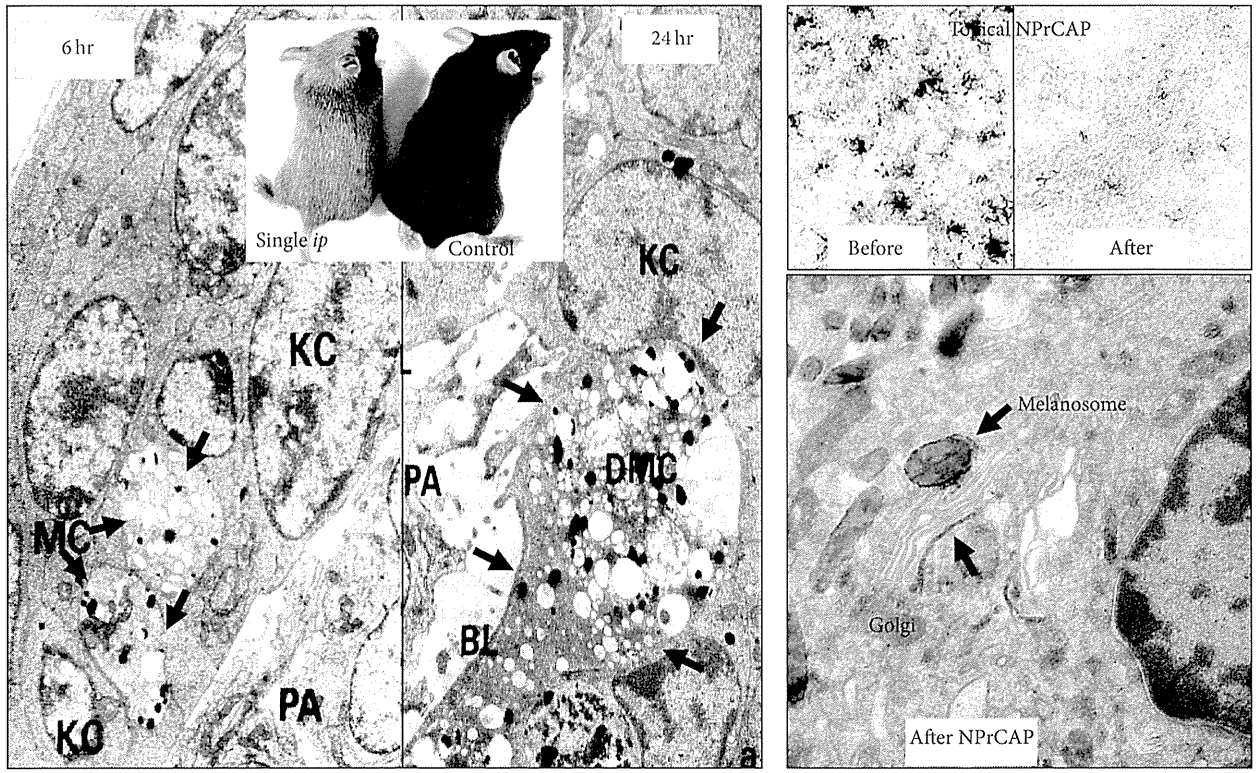


(a) Depigmentation of C57 black mouse hair by a single *ip* administration of NPrCAP or NAcCAP

(b) Depigmentation of black skin by topical NPrCAP

FIGURE 3: Depigmenting effect of NPrCAP. (a) Depigmentation of C57 black mouse hair follicles by a single *ip* administration of NPrCAP or NAcCAP results in complete loss of melanin pigmentation. Entire coat color changes to silver from black. Electron microscopic observation reveals selective degradation of melanocytes and melanogenic organelles such as early-stage melanosomes at 6 hr after administration. At 24 hr after administration, these melanocytes reveal total degradation. (b) Depigmentation of black skin after topical application of NPrCAP. There is a marked decrease of melanocyte populations after topical application. Electron microscopic observation indicates selective accumulation of NPrCAP in the tyrosinase areas such as in melanosomes and Golgi apparatus as indicated by the deposition of electron dense materials (see arrows).

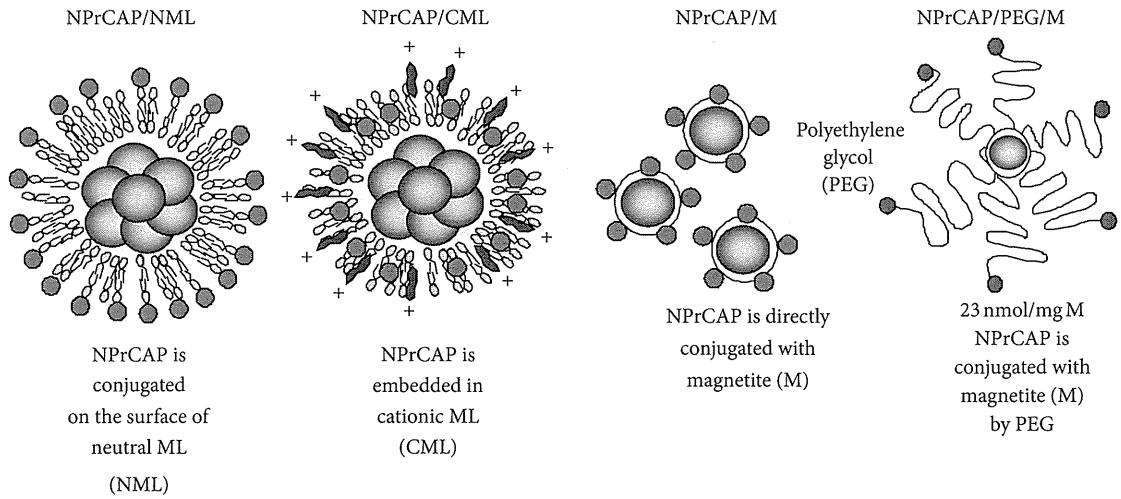
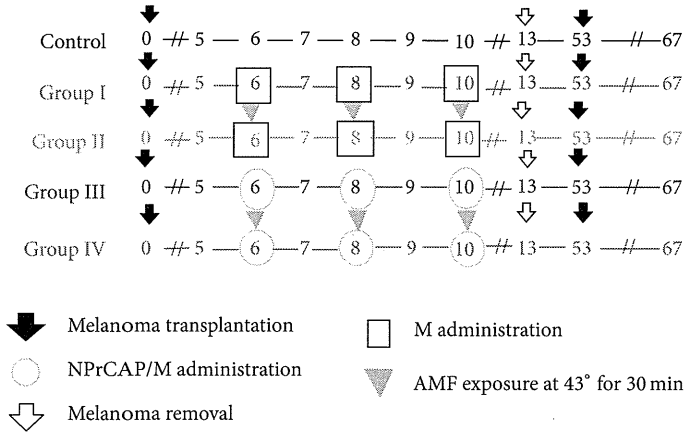
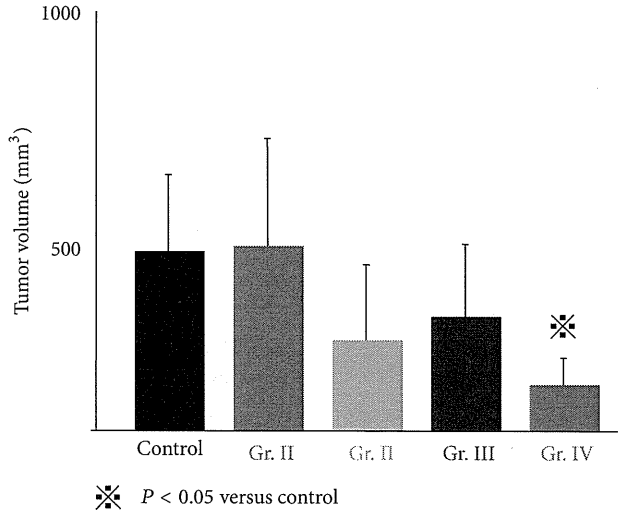


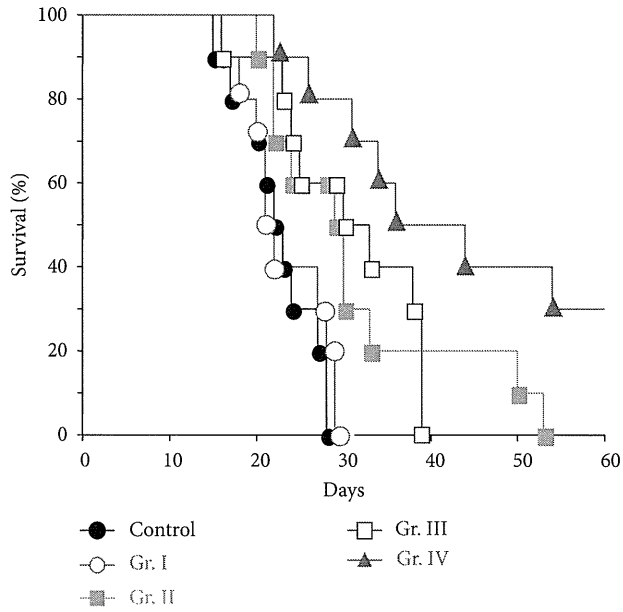
FIGURE 4: Conjugates of NPrCAP/magnetite nanoparticles for developing melanogenesis-targeted melanoma nanomedicine.



(a) Protocols of Groups I, II, III, and IV of experimental mice



(b) Tumor volumes of rechallenge B16F1 melanoma transplants



(c) Kaplan-Meier survival after tumor rechallenge

FIGURE 5: Melanoma growth and survival of melanoma-bearing mice by CTI therapy using NPrCAP/M with and without AMF exposure. (a) Experimental protocols. (b) Tumor volumes of rechallenge melanoma transplants on day 13 of after transplantation. (c) Kaplan-Meier survival of melanoma-bearing mice after treatment following experimental protocols of Figure 5(a).

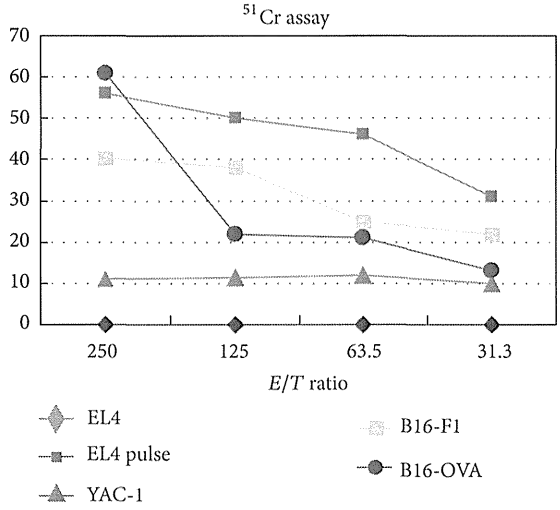


FIGURE 6: Hyperthermia of melanoma cells using B16OVA cells for induction of CTL in CTI therapy. Cytotoxic activity of spleen cells after CTI therapy against B16OVA cells, B16F1 cells, EL4 cells, EL4 cells pulsed with SL8 peptide (OVA-immunodominant peptide), or YAC-1 cells was determined by standard ^{51}Cr-release assay. B16OVA cells were subjected to hyperthermia using NPrCAP/M with AMF exposure *in vitro*.

4.2. T-Cell Receptor Repertoires of Tumor-Infiltrating Lymphocytes by Conjugates of NPrCAP and Magnetite Nanoparticles with Heat Exposure (Hyperthermia). It is clear now from our previous studies [22, 41] that conjugates of NPrCAP/magnetite nanoparticles (NPrCAP/M) with heat treatment (hyperthermia) can successfully induce the growth inhibition of primary and secondary melanoma transplants. It is also found that NPrCAP/M with hyperthermia elicited the response of cytotoxic T lymphocyte (CTL) via the release of HSP-peptide complex from degraded tumor cells [43] (Figure 6). In addition, $CD8^+$ T cells were observed within B16 melanoma nodules after hyperthermia using NPrCAP/M [37]. TIL reactivity to antigen is mediated via T-cell receptors (TCRs) consisting of α and β chains. We studied the TCR repertoire after hyperthermia using NPrCAP/M in order to further understand the T-cell response to melanoma after hyperthermia using NPrCAP/M [45]. We found that TCR repertoire was restricted in TILs, and the expansion of $V\beta11^+$ T cells was preferentially found. DNA sequences of the third complementarity determining regions were identified. This approach is based on subcutaneous melanoma transplantation in the hind foot pad, which confines the DLN to the inguinal and popliteal lymph nodes. Melanoma growth was significantly suppressed by the treatment of NPrCAP/M-mediated hyperthermia. $CD8^+$ T cells were observed substantially around the tumor and slightly within the tumor, while few and no $CD8^+$ T cells were observed around and within the tumor of nontreated mice.

In addition, significant enlargement of inguinal DLNs was observed in all of tumor-bearing mice including nontreated mice and NPrCAP/M-injected mice. The number of $CD8^+$ T cells in inguinal DLNs increased significantly in the mice treated with NPrCAP/M-mediated hyperthermia.

5. Melanocytotoxic and Immunogenic Properties of NPrCAP without Hyperthermia

5.1. Induction of Apoptosis, Reactive Oxygen Species (ROS), and Tumor-Specific Immune Response by NPrCAP Administration Alone. In our animal study, those animals bearing B16F1 and B16F10 melanoma cells showed, to certain degree, rejection of second re-challenge melanoma transplantation by administration of both NPrCAP alone and NPrCAP/M minus AMF exposure [46]. Our working hypothesis for this finding is that there is a difference in the cytotoxic mechanism and immunogenic property of NPrCAP/M between experimental groups with and without hyperthermia by AMF exposure. The animals with NPrCAP/M without AMF exposure resulted in non-necrotic, apoptotic cell death. The animals with NPrCAP/M plus AMF exposure, on the other hand, resulted in nonapoptotic, necrotic cell death with immune complex production of melanoma peptide as well as Hsp70 and a small amount of Hsp 90.

To further examine the mechanism of the cell death induced by NPrCAP, those cells treated with NPrCAP alone were subjected to flow cytometric analysis, caspase 3 assay, and TUNEL staining [46]. The sub-G1 fraction was increased in the NPrCAP-treated B16F1 cells, comparable to TRAIL-exposed B16F1, but not in the NPrCAP-treated non-melanoma cells (NIH3T3, RMA) or nonpigmented melanoma cells (TXM18) (Figure 7). The luminescent assay detected caspase 3/7 activity in the NPrCAP-treated B16F1 cells remarkably increased (35.8-fold) compared to that in the nontreated cells. NIH3T3, RMA, and TXM18 cells treated with TRAIL showed 10.6-, 7.1-, and 5.8-fold increases of caspase 3/7 activation compared to the control, respectively, whereas those with NPr-4-*S*-CAP showed increases of 4.1-, 1.4-, and 1.8-fold, respectively. The number of TUNEL-positive cells was significantly increased only in the B16F1 tumor treated with NPrCAP. This increase was not observed in the B16F1 tumor without NPrCAP or in the RMA tumors with or without NPrCAP. The findings indicate that NPrCAP induces apoptotic cell death selectively in melanoma cells.

5.2. Melanocytotoxic and Immunogenic Properties of NPrCAP Compared to Monobenzyl Ether Hydroquinone. Monobenzyl ether of hydroquinone has long been known to produce the skin depigmentation at both the drug-applied area by direct chemical reaction with tyrosinase and the non-applied distant area by immune reaction with still unknown mechanism [43, 48–50]. The melanogenesis-related cytotoxicity primarily derives from tyrosinase-mediated formation of dopaquinone and other quinone intermediates, which produce ROSs such as superoxide and H_2O_2 [4, 31, 32, 51]. This unique biological property of melanin intermediates not only causes cell death, but also may produce immunogenic properties. We postulated that the cytotoxic action of NPrCAP appears to involve two major biological processes. One is

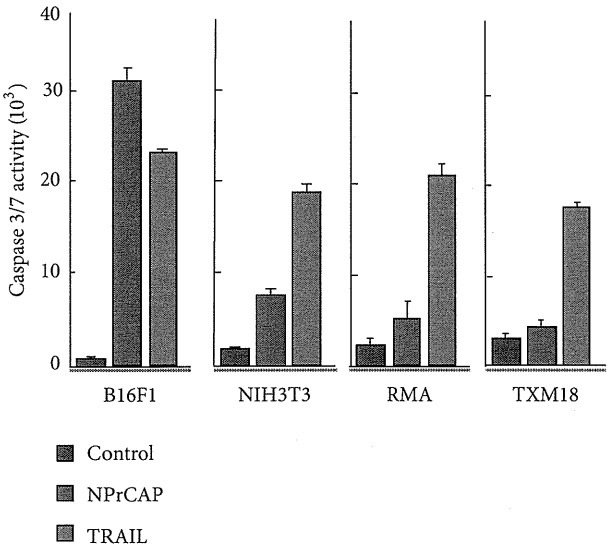


FIGURE 7: NPrCAP-mediated apoptotic cell death of B16F1 melanoma cells. Assay of caspase 3/7 in cells treated with NPrCAP or TRAIL. Cells were cultured in the presence of NPrCAP, TRAIL, or propylene glycol in 96-well plates and then processed for measurement of caspases 3 and 7 using a Caspase-Glo3/7 assay kit. From Ishii-Osai et al. [46].

cytostatic process which derives from the DNA synthesis inhibition through the interaction of quinone and free radicals with SH enzymes and thymidine synthase. Another is the cytocidal process by damage of DNA and mitochondrial ATP through oxidative stress and interaction with SH-enzyme [10]. They bind protein disulphide isomerase [52].

Monobenzyl ether form of hydroquinone was shown to produce a reactive *ortho*-quinone generated by tyrosinase-catalyzed oxidation and self-coupling and thiol conjugation reactions [53]. It was also shown to induce cell death without activating the caspase cascade or DNA fragmentation, indicating that the death pathway is non-apoptotic [53, 54]. It was further suggested that monobenzyl ether hydroquinone induced the immunogenicity to melanocytes and melanoma cells by forming quinone-haptens to tyrosinase protein and by inducing the release of tyrosinase and melanoma antigen recognized by T cells-1 (MART-1) containing $CD63^+$ exosomes following melanosome oxidative stress induction. The drug further augmented the processing and shedding of melanocyte differentiation antigens by inducing melanosome autophagy and enhanced tyrosinase ubiquitination, ultimately activating dendritic cells, which induced cytotoxic melanoma-reactive cells. These T cells eradicated melanoma *in vivo* [54, 55].

5.3. Development of Vitiligo during Melanoma Immunotherapy and Activation of NPrCAP by Tyrosinase to Form Possible Antigen Peptides. Advanced melanoma patients and melanoma patients treated by vaccine immunotherapy often reveal vitiligo-like changes of the skin. Interestingly, this vitiligo development is associated with a superior prognosis in melanoma patients [56]. Although there have been several separate theories for the pathogenesis of vitiligo, the haptenation theory has recently been put forth to explain the molecular mechanism of monobenzone-induced skin depigmentation [54, 57, 58]. Westerhof et al. proposed the haptenation theory in which increased intracellular H_2O_2 could trigger the increased turnover of elevated levels of surrogate substrates of tyrosinase, resulting in melanocyte-specific T-cell responses [57, 59]. According to this hypothesis, tyrosinase could be recognized as a melanoma-specific tumor antigen in relation to the systemic immune responses.

Phenolic substrates as prohaptens are oxidized by tyrosinase to produce *ortho*-quinones, which act as haptens that covalently bind to tyrosinase or other melanosomal proteins to generate possible neoantigens [44, 53, 54]. These neo-antigens, in turn, trigger an immunological response cascade that results in a melanocyte-specific delayed-type hypersensitivity reaction leading to melanocyte elimination to produce depigmentation in vitiligo and melanoma rejection. We examined the tyrosinase-mediated oxidation of NPrCAP and its subsequent binding to sulfhydryl compounds (thiols) in NPrCAP-treated melanoma tissues and demonstrated that NPrCAP is oxidized by tyrosinase to form a highly reactive *ortho*-quinone, (*N*-propionyl-4-*S*-cysteaminylcatechol, NPrCAQ; Figure 8), which then binds covalently to biologically relevant thiols including proteins through the cysteine residues. *In vitro* and *in vivo* studies were also conducted to prove the binding of the quinone-hapten NPrCAQ to proteins. The thiol adducts were analyzed after acid hydrolysis as 5-*S*-cysteaminyl-3-*S*-cysteinylcatechol (CA-CysC) (Figure 8). Our results specifically provided evidence that NPrCAP is oxidized by tyrosinase to an *ortho*-quinone, NPrCAQ, which is highly reactive yet stable enough to survive and then interact with biologically relevant thiols to form covalent adducts. The activation of NPrCAP to NPrCAQ by tyrosinase and the subsequent binding to proteins through cysteine residues were also demonstrated in the *in vitro* and *in vivo* experiments. Our finding was the first demonstration that the quinone-protein adduct formation actually takes place in melanoma cells and melanoma tissues through the tyrosinase-mediated mechanism. Furthermore, 60–80% of the NPrCAQ-thiol adducts were found in the protein fraction in melanoma cells and in the tumors. This is surprising when we consider the much lower reactivity of protein sulfhydryl groups compared with those in small thiols such as cysteine [60, 61]. The remaining nonprotein SH adducts were produced by the reaction of NPrCAQ with free cysteine or glutathione as a detoxifying mechanism. In this connection, it was previously shown that the depletion of glutathione augmented the melanocytotoxicity and antimelanoma effects of NAcCAP [62].

According to the potent melanoma immunotherapy theory using monobenzone [54, 55, 57–59], tyrosinase appears to trigger melanoma regression. Tyrosinase oxidation of monobenzone produces a highly reactive quinone-hapten [44, 54] and ROS concurrently [54]. The quinone-hapten binds to cysteine residues in tyrosinase or other melanosomal proteins thereby generating possible neoantigen, which activate hapten-reactive $CD8^+$ T-cells. The latter cells kill monobenzone-exposed melanocytes expressing haptenated

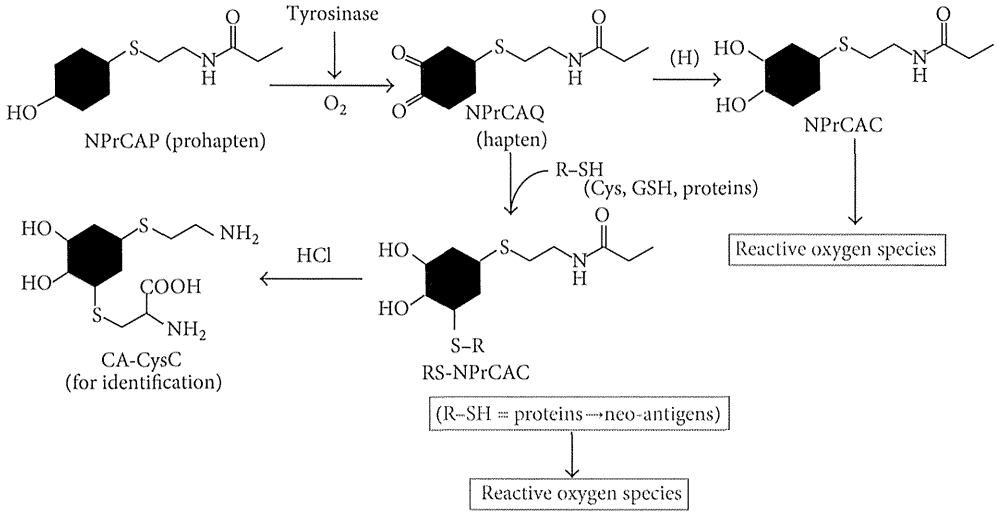


FIGURE 8: Tyrosinase activation of NPrCAP (prohapten) and binding of the quinone-hapten NPrCAQ with proteins thorough cysteine residues. Oxidation of NPrCAP with tyrosinase produces the quinone NPrCAQ, which is reduced to the catechol NPrCAC or binds to thiols (cysteine, glutathione, melanosomal proteins). The production of NPrCAQ-thiol adducts can be confirmed by the detection of CA-CysC after acid hydrolysis. NAcCys-NPrCAC is produced by the addition reaction of NAcCys (R-SH) with NPrCAQ. From Ito et al. [47].

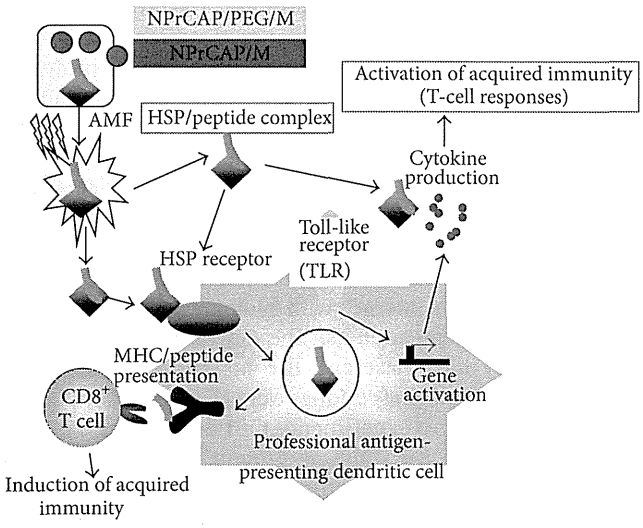


FIGURE 9: Scheme of intracellular hyperthermia using NPrCAP /PEG/M or NPrCAP/M with AMF exposure. NPrCAP/PEG/M nanoparticles are selectively incorporated in melanoma cells. Intracellular hyperthermia can induce necrotic cell death, and adjacent live melanoma cells suffer heat shock, resulting in increased level of intracellular HSP-peptide complexes. Repeated hyperthermia turns heat-shocked cells to necrotic cells, leading to the release of HSP-peptide complexes into extracellular milieu. The released HSPs-peptide complexes are taken up by dendritic cells (DCs). Then, DCs migrate into regional lymph nodes and cross-present HSP chaperoned antigenic peptides to $CD8^+$ T cells in the context of MHC class I molecules, thereby inducing antimelanoma cytotoxic $CD8^+$ T cells.

antigens on their surface, further liberating melanocyte antigens for presentation by dendritic cells. Finally, the antigen-specific T-cell response is induced and propagated [54, 57–59]. The ROS generated also causes damage to melanosomes leading to the presentation of melanosome-derived antigens and the induction of antigen-specific T-cell responses [58].

These immunological events can also be expected to occur for our NPrCAP because the involvement of $CD8^+$ T cells and the production of ROS in NPrCAP-treated melanoma cells were demonstrated in our previous study [46]. We expect the production of NPrCAC through redox exchange in melanoma cells and the subsequent production of ROS from the catechol because the closely related catechol, 4-*S*-cysteaminylcatechol, was shown to produce superoxide radicals (which are rapidly converted to hydrogen peroxide) [63]. The thiol adduct RS-NPrCAC, as a catechol, may also contribute to the production of ROS.

6. Summary and Conclusion

Several clinical trials using melanoma peptides or an antibody that blocks cytotoxic T-lymphocyte-associated antigen on lymphocytes have been shown to improve overall melanoma survival [64–66]. Promising oncogene-targeted melanoma therapy has also been successfully introduced recently [67].

Our study may however indicate that exploitation of a specific biological property to cancer cells can be another approach for developing novel melanoma-targeted drugs which can also trigger the production of melanoma-targeted *in situ* vaccine. Our approach using melanogenesis substrate and magnetite nanoparticles is based upon the expectation of (i) direct killing of melanoma cells by chemotherapeutic and thermo-therapeutic effect of melanogenesis-targeted drug (NPrCAP/M) and (ii) indirect killing by immune reaction (*in situ* peptide vaccine) after exposure to AMF. It is hoped from these rationales that a tumor-specific DDS is developed by NPrCAP, and selective cell death can be achieved by exposure of conjugates of NPrCAP/M nanoparticles to AMF. Hyperthermia increases the expression of intracellular HSPs which

is important in and necessary for the induction of antitumor immunity [41, 68]. Overexpression of HSPs increases tumor immunogenicity by augmenting the chaperoning ability of antigenic peptides and presentation of antigenic peptides in MHC class I molecules [39, 69]. In this process professional antigen-presenting dendritic cells play unique and important roles in taking up, processing, and presenting exogenous antigens in association with MHC class I molecules. Our study indicated that combination of melanogenesis substrate, NPrCAP, and local magnetite nanoparticles with hyperthermia could induce *in situ* a form of vaccine against tumor cells and may be effective not only for primary melanoma but also for distant secondary metastases (Figure 9).

Interestingly we found that NPrCAP by itself has potent chemotherapeutic and immune-adjuvant effects. It was demonstrated that the phenol NPrCAP, as a prohapten, can be activated in melanoma cells by tyrosinase to the reactive quinone-hapten NPrCAQ which binds to melanosomal proteins through their cysteine residues to form possible neo-antigens, thus triggering the immunological response (Figure 8).

Abbreviations

AMF: Alternating magnetic field
BSA: Bovine serum albumin
BSO: Buthionine sulfoximide
CA-CysC: 5-*S*-cysteaminyl-3-*S*-cysteinylcatechol
CAP: Cysteaminylphenol
CDR3: Third complementarity determining region
CML: Cationic magneto-liposome
CTI therapy: Chemothermoimmunotherapy
CTL: Cytotoxic T lymphocyte
DCs: Dendritic cells
DDS: Drug delivery system
DLNs: Draining lymph nodes
HSP/Hsp: Heat shock protein
IL: Interleukin
M: Magnetite nanoparticle
mAb: Monoclonal antibody
MART-1: Melanoma antigen recognized by T cells-1
MCLs: Magnetite cationic liposomes
MC1R: Melanocortin 1 receptor
MHC: Major histocompatibility complex
MITF: Microphthalmia transcription factor
ML: Noncationic magnetoliposome
MSH: Melanocyte stimulating hormone
NAcCAP: N-acetyl-4-S cysteaminylphenol
NDLN: Nondraining lymph node
NPrCAC: N-propionyl-4-S cysteaminylcatechol
NPrCAP: N-propionyl-4-S cysteaminylphenol
NPrCAQ: N-propionyl-4-S-cysteaminylquinine
Nrf2: NF-E2-related factor 2
OVA: Ovu-albumin
PEG: Polyethylene glycol
ROS: Reactive oxygen species
RT-PCR: Reverse transcription polymerase chain reaction
TILs: Tumor-infiltrating lymphocytes
TCRs: T-cell receptors
TRP-2: Tyrosinase-related protein-2.

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FULL PAPER

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NY-ESO-1 antibody as a novel tumour marker of gastric cancer

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Background: NY-ESO-1 antibodies are specifically observed in patients with NY-ESO-1-expressing tumours. We analysed whether the NY-ESO-1 humoral immune response is a useful tumour marker of gastric cancer.

Methods: Sera from 363 gastric cancer patients were screened by enzyme-linked immunosorbent assay (ELISA) to detect NY-ESO-1 antibodies. Serial serum samples were obtained from 25 NY-ESO-1 antibody-positive patients, including 16 patients with curative resection and 9 patients who received chemotherapy alone.

Results: NY-ESO-1 antibodies were detected in 3.4% of stage I, 4.4% of stage II, 25.3% of stage III, and 20.0% of stage IV patients. The frequency of antibody positivity increased with disease progression. When the NY-ESO-1 antibody was used in combination with carcinoembryonic antigen and CA19-9 to detect gastric cancer, information gains of 11.2% in stages III and IV, and 5.8% in all patients were observed. The NY-ESO-1 immune response levels of the patients without recurrence fell below the cutoff level after surgery. Two of the patients with recurrence displayed incomplete decreases. The nine patients who received chemotherapy alone continued to display NY-ESO-1 immune responses.

Conclusion: When combined with conventional tumour markers, the NY-ESO-1 humoral immune response could be a useful tumour marker for detecting advanced gastric cancer and inferring the post-treatment tumour load in seropositive patients.

Gastric cancer is the second most common cause of cancer-related death worldwide (Health and Welfare Statistics Association: Tokyo, 2006; Katanoda and Yako-Suketomo, 2009). Although complete removal of the tumour by surgical resection is an ideal treatment option for patients with gastric cancer, many patients with advanced-stage gastric cancer need to be treated with intensive chemotherapy. Gastric cancer patients exhibit high relapse rates even after curative surgery and unresponsiveness to chemotherapy, resulting in dismal survival rates (Sasako *et al*, 2011). Several methods for the prediction and early detection of subclinical 'minimal residual cancer' after surgery (Austrup *et al*, 2000; Klein *et al*, 2002) or relapse have been developed, for example, peritoneal lavage, positron emission tomography, gene profiling, and so on. (Motoori *et al*, 2006; Makino *et al*, 2010; Graziosi *et al*, 2011), reliable markers that can specifically reflect gastric cancer disease status have not been determined.

Analysing serum level of tumour markers is employed for cancer detection, monitoring patients' disease status, and prognosis prediction. Several organ-specific tumour markers are used in the clinic, for example, prostate-specific antigen and prostatic acid

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phosphatase for prostate cancer (Seamonds *et al*, 1986; Ferro *et al*, 1987) and protein induced by vitamin K absence-II for liver cancer (Fujiyama *et al*, 1986). As no gastric cancer-specific markers have been determined, a combination of several nonspecific tumour markers, for example, carcinoembryonic antigen (CEA), CA19-9,and so on, is merely applicable for monitoring treatment efficacy, but not the diagnosis of gastric cancer (Takahashi *et al*, 1995, 2003). Carcinoembryonic antigen and CA19-9 are found in the sera of 20–60% of gastric cancer patients, and their expression levels in gastric cancer are related to clinical events, such as relapse (Kodera *et al*, 1996). Carcinoembryonic antigen value, in particular, is indicative of the formation of a large tumour, liver or peritoneal metastasis, and/or a high risk of relapse and poor prognosis (Ikeda *et al*, 1993; Yamamoto *et al*, 2004). However, as CEA, a cell surface-anchored glycoprotein, is expressed in normal cell membranes, 5% of CEA-positive cases are pseudopositives, that is, caused by heavy smoking, endometriosis, and ageing, and so on. (Alexander *et al*, 1976), suggesting the importance of novel markers for gastric cancer.

NY-ESO-1 antigen, a cancer/testis (CT) antigen, was originally identified in oesophageal cancer by serological expression cloning using autologous patient serum and has been shown to be strongly immunogenic. Spontaneous NY-ESO-1 antibody production is often observed in patients with NY-ESO-1-expressing tumours, for example, 9.4% of melanoma patients, 12.5% of ovarian cancer patients, 7.7–26.5% of breast cancer patients, 4.2–20.0% of lung cancer patients, and 52% of prostate cancer patients, but has not been detected in non-cancerous donors (Stockert *et al*, 1998; Nakada *et al*, 2003; Türeci *et al*, 2006; Chapman *et al*, 2007; Isobe *et al*, 2009; Gati *et al*, 2011). Thus, it is possible that the NY-ESO-1 humoral immune response could be used as a serological marker for detecting these cancers and to facilitate the clinical management of some patients with particular types of cancer (Gnjatic *et al*, 2006). Jäger *et al* (1999) found that the change in the NY-ESO-1 humoral immune response reflected the overall tumour load in 10 out of 12 patients with various cancers. However, there is ongoing controversy regarding the association between the NY-ESO-1 immune response and prognostic criteria (Yuan *et al*, 2011). To address these issues in gastric cancer, we investigated the clinical usefulness of the NY-ESO-1 humoral immune response for diagnosis, monitoring, and relapse prediction in gastric cancer patients.

MATERIALS AND METHODS

Serum sample and tissue specimen collection from gastric cancer patients. In all, 363 patients with histologically confirmed gastric cancer, who underwent surgical resection or chemotherapy at one of four institutions between 2004 and 2011, were included in this study after providing written informed consent. Serum samples were obtained from the 363 patients during their admission to hospital for surgical treatment and/or chemotherapy, and afterwards, serial serum samples were obtained at each follow-up visit from 25 patients who displayed NY-ESO-1 humoral immune responses. All serum samples were collected as surplus samples after routine blood tests and stored. Fixed and frozen gastric cancer tissue samples were obtained from 60 out of 363 patients during surgery and stored. The samples were subsequently subjected to expression analysis. Information regarding blood test results, tumour stage, histological type, depth of invasion, lymph node metastasis, and distant metastasis, which were obtained from pathological examinations and CT scans, were collected from the relevant patient databases. Serum samples obtained from 50 healthy donors were used as controls. This study was approved by the institutional review boards of Osaka University Hospital, Toyonaka Municipal Hospital, Ikeda City Hospital, and Minoh City Hospital.

Reverse transcription–polymerase chain reaction. Total cellular RNA was extracted from the frozen tissue using TRIZOL reagent (Invitrogen, Carlsbad, CA, USA). The total RNA (1 μg) was subjected to the reverse transcription (RT) in 20 μl buffer with oligo-$(dT)_{15}$ primer using a RT system (Promega, Madison, WI, USA). Conventional polymerase chain reaction (PCR) was performed in a 25-μl reaction mixture containing 1 μl of cDNA template, 500 nM of each primer, and 1 U of *Taq* DNA polymerase (AmpliTaq Gold, Roche Molecular Systems, Pleasanton, CA, USA) in the following conditions: one cycle of 95 °C for 12 min; followed by 35 cycles of 94 °C for 1 min, 60 °C for 1 min, and 72 °C for 1.5 min; and then a final step of 72 °C for 10 min. The sequences of the primers for *NY-ESO-1* were as follows: ESO1-1, 5′-AGTTC TACCTCGCCATGCCT-3′; and ESO1-2, 5′-TCCTCCTCCAGC GACAAACAA-3′. The integrity of each RNA sample was verified by performing RT–PCR for *porphobilinogen deaminase* (*PBGD*). The PCR products were subjected to electrophoresis on a 2% agarose gel and visualised with ethidium bromide.

Immunohistochemistry. Formalin-fixed, paraffin-embedded tissues were used for the immunohistochemistry (IHC) analyses. Slides were incubated with the primary antibody overnight at 4 °C. The monoclonal antibody E978, which was previously generated by our group, was used to detect NY-ESO-1. The slides were then subjected to a heat-based antigen retrieval technique by immersing them in a preheated buffer solution (hipH solution; Dako, Carpinteria, CA, USA). A polymer-based antibody detection system (PowerVision; Leica Microsystems, Buffalo Grove, IL, USA) was used as the secondary reagent, and 3,3-diaminobenzidine tetrahydrochloride (Liquid DAB; Biogenex, San Ramon, CA, USA) was used as the chromogen. Normal adult testis tissue as a positive control and appropriate negative controls were included for each case.

Enzyme-linked immunosorbent assay. A measure of 100 μl of 1 μg ml^{-1} recombinant protein in coating buffer (pH 9.6) were added to each well of 96-well PolySorp immunoplates (Nunc, Roskilde, Denmark) and incubated overnight at 4 °C. The plates were then washed with PBS and blocked with 200 μl per well of 5% FCS/PBS for 1 h at room temperature. After being washed again, 100 μl of serially diluted serum were added to each well and incubated for 2 h at room temperature. Then, after extensive washing, goat anti-human IgG (Medical & Biological Laboratories, Nagoya, Japan) was added to the wells as a secondary antibody, and the plates were incubated for 1 h at room temperature. The plates were washed again, and the signals were developed with 100 μl per well of 0.03% *o*-phenylene diamine dihydrochloride, 0.02% hydrogen peroxide, and 0.15 M citrate buffer, and absorbance was read at 490 nm using an enzyme-linked immunosorbent assay (ELISA) reader (Benchmark Microplate Reader; Bio-Rad, Hercules, CA, USA). Ovalbumin (OVA; Sigma, St Louis, MO, USA) was used as the control protein. Levels of NY-ESO-1 humoral response were assessed using optical density (OD) values.

CEA and CA19-9. Serum CEA and CA19-9 levels were measured at each hospital's clinical laboratory department. Carcinoembryonic antigen and CA19-9 positivity were defined as serum levels of CEA and CA19-9 of >5.0 ng ml^{-1} and >37 U ml^{-1}, respectively.

Statistical analysis. Fisher's exact test was used to assess the associations between NY-ESO-1 antibody expression and clinicopathological parameters. Kaplan–Meier curves were plotted to assess the effect of the NY-ESO-1 antibody on overall survival. Survival curves were compared using the log-rank test.

Table 1. Frequencies of NY-ESO-1 antibody, CEA, and CA19-9 in gastric cancer patients

Stage	NY-ESO-1 Ab	CEA	CA19-9	CEA and/or CA19-9	CEA and/or CA19-9 and/or NY-ESO-1 Ab
I	6/176 (3.4)	24/176 (13.6)	6/176 (3.4)	27/176 (15.3)	31/176 (17.6)
II	2/45 (4.4)	8/45 (17.8)	7/45 (15.6)	11/45 (24.4)	12/45 (26.6)
III	17/67 (25.3)	22/67 (32.9)	11/67 (16.4)	25/67 (37.3)	35/67 (52.2)
IV	16/75 (20.0)	23/75 (30.7)	30/75 (40.0)	40/75 (53.3)	46/75 (61.3)
I+II	8/221 (3.6)	32/221 (14.5)	13/221 (5.9)	38/221 (17.2)	43/221 (19.5)
III+IV	33/142 (23.2)	45/142 (31.7)	41/142 (28.9)	65/142 (45.8)	81/142 (57.0)
Total	41/363 (11.1)	77/363 (21.2)	54/363 (14.9)	103/363 (28.4)	124/363 (34.2)

Abbreviations: Ab = antibody; CA = carbohydrate antigen; CEA = carcinoembryonic antigen. Values within parentheses are percentages.

RESULTS

Determination of NY-ESO-1 humoral immune response positivity. We first determined the OD cutoff value for NY-ESO-1 humoral immune response positivity. When the serum samples from the 50 healthy donors were examined for reactivity to the NY-ESO-1 recombinant protein by ELISA, their OD values ranged from 0.08 to 0.20, and their mean and standard deviation values were 0.15 and 0.05, respectively, at a dilution of 1 : 200. Thus, NY-ESO-1 humoral immune response positivity was defined as an OD value of >0.25 at a dilution of 1 : 200 (95% accuracy level) and >3 times of the OD value against control protein (OVA).

NY-ESO-1 humoral immune responses of gastric cancer patients. Serum samples were obtained from 363 gastric cancer patients, including 176 stage I, 45 stage II, 67 stage III, and 75 stage IV patients at admission (Table 1). The NY-ESO-1 antibody was detected in 3.4% (6 of 176) of stage I, 4.4% (2 of 45) of stage II, 25.3% (17 of 67) of stage III, and 20.0% (16 of 75) of stage IV gastric cancer patients, resulting in an overall detection rate of 11.1% (41 of 363). An analysis of the gastric cancer patients' characteristics found that NY-ESO-1 antibody positivity was significantly correlated with gender (male > female) and tumour progression (Table 2). In particular, the patients with progressive gastric cancer involving deeper tumour invasion, positive lymph node metastasis, positive distant metastasis, or a higher clinical stage tended to produce the NY-ESO-1 antibody.

Analysis of NY-ESO-1 antigen expression. *NY-ESO-1* mRNA and NY-ESO-1 protein expression were analysed by RT–PCR and IHC, respectively, in gastric cancer tissues obtained from 60 patients for whom both frozen and formalin-fixed specimens were available, including 12 stage I, 12 stage II, 20 stage III, and 16 stage IV patients (Table 3). *NY-ESO-1* mRNA was detected in six specimens. NY-ESO-1 was immunohistochemically detected in 19 specimens, including 6 and 13 that were positive and negative for *NY-ESO-1* mRNA, respectively. Most of the specimens displayed a heterogeneous staining pattern (data not shown).

NY-ESO-1 antibody and antigen expression. We analysed the frequency of NY-ESO-1 antibody positivity in gastric cancer patients in whom NY-ESO-1 antigen expression was or was not detected by RT–PCR or IHC. As shown in Table 3, 9 out of the 60 gastric cancer patients whose specimens were available for expression analysis possessed the NY-ESO-1 antibody in their sera. The NY-ESO-1 antibody was detected in 8 of 19 (42.1%) patients with IHC-positive gastric cancer and 5 of 6 (83.3%) patients with RT–PCR (and IHC)-positive gastric cancer, whereas only 1 of 41 patients in whom both RT–PCR and IHC analysis produced negative results displayed an NY-ESO-1 humoral immune responses.

Table 2. Relationship between NY-ESO-1 antibody positivity and clinicopathological features in gastric cancer patients

Variable	NY-ESO-1 Ab		*P*-value*
	Negative	Positive	
Gender			
Male	223 (86.4)	35 (13.6)	0.04307
Female	99 (94.3)	6 (5.7)	
Age (years)			
>65	178 (88.6)	23 (11.4)	0.9209
<65	144 (88.9)	18 (11.1)	
Histological type			
Differentiated	143 (89.4)	17 (10.6)	0.5605
Undifferentiated	132 (87.4)	19 (12.6)	
Depth of tumour invasion			
cT1–T2	193 (92.8)	15 (7.2)	0.0044
cT3–T4	129 (83.2)	26 (16.8)	
Lymph node metastasis			
Negative	196 (97.0)	6 (3.0)	<0.001
Positive	126 (78.3)	35 (21.7)	
Distant metastasis			
Negative	277 (91.1)	27 (8.9)	<0.001
Positive	45 (76.3)	14 (23.7)	
Stage			
I–II	213 (96.4)	8 (3.6)	<0.001
III–IV	109 (76.8)	33 (23.2)	

Abbreviations: Ab = antibody. Fisher's exact test was used for the statistical analysis. Values within parentheses are percentages.

Frequencies of NY-ESO-1 humoral immune responses and conventional tumour markers in gastric cancer patients. The frequency of the NY-ESO-1 humoral immune response was compared with those of conventional tumour markers in gastric

Table 3. Frequency of NY-ESO-1 antibody positives in gastric cancer patients in whom the NY-ESO-1 antigen was or was not detected by IHC or RT–PCR

	IHC		
	Positive	**Negative**	**Total**
mRNA			
Positive	5/6 (83.3)	0/0 (0.0)	5/6 (83.3)
Negative	3/13 (23.1)	1/41 (2.4)	4/54 (7.4)
Total	8/19 (42.1)	1/41 (2.4)	9/60 (15.0)

Abbreviations: IHC = immunohistochemistry; RT–PCR = reverse transcription–polymerase chain reaction. Frozen and formalin-fixed tissue specimens from 60 patients, including 12 stage I, 12 stage II, 20 stage III, and 16 stage IV patients, were analysed. All stage IV patients had previously undergone surgical treatment. Values within parentheses are percentages.

cancer patients. The serum CEA and CA19-9 levels of 363 gastric cancer patients were measured at admission (Table 1). Carcinoembryonic antigen and CA19-9 positivity were observed in 21.2% (77 of 363) and 14.9% (54 of 363) of the gastric cancer patients, respectively, and, except for CA19-9 in the stage III patients, they displayed higher frequencies than the NY-ESO-1 humoral immune response in all stages of the disease. We then analysed whether the addition of the NY-ESO-1 humoral immune response to CEA and CA19-9 increased the diagnostic frequency of gastric cancer. The combined use of CEA and CA19-9 tests produced positivity rates of 15.3% (27 of 176) in stage I, 24.4% (11 of 45) in stage II, 37.3% (25 of 67) in stage III, and 53.3% (40 of 75) in stage IV gastric cancer patients, resulting in an overall positivity rate of 28.4% (103 of 363). When the NY-ESO-1 humoral immune response was added to these two conventional tumour markers, the positivity rates of all stages increased, resulting in information gains of 14.9% (from 25 to 35 patients; 10 of 67) in stage III and 11.2% (from 65 to 81 patients; 16 of 142) in stage III and IV gastric cancer patients.

Changes in the NY-ESO-1 humoral immune responses of the patients during their clinical courses. Serial serum samples were obtained from 25 gastric cancer patients who displayed positive NY-ESO-1 antibody at admission, and the changes in their NY-ESO-1 humoral immune responses were examined throughout their clinical courses. In all, 6 stage I, 2 stage II, and 8 stage III patients received curative surgical treatment, and 14 did not suffer recurrence. The NY-ESO-1 immune response levels of the patients who did not suffer recurrence decreased after treatment and had fallen below the cutoff level by 9 months after surgery in most cases and did not subsequently increase (Figure 1). The half-lives of their NY-ESO-1 humoral immune response levels were 1.5, 1.6, 2.1, 3.2, and 6.6 months in the stage I patients; 3.0 and 4.0 months in the stage II patients; and 1.6, 1.9, 2.3, 3.0, 3.2, 4.1, and 6.7 months in the stage III patients (mean: 3.0 months). On the other hand, the two patients who underwent curative surgery but subsequently suffered recurrence, M-2 (stage I) and M-11 (stage III), displayed not only incomplete decreases in their NY-ESO-1 humoral immune response levels but also their subsequent restoration to pretreatment levels (Figure 1 and Figure 2A and B). In a comparison between the patients' conventional tumour marker levels and their NY-ESO-1humoral immune response levels, we found that the changes in their CEA and CA19-9 levels were consistent with their NY-ESO-1 immune response levels in patient M-2, whereas patient M-11 was negative for both CEA and CA19-9 throughout their clinical course. Nine stage IV patients who received chemotherapy alone maintained high NY-ESO-1 humoral immune response levels throughout their clinical courses,

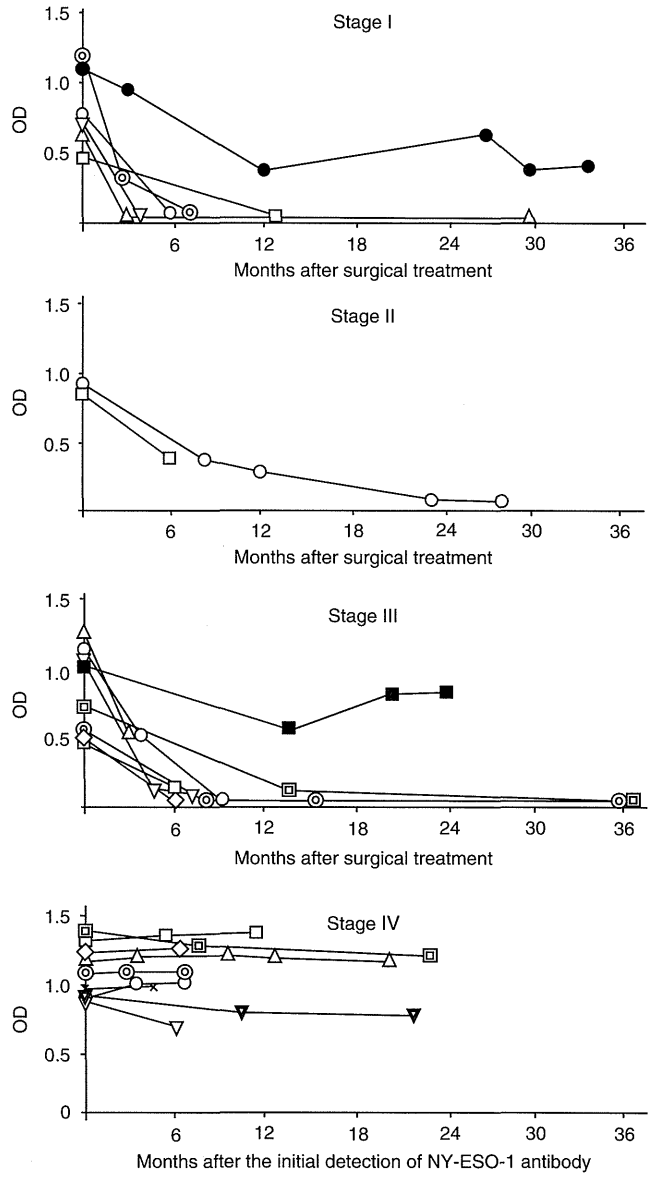

Figure 1. Change in the NY-ESO-1 humoral immune responses of gastric cancer patients after treatment. The serum NY-ESO-1 humoral immune responses of patients with stage I, II, III, or IV gastric cancer in whom NY-ESO-1 antibody production was detected before surgical treatment or chemotherapy were serially analysed. In all, 6 stage I, 2 stage II, and 8 stage III patients received curative surgery, and only 2 patients (●, ■) suffered recurrence. Other 14 patients did not suffer recurrence. Nine patients with stage IV gastric cancer received chemotherapy alone after the initial detection of NY-ESO-1 antibody. Each mark represents a patient. Optical density (OD) values were measured at a serum dilution of 1 : 200.

including some patients who achieved partial tumour responses after chemotherapy (Figure 1).

Prognostic value of the NY-ESO-1 humoral immune response in gastric cancer. The prognostic value of the NY-ESO-1 immune response was evaluated in gastric cancer patients. An analysis of the cumulative overall survival of the gastric cancer patients indicated that there was no difference in the survival rates of the patients who did and did not display positive NY-ESO-1 humoral immune responses (Figure 3A). However, among the patients with higher stage gastric cancer, overall survival was better in the patients in whom NY-ESO-1humoral immune responses were

detected, although the difference was not significant (Figure 3B). NY-ESO-1 protein expression, as detected by IHC, did not affect the overall survival rate (data not shown).

DISCUSSION

NY-ESO-1 antibody was detected in 23.2% of stage III and IV gastric cancer patients, and the combinatorial use of the NY-ESO-1 antibody with CEA and CA19-9 as tumour markers increase the percentage of tumour detection from 45.8 to 57.0%. As the frequency of NY-ESO-1 humoral immune response was relatively low in the patients with early-stage gastric cancer, analysing serum NY-ESO-1 antibody levels alone might not be useful for screening for early-stage gastric cancer. Nevertheless, the expression of NY-ESO-1, a CT antigen, is restricted to tumour tissues and NY-ESO-1 antibody is only detectable in patients with NY-ESO-1-expressing tumours (Stockert *et al*, 1998), indicating the highly specific nature of NY-ESO-1 humoral immune responses in cancer patients. Given that NY-ESO-1 expression by malignant cells is required for antibody induction (Stockert *et al*, 1998), the detection of NY-ESO-1 antibody would be helpful for diagnosing malignancy, although extensive analysis of serum samples from patients with non-cancerous disease, for example, liver or renal disorders, autoimmune diseases, and so on, would be necessary to confirm. In our expression analysis, more NY-ESO-1-positive cases were detected by IHC (19 of 60) than by RT–PCR (6 of 60). This was probably due to the heterogeneous expression of NY-ESO-1 in gastric cancer and the fact that a limited number of biopsy samples were used for the RT–PCR, whereas multiple slices from whole tumour specimens were used for the IHC. Extensive IHC analysis should be used for NY-ESO-1 expression studies of gastric cancer.

We detected a correlation between the NY-ESO-1 humoral immune response levels and the clinical outcome after therapy in gastric cancer patients. The patients who underwent surgery and did not suffer a subsequent relapse displayed consistent decreases in their NY-ESO-1 humoral immune response levels or even the complete disappearance of the NY-ESO-1 antibody from their sera. It is generally accepted that constant immunological stimulation is necessary to maintain a strong humoral immune response (Jager *et al*, 1999). Thus, reduction of antigen doses by the removal of NY-ESO-1-expressing tumour is one possible reason for the observed decreases in these patients' NY-ESO-1 humoral immune response levels after surgery. Patients M-2 and M-11, in whom NY-ESO-1 humoral immune responses remained high for 1 year after surgery and increased thereafter, may have a subclinical residual disease of the so-called 'minimal residual cancer' (Austrup *et al*, 2000; Klein *et al*, 2002) after curative surgery. Local recurrent tumours of 23 and 25 mm in diameter subsequently developed in M-2 and M-11, respectively, suggesting that even a small tumour burden is sufficient to stimulate antibody production. Patient M-2 showed a partial decrease in their NY-ESO-1 humoral immune response levels after the resection of the relapsed tumour, and we are carefully observing the progression of this tumour.

Nine patients with stage IV gastric cancer received chemotherapy alone. Among them, six patients displayed stable disease, two

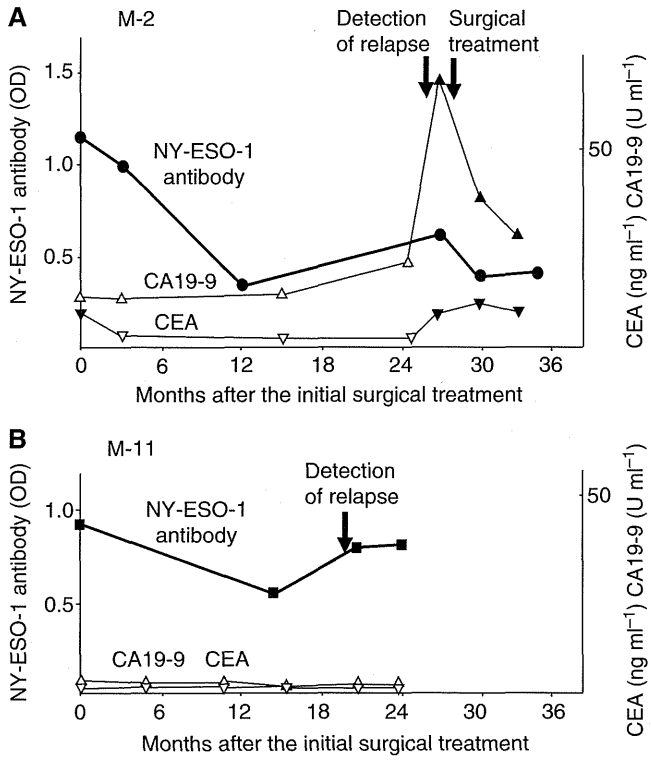

Figure 2. NY-ESO-1 humoral immune response, CEA, and carbohydrate antigen (CA)19-9 levels of patients who relapsed after curative surgery. The NY-ESO-1 humoral immune response (●, ■; Figure 1), CEA (▽), and CA19-9 (△) levels of two patients, M-2 (stage I) (**A**) and M-11 (stage III) (**B**), who underwent curative surgery but subsequently suffered recurrence, were serially analysed. OD values were measured at a serum dilution of 1 : 200. The closed marks indicate CEA or CA19-9 positivity.

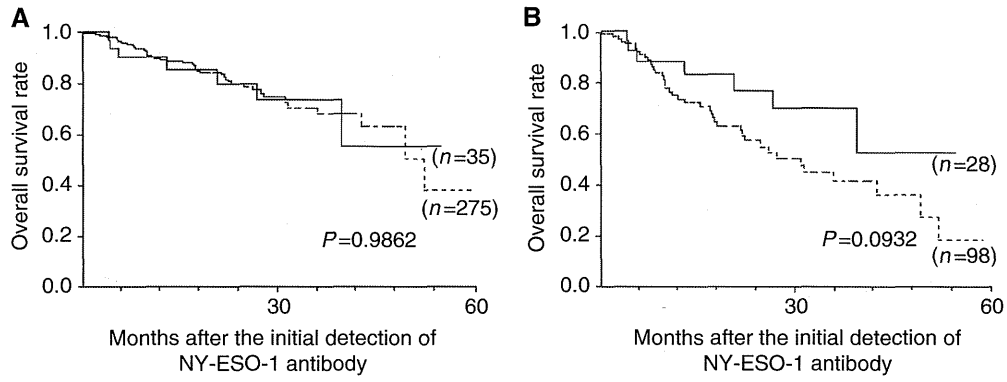

Figure 3. Prognostic role of NY-ESO-1 antibody in gastric cancer patients. The cumulative overall survival rate was analysed in all patients ($n=310$; **A**) and stage III and IV ($n=126$; **B**) gastric cancer patients in whom NY-ESO-1 antibodies were (continuous line) and were not detected (dotted line). The detection of NY-ESO-1 protein by IHC analysis did not affect the overall survival rate (data not shown). Survival curves were plotted using the Kaplan–Meier method. The log-rank test was used for comparisons between groups. *P*-values <0.05 were considered significant.

patients displayed progressive disease, and one patient (M-19) achieved a partial response. Serial analysis of the NY-ESO-1 humoral immune responses of these nine patients including M-19 showed that they barely changed throughout their clinical courses, suggesting that even small tumours are enough to provoke strong NY-ESO-1 humoral immune responses. In this regard, the NY-ESO-1 humoral immune response might not be suitable as a clinical marker for palliative therapy.

We have performed serial cancer vaccine clinical trials with NY-ESO-1 because of its strong immunogenicity and high specificity (Uenaka *et al*, 2007; Wada *et al*, 2008; Kakimi *et al*, 2011). The NY-ESO-1humoral immune response could be a reliable marker of the induction of immune response, as well as for predicting clinical responses in these trials. Furthermore, antibody-based examinations detected both intra- and intermolecular antigen spreading in the sera of patients who had been vaccinated with NY-ESO-1 protein (Kawada *et al*, 2012), suggesting the possible correlation of NY-ESO-1 humoral immne responses and clinical status. In addition, we have started a phase I study of vaccination with NY-ESO-1 protein mixed with Hiltonol (Poly ICLC), Picibanil (OK-432), and Montanide (ISA-51) in patients with NY-ESO-1-expressing cancers (UMIN000007954). Furthermore, NY-ESO-1 vaccine involving modulators of immune checkpoints, for example, anti-CTLA4 antibody and anti-PD-1 antibody, and reagents that are antagonistic to regulatory T cells, for example, anti-CCR4 antibody (Pardoll, 2012) should be considered.

Recently, the antibody against p53, another tumour antigen, has been recognised as a useful tumour marker (Lubin *et al*, 1995). Shimada *et al* (2000)) reported that p53 antibody was detected in 35% of serum samples from patients with *in situ* oesophageal cancer and that it disappeared after endoscopic mucosal resection, proposing that p53 antibody is useful for the early detection and subsequent monitoring of oesophageal cancer. In addition, Müller *et al* (2006) reported that p53 antibody was found in 23.4% of serum samples from cancer patients with 100% accuracy and was correlated with poor prognosis in hepatocellular carcinoma and breast cancer.

Here, we have demonstrated that the NY-ESO-1 humoral immune response could also be valuable as a marker for detecting advanced gastric cancer and inferring whether residual tumour cells remain after treatment, although its frequency in gastric cancer is not very high. We have started a prospective multi-institutional clinical study of NY-ESO-1 humoral immune responses in higher stage gastric cancer patients. In this new study, the NY-ESO-1 humoral immune responses of approximately 100 patients who relapsed after curative surgery will be serially analysed and then followed up. This trial has been registered as UMIN000007925 in Japan.

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LETTER

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T_{reg} induction by a rationally selected mixture of Clostridia strains from the human microbiota

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Manipulation of the gut microbiota holds great promise for the treatment of inflammatory and allergic diseases[1,2]. Although numerous probiotic microorganisms have been identified[3], there remains a compelling need to discover organisms that elicit more robust therapeutic responses, are compatible with the host, and can affect a specific arm of the host immune system in a well-controlled, physiological manner. Here we use a rational approach to isolate $CD4^+FOXP3^+$ regulatory T (T_{reg})-cell-inducing bacterial strains from the human indigenous microbiota. Starting with a healthy human faecal sample, a sequence of selection steps was applied to obtain mice colonized with human microbiota enriched in T_{reg}-cell-inducing species. From these mice, we isolated and selected 17 strains of bacteria on the basis of their high potency in enhancing T_{reg} cell abundance and inducing important anti-inflammatory molecules—including interleukin-10 (IL-10) and inducible T-cell co-stimulator (ICOS)—in T_{reg} cells upon inoculation into germ-free mice. Genome sequencing revealed that the 17 strains fall within clusters IV, XIVa and XVIII of Clostridia, which lack prominent toxins and virulence factors. The 17 strains act as a community to provide bacterial antigens and a TGF-β-rich environment to help expansion and differentiation of T_{reg} cells. Oral administration of the combination of 17 strains to adult mice attenuated disease in models of colitis and allergic diarrhoea. Use of the isolated strains may allow for tailored therapeutic manipulation of human immune disorders.

$CD4^+FOXP3^+$ T_{reg} cells are present most abundantly in the intestinal mucosa at steady state, and contribute to intestinal and systemic immune homeostasis[4–7]. In germ-free mice, the frequency of colonic T_{reg} cells and levels of IL-10 expression by T_{reg} cells are markedly reduced[4–7]. We have shown previously that a combination of Clostridia strains isolated from conventionally reared mice potently affect the number and function of $CD4^+FOXP3^+$ T_{reg} cells in mouse colonic lamina propria[4]. In an attempt to enable clinical translation of our previous findings, we aimed to identify T_{reg}-cell-inducing bacterial strains derived from the human microbiota (see Supplementary Fig. 1 for a summary of the procedure).

We obtained a human stool sample from a healthy Japanese volunteer. Because we previously reported that the chloroform-resistant fraction of mouse gut microbiota was enriched in T_{reg}-cell-inducing species[4], the stool sample was either untreated or treated with chloroform and orally inoculated into IQI/Jic germ-free mice. Each group of ex-germ-free (exGF) mice was separately housed for 3–4 weeks in vinyl isolators to avoid further microbial contamination. Although a recent study showed that the human microbiota had no impact on the immune responses in the mouse small intestine[8], we observed a significant increase in the percentage of $FOXP3^+$ T_{reg} cells among $CD4^+$ T cells in the colons of exGF mice inoculated with untreated human faeces compared with germ-free mice (Fig. 1a and Supplementary Fig. 2). Notably, a more pronounced increase was observed in the colons of exGF mice inoculated with chloroform-treated human faeces (Fig. 1a). These findings suggest that the human intestinal microbiota contains T_{reg}-cell-inducing bacteria, and that they are enriched in the chloroform-resistant fraction. We also examined the effects of human faeces inoculation on colonic IL-17- and IFN-γ-expressing $CD4^+$ cells (T_H17 and T_H1 cells). In exGF mice inoculated with untreated or chloroform-treated human faeces, the frequency of T_H1 cells was unchanged compared with germ-free mice (Fig. 1b). By contrast, there

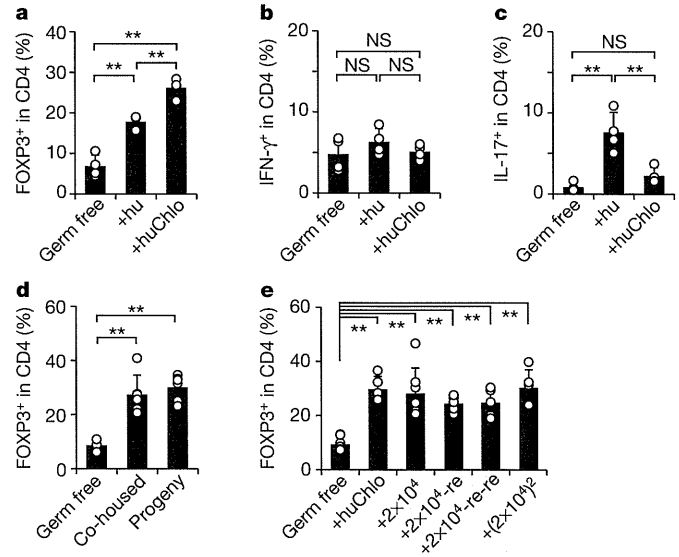

Figure 1 | T_{reg} cell accumulation in germ-free mice induced by inoculation with human microbiota. a–e, The percentages of $FOXP3^+$, $IL\text{-}17^+$ and $IFN\text{-}\gamma^+$ cells within the $CD4^+$ cell population in the colon lamina propria of the indicated mice are shown (see also Supplementary Fig. 2). Circles represent individual animals. The height of the black bars indicates the mean. All experiments were performed more than twice with similar results. Error bars indicate s.d. $^{**}P < 0.01$; NS, not significant. +hu, exGF mice inoculated with untreated human faeces; +huChlo, exGF mice inoculated with chloroform-treated human faeces. (See the main text for further definitions of *x*-axis labels.)

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was a significant accumulation of T_H17 cells in the colons of exGF mice inoculated with untreated human faeces (Fig. 1c and Supplementary Fig. 2). Notably, the capacity of human faeces to induce T_H17 cells was greatly diminished after treatment with chloroform (Fig. 1c). These results indicate that the chloroform-sensitive bacterial fraction in the human stool tested contained T_H17-cell-inducing bacteria, whereas the chloroform-resistant bacteria preferentially promoted T_{reg} cell accumulation in the colon.

To investigate whether T_{reg} cell induction by the chloroform-resistant fraction of human intestinal bacteria is transmissible, adult germ-free mice were co-housed with exGF mice inoculated with chloroform-treated human faeces for 4 weeks. Co-housed mice showed a significant increase in the frequency of colonic T_{reg} cells (Fig. 1d). In addition, the progeny of exGF mice inoculated with chloroform-treated human faeces also showed increased numbers of T_{reg} cells (Fig. 1d). Therefore, T_{reg} cell induction by human intestinal bacteria is horizontally and vertically transmissible. Oral inoculation of germ-free mice with 2×10^4-fold diluted caecal samples from exGF mice inoculated with chloroform-treated human faeces fully induced the accumulation of T_{reg} cells in the colon lamina propria, suggesting that abundant rather than minor members of the intestinal microbiota in exGF mice inoculated with chloroform-treated human faeces drive the observed induction of T_{reg} cells (Fig. 1e). The T_{reg}-cell-inducing microbiota in mice inoculated with the 2×10^4-fold diluted sample ($+2 \times 10^4$ mice) was a stable community, because serial oral inoculation of caecal contents from these mice equally induced the accumulation of T_{reg} cells in secondary ($+2\times10^4$-re mice) and tertiary recipients ($+2\times10^4$-re-re mice) (Fig. 1e). To minimize nonessential components of the microbiota for T_{reg} cell induction, the caecal contents of $+2 \times 10^4$ mice were again diluted 2×10^4-fold and orally inoculated into another set of germ-free mice ($+(2 \times 10^4)^2$ mice). The $+(2 \times 10^4)^2$ mice had a marked accumulation of T_{reg} cells in the colon (Fig. 1e). These results suggested that we succeeded in obtaining mice colonized with a relatively restricted and stable community of bacterial species enriched for T_{reg} cell inducers.

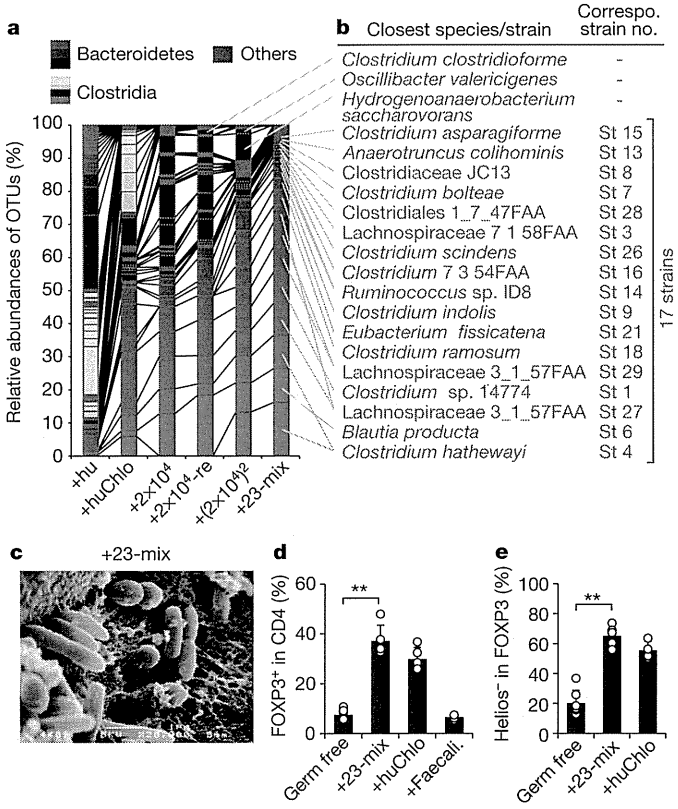

Figure 2 | Assessment of microbiota composition and isolation of T_{reg}-cell-inducing strains. a, b, Pyrosequencing of 16S rRNA genes was performed on caecal contents from the indicated mice. Relative abundance of OTUs (%) in the caecal bacterial community in each mouse (**a**), and the closest species/strain in the database and the corresponding isolated strain number for the indicated OTU (**b**) are shown. **c**, SEM showing the proximal colon of +23-mix mice. Original magnification, ~×20,000. **d, e**, The percentages of $FOXP3^+$ cells within the $CD4^+$ cell population (**d**) and $Helios^-$ cells in $CD4^+FOXP3^+$ cells (**e**) in the colon of the indicated mice. Circles represent individual animals. All experiments were performed more than twice with similar results. Error bars indicate s.d. $^{**}P < 0.01$.

The composition of the gut microbiota in mice treated with human samples was analysed by 16S ribosomal RNA (rRNA) gene amplicon sequencing using a 454 sequencer. Quality filter-passed sequences (3,000 reads for each sample) were classified into operational taxonomic units (OTUs) based on sequence similarity (>96% identity). The numbers of detected reads and closest known species for each OTU are shown in Supplementary Table 1, and the relative abundance of OTUs in each caecal sample is shown in Fig. 2a. As expected, the OTU profiles of mice treated with human faeces were quite different from those of conventional specific pathogen-free (SPF) mice (Supplementary Fig. 3). In mice inoculated with untreated human faeces, OTUs belonging to Bacteroidetes accounted for about 50% of the caecal microbial community (Fig. 2a). By contrast, most OTUs in exGF mice inoculated with chloroform-treated human faeces were related to Clostridia species. Most bacteria in $+2 \times 10^4$, $+2 \times 10^4$-re and $+(2 \times 10^4)^2$ mice had 16S rRNA gene sequence similarities with about 20 species of Clostridia, listed in Fig. 2b.

To isolate bacterial strains with T_{reg}-cell-inducing capabilities, we cultured caecal contents from $+2 \times 10^4$, $+2 \times 10^4$-re and $+(2 \times 10^4)^2$ mice *in vitro* and picked 442 colonies. BLAST searches of 16S rRNA gene sequences of the isolated colonies revealed that 31 strains in total were present, all of which were Clostridia (Supplementary Fig. 4). Of the 31 strains, we selected 23 that had less than 99% 16S rRNA gene sequence identity to any of the other 30 strains (Supplementary Fig. 4). We then individually cultured the 23 strains, mixed them in equal amounts, and orally inoculated the mixture into germ-free IQI mice (+23-mix mice). Numerous rod- and round-shaped bacteria were observed by scanning electron microscopy (SEM) on the epithelial cell surface in +23-mix mice (Fig. 2c), and the size and appearance of the caeca were quite different from those in germ-free mice, indicating successful colonization (Supplementary Fig. 5a). Pyrosequencing of 16S rRNA genes revealed that the caecal microbiota composition in +23-mix mice was quite similar to that in $+(2 \times 10^4)^2$ mice (Fig. 2a). In +23-mix mice, we observed efficient induction of T_{reg} cells in the colonic lamina propria (Fig. 2d). The magnitude was comparable to that observed in exGF mice inoculated with chloroform-treated human faeces and much higher than that in mice colonized with *Faecalibacterium prausnitzii*, a human Clostridia strain well known for enhancing regulatory cell functions[9] (Fig. 2d). Most T_{reg} cells in +23-mix mice expressed low levels of Helios (also known as IKZF2), indicating antigen-experienced cells (Fig. 2e, Supplementary Fig. 5b and ref. 10).

Only 17 strains listed in Fig. 2b and Supplementary Fig. 4 were detected in +23-mix mice by 16S rRNA gene sequencing, indicating that these 17 strains may be sufficient to induce T_{reg} cells. Indeed, we found that the mixture of 17 strains (17-mix) induced $FOXP3^+$ T_{reg} cells to a similar extent as the 23-mix (Fig. 3a). The increase in T_{reg} cells induced by the 17-mix was reproducibly observed in exGF mice of different genetic backgrounds (IQI, BALB/c and C57BL/6) (Fig. 3a). Moreover, the mix was effective in other rodents: the frequency of colonic T_{reg} cells in exGF rats inoculated with 17-mix was significantly higher than that in germ-free rats and comparable to that in SPF rats (Fig. 3a). The colonization with 17-mix induced a significant increase in the frequency of $IL\text{-}10^+$ and/or $ICOS^+$ cells within the T_{reg} cell population, as revealed by analysis of exGF IL-10 reporter mice ($Il10^{Venus}$ mice, ref. 4) colonized with the 17-mix (Fig. 3b). Furthermore, $IL\text{-}10^+$ T_{reg} cells in

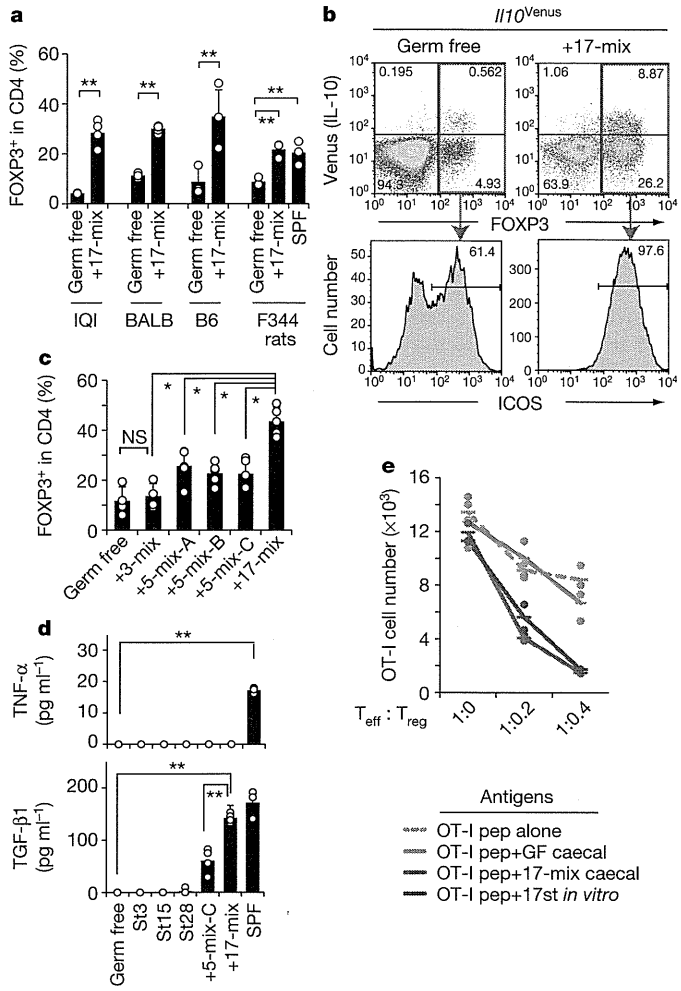

Figure 3 | Characterization of 17 T_{reg}-cell-inducing strains. **a**, The percentages of $FOXP3^+$ cells within the $CD4^+$ cell population in the colon lamina propria of the indicated mice and rats. **b**, The expression of Venus (IL-10) and FOXP3 by the gated colonic lamina propria $CD4^+$ cells, and ICOS expression by $CD4^+FOXP3^+$ cells in exGF *Il10*^{Venus} mice colonized with or without 17-mix. **c**, Percentages of $FOXP3^+$ cells within the $CD4^+$ cell population in IQI exGF mice colonized with the indicated mix. **d**, The production of TNF-α and TGF-β1 in HCT8 cells stimulated with caecal extracts from the indicated mice. **e**, $CD8^+$ T cells from OT-I mice (T_{eff}) and the indicated ratio of colon lamina propria $CD4^+CD25^+$ cells from +17-mix mice (T_{reg}) were incubated with $CD11c^+$ cells pulsed with OT-I peptide alone or in combination with autoclaved caecal contents from +17-mix mice (+17-mix caecal), germ-free mice (+GF caecal), or autoclaved 17 strains cultured *in vitro* (+17 st *in vitro*). Depicted data represent average of duplicates (see also Supplementary Fig. 9c). Circles in **a**, **c**–**e** represent samples from individual animals. All experiments were performed more than twice with similar results. Error bars indicate s.d. $^{**}P < 0.01$; $^{*}P < 0.05$; NS, not significant.

+17-mix mice expressed high levels of CTLA4 (Supplementary Fig. 5c). Because IL-10 and CTLA4 are essential for the immunosuppressive activity of T_{reg} cells[11,12], and ICOS is required for the T_{reg}-cell-mediated suppression of T_H2 responses[13], these results suggest that the mixture of 17 strains affects both the number and function of T_{reg} cells in the colon. Next, we monocolonized germ-free mice with one of each of the 17 individual strains to determine their individual T_{reg} cell induction capability. The monocolonized exGF mice exhibited low to intermediate levels of T_{reg} cell induction with inter-individual variability (Supplementary Fig. 6a). As expected, none of the strains induced T_H17 cells in the monocolonized mice (Supplementary Fig. 6b). We also examined T_{reg} cell induction by subsets of the 17-mix (randomly selected combinations of 3–5 strains: 3-mix, 5-mix-A, 5-mix-B, and 5-mix-C, see Supplementary Fig. 4). Although all tested combinations of 5-mix induced increases in the frequency of T_{reg} cells, the magnitude was substantially lower than that observed in +17-mix mice (Fig. 3c). Therefore, it is likely that the 17 strains act synergistically to amplify the induction of T_{reg} cells in a microbial-community-dependent fashion.

To investigate the mechanism for the T_{reg} cell induction by the community of 17 strains, we incubated various human and mouse intestinal epithelial cell lines and primary cells with aqueous extracts from caecal contents from the +17-mix mice, and assessed the production of the active form of TGF-β1, a key cytokine for the differentiation and expansion of T_{reg} cells. The caecal extracts from +17-mix mice routinely elicited TGF-β1, but not IL-6 and TNF-α, production, and the magnitude was significantly higher than that elicited by caecal extracts from single-strain or 5-mix-colonized mice (Fig. 3d and Supplementary Fig. 7). The induction of TGF-β1 was not inhibited by pre-treatment of the caecal extracts with a protease or nuclease (Supplementary Fig. 7c). Short-chain fatty acids (SCFAs) are protease- and nuclease-insensitive and have been associated with regulation of host immune homeostasis[14]. Quantitative analysis of SCFAs in caecal contents from +17-mix mice showed a significantly higher concentration of acetate, propionate, butyrate and isobutyrate than that in single-strain or 5-mix-colonized mice (Supplementary Fig. 8a). Furthermore, a mixture of sodium salts of these SCFAs elicited TGF-β1 production in epithelial cells to a level similar to that seen when the cells were stimulated with caecal extracts from +17-mix mice (Supplementary Fig. 8b). Therefore, the community of 17 strains cooperatively produces SCFAs that can elicit a TGF-β response, and this activity may contribute to the differentiation and expansion of T_{reg} cells. We also investigated whether the 17 strains provide bacterial antigens to T cells. To do this, we addressed the antigen specificity of T_{reg} cells accumulated in +17-mix mice using a cognate antigen-driven suppression assay. $CD4^+CD25^+$ lamina propria T cells from +17-mix mice substantially inhibited the OT-I ovalbumin (OVA) peptide-driven proliferation of OT-I CD8 T cells, and this suppression was markedly enhanced in the presence of autoclaved caecal content from +17-mix mice or autoclaved 17 strains cultured *in vitro*, but not in the presence of OT-II OVA peptide or caecal content from germ-free mice (Fig. 3e and Supplementary Fig. 9). These results are consistent with previous reports[15,16] and suggest that some fraction of colonic lamina propria T_{reg} cells in +17-mix mice is specific to the 17 strains of Clostridia. Next, we assessed the kinetics of T_{reg} cell accumulation and their expression of Ki67, a cell-cycle-associated nuclear protein, and gut-homing-associated molecules CD103 and β7 integrin. We observed a marked increase in the proportion of Ki67, CD103 and β7 expressing cells by 1 week after inoculation with the 17-mix (Supplementary Figs 10 and 11). Collectively, these observations indicate that the 17 strains provide SCFAs, bacterial antigens and probably other factors, which together contribute to differentiation, expansion and colonic homing of T_{reg} cells.

To define the identity of the 17 bacterial strains fully, we sequenced their genomes (Supplementary Fig. 12). Phylogenetic comparison of the 17 strains using ribosomal multi locus sequencing typing (rMLST) revealed that the 17 strains belong to bacterial species falling within clusters XIVa, IV and XVIII of Clostridia as defined previously[17] (in a recent taxonomy, members of cluster XVIII Clostridia were reclassified in the class Erysipelotrichi) (Supplementary Fig. 13). The genome sequencing also revealed that the 17 strains all lack strong virulence-related genes such as collagenase and phospholipase C, often identified in pathogenic Clostridia species (Supplementary Table 2). We then examined the relative abundance of the 17 strains in healthy and ulcerative colitis human subjects using draft genome sequences of the 17 strains and publicly available human microbiome genomes generated through the MetaHIT project[18]. Ulcerative colitis subjects showed a tendency towards a reduction of the 17 strains, and 5 out of the 17 strains were significantly reduced in ulcerative colitis patients (Supplementary Fig. 14).

To evaluate the potential benefits of supplementation with the 17 strains, 17-mix or control PBS was orally administered into adult SPF

mice every 2 or 3 days (SPF + 17-mix or SPF + ctrl, respectively). We confirmed a significant increase in the frequency of colonic T_{reg} cells in SPF + 17-mix mice compared with SPF + ctrl mice after 3 weeks of treatment (Fig. 4a). While being continuously treated with 17-mix or control, mice were subjected to the OVA-induced allergic diarrhoea model[19]. The occurrence and severity of diarrhoea and the OVA-specific serum IgE levels were significantly reduced in SPF + 17-mix mice relative to control mice (Fig. 4b–d). The protective effect of 17-mix was significantly attenuated by treatment of mice with a T_{reg}-cell-depleting anti-CD25 antibody (Supplementary Fig. 15). We also subjected mice to an experimental colitis model induced by trinitrobenzene sulphonic acid (TNBS)[20]. SPF + 17-mix mice showed less severe colon shortening and milder histological disease features, accompanied by lower mortality

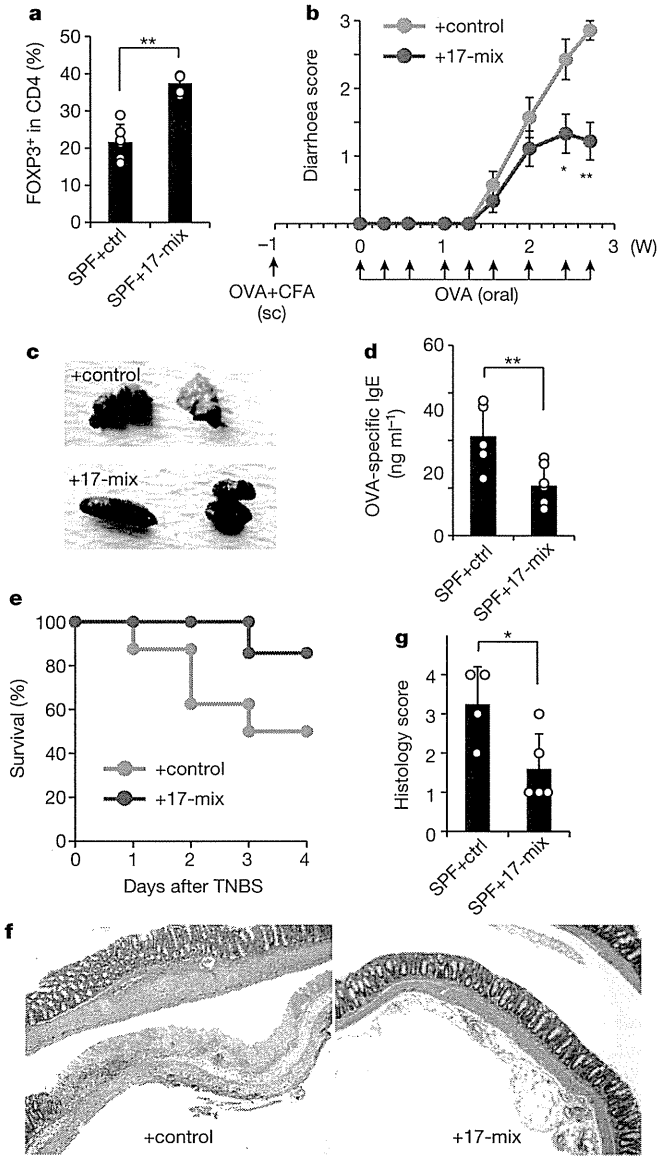

Figure 4 | Treatment with 17-mix suppresses experimental colitis models. **a**, The percentages of $FOXP3^+$ cells within the $CD4^+$ cell population in SPF + 17-mix or SPF + ctrl mice. **b–d**, SPF + 17-mix ($n = 9$) and SPF + ctrl ($n = 7$) mice were subjected to OVA-induced diarrhoea. The diarrhoea score (**b**; see Methods for definition), representative photographs of faeces (**c**), and OVA-specific IgE levels in the sera (**d**) are shown. sc, subcutaneous. **e–g**, SPF + 17-mix ($n = 8$) and SPF + ctrl ($n = 7$) were treated with TNBS. Animal survival (**e**), haematoxylin and eosin staining (original magnification, ×10) (**f**), and histology score of the distal colon (**g**) on day 4 after TNBS administration are shown. Data are representative of two independent experiments. Error bars indicate s.d. $^{**}P < 0.01$; $^{*}P < 0.05$.

than control mice (Fig. 4e–g and Supplementary Fig. 16a). In keeping with this clinical outcome, there was significantly increased expression of *Foxp3* and *Tgfb1* mRNA in SPF + 17-mix mice compared with control mice, as well as a tendency towards a reduction of inflammatory cytokine transcripts (Supplementary Fig. 16b). Identical suppression of colitis by 17-mix was also observed in an adoptive transfer model, in which germ-free SCID mice were orally inoculated with faeces from SPF mice together with or without 17-mix and then transferred with $CD4^+CD45RB^{hi}$ T cells (Supplementary Fig. 17).

The clinical track record of efficacy of single-strain probiotics has been modest. It has been postulated that a collection of functionally distinct bacterial species rationally selected from the human gut microbiota may be more effective than single strains in preventing/treating disease[21]. In the present study, we isolated 17 strains within Clostridia clusters XIVa, IV and XVIII from a human faecal sample; these strains affect T_{reg} cell differentiation, accumulation and function in the mouse colon. It remains to be seen whether the 17 strains will have similar effects in the human intestine; however, a decreased prevalence of Clostridia clusters XIVa and IV in faecal samples from patients with inflammatory bowel disease and atopy[22–24] may suggest that supplementation with the 17-strain bacterial community might counterbalance dysbiosis, induce T_{reg} cells and aid in the management of allergic and inflammatory conditions.

METHODS SUMMARY

Experiments were performed with authorization from the Institutional Review Board for Human Research at RIKEN Yokohama Research Institute. Human stool from a healthy volunteer (Japanese, male, age 31 years) was obtained with informed consent. The sample was mixed with or without chloroform, and the aliquots were inoculated into germ-free IQI mice. Detailed procedures for lamina propria lymphocyte analysis, isolation of bacteria, extraction of bacterial DNA and sequencing are described in Methods. Statistical analysis was performed using the Student's *t*-test.

Full Methods and any associated references are available in the online version of the paper.

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Author Contributions K.Ho. planned experiments, analysed data and wrote the paper together with B.O. and M.H.; K.A. and T.Tano. performed immunological analyses and bacterial cultures together with Y.N., S.N. and H.M.; W.S., K.O., S.K. and M.H. performed bacterial sequence analyses; K.M. and S.U. provided essential materials; H.N., T.S. and S.S. supervised the T_{reg} cell suppression assay; S.F., K.Ha., H.O., T.Tani., J.V.F. and P.W. were involved in data discussions.

Author Information All genome sequence data are deposited in DDBJ BioProject ID PRJDB521-543. Reprints and permissions information is available at www.nature.com/reprints. The authors declare competing financial interests: details are available in the online version of the paper. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to M.H. (hattori@k.u-tokyo.ac.jp) or K.Ho. (kenya@rcai.riken.jp).