Targeting mutant p53-expressing tumours with a T cell receptor-like antibody specific for a wild-type antigen

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Accumulation of mutant p53 proteins is frequently found in a wide range of cancers. While conventional antibodies fail to target intracellular proteins, proteosomal degradation results in the presentation of p53-derived peptides on the tumour cell surface by class I molecules of the major histocompatibility complex (MHC). Elevated levels of such p53-derived peptide-MHCs on tumour cells potentially differentiate them from healthy tissues. Here, we report the engineering of an affinity-matured human antibody, P1C1TM, specific for the unmutated p53125-134 peptide in complex with the HLA-A24 class I MHC molecule. We show that P1C1TM distinguishes between mutant and wild-type p53 expressing HLA-A24+ cells, and mediates antibody dependent cellular cytotoxicity of mutant p53 expressing cells in vitro. Furthermore, we show that cytotoxic PNU-159682-P1C1TM drug conjugates specifically inhibit growth of mutant p53 expressing cells in vitro and in vivo. Hence, p53-associated peptide-MHCs are attractive targets for the immunotherapy against mutant p53 expressing tumours.
The p53 transcription factor plays an important role in response to cellular stress and is crucial in the protection against cancer development. Through the regulation of genes involved in DNA repair, cell cycle arrest and apoptosis, p53 ensures genetic integrity by preventing the accumulation of aberrations that would otherwise lead to malignancy and oncogenesis. In the absence of stress signals, p53 is kept at low levels by the continuous ubiquitination by E3 ubiquitin ligases such as MDM2, and degradation by the proteasome. Stress signals result in post-translational modifications of the p53 protein, leading to the increased stability of the p53 protein by disrupting the p53-MDM2 interaction, and the subsequent activation of p53. The p53 gene is the most commonly mutated gene found in human malignancies. While frame-shift and nonsense mutations have been observed, missense mutations resulting in single amino acid changes in the DNA-binding domain make up the majority of tumour-associated mutations. Studies have further identified six “hotspot” positions in the DNA-binding domain at Arg175, Gly245, Arg248, Arg249, Arg273 and Arg282 that are the most frequently mutated. These mutations are known to increase the stability of the mutant proteins and also disrupt the native formation of the p53 protein, resulting in the inability to recognize and bind the cognate p53 response elements, while suppressing wild-type p53 and other p53 family members, and thus impairing tumour-suppressive function and promoting oncogenesis.

CD8+ T cells recognize short peptide epitopes presented on the cell surface of tumour cells in complex with a class I protein of the major histocompatibility complex (MHC) via their T cell receptors (TCRs). Proteins expressed by the tumour cells are continuously degraded and presented as a peptide-MHC (pMHC) antigen to stimulate anti-tumour CD8+ T cell responses. The ability to target such pMHCs has been achieved by soluble TCRs or antibodies with TCR-like recognition, termed TCRL (TCRL) or TCR mimic antibodies, with great therapeutic potential. Elevated p53 levels in tumours expressing mutant p53 may result in higher levels of presentation of p53-derived peptides by MHC molecules. Peptides containing mutant sequences are rare due to the MHC-binding restrictions; however, elevated levels of MHCs presenting wild-type p53 peptide sequences can potentially differentiate malignant expressing mutant p53 from healthy cells expressing wild-type p53.

Here, we report the engineering of a TCRL antibody, P1C1TM, specific for a wild-type p53125-134 peptide presented by the HLA-A24:02 (HLA-A24) MHC allele. We show that P1C1TM can differentiate between mutant and wild-type p53-expressing HLA-A24+ cell lines based on the differences in the antigen expression level. Its implications and potential applications for cancer therapy are discussed.

**Results**

**Isolation of p53125-134/HLA-A24-specific antibodies.** A human Fab library consisting of 3 × 1010 M13 phagemids were used for the isolation of p53125-134/HLA-A24-specific antibodies. Negative selection against a control pMHC and streptavidin beads was done prior to positive selection to reduce non-specific clones. After three rounds of biopanning, 36 single Fab clones were selected based on their specific binding to p53125-134/HLA-A24 over the control pMHC in an enzyme-linked immunosorbent assay (ELISA). DNA fingerprinting and subsequent sequencing identified four unique clones, P1H4, P1B11, P1A8 and P1C1. The four clones were expressed in immunoglobulin G1 (IgG1) form and assessed for their specificities to the p53125-134/HLA-A24 pMHC by ELISA. Clones P1H4 and P1C1 showed the strongest binding to p53125-134/HLA-A24 pMHC, but P1C1 showed the least non-specific binding to the control pMHC (Fig. 1a).

**Characterization of TCRL antibody P1C1.** The binding specificity of clone P1C1 was further characterized using an HLA-A24+ but p53-null SaoS2 cell line. Cells were either unpulsed or pulsed with various known HLA-A24-restricted peptides and stained with 10 µg mL⁻¹ of antibody. P1C1 showed insignificant binding to unpulsed SaoS2 cells, while strong binding was observed only to p53125-134-pulsed SaoS2 but not to control peptides (Fig. 1b). SaoS2 cells pulsed with varying concentrations of p53125-134 peptide showed that P1C1 bound cells pulsed with at least 400 nM of peptides (Fig. 1c). Next, SaoS2 cells pulsed with 10 µM peptides were stained with varying concentrations of P1C1 (Fig. 1d). P1C1 staining was observed at an antibody concentration of at least 10 ng mL⁻¹.

To reduce potential immunogenicity of the lead P1C1 antibody, site-directed mutagenesis was performed to convert the backbone to germline-like sequences (IGHV4-31*03, IGLV1-40*01) (Supplementary Fig. 1a). The germline P1C1, P1C1gl, demonstrated similar binding profile as the parental P1C1 antibody (Supplementary Fig. 1b). Lastly, the ability of P1C1 to recognize and bind HLA-A24+ HT29 cells that express high levels of mutant p53 was examined. However, minimal staining was seen even with 10 µg mL⁻¹ of antibodies (Fig. 1e). Together, the data suggested that P1C1 bound with relatively low affinity.

**Affinity maturation of P1C1.** A potential challenge to the use of soluble TCRs or TCRL antibodies is the relatively low number of specific pMHC complexes available for binding per cell as compared to other traditional antibody targets, for example, HER2. Hence we proceeded to improve the affinity of P1C1 by generating four separate libraries in which the complementarity-determining regions (CDRs) 1 and 3 of the heavy and light chains were randomized with degenerate codons encoding for the wild-type amino acid and a restricted diversity that includes primarily Ala, Ser, Tyr and Asp (Table 1)21. Glycine and tryptophan residues were left unchanged to preserve structural integrity. The constructed CDR1heavy, CDR3heavy, CDR1light and CDR3light libraries contained 6.7 × 10⁷, 4.5 × 10⁷, 3.8 × 10⁸ and 1.4 × 10⁸ clones, respectively, covering the theoretical maximum diversity of each library of 256, 1.6 × 10⁸, 3.8 × 10⁸ and 1.6 × 10⁸, respectively. Individual clones from the CDR1heavy library were directly screened for specificity by ELISA due to its small theoretical diversity, while the CDR3heavy, CDR1light and CDR3light were subjected to three rounds of biopanning with increased stringency to enrich for stronger binders. While no improved binders were isolated from the CDR3light library, 28, 15 and 11 unique and dominant clones were identified from CDR1heavy, CDR3heavy and the CDR1light libraries, respectively, and were assessed by biolayer interferometry for improved off-rate. Subsequently, clones 2E3, 1E11 and 1G7 were identified as the best binders from the CDR1heavy, CDR3heavy and the CDR1light libraries, respectively.

**Characterization of P1C1 affinity-matured mutants.** Clones 2E3, 1E11 and 1G7 and a triple mutant (P1C1TM) incorporating the mutations in the three CDRs (Fig. 2a) were expressed in IgG1 format and their binding affinities were analysed by ELISA. 1E11 showed the biggest improvement in binding, while 2E3 and 1G7 exhibited similar binding affinity as P1C1gl (Fig. 2b). Interestingly, P1C1TM had significantly higher binding affinity, measured at around 5 nM (Fig. 2c), suggesting that the mutations in the individual CDRs had a synergistic effect when combined. Affinity measurements of the P1C1gl antibody (Kd = 116 nM) and the single mutants confirmed that 1E11 (20 nM) had the
most significant improvement in binding affinity as compared to 2E3 (69 nM) and 1G7 (66 nM) (Supplementary Fig. 2).

The specificity of P1C1TM was next analysed by flow cytometry. SaoS2 cells were pulsed with a panel of HLA-A24-restricted peptides and stained with 10 µg mL⁻¹ of antibody. The specificity of the affinity-matured clone was observed when cells were pulsed with p53125–134 (Fig. 2d). SaoS2 cells were then pulsed with a range of concentration of p53125–134 peptides and stained with 10 µg mL⁻¹ of antibody. As compared to the parent P1C1 clone, staining by P1C1TM was observed when cells were pulsed with peptides at as low as 16 nM (Fig. 2e).

Fine specificity of P1C1TM. To better understand the specificity and possible cross-reactivity of P1C1 and importantly the affinity-matured P1C1TM, the fine specificity of P1C1gl and P1C1TM was first assessed by alanine scanning mutagenesis of the p53125–134 peptides. Position 5 was left unchanged, as it is an alanine in the wild-type sequence. Figure 3a shows that alanine substitutions at positions 3, 4, 6 and 8 significantly reduced the binding of P1C1 compared to wild-type p53125–134 peptide-pulsed cells. Reduction of P1C1 binding observed was not due to reduced peptide binding to the HLA-A24 molecule itself as the mutant peptides, except P2A and P10A, were still able to rescue HLA-A24 complexes in a ultraviolet (UV)-peptide exchange ELISA (Supplementary Fig. 3a). Positions 2 and 10 are known anchor residues that are important for the peptide’s binding to HLA-A2443; thus, substitutions to alanine reduced their ability to form stable pMHC and thus also abolished P1C1gl binding. Affinity-maturated P1C1TM exhibited reduced sensitivity to the positions 3, 4 and 6 but not to position 8 (Fig. 3a). This suggests that the CDR loops targeted in our affinity maturation strategy play a role in binding the central residues of the p53125–134 peptide.

We next looked at possible cross-reactive epitopes in other proteins encoded in the human genome. A motif search of the Kyoto Encyclopaedia of Gene and Genomes (KEGG) database followed by HLA-A24-binding affinity prediction by NetMHC 3.0 identified several potential cross-reactive peptides (Fig. 3b). The ability of these peptides, as well as the murine homologue p53119–128 that differs only at positions 5 and 9, to be presented by HLA-A24 was assessed by UV-peptide exchange HLA-stability ELISA. All 15 peptides tested rescued cleaved UV-cleavable HLA-A24 pMHCs to similar or better extent than the p53125–134 peptide.
Table 1 Degenerate codons and their corresponding amino acid sequences of CDR libraries designed for affinity maturation.

| Heavy CDR1 | Degenerate codon | Heavy CDR3 | Degenerate codon | Light CDR1 | Degenerate codon | Light CDR3 | Degenerate codon |
|------------|------------------|------------|------------------|------------|------------------|------------|------------------|
|            | K M T G G T G G T K M T K M T T G G K M T |            | K M W D M T K H T G G A K M T K H T K M T B M T |            | Y/A/D/S/N/T G Y/A/D/S Y/A/D/ S |            | Y/A/D/S/N/T G Y/A/D/S Y/A/D/S/D/T/I/Y/A/D/ S |
|            | S               |            | N/T             | S          | D M T G G G K M T K M T K M T D M T |            | S      |
|            |                 |            | V               |             | S/E             |            | S/E             |
|            |                 |            | H/P             |             | S              |            | S              |

Diversity of CDR1 and CDR3 libraries of the heavy and light chains of P1C1. Residues of the respective CDR loops were identified by Kabat definition and randomized with degenerate codons encoding for the original amino acid or a limited diversity consisting of Ala, Ser, Tyr or Asp. Gly and Typ were left unchanged to preserve structural integrity. Theoretical diversity of the heavy-chain CDR1, 3 and light-chain CDR1, 3 libraries are calculated to be 256, 1.6 × 10^5, 3.8 × 10^8 and 1.6 × 10^7.

Detection of endogenously processed p53_125–134/A24 pMHC. Next, we studied and compared the physiological expression and levels of p53_125–134/A24 pMHC complexes on tumour cell lines with various p53 statuses, using the affinity-matured P1C1TM. Three HLA-A24+ cell lines, the osteosarcoma SaoS2 (p53null), hepatocellular carcinoma HepG2 (p53wt) and colon adenocarcinoma HT29 (p53R273H) express different levels of p53 (Supplementary Fig. 4). Staining of these three cell lines with P1C1TM show that levels of p53_125–134/A24 pMHCs detected by P1C1TM correlated with the level of p53 expression (Supplementary Fig. 5a, 5b). Staining of A24+ PBMCs with P1C1TM was not observed in both A24+ and A24− PBMCs from various donors (Supplementary Fig. 5a). Staining of A24+ PBMCs was observed only when PBMCs were pulsed with the p53_125–134 peptide (Fig. 4d).

However, upregulation of p53 has been reported in activated T lymphocytes. Indeed, in our hands, we found that T cells activated with anti-CD3 and anti-CD28 agonists showed an increase in intracellular p53 (Supplementary Fig. 4c). Subsequently, staining by P1C1TM was observed only in the activated A24+ T cells but not in resting A24+ T cells or A24− T cells (Fig. 4e). This was further shown in four other A24+ and two other A24− donors (Supplementary Fig. 5b).

Next, we treated HT29, A549-A24 and MCF7-A24 cells with 100 U mL⁻¹ interferon-γ, a cytokine known to upregulate the MHC class I expression and the components of the antigen-processing pathways. 5 µM nutlin, a small molecule that inhibits the Mdm2-p53 interaction or 5 µM of the proteasome inhibitor MG132 (bortezomib). Changes in levels of HLA-A24, intracellular p53 and p53_125–134/A24 complexes were then monitored after treatment (Supplementary Fig. 6). Expectedly, levels of HLA-A24 detected on the surface increased significantly in HT29 cells treated with interferon-γ, resulting in a similar increase in P1C1TM staining. Expressions of the HLA-A24 heavy-chain molecule are constitutive in both the transduced A549−24 and MCF7-A24 cells and thus are not expected to be regulated by interferon-γ directly. However, A24 expression significantly increased in the A549−A24 cells upon interferon-γ treatment accompanied by an observed increase in P1C1TM staining (Supplementary Fig. 6b). This was less evident in MCF7-A24 cells, as both A24 and P1C1TM staining were only modestly

peptide (Supplementary Fig. 3b); thus, all peptides are able to form stable HLA-A24 pMHCs. However, P1C1TM binding was only detected with the cognate p53_125–134/A24 pMHC but not with any of the predicted cross-binders (Fig. 3c). Similarly, P1C1TM binding was seen only with SaoS2 cells pulsed with 10 µM of p53_125–134 peptides, but not with the other peptides (Fig. 3d). Hence, P1C1TM exhibits a high specificity for the p53_125–134 peptide/HLA-A24 pMHC.
increased after treatment with interferon-γ. No increase in intracellular p53 was observed in all the treated cells.

HT29 cells treated with nutlin did not result in any change in the level of intracellular p53 or surface p53 peptide/MHC complexes (Supplementary Fig. 6b). Interestingly, MCF7-A24 cells treated with nutlin resulted in almost 2-fold increase in intracellular p53, but no significant increase was seen in surface p53 peptide/MHC complexes. Treatment with MG132 and other proteasome inhibitors is known to result in significant reductions of the germline sequence converted P1C1 (P1C1gl), individual mutants and triple mutant (P1C1TM) was done by ELISA. Soluble biotinylated recombinant p53125–204 pMHCs were incubated with immobilized antibodies and detected with HRP-conjugated streptavidin.

**Fig. 2** Affinity maturation of TCRL antibody P1C1. **a** CDRI and 3 of the heavy chain and CDRI of the light chain of the original P1C1gl clone and the affinity-matured clones are shown. Mutants 2E3, 1E11 and 1G7 were identified by off-rate comparison for improved binding from the heavy-chain CDRI, CDRI and light-chain CDRI libraries, respectively. Mutations were sequenced and combined in a single triple mutant, P1C1TM. **b** Comparison of the binding affinities of the germline sequence converted P1C1 (P1C1gl), individual mutants and triple mutant (P1C1TM) was done by ELISA. Soluble biotinylated recombinant p53125–134/A24 pMHCs were incubated with immobilized antibodies and detected with HRP-conjugated streptavidin. **c** Binding kinetics of P1C1TM was analysed by surface plasmon resonance. Soluble recombinant p53125–134/A24 pMHCs was flowed over P1C1TM antibodies captured on an anti-human IgG-coated sensor chip at a range of concentrations between 200 and 2 nM. Binding specificity and avidity of P1C1TM to p53125–134/A24 pMHCs on cells were evaluated with SaoS2 cells pulsed with either a panel of six known A24 peptides, including p53125–134 (d) or a range of p53125–134 peptide concentrations (e). Data are representative of two or more experiments.

Antibody-dependent cellular cytotoxicity potential of P1C1TM. We evaluated P1C1’s ability to mediate antibody-dependent cellular cytotoxicity (ADCC) against mutant p53 expressing tumour cells. Both P1C1gl and P1C1TM were able to mediate ADCC of unpulsed HT29 cells in a dose-dependent manner (Fig. 5a). However, while no significant cytotoxicity was observed in the control MDA-MB-231 cell line, toxicity was also low in the HLA-A24 transduced MDA-MB-231 (231-A24) cell line (Fig. 5b). The efficiency of ADCC is dependent on the level of antibodies bound to target cells<sup>29</sup>; thus, the lack of cytotoxicity observed in the 231-A24 cell line may be due to the relatively lower level of presentation of p53125–134/A24 pMHC complexes (Fig. 4). This is further confirmed when enhanced P1C1gl- and P1C1TM-mediated ADCC was observed after HT29 and 231-A24 cells were pulsed with 10 μM peptides (Fig. 5a, b).
The substitutions of leucine 234 and leucine 235 to alanines (LALA) in the antibody Fc region have been shown to reduce the binding affinity to Fcγ receptors, resulting in poorer ADCC\(^{30}\). Introduction of the LALA mutations to P1C1TM (P1C1TM-LALA) showed unchanged binding properties (Fig. 5c), but cytotoxicity was significantly reduced (Fig. 5d). Thus, these data show that the TCRL antibody P1C1TM can facilitate immune cell-mediated cytotoxicity on cells presenting high levels of p53\(_{125-134}\)/A24 pMHCS.

**In vitro growth inhibition by P1C1TM antibody–drug conjugates.** We next explored the potential use of P1C1TM as an antibody–drug conjugate (ADC) to deliver a cytotoxic payload to tumours expressing mutant p53. First, P1C1TM was conjugated with a pH-dependent dye pHrodo Red to evaluate the kinetics of internalization. HT29 cells were incubated with the pHrodo Red–with a pH-dependent dye pHrodo Red to evaluate the kinetics of tumours expressing mutant p53. First, P1C1TM was conjugated intensity of the conjugated dye was indicative of the level of internalization of the conjugated dye was indicative of the level of internalization of P1C1TM. Figure 6a shows that P1C1TM was internalized as rapidly as 30 min after incubation at 37°C, whereas no internalization of P1C1TM was observed when cells were incubated on ice. Next, an indirect killing assay was carried out using four different secondary ADCs. HT29 cells were first incubated with P1C1TM before equimolar of secondary antibodies conjugated to either tubulin inhibitor monomethyl auristatin E (MMAE), DNA-alkylating agents PNU-159682 and pyrrolobenzodiazepine (PBD) or the RNA polymerase inhibitor α-amanitin (AAMT) were added. Cytotoxicity to HT29 cells was observed when cells were treated with DNA-alkylating agents, but not when cells were with treated with AAMT or MMAE. Non-specific toxicity to HT29 cells was observed when treated with highest dose of 10\(\mu\)g mL\(^{-1}\) of PNU-159682, but not PBD-conjugated secondary antibody. However, >50% cytotoxicity was observed with 0.1\(\mu\)g mL\(^{-1}\) PNU-conjugated secondary antibodies, while similar levels of cytotoxicity were observed only with 10\(\mu\)g mL\(^{-1}\) of PBD-conjugated secondary antibodies (Fig. 6b).

P1C1TM was subsequently conjugated with PNU-159682 (P1C1TM-PNU). A binding comparison of P1C1TM and the ADC P1C1TM-PNU showed that the conjugation did not affect P1C1TM’s specificity (Fig. 6c). A panel of HLA-A24-expressing cell lines incubated with either P1C1TM or P1C1TM-PNU were assessed for viability after 72 h. The cell lines expressing wild-type p53 experienced toxicity only when treated with a high concentration of P1C1TM-PNU. However, mutant p53-expressing cell lines HT29 and MDA-MB-231-A24 appeared to be much more sensitive to P1C1TM-PNU with toxicity observed with doses as low as 0.1\(\mu\)g mL\(^{-1}\).
As activated T cells present elevated levels of p53-derived peptide-MHC complexes, we next looked at the effect of PICITM-PNU on activated T cells. At the highest dose tested (3 µg mL⁻¹), PICITM-PNU was able to inhibit the growth of T cells from both A24⁺ and A24⁻ donors. However, activated A24⁺ T cells treated with 1 µg mL⁻¹ PICITM-PNU had notably higher levels of growth inhibition than A24⁻ T cells (Supplementary Fig. 8), whereas no significant growth inhibition was observed when the T cells were treated with lower concentrations of PICITM-PNU.

**In vivo efficacy of PICITM-PNU ADC.** To assess the anti-tumour efficacy of the ADC PICITM-PNU in vivo, HT29 and HepG2 xenografts were introduced into NSG mice. Mice were given a single dose of 1 or 0.3 mg kg⁻¹ of PICITM-PNU or a 1 mg kg⁻¹ of unconjugated PICITM as control 9 days post tumour introduction, when tumour sizes reached ~100 mm³. Single doses of 1 or 0.3 mg kg⁻¹ of PICITM-PNU or 1 mg kg⁻¹ of unconjugated PICITM were well tolerated in the treated mice with no observed changes in either behaviour nor body weight. In mice harbouring HT29 xenografts, treatment with 1 mg kg⁻¹ but not 0.3 mg kg⁻¹ of PICITM-PNU resulted in a 70% inhibition of tumour growth (Fig. 7a). Control of tumour growth was observed even beyond 30 days post treatment. However, in mice harbouring HepG2 xenografts, tumour growths of mice treated with 1 or 0.3 mg kg⁻¹ of PICITM-PNU were identical to the control group treated with 1 mg kg⁻¹ of unconjugated PICITM antibodies (Fig. 7a). Hence, the anti-tumour efficacy of...
P1C1TM-PNU was specific for cells expressing mutant p53 and presenting high levels of p53$_{125-134}$/A24 pMHCs both in vitro and in vivo. In a separate experiment, mice bearing HT29 xenografts were treated with an initial dose of 1 mg kg$^{-1}$ followed by two subsequent weekly doses of 0.3 mg kg$^{-1}$. However, while inhibition of tumour growth was observed, mice exhibited reductions in fitness after the third dose of ADCs and eventually succumbed to the treatment (Fig. 7b).

Lastly, we studied the efficacy of P1C1TM-PNU in a pulmonary HT29 metastatic model. HT29 cells were introduced into NSG mice by intravenous tail injection and treated with either 1 mg kg$^{-1}$ P1C1TM-PNU or 1 mg kg$^{-1}$ P1C1TM 24 h after introduction of tumour cells. Twenty days post tumour introduction, mice treated with P1C1TM only lost significant body weight. By day 40, 40% of the control-treated mice had to be sacrificed. On the other hand, mice treated with P1C1TM-PNU had delayed onset of disease and 80% of the mice were able to survive 60 days post tumour introduction before succumbing to disease (Fig. 7c).

In vivo evaluation of off-target toxicity of P1C1TM-PNU. While P1C1TM-PNU showed specific cytotoxicity only to HT29 xenografts presenting high levels of p53$_{125-134}$/A24 pMHCs in vivo, its potential in vivo off-target toxicity to healthy tissues had to be addressed. Hence, we treated HLA-A24 transgenic mice with P1C1TM-PNU with a single therapeutic dose of 1 mg kg$^{-1}$ or left untreated. No evidence of toxicity was observed as judged by their behaviour and weight changes. Pathological evaluation of major organs was then performed 20 days after treatment (Supplementary Table 1). Most organs, including the heart, kidney and bone marrow, exhibited no evident changes in histology (Supplementary Fig. 9). Overall analysis by the pathologist concluded no significant cytotoxicity posed by P1C1TM-PNU.

Discussion

The selection of a suitable antigen is crucial to the safety and efficacy of any targeted immunotherapy. The ideal tumour-associated antigen (TAAs) would be one that differentiates tumours from normal healthy tissues with great specificity. Unfortunately, a great number of such TAAs are intracellular proteins that are not targetable by conventional antibodies.$^{31,32}$ However, the continuous degradation of such proteins into peptides and their presentation as peptide-MHC complexes, recognized by TCRs expressed by CD8$^+$ T cells, are attractive targets for targeted therapeutics with TCR specificity.

The prevalence of mutations in the p53 gene in the majority of cancers makes targeting p53 an approach with potentially broad applicability.$^{33,34}$ Furthermore, missense mutations resulting in the accumulation of the p53 protein in tumours but not in healthy cells can lead to higher levels of p53-derived pMHC
complexes on the surface of the tumour cells. While the MHC complexes may present p53-derived peptides harbouring mutant sequences, these are rare due to strict MHC class I restrictions. However, several immunoergic wild-type p53 peptides that are present in both wild-type and mutant p53 proteins have been reported, leading to the development of therapeutic strategies targeting these wild-type p53 pMHCs, including peptide vaccines, soluble TCRs, and more recently a murine TCR mimic antibody, T1-116C, specific for the wild-type p53. Li et al. previously reported an antibody, T1-116C, which binds to certain mutant p53 molecules, but fails to mediate the degradation of these mutant proteins, leading to the accumulation of mutant p53 proteins. Importantly, no staining was observed in cells that expressed wild-type p53 but only minimal on those expressing wild-type p53. Hence, there was no correlation between the level of binding of T1-116C and the mutation status of p53, as significant staining was observed in cell lines expressing wild-type p53 and even on HL-60 cells that do not express p53 or HLA-A2. In our hands, we observed that P1C1TM binding was strong on cell lines expressing either wild-type or mutant p53. Hence, there was no correlation between the level of binding of T1-116C and the mutation status of p53, as significant staining was observed in cell lines expressing wild-type p53 and even on HL-60 cells that do not express p53 or HLA-A2. In our hands, we observed that P1C1TM binding was strong on cell lines expressing wild-type p53, but only minimal on those expressing wild-type p53. Importantly, no staining was observed in cells that expressed HLA-A24 but not p53. The degradation of wild-type p53 through the ubiquitin proteolysis pathway is dependent on its interaction with the E3 ligase, MDM2. Mutations in p53 have been classified as contact mutants, in which mutations affect the DNA-binding function of p53, and contact mutants whereby the mutation results in unfolding or aggregation of the protein. The structure of the p53 molecule affects the way it interacts with MDM2, hence while MDM2 is still able to interact with certain mutant p53 molecules, it fails to mediate the degradation of these mutant proteins, leading to the accumulation of mutant p53 proteins. HT29 cells harbour the R273H contact mutation that leads to reduced DNA binding without

![Image](https://example.com/image.png)

Fig. 6 Antibody-drug conjugate P1C1TM-PNU mediates cytotoxicity of tumour cells presenting p53 residues 125–134/HLA-A24 pMHC. a, P1C1TM and an isotype control human IgG1 was conjugated with the pH-dependent dye, pHrodo Red and incubated with HT29 cells at 37 °C or on ice. At different time points, cells were washed with cold PBS and internalization of pHrodo Red-labelled antibodies was assessed by flow cytometry. Data are representative of two independent experiments. b, HT29 cells were incubated with varying concentrations of P1C1TM in the presence or absence of four different anti-human IgG secondary antibody-drug conjugates in equimolar ratios. Cell viability was assessed after 3 days by an MTS assay. Data are means of triplicates ± SEM. c, Cytotoxic drug PNU-159682 was directly conjugated to P1C1TM (P1C1TM-PNU) and assessed for binding to HT29 and HepG2 cells by flow cytometry. Data are representative of two independent experiments. Antibody staining was observed in cell lines expressing either wild-type or mutant p53. Hence, there was no correlation between the level of binding of T1-116C and the mutation status of p53, as significant staining was observed in cell lines expressing wild-type p53 and even on HL-60 cells that do not express p53 or HLA-A2. In our hands, we observed that P1C1TM binding was strong on cell lines expressing mutant p53, but only minimal on those expressing wild-type p53. Importantly, no staining was observed in cells that expressed HLA-A24 but not p53.
compromising the p53 protein structure\textsuperscript{44,45}. In addition, it has been shown that the mutant p53 in HT29 cells continues to be associated with Mdm2 albeit at lower affinity\textsuperscript{43}. Here we show that p53\textsubscript{125–134}/HLA-A24 complexes were readily detected on the surface of HT29 cells by P1C1TM. Treatment of HT29 cells with the MDM2 inhibitor, nutlin, did not result in any change in the level of intracellular p53 or surface p53 peptide/MHC complex. This is in agreement with previously reported observation that the mutant p53 in HT29 cells continues to be associated with MDM2, the E3 ligase had no effect on p53 stability\textsuperscript{43}. However, when HT29 cells were treated with interferon-γ, surface levels of HLA-A24 increased along with a similar increase in P1C1TM staining. Also, treatment of HT29 cells with the proteasome inhibitor MG132 yielded a decrease in surface levels of HLA-A24 and a concomitant decrease in P1C1TM staining, as a result of the reduction in proteasomal activity required to generate peptides that stabilize the pMHC complexes on the surface of the cells. Thus, collectively these data show that while mutant p53 accumulate to high levels intracellularly in tumour cells, continuous degradation through MDM2-independent, proteasome-dependent pathways generates peptides that are eventually presented on the cell surface as pMHC complexes and are attractive targets for immunotherapy.

The specificity of the TCRL antibodies for the peptide presented is critical for the safety and efficacy of TCRL antibody-based therapeutics. Unlike TCRs, an antibody’s specificity is not naturally restricted to pMHC complexes. Structural studies and comparisons by various groups have observed that antibodies with TCRL specificity do not necessarily recognize and bind the target pMHC in the conserved TCRL orientation\textsuperscript{46–48}. The orientation and the fine specificity of the antibody for the pMHC will determine the level of off-target cross-reactivity\textsuperscript{46}. Thus, it is of great importance that we validated P1C1TM’s fine specificity for the p53\textsubscript{125–134}/HLA-A24 pMHC. We showed that P1C1TM interacted effectively with the central residues of the p53\textsubscript{125–134} peptide and exhibited almost undetectable cross-reactivity to numerous peptides with homologous sequences. Interestingly, P1C1TM does not bind the murine p53\textsubscript{125–134} peptide that differs only at positions 5 and 9. Structural studies are underway to elucidate the molecular interactions and fine specificity between P1C1TM and the p53\textsubscript{125–134}/HLA-A24 pMHC.

Antibodies with TCRL specificity have been observed to have anti-tumour activity in vitro and in vivo\textsuperscript{7,8,11,13–15}. Similarly, we demonstrated that P1C1TM was able to mediate ADCC of mutant p53-expressing cells presenting high levels of p53\textsubscript{125–134}/HLA-A24 complexes. Alternatively, antibodies can be armed with cytotoxic payloads, such as DNA-damaging drugs and inhibitors of tubulin polymerization, for delivery to tumour cells, resulting in their cytotoxicity. The kinetics of internalization and expression levels of the target antigen are two important points for consideration for the development of such ADCs\textsuperscript{49}. Constitutive internalization of pMHCs by a clathrin- and dynamin-independent mechanism have been reported\textsuperscript{50}, favouring the development of TCRL ADCs. Hence, we explored the possibility

\begin{figure}
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\caption{P1C1M-PNU limits growth of mutant p53-expressing HT29 xenograft in vivo. HLA-A24 expressing a HT29 (p53\textsuperscript{R273H}) and HepG2 (p53\textsuperscript{wt}) cells were injected subcutaneously into NSG mice. After tumours reached an average size of \( \geq 100\) mm\(^2\), mice were treated with a single dose of either P1C1TM-PNU ADC (1 or 0.3 mg kg\(^{-1}\)) or unconjugated P1C1TM only (1 mg kg\(^{-1}\)) as indicated by the arrows. P1C1TM-PNU significantly delayed tumour growth as compared to the unconjugated P1C1TM (* represents significant differences with \( p \leq 0.05\). Statistical analyses were done by Student's t test with Holm-Sidak method of multiple comparison.). b Mice bearing HT29 (p53\textsuperscript{R273H}) tumours were treated with a loading dose of 1 mg kg\(^{-1}\) of either P1C1TM-PNU or unconjugated P1C1TM only, followed by two more doses at 0.3 mg kg\(^{-1}\) 7 days apart. c HT29 cells were introduced i.v. into NSG mice and treated with 1 mg kg\(^{-1}\) P1C1TM-PNU or unconjugated P1C1TM 24 h post introduction. P1C1TM-PNU-treated mice showed significantly prolonged survival compared to unconjugated P1C1TM-treated mice (* \( p = 0.0339\). Statistical analysis was done with a log-rank test for survival.). Tumour sizes were calculated as \( V = \frac{1}{2}(\text{length} \times \text{width}^2)\). Data are means of triplicates ± SEM.}
\end{figure}
of targeting mutant p53-expressing tumours with a p53-derived pMHC-specific TCRL ADC. However, tumour-specific pMHCs are low in density on the surface of target cells as compared to the current ADC targets such as HER2. A comparative efficacy study between two separate ADCs, based on the same anti-Her2 antibody trastuzumab, showed that the selection of appropriate drugs may overcome the low antigen density. A recent study also showed that in addition to direct anti-tumour effects, the anti-Her2 targeting ADC was able to enhance anti-tumour immune responses through the activation of antigen-presenting cells and also increasing MHC class I expression on tumour cells. Such an adjuvant effect could potentiate the efficacy of TCRL ADCs. Lowe et al.33 and others have demonstrated that TCRL ADCs conjugated with duocarmycin and immunotoxins were capable of mediating cytotoxicity in tumour cells expressing low levels of target pMHC complexes.10,40

Consistent with these studies, we observed the ability of the p5325,134/A24-HLA-A24-specific PIC1TM antibody to specifically deliver the DNA-targeted cytotoxic drugs PDB and PNU-159682, to inhibit mutant p53+ tumour cell growth in vitro. Importantly, the cytotoxic effects of PIC1TM-PNU ADCs were restricted to only mutant p53-expressing colorectal cancer cells in vivo models of colon adenocarcinoma. Cytotoxicity was also observed in activated T cells treated with high concentrations of PIC1TM-PNU. This may be due to differences in sensitivity to the PNU-159682 as Jurkat T cells were found to be >5-fold more sensitive to PNU-159682 than to HT22 cells.43 Resting T cells do not present significant levels of p5325,134/A24-HLA-A24 complexes and thus may more accurately represent healthy tissues in vivo. Furthermore, our study with HLA-A24 transgenic mice, where all tissues potentially present a myriad of HLA-A24-restricted pMHCs, showed that the therapeutically effective dose was well tolerated with minimal pathology observed. While the HLA-A24 antigen repertoires presented by human and mouse are different, this study nevertheless provided further specificity evidence at the proteome scale. Hence, in our proof-of-concept studies described, while therapeutic effects were modest, we observed evidences of specificity and safety of targeting p53-derived pMHCs with a TCRL ADC. Further improvements in the efficacy and safety of the drug conjugate can be achieved with better selection of linker, payload and conjugation chemistry.19,55

Over-expression of the p53 protein in tumour cells allows p53-derived pMHCs to be potential targets for immunotherapy.7,18,35,56–59 However, the specificity of immunotherapeutic strategies against such antigens is highly dependent on the threshold level of antigens required for binding and activation.30,32,60 Tumour-associated pMHCs are relatively low in density, but are known to be arranged as clusters on the cell surface,61 facilitating the binding of multivalent molecules such as antibodies. We speculate that the ability of our PIC1TM to discern the subtle differences in antigen levels may be attributed to its moderate rather than high affinity. This has been proven to be crucial in the reduction of on-target/off-tumour non-specificity, especially in chimeric antigen receptor (CAR) T cells.20,62–64 In a recent study by Liu et al.65 affinity-tuned anti-Her2 CAR-T cells demonstrated dramatically different efficacy and toxicity profiles against tumour and normal tissues. Similarly, PIC1TM may strike such affinity balance to achieve discrimination between cells expressing wild-type and mutant p53, presenting a promising therapeutic strategy for a wide range of cancers.

**Methods**

**Cells line and reagents.** MDA-MB-231 (expressing p53 with a mutation at Arg280 to a Lys, i.e. p53R280K) (cat. #HTB-20), A549 (wild-type p53 expressing, i.e. p53WT) (cat. #CCL-185), BT474 (p53R280K) (cat. #HTB-20), MCF7 (p53WT) (cat. #HTB-22) and HepG2 cells (p53WT) (cat. #HB-8065) were purchased from ATCC. Cells were cultured in either RPMI-1640 or Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% foetal bovine serum and 1% penicillin/streptomycin in humidified CO2 (5%) incubator at 37 °C. The HLA-A24-62 heavy chain was cloned into the pLVX-IRE-S-Zagreens1 lentiviral expression vector and lentivirus particles were produced according to the manufacturer’s protocol (Clontech Laboratories Inc.). MDA-MB-231, A549, BT474 and MCF7 cells were then transduced, and the successfully transduced cells were selected by fluorosence-activated cell sorting for Zagreens1 green fluorescent protein expression. (p53R280K deletion of TP53 gene, i.e. p53WT) and HT22 (expressing p53 with a mutation at position Arg282 to His, i.e. p53R282H) cells were a kind gift from Dr. Wang Bei (Sigin, A*STAR) and Dr. Lee-Ann Hwang (p53Lab, A*STAR), respectively. The cells were cultured as described above in DMEM and McCoy, respectively. Nudtin and MG132 were kind gifts from Prof. Sir David Lane (p53Lab, A*STAR) and recombinant interferon-γ was purchased from Milltenyi Biotec. Cell lines are routinely checked for mycoplasma contamination.

The p53 states of cell lines used can be found on the TP53 database (http://p53.fr).

**Ethics.** All experiments involving animals were performed in accordance with guidelines approved by the Institutional Animal Care and Use Committee of the Biological Resource Centre (BRC), Agency for Science, Technology and Research (A*STAR), Singapore. Use of the apheresis blood from healthy donors for this study was approved by the National University of Singapore Institutional Review Board (IRB reference number: 2017/2512).

**Pepitides.** All peptides were synthesized by Genescript USA Inc. at >90% purity and dissolved in dimethyl sulphoxide at 40 mg mL−1 and frozen at −80 °C. The peptides hTERT254–322 and hTERT466–480, WT1125–134, WT1125–134, p53125–124 and p53125–212 were all previously described by various groups as HLA-A24-binding peptides.17,56,68–68 Biotinylated and non-biotinylated p5325,134/A24 pMHC and control peptide-MHCs were synthesized either by UV-peptide exchange or refolded in recombinant HLA-A24 heavy chain and β2-microglobulin. Briefly, peptide exchange was performed by exposing 2.1 μM recombinant HLA-A24 conditional peptide-MHC to 15 min UV irradiation in the presence of 50 μM peptides in phosphate-buffered saline (PBS) on ice, followed by an hour incubation at 37 °C. Refolding of recombinant peptide-HLA-A24 complexes was done in vitro by the rapid dilution of protein inclusion bodies of the α-chain of HLA-A24 and β2-microglobulin in the presence of peptides. Refolded complexes were then concentrated, dialysed, biotinylated and lastly purified by size-exclusion chromatography on an Äkta Pure Fast Protein Liquid Chromatography system (GE Healthcare).

**Biopanning for p5325,134/A24-specific TCRL antibodies.** Biopanning of a naive human Fab phage display library (Humanx Pte Ltd) was carried out to isolate p5325,134/A24-specific TCRL antibodies. Briefly, the phage library is first subjected to negative selection with uncoated M280 streptavidin magnetic beads (Life Technologies) before incubating with soluble biotinylated p53125,134/A24 pMHCs. The concentration of p53125,134/A24 pMHC used was 100 nM in the first round, 50 nM in the second round and 10 nM in the third round. The input phage in the first round was 1010 and 1011 CFU for the subsequent rounds. Bead-bound phages were washed with washing buffer (PBS with 0.1% Tween-20) with increasing stringency from 4 times to 20 times from round 1 to round 3. Phage eluted with 100 nM triethylamine was used to infect TG1 E. coli cells (Lucigen) and rescued into M13K07 helper phage (New England Biolabs). A single biopanning, Fabbs of selected clones were eluted in HB2151 E. coli cells for screening by ELISA. Unique p53125,134/A24 pMHC-binding clones were subsequently identified by DNA fingerprinting with EnvRI restriction digest (New England Biolabs) and DNA-sequencing.

**Enzyme-linked immunosorbant assay.** Screening of p53125,134/A24-specific antibody leads was performed by sandwich ELISA. The 96-well plates were pre-coated with 5 μg mL−1 NeutriAvidin (Thermo Fisher Scientific, cat. #31000) in 50 mM carbonate buffer pH 9.6. Fab or IgG bound to NeutriAvidin-captured biotinylated peptide-MHC was detected by incubation with horseradish peroxidase-conjugated anti-human IgG Fab’2 (cat. #109-036-097) or anti-human IgG Fc secondary antibody (cat. #109-035-098) (Jackson ImmunoResearch, both in 1 in 3000 dilution), respectively. Bound antibody was observed upon incubation with 3, 3′, 5, 5′-tetramethylbenzidine (SureMoids) substrate was quenched with 1 M HCl and absorbance (Abs) was quantified at 450 nm on the EnSpire microplate reader (Perkin Elmer).

Binding affinities of p53125,134/A24-specific antibodies were characterized by cells ELISA as described above, but with wells coated with antibodies. Subsequently, biotinylated peptide-MHCs that bind to the coated IgG were detected with horseradish peroxidase (HRP)-conjugated streptavidin (BioLegend, cat. #405210, 1 in 3000 dilution).

Stability of the HLA-A24 complexes was determined by a stability ELISA. Briefly, 96-well half area plate (Corning) was coated with 2 μg mL−1 NeutriAvidin (Thermo Fisher Scientific) and used to capture 1.6 nM biotinylated peptide-exchanged HLA-A24 pMHC. Peptide-rescued HLA-A24 pMHC complexes were
then probed with 1 µg mL⁻¹ anti-f2-microglobulin antibody (Clone T999, BD PharMingen), followed by HRP-conjugated anti-mouse IgG secondary antibody (Jackson Immunoresearch, 1 in 3000 dilution).

IgG expression and purification. p5325-124/A24-specific antibodies were refor-
matted from Fabs into human IgG1 by cloning into the pTT5 vector and expressed in HEK293-6E cells as previously described by Durocher et al.⁴⁰ Construction of the triple mutant P1C1 and PICITM-LALA were done by combining mutations from individual clones into the P1C1 wild-type sequence using the Quikchange Lightning Multi Site-directed Mutagenesis Kit (Stratagem). Both the vector and cells were obtained from National Research Council of Canada. Antibodies were purified from the culture supernatant using Protein G resin (Merck Millipore).

Flow cytometry. Binding avidity and specificity of p5325-124/A24-specific anti-
bodies were analysed by pulsing SaoS2 cells in the presence of wild-type p53 peptide (P1C1) or the restricted peptides P1C1-126-140 and P1C1-205-219. Pulsed cells were then washed with staining buffer (1% bovine serum albumin (BSA) in PBS) to remove excess peptides followed by staining with p5325-124/A24-specific antibodies. Bound antibodies were detected by incubation with 1 µg mL⁻¹ polyclonal Alexa Fluor 647-conjugated goat anti-human IgG secondary antibodies (anti-human IgG-AF647) (Life Technologies, cat. #A-21445). Staining of endo-
genous p5325-124 was done with the HLA-A24+ cell lines HT29, HepG2 and SaoS2, and the HLA-A24-transduced and -untransduced cell lines A549, MDA-
MB-231, BT474 and MCF7. PBMCs were isolated from buffy coats obtained from the Blood Bank of Health Sciences Authority (Singapore), using Ficol-Hypaque density gradient centrifugation. Target cells HT29, MDA-MB-231 and 219-A24 were pulsed or unpulsed with p5325-124 peptides and incubated with TCRL antibodies at vincristine concentrations and PBMCs at an effecter/target ratio of 15:1 for 16 h. Cytotoxicity was then measured by lactate dehydrogenase (LDH) release in the supernatants with CytoTox 96 Non-Radioactive Cytotoxicity Assay Kit (Promega) according to the manufacturer’s instructions.

ADCC assay. PBMCs were isolated from buffy coats obtained from the Blood Bank of Health Sciences Authority (Singapore), using Ficol-Hypaque density gradient centrifugation. Target cells HT29, MDA-MB-231 and 219-A24 were pulsed or unpulsed with p5325-124 peptides and incubated with TCRL antibodies at vincristine concentrations and PBMCs at an effecter/target ratio of 15:1 for 16 h. Cytotoxicity was then measured by lactate dehydrogenase (LDH) release in the supernatants with CytoTox 96 Non-Radioactive Cytotoxicity Assay Kit (Promega) according to the manufacturer’s instructions.

ADC cytotoxicity assay. Anti-human secondary antibody–drug conjugated with the cytotoxic drugs MMAE (anti-HuFc-MMAE, cat. #AH-102A), PNU-159682 (anti-HuFc-PNU-159682, cat. #AH-102PN), pyrrolobenzodiazepine (anti-HuFc-
PBD, cat. #AH-106BP) and AAMT (Fab-anti-HuFc-AAMT, cat. #AH-205AM) were purchased from Moradec. PICITM was conjugated to PNU-159682 via a vc-
PAB linker, to a DAR of about 4.1 (Levena Biopharma). For indirect killing assays, target cells (20,000 per well) were first incubated with PICITM at various con-
centrations for 30 min before the addition of the secondary antibodies at equimolar concentrations. For direct killing assays, target cells (20,000 per well) were incu-
bated with PICITM-PNU at various concentrations. Assays were incubated for 3 days at 37 °C and viable cells were quantified with the either the CellTiter 96® AQueous One Solution Cell Proliferation Kit (Promega) or the alamarBlue(Calbiochem). Antibody sensitivity was performed by pulsing SaoS2 cells in the presence of wild-type p53 (TYSPPLNKLF), or a panel of A24-restricted peptides for 1 h at 37 °C prior to staining. Bodies were analysed by pulsing SaoS2 cells in the presence of wild-type p53

Affinity maturation. Affinity maturation of the selected clone P1C1 was per-
formed by phage display using a library in which restricted CDR diversity was introduced (Vex 4.1). Briefly, the library was constructed such that the CDRs of the heavy and light chains were randomized with a degenerate codon that encoded 4 main amino acids (tyrosine, alanine, aspartate and serine). Biopanning was per-
formed as previously described with increased stringency by decreasing the binding p53 peptide-MHC concentration from 20 to 1 and 0.2 nM in the first, second and third round of panning, respectively. Additionally, to remove rapidly dissociating clones, the bead-bound phages were subjected to dissociation in increasing number of washes and temperature of wash buffer with each sequential round of panning. Positive clones were identified by ELISA as previously described, and DNA sequences were further isolated and analyzed. Cytotoxicity was determined using the FACSVerse® (Becton Dickinson) and analyzed with the FlowJo Software.

Animals. NSG mice were purchased from InVivos Pte Ltd and bred under specific pathogen-free conditions. HLA-A24 transgenic mice were purchased from Taconic Biosciences. These mice carry a transgenic consisting fragments of the human HLA-
A*24:02 gene and mouse H2-Kk gene, which encodes a chimeric 1 molecule consisting of the human HLA-A24 leader, α1 and α2 domains ligated to the murine α3, transmembrane and cytoplasmic H2-Kk domains.

In vivo specificity of p53/A24-specific TCRL antibody. Six- to eight-week-old NSG mice were subcutaneously implanted with 2.5 x 10⁶ HT29 in the lower right flank and 5 x 10⁶ MDA-MB-231 (or 2.5 x 10⁶ SaoS2) in the lower left flank. Tumour volume was determined using an external caliper measuring the greatest longitudinal diameter (length) and the greatest transverse diameter (width). Tumour volume (V) was calculated using the modified ellipsoid formula V = ½ lwi x wi x 1+ wi x wi x wi (length x width x height). Antibodies used for imaging were conjugated with Alexa Fluor 680 using Invitrogen’s AAVK Rapid Antibody Labelling Kit. Upon establishment of tumours (≥ 100 mm³), 50 µg of Alexa Fluor 680 labelled PICITM IgG was intra-
venously injected through the tail vein of each mouse. Binding of the labelled antibody to tumours was detected using the IVIS® Imaging System (Perkin Elmer) 48 and 120 h post antibody administration and quantified by the Living Image 3.2 software.

In vivo efficacy of p53/A24-specific TCRL ADC. Six- to eight-week-old NSG mice were subcutaneously implanted with 2.5 x 10⁶ HT29 in the lower right flank and 5 x 10⁶ MDA-MB-231 (or 2.5 x 10⁶ SaoS2) in the lower left flank. Tumour volume was determined using an external caliper measuring the greatest longitudinal diameter (length) and the greatest transverse diameter (width). Tumour volume (V) was calculated using the modified ellipsoid formula V = ½ lwi x wi x 1+ wi x wi x wi (length x width x height). Antibodies used for imaging were conjugated with Alexa Fluor 680 using Invitrogen’s AAVK Rapid Antibody Labelling Kit. Upon establishment of tumours (≥ 100 mm³), 50 µg of Alexa Fluor 680 labelled PICITM IgG was intra-
venously injected through the tail vein of each mouse. Binding of the labelled antibody to tumours was detected using the IVIS® Imaging System (Perkin Elmer) 48 and 120 h post antibody administration and quantified by the Living Image 3.2 software.

In vivo cross-reactivity and toxicity of p53/A24-specific TCRL antibody. Six-
to eight-week-old HLA-A24 transgenic mice were intravenously injected a single dose of PICITM-PNU or PICITM antibody (n = 5 per group). Body weight and fitness of mice were monitored thrice weekly. Mice were sacrificed 20 days post tumour injection for histopathological studies (Advanced Molecular Pathology Laboratory, A*STAR).

Statistical analysis. All data were compiled and analysed by GraphPad Prism 6.0. Data presented are means ± SEM and non-linear regression analyses were used to fit curves. Flow cytometry binding data are representative of three or more inde-
pendent experiments. Due to variability in PBMC donors, ADC assays were not
statistically comparable, but data presented are means of triplicates in individual experiments representative of three or more independent experiments. Statistical analysis of animal experiments were done by Student’s t test with Holm–Šidák method of multiple testing correction and statistical significance was defined with α < 0.05. Statistical analysis of the animals treated in the metastatic model was done by a log-rank (Mantel–Cox) test.

**Reporting summary** Further information on research design is available in the Nature Research Reporting Summary linked to this article.

**Data availability** The source data underlying Figs. 1–3, 5–7 and Supplementary Figs. 3, 4, 6 and 8 are provided as a Source Data file. All the other data supporting the findings of this study are available in the article and its supplementary information files and from the corresponding author upon reasonable request. A reporting summary for this article is available as a Supplementary Information file. PICI and the affinity-matured mutants are proprietary and can only be obtained through a Materials Transfer Agreement.

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Author contributions
L.L., A.G. and C.W. designed the research, analysed the data and wrote the paper. L.L. and A.G. designed and carried out the antibody engineering. L.L., A.G., J.K. and S.L. carried out the in vitro experiments. A.G. carried out the mouse experiments.

Competing interests
The authors declare no competing interests.

Additional information
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