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Concise Communication

Immunology

Oncology

The TP53 gene, encoding the critical p53 tumor suppressor, is the most commonly mutated gene in cancer. Intratumoral T cell responses to mutations occurring frequently at certain TP53 positions, termed hot spots, have not been systematically studied. The 8 most commonly mutated positions in TP53 were found in 33 (24%) of 140 common epithelial tumors analyzed. A TP53-specific screening assay was developed to evaluate T cell responses to these p53 neoepitopes presented through intracellular (tandem minigene) and extracellular (pulsed peptide) pathways on autologous antigen-presenting cells expressing all human leukocyte antigen (HLA) class I and II molecules. Tumor-infiltrating lymphocytes (TILs) from 11 patients recognized autologous p53 neoantigens, which accounted for 8% and 39% of all patients sequenced ($n = 140$) and screened ($n = 28$), respectively. These responses were restricted by a variety of HLA restriction elements, including common class I (A*02:01) and class II (DPB1*02:01 and DRB1*13:01) alleles. T cell receptors (TCRs) were identified from TP53 mutation–reactive helper (CD4) and cytotoxic (CD8) T cells, and TIL and TCR gene–engineered T cells recognized tumor cell lines endogenously expressing HLA and mutant TP53. Thus, the most commonly mutated gene in cancer, TP53, appears to be immunogenic and represents an attractive candidate for evaluating targeted immune cancer therapies. Introduction The adoptive cell transfer of select tumor-infiltrating lymphocytes (TILs) for personalized immunotherapy can mediate [...]

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The *TP53* gene, encoding the critical p53 tumor suppressor, is the most commonly mutated gene in cancer. Intratumoral T cell responses to mutations occurring frequently at certain *TP53* positions, termed hot spots, have not been systematically studied. The 8 most commonly mutated positions in *TP53* were found in 33 (24%) of 140 common epithelial tumors analyzed. A *TP53*-specific screening assay was developed to evaluate T cell responses to these p53 neoepitopes presented through intracellular (tandem minigene) and extracellular (pulsed peptide) pathways on autologous antigen-presenting cells expressing all human leukocyte antigen (HLA) class I and II molecules. Tumor-infiltrating lymphocytes (TILs) from 11 patients recognized autologous p53 neoantigens, which accounted for 8% and 39% of all patients sequenced ($n = 140$) and screened ($n = 28$), respectively. These responses were restricted by a variety of HLA restriction elements, including common class I (A*02:01) and class II (DPB1*02:01 and DRB1*13:01) alleles. T cell receptors (TCRs) were identified from *TP53* mutation-reactive helper (CD4) and cytotoxic (CD8) T cells, and TIL and TCR gene-engineered T cells recognized tumor cell lines endogenously expressing HLA and mutant *TP53*. Thus, the most commonly mutated gene in cancer, *TP53*, appears to be immunogenic and represents an attractive candidate for evaluating targeted immune cancer therapies.

Introduction

The adoptive cell transfer of select tumor-infiltrating lymphocytes (TILs) for personalized immunotherapy can mediate long-term objective tumor regressions in patients with metastatic melanoma, bile duct, cervix, colon, and breast cancers (1–5). These clinical responses are likely mediated by recognition of mutated gene products termed neoantigens because they are absent in normal tissues. Intratumoral T cell responses to mutated neoantigens identified thus far have been unique to the patient, i.e., they are private mutations not identified in other patients' tumors, except for a neoepitope arising from the KRAS^{G12D} driver mutation (1, 3). However, it is largely unknown whether naturally occurring T cells within tumors recognize other shared mutated neoantigens expressed by cancers of unrelated patients.

Across all cancer types, *TP53* is the most commonly mutated gene, but mutations occur throughout the gene with preference in the DNA binding domain (6, 7). Therapeutic approaches evaluating small-molecule inhibitors and T cells targeting both WT

and mutant p53 tumors in mouse models have been described (8–11). Preliminary studies provided some evidence that mutated *TP53* could be recognized by peripheral blood T cells after in vitro stimulation and in vivo vaccination (12–14). However, evidence of immune responses to mutated *TP53* within human tumors is limited even though most tumors will express a *TP53* mutation. We previously reported patient-specific neoantigen screening in 7 patients with metastatic ovarian cancer, 2 of whom had mutated *TP53* neoantigens restricted by HLA-DRB3*02:02 (15, 16). Here, we developed a novel strategy to systematically and comprehensively analyze intratumoral T cell responses to defined *TP53* hot spot mutations independent of other tumor mutations in 133 new patients with multiple tumor types. The goal was to translate these cells or their TCR genes into broadly applicable adoptive cellular therapies for common epithelial malignancies.

Results and Discussion

Patients with metastatic epithelial cancers were enrolled in clinical trials (NCT01174121, NCT02133196, and NCT01585428) at the National Cancer Institute (NCI). Metastases were resected for growth of TILs from fragment cultures in high-dose interleukin 2 (IL-2), and peripheral blood lymphocytes (PBLs) were collected for germline sequencing controls and to generate autologous antigen-presenting cells (APCs) for screening assays. A total of 140 patients diagnosed with cancers of the bile duct, breast, colon, cervix, endometrium, gastroesophagus, head and neck, lung, ovary, pancreas, and rectum were evaluated. Of these patients, 91 (65%) had a tumor that expressed a mutation in *TP53* (Figure 1A and Supplemental Table 1;

► Related Commentary: p. 949

Conflict of interest: PM, AP, PFR, MRP, WL, SAR, and DCD have a patent application (no. 62/565,383) for TCRs described in this manuscript. PM, SAR, and DCD have a patent application (no. 62/565,464) for methods described in this manuscript.

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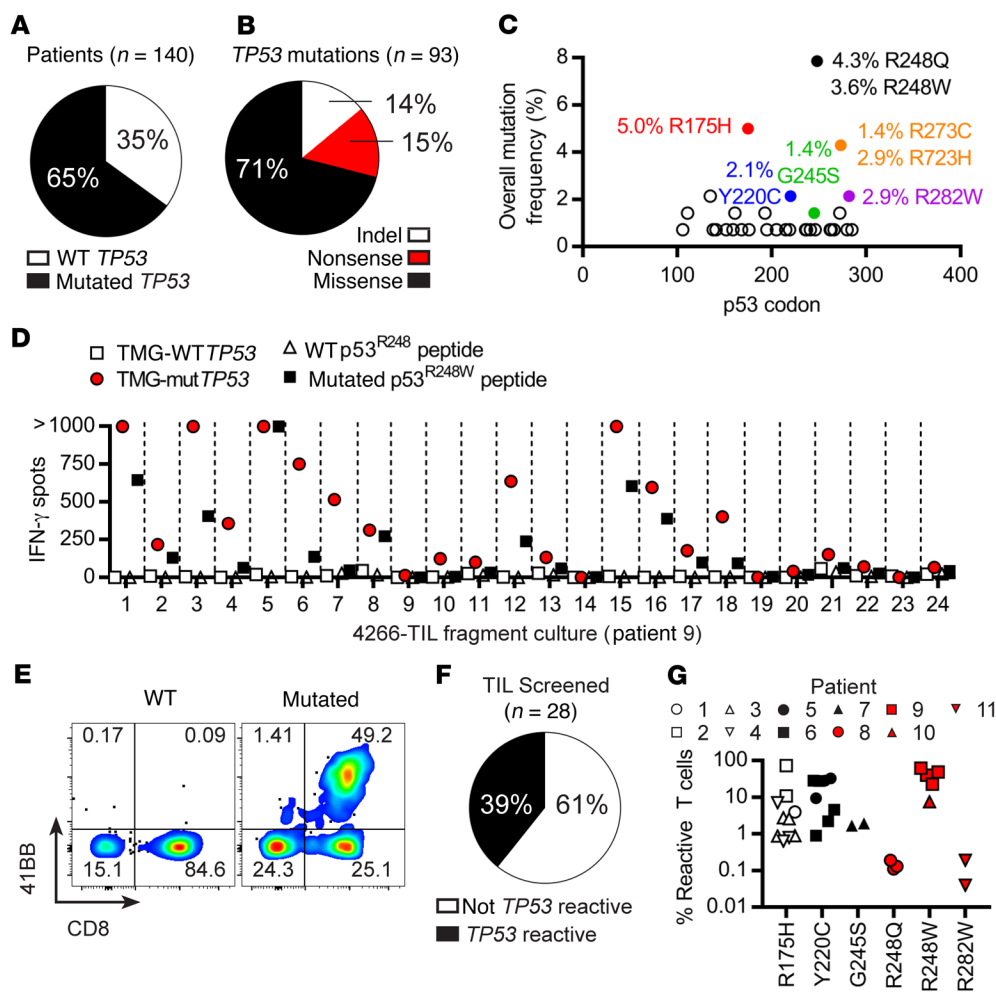


Figure 1. *TP53* hot spot mutations are immunogenic and elicit intratumoral T cell responses to *p53* neoantigens. (A) Patients with a tumor expressing a *TP53* mutation. (B) Classification of *TP53* mutations from resected tumors. (C) The overall frequencies of each missense mutation in *TP53* from all patients sequenced by *p53* codon where the specific amino acid change frequency is given for selected mutations. (D) Screening results from patient 9 as measured by IFN- γ ELISPOT. (E) Expression of 41BB from patient 9's TIL culture 1 in response to *TP53* TMGs. (F) Overall frequency of TIL responses to mutated *TP53*, which include TMG, peptide, or both (Supplemental Table 5). (G) Frequencies of verified positive TIL responses from selected fragment cultures (some patients had >1) to *p53* R175H, Y220C, G245S, R248Q, R248W, and R282W neoantigens. The upregulation of 41BB minus the background in response to mutated *p53* peptide (CD4) or TMG (CD8) is reported. Types of T cell responses can be found in Table 1.

supplemental material available online with this article; <https://doi.org/10.1172/JCI123791DS1>). A total of 93 nonsynonymous *TP53* mutations were detected, including 14 insertions or deletions (indels), 13 nonsense mutations, and 66 missense mutations (Figure 1B). Tumors from 33 patients, representing 24% of all patients evaluated, possessed mutations within codons 175, 220, 245, 273, and 282 (Figure 1C and Supplemental Tables 1 and 2), positions that corresponded to previously identified *TP53* hot spots in a wide variety of cancer types (Supplemental Tables 3 and 4). There was no explicit bias toward a particular disease type in patient accrual at the NCI Surgery Branch during this time even though there was a preponderance of colorectal patients enrolled in the protocol. Studies of fresh tumor with available RNA-seq also demonstrated high levels of *TP53* gene expression, regardless of *TP53* mutation status (Supplemental Table 1). The high levels of *TP53* gene expression for the targeted mutations, which ranged between the 89th and 100th percentile of all expressed transcripts, raised the possibility that one or more of the peptides arising from these common mutations might be immunogenic in these patients.

The Catalogue of Somatic Mutations in Cancer (COSMIC) database (<https://cancer.sanger.ac.uk/cosmic>) showed that the 12 most common *TP53* hot spot mutations across all tumor types were R175H, Y220C, G245S, G245D, R248L, R248Q,

R248W, R249S, R273C, R273H, R273L, and R282W. A novel high-throughput technique was developed using minigenes encoding 25 amino acid peptides containing each *TP53* mutation and flanked by 12 amino acids of WT sequence. The peptides were concatenated in tandem to generate a tandem minigene (TMG) as has been performed in testing patient-specific neoepitopes (Supplemental Figure 1) (2, 3, 5). A similar TMG encoding the corresponding WT sequences was also generated to facilitate analysis of T cell specificity to the mutated variants. The TMGs were synthesized de novo as DNA, cloned into an expression vector plasmid DNA, and in vitro transcribed into mRNA. The same 25 amino acid sequences (WT or mutated) were also synthesized as individual peptides at greater than 95% purity using high-performance liquid chromatography. Immature dendritic cells (APCs) were generated from the autologous patients' peripheral blood and were either electroporated with TMGs (irrelevant, WT, or mutated *TP53*; 12–16 minigenes each) or pulsed with individual WT or mutated peptides corresponding only to the autologous *TP53* mutation. Cocultures of TIL fragment cultures and prepared APCs were incubated overnight at 37°C, and T cell responses were measured by OX40 and 41BB upregulation on the cell surface (flow cytometry) and secretion of interferon- γ (IFN- γ) using an enzyme-linked immunospot (ELISPOT) assay. The upregulation of 41BB was more consistent

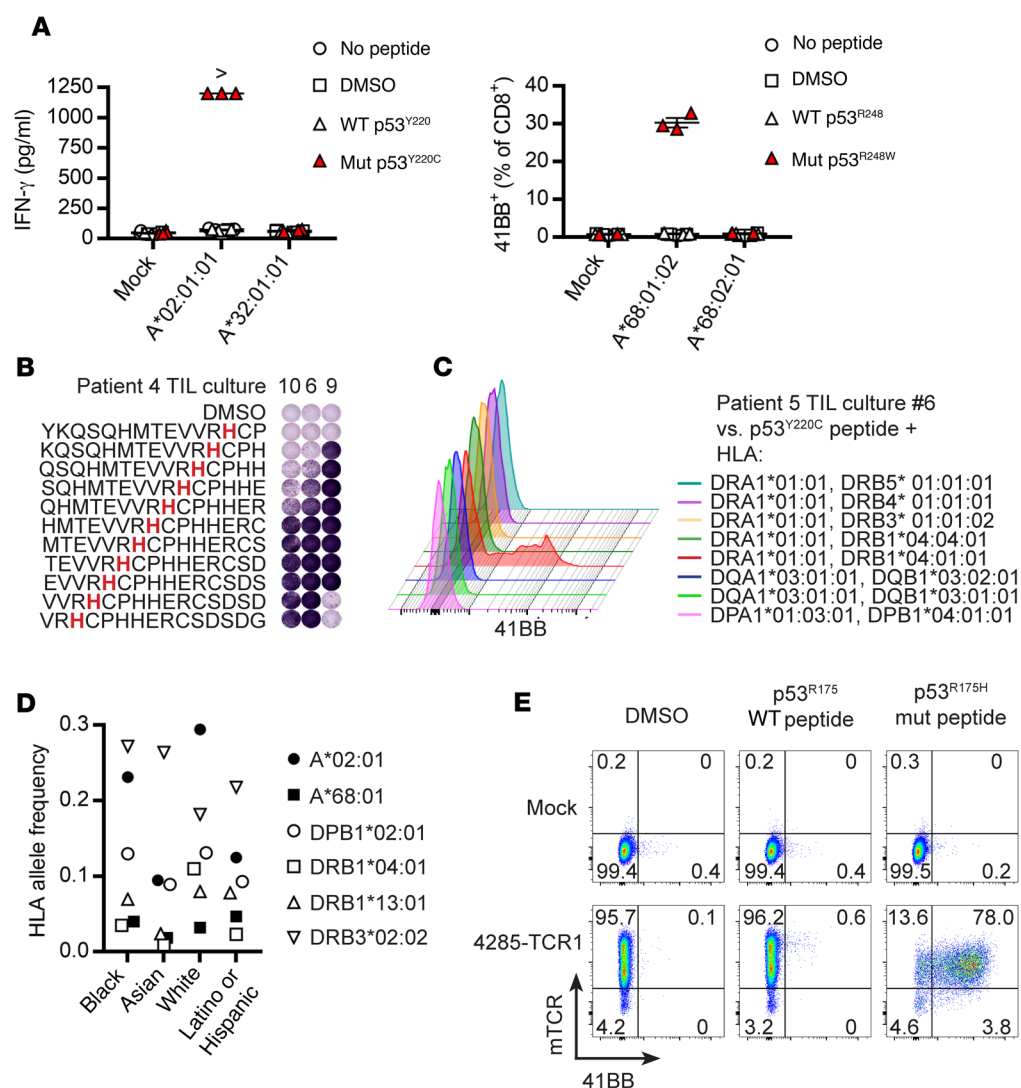


Figure 2. Immunogenic TP53 hot spot mutations could be restricted by common HLA alleles and targeted by TCRs isolated from p53 neoantigen-reactive TILs. (A) HLA class I restriction of p53^{R248W} (patient 5, TIL culture 4259-F1) and p53^{R248W} (patient 9, TIL culture 4266-F1) neoepitopes. Data are mean \pm SEM, $n = 3$ technical replicates. > indicates 1,250 pg/ml IFN- γ . (B) Peptide mapping of CD4⁺ T cell responses from patient 4 to p53^{R175H} neoepitopes. (C) HLA class II restriction for p53^{R248W} neoantigen from patient 5. (D) HLA allele frequencies from selected populations (<http://www.allelefrequencies.net/default.asp>). (E) Specificity of HLA-DRB*13:01/p53^{R175H}-reactive TCR to 25 amino acid p53^{R175H} peptides.

and robust than OX40 for assessing CD4 T cell reactivities and was thus reported (Supplemental Figure 2). Negative controls for screening were dimethyl sulfoxide (DMSO; peptide vehicle) and T cells only (media), and a mixture of phorbol myristate acetate (PMA) and ionomycin as positive control. A positive response in the initial screening was measured if the IFN- γ secretion and/or 41BB expression was more than twice background (Supplemental Table 5). Selected TIL fragment cultures with the most cells and robust responses were then rescreened in additional independent experiments where they had to display at least 10-fold higher avidity to the mutated gene relative to the relevant WT counterpart to be considered positive.

Patient 9 exemplified the speed at which mutated TP53 neoantigen-reactive T cells could be identified for possible cell-based treatment. Lung metastases were resected from this patient, 24 fragment cultures were grown, the screen was performed, and p53^{R248W} T cell responses were identified 34 days after surgery (Figure 1, D and E). Secretion of IFN- γ was off scale (>1000 spots) for TIL cultures 1, 3, 5, and 15 when cocultured with the mutated TP53 TMG (Figure 1D, red circles), and this was specific for the p53^{R248W} peptide (Figure 1D, closed squares). Upregulation of 41BB in response to the p53^{R248W} neoantigen

demonstrated this was a CD8⁺ T cell response (Figure 1E). Autologous PBLs and TILs were available for 28 of 33 patients (Supplemental Table 2). In aggregate, 39% ($n = 11$) of patients displayed a T cell response to one of the TP53 hot spot mutations under investigation (Figure 1F), and there was a wide range of TP53-reactive T cell frequencies found either through IFN- γ secretion, 41BB upregulation, or both in response to TMG, peptide, or both (Figure 1G and Supplemental Table 5). T cell responses were readily detected against R175H, Y220C, G245S, R248Q, R248W, and R282W with a range of frequencies (0.1%–80% of CD3⁺ 41BB⁺ cells) in the 11 patients with mutated TP53-reactive T cells (Figure 1G and Supplemental Figures 3–8).

We next evaluated the HLA restriction elements for these TP53 neoantigens by transfecting DNA plasmids encoding HLA alleles into the COS7 cell line (15). TILs from patients 1 and 5 recognized HLA-A*02:01 neoepitopes containing p53^{R175H} and p53^{R248W}, respectively, and TILs from patient 9 recognized an HLA-A*68:01-restricted neoepitope containing p53^{R248W} (Figure 2A and Supplemental Figure 9). Of note, the only shared mutated TP53 neoepitope we identified was the HMTEVVRHC peptide, which was recognized in the context of HLA-A*02:01 by TILs from patients 1 and 2 (Table 1). To identify HLA class

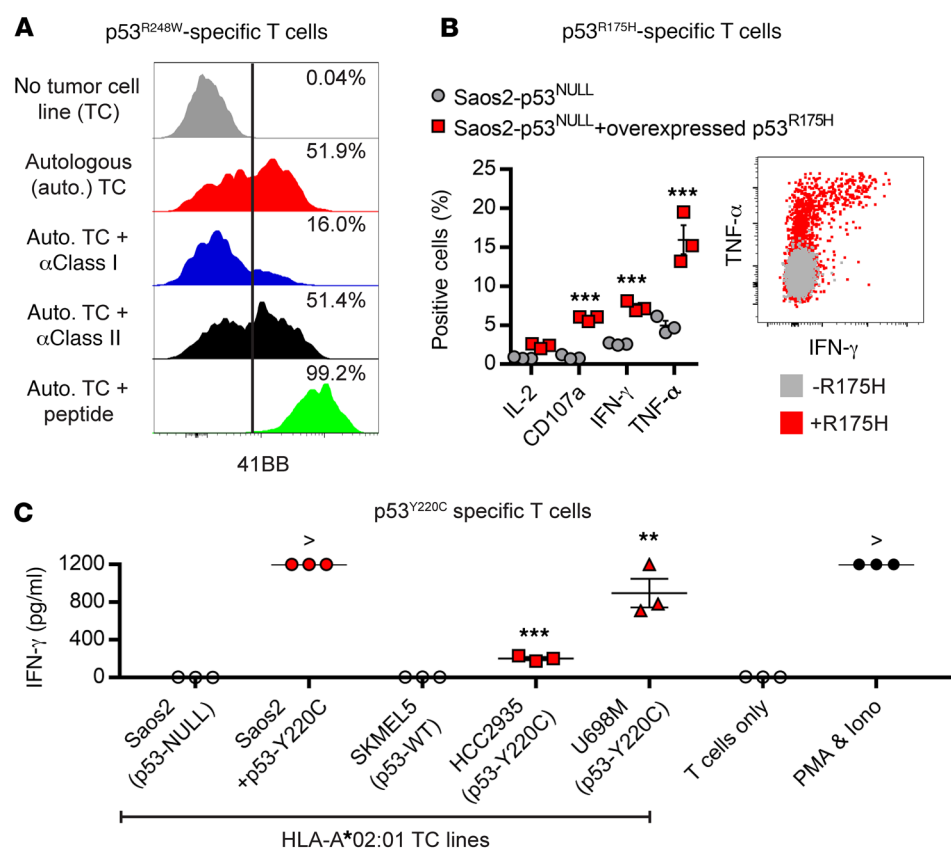


Figure 3. Tumor cells naturally process and present p53 neoantigens to T cells. (A) Expression of 41BB on CD8⁺ T cells expressing HLA-A*68:01/p53^{R248W} neoantigen-specific TCR following coculture with autologous tumor cell line (TC), which was incubated with anti-HLA class I or II blocking antibodies or p53^{R248W} peptide. (B) Intracellular cytokine staining of cocultures of CD8⁺ T cells expressing HLA-A*02:01/p53^{R175H} neoantigen-specific TCR and Saos2 cells alone (HLA-A*02:01+ and p53^{NULL}; gray circles on left graph or gray dots on right plot) or overexpressing p53^{R175H} full-length protein (red squares on left graph or red dots on right plot). (C) Secretion of IFN-γ in cocultures of HLA-A*02:01/p53^{Y220C} neoantigen-specific T cells (CD8⁺-enriched TIL culture 4259-F1 from patient 5) with HLA-A*02:01-positive tumors from unrelated donors and Saos2 tumor cells overexpressing full-length p53^{Y220C} protein. > indicates 1,250 pg/ml IFN-γ. Data in panels B and C are mean ± SEM, *n* = 3 technical replicates, and 2-tailed Student's *t* tests were performed for statistical analyses (***P* < 0.01 and ****P* < 0.001).

II-restricted minimal neoepitopes within the 25 amino acid p53 neoantigen, we tested peptides containing 15 amino acids that overlapped in 14 amino acids, similar to a previous study evaluating patient-specific neoepitopes where we identified HLA-DRB3*02:02-restricted p53^{Y220C} (HNYMCMSSCMGSMN) and p53^{G245S} (NTFRHSVVPCEPPE) neoantigens from patients 6 and 7, respectively (15). For example, TIL cultures 4285-F9 and 4285-F6/4285-F10 from patient 4 responded to peptides containing the EVVRHCPH and VRHCPHER core sequences, respectively (Figure 2B). Both of these p53^{R175H} peptides were restricted by HLA-DRB1*13:01, and the CD4⁺ T cell response to p53^{R248W} from patient 10 was restricted by HLA-DPB1*02:01 (Supplemental Figures 10–12). CD4⁺ and CD8⁺ T cells elicited reactivity to the HLA-DRB1*04:01-restricted peptide RNT-FRHSVVPCE (Figure 2C) and to the HLA-A*02:01-restricted peptide VVPCEPPEV (Figure 2A) p53^{Y220C} neoepitopes, respectively, from patient 5. Some of the HLA alleles that presented the immunogenic p53 neoantigens in our patient cohort were frequent in Black, Asian, White, and/or Latino and Hispanic American populations (Figure 2D), as measured by allele frequency, which is roughly half the population frequency (allele frequency of 0.1 corresponds to ~20% of the population), indicating that a diverse cohort of patients could benefit from mutated *TP53*-specific T cell immunotherapy.

A library of p53 neoantigen-specific TCRs was also generated following coculture of TILs with p53 neoantigen, sorting for 41BB⁺ cells, and single-cell reverse transcriptase PCR with TCR gene-specific primers (17). Peripheral blood T cells were genetically modified with TCRs using the nonviral Sleeping

Beauty transposon/transposase system (18), and TCR-transposon T cells were cocultured with target p53 neoepitopes. We previously identified p53^{Y220C}- and p53^{G245S}-reactive TCRs with a common restriction element (HLA-DRB3*02:02) from patients 6 and 7 (15). In this study, we isolated TCRs specific for p53^{R175H}/HLA-A*02:01 (patient 1), p53^{R175H}/HLA-DRB1*13:01 (patient 4), p53^{Y220C}/HLA-DRB1*04:01 (patient 5), p53^{R248W}/HLA-A*68:01 (patient 9), and p53^{R248W}/HLA-DPB1*02:01 (patient 10) (Figure 2E and Supplemental Figures 9 and 11–16). A total of 9 TCRs recognizing 7 different p53 neoepitopes have been identified, which were likely unique as they were not found in the NCBI (<https://www.ncbi.nlm.nih.gov/>) and VDJDB (<https://vdjdb.cdr3.net/>) databases (Supplemental Table 6). Thus, off-the-shelf TCR gene therapy could be used for patients with tumors expressing the combination of one of the above HLA and p53 neoantigen combinations.

We evaluated the ability of mutated *TP53*-specific T cells to recognize naturally processed p53 neoepitopes. An autologous tumor cell line generated from mouse xenografts in immunocompromised mice with endogenous expression of both mutated p53^{R248W} and HLA-A*68:01 (Supplemental Figure 17) was recognized by HLA-A*68:01/p53^{R248W}-reactive T cells from patient 9, which was inhibited by class I-blocking antibody (Figure 3A). Saos2 is a *TP53* knockout (p53^{NULL}) tumor cell line commonly used to study *TP53* biology that naturally expresses HLA-A*02:01. Cocultures of HLA-A*02:01/p53^{R175H}-specific CD8⁺ T cells from patient 1 with Saos2 overexpressing p53^{R175H} demonstrated significant specific intracellular expression of IFN-γ, tumor necrosis factor-α (TNF-α), and CD107a, which is indicative of

Table 1. Patients with T cell responses to TP53 hot spot mutations

Patient	Age/Sex	Cancer type	Tumor no.	TP53 mutation	T cell type	TP53 neoepitope (mutation)	HLA restriction
1	52/M	Colon	4141	R175H	CD8	HMTEVVRHC	A*02:01
2	36/F	Colon	4196	R175H	CD8	HMTEVVRHC	A*02:01
3	55/M	Colon	4252	R175H	CD4	YKQSQHMTEVVRHCPHHERCSDSDG	Class II
4	46/M	Colon	4285	R175H	CD4	VVRHCPHHERCSDSD QHMTTEVVRHCPHHER	DRB1*13:01
5	44/F	Colon	4259	Y220C	CD4 CD8	RNTFRHSVVVPCE VVPCEPEV	DRB1*04:01 A*02:01
6	39/F	Ovary	4149	Y220C	CD4	NTFRHSVVVPCEPE	DRB3*02:02
7	58/F	Ovary	4127	G245S	CD4	HYNYMCSNCSGCMN	DRB3*02:02
8	69/F	Colon	4268	R248Q	CD4 CD8	YMCNSSCMGGMNQRPILTIITLED	Class I and II
9	41/F	Colon	4266	R248W	CD8	SSCMGGMNWR	A*68:01
10	49/M	Rectal	4273	R248W	CD4	SCMGGMNWRPILTI	DPB1*02:01
11	66/F	Pancreas	4270	R282W	CD4	FEVRVCACPGROWRTEENLRKKGE	Class II

The type of T cell response, reactive p53 neoepitope (minimal when possible), and HLA restriction for each patient. Some T cell responses were of insufficient frequency to identify the specific HLA restriction; thus, the HLA family (class I or II) is denoted.

T cell degranulation and tumor cytolysis, compared with cocultures with parental Saos2 cells (Figure 3B). CD4⁺ T cells also degranulated in response to p53^{R175H}, p53^{Y220C}, and p53^{R248W} neoepitopes, indicating that these cells may have the capability to directly lyse tumor cells (Supplemental Figures 18–20). A panel of HLA-A*02:01-expressing tumor cells from unrelated donors was cocultured with HLA-A*02:01/p53^{Y220C}-reactive T cells from patient 5, which secreted significant IFN-γ in response to HCC2935 and U698M tumor cell lines with endogenous expression of p53^{Y220C} and Saos2 overexpressing p53^{Y220C}, but lacked IFN-γ secretion in cocultures with Saos2 (p53^{NULL}) and SKMEL5 (WT TP53) tumor cell lines (Figure 3C). These data indicate that TP53 mutational hot spot mutations can be processed and presented on the surfaces of tumor cells in the context of relevant HLA molecules and can be recognized by T cells.

Both CD4⁺ and CD8⁺ T cells responded to mutated TP53 neoantigens, and 2 of the previously reported ovarian cancer patients were included with the 9 new responses to accurately describe the totality of our experience detecting T cell responses to mutated TP53 in 11 patients (Table 1). Our previous experience in detecting TP53 mutation-specific T cells in ovarian cancer (15, 16) was expanded in this study to colorectal and pancreas cancers and 4 more TP53 hot spot mutations were determined to be immunogenic in the context of 5 new HLA restrictions. This led to an additional 7 mutated TP53-specific TCRs for the potential benefit of future patients with matching HLA and TP53 mutations. Moreover, once it was determined that the tumor expressed a TP53 hot spot mutation, TIL screening could occur because the TMG and peptides were pre-prepared, thus eliminating the time and cost associated with personalized neoantigen screening. It is currently unknown why T cell responses were frequently observed to p53 neoepitopes, but high TP53 expression (Supplemental Table 1) or increased stability of the mutant p53 protein could influence their immunogenicity (19). Individual codon substitutions also appeared to differ in terms of the frequency with which they gave rise to an immunogenic epitope (Supplemental Table 7), although the sample size was small, and future studies can evaluate

this in more detail with a larger patient cohort. The frequency of these responses was likely to be influenced by the HLA alleles expressed by patients' cells. Two, three, or six patients had both, only CD8⁺, or only CD4⁺ T cell responses to mutated TP53, respectively (Table 1), suggesting that there are complex dynamics driving the immunogenicity of TP53 hot spot mutations, which may include expression of HLA on the tumor or APC, TCR avidity, and T cell trafficking to the tumor microenvironment.

The observation that 11 of 28 patients (39%) recognized an autologous p53 neoepitope suggests that

TP53 mutations may be of value in developing cell transfer immunotherapy. CD4⁺ or CD8⁺ TILs selected for IFN-γ secretion or 41BB upregulation in response to KRAS and passenger mutations have been grown to large numbers and infused into cancer patients, which resulted in long-term objective tumor regressions of breast cancer, colon cancer, and cholangiocarcinoma (1, 2, 4), and suggests that targeting TP53 mutations could result in similar clinical benefit. Our focused TP53 screening approach could also be a model for targeting other high-value mutated tumor neoantigens present in large numbers of unrelated patients, e.g., KRAS, PIK3CA, and EGFR. Neoantigen loss through T cell-selective pressure in vivo could be a mechanism of resistance, especially when targeting tumor suppressors. However, the TP53 hot spot mutations may not follow this general rule, as the WT TP53 allele is typically lost through loss of heterozygosity (Supplemental Table 1), and the TP53 mutation can inactivate WT p53 tumor suppressor function and sometimes also have gain-of-function activity, which may be critical for tumor survival (20). We plan to directly test the ability of T cells specific for TP53 mutations (TILs and TCRs) to eliminate metastatic cancer in clinical trials at the NCI Surgery Branch.

Methods

Antibodies used in this study can be found in Supplemental Table 8. Additional methods are in the Supplemental Material.

Study approval. Written, informed consent was granted for all patients prior to enrollment in National Institutes of Health protocols (NCT01174121, NCT02133196, and NCT01585428), which were approved by the Institutional Review Board. Studies in mice were approved by the National Institutes of Health Institutional Animal Care and Use Committee.

Author contributions

PM, SAR, and DCD conceptualized and developed the project. PM, AP, PFR, MRP, BCP, LJ, JJG, ZY, AS, ET, VH, WL, and DCD designed and performed experiments and analyzed data. RPTS supervised

patient samples and NPR supervised mouse studies. PM, PFR, SAR, and DCD wrote the manuscript, and all authors edited and approved the manuscript.

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