Advances in Brief

Characterization of HLA-A3-restricted Cytotoxic T Lymphocytes Reactive against the Widely Expressed Tumor Antigen Telomerase[1]

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Abstract

Purpose: We have reported previously that the telomerase catalytic subunit, human telomerase reverse transcriptase (hTERT), is a widely expressed tumor-associated antigen recognized by CTLs. A nine-amino acid peptide derived from hTERT binds strongly to HLA-A2 antigen and elicits CTL responses against a broad panel of hTERT$^+$ tumors (but not hTERT$^+$ hematopoietic progenitor cells). The applicability of hTERT as a potential target for anticancer immunotherapy would be widened by the identification of epitopes restricted to other common HLA alleles, such as HLA-A3 antigen.

Experimental Design: Using a method of epitope deduction, HLA-A3-restricted peptide epitopes were screened from hTERT and tested for immunogenicity in a human *in vitro* T-cell system.

Results: The hTERT peptide K973 was used to generate specific CD8$^+$ CTLs from HLA-A3$^+$ cancer patients and healthy individuals. These CTLs lysed hTERT$^+$ tumors from multiple histologies in an MHC-restricted fashion, suggesting that the epitope is naturally processed and presented by tumors. In contrast, highly enriched HLA-A3$^+$ CD34$^+$ peripheral blood progenitor cells or activated T cells were not lysed.

Conclusion: Given the expression of HLA-A2 and HLA-A3 antigen in the general population, these findings extend the potential applicability of hTERT as a therapeutic target to >60% of all cancer patients. The characterization of hTERT as a polyepitope, polyallelic tumor-associated antigen may provide an approach for circumventing therapy-induced resistance potentially mediated by antigenic- and allelic-loss tumor escape mutants.

Introduction

Although the discovery of human TAAs[3] has revitalized efforts to develop antigen-specific vaccines and adoptive T-cell immunotherapy (1, 2), a major limitation has been the lack of universal TAAs applicable to the majority of patients with common cancers (3). Using a method of epitope deduction, we recently identified a widely expressed HLA-A2-restricted epitope derived from the telomerase catalytic subunit (hTERT) that triggers T-cell responses against a broad range of hTERT$^+$ tumors (4). A clinical trial targeting this epitope for therapeutic vaccination is under way at our institution. hTERT is expressed by the vast majority of human cancers but rarely in normal cells (5). Furthermore, its expression has been directly linked to tumor development (6, 7) such that inhibition of telomerase in telomerase-positive human tumors leads to growth arrest (8–10). Most TAAs described previously are not known to play a role in oncogenesis or tumor growth, and as such, TAA-specific immunotherapy has been susceptible to the appearance of antigen-deficient mutant tumors that are nevertheless clinically progressive (11–13). In addition, targeting a single HLA restriction element carries the risk of inducing immune escape by tumor down-regulation of the particular HLA allele (14–19).

In this study, we extended our search for hTERT-derived immunogenic peptides to HLA-A3 antigen because this allele is expressed by 15–25% of patients and identify one such peptide that can trigger HLA-A3-restricted CTLs that kill hTERT$^+$ tumors from multiple histologies.

Materials and Methods

Donor and Patient Samples. PBMCs were obtained by leukapheresis or phlebotomy from cancer patients and normal donors after informed consent. Primary tumor cells were obtained following biopsy of a patient with a lymph node heavily involved with NHL. Mobilized CD34-enriched peripheral blood

Table 1 Binding of hTERT and control peptides to human HLA-A3 antigen

Name	Sequence	Protein	Position (amino acid)	Score[a]	Fluorescence index[b]
K973	KLFGVLRLK	hTERT	973	101	3.5
G804	GLFDVFLRF	hTERT	804	270	0.0
R535	RLREEILAK	hTERT	535	90	0.0
I265	ILRGSVAHK (positive control)	Influenza NP	265	90	3.9
I540	ILAKFLHWL (HLA-A2 peptide)	hTERT	540	0	0.0

[a] Calculated score in arbitrary units based on http://bimas.dcrt.nih.gov/molbio/hla_bind.
[b] (Mean fluorescence with peptide − mean fluorescence without peptide)/(Mean fluorescence without peptide); results are representative of three experiments.

cells were obtained from a patient with sarcoma scheduled for autologous stem cell transplantation and purified by cell sorting. Our Internal Review Board approved the protocols and informed consent procedures used to obtain all patient samples. Cell lines were from American Type Culture Collection (Manassas, VA) or as described (4).

Peptides and Cytokines. Peptides were purchased from Sigma Chemical Co. (Genosys Biotechnologies, Woodlands, TX). Four hTERT-derived peptides used in this study are listed in Table 1. The peptide I265 (ILRGSVAHK) is derived from the nucleoprotein of influenza A (20), and the peptide I476 (ILKEPVHGV) is derived from the HIV *RT-pol* gene (21). Recombinant human flt-3 ligand, IL-3, IL-6, and granulocyte-CSF were purchased from Peprotech (Rocky Hill, NJ), and recombinant human erythropoietin was purchased from R&D Systems (Minneapolis, MN).

Generation and Evaluation of CTL and T Cells. CTLs were generated as described previously (4, 22). CTLs as effector cells were used after the third or fourth restimulation in chromium release assays for which specific lysis was calculated as described (4). SD was <5%.

T2A3 Binding Assay. Transporter associated with antigen processing-deficient T2 cells (American Type Culture Collection) transfected with the *HLA-A3* gene (Ref. 23; a kind gift of Dr. P. Cresswell, Yale University Medical Center, New Haven, CT) were used to measure peptide binding to HLA-A3 antigen. After peptide pulsing overnight, HLA-A3 antigen expression was measured by flow cytometry using an anti-HLA-A3 mAb (One Lambda, Canoga Park, CA), followed by FITC-conjugated F(ab′)$_2$ goat antimouse IgM (Zymed, South San Francisco, CA).

ELISPOT Analysis. PBMCs at 2×10^5 cells/well were added to ImmunoSpot plates (Cellular Technology, Cleveland, OH) precoated with 10 µg/ml of anti-IFN-γ mAb (Mabtech, Nacka, Sweden) in the presence or absence of peptide overnight at 37°C. After washing, wells were then incubated with 1 µg/ml biotin-conjugated anti-IFN-γ mAb (Mabtech), followed by streptavidin-alkaline phosphatase (Mabtech). Spots were developed with 5-bromo-4-chloro-3-indolyl-phosphate and nitroblue tetrazolium color development substrate (Promega). Spots were counted using an ImmunoSpot microscope reader (Cellular Technology).

Evaluation of Telomerase Activity and hTERT *in Situ* Hybridization. TRAP assays were performed as described previously (24). *In situ* hybridization for hTERT mRNA was performed using methods (25) and probes (26) described previously.

Results

Identification of an HLA-A3-restricted Epitope from hTERT. To determine whether hTERT-derived peptides bind to HLA-A3 antigen, the hTERT sequence was reviewed for peptides that could potentially bind this allele using a peptide motif scoring system.[4] Three peptides were synthesized and evaluated for binding to HLA-A3 antigen using transporter associated with antigen processing-deficient T2 cells transfected with the *A3* gene (Table 1). All three peptides received predictive scores similar to the positive control peptide (I265) derived from influenza A nucleoprotein, but only peptide K973 and the control peptide bound in the T2A3 assay.

CTLs were then generated *ex vivo* using purified CD8^{+} T cells by priming with peptide-pulsed autologous dendritic cells, followed by weekly restimulation with autologous CD40-activated B cells, according to the same culture system we described previously for the generation of HLA-A2-restricted hTERT-specific CTLs from normal donors (4). PBMCs used for CTL generation were obtained from two HLA-A3[+] cancer patients and three HLA-A3[+] healthy individuals. One patient was a 69-year-old man with metastatic melanoma whose treatment history included chemotherapy and granulocyte/macrophage-CSF therapy. The second patient was a 71-year-old woman with metastatic ovarian cancer whose prior treatments included chemotherapy and a Phase I autologous tumor cell vaccine.

On the basis of the lysis of T2A3 targets pulsed with but not without the relevant peptide, K973-specific CTLs were demonstrated for two of two cancer patients and two of three normal donors (Fig. 1). CTLs specific for influenza I265 were also generated from three of three donors tested (one cancer patient and two healthy individuals). K973-specific CTLs (and I265-specific CTLs as a control) were then tested for cytotoxicity against a panel of tumor cell lines with diverse histological origins to determine whether the K973 hTERT peptide is naturally processed and presented by tumors. Each tumor cell expressed equal amounts of surface MHC class I antigen, as determined by flow cytometry, but only certain lines were HLA-A3 positive (data not shown). All cell lines demonstrated telomerase activity (Fig. 2). As shown in Fig. 3, *A–C*, K973-specific CTLs killed telomerase-positive tumors from a range of histologies, including multiple myeloma, lung carcinoma, and melanoma. HLA-A3-negative cell lines were not lysed. Tumor-specific CTLs were generated for two of two cancer patients and

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[4] Available on the Internet (http://bimas.dcrt.nih.gov/molbio/hla_bind/).
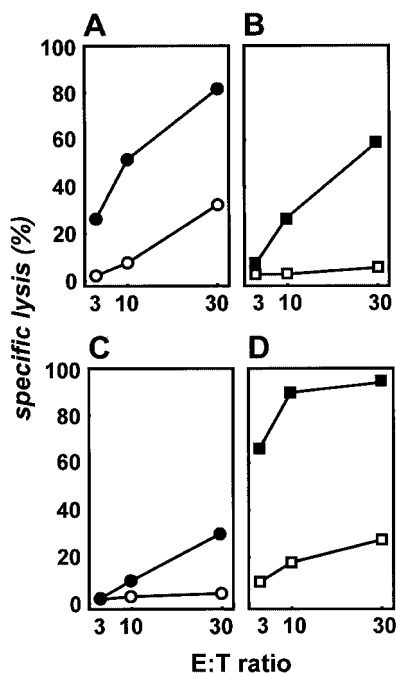
Fig. 1 Cytotoxicity of CTLs against peptide-loaded T2A3 targets. CTLs were generated against the K973 hTERT-derived peptide (*A* and *C*) or the I265 peptide (*B* and *D*) derived from influenza A from a normal donor (*A* and *B*) or the patient with melanoma (*C* and *D*). Lysis of target cells incubated with the relevant peptide (*solid symbols*) but not the irrelevant peptide (*open symbols*) was observed.

Fig. 2 Telomerase activity of tumor cells and normal cells tested as CTL targets. Cytosolic cellular extracts were assayed for telomerase activity. A 36-bp internal control PCR product (*IC*) was seen for each reaction. As a negative control, each extract was heat treated (*HT*) to inactivate telomerase.
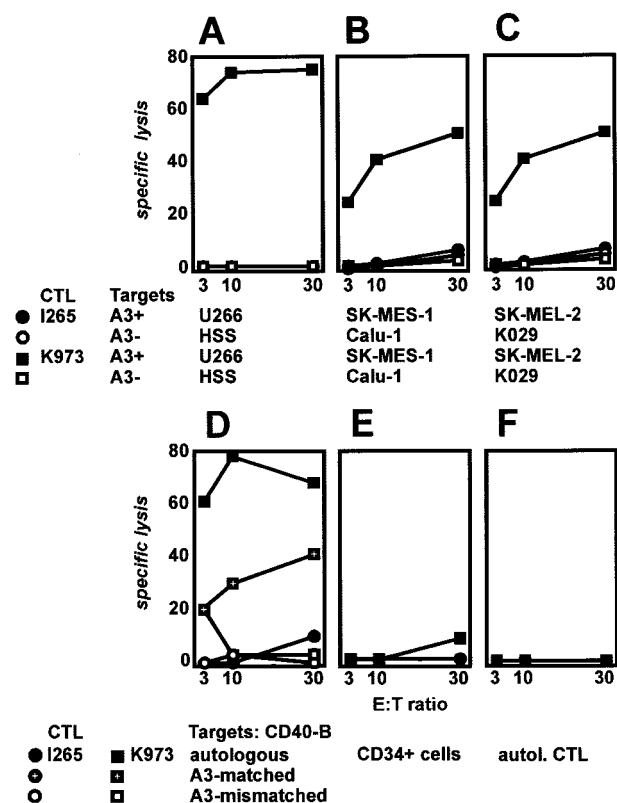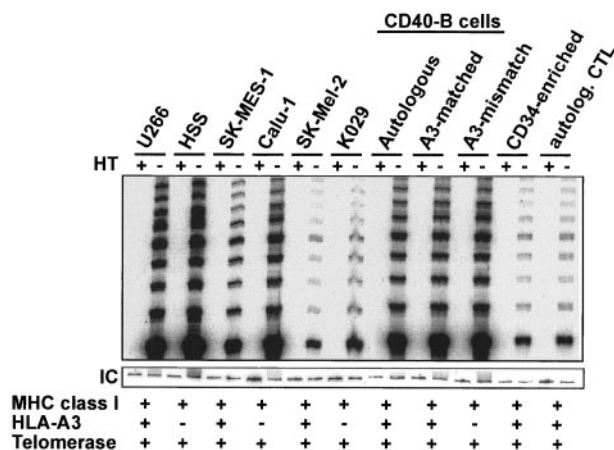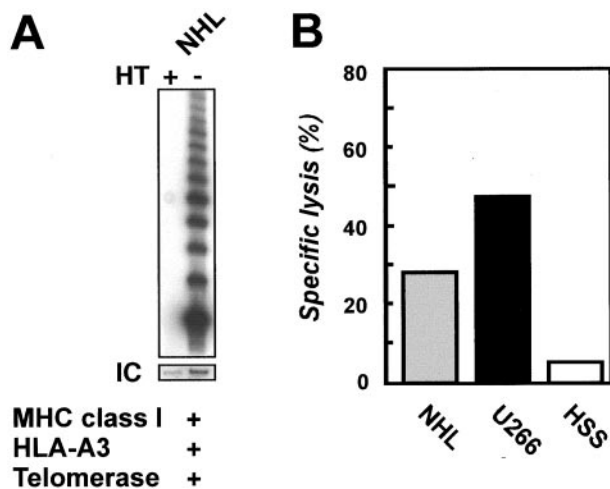

Fig. 3 Cytotoxicity of K973 hTERT-specific, HLA-A3-restricted CTLs. K973-specific CTLs lysed myeloma (*A*), carcinoma (*B*), and melanoma (*C*) cell lines that expressed both HLA-A3 antigen and telomerase activity. Influenza NP I265-specific CTLs did not lyse any target. *D,* K973-specific CTLs lysed autologous CD40-B cells and HLA-A3-matched CD40-B cells but not HLA-A3-negative CD40-B cells. Neither K973-specific nor I265-specific CTLs lysed HLA-A3^{+} CD34^{+} peripheral blood cells (*E*) or autologous CTLs (*F*), despite the observation that these normal target cells are telomerase positive (Fig. 2). Mean results of one experiment of one (*D* and *F*) or two (*A, B, C,* and *E*) performed using a patient CTL line are shown. Tumor specific cytotoxicity was similarly observed for CTLs from a second cancer patient and one normal donor. No cytotoxicity against HLA-A3^{+} CD34^{+} cells was seen in an additional experiment with K973-specific CTLs from a normal donor.

ratio of 20:1 and effector:hot target ratio of 30:1, K973-pulsed T2A3 cells inhibited U266 lysis 29% compared with influenza I265-pulsed T2A3 cells.

K973-specific CTLs were also tested against freshly isolated, primary tumor cells. NHL cells were obtained from an HLA-A3[+] patient and confirmed to be telomerase positive by TRAP (Fig. 4*A*). K973-specific CTLs lysed these primary NHL cells, as they did HLA-A3^{+} U266 cells, but did not lyse HLA-A3-negative HSS cells (Fig. 4*B*).

As a further evaluation of HLA-A3 restriction, CD40-B cells from several donors were used as targets. Although freshly isolated peripheral blood B cells are telomerase negative, they become telomerase positive after several weeks of *in vitro* stimulation with CD40L (Fig. 2). K973-specific CTLs lysed HLA-A3-matched CD40-B cells (either autologous or from an HLA-A3[+] breast cancer patient) but did not lyse CD40-B from

one of three normal donors. Influenza I265-specific CTLs demonstrated no cytotoxicity against any of these targets, further demonstrating the specificity of CTLs generated in our system. In cold-target inhibition experiments, incubation with T2A3 cells pulsed with K973 peptide inhibited the lysis of chromium-labeled U266 cells by K973-specific CTLs. At a cold:hot target

Fig. 4 Cytotoxicity of K973-specific CTLs against primary tumor cells. *A*, NHL cells obtained after lymph node biopsy of an HLA-A3-positive patient demonstrated telomerase activity. *HT,* heat treated. *B*, K973-specific CTLs from one donor tested lysed NHL cells and HLA-A3[+] telomerase-positive U266 cells but not HLA-A3-negative, telomerase-positive HSS cells. One representative experiment of two performed is shown at an E:T ratio of 30:1.

an HLA-A3-negative donor (Fig. 3*D*). No lysis of CD40-B cells was observed with NP265-specific CTLs.

Evaluation of K973-specific Cytotoxicity against Normal Telomerase-positive Cells. A major concern of using hTERT as a TAA is the potential cytolysis of rare normal cells that express this antigen, including hematopoietic progenitor cells and activated lymphocytes (4). Similar to our findings for the HLA-A2-binding hTERT epitope, K973-specific CTLs failed to lyse highly enriched (>90%) HLA-A3^{+} CD34^{+} peripheral cells obtained after sorting PBMCs mobilized from a patient undergoing autologous stem cell transplantation (Fig. 3*E*). These cells are >95% CD38^{+} and express high levels of telomerase activity; CD34^{+} CD38^{−} cells have low telomerase activity (27).

We next examined activated CD34^{+} hematopoietic progenitor cells as targets for hTERT-specific CTLs. Mobilized CD34^{+} peripheral blood cells are quiescent and can be induced to express higher levels of telomerase activity following stimulation *in vitro* with certain cytokine mixtures (28). Purified CD34^{+} peripheral blood cells from an HLA-A3^{+} patient were treated *in vitro* as described (28) for 5 days with flt-3 ligand (20 ng/ml), IL-3 (50 ng/ml), IL-6 (20 ng/ml), erythropoietin (6 units/ml), and granulocyte-CSF (100 ng/ml). Microscopic analysis revealed an increased cell diameter after activation, and TRAP analysis demonstrated strong telomerase activity (data not shown). For two CTL lines raised using the K973 peptide, specific lysis of these activated CD34^{+} cells at an E:T ratio of 30:1 was 3.4 and 10.8%, respectively, compared with the positive control lysis of U266 cells at 81.8 and 62.7%. Thus, CD34^{+} remained refractory to lysis, even after cytokine stimulation.

Because potential autocytotoxicity of hTERT-positive CTLs might lead to fratricide (29) within our hTERT CTL cultures, we also tested autologous CTLs as targets. Although autologous CTLs were telomerase positive (Fig. 2), K973-specific CTLs failed to lyse these targets (Fig. 3*F*). Identical results were obtained when chromium release assays were extended to 8 h (data not shown). As with stem cells, these findings may reflect relatively low protein levels of hTERT (a hypothesis that could eventually be tested with an anti-hTERT mAb), or alternatively, may indicate that hTERT is not properly processed and presented in certain normal cells. It is unlikely that autologous CTLs are merely resistant to CTL lysis because autologous CTLs pulsed with I265 peptide are readily killed by autologous I265-specific CTLs (data not shown). Similarly, hematopoietic stem cells become targets for CTLs if first pulsed with the relevant peptide (4). CD40-activated B cells, in either the HLA-A2 or HLA-A3 antigen system, represent the only cell other than tumor cells that undergo hTERT-specific lysis (Ref. 4 and Fig. 3*D*).

Evaluation of Baseline PBMC Reactivity against the K973 Peptide. Because the K973 hTERT epitope was identified by deduction and not by a classical analysis of patients' antitumor T-cell responses, it is not known whether hTERT triggers a K973-specific, HLA-A3-restricted natural *in vivo* T-cell response during tumor progression. IFN-γ ELISPOT analyses were therefore performed on the available PBMCs from the melanoma patient and two of the healthy individuals to identify and enumerate recirculating K973-specific CD8^{+} cells. *In situ* hybridization performed on the patient's tumor biopsy material demonstrated homogeneous hTERT expression (data not shown). For each of the three donors, no positive spots were observed. There were also no spots in response to the HLA-A2-binding HIV peptide RT-pol I476, but 15–20 spots/well were observed in response to the positive control influenza peptide I276. These data corroborate our more extensive experiments evaluating baseline PBMC reactivity in HLA-A2[+] patients and normal donors against the I540 hTERT peptide; in 9 tumor-bearing patients and 10 healthy donors, no hTERT-specific T cells were identified above the threshold detection frequency of tetramer or ELISPOT analyses.[5] These data suggest that unlike certain melanoma antigens (30–32), there is no expanded pool of hTERT-specific CTLs at baseline, even in the setting of active neoplasia.

Discussion

In this study, we identify an HLA-A3-binding peptide epitope derived from the telomerase catalytic subunit hTERT that triggers MHC-restricted CTL lysis of hTERT^{+} tumors. Specific CTLs were induced *ex vivo* from both advanced cancer patients and normal donors. In contrast, hematopoietic CD34^{+} progenitor cells and activated T cells, two important examples of rare normal cells that express telomerase activity, were not lysed by specific CTLs. Combined with our previous identification of an immunogenic HLA-A2-binding hTERT peptide (4), these findings support efforts to test the feasibility of hTERT as a potential therapeutic target for the majority of patients across a wide range of cancer types.

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[5] R. H. Vonderheide and J. L. Schultze, unpublished observations.

The characterization of multiple hTERT epitopes with different HLA restriction elements broadens the clinical potential of this target antigen in two important ways:

(*a*) Targeting common HLA alleles increases the number of patients potentially eligible for hTERT-directed immunotherapy. The HLA-A2 antigen is expressed by 50% of our patient population, and HLA-A3 antigen is expressed by 15–25% of patients. Coexpression is found in nearly 10% of patients. Thus, >60% of all cancer patients could already be considered for therapies that target the two hTERT epitopes identified thus far. This percentage of patients would be further increased by the identification of additional hTERT peptides that bind to other common HLA alleles. For example, three other alleles (HLA-A1, HLA-A11, and HLA-A24 antigens) in addition to HLA-A2 and HLA-A3 antigens encompass >90% of the North American population.

(*b*) The characterization of multiple hTERT epitopes with multiple HLA restriction elements helps address the issue of immune escape that has limited previous antigen-specific T-cell therapies. Tumor loss of a single HLA allele is a significant component of MHC alterations described in cancer cells (14–19). As emphasized in these studies, staining of cells with a mAb recognizing a monomorphic determinant of HLA class I antigens does not detect selective loss of HLA class I allospecificities. Thus, in strategies targeting both of the hTERT epitopes, tumor loss of only HLA-A2 or HLA-A3 antigens would not be sufficient for immune escape in patients who express both alleles. Although this percentage is only 10% for HLA-A2 and HLA-A3 antigens, the identification of epitopes for other HLA alleles would increase the percentage of patients with allelic overlap.

Targeting multiple peptides derived from hTERT may also minimize immune escape because of loss or mutation of antigen expression. In contrast to other TAAs not critically involved in tumor growth and development (11–13), inhibition of hTERT activity in human tumors that express telomerase activity leads to growth arrest without the appearance of telomerase-negative clones (8). Thus, tumor deletion, mutation, or down-regulation of hTERT, as a potential consequence of anti-hTERT immunotherapy, might be detrimental *per se* to sustained tumor growth.

An issue meriting careful consideration is the potential risk of inducing autoimmunity in patients treated with hTERT-specific therapies. Our *in vitro* findings suggest that hTERT is a poor autoantigen in hematopoietic stem cells and activated T blasts. Although experiments in animals, such as immunization of mice with murine TERT or TERT-derived peptides, will be important in further preclinical evaluations, these experiments will need to address the significant differences between mouse and human telomerase biology (33, 34). The evaluation of autoimmunity is an important end point in the current hTERT-specific vaccine trial.

Finally, our findings support the notion that genes broadly implicated in tumor growth and development can be evaluated as polyantigenic, polyallelic tumor antigens. Advances in molecular genetics and the analysis of differential gene expression have revealed increasing numbers of molecules selectively involved in the neoplastic process (35). We propose that these molecules, if presented by tumor cells on MHC, may serve as immune targets for which mutation or loss as a means of immune escape may be incompatible with sustained tumor growth. The identification and clinical combination of multiple tumor antigens with both critical roles in oncogenesis and broad, selective expression in cancer would be important for the development of antitumor immunotherapy.

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