Development of a T Cell Receptor Mimic Antibody against Wild-Type p53 for Cancer Immunotherapy

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Abstract

The tumor suppressor p53 is widely dysregulated in cancer and represents an attractive target for immunotherapy. Due to its intracellular localization, p53 is inaccessible to classical therapeutic monoclonal antibodies, an increasingly successful class of anti-cancer drugs. However, peptides derived from intracellular antigens are presented on the cell surface in the context of major histocompatibility class I (MHC I), and can be bound by T cell receptors (TCRs). Here, we report the development of a novel antibody, T1-116C, that acts as a TCR mimic to recognize an HLA-A*0201-presented wild-type p53 T cell epitope, p53_{65-73} (RMPEAAPPV). The antibody recognizes a wide range of cancers, does not bind normal peripheral blood mononuclear cells, and can activate immune effector functions to kill cancer cells in vitro. In vivo, the antibody targets p53_{65-73} peptide-expressing breast cancer xenografts, significantly inhibiting tumor growth. This represents a promising new agent for future cancer immunotherapy.

Introduction

Classical therapeutic antibodies commonly target cell surface or secreted antigens but are unable to access intracellular proteins. However, intracellular proteins are degraded by proteasome-dependent and independent mechanisms, resulting in the generation of peptides for surface presentation by major histocompatibility complex (MHC) class I(1). This presentation of peptides derived from intracellular proteins on the cell surface is part of the normal cellular process enabling the recognition of intracellular antigens by the immune system, in particular CD8+ T cells whose T cell receptors (TCRs) bind the MHC class I-presented peptides to enable killing of cells expressing foreign antigens. Antibodies mimicking this ability of T cells to recognize MHC class I-presented peptides, so-called TCR mimic (TCRm) or TCR-like...
antibodies, have been generated against several intracellular antigens presented by common human leukocyte antigen (HLA) haplotypes such as HLA-A*0201 (HLA-A2) and have demonstrated potential therapeutic efficacies in various models (2-4).

One of the most extensively studied tumor-associated antigens is the tumor suppressor p53, whose widespread deregulation and involvement in malignant transformation make it an almost universal target for the immunotherapy of cancer (5). p53-derived peptides have been investigated as targets in various immunotherapy strategies including vaccines, recombinant TCRs and TCRm antibodies (6-8). Missense mutations in TP53 commonly lead to an accumulation of p53 protein in the cytosol, which leads to enhanced processing (of both wild-type and mutant peptides) by the antigen processing machinery (9). Evidence from studying the humoral immune responses in cancer patients is that they recognize both wild-type and mutant p53 epitopes, without mutant p53 containing immunodominant epitopes (10). The diversity of TP53 mutations, scarcity of mutant p53-derived T cell epitopes (11,12) and alternative mechanisms that regulate the wild-type p53 protein make immunotherapeutic strategies targeting wild-type p53 epitopes more broadly applicable and thus these have been actively pursued in a clinical setting (13-15).

Here we report the production of a novel TCRm antibody targeting a wild-type p53-derived peptide and its potential application in tumor immunotherapy.

Materials and Methods

Cell culture

The following cell lines were purchased from American Type Culture Collection (ATCC) in 2014: A2058, AU565, CALU6, COR-L23, G361, Hs-695T, MDA-MB-231, NCI-H1299, NCI-H1395, NCI-H1930, NCI-H1975, NCI-H2087, and PANC-1. Colo-205 was purchased from ATCC in 2015. Cell lines
purchased from German Collection of Microorganisms and Cell Cultures (DSMZ): OCI-Ly1 (2004), OCI-Ly8 (2004), SW480 (2004), Granta-519 (prior to 2000), and KM-H2 (prior to 2000). MDA-MB-453, MDA-MB-468, T47D, and MCF-7 were obtained from Cancer Research UK Claire Hall Laboratories (London, UK) in 2004. CCRF-CEM, HUT 78, KARPAS-299, and RPMI 8402 were from Georges Delsol (Toulouse, France) between 2000-2004. Daudi and Jurkat were obtained from the Sir William Dunn School of Pathology (University of Oxford, UK) prior to 2000. OCI-Ly3 and SU-DHL-6 were from Dr Eric Davis (NIH, USA) in 2000. MO1043 was obtained from Prof Riccardo Dalla-Favera (Columbia University, USA) in 2014, T2 and HL-60 from Prof Alain Townsend (University of Oxford, UK) prior to 2000, and 143B from Dr Judy Bastin (University of Oxford, UK) in 2015. MOLT-4 was obtained from Neckar Hospital, Paris prior to 2000. Colo-678 was from Prof Walter Bodmer (University of Oxford, UK) in 2013. Thiel was from Prof Diehl (University of Cologne, Germany). FL-18 was from Shirou Fukahara (Kyoto University, Japan) prior to 2000. SU-DHL-1 was from Dr Steve Morris (St Jude's, Memphis, USA) prior to 2000.

Hematological cell lines were cultured in RPMI containing 10% fetal bovine serum (Life Technologies, #10082147), and others in DMEM containing 10% serum, supplemented with penicillin/streptomycin (100U/ml) and L-glutamine (2mM). The cells were cultured in 37°C incubators containing 5% CO₂. Experiments were performed using cells within maximum of 15 passages after thawing. The cell lines undergo periodic testing to ensure freedom from mycoplasma contamination using PlasmoTest Mycoplasma Detection kit (Invitrogen). MDA-MB-231 and Thiel were recently authenticated using STR profiling by LGC Standards, UK, we experimentally performed the HLA-A2 and p53 expression profiling 'in house'.
Generation of HLA-A2 tetramers

A bacterial expression construct encoding the human HLA-A*0201 extracellular domain (amino acids 24-293) fused with a C-terminal BirA biotinylation sequence (LNDIFEAQKIEWH), and separate construct expressing mature human β2 microglobulin (β2m, amino acids 21-119), were each generated and transformed into competent Escherichia coli strain BL21(DE3). Protein expression was induced by addition of 0.5mM IPTG in low-salt LB medium (1% Tryptone, 0.5% Yeast extract and 0.5% NaCl w/v), and insoluble inclusion bodies containing the recombinant proteins were purified using BugBuster (Merck, #70750-3), according to the manufacturer's instructions. Peptides were synthesized by the peptide synthesis facility in the Weatherall Institute of Molecular Medicine (University of Oxford).

HLA-A2 tetramers were generated as previously described(16, 17). Briefly, HLA-A*0201 (15mg), β2m (12.5mg) and peptide (5mg) were added into 500ml of refolding buffer (100mM Tris.Cl pH8.0, 400mM L-Arginine, 2mM EDTA, 5mM reduced-glutathione, 0.5mM oxidized-glutathione, and 0.1mM PMSF) and refolded for 48h. The refolding complex was concentrated and buffer exchanged to 10mM Tris-HCl pH8.0, then biotinylated with BirA protein biotin ligase (Avidity LLC, #BirA500). Biotinylated protein was then separated using an Akta Purifier FPLC with a Sephadex 75 column and HLA-A2/β2m/peptide monomers were isolated and subsequently stored at -80°C. Aliquots were thawed and tetramerized with Extravidin (Sigma-Aldrich, #E2511) on use.

Generation of anti-p53 TCRm monoclonal antibodies

All in vivo work was approved by local ethics review committee and governed by appropriate Home Office establishment, project and personal licenses. MF1 mice (6-8 week old females) were immunized with the HLA-A*0201/p53 tetramers 4 times with 100 μg tetramer at 10 day intervals and fusions were performed two days after
the final immunization. A standard fusion protocol was followed (18) with NS0 murine
myeloma cells as the fusion partner, and hybridomas were grown out under
hypoxanthine, aminopterin and thymidine (HAT) selection. Hybridoma supernatants
were screened for the presence of secreted antibodies specifically, or preferentially,
recognizing the immunizing tetramer rather than a control tetramer by ELISA.
Positive hybridoma colonies were expanded and cloned by limiting dilution for further
validation.

Production of purified antibodies
Production of purified TCRm antibodies from hybridoma supernatant was achieved
by culturing hybridoma cells in serum-free medium to extinction, or in CL350
bioreactors (Sigma-Aldrich, #Z688037), followed by protein A or protein G purification
of immunoglobulin.
Endotoxin-free recombinant T1-116C antibody (mlgG1) production, and its isotype
switching (mlgG2a or hlgG1), were outsourced to Absolute Antibody Ltd after the
antibody variable region cDNAs were cloned based on a published method (19).
Briefly, T1-116C heavy and light chains were cloned into pUVE vectors, then
transiently transfected into ABS293 cells. Culture supernatants were harvested and
antibody purified through Protein A affinity chromatography. Purified antibody was
analyzed by SDS-PAGE and endotoxin level was determined by LAL chromogenic
endotoxin assay (Thermo Scientific, #88282).

T2 cell binding assay
TAP-deficient T2 cells cultured at logarithmic phase were pulsed with peptides at
100mM (or a range of lower concentrations for peptide titration experiments) for 12-
16h in a flat bottom 96-well tissue culture plate under standard cell culture conditions.
Cells were then harvested and stained with TCRm antibodies and/or HLA-A2-specific
mAb BB7.2 (Abcam, #ab74674), followed by APC conjugated goat anti-mouse
secondary antibody (eBioscience, #17-4010-82). Samples were washed with FACS wash buffer (2% FBS in PBS + 0.1% sodium azide) then fixed with 1% paraformaldehyde (in PBS) and acquired with a FACSCalibur (BD Biosciences).

Western blotting

Whole cell lysates were prepared using Mammalian Protein Extraction Reagent (Thermo Scientific, 78503) containing a nuclease to degrade any nucleic acids and additional protease and phosphatase inhibitors. Protein concentrations were quantified using BCA assay (Thermo Scientific, 23227). 30μg whole cell lysates were resolved on 10% polyacrylamide gels and transferred to Protran™ nitrocellulose membranes (GE Healthcare, 15269794). Membranes were blocked in 5% (w/v) low fat milk in PBS for 1 hour at RT, and were then incubated with primary antibodies overnight at 4°C diluted in 5% (w/v) low fat milk in PBS (mouse anti-p53 (DO-1, Santa Cruz Biotechnology, sc-126, 1ug/ml); mouse anti-p53 (DO-7, Santa Cruz Biotechnology, sc-47698, 1ug/ml); mouse anti-p53 (Pab1801, Santa Cruz Biotechnology, sc-98, 1ug/ml); mouse anti-β-Actin (Sigma, clone AC-15) 1:20,000). This was followed by washing of the membranes in PBS (three washes) and PBS-Tween (0.1% v/v, one wash) at RT (5 minutes each wash) then incubation in secondary antibody solution (goat anti-mouse IgG-HRP (Dako, P0447) diluted 1/5000 in 5% (w/v) low fat milk in PBS) for 1 hour at RT. After washing as above, antibody binding was detected using ECL reagent (GE Healthcare, RPN2106) and visualized with a G:BOX ChemiXRQ imaging system (Syngene).

Mass spectrometry identifying HLA-I associated peptides

Sample processing and data analysis were carried out as previously described(20). Briefly, 10⁹ MDA-MB-231 and MCF-7 cells were lysed and cleared by centrifuging at 300g for 10min at 4°C to remove nuclei, followed by 15000g for 45min at 4°C to pellet other insoluble material. HLA complexes were captured by rotating 1ml W6/32
conjugated immunoresin (2.5mg/ml) with the cleared lysates overnight at 4°C. Beads were re-packed in the column and washed by using subsequent runs of ice-cold 50mM Tris buffer (pH 8.0) containing first 150mM NaCl and 0.005% NP40, then 150mM NaCl, followed by 400mM NaCl and lastly just 50mM Tris buffer. HLA-peptide complexes were eluted by using 5ml ice-cold 10% acetic acid and dried. Samples were analyzed on an Ultimate 3000 HPLC system (Thermo Scientific) online coupled to a Q-Exactive Hybrid Quadrupole-Orbitra Mass Spectrometer (Thermo Scientific). Raw data were analyzed using Peaks 7.5 (Bioinformatics solutions) with a database containing all annotated human SwissProt entries.

Quantitation of antibody molecules bound per target cell

Cell lines or T2 cells pulsed with the RMPEAAPPV peptide at 0.5-100µM concentrations or the Flu peptide at 100µM were stained with PE-conjugated T1-116C mAb (mAb:PE = 1:1) or an isotype matched control antibody at 10µg/ml for 30min on ice. Cells were washed with FACS Wash buffer then fixed with 1% paraformaldehyde before being analyzed with a FACSCalibur (BD Biosciences). QuantiBRITE-PE beads (BD Biosciences, #340495) were acquired in parallel and correlation between geometric means (corrected to remove background binding to isotype control antibody) and PE molecules/beads of the four QuantiBRITE bead populations was established according to the manufacturer’s instructions. Number of T1-116C-PE antibody molecules bound per cell was calculated based on the correlation formula and subtraction of background from negative cells.

Complement Dependent Cytotoxicity (CDC) Assay

1x10^5 cells were opsonized with antibody for 15min at RT in a flat-bottom 96-well plate. Human serum was added to a final volume of 10% and incubated for 30min at 37°C. Cells were transferred to a FACS tube where 10µL propidium iodide (PI) solution (10µg/mL in PBS) was added prior to data acquisition. Percentage cell
death was defined as the percentage PI+ cells of the total cell population. Means from duplicate wells from each condition were calculated.

**Antibody Dependent Cellular Phagocytosis (ADCP) Assay**

Mouse bone marrow derived macrophages (BMDM) were differentiated from the bone marrow of WT BALB/c female mice and cultured for 7-10 days in the presence of 20% L929 conditioned media (containing M-CSF). 5x10⁴ BMDM per well were plated in a flat-bottom 96-well plate the day before the assay was performed as previously described(21). In brief, target cells were labeled with Carboxyfluorescein succinimidyl ester (CFSE) at RT before being washed once in RPMI media. The CFSE labeled cells were opsonised with antibody for 30min at 4°C, washed once and then 2.5x10⁵ opsonized target cells added to the BMDM and left to co-culture at 37°C for 1h. The BMDM were labeled with anti-F4/80-APC (AbD Serotec, #MCA497APC) and the wells washed with PBS, before removal and analysis of the cells on FACSCalibur (BD Biosciences). Percentage phagocytosis was defined as the percentage of CFSE+F4/80+ cells of the total F4/80+ population. Means from triplicate wells from each condition were calculated.

**Antibody Dependent Cellular Cytotoxicity (ADCC) Assay**

Human peripheral blood mononuclear cells (PBMC) isolated by density gradient centrifugation were sourced from the National Blood Service and studies were conducted under ethical approval from the NRES Committee South Central – Oxford B (C06.216). Target cells were labeled with calcein AM (Life Technologies, #C1430) and suspended in RPMI. The labeled cells were opsonized with antibody for 30min at 4°C before washing once in RPMI media. The target cells and PBMC effector cells were co-cultured at a 50:1 (Effector:Target) ratio for 4h at 37°C. The cells were pelleted by centrifugation (1500rpm for 5min), the supernatant transferred to a white 96-well plate, and read using a Varioskan Flash (Thermo Scientific) to record calcein
release (excitation wavelength 485nm; emission wavelength 530nm). Per cent of maximum lysis was defined as the calcein release compared to the response recorded when cells were treated with 4% TritonX-100 solution. Means from triplicate wells from each condition were calculated.

**Antibody radiolabeling**

T1-116C-mIgG2a and an isotype control antibody (Absolute Antibody Ltd) were radiolabeled with $^{111}$In as previously described(22). Briefly, 500μg of T1-116C or isotype control antibody was dissolved in 0.1M sodium bicarbonate aqueous buffer (pH 8.2) before adding a 20-fold molar excess of 2-(4-isothiocyanatobenzyl)-diethylenetriaminepentaacetic acid ($\text{p-SCN-Bn-DTPA}$; Macrocyclics) and incubating for 1h at 37°C. The DTPA-conjugated antibody was subsequently purified using a Sephadex G50 gel filtration column and radiolabeled using $^{111}$In-chloride (1MBq per 1μg of IgG). The protein was further purified by Sephadex G50 size exclusion chromatography. Radiochemical purity was determined by instant thin layer chromatography (iTLC) as >95%.

**In vivo imaging and biodistribution**

Female BALB/c $\text{nu/nu}$ mice (Charles Rivers Laboratories) were injected subcutaneously on their flanks with $1\times10^6$ MDA-MB-231 or MDA-MB-468 breast cancer cells. $^{111}$In-labeled T1-116C or mlgG2a isotype control antibody (5MBq, 5μg) was administered intravenously when tumor sizes reached 120mm$^3$ at day 20, and SPECT/CT imaging was performed at 24, 48, 72h after injection, using a Bioscan NanoSPECT/CT. Volume-of-interest analysis was performed on SPECT images using the Inveon Research Workplace software package (Siemens). After imaging at 72h post injection, animals were sacrificed and selected organs were removed, rinsed, blot dried, weighed, and the amount of $^{111}$In in each tissue was measured.
using an automated gammacounter. Uptake of $^{111}$In was expressed as the percentage of the injected dose per gram of tissue (%ID/g).

**Tumor in vivo growth experiments**

MDA-MB-231 cells (1×10$^7$) in 100μl Matrigel were injected subcutaneously into the flank of BALB/c nu/nu mice (Crl:NU-Foxn1$^{nu}$, 6-8 weeks female, weight 15-22g, Charles River Laboratories). Animals were randomly grouped and antibodies or PBS was administered twice a week (10mg/kg for Ab and 200μl for PBS) by intraperitoneal injection. Tumor sizes were calculated as length x width x height x $\pi / 6$. Geometric Mean Diameter (GMD) was calculated as $(L \times W \times H)^{1/3}$. Student $t$ test was used to evaluate the growth curves.

**Results**

**Generation of p53/HLA-A2 murine monoclonal antibodies**

A peptide derived from an N-terminal region of wild-type p53 that is rarely mutated was selected to enable targeting of the maximal number of potential patients, including those carrying the most common mutations leading to premature termination of p53 translation (R196X and R213X). This p53$^{65-73}$ peptide RMPEAAPPV (p53RMP) has also been proven to have endogenous presentation(23,24) and has been tested in clinical trials of p53 vaccines without patients experiencing any adverse side effects(7). HLA-A2/p53RMP tetramers were produced and shown to be able to display the p53RMP peptide to T cells (Supplementary Fig.1). These tetramers were used as the immunogen to generate TCRm mAbs recognizing p53RMP presented by HLA-A2 using classical hybridoma technology. Hybridoma supernatants were screened for reactivity against the immunizing p53RMP tetramer, and for specificity by their lack of binding to a tetramer comprising HLA-A2 with a non-related peptide derived from influenza A virus M1
protein (Flu), by ELISA. Unsurprisingly the majority of the antibodies failed to
demonstrate specificity for the p53RMP containing tetramer and thus recognized the
MHC portion of the complex (a representative example is illustrated in
Supplementary Fig.2).
The T1-116C hybridoma stably secreted antibodies recognizing the immunizing p53
tetramer but not the control tetramer by ELISA (Supplementary Fig.2). Antibody
binding specificity towards the p53RMP peptide was further validated on the surface
of human T2 lymphoblast cells. T2 cells are deficient in the transporter associated
with antigen processing (TAP) and pulsing them with an HLA-A2-binding peptide
stabilizes the HLA-A2/peptide complex on the cell surface. T1-116C antibodies
stained the cell surface of T2 cells pulsed with the target p53RMP peptide but not T2
cells pulsed with the Flu peptide, survivin and HCMV peptides or non-target peptides
derived from p53 (Fig.1a).

The T1-116C antibody was protein A purified from the hybridoma supernatants and
was further tested for a dose response in its binding to the p53RMP/HLA-A2 complex
on the cell surface. T2 cells pulsed with the p53RMP peptide showed increased T1-
116C binding when the antibody concentration increased; this was saturated at 5
μg/ml (Fig.1b). Likewise, increasing peptide concentrations in the T2 cell assay also
enabled increased T1-116C binding (Fig.1c). On both occasions, T1-116C binding
was proportionally lower than that of the BB7.2 antibody, which detects HLA-A2
expression on the cell surface independently of the peptide being presented.

T1-116C binding is predominantly restricted to cancer cell lines with HLA-A2
and p53 expression

Having validated the specificity of T1-116C binding, we investigated whether the mAb
could recognize the naturally processed p53RMP peptide presented on the surface
of cancer cells. A panel of 39 cancer cell lines derived from various tissues were
tested for T1-116C mAb binding, and representative staining is shown in Fig.2a. As summarized in Table 1, the T1-116C antibody was able to label cell lines derived from a variety of different cancer subtypes including lung cancer, osteosarcoma, colon cancer, breast cancer, melanoma, pancreatic cancer and hematological malignancies including chronic lymphocytic leukemia, follicular lymphoma, mantle cell lymphoma and diffuse large B-cell lymphoma. The T1-116C antibody immunolabeling was almost exclusively restricted to HLA-A2+ and p53+ cancer cell lines, staining 68.2% (15/22) of the HLA-A2+ cell lines (21 of which had confirmed p53 protein expression) but only one of the 17 HLA-A2- cell lines (11 of which expressed detectable p53 protein) (Fig.2b and Supplementary Fig.3). There was no T1-116C labeling of the HLA-A2+ Thiel cell line in which p53 protein expression was undetectable by either Western blotting (Fig.2b) or immunocytochemistry (data not shown). However, HL-60 cells lacked both HLA-A2 and p53 protein expression and were bound by the T1-116C antibody (Supplementary Fig.3). The epitope bound by T1-116C on HL-60 cells is as yet unknown, but the binding does not seem to represent epitope independent binding by the Fc receptors expressed on HL-60 cells, as control antibodies with the same isotype did not bind.

Neither the level of p53 protein nor transcript expression was an accurate indicator of the intensity of T1-116C staining (Fig.2b, Table 1, Supplementary Fig.4). This is consistent with reports of p53 turnover, rather than steady-state levels, determining the presentation of epitopes by MHC class I to CTLs(25). Proteasome inhibition using the inhibitor bortezomib significantly increased the levels of detectable p53 protein after 24 hours in NCI-H1395 cells (Supplementary Fig.5). This demonstrates that p53 is normally actively being turned over in these cells and is consistent with this leading to p53 peptide presentation and strong T1-116C staining despite low levels of the p53 protein. T1-116C was able to recognize cell lines with either wild-type p53 or a variety of different TP53 mutations. Interestingly, three (MDA-MB-435, MCF-7 and KMH2) of the six HLA-A2+/p53+ cell lines that were not stained by T1-116C had been...
reported in the International Agency for Research on Cancer (IARC) database as having wild-type TP53 and only expressed low levels of the protein.

To further confirm that the p53RMP peptide is endogenously presented on cancer cells bound by T1-116C, we used mass spectrometry (MS) to identify HLA class I molecule-associated peptides from two breast cancer cell lines: MDA-MB-231, which is recognized by T-116C, and MCF-7, which is not. The cells were lysed and immunoprecipitated with a pan-HLA class I antibody W6/32. Peptides associated with HLA class I complexes were isolated by high performance liquid chromatography (HPLC) and their identities analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS). No p53-derived peptide was identified from MCF-7 cells, whereas 4 such peptides were detected from MDA-MB-231 cells: KLLPENNVL (24-32), RMPEAAPRV (65-73), GLAPPQHLIRV (187-197), and LLGRNSFEV (264-272) (Fig.3a/b). All 4 peptides have been reported previously and two of them, GLAPPQHLIRV (187-197) and LLGRNSFEV (264-272), have been targeted by various immunotherapies(13,26). The T1-116C target peptide isolated from MDA-MB-231 had the sequence RMPEAAPRV instead of RMPEAAPPV, reflecting a germline polymorphism at codon 72 reported to be associated with altered apoptosis-inducing function and hence increased cancer susceptibility(27). The change of proline at codon 72 to an arginine did not affect the binding of T1-116C as demonstrated by flow cytometry of a T2 stabilization assay (Fig.3c).

The expression of p53 in normal tissues has been linked to radiation-sensitivity, with hematopoietic tissues being among the normal adult tissues exhibiting the highest levels of p53 protein(28,29). Normal circulating peripheral blood mononuclear cells (PBMCs) have also been demonstrated to express the p53 protein(30). Flow cytometry analysis was performed to investigate whether normal PMBCs presented sufficient copies of the wild-type p53RMP peptide to enable binding of the T1-116C antibody. 13/14 (92.9%) PBMC preparations from HLA-A2* donors were negative for...
T1-116C staining (Supplementary Fig.6a). The single positive donor (Buf21), who only exhibited weak staining, had an abnormally high expansion of granulocytes (Supplementary Fig.6b), which may be indicative of some potential abnormality. A non-exhaustive list of potential health problems associated with such granulocytosis includes leukemia, bacterial infection and autoimmune disorders. These data indicate that the T1-116C antibody discriminates between p53+/HLA-A2+ normal and tumor cells. This is consistent with reports from studies using T cells which indicated that malignant cells have increased p53 epitope presentation(25,31,32).

Quantification of T1-116C binding to cancer cell lines and demonstration of engagement with immune effector cells to enable cell killing in vitro

The number of available epitopes present on the cell surface for antibody binding is an important determinant of therapeutic antibody activity(33). A standard curve of PE-coupled calibration beads (QuantiBRITE PE beads) was used to estimate the number of PE-conjugated T1-116C antibodies bound to the surface of peptide-pulsed T2 cells and cancer cell lines (Supplementary Table 1). T2 cells were pulsed with increasing concentrations of the p53RMP peptide. Approximately >150 bound T1-116C molecules per cell were detectable above background levels in this assay. This is comparable to p53\textsubscript{264-272}/HLA-A2 TCR binding (200-300 binding sites per cell) detected using a soluble TCR with the same assay system(34). The tested cancer cell lines bound between 500 – 15,000 T1-116C-PE molecules per cell.

The original T1-116C mAb was a murine IgG1/κ isotype. For further functional studies, a human IgG1 chimeric antibody (hlG1) and a mouse IgG2a (mlG2a) antibody were generated by transferring the heavy and light chain variable regions of T1-116C into hlG1 and mlG2a backbones, respectively. The recombinantly produced antibodies retained the binding specificity of the original antibody purified from hybridoma supernatant (Fig.4a).
Several TCRm antibodies against cancer targets have been shown to have in vivo activity against tumors by mediating immune effector mechanisms such as complement-dependent cytotoxicity (CDC), antibody dependent phagocytosis (ADCP) and/or antibody-dependent cellular cytotoxicity (ADCC)(4,35,36). The ability of a chimeric T1-116C antibody with a human IgG1 Fc domain to engage human immune effector cells was tested against B-cell lymphoma cell lines displaying high T1-116C binding, with rituximab (anti-CD20) used as a positive control (Fig.4b). The T1-116C antibody was able to engage immune effector cells to kill both OCI-Ly1 and OCI-Ly8 B-cell lymphoma cell lines by ADCP, albeit less effectively than rituximab, with the highest dose (10µg/ml) exhibiting the greatest effect. The T1-116C antibody did not convincingly demonstrate significant killing by ADCC. Intriguingly, the CDC killing mediated by T1-116C against OCI-Ly8 cells was higher than that achieved with rituximab at the two higher antibody concentrations.

**T1-116C binds to and inhibits the growth of breast cancer xenografts in vivo**

Antibody biodistribution in vivo gives a good indication of antibody uptake and clearance, including specific targeting to the tumor. For this purpose, T1-116C (mlgG2a) was conjugated to the metal ion chelator pSCN-BnDTPA, which allowed radiolabeling with $^{111}$In chloride. The biodistribution of the radiolabeled antibody following intravenous administration(37) was compared to that of a non-targeting isotype control antibody in athymic mice bearing breast cancer xenografts that bind T1-116C in vitro, (MDA-MB-231) or those that lack in vitro T1-116C binding (MDA-MB-468) using Single Photon Emission Computed Tomography (SPECT) (Fig.5a). Radiolabeled T1-116C and the isotype control followed a pattern of blood clearance, and tumor and tissue uptake that is consistent with other radiolabeled whole antibodies(37). Initially, the radiolabeled antibodies were observed in the blood, as indicated by the high signal in the heart, the carotid arteries and the well-perfused...
The amount of radiolabeled antibody in the blood then gradually decreased over time, while uptake in the MDA-MB-231 tumor increased to over 25 per cent of the injected dose per gram (%ID/g) at 72 h post injection (Fig. 5b), thus increasing tumor-to-blood ratio, as indicated by tumor-to-heart uptake levels (Fig. 5c). MDA-MB-231 tumors, but not the MDA-MB-468 tumors showed higher uptake of T1-116C (p<0.001 at 48 h post injection) compared to the isotype control antibody. Biodistribution after dissection (Fig. 5d) confirmed a significantly higher uptake of radiolabeled T1-116C in MDA-MB-231 tumor tissues compared to normal tissues (p<0.0001), compared to MDA-MB-468 (p<0.0001) and compared to the control antibody (p<0.0001).

To investigate whether T1-116C antibody has any effect on in vivo tumor growth, recombinant T1-116C in either hlgG1 or mlgG2a formats were tested for their ability to prevent the engraftment of MDA-MB-231 tumors in BALB/c nu/nu mice (10mg/kg, twice weekly). The T1-116C mlgG2a format antibody significantly inhibited tumor growth in vivo (P<0.0001) (Fig.6a). The hlgG1 format T1-116C antibody did not significantly affect tumor growth. Although hlgG1 can bind all activating murine FcγRs, it has been reported to be less potent than mlgG2a antibodies in mouse models(38), which likely contributes to the differences observed.

The T1-116C mlgG2a antibody was further tested for its ability to prevent the growth of established MDA-MB-231 tumors in BALB/c nu/nu mice (Fig.6b). Compared to an isotype matched control antibody (anti-fluorescein) or PBS carrier alone, the T1-116C antibody significantly reduced the growth rate of MDA-MB-231 tumors (P<0.0001).

**Discussion**

p53 expression and epitope presentation can be affected by multiple mechanisms, including MDM2 overexpression, human papilloma virus infection and p14ARF mutations(39). Generally tumor cells are found to have higher copy numbers of wild-
type p53 peptide-MHC class I complexes than normal cells(25,31,32). This is partly
due to the increased turnover and thus processing of p53 in tumor cells(25) and
partly due to the low levels of p53 in normal cells(40,41). Consequently, CTLs
recognizing wild-type p53 can discriminate between p53+ tumor cells and normal
tissues(42). Both CTLs and T helper (Th) cells directed against wild-type p53 have
eradicated tumors in vivo without damage to normal tissues(43,44). Importantly, high
tumor levels of p53 are not a prerequisite for tumor killing by CTLs targeting wild-type
p53 peptides, and such CTLs were able to kill tumors expressing low-level p53
protein. Interestingly, T cell recognition of tumors without detectable p53 protein
expression has been reported within the context of human papilloma virus infection,
where enhanced p53 proteasomal degradation occurs(45). Thus, both wild-type and
mutated p53 are among the top tumor antigens prioritized by a National Cancer
Institute pilot project to accelerate translational research(5).

Vaccination studies utilizing a variety of wild-type p53 peptides (HLA-A2 restricted
peptides comprising p53 amino acids 65-73, 149-157, 187-197, 217-255 and 264-
272) and different vaccine delivery systems have been taken through to clinical
trials(14,15). While the vaccines were safe, able to induce anti-p53 immune
responses, and some patients achieved stable disease, we are unaware of clinical
responses with a significant reduction in tumor burden, which is consistent with most
cancer vaccination studies, and is largely due to the immunosuppressive nature of
the tumor microenvironment(14,15). It is estimated that for CTL killing, the optimal
number of binding sites on target cells is between 80-120, while higher
concentrations (500-700) induce T cell hyporesponsiveness(46,47). Here, we
detected 500-15,000 T1-116C molecules being bound to cancer cell lines and the
potential contribution that this might make to T cell unresponsiveness to the p53RMP
epitope could be further investigated in vaccination studies. The high number of T1-
116C molecules bound per cell is comparable to those bound by a TCRm antibody
against the melanoma differentiation antigen tyrosinase(47). Fortunately, it is
generally accepted that antibody-mediated function correlates positively with the number of their binding sites on target cells.(33).

TCRm antibodies circumvent the processes of immune cell priming and maturation, can directly recognize and bind peptide-presenting targets, and subsequently induce cytotoxicity through the components of the innate immune system such as natural killer cells, complement and macrophages. As demonstrated in this study, TCRm T1-116C raised against the wild-type p53RMP peptide elicited all of these functions and impaired tumor growth in vivo, indicating promising therapeutic efficacy in a preclinical model of aggressive triple receptor negative breast cancer, a malignancy that urgently needs improved therapeutic options.

Similarly to TCRs, TCRm antibodies possess the ability to recognize multiple epitopes that have similar structures(4,48). Such cross-reactivity potentially poses a risk for future clinical applications. We have so far observed that T1-116C binding requires both HLA-A2 and p53 expression in the cell lines we tested with the exception of the promyelocytic leukemia cell line, HL-60. This cell line is reported to express HLA-A*0101, HLA-B*5701, and HLA-C*0602(49), and does not have detectable p53 expression. We have ruled out Fc receptor binding and cell line misidentification, and have confirmed the lack of HLA-A2 expression on our lab stock of the cell line. Considering that no other HLA-A2- or p53- cell lines, nor normal PBMC samples, showed significant binding by this antibody, the ligand(s) bound on HL-60 is evidently not widely expressed. Further investigations are underway to characterize the amino acid dependency of T1-116C binding within the p53RMP peptide and to identify whether peptides derived from antigens other than p53 may also be recognized and thus provide an explanation for this potential off-target binding. However, in MDA-MB-231 cells, the p53RMP peptide was experimentally demonstrated to be co-immunoprecipitated with MHC class I by mass spectrometry analysis of bound peptides, demonstrating its availability as a target epitope in the
cell line used for the in vivo study. Interestingly, crystallisation of the ESK1 TCRm antibody bound to its antigen Wilms tumor 1 (WT1)/HLA-A2 recently demonstrated that the antibody bound its target differently to TCRs and indeed exhibited binding to multiple HLA-A*02 subtypes(50).

Murine antibodies cannot be repeatedly administered in man because of the development of immune responses against murine immunoglobulin epitopes. To enable T1-116C re-administration in patients we humanized and de-immunized the antibody and showed that the recombinant hT1-116C antibody retains similar in vitro binding specificity to the original murine reagent (unpublished data). While immune effector functions engaged by the naked T1-116C might give the antibody sufficient potency against B-cell lymphomas, where antibodies against highly-expressed B-cell differentiation antigens have proven effective, there may be additional arming strategies needed for efficacy in solid tumors. Dahan and Reiter(2) have recently comprehensively reviewed the mechanisms of action whereby TCRm antibodies can be used to target tumors. In general their indications for therapeutic targeting of other agents are similar to those for TCRs, particularly following the successful engineering of high affinity recombinant TCRs to overcome their naturally low affinity. These include the use of TCRm antibodies to deliver drugs or toxins, and their potential as a targeting moiety for tumor targeting viruses. Importantly these approaches do not require a competent immune system and thus will be suitable for immunosuppressed patients lacking both immune effectors and T cells. TCRm antibodies also have the potential to be used as the targeting agent on chimeric antigen receptor (CAR) engineered T cells, without any potential for recombining with endogenous TCRs. Future studies of multiple tumor models and normal tissue cross-reactivity profiling are required to evidence sufficient efficacy and specificity, but we believe this p53RMP TCRm antibody represents a promising new agent for future cancer immunotherapy.
Acknowledgements

We would like to thank Professor Adrian Harris and Dr Massimo Masiero for helpful
discussions and Jose Orta for technical support.
References


Antigenic modulation limits the effector cell mechanisms employed by type I anti-CD20 monoclonal antibodies. Blood 2015;125:1901-9

Imaging DNA damage in vivo using gammaH2AX-targeted immunoconjugates. Cancer Res 2011;71:4539-49


Table 1. Tumor cell expression of HLA-A2, p53 and their T1-116C binding.

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1. Acute Promyelocytic leukemia (APC), Chronic lymphocytic leukemia (CLL), follicular lymphoma (FL), mantle cell lymphoma (MCL), diffuse large B-cell lymphoma (DLBCL), classical Hodgkin lymphoma (cHL), Burkitt lymphoma (BL), T cell acute lymphoblastic leukemia (T-ALL), cutaneous T cell lymphoma (CTCL), ALK+ anaplastic large cell lymphoma (ALCL). HLA-A2 expression was detected by BB7.2 mAb staining. TP53 status is indicated with the original amino acid, codon position, and alteration; data were retrieved from the IARC TP53 database (http://p53.iarc.fr/CellLines.aspx). WT refers to wild-type TP53, WT/NULL and WT/MUT indicates either null or mutated TP53 reported as well as wild-type in IARC TP53 database. p53 protein expression was detected by Western blotting, and T1-116C staining was tested by FACS.
**Figure legends**

**Fig.1** Binding of the T1-116C TCRm antibody to p53RMP/HLA-A2 complexes on live cells detected by flow cytometry. (a) T2 cells pulsed with p53RMP peptide at 100 μM were stained with TCRm antibody supernatants and detected by an APC-conjugated anti-mouse secondary antibody. A panel of irrelevant peptides, including others derived from p53, were used as negative controls. (b) T2 cells pulsed with p53RMP peptide at 100 μM were stained with the purified T1-116 antibody at the indicated concentrations. HLA-A2 specific mAb BB7.2 was used in parallel to detect HLA-A2 expression. Mean fluorescence intensities (MFIs) of the staining were plotted on the right panel (for clarity the left panel does not contain all the tested concentrations). (c) T2 cells pulsed with the p53RMP peptide at various concentrations were stained with the T1-116C antibody and the BB7.2 antibody against HLA-A2 at 10 μg/ml. MFIs of the staining were plotted on the right panel. Flu peptide pulsing at 100 μM was used as a negative control.

**Fig.2** T1-116C cell surface binding of cancer cells is typically HLA-A2 and p53-restricted. (a) Cultured cancer cell lines were stained with the T1-116C antibody for FACS analysis. The cell lines recognized by T1-116C are commonly both positive for HLA-A2 and p53. T1-116C does not stain the majority of cell lines that are HLA-A2 negative, regardless of their p53 expression status. (b) p53 protein expression in cancer cells detected by p53 mAbs DO-1, DO-7 and Pab1801. The status of p53 expression and mutation, as well as HLA-A2 expression and T1-116C staining is summarized at the bottom of the panel. A ‘?’ signifies that the TP53 mutation status is unknown.

**Fig.3** MDA-MB-231 cells present p53RMP peptide that is detectable by T1-116C mAb. (a) Diagram illustrating the p53-derived peptides identified by mass spectrometry after MHC class I immunoprecipitation from MDA-MB-231 cells. Black bars indicating the position of the peptides on p53 protein, and corresponding peptide
sequences are labeled underneath the bars. TA, transactivation; PR, proline-rich; DBD, DNA binding domain; TET, tetramerization domain; Reg, regulatory region. (b) MS/MS spectrum of p53RMP detected from MDA-MB-231 breast cancer cells. The detected mass over charge ratio [M+2p]2+/2 (p: protons) of the doubly charged peptide ion and the theoretical peptide mass [M] of the peptide are stated above the spectrum. All fragments that have been detected are indicated in the peptide sequence. Most abundant fragment ions are assigned in the spectrum. Fragment ions are annotated as follows: b: N-terminal fragment ion; y: C-terminal fragment ion; y++: doubly charged C-terminal peptide ion, -NH3: ammonia loss. (c) T1-116C binds both versions of p53RMP peptides in a T2 stabilization assay. The amino acid affected by the MDA-MB-231 p53 polymorphism is highlighted in bold.

**Fig.4** The p53 TCRm T1-116C antibody can engage immune effector functions to achieve target cell killing. (a) Validation of recombinantly expressed T1-116C antibodies. T2 cells pulsed with p53RMP or Flu peptide, alone with OCI-Ly1 and OCI-Ly8 lymphoma cells, were stained with the original T1-116C (hybridoma purified), and recombinant T1-116C in mlG1, mlG2a, and hlG1 isotypes. APC-conjugated anti-mouse or anti-human secondary antibodies were used to visualise the staining for flow cytometry analysis. Mean fluorescence intensities (MFIs) of T1-116C staining were displayed for each plot. (b) Cytotoxicity of T1-116C against the B-cell lymphoma cell line OCI-Ly8 through immune effector functions. A human IgG1 chimeric form of T1-116C, at increasing concentrations (μg/ml), was used to induce human PBMC to exert antibody-dependent cell-mediated cytotoxicity (ADCC) (effector:target [E:T] ratio = 50:1), mouse bone marrow-derived macrophage (BMDM)-mediated ADCP (T:E=5:1), or human serum complement (10% v/v) mediated CDC against OCI-Ly8 cells. The anti-CD20 mAb Rituximab was used as a positive control. Herceptin was used as an isotype control antibody (Ctrl) at 10μg/ml. One of three representing results was shown. Similar levels of ADCC and ADCP were observed against the B-cell lymphoma cell line OCI-Ly1 (data not shown).
Fig. 5 T1-116C antibody biodistribution in athymic mice bearing MDA-MB-231 (a) or MDA-MB-468 (b) xenografts. Female BALB/c nu/nu mice were subcutaneously inoculated with $1 \times 10^6$ tumor cells that were allowed to grow until they reached 120 mm$^3$ at day 20. $^{111}$In-labeled T1-116C (n=2) or an isotype control (n=3) was administered intravenously and SPECT/CT imaging performed at various times. Coronal (top) and transaxial (bottom) sections of SPECT images show high tumor uptake in athymic mice bearing MDA-MB-231 compared to MDA-MB-468 xenografts (a). Tumor uptake (b), and the ratio of antibody radio signals between tumor and heart (c) were calculated through volume of interest (VOI) analysis on SPECT images. (d) Biodistribution after dissection at 72 h post injection. (*: $P<0.05$; **: $P<0.01$; ***: $P<0.001$; ****: $P<0.0001$ by ANOVA).

Fig. 6 The p53 TCRm T1-116C Ab inhibits tumor growth in vivo. (a) T1-116C prevents engraftment of a triple receptor negative breast cancer xenograft in vivo. $1 \times 10^7$ human breast cancer MDA-MB-231 cells were injected subcutaneously into in BALB/c nu/nu mice (n = 10 per group). T1-116C in two formats, a murine IgG2a isotype (mlgG2a) versus a human IgG1 isotype (hlgG1), or PBS carrier alone, was administered twice a week (10 mg/kg) starting from the time of tumor inoculation. (b) The T1-116C antibody delays MDA-MB-231 xenograft tumor growth. MDA-MB-231 cells were inoculated as described above and the tumors were allowed to grow with treatment starting at day 14 when the average tumor sizes reached 150 mm$^3$. Mice were divided into groups having similar average tumor sizes and distributions (n = 9). T1-116C-mlgG2a and an isotype control antibody were injected twice a week (10 mg/kg) i.p. until the end of the experiment. Tumor sizes were calculated as length x width x height x $\pi / 6$. 

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**Figure 1**

(a) Graph showing the percentage of max response to different peptides at various concentrations of T1-116C.

(b) Graph showing the mean fluorescence intensity (MFI) of BB7.2 and T1-116C in response to different concentrations of T1-116C.

(c) Graph showing the MFI of BB7.2 and T1-116C in response to different concentrations of p53RMP and Flu.
Figure 2

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</table>

| p53+ | + | + | + | + | + | - | - | - | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| HLA-A2 | + | + | + | + | - | + | + | - | - | + | + | + | + | + | + | + | + | + | + | - | - | + |
| T1-116C | + | + | + | + | - | - | - | - | + | + | + | + | + | + | + | - | - | - | + | - | - | + |
Figure 3

Author Manuscript Published OnlineFirst on March 31, 2017; DOI: 10.1158/0008-5472.CAN-16-3247
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

a

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<th>DBD</th>
<th>TET</th>
<th>Reg</th>
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<td>KLPENNVL (24-32)</td>
<td>RMPEAAPPV (65-73)</td>
<td>GLAPPQHLIRV (187-197)</td>
<td>LLGRNSFEV (264-272)</td>
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</table>

b
detected mass [M+2H]^{2+}: 513.78 m/z
theoretical mass [M]: 1025.54 Da

\[
\begin{array}{cccccccc}
\text{y}_8 & \text{y}_7 & \text{y}_6 & \text{y}_5 & \text{y}_4 & \text{y}_3 & \text{y}_2 & \text{y}_1 \\
b_1 & b_2 & b_3 & b_4 & b_5 & b_6 & y_7 & y_8 \\
\end{array}
\]

RMPEAPPV

RMPPEAPPV

b_1 b_2 b_3 b_4 b_5 b_6 y_7 y_8


c

No peptide
p53-RMPEAAPPV
p53-RMPEAAPPV

% of max

T1-116C
Figure 4

(a) 

T2 cells

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<td>OCI-Ly8</td>
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Hybridoma Purified

Recombinant mlgG1

mlgG2a

hlgG1

Isotype

T1-116C

(b) 

% ADC

% ADCP

% CDC

T1-116C

Rituximab
Figure 6

(a) PBS, T1-116C-hlgG1, T1-116C-mlgG2a

(b) Isotype, T1-116C

Tumour volume (mm²) vs. Time (days)
Development of a T Cell Receptor Mimic Antibody against Wild-Type p53 for Cancer Immunotherapy

Demin Li, Carol Bentley, Amanda Anderson, et al.

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