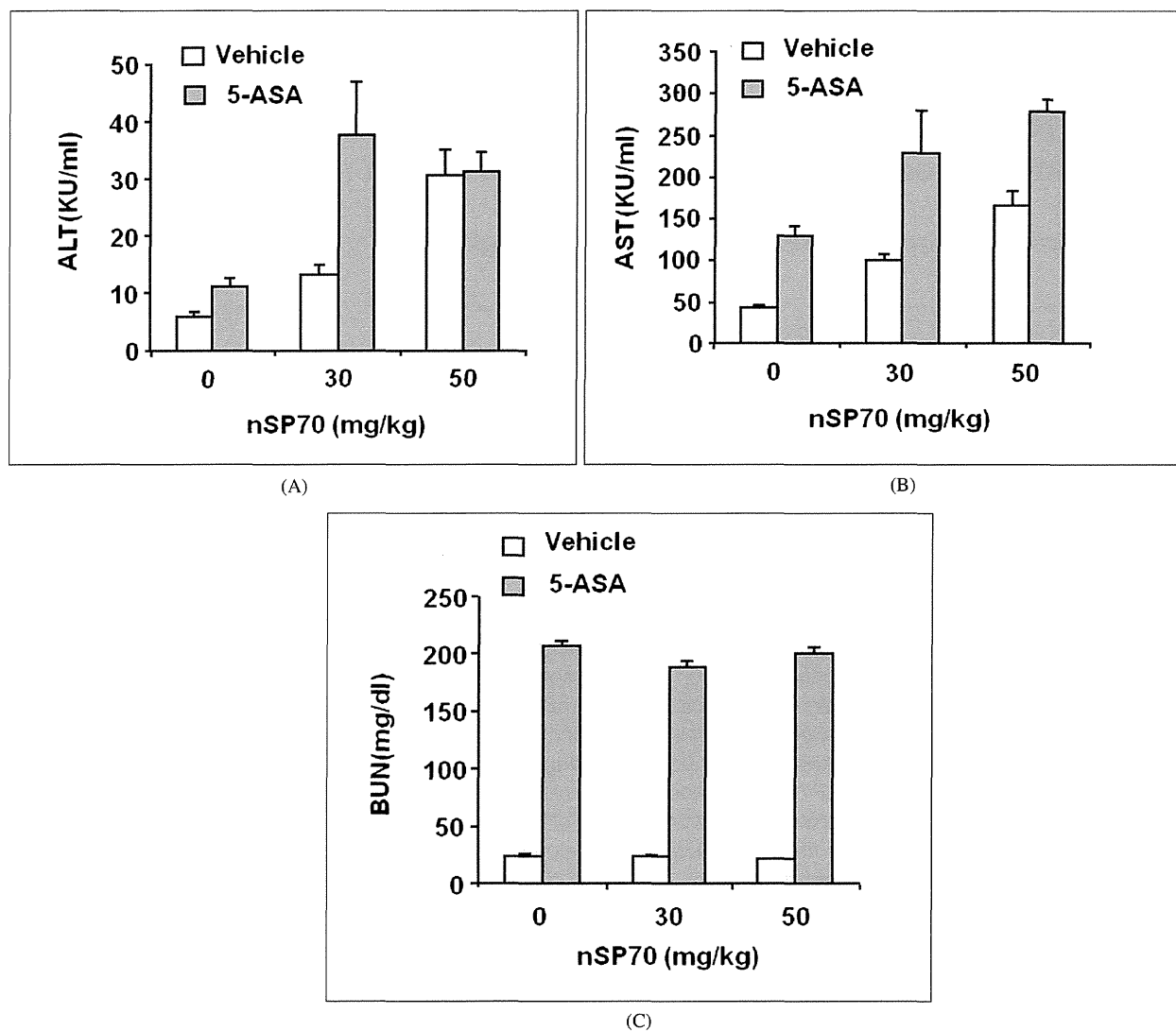


Fig. 1: Effect of nSP70 on 5-aminosalicylic acid (5-ASA)-induced toxicity. Mice were injected intraperitoneally with 5-ASA at 0 (open column) or 500 mg/kg (gray column) and intravenously with nSP70 at the indicated doses. After 24 h, the serum was collected. Shown are the levels of ALT (A), AST (B), and BUN (C). Data are means  $\pm$  SEM (n = 4)

tions between nSP70 and chemicals in their administration and absorption, nSP70 and chemicals were administered intravenously and intraperitoneally, respectively. Administration of nSP70 alone has been shown to cause liver injury but not kidney injury (Nishimori et al. 2009b). Also, in this study, nSP70 did not enhance kidney injury induced by 5-aminosalicylic acid or tetracycline, two drugs known to be nephrotoxic (Grisham et al. 1992; Kunin 1971). The renal toxicity of cisplatin, another nephrotoxic chemical, was unaffected by nSP70 (Nishimori et al. 2009a). Like 5-aminosalicylic acid, tetracycline, and acetaminophen (Chun et al. 2009; Herzog and Leuschner 1995; Kunin 1971), nSP70 is hepatotoxic (Nishimori et al. 2009b), and we showed here that its co-administration synergistically enhanced liver injury. These findings indicate that nSP70 may enhance the toxicity of certain chemicals. Therefore, it will be important to assess the tissue-specific risk of nano-sized materials.

The nSP70 particles had a lethal effect when combined with tetracycline. The 50% lethal dose of tetracycline is 318 mg/kg by intraperitoneal injection in mice. A previous report showed that 100 mg/kg nSP70 is lethal in 100% of mice (Nishimori et al.

2009b). A single injection of tetracycline (100 mg/kg) or nSP70 (30 or 50 mg/kg) alone was not lethal in this study but a combination of the two was. Co-administration of cisplatin and nSP70 showed a similar synergistic lethal effect. This could be due to an interaction between nSP70 and serum albumin. Tetracycline in the bloodstream can bind to albumin (Popov et al. 1972; Powis 1974). Likewise, serum albumin adsorbs onto nano-sized silica particles (Dutta et al. 2007). When injected intravenously, 100-nm anionized albumin-modified liposomes are taken up by hepatic endothelial cells and Kupffer cells (Kamps et al. 1997), which normally clear chemically modified albumin (Jansen et al. 1991). Thus, tetracycline-bound serum albumin may adsorb onto nSP70, causing it to be taken up by the hepatic endothelial cells and Kupffer cells in the liver where it may accumulate and cause lethal liver damage.

Indirect interactions between chemicals and nano-sized particles mediated by serum albumin may be useful for estimating the toxicity of nano-sized materials. In this study, co-treatment of mice with nSP70 (50 mg/kg) and tetracycline decreased BUN levels compared to tetracycline alone or nSP70 (30 mg/kg) and tetracycline. A similar decrease in BUN levels

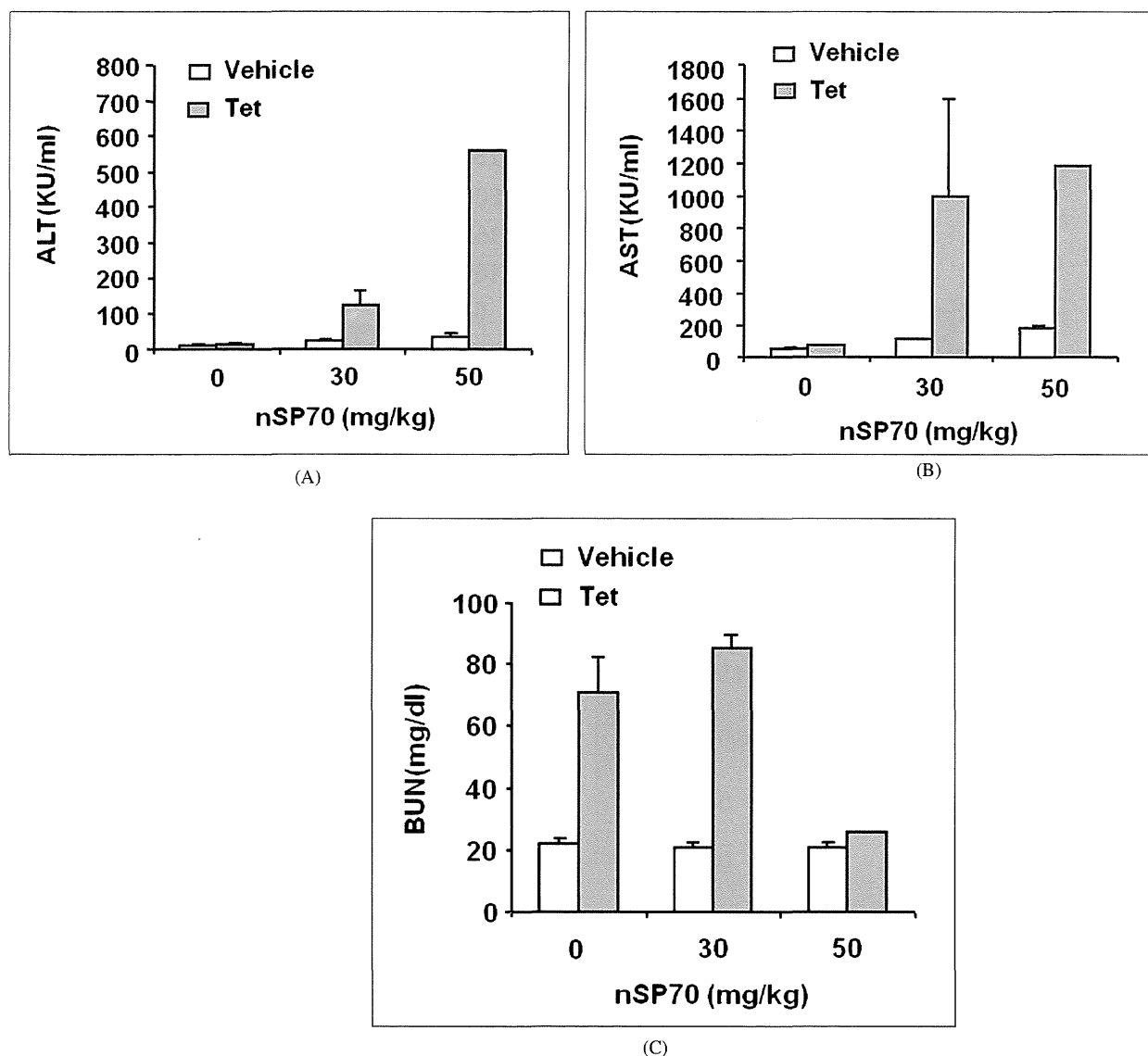


Fig. 2: Effect of nSP70 on tetracycline (Tet)-induced toxicity. Mice were injected intraperitoneally with Tet at 0 (open column) or 100 mg/kg (gray column) and intravenously with nSP70 at the indicated doses. After 24 h, the serum was collected. Shown are the levels of ALT (A), AST (B), and BUN (C). One of 4 mice died when co-treated with nSP70 (30 mg/kg) and Tet (100 mg/kg), and 2 of 4 mice died when co-treated with nSP70 (50 mg/kg) and Tet (100 mg/kg). Data are means or means  $\pm$  SEM ( $n=2-4$ )

was also reported in mice co-treated with nSP70 and cisplatin (Nishimori et al. 2009a). However, the mechanism by which these decrease the BUN level remains to be determined.

In conclusion, we found that nSP70 cause synergistic toxicity when combined with some clinically used drugs, although the synergistic effects differ between chemicals. One combination was lethal, and the others resulted in tissue injury. These studies suggest that evaluation of possible synergistic adverse effects with pharmaceutical drugs may be important for assessing the safety of nano-sized particles.

## 4. Experimental

### 4.1. Materials

The nSP70 nanoparticles were obtained from Micromod Partikeltechnologie GmnH (Rostock, Germany). The mean diameter of the particles, as analyzed by a Zetasizer (Sysmex Co., Kobe, Japan), was 55.7 nm, and the particles were spherical and nonporous. The particles were stored at 25 mg/ml as an aqueous suspension. The suspensions were thoroughly dispersed by soni-

cation before use and diluted in water. An equal volume of solution was injected for each treatment. Acetaminophen, tetracycline, and trazodone were dissolved in saline solution, and 5-aminosalicylic acid was suspended in 1% sodium salt of carboxy methyl cellulose. All reagents were of research grade.

### 4.2. Animals

Eight-week-old BALB/c male mice were purchased from Shimizu Laboratory Supplies Co., Ltd. (Kyoto, Japan). Mice were maintained in controlled environment ( $23 \pm 1.5^\circ\text{C}$ ; 12-h light/12-h dark cycle) with free access to standard rodent chow and water. The mice were given 1 week to adapt before experiments. All of the experimental protocols complied with the ethical guidelines of the Graduate School of Pharmaceutical Sciences, Osaka University.

### 4.3. Biochemical analysis

Serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), and blood urea nitrogen (BUN) were measured using commercially available kits according to the manufacturer's protocols (WAKO Pure Chemical, Osaka, Japan).

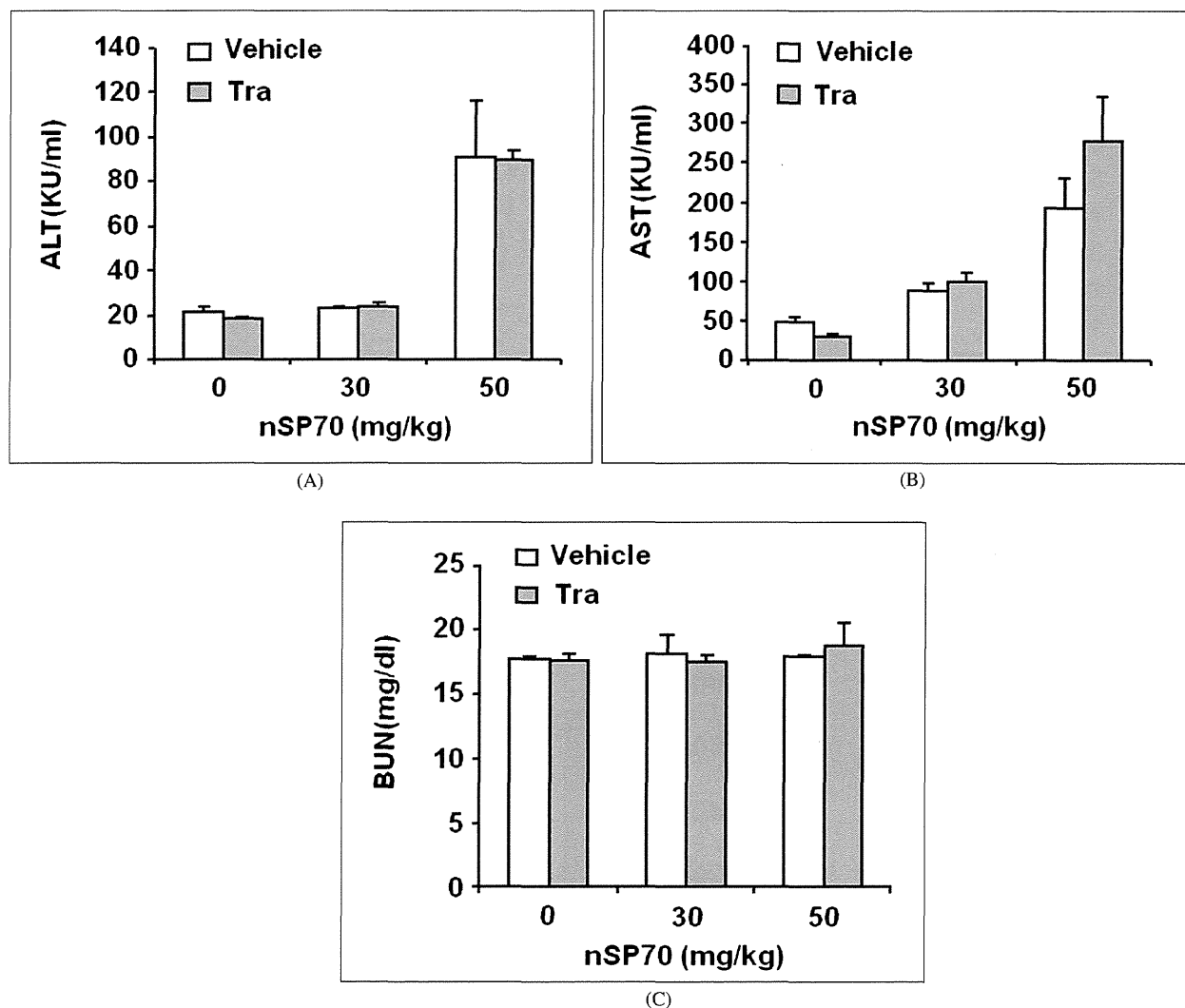


Fig. 3: Effect of nSP70 on trazodone (Tra)-induced toxicity Mice were injected intraperitoneally with Tra at 0 (open column) or 100 mg/kg (gray column) and intravenously with nSP70 at 30 or 50 mg/kg. After 24 h, the serum was collected. Shown are the levels of ALT (A), AST (B), and BUN (C). Data are means  $\pm$  SEM (n=4)

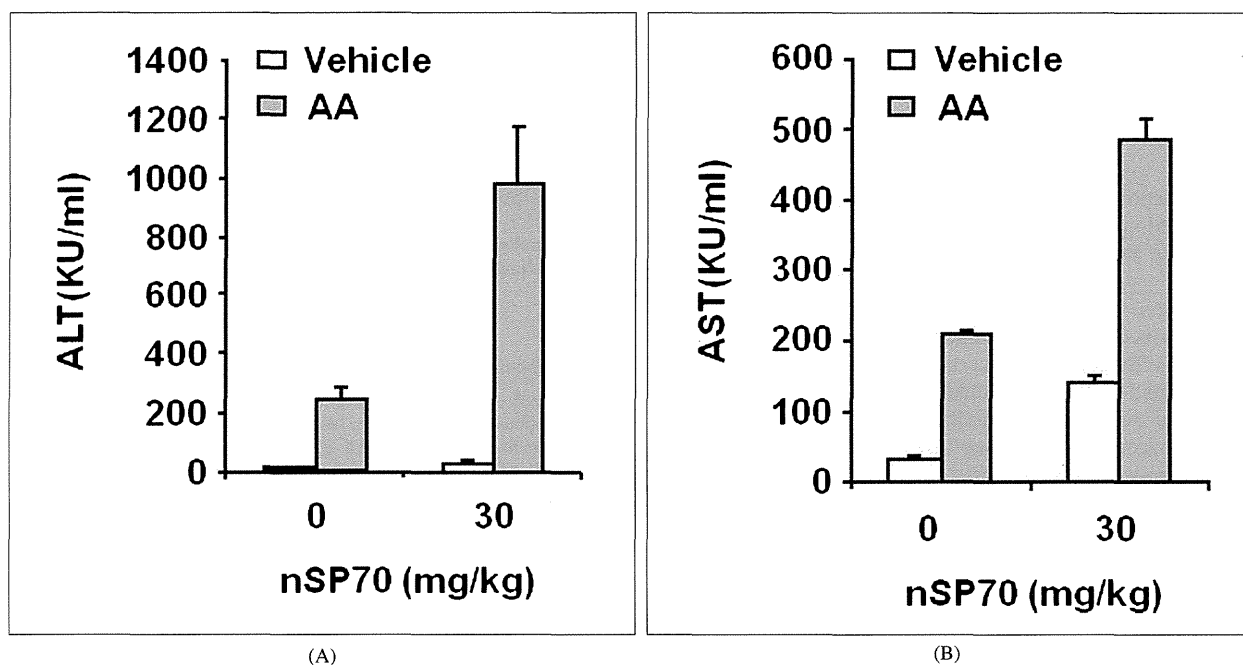


Fig. 4: Effect of nSP70 on acetaminophen (AA)-induced toxicity Mice were injected intraperitoneally with AA at 0 (open column) or 500 mg/kg (gray column) and intravenously with nSP70 (30 mg/kg). After 24 h, the serum was collected. Shown are the levels of ALT (A) and AST (B). Data are means  $\pm$  SEM (n=4)

Acknowledgements: The authors thank all members of our laboratory for their useful comments and discussion. This study was supported by a grant from the Ministry of Health, Labor, and Welfare of Japan.

## References

- Ali MM, Frei E, Straub J, Breuer A, Wiessler M (2002) Induction of metallothionein by zinc protects from daunorubicin toxicity in rats. *Toxicology* 179: 85–93.
- Byrne JD, Baugh JA (2008) The significance of nanoparticles in particle-induced pulmonary fibrosis. *McGill J Med* 11: 43–50.
- Chun LJ, Tong MJ, Busuttill RW, Hiatt JR (2009) Acetaminophen hepatotoxicity and acute liver failure. *J Clin Gastroenterol* 43: 342–349.
- Deltenre P, Berson A, Marcellin P, Degott C, Biour M, Pessayre D (1999) Mesalazine (5-aminosalicylic acid) induced chronic hepatitis. *Gut* 44: 886–888.
- Dutta D, Sundaram SK, Teegarden JG, Riley BJ, Fifield LS, Jacobs JM, Addleman SR, Kaysen GA, Moudgil BM, Weber TJ (2007) Adsorbed proteins influence the biological activity and molecular targeting of nanomaterials. *Toxicol Sci* 100: 303–315.
- Grisham MB, Ware K, Marshall S, Yamada T, Sandhu IS (1992) Prooxidant properties of 5-aminosalicylic acid. Possible mechanism for its adverse side effects. *Dig Dis Sci* 37: 1383–1389.
- Herzog R, Leuschner J (1995) Experimental studies on the pharmacokinetics and toxicity of 5-aminosalicylic acid-O-sulfate following local and systemic application. *Arzneimittelforschung* 45: 300–303.
- Jansen RW, Molema G, Harms G, Kruijt JK, van Berkel TJ, Hardonk MJ, Meijer DK (1991) Formaldehyde treated albumin contains monomeric and polymeric forms that are differently cleared by endothelial and Kupfer cells of the liver: evidence for scavenger receptor heterogeneity. *Biochem Biophys Res Commun* 180: 23–32.
- Jin CY, Zhu BS, Wang XF, Lu QH (2008) Cytotoxicity of titanium dioxide nanoparticles in mouse fibroblast cells. *Chem Res Toxicol* 21: 1871–1877.
- Kamps JA, Morselt HW, Swart PJ, Meijer DK, Scherphof GL (1997) Massive targeting of liposomes, surface-modified with anionized albumins, to hepatic endothelial cells. *Proc Natl Acad Sci U S A* 94: 11681–11685.
- Kovacic P (2005) Role of oxidative metabolites of cocaine in toxicity and addiction: oxidative stress and electron transfer. *Med Hypotheses* 64: 350–356.
- Kunin CM (1971) Hepatorenal toxicity of tetracycline. *Minn Med* 5: 532–533.
- Margetts PJ, Churchill DN, Alexopoulou I (2001) Interstitial nephritis in patients with inflammatory bowel disease treated with mesalamine. *J Clin Gastroenterol* 32: 176–178.
- Nishimori H, Kondoh M, Isoda K, Tsunoda S, Tsutsumi Y, Yagi K (2009a) Influence of 70 nm silica particles in mice with cisplatin or paraquat-induced toxicity. *Pharmazie* 64: 395–397.
- Nishimori H, Kondoh M, Isoda K, Tsunoda S, Tsutsumi Y, Yagi K (2009b) Silica nanoparticles as hepatotoxicants. *Eur J Pharm Biopharm* 72: 496–501.
- Popov PG, Vaptzarova KI, Kossekova GP, Nikolov TK (1972) Fluorometric study of tetracycline-bovine serum albumin interaction. The tetracyclines—a new class of fluorescent probes. *Biochem Pharmacol* 21: 2363–2372.
- Powis G (1974) A study of the interaction of tetracycline with human serum lipoproteins and albumin. *J Pharm Pharmacol* 26: 113–118.
- Sharma CS, Sarkar S, Periyakaruppan A, Barr J, Wise K, Thomas R, Wilson BL, Ramesh GT (2007) Single-walled carbon nanotubes induces oxidative stress in rat lung epithelial cells. *J Nanosci Nanotechnol* 7: 2466–2472.
- Xie G, Sun J, Zhong G, Shi L, Zhang D (2009) Biodistribution and toxicity of intravenously administered silica nanoparticles in mice. *Arch Toxicol*, in press.
- Xu JJ, Henstock PV, Dunn MC, Smith AR, Chabot JR, de Graaf D (2008) Cellular imaging predictions of clinical drug-induced liver injury. *Toxicol Sci* 105: 97–105.
- Yang ST, Wang X, Jia G, Gu Y, Wang T, Nie H, Ge C, Wang H, Liu Y (2008) Long-term accumulation and low toxicity of single-walled carbon nanotubes in intravenously exposed mice. *Toxicol Lett* 181: 182–189.
- Ye Y, Liu J, Xu J, Sun L, Chen M, Lan M (2010) Nano-SiO<sub>2</sub> induces apoptosis via activation of p53 and Bax mediated by oxidative stress in human hepatic cell line. *Toxicol In Vitro* 24: 751–758.





## Modifying the antigen-immunization schedule improves the variety of monoclonal antibodies obtained from immune-phage antibody libraries against HIV-1 Nef and Vif

Mai Yoshikawa,<sup>1,†</sup> Yohei Mukai,<sup>1,†</sup> Shin-ichi Tsunoda,<sup>2</sup> Yasuo Tsutsumi,<sup>1,2</sup> Yasuo Yoshioka,<sup>1,3</sup> Naoki Okada,<sup>1</sup> and Shinsaku Nakagawa<sup>1,\*</sup>

Department of Biotechnology and Therapeutics, Graduate School of Pharmaceutical Sciences, Osaka University, 1-6 Yamadaoka, Suita, Osaka 565-0871, Japan,<sup>1</sup> Laboratory of Biopharmaceutical Research, National Institute of Biomedical Innovation (NiBio), Osaka 567-0085, Japan,<sup>2</sup> and The Center for Advanced Medical Engineering and Informatics, Osaka University, 1-6 Yamadaoka, Suita, Osaka 565-0871, Japan<sup>3</sup>

Received 2 August 2010; accepted 25 December 2010  
Available online 28 January 2011

**Immune phage antibody libraries are an attractive technology for isolating antigen-specific monoclonal antibodies (mAbs). Here we show that the immunization schedule affects the immune phage antibody library properties. We subcutaneously (s.c.) administered HIV-1 Nef and Vif antigens with different schedules (25  $\mu\text{g} \times 2$  s.c. and 10  $\mu\text{g} \times 3$  s.c.). The variety of isolated mAbs in 25  $\mu\text{g} \times 2$  s.c. groups (Nef: 11 clones, Vif: 9 clones) was superior to that in the 10  $\mu\text{g} \times 3$  s.c. groups (Nef: 2 clones, Vif: 1 clone). This finding suggests that it is important to optimize the immunization schedule for isolating a wide variety of mAbs.**

© 2011, The Society for Biotechnology, Japan. All rights reserved.

[**Key words:** Phage display system; Monoclonal antibody; HIV-1; Antibody library; Immunization; Drug delivery; Targeting; Antibody therapy]

Phage antibody libraries are an extremely attractive technology that enables rapid isolation of monoclonal antibodies (mAbs) toward the development of therapeutic agents and basic research tools (1). Immune phage antibody library, constructed from cDNA derived from B-cells in the spleen of immunized animals, in particular, allows for an isolation of the desired mAbs with high antigen affinity and specificity (2). This library contains the antibody repertoires reconstructed from B-cells of immunized animals. Therefore, the diversity of the antigen specific B-cell repertoire is important to obtain a large variety of the desired mAbs. In general, after immunization, activated B-cells individually produce antigen-specific mAbs. Excess immunization, however, enhances the survival of specific B-cells that express mAbs with high affinities to antigens, resulting in a limited antigen-specific B-cell repertoire in immunized animals (3). To obtain a large variety of mAbs, optimizing the strength of the immunization is promising to retain the diversity of antigen specific B-cells *in vivo*.

A large variety of antigen-specific mAbs is preferable for developing therapeutic agents as well as for many applications in basic research (4,5). In particular, certain types of mAbs, such as neutralizing mAbs, signaling mAbs, and intracellular mAbs (intrabody), are useful, but difficult to obtain because of their rarity. These specialized mAbs must be screened from various candidate mAbs (6). Therefore, a method to identify a large variety of mAbs is highly desirable to accelerate the discovery of specialized mAbs.

HIV-1 encoded Nef and Vif proteins have an important role in HIV-1 viral replication and infectivity (7). Computer-based drug design against HIV enzymes has been successfully achieved, but is not adequate for complete therapy because of some drug resistance. HIV-1 Nef and Vif are also considered attractive targets for anti-HIV therapy. Computer-based drug design for Nef and Vif is also difficult, however, because these targets do not have a clear active domain for the design of specific drug-like enzymes. Therefore, it is thought that intrabody-based therapy will be a promising approach for therapy against these proteins (7,8). A large variety of mAbs will be necessary, however, to isolate the intrabody that can inhibit the viral activity of HIV.

Here we analyzed a variety of single-chain antibody variable domain fragments (scFvs) obtained from different immunization schedules in the scFv phage display using Nef and Vif antigens. We immunized mice with Nef or Vif using different protocols and assessed the amplification efficiency of their genes of variable domains (V genes). We then constructed anti-Nef and anti-Vif scFv phage libraries and estimated the variety of scFvs obtained from these phage libraries. The findings of the present study provide critical information to enable isolation of a wide variety of mAbs from immune phage antibody libraries. These findings will also contribute to the discovery of specialized mAbs useful for both future therapies and advanced basic research.

### MATERIALS AND METHODS

**Immunization with Nef and Vif recombinant protein** On day 0, 6-week-old female BALB/c mice were subcutaneously (s.c.) injected with 25  $\mu\text{g}$  or 10  $\mu\text{g}$  of antigens as 100- $\mu\text{l}$  emulsions with a Titer Max Gold adjuvant (Sigma-Aldrich Corp., St. Louis, MO, USA). For these immunizations, we used purified recombinant HIV-1 Nef and HIV-1 Vif proteins

\* Corresponding author. Tel.: +81 6 6879 8175; fax: +81 6 6879 8179.  
E-mail addresses: yohe@mks.osaka-u.ac.jp (Y. Mukai), nakagawa@phs.osaka-u.ac.jp (S. Nakagawa).

<sup>†</sup> The first two authors contributed equally to this work.

(Immunodiagnosics, Inc., Woburn, MA) as antigens. On day 14, the immunizations were repeated in the same manner. The group administered with 10  $\mu$ g received an additional immunization on day 28. Serum samples were collected on days 14, 21, and 35, and IgG titers were measured using Nef- or Vif-immobilized capture ELISA.

**Calculation of antibody titer in collected serum** Nef or Vif recombinant protein (Immunodiagnosics, Inc.) was immobilized on immunoassay plates (Nalge Nunc International, Rochester, NY, USA) at 4°C for 8 h. The plates were blocked with 4% Block Ace (Dainippon Sumitomo Pharma Co., Ltd, Osaka, Japan) at 37°C for 2 h. Serum samples were diluted with 0.4% Block Ace, and then reacted with immobilized antigens for 1 h at room temperature. Anti-mouse IgG horseradish peroxidase (HRP) conjugate (Sigma-Aldrich Corp.) was reacted for 1 h at room temperature, and then HRP activity was assessed by a 3,3',5,5'-tetramethylbenzidine (TMB) substrate (Thermo Fisher Scientific Inc., Rockford, IL, USA). The reaction was stopped by 1 N H<sub>2</sub>SO<sub>4</sub>, and then absorbance was measured at 450 nm and 655 nm as a reference. Antibody titers were calculated as Log<sub>2</sub> titer. Log<sub>2</sub> titer indicates a maximum serum dilution value (2<sup>n</sup>) that can induce the antigen-specific binding potency.

**Construction of the phage antibody library** Immunized mice were killed on day 21 (25  $\mu$ g  $\times$  2 s.c. groups) or on day 35 (10  $\mu$ g  $\times$  3 s.c. groups). Spleen and bone marrow cells were collected separately from immunized mice. The phage antibody library was constructed as described previously (9) with a slight modification. Briefly, variable regions of immunoglobulin light-chain (VL) and heavy-chain (VH) genes were amplified by polymerase chain reaction (PCR) using an original primer set (9). VL and VH cDNAs were linked by PCR to synthesize the scFv gene library. The cDNAs were amplified by the following primers: Y15-2 (5'-GTTCTTTCTATGCGGCCAGCCGGC-CATGGCC-3') and Y16-2 (5'-ATCCGATACGGACCGCCGACCTGCGGCCGC-3') by cycling 35 times at 96°C for 60 s, 65°C for 60 s, and 68°C for 60 s. The amplified scFv gene library was digested by *Nco* I and *Not* I, and then cloned into pCANTAB5E. The products were transformed into *E. coli* TG1 strain, and these TG1 cells were used in the phage preparation. The library size was estimated from the number of colonies formed in this transformation. Preparation of the phage library was described previously (9).

**Affinity panning** Anti-FLAG M2 antibody (Sigma-Aldrich Corp.) and Nef and Vif recombinant protein (Immunodiagnosics, Inc.) were immobilized on immunoassay plates (Nalge Nunc International) at 4°C for 8 h. The plates were blocked by 10% skim milk (Becton Dickinson and Company, Franklin Lakes, NJ, USA) containing 25% glycerol at 37°C for 2 h. Each phage library was reacted with an immobilized anti-FLAG M2 antibody for 1 h at 4°C. After washing with 0.05% Tween 20 (Sigma-Aldrich Corp.), the FLAG-positive phages were rescued using 20 mM glycine-HCl buffer (pH = 2.0), and then neutralized in a 1 M Tris-HCl buffer (pH = 8.0). The eluent was reacted with immobilized Nef or Vif protein for 1 h at 4°C. The antigen-binding phages were rescued by 0.1 N HCl, and then neutralized in a 1 M Tris-HCl buffer (pH = 8.0). The obtained phages were amplified by infecting to the exponential growth of TG1. This panning step was repeated five times. The phage titer was measured by counting the number of colonies on Petrifilm™ *E. coli* count plates (3M Company, Two Harbors, MN, USA).

**Phage ELISA** One hundred fifty TG1 colonies were picked up from the plates after each panning. They were grown in 96-well plates at 37°C, and then monoclonal phages were produced from each TG1 by the infection of M13KO7 helper phages. The amplified phages were then blocked with 2% Block Ace (Dainippon Sumitomo Pharma Co.) at 4°C for 1 h and then added to an immunoassay plate coated with the antigens. Plates were then incubated for 1 h, with agitation at 250 rpm, and washed three times with phosphate buffered saline/0.1% Tween 20, and finally incubated with HRP-conjugated anti-M13 monoclonal antibody (Amersham Bioscience). HRP activity was assessed by the TMB substrate (MOSS, Inc.). The reaction was stopped by 1 N H<sub>2</sub>SO<sub>4</sub>, and then the absorbance was measured at 450 nm and 655 nm as a reference.

## RESULTS

**Amplification of the VL and VH gene libraries** Mice were immunized with HIV-1 Nef or Vif antigens at 10  $\mu$ g or 25  $\mu$ g each. Antibody induction was observed after the 2nd immunization (Table 1, day 21). The mice receiving 25  $\mu$ g were killed on day 21 (25  $\mu$ g  $\times$  2 s.c. group). Because the antibody titers of mice receiving 10  $\mu$ g were not sufficient, we

TABLE 1. Antibody titers induced in Nef or Vif-immunized mice.

Antigen	Dose	Log <sub>2</sub> titer (2 <sup>n</sup> )		
		Day 14	Day 21	Day 35
Nef	10 $\mu$ g	<6	9	13
	25 $\mu$ g	<6	12	N.T.
Vif	10 $\mu$ g	<6	10	13
	25 $\mu$ g	<6	12	N.T.

Nef or Vif recombinant antigen (10  $\mu$ g or 25  $\mu$ g) was injected into 5 mice on day 0. On day 14, the same dose of antigen was injected into the same mice. For the 10- $\mu$ g immunization groups, an additional immunization was performed on day 28. Antigen titers from the collected serum on days 14, 21, and 35 were measured by ELISA. Log<sub>2</sub> titer indicates maximum serum dilution value (2<sup>n</sup>) that can induce the antigen specific binding potency. N.T., not tested.

performed a 3rd immunization in mice administered with 10  $\mu$ g. After the 3rd immunization, sufficient antibody titers were observed in the groups receiving 10  $\mu$ g (Table 1, day 35) and then the mice were killed on day 35 (10  $\mu$ g  $\times$  3 s.c. group).

The VL and VH genes were amplified from cDNA derived from the spleens and bone marrow cells of immunized mice using a primer mix including thousands of independent primers (9). In both immunizations of Nef and Vif, the amplifications of V genes were more efficient in the 25  $\mu$ g  $\times$  2 s.c. groups than in the 10  $\mu$ g  $\times$  3 s.c. groups (Fig. 1). The yields of V genes in the 25  $\mu$ g  $\times$  2 s.c. groups were approximately 10 times higher than those in the 10  $\mu$ g  $\times$  3 s.c. groups. In each group, VH and VL genes were linked by PCR, and then the obtained scFv genes were ligated in the pCANTAB5E vector.

**Quality checks of immune scFv phage libraries** These plasmid libraries were transformed into TG1, and the diversity of library was estimated from the number of colonies formed in the transformation. Each library contained a 200 to 700 million scFv repertory. DNA sequence analysis of 20 clones picked randomly from each library indicated that each library comprised independent clones with different sequences (data not shown). We produced phage antibody libraries from these TG1s, and then evaluated the affinity of each phage library against each antigen by phage ELISA. The results suggested that both phage libraries from mice immunized with Nef had almost the same affinity against the immobilized Nef (Fig. 2A). Similar results were obtained in the anti-Vif phage libraries (Fig. 2B). The results suggested that the whole affinity of each phage library did not depend on the immunization schedule. These results are also consistent with the results of the antibody values in the serums shown in Table 1.

**Selection and identification of the antigen-specific scFvs** Anti-Nef or anti-Vif scFv displaying phages were concentrated by repeating the affinity panning against Nef or Vif, respectively. The output/input ratios were increased similarly in all groups during 5 panning steps (Figs. 3A, B). The output/input ratio indicates the concentration of the phages displaying anti-Nef or anti-Vif scFvs. The results showed that the efficiency in concentrating the desired phages also did not depend on their immunization schedules.

To identify antigen-specific scFvs, 150 colonies were randomly picked up after the 3rd, 4th, and 5th panning. The affinities of monoclonal phages against Nef or Vif were then assessed by phage ELISA. Many scFvs showed antigen-specific affinity in all groups. We picked up clones with the antigen-specific affinity, and then confirmed their sequences to analyze the variety of the obtained scFvs. From the 25  $\mu$ g  $\times$  2 s.c. groups, we could identify different scFvs (11 scFvs for Nef and 9 scFvs for Vif) (Table 2). On the other hand, from the 10  $\mu$ g  $\times$  3 s.c. groups, the variety of the obtained scFvs was extremely limited (2 scFvs for Nef and 1 scFv for Vif) (Table 2). We also confirmed that scFvs from

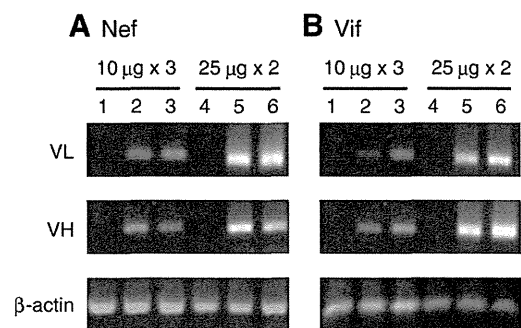


FIG. 1. Amplification of VL and VH gene repertoires from Nef or Vif-immunized mice. VL and VH gene repertoires were amplified from cDNAs that were synthesized from spleen and bone marrow-derived mRNAs from Nef or Vif-immunized mice. VL and VH genes were obtained by PCR from 1  $\mu$ l of (A) Nef or (B) Vif-immunized mice cDNA using 1  $\mu$ l (Lanes 1 and 4); 2  $\mu$ l (Lanes 2 and 5); or 4  $\mu$ l (Lanes 3 and 6) of our original mix primers.  $\beta$ -Actin gene was amplified with the same protocol as a positive control.

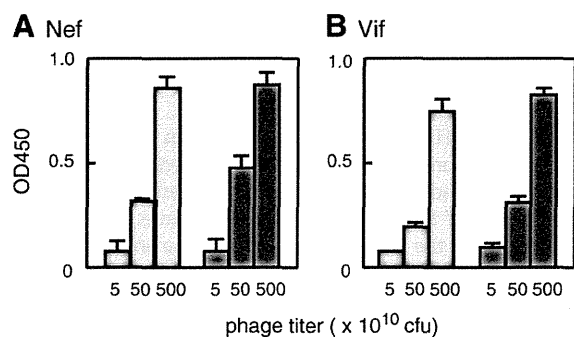


FIG. 2. The binding potency of Nef or Vif immunized phage libraries. The binding potency of the Nef (A) or Vif (B) immunized phage library was evaluated by phage ELISA against immobilized Nef or Vif recombinant antigen, respectively. Phage libraries constructed from mice immunized with 10  $\mu\text{g}$  antigen  $\times 3$  (grey column) or 25  $\mu\text{g}$  antigen  $\times 2$  (black column) were used in this experiment.  $5 \times 10^{10}$ – $5 \times 10^{12}$  colony forming unit (cfu) phage solutions were added to the immobilized antigens and detected by horseradish peroxidase-linked anti-M13 antibody.

the 10  $\mu\text{g}$   $\times 3$  s.c. groups were included in the 25  $\mu\text{g}$   $\times 2$  s.c. groups. The obtained scFvs were all identified after the 3rd panning, and the variety of scFvs was decreased during the 4th and 5th panning (Table 2). These results suggested that phage libraries in the 25  $\mu\text{g}$   $\times 2$  s.c. groups had a greater diversity of scFvs than those in the 10  $\mu\text{g}$   $\times 3$  s.c. groups.

## DISCUSSION

These findings of the present study indicated that the diversity of antigen specific antibodies displayed on a phage library greatly depended on the immunization protocol. In the immunization, antibody values in the serums of 10  $\mu\text{g}$   $\times 3$  s.c. and 25  $\mu\text{g}$   $\times 2$  s.c. groups were similar, but the efficiencies of the amplification in the 25  $\mu\text{g}$   $\times 2$  s.c. groups were greater than those in the 10  $\mu\text{g}$   $\times 3$  s.c. groups. In this step, we used a mixture of primers to amplify almost all antibody repertoires (9). Therefore, these results indicated that the diversity of antibody-producing B-cells in the 10  $\mu\text{g}$   $\times 3$  s.c. groups was extremely limited. Indeed, after the selections, we obtained only 2 anti-Nef scFvs and 1 anti-Vif scFv in the 10  $\mu\text{g}$   $\times 3$  s.c. groups despite obtaining 11 anti-Nef scFvs and 9 anti-Vif scFvs in the 25  $\mu\text{g}$   $\times 2$  s.c. groups. A previous report showed that increased immunization frequency and timespan enhance the survival of B-cells that express the antibody with high affinity, resulting in a small antibody repertoire in immunized animals (3). Our result showed that this machinery is also important for the construction of an immune phage antibody library, and the induction of a high antibody titer in a short

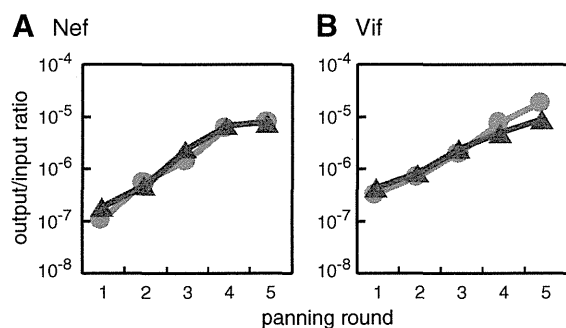


FIG. 3. Concentration of anti-Nef or Vif mAb displaying phages by affinity panning. Anti-Nef or Vif scFv-displaying phages were concentrated by affinity panning against immobilized Nef or Vif recombinant antigens, respectively. The output/input ratio (output phage titer/input phage titer) of the (A) Nef- or (B) Vif-immunized library was calculated and plotted. Grey circles indicate the output/input ratio of the 10  $\mu\text{g}$   $\times 3$  s.c. groups, and black triangles indicate that of the 25  $\mu\text{g}$   $\times 2$  s.c. groups.

TABLE 2. The diversity of isolated scFvs from each phage antibody library.

Antigen		Nef		Vif	
Dose		10 $\mu\text{g}$	25 $\mu\text{g}$	10 $\mu\text{g}$	25 $\mu\text{g}$
Clones	3rd	2	11	1	9
	4th	1	3	1	3
	5th	1	1	1	1
	Unique	2	11	1	9

One hundred fifty clones were randomly picked from 3rd, 4th, and 5th panning output repertoires. The binding potencies of these clones were assessed by phage ELISA. The variety of isolated clones was assessed by sequencing positive clones in phage ELISA after each panning round. "Unique" indicates the variety of unique clones obtained from through 3rd to 5th panning.

period will be helpful to obtain the large variety of antibodies from an immune phage antibody library.

Nef and Vif, the model targets in this study, are attractive targets for anti-HIV therapy in HIV-infected T-cells (7). Antibodies that can work in the cell are known as intrabodies, which have recently received a lot of attention (10). To target these intracellular molecules, it is necessary to isolate antibodies that fold and function properly in a reduced intracellular environment (11). Because these antibodies are rare, however, it is important to screen from high variety of antibodies that recognize the same target. From this point of view, anti-Nef and anti-Vif scFvs identified in this report will be useful as intrabody candidates.

In conclusion, the immunization protocol affects the variety of antibodies identified from phage antibody libraries. We expect that our findings will allow for the isolation of a large variety of antibodies that will be helpful for screening any desired antibodies and contribute to the progress of biotechnology for the development of antibody-based medicine and other basic protein research.

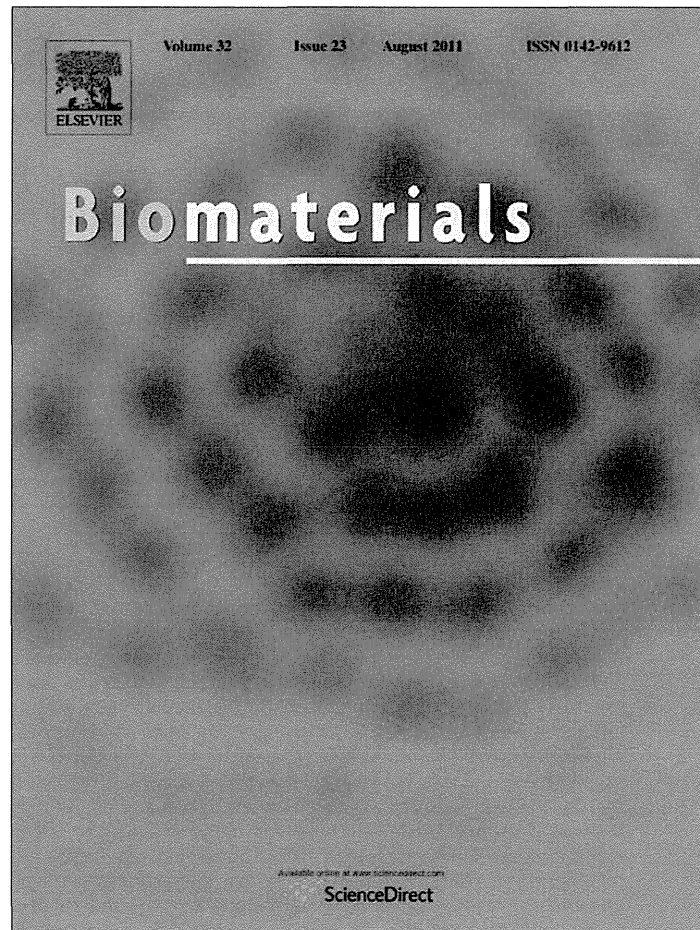
## ACKNOWLEDGMENTS

This study was supported by Research Fund Project on Health Sciences focusing on Drug Innovation from the Japan Health Sciences Foundation, and in part by a Grant-in-Aid for Scientific research (B) and Grant-in-Aid for Scientific Research on Innovative Areas and from the Ministry of Education, Culture, Sports, Science, and Technology of Japan (MEXT) and Japan Society for the Promotion of Science (JSPS).

## References

- McCafferty, J., Griffiths, A. D., Winter, G., and Chiswell, D. J.: Phage antibodies: filamentous phage displaying antibody variable domains, *Nature*, **348**, 552–554 (1990).
- Rojas, G., Almagro, J. C., Acevedo, B., and Gaviñondo, J. V.: Phage antibody fragments library combining a single human light chain variable region with immune mouse heavy chain variable regions, *J. Biotechnol.*, **94**, 287–298 (2002).
- Honjo, T. and Habu, S.: Origin of immune diversity: genetic variation and selection, *Annu. Rev. Biochem.*, **54**, 803–830 (1985).
- Adams, G. P. and Weiner, L. M.: Monoclonal antibody therapy of cancer, *Nat. Biotechnol.*, **23**, 1147–1157 (2005).
- Griffiths, A. D. and Duncan, A. R.: Strategies for selection of antibodies by phage display, *Curr. Opin. Biotechnol.*, **9**, 102–108 (1998).
- Nieri, P., Donadio, E., Rossi, S., Adinolfi, B., and Podesta, A.: Antibodies for therapeutic uses and the evolution of biotechniques, *Curr. Med. Chem.*, **16**, 753–779 (2009).
- Richter, S. N., Frasson, I., and Palu, G.: Strategies for inhibiting function of HIV-1 accessory proteins: a necessary route to AIDS therapy, *Curr. Med. Chem.*, **16**, 267–286 (2009).
- Fackler, O. T., Alcover, A., and Schwartz, O.: Modulation of the immunological synapse: a key to HIV-1 pathogenesis, *Nat. Rev. Immunol.*, **7**, 310–317 (2007).
- Imai, S., Mukai, Y., Nagano, K., Shibata, H., Sugita, T., Abe, Y., Nomura, T., Tsutsumi, Y., Kamada, H., Nakagawa, S., and Tsunoda, S.: Quality enhancement of the non-immune phage scFv library to isolate effective antibodies, *Biol. Pharm. Bull.*, **29**, 1325–1330 (2006).
- Lo, A. S., Zhu, Q., and Marasco, W. A.: Intracellular antibodies (intrabodies) and their therapeutic potential, *Handb. Exp. Pharmacol.*, **181**, 343–373 (2008).
- Worn, A., Auf der Maur, A., Escher, D., Honegger, A., Barberis, A., and Pluckthun, A.: Correlation between in vitro stability and in vivo performance of anti-ICGN4 intrabodies as cytoplasmic inhibitors, *J. Biol. Chem.*, **275**, 2795–2803 (2000).

Provided for non-commercial research and education use.  
Not for reproduction, distribution or commercial use.



This article appeared in a journal published by Elsevier. The attached copy is furnished to the author for internal non-commercial research and education use, including for instruction at the authors institution and sharing with colleagues.

Other uses, including reproduction and distribution, or selling or licensing copies, or posting to personal, institutional or third party websites are prohibited.

In most cases authors are permitted to post their version of the article (e.g. in Word or Tex form) to their personal website or institutional repository. Authors requiring further information regarding Elsevier's archiving and manuscript policies are encouraged to visit:

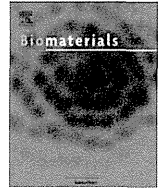
<http://www.elsevier.com/copyright>



ELSEVIER

Contents lists available at ScienceDirect

Biomaterials

journal homepage: [www.elsevier.com/locate/biomaterials](http://www.elsevier.com/locate/biomaterials)

## Fine tuning of receptor-selectivity for tumor necrosis factor- $\alpha$ using a phage display system with one-step competitive panning

Yasuhiro Abe<sup>a</sup>, Tomoaki Yoshikawa<sup>a,b</sup>, Masaki Inoue<sup>a</sup>, Tetsuya Nomura<sup>a</sup>, Takeshi Furuya<sup>a,b</sup>, Takuya Yamashita<sup>a,b</sup>, Kazuya Nagano<sup>a</sup>, Hiromi Nabeshi<sup>a,b</sup>, Yasuo Yoshioka<sup>a,c</sup>, Yohei Mukai<sup>a,b</sup>, Shinsaku Nakagawa<sup>b,c</sup>, Haruhiko Kamada<sup>a,c</sup>, Yasuo Tsutsumi<sup>a,b,c</sup>, Shin-ichi Tsunoda<sup>a,b,c,\*</sup>

<sup>a</sup>Laboratory of Biopharmaceutical Research, National Institute of Biomedical Innovation, 7-6-8 Saito-Asagi, Ibaraki, Osaka 567-0085, Japan

<sup>b</sup>Graduate School of Pharmaceutical Sciences, Osaka University, 1-6 Yamadaoka, Suita, Osaka 565-0871, Japan

<sup>c</sup>The Center of Advanced Medical Engineering and informatics, Osaka University, 1-6 Yamadaoka, Suita, Osaka 565-0871, Japan

### ARTICLE INFO

#### Article history:

Received 24 March 2011

Accepted 5 April 2011

Available online 5 May 2011

#### Keywords:

Cytokine  
Molecular biology  
Protein  
Affinity  
Bioactivity

### ABSTRACT

Tumor necrosis factor- $\alpha$  (TNF) is one of the attractive targets for the development of anti-inflammatory and anti-tumor drugs, because it is an important mediator in the pathogenesis of several inflammatory diseases and tumor progression. Thus, there is an increasing need to understand the TNF receptor (TNFR1 and TNFR2) biology for the development of TNFR-selective drugs. Nonetheless, the role of TNFRs, especially that of TNFR2, remains poorly understood. Here, using a unique competitive panning, we optimized our phage display-based screening technique for isolating receptor-selective TNF mutants, and identified several TNFR2-specific TNF mutants with high TNFR2 affinity and full bioactivity via TNFR2. Among these mutants, the R2-7 clone revealed very high TNFR2-selectivity ( $1.8 \times 10^5$  fold higher than that for the wild-type TNF), which is so far highest among the reported TNFR2-selective TNF mutants. Because of its high TNFR2-selectivity and full bioactivity, the TNF mutant R2-7 would not only help in elucidating the functional role of TNFR2 but would also help in understanding the structure-function relationship of TNF/TNFR2. In summary, our one-step competitive panning system is a simple, useful and effective technology for isolating receptor-selective mutant proteins.

© 2011 Elsevier Ltd. All rights reserved.

### 1. Introduction

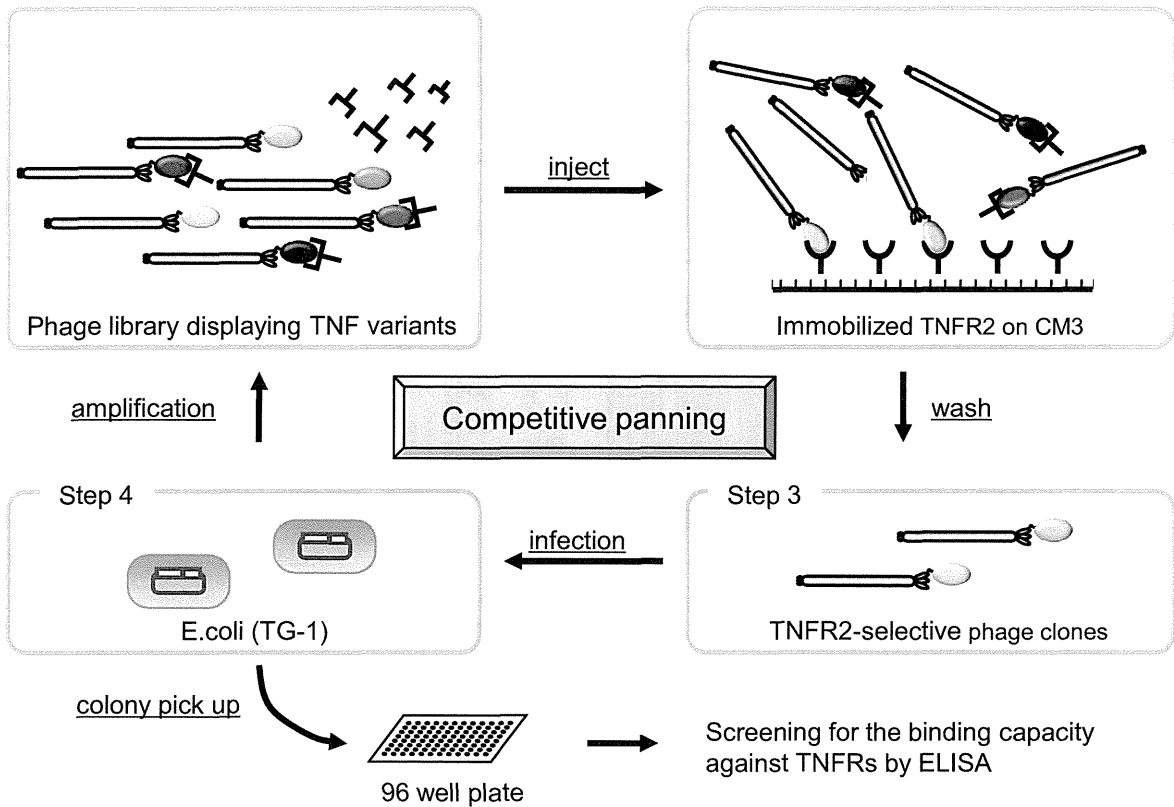
Tumor necrosis factor- $\alpha$  (TNF) is a major inflammatory cytokine that plays a central role in host defense and inflammation via two receptor subtypes, TNF receptor (TNFR)1 and TNFR2 [1,2]. Elevated serum levels of TNF correlates with the severity and progression of the inflammatory diseases such as rheumatoid arthritis (RA), inflammatory bowel disease, septic shock, multiple sclerosis and hepatitis [3–5]. Currently, TNF-neutralization therapies have proven successful for the treatment of RA [4,6,7]. However, these therapies can cause serious side effects, such as tuberculosis, because TNF-dependent host defense functions are also inhibited [8,9]. Therefore, understanding the function of TNF/TNFRs is important for optimal therapy of various TNF-related autoimmune

diseases. TNFR1 is constitutively expressed in most tissues and seems to be the key mediator of TNF signaling [10,11]. In contrast, the expression of TNFR2 is more restricted and is found mainly on certain T-cell subpopulations [12], endothelial cells, cardiac myocytes [13] and neuronal tissue [14,15]. Recent studies suggested that TNFR2 signaling is associated with T-cell survival [16], cardioprotection [17,18], remyelination [19], and survival of some neuron subtypes [20,21]. Although the two TNFRs have been shown to have distinct functions in some cells [22], the physiological significance of the presence of both receptors is not fully understood. Especially TNFR2-induced signaling remains elusive and need further investigation.

In order to understand the mechanism of TNFRs, we have investigated the relationship between the biological activities and structural properties of a large number of TNF mutants by phage-display technique [23,24]. However, screening efficiency of isolating TNFR2-selective TNF mutants using this technique is extremely low, and it is difficult to prepare large repertoire of TNFR2-selective TNF mutants for the structure-activity relationship study. In our previous study, we screened 500 phage clones

\* Corresponding author. Laboratory of Biopharmaceutical Research, National Institute of Biomedical Innovation, 7-6-8 Saito-Asagi, Ibaraki, Osaka 567-0085, Japan. Tel.: +81 72 641 9814; fax: +81 72 6419817.

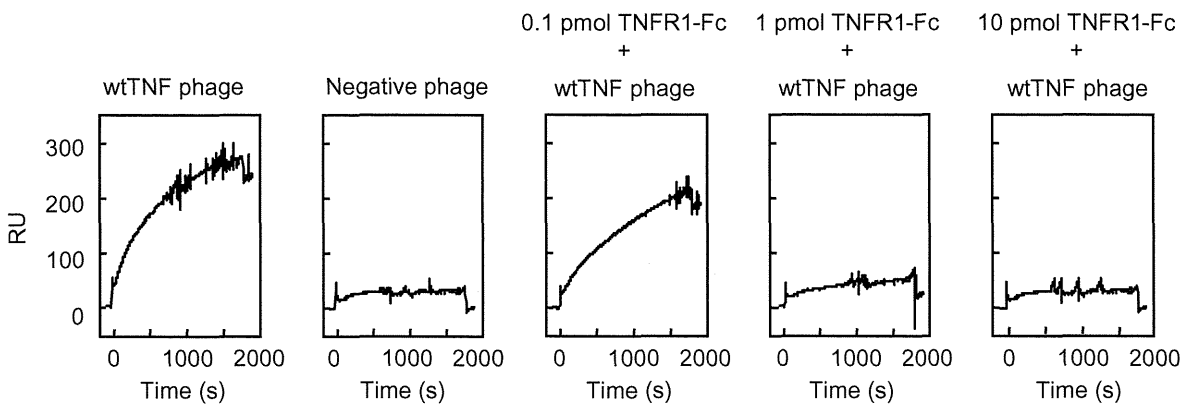
E-mail address: [tsunoda@nibio.go.jp](mailto:tsunoda@nibio.go.jp) (S.-i. Tsunoda).



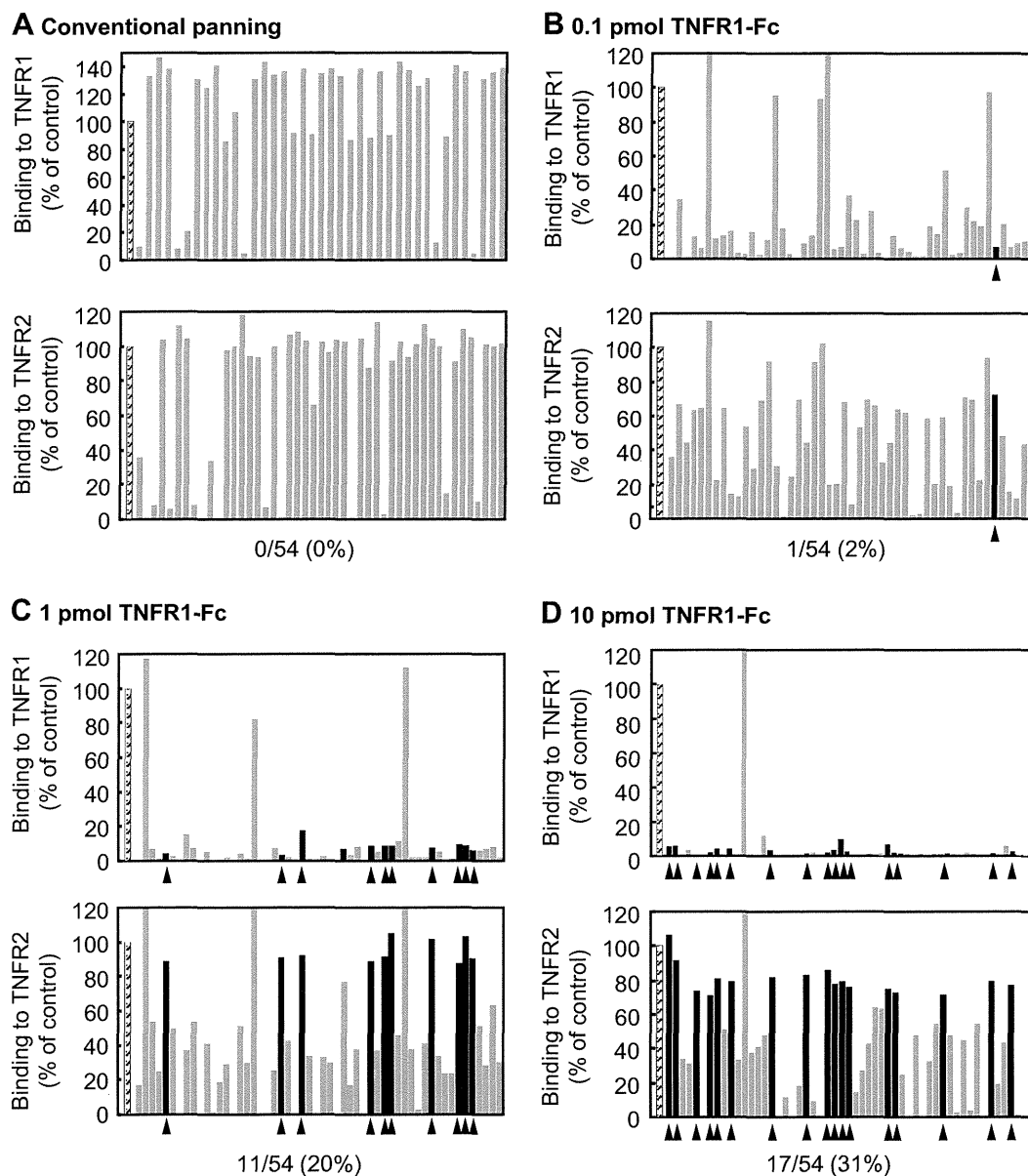
**Fig. 1.** Screening scheme for isolating TNFR2-selective TNF mutants using competitive panning. To concentrate TNFR2-selective mutant TNFs, phage libraries were pre-incubated with TNFR1 Fc chimera (TNFR1-Fc), and subsequent biopanning against the TNFR2 was carried out in the presence of TNFR1-Fc using the BIAcore biosensor. After several rounds of panning, phage clones were isolated and screened by ELISA.

for isolating TNFR2-selective mutants using the conventional panning method [23]. Out of the 500 clones, only 2 clones showed selectivity for TNFR2 binding that was 10-times higher than the wild-type TNF (wtTNF). Furthermore, bioactivities of these two TNFR2 selective TNF mutants were lower than that of wtTNF (<30%). To improve the screening efficacy, we optimized our phage display-based cytokine mutagenesis technology [25] with an unique competitive panning technique for identifying TNFR2-specific TNF mutants with higher affinity and bioactivity. In this

competitive panning technique, phage libraries were pre-incubated with TNFR1 Fc chimera (TNFR1-Fc), and subsequent biopanning against the TNFR2 was carried out in the presence of TNFR1-Fc using the BIAcore biosensor. Since TNFR1-binding clones could not bind to TNFR2 due to steric hindrance, TNF mutants binding only to TNFR2 were selectively enriched with high efficiency. Using this optimized competitive panning technique, we have identified TNFR2-selective TNF mutants with full bioactivity via TNFR2.



**Fig. 2.** Optimization of competitive panning using BIAcore biosensor. 0.1 pmol, 1 pmol or 10 pmol of human TNFR1-Fc was mixed with  $1 \times 10^{10}$  CFU phages displaying wtTNF for 2 h at 4 °C, and the mixture was passed over the TNFR2-immobilized CM3 sensor chip and real-time biomolecular interaction analyses were performed with BIAcore biosensor. Anti-CD25 single chain Fv-displaying phage was used as a negative control.



**Fig. 3.** Determination of relative affinities of mutant TNFs for TNFR1 or TNFR2 by capture ELISA. *E. coli* supernatant containing a TNF mutant (gray bar) from each panning conditions, in which phages were premixed with (A) none, (B) 0.1 pmol, (C) 1 pmol and (D) 10 pmol of TNFR1-Fc, were applied to the TNFR1-Fc or TNFR2-Fc immobilized plate and detected with biotinylated polyclonal anti-TNF antibody. wtTNF was used as a positive control (hatched bar). Affinities of TNFR2-selective clones (black bar) for TNFR2 was more than 70% of that of the wtTNF, and that for TNFR1 was less than 30% of that of the wtTNF.

**2. Materials and methods**

**2.1. Cells**

HEP-2 cells, a human fibroblast cell line, were provided by Cell Resource Center for Biomedical Research (Tohoku University, Sendai, Japan) and were maintained in RPMI 1640 (Sigma–Aldrich Japan, Tokyo, Japan) supplemented with 10% bovine fetal serum (FBS) 1 mM sodium pyruvate, 50 mM 2-mercaptoethanol, and antibiotics. hTNFR2/mFas-PA cells are preadipocytes derived from TNFR1<sup>-/-</sup>R2<sup>-/-</sup> mice expressing a chimeric receptor, the extracellular and transmembrane domain of human TNFR2, and intracellular domain of mouse Fas; these cells were cultured in RPMI 1640 supplemented with 10% FBS, 5 μg/ml Blasticidin S HCl (Invitrogen, Carlsbad, CA), and antibiotics [26].

**2.2. Library construction**

Protocol for the construction of phage-display library displaying structural mutants of human TNF has been described previously [23]. In brief, multiple

mutations were introduced into the wtTNF gene by PCR to randomly replace the codons of 6 amino acid residues at positions 29, 31, 32, 145, 146 and 147, respectively, of the TNF protein. The PCR product was digested with the restriction enzymes Hind III and Not I, and ligated into the Hind III/Not I digested pY03' phagemid vector for displaying the TNF mutants on the phage surface as g3p-fusion proteins.

**2.3. Optimization of competitive panning using BIAcore biosensor**

Human TNFR2-Fc (R&D systems, Minneapolis, MN) was diluted to 50 μg/ml in 10 mM sodium acetate buffer (pH 4.5) and immobilized onto a CM3 sensor chip using an amine coupling kit (GE Healthcare, UK), which resulted in an increase of 5000–6000 resonance units (RU). 0.1 pmol, 1 pmol or 10 pmol of human TNFR1-Fc (R&D systems) was mixed with 100 μl of wtTNF-displaying phage (1 × 10<sup>11</sup> CFU/ml) for 2 h at 4 °C, and the mixture was passed over the TNFR2-immobilized CM3 sensor chip at a flow rate of 3 μl/min. The binding kinetics of the mixtures to TNFR2-Fc were analyzed by BIAcore 2000 (GE Healthcare).

**Table 1**  
Amino acid sequences of wtTNF and TNFR2-selective TNF mutants.

Clone	Residue position					
	29	31	32	145	146	147
wtTNF	L	R	R	A	E	S
R2-6	L	R	R	H	E	D
R2-7	V	R	R	D	D	D
R2-8	L	R	R	N	D	D
R2-9	L	R	R	T	S	D
R2-10	L	R	R	Q	D	D
R2-11	L	R	R	T	D	D
R2-12	L	R	R	D	G	D
R2-13	L	R	R	D	E	D

#### 2.4. Selection of phage displaying TNFR2-selective TNF mutants by competitive panning

$1 \times 10^{10}$  CFU phages displaying TNF mutants were pre-incubated for 2 h at 4 °C, with serially diluted TNFR1-Fc. The mixtures were injected at 3  $\mu$ /min over the sensor chip. After injection, the sensor chip was washed using the rinse command for 3 min. Elution was carried out using 20  $\mu$ l of 10 mM glycine-HCl (pH 2.0) and the eluted phage was neutralized with 1 M Tris-HCl (pH 6.9). The recovered phages were amplified by infection of *E. coli* strain TG1 (Stratagene, La Jolla, CA), which allow read-through of the amber stop codon located between the TNF and g3p sequences of pY03' phagemid vector. These steps were repeated twice. After final round of panning, the phage mixture was used to infect *E. coli* and plated on LB agar/ampicillin plates. Single clones of transfected TG1 were randomly picked from the plate and each colony was grown in 2-YT medium with ampicillin (100  $\mu$ g/ml) and glucose (2% w/v) at 37 °C until the OD<sub>600</sub> of the culture medium reached 0.4. Each culture was centrifuged, the supernatants were removed, and fresh 2-YT media with ampicillin (100  $\mu$ g/ml) was added to each *E. coli* pellet. After incubation for 6 h at 37 °C supernatants were collected and used to determine affinity for TNFRs by capture ELISA as described previously [24]. After the procedure, the phagemid vectors were sequenced using a Big Dye Terminator v3.1 kit and ABI PRISM 3100 (Applied Biosystems Ltd., Pleasanton, CA).

#### 2.5. Expression and purification of TNF mutants

Preparation of purified recombinant protein was described previously [25]. In brief, TNF mutants recombined into pYas1 vector, under the control of T7 promoter, were produced in *E. coli* (BL21 $\lambda$ DE3). Mutant TNFs recovered from inclusion body, which were washed in Triton X-100 and solubilized in 6 M guanidine-HCl, 0.1 M Tris-HCl, pH 8.0, and 2 mM EDTA. Solubilized protein was adjusted to 10 mg/ml and was reduced with 10 mg/ml dithioerythritol for 4 h at RT and refolded by 100-fold dilution in a refolding buffer (100 mM Tris-HCl, 2 mM EDTA, 1 M arginine, and oxidized glutathione (551 mg/L)). After dialysis with 20 mM Tris-HCl, pH 7.4, containing 100 mM urea, active trimeric proteins were purified by Q-Sepharose (GE Healthcare) chromatography and size-exclusion chromatography (Superose 12; GE Healthcare).

#### 2.6. Analysis of binding kinetics using surface plasmon resonance (SPR)

The binding kinetics of the wtTNF and TNF mutants were analyzed by the SPR technique (BIAcore 2000; GE Healthcare). TNFR1-Fc or TNFR2-Fc were separately

immobilized on to CM5 sensor chip, resulting in an increase of 3000–3500 RU. During the association phase, wtTNF or TNF mutants diluted in running buffer (HBS-EP) at 156.8, 52.3, 17.4, 5.8 or 1.9 nM were passed over the immobilized TNFR2 for 2 min at a flow rate of 20  $\mu$ l/min. During the dissociation phase, HBS-EP was run over the sensor chip for 1 min at a flow rate of 20  $\mu$ l/min. The SPR measurements for TNFR1 were performed using much higher concentrations of TNF mutants (392.1, 130.7, 43.6, 14.5 or 4.8 nM). The data were analyzed globally with BIAevaluation 3.1 software (GE Healthcare) to apply a 1:1 Langmuir binding model. The obtained sensorgrams were fitted globally over the range of injected concentrations and simultaneously over the association and dissociation phases.

#### 2.7. In vitro assessment of bioactivity via TNFR1 or TNFR2 with TNF mutants

HEp-2 cells were seeded at  $4 \times 10^4$  cells/well in 96-well plates and incubated for 18 h with serially diluted wtTNF (Peprotech, Rocky Hill, NJ) or TNF mutants in the presence of 50 mg/ml cycloheximide. After incubation, cell survival was determined by methylene blue assay as described previously [25]. In the case of analyzing TNFR2-mediated biological activity, hTNFR2/mFas-PA were seeded on 96-well micro titer plates with a density of  $1.5 \times 10^4$  cells/well in culture medium. Serial dilutions of wtTNF (Peprotech) and TNF mutants were prepared with 1  $\mu$ g/ml cycloheximide and added to each well. After 48 h-incubation at 37 °C, the cell viabilities were analyzed using a WST-8 assay kit (Nacalai Tesque) according to the manufacturer's instructions.

### 3. Results

#### 3.1. Optimization of one-step competitive panning protocol

To improve identifying TNFR2-selective TNF mutants with better bioactivity, we have introduced a step to remove the TNFR1-binding phages from the library by competitive panning using TNFR1-Fc. We postulated that TNFR1-binding clones could be eliminated when panning for the TNFR2-binding clones is performed in the presence of TNFR1 protein (see Fig. 1). Although an immunoplate or immunotube is commonly used for the panning [27–29], these techniques cannot make real-time observation of the interaction between phage library and receptor, and are difficult to automate and control the precise settings. Therefore, we first utilized the BIAcore biosensor and optimized the concentration of TNFR1-Fc required for eliminating the TNFR1-binding clones. Serially diluted human TNFR1-Fc was mixed with  $1 \times 10^{10}$  CFU phages displaying wtTNF, and the binding avidity of the phage-displayed wtTNF for TNFR2 was assessed using a BIAcore biosensor. As shown in Fig. 2, TNFR1-Fc inhibited the binding of phage-displayed wtTNF to TNFR2 in a dose-dependent manner. 10 pmol of TNFR1-Fc virtually abolished the binding of wtTNF not only to TNFR2 (last panel in Fig. 2) but also the binding of wtTNF to TNFR1 (data not shown). These results clearly suggest that 10 pmol of TNFR1-Fc would be sufficient for competitively subtract unwanted TNFR1-binding phage clones from a phage library displaying structural TNF mutants.

**Table 2**  
Binding kinetics of TNFs to TNFR1 and TNFR2.

	TNFR1				TNFR2			
	$k_{on}^a$ ( $10^6$ M <sup>-1</sup> s <sup>-1</sup> )	$k_{off}^b$ ( $10^{-4}$ s <sup>-1</sup> )	$K_D^c$ ( $10^{-10}$ M)	Relative <sup>d</sup> (%)	$k_{on}^a$ ( $10^6$ M <sup>-1</sup> s <sup>-1</sup> )	$k_{off}^b$ ( $10^{-4}$ s <sup>-1</sup> )	$K_D^c$ ( $10^{-10}$ M)	Relative <sup>d</sup> (%)
wtTNF	0.45	1.3	2.9	100.0	2.0	12.1	6.1	100.0
R2-6	0.79	54.5	68.8	4.2	3.2	7.8	2.4	251.4
R2-7	0.44	116.0	262.0	1.1	2.1	7.4	3.6	169.7
R2-8	1.22	50.3	41.1	7.1	3.1	6.6	2.1	291.0
R2-9	1.19	50.1	42.3	6.9	3.8	12.6	3.3	185.2
R2-10	0.67	43.9	63.7	4.6	2.2	5.3	2.4	253.5
R2-11	0.81	87.5	108.	2.7	2.3	5.4	2.3	264.5
R2-12	1.36	98.8	72.6	4.0	4.1	10.6	2.6	235.0
R2-13	0.97	104.0	107.0	2.7	2.9	8.2	2.9	212.2

Kinetic parameters for each TNF were calculated from the respective sensorgram by BIAevaluation 3.1 software, and taking into consideration that the TNF binds as a trimer.

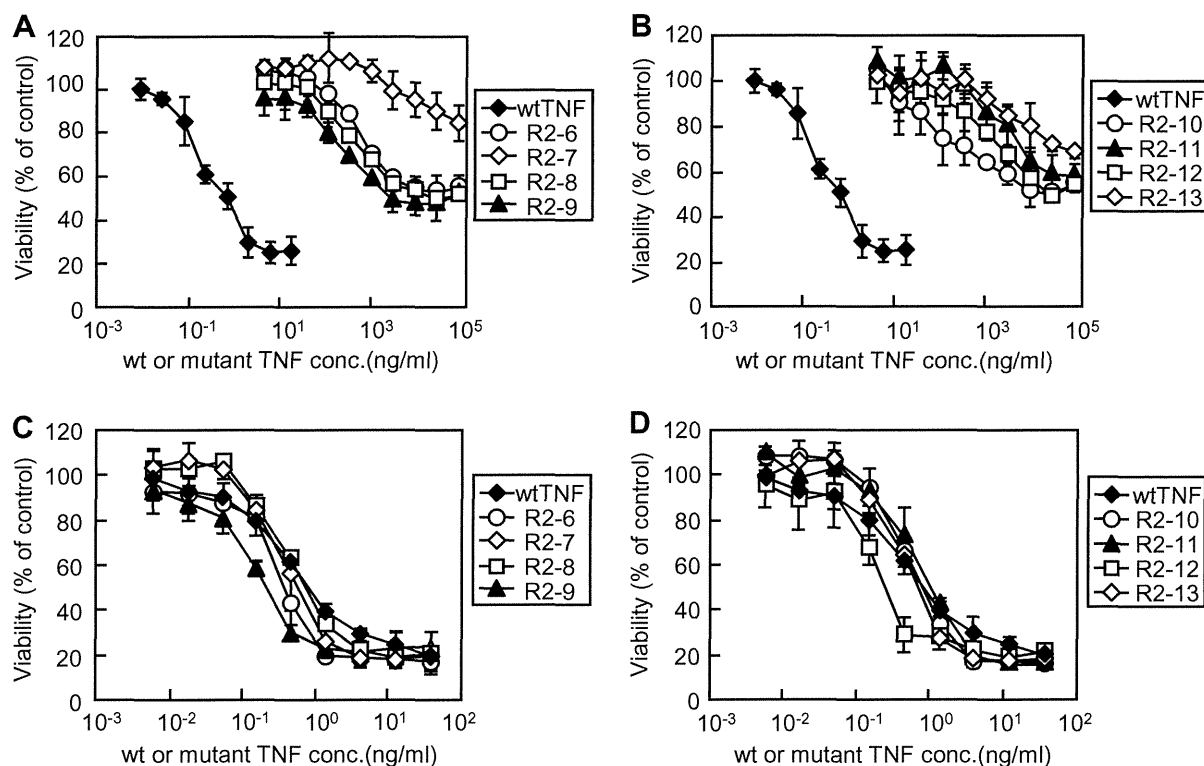
<sup>a</sup>  $k_{on}$  is the association kinetic constant.

<sup>b</sup>  $k_{off}$  is the dissociation kinetic constant.

<sup>c</sup>  $K_D$  is the equilibrium dissociation constant ( $K_D = k_{off}/k_{on}$ ).

<sup>d</sup> Relative values were calculated from the  $K_D$  (wtTNF)/ $K_D$  (TNF mutants)  $\times$  100.





**Fig. 4.** In vitro bioactivity assay of TNF mutants via TNFR1 or TNFR2. The bioactivity of mutant TNFs via TNFR1 or TNFR2 were measured by cytotoxicity assay against HEP-2 cells (A and B) or hTNFR2/mFas-PA (C and D), respectively. Each point represents the mean  $\pm$  S.D. of triplicate measurements.

**3.2. Selection of TNFR2-selective TNF mutants by one-step competitive panning**

To concentrate TNFR2-selective mutant TNFs, the TNF structural mutant displaying phage library was subjected to two rounds of conventional panning or competitive panning against TNFR2 using the BIAcore biosensor. After the second round of panning, *Escherichia coli* (TG1) supernatants of 54 randomly picked clones from each panning procedure were further screened by capture ELISA to analyze their binding specificities for each TNFR (Fig. 3). Consequently, we obtained numerous clones with high-affinity for TNFR2 under all panning conditions. Binding avidities of these clones for TNFR1 tended to decrease depending on the concentration of TNFR1-Fc used for premixing.

However, binding avidity of a TNFR2-selective clone, which binds only to TNFR2 (Fig. 3, black bar), tended to increase depending on the concentration of TNFR1-Fc used for premixing. Almost all clones obtained from the conventional and competitive panning with 0.1 pmol of TNFR1-Fc (Fig. 3A and B, respectively) bound to TNFR1, and the panning efficiency for isolating the TNFR2-selective TNF mutants was <2%. In contrast, clones obtained from the subtracted panning with 1 or 10 pmol of TNFR1-Fc (Fig. 3C and D, respectively) contained many TNFR2-selective TNF mutants (>20%). From these panned clones, we eventually identified eight candidate agonists that selectively and strongly bound to the TNFR2. Amino acid sequences of these eight candidate TNFR2-selective TNF mutants are shown in Table 1. TNFR2-selective mutants were mutated near residue 145 and

**Table 3**  
In vitro bioactivities of TNF mutants via TNFR1 or TNFR2.

	TNFR1 <sup>a</sup>		TNFR2 <sup>b</sup>		TNFR2/TNFR1 <sup>c</sup>
	EC50 <sup>e</sup> (ng/ml)	Relative Activity <sup>d</sup> (%)	EC50 <sup>e</sup> (ng/ml)	Relative activity <sup>d</sup> (%)	
wtTNF	0.6	100	0.56	100	1.0
R2-6	$8.1 \times 10^3$	$7.3 \times 10^{-3}$	0.39	144	$2.0 \times 10^4$
R2-7	$>1.0 \times 10^5$	$<6.0 \times 10^{-4}$	0.51	110	$1.8 \times 10^5$
R2-8	$4.6 \times 10^3$	$1.2 \times 10^{-2}$	0.67	84	$7.0 \times 10^3$
R2-9	$2.1 \times 10^3$	$2.8 \times 10^{-2}$	0.21	267	$9.5 \times 10^3$
R2-10	$1.1 \times 10^4$	$5.4 \times 10^{-3}$	0.72	78	$1.4 \times 10^4$
R2-11	$6.7 \times 10^4$	$8.9 \times 10^{-4}$	0.95	59	$6.6 \times 10^4$
R2-12	$2.6 \times 10^4$	$2.2 \times 10^{-3}$	0.23	243	$1.1 \times 10^5$
R2-13	$>1.0 \times 10^5$	$<6.0 \times 10^{-4}$	0.63	89	$1.5 \times 10^5$

<sup>a</sup> Bioactivities of the wtTNF and TNF mutants via TNFR1 were measured by determining the TNF-induced cytotoxicity in HEP-2 cells.  
<sup>b</sup> Bioactivities of the wtTNF and TNF mutants via TNFR2 were measured by determining the TNF-induced cytotoxicity in hTNFR2/mFas-PA.  
<sup>c</sup> Experimental data were analyzed by a logistic regression model to calculate the mean effective concentration (EC50).  
<sup>d</sup> Relative activities were calculated from the EC50 (wtTNF)/EC50 (TNF mutants).  
<sup>e</sup> Selectivity for TNFR2 was calculated from the ratio of the relative activity (via TNFR2)/relative activity (via TNFR1).

conserved near residue 30. These findings indicate that the amino acid residues near position 30 are an essential for TNFR2 binding.

### 3.3. Binding kinetics of TNFR2-selective TNF mutants

To investigate the properties of eight TNFR2-selective TNF mutants in detail, we prepared recombinant protein using the previously described methods [30,31]. TNF mutants expressed as an inclusion body in *E. coli* (BL21λDE3) were denatured and refolded. Then, active TNF mutants were purified by ion-exchange and gel-filtration chromatography. TNF mutant purity was greater than 90% in sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and all mutants were confirmed to form homotrimers in the same manner as the wtTNF by gel-filtration analysis (data not shown). To analyze the binding properties of these TNFR2-selective TNF mutants, we determined their binding dissociation constants (kinetic on- and off-rates) for TNFR1 and TNFR2, respectively, in detail using the surface plasmon resonance technique (Table 2). Our analysis showed that all eight mutant TNFs bound to the TNFR2 with high affinity; in contrast, they bound to the TNFR1 with greatly reduced affinity (typically between 1 and 7% of the wtTNF affinity). The dissociation constants ( $K_D$ ) of these mutants for TNFR2 were between  $2.1\text{--}3.6 \times 10^{-10}$  M, and their relative affinities for TNFR2 were between 169 and 291% of that of the wtTNF. Thus, using the competitive panning technique we successfully obtained a large repertoire of TNFR2-selective TNF mutants with different binding parameters (on- and off-rates and dissociation constants).

### 3.4. Bioactivities of TNFR2-selective TNF mutants

To examine the bioactivity of these TNF mutants via TNFR1, we subsequently performed a cytotoxicity assay using HEp-2 cells (Fig. 4A and B). All TNF mutants (R2-6 ~ R2-13) showed almost no cytotoxicity, and the bioactivity was much lower than that of the wtTNF. Next, we evaluated the TNFR2-mediated activity of TNF mutants using the hTNFR2/mFas-PA, which were previously constructed in our laboratory [26]. The TNFR2-mediated bioactivities of these 8 mutant TNF proteins were at least same or higher than that of the wtTNF (Fig. 4C and D). As a negative control, we determined TNF cytotoxicity in parental TNFR1<sup>-/-</sup>R2<sup>-/-</sup> preadipocytes and observed no wtTNF- or mutant TNF-mediated cell death (data not shown). Results of the cytotoxicity assay are summarized in Table 3. R2-7, the most highly TNFR2-selective mutant, exhibited  $1.8 \times 10^5$  fold higher TNFR2-selectivity than that for the wild-type TNF.

## 4. Discussion

Recently, it was revealed that the two TNFRs worked together by crosstalk signaling, which suggested that the TNF-mediated signaling in the presence of both TNF receptors actually correlates with their physiological functions [32–34]. To understand the mechanism as well as to analyze the structure–function relationship of the TNFRs, several attempts were made in the past to create TNFR-specific mutant TNFs by conventional site-directed mutagenesis methods (such as Kunkel's method) [35–37]. However, these attempts were not very successful in yielding a desired TNF mutant having high receptor specificity and full bioactivity. For example, the TNFR2-binding affinity of the double mutant D143N-A145R was about 5–10 fold less than the wtTNF [38]. To overcome these problems, we applied phage-display technique and optimized panning method using the BIAcore biosensor (Fig. 1). Using an adequate amount of selective competitive inhibitor (>1 pmol TNFR1-Fc), this one-step competitive panning is ten times more efficient for screening TNFR2-selective TNF mutants, suggesting the competitive panning technology described here is a simple and effective screening method for fine-tuning TNF receptor-selectivity (Fig. 3). As a result of

screening, we obtained successfully obtained TNFR2-selective TNF mutants with full bioactivity via TNFR2 (Table 3). Because of its high TNFR2-selectivity and full bioactivity, the TNF mutant R2-7 would help in elucidating the functional role of TNFR2.

One advantage of our phage-display-based technique is that it can be used to obtain the sequence information of many mutants [39,40]. It was previously shown by site-specific mutagenesis technique that mutations at positions 29, 31 and 32 (L29S, R31E and R32W) remarkably reduced the TNF's affinity for binding to TNFR2 [35,37,38]. For most of the TNFR2-selective TNF mutants, amino acids at positions 29, 31 and 32 were indeed identical (except for the R2-7 mutant which contained a conserved L to V substitution at position 29) to those of the wtTNF (Table 1), which is consistent with the previously reported idea that these three amino acids play critical roles in maintaining the binding between the TNF and TNFR2. The amino acid sequence at positions 145, 146 and 147 of the TNFR2-selective TNF mutants were, however, very different from those of the wtTNF. For example, the amino acid residue at position 145 of the TNF mutants R2-7, R2-12 and R2-13 contained an Asp residue in place of the Ala residue, and all of them showed high TNFR2 selectivity. Structural analysis and mutagenesis studies suggested that the loop containing the residues 145–147 is involved in the receptor binding [41–43]. Since Asp is a comparatively large residue, we speculated that this substitution could lead to a steric hindrance disrupting the interaction between the TNFR1 and TNFR2-selective mutants, which may be why they are less TNFR1-selective. However, why this replacement would increase the selectivity for TNFR2 is unclear at this moment. Currently, we are working on determining the structure of the TNF/TNFR2 complex by X-ray crystallography [44] so that structure–activity relationship studies could be initiated in the near future. Additionally, this structural information, in combination with bioinformatics technology, will be useful for designing TNFR2-selective inhibitors (peptide mimics and chemical compounds).

## 5. Conclusions

In this study, we optimized our phage display-based screening using a unique competitive panning technique, which is ten times more efficient for screening TNFR2-selective TNF mutants compared to the conventional panning method. As a result of screening, we have succeeded in isolating several TNFR2-specific TNF mutants with high TNFR2 affinity and full bioactivity via TNFR2. Further analysis of the relationship between the structure and bioactivity of the TNF mutants would offer highly valuable and useful information regarding the TNF/TNFR biology. In conclusion, our fine-tuned competitive panning system is a simple and effective technology for isolating receptor-selective mutant proteins.

## Acknowledgment

This study was supported in part by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan, and from the Japan Society for the Promotion of Science (JSPS). This study was also supported in part by Health Labour Sciences Research Grants from the Ministry of Health, Labor and Welfare of Japan, and by Health Sciences Research Grants for Research on Publicly Essential Drugs and Medical Devices from the Japan Health Sciences Foundation.

## References

- [1] Aggarwal BB. Signalling pathways of the TNF superfamily: a double-edged sword. *Nat Rev Immunol* 2003;3(9):745–56.
- [2] Szlosarek PW, Balkwill FR. Tumour necrosis factor (alpha): a potential target for the therapy of solid tumours. *Lancet Oncol* 2003;4(9):565–73.

[3] Aderka D, Engelmann H, Maor Y, Brakebusch C, Wallach D. Stabilization of the bioactivity of tumor necrosis factor by its soluble receptors. *J Exp Med* 1992; 175(2):323–9.

[4] Feldmann M, Maini RN. Lasker Clinical Medical Research Award. TNF defined as a therapeutic target for rheumatoid arthritis and other autoimmune diseases. *Nat Med* 2003;9(10):1245–50.

[5] Muto Y, Nouri-Aria KT, Meager A, Alexander GJ, Eddleston AL, Williams R. Enhanced tumour necrosis factor and interleukin-1 in fulminant hepatic failure. *Lancet* 1988;2(8602):72–4.

[6] Thorbecke GJ, Shah R, Leu CH, Kuruvilla AP, Hardison AM, Palladino MA. Involvement of endogenous tumor necrosis factor alpha and transforming growth factor beta during induction of collagen type II arthritis in mice. *Proc Natl Acad Sci U S A* 1992;89(16):7375–9.

[7] Williams RO, Feldmann M, Maini RN. Anti-tumor necrosis factor ameliorates joint disease in murine collagen-induced arthritis. *Proc Natl Acad Sci U S A* 1992;89(20):9784–8.

[8] Gomez-Reino JJ, Carmona L, Valverde VR, Mola EM, Montero MD. Treatment of rheumatoid arthritis with tumor necrosis factor inhibitors may predispose to significant increase in tuberculosis risk: a multicenter active-surveillance report. *Arthritis Rheum* 2003;48(8):2122–7.

[9] Lubel JS, Testro AG, Angus PW. Hepatitis B virus reactivation following immunosuppressive therapy: guidelines for prevention and management. *Intern Med J* 2007;37(10):705–12.

[10] Leist M, Gantner F, Jilg S, Wendel A. Activation of the 55 kDa TNF receptor is necessary and sufficient for TNF-induced liver failure, hepatocyte apoptosis, and nitrite release. *J Immunol* 1995;154(3):1307–16.

[11] Mori L, Iselin S, De Libero G, Lesslauer W. Attenuation of collagen-induced arthritis in 55-kDa TNF receptor type 1 (TNFR1)-IgG1-treated and TNFR1-deficient mice. *J Immunol* 1996;157(7):3178–82.

[12] Ware CF, Crowe PD, Vanarsdale TL, Andrews JL, Grayson MH, Jerzy R, et al. Tumor necrosis factor (TNF) receptor expression in T lymphocytes. Differential regulation of the type I TNF receptor during activation of resting and effector T cells. *J Immunol* 1991;147(12):4229–38.

[13] Irwin MW, Mak S, Mann DL, Qu R, Penninger JM, Yan A, et al. Tissue expression and immunolocalization of tumor necrosis factor-alpha in post-infarction dysfunctional myocardium. *Circulation* 1999;99(11):1492–8.

[14] Dopp JM, Sarafian TA, Spinella FM, Kahn MA, Shau H, de Vellis J. Expression of the p75 TNF receptor is linked to TNF-induced NFkappaB translocation and oxyradical neutralization in glial cells. *Neurochem Res* 2002;27(11):1535–42.

[15] Yang L, Lindholm K, Konishi Y, Li R, Shen Y. Target depletion of distinct tumor necrosis factor receptor subtypes reveals hippocampal neuron death and survival through different signal transduction pathways. *J Neurosci* 2002; 22(8):3025–32.

[16] Ban L, Zhang J, Wang L, Kuhlreiber W, Burger D, Faustman DL. Selective death of autoreactive T cells in human diabetes by TNF or TNF receptor 2 agonism. *Proc Natl Acad Sci U S A* 2008;105(36):13644–9.

[17] Monden Y, Kubota T, Inoue T, Tsutsumi T, Kawano S, Ide T, et al. Tumor necrosis factor-alpha is toxic via receptor 1 and protective via receptor 2 in a murine model of myocardial infarction. *Am J Physiol Heart Circ Physiol* 2007;293(1):H743–53.

[18] Wang M, Crisostomo PR, Markel TA, Wang Y, Meldrum DR. Mechanisms of sex differences in TNFR2-mediated cardioprotection. *Circulation* 2008;118(Suppl. 14):S38–45.

[19] Arnett HA, Mason J, Marino M, Suzuki K, Matsushima GK, Ting JP. TNF alpha promotes proliferation of oligodendrocyte progenitors and remyelination. *Nat Neurosci* 2001;4(11):1116–22.

[20] Faustman D, Davis M. TNF receptor 2 p.thway: drug target for autoimmune diseases. *Nat Rev Drug Discov* 2010;9(6):482–93.

[21] Fontaine V, Mohand-Said S, Hanoteau N, Fuchs C, Pfizenmaier K, Eisel U. Neurodegenerative and neuroprotective effects of tumor Necrosis factor (TNF) in retinal ischemia: opposite roles of TNF receptor 1 and TNF receptor 2. *J Neurosci* 2002;22(7). RC216.

[22] MacEwan DJ. TNF receptor subtype signalling: differences and cellular consequences. *Cell Signal* 2002;14(6):477–92.

[23] Mukai Y, Shibata H, Nakamura T, Yoshioka Y, Abe Y, Nomura T, et al. Structure-function relationship of tumor necrosis factor (TNF) and its receptor interaction based on 3D structural analysis of a fully active TNFR1-selective TNF mutant. *J Mol Biol* 2009;385(4):1221–9.

[24] Shibata H, Yoshioka Y, Ohkawa A, Minowa K, Mukai Y, Abe Y, et al. Creation and X-ray structure analysis of the tumor necrosis factor receptor-1-selective mutant of a tumor necrosis factor-alpha antagonist. *J Biol Chem* 2008;283(2):998–1007.

[25] Yamamoto Y, Tsutsumi Y, Yoshioka Y, Nishibata T, Kobayashi K, Okamoto T, et al. Site-specific PEGylation of a lysine-deficient TNF-alpha with full bioactivity. *Nat Biotechnol* 2003;21(5):546–52.

[26] Abe Y, Yoshioka Y, Kamada H, Shibata H, Nomura T, Minowa K, et al. Simple and highly sensitive assay system for TNFR2-mediated soluble- and transmembrane-TNF activity. *J Immunol Methods* 2008;335(1–2):71–8.

[27] Schwarz M, Rottgen P, Takada Y, Le Gall F, Knackmuss S, Bassler N, et al. Single-chain antibodies for the conformation-specific blockade of activated platelet integrin alphaIIb beta3 designed by subtractive selection from naive human phage libraries. *Faseb J* 2004;18(14):1704–6.

[28] Popkov M, Rader C, Barbas 3rd CF. Isolation of human prostate cancer cell reactive antibodies using phage display technology. *J Immunol Methods* 2004; 291(1–2):137–51.

[29] Eisenhardt SU, Schwarz M, Bassler N, Peter K. Subtractive single-chain antibody (scFv) phage-display: tailoring phage-display for high specificity against function-specific conformations of cell membrane molecules. *Nat Protoc* 2007;2(12):3063–73.

[30] Shibata H, Yoshioka Y, Abe Y, Ohkawa A, Nomura T, Minowa K, et al. The treatment of established murine collagen-induced arthritis with a TNFR1-selective antagonistic mutant TNF. *Biomaterials* 2009;30(34):6638–47.

[31] Shibata H, Yoshioka Y, Ikemizu S, Kobayashi K, Yamamoto Y, Mukai Y, et al. Functionalization of tumor necrosis factor-alpha using phage display technique and PEGylation improves its antitumor therapeutic window. *Clin Cancer Res* 2004;10(24):8293–300.

[32] Wajant H, Pfizenmaier K, Scheurich P. Tumor necrosis factor signaling. *Cell Death Differ* 2003;10(1):45–65.

[33] Weiss T, Grell M, Siemienski K, Muhlenbeck F, Durkop H, Pfizenmaier K, et al. TNFR80-dependent enhancement of TNFR60-induced cell death is mediated by TNFR-associated factor 2 and is specific for TNFR60. *J Immunol* 1998; 161(6):3136–42.

[34] Fotin-Mlecsek M, Henkler F, Samel D, Reichwein M, Hauser A, Parmryd I, et al. Apoptotic crosstalk of TNF receptors: TNF-R2 induces depletion of TRAF2 and IAP proteins and accelerates TNF-R1-dependent activation of caspase-8. *J Cell Sci* 2002;115(Pt. 13):2757–70.

[35] Yamagishi J, Kawashima H, Matsuo N, Ohue M, Yamayoshi M, Fukui T, et al. Mutational analysis of structure-activity relationships in human tumor necrosis factor-alpha. *Protein Eng* 1990;3(8):713–9.

[36] Barbara JA, Smith WB, Gamble JR, Van Ostade X, Vandenebee P, Tavernier J, et al. Dissociation of TNF-alpha cytotoxic and proinflammatory activities by p55 receptor- and p75 receptor-selective TNF-alpha mutants. *Embo J* 1994; 13(4):843–50.

[37] Van Ostade X, Vandenebee P, Everaerd B, Loetscher H, Gentz R, Brockhaus M, et al. Human TNF mutants with selective activity on the p55 receptor. *Nature* 1993;361(6409):266–9.

[38] Loetscher H, Stueber D, Banner D, Mackay F, Lesslauer W. Human tumor necrosis factor alpha (TNF alpha) mutants with exclusive specificity for the 55-kDa or 75-kDa TNF receptors. *J Biol Chem* 1993;268(35):26350–7.

[39] Abe Y, Nomura T, Yoshioka Y, Kamada H, Tsunoda S, Tsutsumi Y. Anti-inflammatory effects of a novel TNFR1-selective antagonistic TNF mutant on established murine collagen-induced arthritis. *Adv Exp Med Biol* 2011;691:493–500.

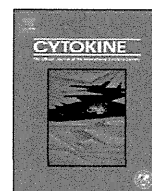
[40] Yoshioka Y, Watanabe H, Morishige T, Yao X, Ikemizu S, Nagao C, et al. Creation of lysine-deficient mutant lymphotoxin-alpha with receptor selectivity by using a phage display system. *Biomaterials* 2010;31(7):1935–43.

[41] Eck MJ, Sprang SR. The structure of tumor necrosis factor-alpha at 2.6 Å resolution. Implications for receptor binding. *J Biol Chem* 1989;264(29):17595–605.

[42] Van Ostade X, Tavernier J, Fiers W. Structure-activity studies of human tumour necrosis factors. *Protein Eng* 1994;7(1):5–22.

[43] Idriss HT, Naismith JH. TNF alpha and the TNF receptor superfamily: structure-function relationship(s). *Microsc Res Tech* 2000;50(3):184–95.

[44] Mukai Y, Nakamura T, Yoshioka Y, Yoshioka Y, Tsunoda S, Nakagawa S, et al. Solution of the structure of the TNF-TNFR2 complex. *Sci Signal* 2010;3(148). ra83.



## Lysine-deficient lymphotoxin- $\alpha$ mutant for site-specific PEGylation

Shogo Narimatsu<sup>a,1</sup>, Yasuo Yoshioka<sup>b,c,\*</sup>, Hikaru Watanabe<sup>a</sup>, Takashi Masano<sup>a</sup>, Tomohiro Morishige<sup>a</sup>, Xinglei Yao<sup>a,d</sup>, Aya Tanabe<sup>a</sup>, Shin-ichi Tsunoda<sup>c,e</sup>, Yasuo Tsutsumi<sup>b,c,f</sup>, Yohei Mukai<sup>a</sup>, Naoki Okada<sup>a</sup>, Shinsaku Nakagawa<sup>a,b,\*</sup>

<sup>a</sup> Laboratory of Biotechnology and Therapeutics, Graduate School of Pharmaceutical Sciences, Osaka University, 1-6 Yamadaoka, Suita, Osaka 565-0871, Japan

<sup>b</sup> The Center for Advanced Medical Engineering and Informatics, Osaka University, 1-6 Yamadaoka, Suita, Osaka 565-0871, Japan

<sup>c</sup> Laboratory of Biopharmaceutical Research, National Institute of Biomedical Innovation, 7-6-8 Saito-Asagi, Ibaraki, Osaka 567-0085, Japan

<sup>d</sup> Institute of Pharmaceutics, Zhejiang University, 388 Yuhangtang Road, Hangzhou 310058, China

<sup>e</sup> Laboratory of Biomedical Innovation, Graduate School of Pharmaceutical Sciences, Osaka University, 7-6-8 Saito-Asagi, Ibaraki, Osaka 567-0085, Japan

<sup>f</sup> Laboratory of Toxicology and Safety Science, Graduate School of Pharmaceutical Sciences, Osaka University, 1-6 Yamadaoka, Suita, Osaka 565-0871, Japan

### ARTICLE INFO

#### Article history:

Received 7 May 2011

Received in revised form 27 July 2011

Accepted 3 August 2011

Available online 25 August 2011

#### Keywords:

Bioconjugation

Cancer immunotherapy

Cytokine

Bioactivity

Protein engineering

### ABSTRACT

The cytokine lymphotoxin- $\alpha$  (LT $\alpha$ ) is a promising anticancer agent; however, its instability currently limits its therapeutic potential. Modification of proteins with polyethylene glycol (PEGylation) can improve their *in vivo* stability, but PEGylation occurs randomly at lysine residues and the N-terminus. Therefore, PEGylated proteins are generally heterogeneous and have lower bioactivity than their non-PEGylated counterparts. Previously, we created phage libraries expressing mutant LT $\alpha$ s in which the lysine residues of wild-type LT $\alpha$  (wtLT $\alpha$ ) were substituted for other amino acids. Here, we attempted to create a lysine-deficient mutant LT $\alpha$  with about the same bioactivity as wtLT $\alpha$  by using these libraries and site-specific PEGylation of the N-terminus. We isolated a lysine-deficient mutant LT $\alpha$ , LT-KO, with almost identical bioactivity to that of wtLT $\alpha$  against mouse LM cells. The bioactivity of wtLT $\alpha$  decreased to 10% following random PEGylation, whereas that of LT-KO decreased to 50% following site-specific PEGylation; PEGylated LT-KO retained five times the bioactivity of randomly PEGylated wtLT $\alpha$ . These results suggest that site-specific PEGylated LT-KO may be useful in cancer therapy.

© 2011 Elsevier Ltd. All rights reserved.

### 1. Introduction

The cytokine lymphotoxin- $\alpha$  (LT $\alpha$ ) is well known for its tumor cell-specific cytotoxicity [1–3]. It also has roles in various immune responses, such as inducing chemokine production from the blood vessel endothelium or stimulating the activation of adhesion molecules [4–6]. Thus, LT $\alpha$  has potential applications in cancer therapy. More recently, studies in LT $\alpha$  knockout mice have revealed that LT $\alpha$  is a master cytokine of lymph histogenesis, suggesting that the underlying mechanism of this cytokine's anti-tumor activity may be novel [7,8]. Schrama et al. showed that systemic administration of LT $\alpha$  to a tumor-bearing mouse leads to the construction of ectopic lymphoid tissue within the tumor and to the strong induction of

tumor immunity in that lymphoid tissue [9,10]. Further detailed studies are necessary, but the concept that LT $\alpha$  builds ectopic lymphoid tissue in a tumor and effectively induces tumor immunity is a promising breakthrough for cancer immune-therapy. To exert its various biological functions, LT $\alpha$  binds to three receptor subtypes: tumor necrosis factor receptor 1 (TNFR1), TNFR2, and herpes virus entry mediator (HVEM). TNFR1 induces an anti-tumor effect and Peyer's patch development, whereas TNFR2 is essential for immune responses to bacteria and viruses [11]. HVEM has some immunomodulating activity, but details are scarce [12]. LT $\alpha$  has long been considered a promising anti-cancer agent, and LT $\alpha$  mutants are under clinical study in some countries [9,13,14]. However, the clinical use of LT $\alpha$  has been limited because of its very low stability and pleiotropic action *in vivo*.

One way to enhance the stability of proteins is to conjugate them to polyethylene glycol (PEG) [15,16]. PEGylation of proteins increases their molecular size, enhances steric hindrance, and improves plasma half-life. The prolonged circulating lifetime in the blood induces the enhanced permeability and retention (EPR) effect, which is derived from the leaky nature of tumor blood vessels and results in increased delivery of conjugates to tumor tissue [17]. In fact, our group previously showed that optimal PEGylation of

\* Corresponding authors. Addresses: The Center for Advanced Medical Engineering and Informatics, Osaka University, 1-6 Yamadaoka, Suita, Osaka 565-0871, Japan. Tel./fax: +81 6 6879 8233 (Y. Yoshioka); Department of Biotechnology and Therapeutics, Graduate School of Pharmaceutical Sciences, Osaka University, 1-6 Yamadaoka, Suita, Osaka 565-0871, Japan. Tel.: +81 6 6879 8175; fax: +81 6 6879 8179 (S. Nakagawa).

E-mail addresses: [yasuo@phs.osaka-u.ac.jp](mailto:yasuo@phs.osaka-u.ac.jp) (Y. Yoshioka), [nakagawa@phs.osaka-u.ac.jp](mailto:nakagawa@phs.osaka-u.ac.jp) (S. Nakagawa).

<sup>1</sup> Each author contributed equally to the work.