Bioconjugate Chemistry

Article

pubs.acs.org/bc

Sensitive Multiplexed Quantitative Analysis of Autoantibodies to Cancer Antigens with Chemically *S*-Cationized Full-Length and Water-Soluble Denatured Proteins

Junichiro Futami,[*,†,#] Hidenori Nonomura,[†,#] Momoko Kido,[†] Naomi Niidoi,[†] Nao Fujieda,[‡,§] Akihiro Hosoi,[‡,§] Kana Fujita,[†] Komako Mandai,[†] Yuki Atago,[†] Rie Kinoshita,[†] Tomoko Honjo,[†] Hirokazu Matsushita,[§] Akiko Uenaka,[∥] Eiichi Nakayama,[∥] and Kazuhiro Kakimi[§]

[†]Department of Medical Bioengineering, Graduate School of Natural Science and Technology, Okayama University, Okayama 700-8530, Japan

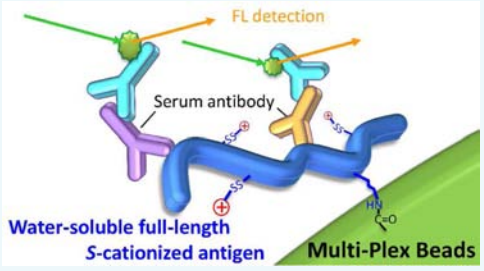
[‡]Medinet Co. Ltd., Yokohama, Kanagawa 222-0033, Japan

[§]Department of Immunotherapeutics, The University of Tokyo Hospital, Tokyo 113-8655, Japan

[∥]Faculty of Health and Welfare, Kawasaki University of Medical Welfare, Kurashiki, Okayama 701-0193, Japan

S *Supporting Information*

ABSTRACT: Humoral immune responses against tumor-associated antigens (TAAs) or cancer/testis antigens (CTAs) aberrantly expressed in tumor cells are frequently observed in cancer patients. Recent clinical studies have elucidated that anticancer immune responses with increased levels of anti-TAA/CTA antibodies improve cancer survival rates. Thus, these antibody levels are promising biomarkers for diagnosing the efficiency of cancer immunotherapy. Full-length antigens are favored for detecting anti-TAA/CTA antibodies because candidate antigen proteins contain multiple epitopes throughout their structures. In this study, we developed a methodology to prepare purified water-soluble and full-length antigens by using cysteine sulfhydryl group cationization (*S*-cationization) chemistry. *S*-Cationized antigens can be prepared from bacterial inclusion bodies, and they exhibit improved protein solubility but preserved antigenicity. Anti-TAA/CTA antibodies detected in cancer patients appeared to recognize linear epitopes, as well as conformational epitopes, and because the frequency of cysteine side-residues on the epitope−paratope interface was low, any adverse effects of *S*-cationization were virtually negligible for antibody binding. Furthermore, *S*-cationized antigen-immobilized Luminex beads could be successfully used in highly sensitive quantitative-multiplexed assays. Indeed, patients with a more broadly induced serum anti-TAA/CTA antibody level showed improved progression-free survival after immunotherapy. The comprehensive anti-TAA/CTA assay system, which uses *S*-cationized full-length and water-soluble recombinant antigens, may be a useful diagnostic tool for assessing the efficiency of cancer immunotherapy.



INTRODUCTION

In recent years, an increasing number of studies have shown that cancer patients produce antibodies against tumor-associated antigens (TAAs) that are detectable in the blood,[1−4] suggesting that these antibodies might be used as biomarkers for cancer screening and as diagnostics for cancer therapy. Within the TAA categories, the cancer/testis antigens (CTAs), a heterogeneous group of proteins typically expressed in the testis with little or no expression in most somatic tissues,[5] are now considered attractive targets for cancer immunotherapy.[6−8] In addition, immune responses to CTAs are utilized as biomarkers to predict prognosis or response to therapy.[9,10] To date, more than 150 CTAs have been identified and their expression studied in numerous cancer types; this information has been incorporated in the cancer/testis (CT) database.[11] In cancer immunotherapy, notable serum antibody responses to several TAAs or CTAs, known as antigen spreading, have been shown to correlate with improved overall survival.[3,12] Thus, quantitative and high-throughput analysis systems to assess antibodies against TAAs or CTAs are desired for cancer diagnosis. In addition to the conventional enzyme-linked immunosorbent assay (ELISA) approaches,[13,14] protein microarray[15−17] and multiplex bead techniques[18] have been applied for measurement of antibodies to TAAs or CTAs.

In order to design reliable antibody detection systems, we should take account of antigen protein properties. The functions of most CTAs, unlike those of many TAAs, are poorly understood even though their overexpression is frequently associated with advanced disease and poorer prognosis.[19] Bioinformatics approaches have helped predict

Received: June 12, 2015
Revised: September 2, 2015
Published: September 10, 2015

DOI: 10.1021/acs.bioconjchem.5b00328
Bioconjugate Chem. 2015, 26, 2076−2084

that a majority of CTAs are intrinsically disordered proteins (IDPs).[20] These IDPs, or IDP regions, lack rigid tertiary structure under physiological conditions in vitro; however, they can adopt a fixed three-dimensional structure after binding to target macromolecules. This conformational flexibility is presumed to be a functional advantage of IDPs that enables them to undergo disorder-to-order transitions upon binding to their biological target and interact with a broad range of binding partners in cells.[21] Indeed, most CTAs are predicted to occupy hub positions in protein−protein interaction networks.[20,22] Consistent with the propensity of IDPs to function in transcriptional regulation and/or cellular signaling, CTAs appeared to function by regulating protein networks to express uncontrolled cancer cellular growth.[20]

Either recombinant proteins or synthetic peptides can be used as antigens to detect serum antibodies. Traditionally, antibody epitopes in proteins are classified further into one of two categories: linear epitopes, which are segments composed of continuous polypeptide chains, or conformational epitopes, which are constituted by several sequentially discontinuous segments on the surface of structurally ordered native antigens.[23] Studies have shown that 90% of B-cell epitopes on native proteins are conformational rather than linear.[24,25] Despite this, a number of epitope analyses of anti-CTA antibodies in patient's sera have successfully identified linear epitopes using overlapping peptides,[9,10,26] probably because most CTAs have little rigid ordered conformation alone. Overlapping peptides covering the entire protein as a panel of linear epitopes were successfully utilized to evaluate the immunological potential of each individual patient.[9,10] Taken together, these findings suggest that recombinant full-length CTA proteins containing comprehensive linear epitopes are preferable antigens to detect polyclonal anti-CTA antibodies.

A physical property of disordered recombinant proteins is their tendency to aggregate during expression, purification, and storage mainly because of hydrophobic intermolecular interactions. Along with this protein aggregation, intermolecular disulfide bond formations are often observed. As most CTAs possess reactive Cys residues in their sequence, suppression of disulfide bond formation is needed to prevent aggregation.

Chemical modification of Cys residues with cationic charges through disulfide bonds or *S*-alkylation has been demonstrated as a powerful strategy to improve the water solubility and storage stability of disordered proteins.[27] For instance, reversible cationization by *S*-alkyldisulfidation with [3-(trimethylammonium)propyl] methanethiosulfonate (TAPS-sulfonate) has been successfully applied to a variety of protein purifications, especially from bacterial inclusion bodies. After purification of TAPS-cationized recombinant proteins in an aqueous buffer, TAPS-proteins can be refolded into biologically active conformations in appropriate redox buffers.[28−31] Furthermore, TAPS-cationized intracellular proteins could be internalized into living cells by adsorption-mediated endocytosis and simultaneously fold into a biologically active structure under the reducing conditions of the cytoplasm.[27,32] This in-cell folding technique can be applied to various transcription factor protein transductions in living cells. Protein cationization with irreversible *S*-alkylation with (3-bromopropyl) trimethylammonium bromide (TAP-Br) is also useful for protein chemical analysis such as peptide mapping and proteomic analysis for poorly soluble proteins.[33]

In this study, we describe a diagnostic system based on a high-throughput Luminex technique for multiplex assays[34] to detect serum antibodies by using water-soluble and full-length Cys sulfhydryl group cationized (*S*-cationized) antigens. Prior to the experiments, we were concerned about the adverse effects of antigen chemical modification on specific binding to antibodies. However, recent structural knowledge-based and in silico analyses of antigen−antibody interface properties have revealed that Cys is the least frequent amino acid residue responsible for antibody−epitope interactions.[35−37] Indeed, we have successfully demonstrated highly sensitive and specific detection of autoantibodies with *S*-cationized antigens. As *S*-cationized antigens are highly water-soluble and show little aggregation, even after long storage, these full-length polypeptides will be a valuable tool for clinical and research-based studies.

RESULTS AND DISCUSSION

Preparation of Water-Soluble *S*-Cationized Antigens. In this study, six different human recombinant full-length antigens with N-terminal His-tags were expressed in *Escherichia coli* by using the pET system. XAGE-1b (GAGED2a, CT12.1a), NY-ESO-1 (CT6.1), p53, WT-1, and gp100 were expressed as inclusion bodies. After solubilization and reduction of inclusion body proteins in 6 M guanidine hydrochloride (GdnHCl) with 30 mM dithiothreitol (DTT), Cys residues were chemically modified with TAPS-sulfonate (Figure 1A). MAGE-A4 (CT1.4) was expressed in soluble fractions and purified with an immobilized metal ion affinity chromatography (IMAC) column. A part of the purified MAGE-A4 protein was also subjected to chemical cationization with TAPS-sulfonate. All *S*-cationized proteins were successfully recovered in an aqueous phase after dialysis against pure water (Figure 1B). Furthermore, cationized proteins were purified to a single sharp peak by reversed-phase HPLC (Figure 1C). After lyophilization of the purified peak fraction, *S*-cationized proteins were solubilized with pure water (Figure 1D). Twelve more different *S*-cationized antigens (Table S1) were prepared using the same procedure.

Specific Binding of Antibody to *S*-Cationized Antigens. To develop an antibody assay system, we evaluated the effect of protein conformation and chemical modification for specific antibody−antigen interactions. As shown in Figure 2, anti-NY-ESO-1 antibodies in the serum of cancer patients who received the NY-ESO-1 vaccine were successfully detected using recombinant NY-ESO-1 protein-immobilized ELISA plates. Although the binding intensities for the TAPS-cationized NY-ESO-1 antigen were somewhat higher than those for the reduced antigen, both antigens showed distinctly different antibody binding activity between healthy subjects and cancer patients. As structurally disordered *S*-cationized antigens possess a linear epitope rather than a conformational epitope, most of the anti-NY-ESO-1 antibodies appeared to recognize linear epitopes. These results are consistent with the numbers of discovered linear epitopes for anti-CTA antibodies that were determined using overlapping peptides. Two of the identified linear epitopes of NY-ESO-1 (regions 61−78 and 163−180) possess Cys residues (Cys 75, 76, 78, and 165);[9] full-length Cys *S*-cationized NY-ESO-1 showed comparable binding to that of reduced NY-ESO-1. These results imply that the side-chain of Cys in epitopes does not exist on an epitope−paratope interface. The binding specificity of serum antibodies was also confirmed by competitive displacement with *S*-cationized excess antigen by using Western blotting (Figure S1).

DOI: 10.1021/acs.bioconjchem.5b00328
Bioconjugate Chem. 2015, 26, 2076−2084

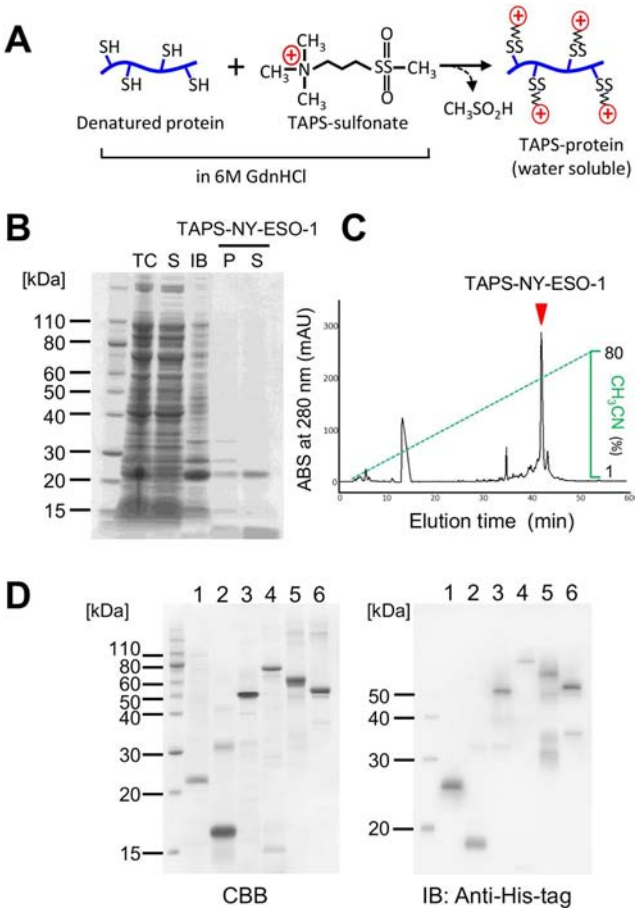


Figure 1. Preparation of *S*-cationized water-soluble antigens for antibody detection. (A) Schematic procedure to prepare TAPS-modified proteins from denatured and reduced proteins. (B) SDS-PAGE analysis along with purification steps of recombinant NY-ESO-1. Equivalent amount (100 μL culture of *E. coli*) of total cellular lysate (TC), soluble fraction (S), or inclusion body (IB) after cell lysis with sonication, and precipitate (P) and soluble (S) fractions of TAPS-modified NY-ESO-1 were loaded after dialysis against water. (C) Purification of water-soluble TAPS-modified NY-ESO-1 on reversed-phase HPLC by linear gradient elution of acetonitrile. (D) Six different *S*-cationized recombinant antigens of NY-ESO-1 (lane 1), XAGE-1b (lane 2), MAGE-A4 (lane 3), gp100 (lane 4), WT-1 (lane 5), and p53 (lane 6) were analyzed using SDS-PAGE stained with Coomassie Brilliant Blue (CBB) or Western blot analysis using anti-His-tag antibody.

Sensitive and Quantitative Detection of Autoantibodies by *S*-Cationized Antigens on Luminex Beads. Studies have shown that the numbers of anti-TAAs and anti-CTAs antibodies increase during the activation of antitumor immune responses. This "antigen spreading"[38] could be a critical biomarker of cancer therapy; thus, comprehensive antibody assay systems are preferable for the evaluation of cancer immunity. In order to develop an antigen-spreading monitoring system, the multiplexed Luminex bead assay system is an attractive platform because it gives precise results with lower sample volumes in a high-throughput format.[34] As shown in Figure 3A, serum antibodies raised in cancer patients were successfully detected with high sensitivity using *S*-cationized antigens immobilized on cross-linked beads by single-plex assays. While 1/100 to 1/1600 dilution of serum is suitable for ELISA (Figure 2), 1/400 to 1/6400 dilution is sufficient for performing the bead assay (Figure 3). Similar highly sensitive antibody detection for NY-ESO-1, XAGE-1b, MAGE-A4, gp100, WT-1, and p53 was demonstrated by six different multiplex assays with *S*-cationized antigen-immobilized beads (Figure 3B). This sensitive multiplexed antibody detection is valuable for clinical tests. According to the standard assay protocol, just 25 μL of diluted serum is required for each assay. If a 10-plex Luminex assay is designed by using ×1600 diluted serum, more than 640 antigens could theoretically be examined with only 1 μL of the patient's serum.

Analysis of Antigen Spreading on Clinical Samples. To test the potency of immunotherapy by evaluating antitumor immune responses, serum anti-CTA (NY-ESO-1, XAGE-1b, and MAGE-A4) antibodies were analyzed with *S*-cationized antigen cross-linked beads. Following autologous $\gamma\delta$ T-cell transfer therapy,[39] an increase in several antibody responses were observed in some patients (Figure 4; Pts 3, 5, and 7), but others showed virtually no response (Figure 4; Pts 1, 8, and 9). Although some samples did not show linearity with the ×400 diluted serum, reasonable signals were obtained with the ×1600 diluted serum. Therefore, we extended the panel of antigens to 16 (BORIS, DPPA2, SPEF2, HSPB9, LY6K, MAEL, MAGEA3, MAGEA4, MAGEA6, NXF2, NY-ESO-1, SSX4, XAGE2, WT1, XAGE-1b, and CEP55) and evaluated the correlation between antibody levels and clinical responses in 14 lung cancer patients. Human serum albumin (HSA) was also conjugated to the beads and used as control. Sera from 14 evaluable patients with advanced lung cancer (10 male and 4 female patients, aged 37−85 years) were examined.[39] Serum antibodies for all 16 antigens, except for the control HSA, were detected in some patients; many of them produced antibodies against multiple antigens. However, the pattern of antibody response varied across patients. As shown in Table S1, the antibody titer of the post-treatment serum was divided by that of the pretreatment sera to obtain a ratio. The average and SD values for HSA in 14 patients were 0.991 and 0.047, respectively. Therefore, we considered the antibody titer increased when the ratio was higher than 1.1 (>mean $\pm$ 2 × SD of control) and the number of antibodies whose titer at ×1600 dilution increased more than 1.1-fold were counted. The 14 patients were divided into two groups: high (n = 7) and low (n = 7) antibody responders in whom the increased antibody production was detected against more than or less than five antigens (median number of positive antigens), respectively (Table S1). As shown in Figure 5, progression-free survival (PFS) of high antibody responders was longer than that of low antibody responders (median PFS was 237 days in high responders and 120 days in low responders; p = 0.0223). Although no antibody against particular antigens was correlated with patient survival as a single agent, the patients whose antibody production increased after autologous $\gamma\delta$ T-cell transfer therapy displayed better prognosis. The prognosis was not correlated with antibodies against particular antigens. Moreover, no correlation between the affinity of antibodies toward their antigen and the patients' survival could be observed. Because the amount of antigens conjugated with beads was fixed and sufficient for detecting few antibodies in the serum, the affinity of antibodies to individual antigens cannot be analyzed using this system. These results indicate that multiplex assays for antibody production might be a biomarker for evaluating patient immunological potential or therapeutic benefit.

Recent studies have elucidated that immunosuppressive networks and immune checkpoints control antitumor immun-

DOI: 10.1021/acs.bioconjchem.5b00328
Bioconjugate Chem. 2015, 26, 2076−2084

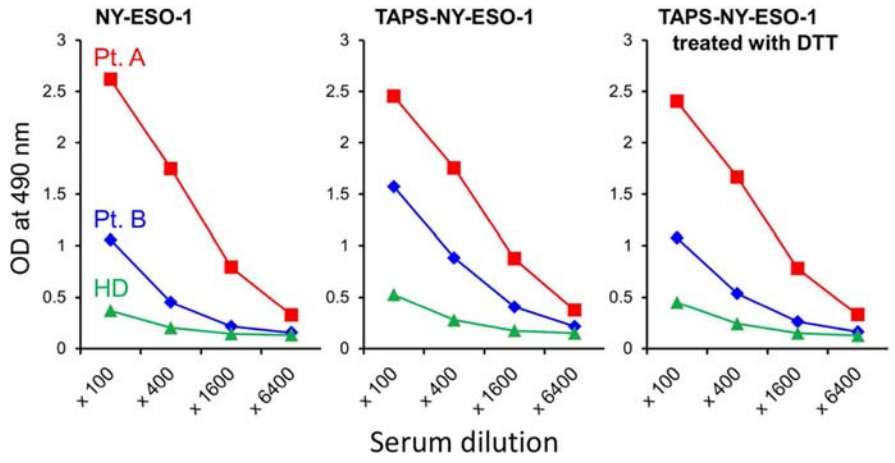


Figure 2. Evaluation of the effects of *S*-cationization on the binding specificity of anti-NY-ESO-1 antibody by ELISA. The sera from cancer patients (Pts A and B) and a healthy donor (HD) from NY-ESO-1f peptide vaccine studies were used for the ELISA. The OD values (490 nm) for the NY-ESO-1 protein and TAPS-NY-ESO-1 with/without DTT at indicated dilutions of sera are shown. Standard deviations of each value were less than 0.02. The experiments were performed at least 3 times with similar results.

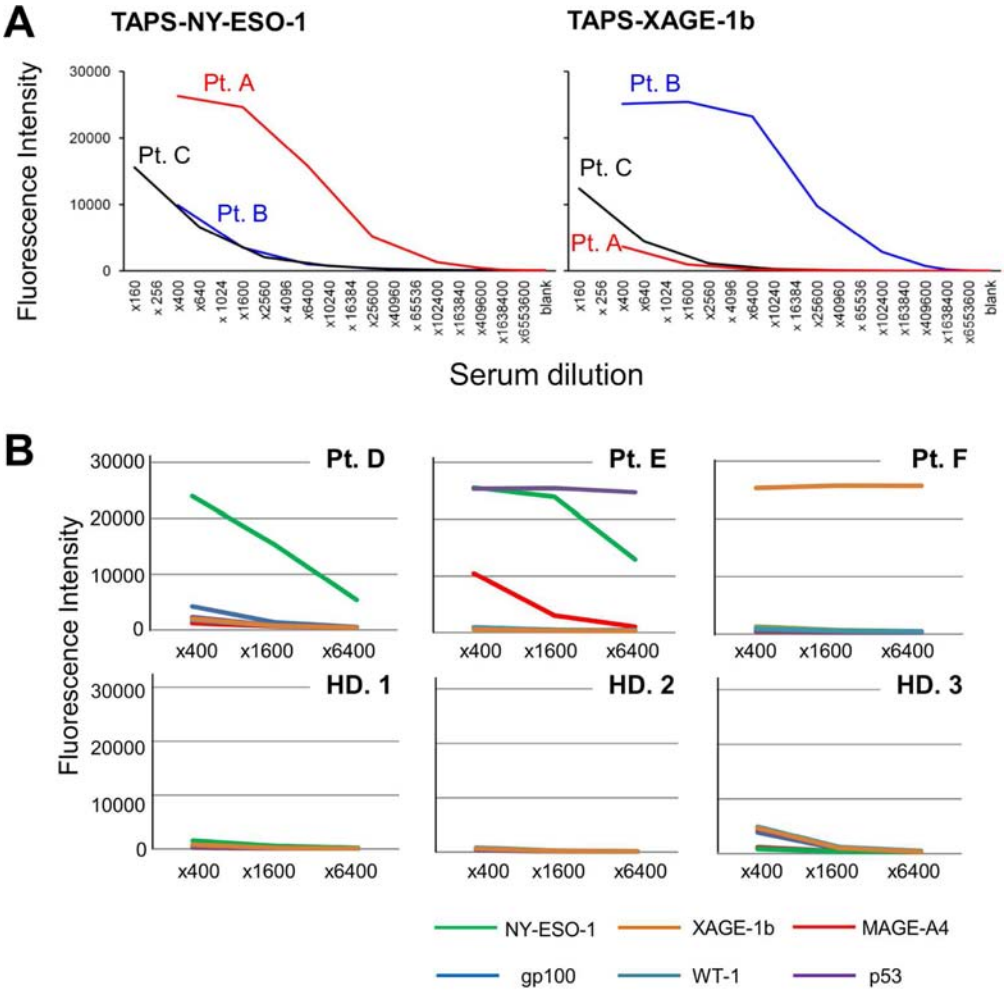


Figure 3. Antibody detection by *S*-cationized antigen-immobilized beads. (A) Single-plex antibody assay with serially diluted serum from cancer patients. The sera from Pts A and B were the same as those used in Figure 2, and were incubated with a single antigen-immobilized bead. (B) Six antigen-multiplexed antibody assays from cancer patients (Pts D–F) and healthy donors (HD 1–3). Sera from Pts D, E, and F on clinical trials were screened for positivity for NY-ESO-1, XAGE-1b, and MAGE-A4 by using ELISA. Anti-NY-ESO-1-positive sera from patient D, anti-MAGE-A4-positive sera from patient E, anti-XAGE-1b-positive sera from patient F, and sera from three healthy donors were analyzed in a multiplexed antibody assay with the mixture of TAPS-modified NY-ESO-1-, XAGE-1b-, MAGE-A4-, gp100-, WT-1, and p53-immobilized beads.

DOI: 10.1021/acs.bioconjchem.5b00328
Bioconjugate Chem. 2015, 26, 2076–2084

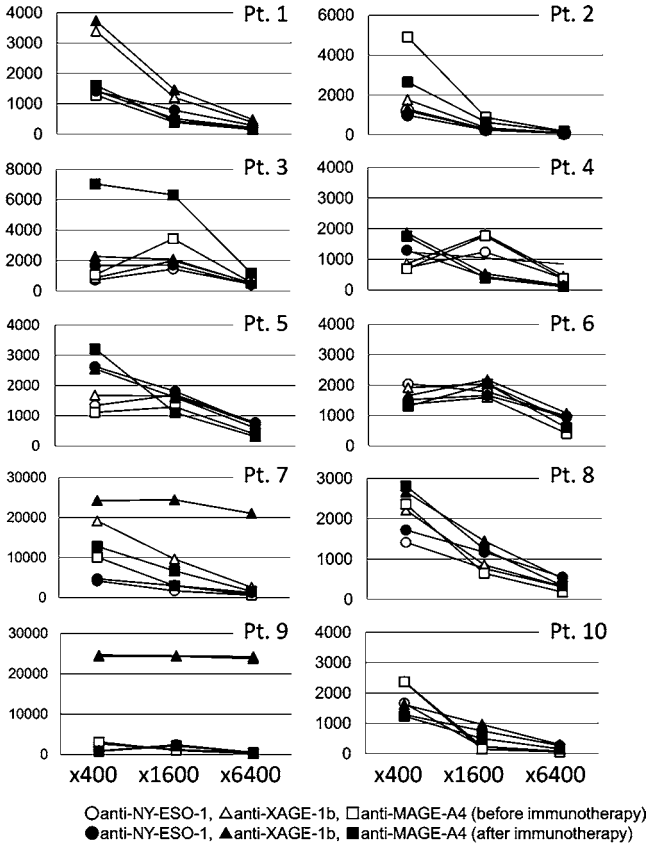


Figure 4. Clinical application of the multiplexed antibody detection system for the analysis of antigen spreading. The sera from lung cancer patients who received autologous γδ T-cell transfer therapy were analyzed using the multiplexed antibody assay with a mixture of TAPS-modified NY-ESO-1 (○), XAGE-1b (△), and MAGE-A4 (□) immobilized beads. The antibody titers before (white symbols) and after the treatment (black symbols) were compared.

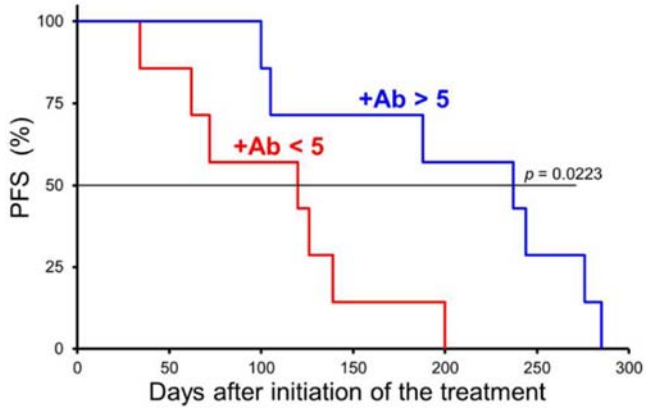


Figure 5. Progression-free survival (PFS) of lung cancer patients who received autologous γδ T-cell transfer therapy. The titers of antibodies against 17 antigens were compared before and after γδ T-cell transfer therapy. The number of antibodies whose titer increased by 1.1-fold were counted (Table S1). The PFS of patients in whom increased antibody production was detected against more than five antigens (+Ab > 5, blue) and less than five antigens (+Ab < 5, red) was compared.

ity, and that their blockage is a successful immunotherapeutic modality against cancer.[40−43] Furthermore, tumor immunogenicity is known to vary greatly between cancers of the same type in different individuals and between different types of cancer.[44] Therefore, to develop effective immunotherapies, reliable biomarkers that can predict which patients can benefit from corresponding immunotherapy are desired. Comprehensive multiplexed antibody detection systems will serve as powerful tools for monitoring antitumor immune responses during therapy and can help evaluate the efficacy of cancer immunotherapies.

Stability of *S*-Cationized Antigen-Immobilized Luminex Beads. The enhanced storage stability of antigen-immobilized Luminex beads also contributes to their availability for diagnostic use. Analysis of the Luminex multiplex assay is based on the principles of flow cytometry; therefore, the assay detector gathers fluorescence intensity from only single beads and does not read data from aggregated beads. As shown in Figure 6A, Cys-protected TAPS-MAGE-A4 significantly suppressed bead aggregation during longer storage compared to native MAGE-A4 immobilized beads. Native MAGE-A4 immobilized on the beads appeared to induce aggregation, probably because of intermolecular disulfide bond formation or higher hydrophobicity along with the denaturation of native conformations (Figure 6B). The *S*-cationized protein solubilization technique presented in this study is a powerful tool not only for preparing water-soluble full-length CTAs, but also for improving the storage stability of antigen-immobilized Luminex beads. Because 156 different CTAs, only 5 do not, possess Cys residues in their sequence, the *S*-cationization strategy is broadly applicable.

Prospective Diagnostic Use of the Multiplexed Antibody Assay for Cancer Immunity. Recently, to develop novel cancer therapies, regulatory authorities require companion diagnostics that provide information essential for the safe and effective use of a corresponding drug or biological product.[45,46] A simple blood test for determining the levels of antitumor immune response has potential to serve as a companion diagnostic for cancer therapy. In this study, we have successfully demonstrated that changes in the serum level of anti-TAAs/CTAs antibodies could be a useful biomarker for clinically effective antitumor immune response (Figure 7). Recently, improved understanding of cancer immunology has given rise to new treatments by combining immunotherapy with standard therapies, such as surgery, radiation therapy, chemotherapy, and molecular targeted therapy.[47] Because clinically measurable immune-mediated antitumor effects generally occur over weeks to months after initial immunotherapy, monitoring immune responses with a simple blood test is valuable for diagnosis during cancer treatments.

Advantages of *S*-Cationized Antigen for Antibody Detection. A number of soluble structurally ordered protein tags,[48] such as glutathione *S*-transferase or maltose-binding protein, are now widely used to improve recombinant protein solubility. In contrast to this current methodology, we employed chemical cationization techniques to enhance the water solubility of disordered proteins. Since this cationization reaction is accompanied by the protection of reactive sulfhydryl groups, *S*-cationized proteins are favored to maintain water solubility without forming disulfide-linked polymers.

The *S*-cationized water-soluble full-length antigens are a preferable resource for multiplexed Luminex beads. Cost-efficient overexpressed recombinant proteins in the form of bacterial inclusion bodies are the best material for preparing *S*-cationized antigens (Figure 1A), and these are easy to purify (Figure 1B,C). As for their ability to detect specific antibodies

DOI: 10.1021/acs.bioconjchem.5b00328
Bioconjugate Chem. 2015, 26, 2076−2084

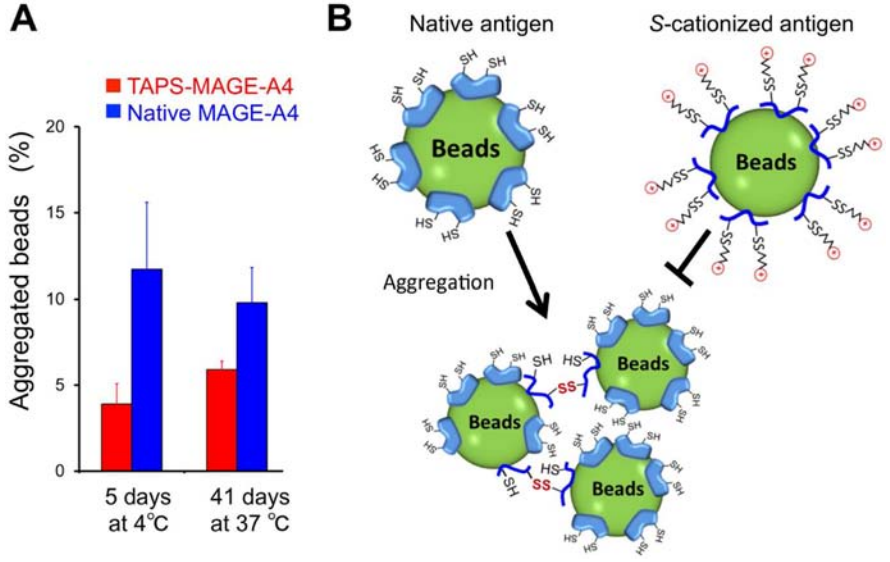


Figure 6. Evaluation of the storage stability of antigen-immobilized beads with native or *S*-cationized conformations. (A) Comparison of aggregation frequency between native MAGE-A4 and TAPS-cationized MAGE-A4 immobilized beads. (B) Putative mechanisms for the enhanced aggregation of native antigen-immobilized beads.

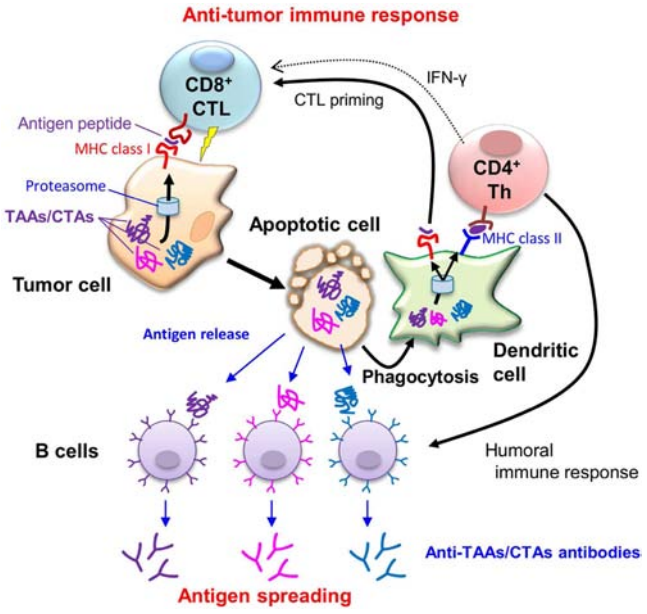


Figure 7. Summary of antitumor immune response involved in TAAs/CTAs. Along with tumor regression effects with cytotoxic T lymphocytes (CTLs), aberrantly expressed TAAs/CTAs are recognized as antigens. Because anti-TAA/CTA antibodies are induced by humoral immune response in vivo, these antibody levels are potential biomarkers that reflect antitumor immune response.

in cancer patients, *S*-cationized conformationally flexible polypeptides successfully worked as antigens, probably because most antibodies recognize linear epitopes without including side residues of Cys in the epitope−paratope interface (Figure 2). Highly sensitive multiplexed antibody assays and higher storage stability were successfully demonstrated with *S*-cationized antigen-immobilized Luminex beads (Figures 3−6). All these advantages make this system an ultimate diagnostic tool to monitor cancer immunity.

EXPERIMENTAL PROCEDURES

Expression of Recombinant Proteins. The cDNAs for antigens encoding XAGE-1b/CT12.1 (Uniprot: Q9HD64), NY-ESO-1/CT6.1 (Uniprot: P78358), p53 (Uniprot: P04637), WT-1 (Uniprot: J3KNN9), HR-gp100[49] (Uniprot: P40967), MAGE-A4/CT1.4 (Uniprot: P43358), BORIS/CTCFL/CT27 (Uniprot: Q8NI51), CEP55/CT111 (Uniprot: Q53EZ4), DPPA2/CT100 (Uniprot: Q7Z7J5), HSPB9/CT51 (Uniprot: Q9BQS6), LY6K/CT97 (Uniprot: Q17RY6), MAEL/CT128 (Uniprot: Q96JY0), MAGE-A3/CT1.3 (Uniprot: P43357), MAGE-A6/CT1.6 (Uniprot: P43360), NXF2/CT39 (Uniprot: Q9GZY0), SPEF2/CT122 (Uniprot: Q9C093), SSX4/CT5.4 (Uniprot: O60224), and XAGE2/CT12.2 (Uniprot: Q96GT9) were cloned into pET28b vectors (Novagen) to express as N-terminal His-tag fused proteins. These plasmid DNAs were used for transformation of chemically competent *E. coli* T7 Express cells (NEB). The freshly prepared colonies were inoculated into small aliquots of lysogeny broth (LB) in the presence of 50 μg/mL kanamycin at 37 °C with shaking. Exponentially growing cells were transferred to a larger volume of terrific broth (TB) and incubated at 37 °C with shaking until an OD_{600} of 0.8. Protein synthesis was then induced with 0.5 mM isopropyl β-D-thiogalactopyranoside (IPTG). Cells expressing each antigen were then incubated with shaking at 37 °C for 3 h. After cultivation, cells were harvested by centrifugation, and the pellets were stored at −80 °C.

Recombinant Protein Purification and Solubilization by *S*-Cationization. Frozen cells expressing recombinant antigens were resuspended in lysis buffer (20 mM Tris-HCl [pH 7.5], 50 mM NaCl, 5 mM $MgSO_4$, and 0.2% Tween-20) and disrupted on ice with a sonicator (Branson, Sonifire250); then Benzonase endonuclease (Novagen) was added. After the lysate was incubated at room temperature for 30 min to digest bacterial nucleic acids, the insoluble fraction was collected by centrifugation for 15 min at 10 000 × *g*. To purify proteins expressed as inclusion bodies, the pellets were resuspended in Milli-Q water using a sonicator, and then collected by centrifugation. Insoluble proteins were solubilized in 6 M GdnHCl containing 0.1 M Tris-HCl (pH 8.0) and 1 mM

DOI: 10.1021/acs.bioconjchem.5b00328
Bioconjugate Chem. 2015, 26, 2076−2084

EDTA. After degassing and purging with nitrogen gas, all Cys residues were reduced with 30 mM of DTT by incubation for 1 h at 37 °C. To prepare the *S*-cationized protein, 70 mM of TAPS-sulfonate (Katayama Chemical) was added to the mixture, and incubated for 30 min at 37 °C. After addition of 0.1 volume of acetic acid and 0.1% of polyethylenimine (PEI; average molecular mass of 600, Wako Chemicals), the mixtures were extensively dialyzed against Milli-Q water. Precipitated protein and bacterial nucleic acids complexed with PEI were removed by centrifugation, and *S*-cationized proteins were recovered in an aqueous phase. The *S*-cationized proteins were further purified with a reversed-phase HPLC column (COSMOSIL Protein-R, Nacalai Tesque) using an acetonitrile linear gradient elution procedure in the presence of 0.1% HCl. The collected peak fraction was lyophilized and stored at −20 °C until use. Soluble MAGE-A4 recombinant protein was purified with an IMAC column, and then subjected to *S*-cationization.

Patients and Sera. Peripheral blood was drawn from patients who were enrolled in the NY-ESO-1f peptide vaccine clinical trial (UMIN-CTR ID: UMIN000001260)[6] or autologous γδ T-cell transfer therapy trial (UMIN-CTR ID: C000000336)[39] and three healthy donors. The participants provided written informed consent under the permission of the ethics committees of The University of Tokyo (Protocol ID: 10044). Sera were stored in a−80 °C freezer until use.

ELISA. Each recombinant protein (1 μg/mL) in a coating buffer (15 mM Na_2CO_3 and 30 mM $NaHCO_3$ [pH 9.6]) was adsorbed onto a 96-well plate (PolySorp immunoplates, Nunc, Roskilde, Denmark) and incubated overnight at 4 °C. After washing with PBS, plates were blocked with 200 μL per well of 5% FCS/PBS for 1 h at room temperature. Then, 100 μL of serially diluted serum was added to each well and incubated for 2 h at room temperature. After extensive washing, horseradish peroxidase-conjugated goat anti-human IgG (Medical & Biological Laboratories, Nagoya, Japan) was added to the wells, and the plates were incubated for 1 h at room temperature. After washing and development, the absorbance at 490 nm was read.

Preparation of *S*-Cationized Antigen-Immobilized Luminex Beads. Purified *S*-cationized antigens were immobilized to Bio-Plex Pro Magnetic COOH Beads (Bio-Rad) using an amine coupling kit (Bio-Rad) according to the manufacturer's instructions. Briefly, carboxyl groups on the beads were activated by EDC/sulfo-NHS to form active esters, and the beads were then conjugated to *S*-cationized proteins by amide bonds. Using a 100 μL suspension of beads (1.25×10^6 beads) and 11 μg of *S*-cationized proteins, more than 70% of immobilized beads were recovered, and more than 90% of the beads showed monodispersion under microscopic observation. Native human serum albumin (HSA, Wako Chemicals) immobilized beads were also prepared as a control.

Bead Assay. Antigen-immobilized beads were blocked with a solution of Block Ace (DS Pharma Biomedical) and washed with Bioplex wash buffer (WB) (Bio-Rad). Block Ace solution was further diluted four times with PBS and used as the dilution buffer (DB). For multiplex analysis, each antigen on immobilized beads was mixed in DB beforehand. The serum was serially diluted in DB; 25 μL of the diluted serum was incubated with 25 μL of 1000 beads for each antigen on immobilized beads in the wells of a 96-well microassay plate (Greiner Bio-One) for 1 h. After extensive washes with WB, 50 μL of a 1/200 dilution of biotin-conjugated anti-human IgG (Vector Laboratories) was added to the wells and incubated for 30 min. A 1/200 dilution of streptavidin-PE (Vector Laboratories) in DB was added to the wells and incubated for 10 min at room temperature. Analysis was performed with MAGPIX xPONENT 4.2 System (Merck Millipore) and the mean fluorescence intensity (MFI) values for each dilution reflected the antibody levels. Before each assay run, the system was calibrated with a MAGPIX Calibration Kit (Luminex) and validated with a MAGPIX Performance Verification Kit (Luminex). MAGPIX Drive Fluid (Luminex) served as the delivery medium for the samples.

Storage Stability of Antigen-Immobilized Luminex Beads. Both native and *S*-cationized MAGE-A4 immobilized beads in storage buffer (Bio-Rad) at pH 7.1 were stored at 4 or 37 °C, and counted on a hemocytometer under microscopic observation. Beads that gathered more than three beads were counted as aggregated, and calculated the aggregation tendency by divided by total number of beads.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.bioconjchem.5b00328.

Table S1 and Figure S1 (PDF)

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: futamij@okayama-u.ac.jp.

Author Contributions

#Equal contribution for first authorship.

Notes

The authors declare the following competing financial interest(s): Okayama University and Medinet Co. Ltd. are applying for patents on the method for producing reagent for antibody detection.

■ ACKNOWLEDGMENTS

This work was supported in part by JSPS KAKENHI (Grant Number 23360370 awarded to J. Futami) and a research program of the Project for Development of Innovative Research on Cancer Therapeutics (P-Direct awarded to K. Kakimi). We thank Emeritus Professor Hidenori Yamada for many helpful discussions.

■ REFERENCES

(1) Chen, H., Werner, S., Tao, S., Zörnig, I., and Brenner, H. (2014) Blood autoantibodies against tumor-associated antigens as biomarkers in early detection of colorectal cancer. *Cancer Lett. 346*, 178−187.

(2) Yao, Y., Fan, Y., Wu, J., Wan, H., Wang, J., Lam, S., Lam, W. L., Girard, L., Gazdar, A. F., Wu, Z., et al. (2012) Potential application of non-small cell lung cancer-associated autoantibodies to early cancer diagnosis. *Biochem. Biophys. Res. Commun. 423*, 613−619.

(3) Gnjatic, S., Ritter, E., Büchler, M. W., Giese, N. A., Brors, B., Frei, C., Murray, A., Halama, N., Zörnig, I., Chen, Y. T., et al. (2010) Seromic profiling of ovarian and pancreatic cancer. *Proc. Natl. Acad. Sci. U. S. A. 107*, 5088−5093.

(4) Chatterjee, M., Mohapatra, S., Ionan, A., Bawa, G., Ali-Fehmi, R., Wang, X., Nowak, J., Ye, B., Nahhas, F. A., Lu, K., et al. (2006) Diagnostic markers of ovarian cancer by high-throughput antigen cloning and detection on arrays. *Cancer Res. 66*, 1181−1190.

DOI: 10.1021/acs.bioconjchem.5b00328
Bioconjugate Chem. 2015, 26, 2076−2084

(5) Scanlan, M. J., Simpson, A. J., and Old, L. J. (2004) The cancer/testis genes: review, standardization, and commentary. *Cancer Immun. 4*, 1.
(6) Kakimi, K., Isobe, M., Uenaka, A., Wada, H., Sato, E., Doki, Y., Nakajima, J., Seto, Y., Yamatsuji, T., Naomoto, Y., et al. (2011) A phase I study of vaccination with NY-ESO-1f peptide mixed with Picibanil OK-432 and Montanide ISA-51 in patients with cancers expressing the NY-ESO-1 antigen. *Int. J. Cancer 129*, 2836−2846.
(7) Wada, H., Isobe, M., Kakimi, K., Mizote, Y., Eikawa, S., Sato, E., Takigawa, N., Kiura, K., Tsuji, K., Iwatsuki, K., et al. (2014) Vaccination with NY-ESO-1 overlapping peptides mixed with Picibanil OK-432 and montanide ISA-51 in patients with cancers expressing the NY-ESO-1 antigen. *J. Immunother. 37*, 84−92.
(8) Caballero, O. L., and Chen, Y. T. (2009) Cancer/testis (CT) antigens: potential targets for immunotherapy. *Cancer Sci. 100*, 2014−2021.
(9) Kawabata, R., Wada, H., Isobe, M., Saika, T., Sato, S., Uenaka, A., Miyata, H., Yasuda, T., Doki, Y., Noguchi, Y., et al. (2007) Antibody response against NY-ESO-1 in CHP-NY-ESO-1 vaccinated patients. *Int. J. Cancer 120*, 2178−2184.
(10) Ohue, Y., Eikawa, S., Okazaki, N., Mizote, Y., Isobe, M., Uenaka, A., Fukuda, M., Old, L. J., Oka, M., and Nakayama, E. (2012) Spontaneous antibody, and CD4 and CD8 T-cell responses against XAGE-1b (GAGED2a) in non-small cell lung cancer patients. *Int. J. Cancer 131*, E649−658.
(11) Almeida, L. G., Sakabe, N. J., deOliveira, A. R., Silva, M. C., Mundstein, A. S., Cohen, T., Chen, Y. T., Chua, R., Gurung, S., Gnjatic, S., et al. (2009) CTdatabase: a knowledge-base of high-throughput and curated data on cancer-testis antigens. *Nucleic Acids Res. 37*, D816−819.
(12) Ohue, Y., Kurose, K., Mizote, Y., Matsumoto, H., Nishio, Y., Isobe, M., Fukuda, M., Uenaka, A., Oka, M., and Nakayama, E. (2014) Prolongation of overall survival in advanced lung adenocarcinoma patients with the XAGE1 (GAGED2a) antibody. *Clin. Cancer Res. 20*, 5052.
(13) Gnjatic, S., Old, L. J., and Chen, Y. T. (2009) Autoantibodies against cancer antigens. *Methods Mol. Biol. 520*, 11−19.
(14) Middleton, C. H., Irving, W., Robertson, J. F., Murray, A., Parsy-Kowalska, C. B., Macdonald, I. K., McElveen, J., Allen, J., Healey, G. F., Thomson, B. J., et al. (2014) Serum autoantibody measurement for the detection of hepatocellular carcinoma. *PLoS One 9*, e103867.
(15) Beeton-Kempen, N., Duarte, J., Shoko, A., Serufuri, J. M., John, T., Cebon, J., and Blackburn, J. (2014) Development of a novel, quantitative protein microarray platform for the multiplexed serological analysis of autoantibodies to cancer-testis antigens. *Int. J. Cancer 135*, 1842−1851.
(16) Gnjatic, S., Wheeler, C., Ebner, M., Ritter, E., Murray, A., Altorki, N. K., Ferrara, C. A., Hepburne-Scott, H., Joyce, S., Koopman, J., McAndrew, M. B., Workman, N., Ritter, G., Fallon, R., and Old, L. J. (2009) Seromic analysis of antibody responses in non-small cell lung cancer patients and healthy donors using conformational protein arrays. *J. Immunol. Methods 341*, 50−58.
(17) Shan, Q., Lou, X., Xiao, T., Zhang, J., Sun, H., Gao, Y., Cheng, S., Wu, L., Xu, N., and Liu, S. (2013) A cancer/testis antigen microarray to screen autoantibody biomarkers of non-small cell lung cancer. *Cancer Lett. 328*, 160−167.
(18) Jia, J., Wang, W., Meng, W., Ding, M., Ma, S., and Wang, X. (2014) Development of a multiplex autoantibody test for detection of lung cancer. *PLoS One 9*, e95444.
(19) Suyama, T., Shiraishi, T., Zeng, Y., Yu, W., Parekh, N., Vessella, R. L., Luo, J., Getzenberg, R. H., and Kulkarni, P. (2010) Expression of cancer/testis antigens in prostate cancer is associated with disease progression. *Prostate 70*, 1778−1787.
(20) Rajagopalan, K., Mooney, S. M., Parekh, N., Getzenberg, R. H., and Kulkarni, P. (2011) A majority of the cancer/testis antigens are intrinsically disordered proteins. *J. Cell. Biochem. 112*, 3256−3267.
(21) Tompa, P., and Csermely, P. (2004) The role of structural disorder in the function of RNA and protein chaperones. *FASEB J. 18*, 1169−1175.
(22) Patil, A., Kinoshita, K., and Nakamura, H. (2010) Hub promiscuity in protein-protein interaction networks. *Int. J. Mol. Sci. 11*, 1930−1943.
(23) Barlow, D. J., Edwards, M. S., and Thornton, J. M. (1986) Continuous and discontinuous protein antigenic determinants. *Nature 322*, 747−748.
(24) Vinion-Dubiel, A. D., McClain, M. S., Cao, P., Mernaugh, R. L., and Cover, T. L. (2001) Antigenic diversity among Helicobacter pylori vacuolating toxins. *Infect. Immun. 69*, 4329−4336.
(25) Huang, J., and Honda, W. (2006) CED: a conformational epitope database. *BMC Immunol. 7*, 7.
(26) Pedersen, J. W., Gentry-Maharaj, A., Fourkala, E. O., Dawnay, A., Burnell, M., Zaikin, A., Pedersen, A. E., Jacobs, I., Menon, U., and Wandall, H. H. (2013) Early detection of cancer in the general population: a blinded case-control study of p53 autoantibodies in colorectal cancer. *Br. J. Cancer 108*, 107−114.
(27) Futami, J., Kitazoe, M., Murata, H., and Yamada, H. (2007) Exploiting protein cationization techniques in future drug development. *Expert Opin. Drug Discovery 2*, 261−269.
(28) Inoue, M., Akimaru, J., Nishikawa, T., Seki, N., and Yamada, H. (1998) A new derivatizing agent, trimethylammoniopropyl methanethiosulphonate, is efficient for preparation of recombinant brain-derived neurotrophic factor from inclusion bodies. *Biotechnol. Appl. Biochem. 28* (Pt 3), 207−213.
(29) Mallorquí-Fernández, G., Pous, J., Peracaula, R., Aymamí, J., Maeda, T., Tada, H., Yamada, H., Seno, M., de Llorens, R., Gomis-Rüth, F. X., and Coll, M. (2000) Three-dimensional crystal structure of human eosinophil cationic protein (RNase 3) at 1.75 A resolution. *J. Mol. Biol. 300*, 1297−1307.
(30) Miura, K., Doura, H., Aizawa, T., Tada, H., Seno, M., Yamada, H., and Kawano, K. (2002) Solution structure of betacellulin, a new member of EGF-family ligands. *Biochem. Biophys. Res. Commun. 294*, 1040−1046.
(31) Newton, D. L., Futami, J., Ruby, D., and Rybak, S. M. (2003) Construction and characterization of RNase-based targeted therapeutics. *Methods Mol. Biol. 207*, 283−304.
(32) Murata, H., Sakaguchi, M., Futami, J., Kitazoe, M., Maeda, T., Doura, H., Kosaka, M., Tada, H., Seno, M., Huh, N., et al. (2006) Denatured and reversibly cationized p53 readily enters cells and simultaneously folds to the functional protein in the cells. *Biochemistry 45*, 6124−6132.
(33) Okazaki, K., Imoto, T., and Yamada, H. (1985) A convenient protein substrate for the determination of protease specificity: reduced and S-3-(trimethylated amino)propylated lysozyme. *Anal. Biochem. 145*, 87−90.
(34) Bjerre, M., Hansen, T. K., Flyvbjerg, A., and Tønnesen, E. (2009) Simultaneous detection of porcine cytokines by multiplex analysis: development of magnetic bioplex assay. *Vet. Immunol. Immunopathol. 130*, 53−58.
(35) Peng, H. P., Lee, K. H., Jian, J. W., and Yang, A. S. (2014) Origins of specificity and affinity in antibody-protein interactions. *Proc. Natl. Acad. Sci. U. S. A. 111*, E2656−2665.
(36) Ramaraj, T., Angel, T., Dratz, E. A., Jesaitis, A. J., and Mumey, B. (2012) Antigen-antibody interface properties: composition, residue interactions, and features of 53 non-redundant structures. *Biochim. Biophys. Acta, Proteins Proteomics 1824*, 520−532.
(37) Kringelum, J. V., Nielsen, M., Padkjær, S. B., and Lund, O. (2013) Structural analysis of B-cell epitopes in antibody:protein complexes. *Mol. Immunol. 53*, 24−34.
(38) Corbière, V., Chapiro, J., Stroobant, V., Ma, W., Lurquin, C., Lethé, B., van Baren, N., Van den Eynde, B. J., Boon, T., and Coulie, P. G. (2011) Antigen spreading contributes to MAGE vaccination-induced regression of melanoma metastases. *Cancer Res. 71*, 1253−1262.
(39) Sakamoto, M., Nakajima, J., Murakawa, T., Fukami, T., Yoshida, Y., Murayama, T., Takamoto, S., Matsushita, H., and Kakimi, K. (2011) Adoptive immunotherapy for advanced non-small cell lung cancer using zoledronate-expanded γδTcells: a phase I clinical study. *J. Immunother. 34*, 202−211.

DOI: 10.1021/acs.bioconjchem.5b00328
Bioconjugate Chem. 2015, 26, 2076−2084

(40) Hodi, F. S., O'Day, S. J., McDermott, D. F., Weber, R. W., Sosman, J. A., Haanen, J. B., Gonzalez, R., Robert, C., Schadendorf, D., Hassel, J. C., et al. (2010) Improved survival with ipilimumab in patients with metastatic melanoma. *N. Engl. J. Med. 363*, 711−723.
(41) Topalian, S. L., Hodi, F. S., Brahmer, J. R., Gettinger, S. N., Smith, D. C., McDermott, D. F., Powderly, J. D., Carvajal, R. D., Sosman, J. A., Atkins, M. B., et al. (2012) Safety, activity, and immune correlates of anti-PD-1 antibody in cancer. *N. Engl. J. Med. 366*, 2443−2454.
(42) Vanneman, M., and Dranoff, G. (2012) Combining immunotherapy and targeted therapies in cancer treatment. *Nat. Rev. Cancer 12*, 237−251.
(43) Marabelle, A., Kohrt, H., Sagiv-Barfi, I., Ajami, B., Axtell, R. C., Zhou, G., Rajapaksa, R., Green, M. R., Torchia, J., Brody, J., et al. (2013) Depleting tumor-specific Tregs at a single site eradicates disseminated tumors. *J. Clin. Invest. 123*, 2447−2463.
(44) Blankenstein, T., Coulie, P. G., Gilboa, E., and Jaffee, E. M. (2012) The determinants of tumour immunogenicity. *Nat. Rev. Cancer 12*, 307−313.
(45) Olsen, D., and Jørgensen, J. T. (2014) Companion diagnostics for targeted cancer drugs - clinical and regulatory aspects. *Front. Oncol. 4*, 105.
(46) US Food and Drug Administration (2014) *In Vitro Companion Diagnostic Devices. Guidance for Industry and Food and Drug Administration Staff*, www.fda.gov/downloads/medicaldevices/deviceregulationandguidance/guidancedocuments/ucm262327.pdf.
(47) Chen, D. S., and Mellman, I. (2013) Oncology meets immunology: the cancer-immunity cycle. *Immunity 39*, 1−10.
(48) Costa, S., Almeida, A., Castro, A., and Domingues, L. (2014) Fusion tags for protein solubility, purification and immunogenicity in Escherichia coli: the novel Fh8 system. *Front. Microbiol. 5*, 63.
(49) Gelbart, Y., Frankenburg, S., Pinchasov, Y., Krispel, S., Eliahu, D., Drize, O., Morag, E., Bartfeld, D., Lotem, M., Peretz, T., et al. (2004) Production and purification of melanoma gp100 antigen and polyclonal antibodies. *Protein Expression Purif. 34*, 183−189.

DOI: 10.1021/acs.bioconjchem.5b00328
Bioconjugate Chem. 2015, 26, 2076−2084