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Priority Report

Cancer Research

Acquired Immune Resistance Follows Complete Tumor Regression without Loss of Target Antigens or IFNγ Signaling

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Abstract

Cancer immunotherapy can result in durable tumor regressions in some patients. However, patients who initially respond often experience tumor progression. Here, we report mechanistic evidence of tumoral immune escape in an exemplary clinical case: a patient with metastatic melanoma who developed disease recurrence following an initial, unequivocal radiologic complete regression after T-cell–based immunotherapy. Functional cytotoxic T-cell responses, including responses to one mutant neoantigen, were amplified effectively with therapy and generated durable immunologic memory. However, these immune responses, including apparently effective surveillance of the tumor mutanome, did not prevent recurrence. Alterations of the MHC class I antigen-processing and presentation machinery (APM) in resistant cancer cells, but not antigen loss or impaired IFNγ signaling, led to impaired recognition by tumor-specific $CD8^+$ T cells. Our results suggest that future immunotherapy combinations should take into account targeting cancer cells with intact and impaired MHC class I–related APM. Loss of target antigens or impaired IFNγ signaling does not appear to be mandatory for tumor relapse after a complete radiologic regression. Personalized studies to uncover mechanisms leading to disease recurrence within each individual patient are warranted. *Cancer Res; 77(17); 4562–6. ©2017 AACR.*

Introduction

Immunotherapy of cancer frequently results in durable clinical responses. However, a significant fraction of patients who initially respond will experience renewed tumor progression. So far, it is reported that about 30% of patients responding to checkpoint inhibitors (1, 2) and about 60% of patients responding to cellular immunotherapy with tumor-infiltrating lymphocytes (TIL; ref. 3) will experience disease progression, respectively, within 2 years and within 5 years. With increasing availability of immunotherapies, disease progression after initial clinical response or treatment-induced immune resistance is rapidly emerging as a major hurdle in oncology practice (4).

It is generally believed that processes leading to acquired immune resistance would practically overlap to mechanisms involved in naturally acquired resistance during primary immunoediting (5). Current knowledge on disease progression after initial response to immunotherapy is derived from comprehensive animal models, or clinical anecdotes that associate certain disease features to renewed disease progression (4). In a recent publication, Verdegaal and colleagues (6) elegantly show how immunogenic neoantigens can be lost or downregulated during tumor progression. In this study, the authors used multiple samples from two patients with melanoma, including one patient treated with adoptive cellular therapy before collection of a progressing lesion. However, none of the samples analyzed was obtained after a true immune-mediated tumor regression, but rather during the natural progression of human melanoma or very short disease stabilization following T-cell–based immunotherapy. Therefore, the relevance of these findings may be limited to the situation of prolonged immunologic interactions of tumors with the adaptive immune system, in the absence of elimination of large tumor masses. In another study, Zhao and colleagues (7) described the case of

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a patient with metastatic melanoma and multiple recurrent lesions, where poorly immunogenic melanoma phenotypes evolve toward T-cell resistance by independent genetic events, leading to MHC class I loss/deficiency. Again, this patient was treated with various forms of immunotherapy but did not appear to experience a clear immune-mediated tumor regression. In contrast, novel cancer immunotherapies frequently induce tumor regressions developing in few weeks to few months but, however, in a significant fraction of patients this initial clinical response does not last. Zaretsky and colleagues (8) described four patient cases who progressed while receiving anti–PD-1 immunotherapy, after an initial partial tumor regression. They identified defective IFNγ signaling and inactivation of β2 microglobulin as mechanisms of acquired resistance to immunotherapy.

Here, we present mechanistic evidence of clinical tumor immune escape in an exemplary clinical case of a patient with metastatic melanoma, who developed disease recurrence following an unequivocal initial radiologic complete tumor regression to cellular immunotherapy with autologous TILs. Given the nature of this treatment, the infusion product, which induced tumor regression, could be studied in detail with antigen-specific assays and tests of direct tumor recognition and killing. Disease recurrence was not associated with cancer cells that lost target antigens or had impaired IFNγ signaling, but with cancer cells with defective MHC class I antigen-processing and presentation machinery (APM).

Materials and Methods

Patient treatment and clinical specimens

All the procedures were approved by the Scientific Ethics Committee for the Capital Region of Denmark. Written informed consent was obtained from the patient according to the Declaration of Helsinki. HLA class I genotyping of a normal cell sample was performed by the Rigshospitalet (Copenhagen, Denmark) HLA typing service, and confirmed with whole-exome sequencing as A*01:01, A*03:01, B*15:01, B*40:01, C*03:04 (homozygous). The patient MM909.11 was treated in the context of the clinical trial NCT00937625, and TILs were prepared accordingly as described in ref. 9. Melanoma cell lines were established as described in ref. 10 at the Center for Cancer Immune Therapy, Herlev Hospital, Denmark.

TILs were established according to standard methods, as described by Ellebæk and colleagues (9). Briefly, TILs were initially isolated and minimally expanded (mcTILs, as in; ref. 10) from tumor fragments in standard complete medium supplemented with IL2 (6,000 IU/mL IL2, Proleukin, Novartis) and subsequently were massively expanded according to the Rapid Expansion Protocol (REP). PBMCs were isolated from a blood sample with gradient centrifugation, and stored according to local standard operating procedures. All melanoma cell lines, were generated by serial passage of adherent cells released from tumor fragments and cultured in RPMI1640 (Life Technologies) supplemented with 10% FCS (Life Technologies), as described previously (11).

The time points for collection of tumor samples (used for generation of TILs and melanoma cell lines) and PBMCs are indicated in Fig. 1A. Briefly, tumor #1 was resected in February 2011, and both TIL#1 and Mel#1 were established from this sample; TIL#1 were infused in July 2011, and this treatment resulted in a complete response according to RECIST 1.0; PBMCs were collected at serial time points; tumor #2, first identified with a PET/CT scan in August 2012 was resected in September 2012, and both TIL#2 and Mel#2 were established from this sample.

Analysis of T-cell responses

All peptides were obtained from the NKI peptide synthesis facility (Amsterdam, the Netherlands) or Pepscan (Lelystad, the Netherlands). Combinatorial encoding with peptide(p)-MHC multimers was carried out with TIL#1 and TIL#2 to identify T-cell–specific for shared antigens, as described in ref. 12. One initial screening to detect $CD8^+$ T cells in TIL#1 recognizing mutant antigens for the patient specific HLA alleles of Mel#1 was carried out as described in Supplementary Methods and previously used in ref. 13. Additional focused screenings to identify whether mutant antigens privately expressed in Mel#1 were recognized was conducted by stimulating TIL#1 and TIL#2 with short peptides (8–11 mers) in overnight IFNγ ELISPOT assays. ELISPOT were carried out as previously described (10), using 23 additional potential private neoepitopes of Mel#1, identified as described below (9 other private neo-epitopes were already tested in the primary screening, and thus were not analyzed). Prediction of neoepitopes is described in the Supplementary File. Positive responses were confirmed with intracellular staining of TNF and IFNγ after stimulation of TIL#1 or TIL#2 (criteria for evaluation of positive responses were as in; ref. 11). Responses were observed to the peptides RLSNRLLLR (TAG_{RLS}, HLA-A3, >2% of $CD8^+$ T cells in TIL#2) and three of four tested peptides derived from the LRCH1 p.E672K mutation (CLPHHILEKK, HLA-A*03:01, >2% of $CD8^+$ T cells in TIL#2; KLCLPHHILEK, HLA-A*03:01, >2% of $CD8^+$ T cells in TIL#2; LEKKGLVKVGI, HLA-B*40:01, >0.05% of $CD8^+$ T cells in TIL#2). Because the highest responses were detected after stimulation with the mutant CLPHHILEKK peptide (data not shown), all additional experiments with recognition of the $LRCH1_{E>K}$ mutation were carried out with this reagent. Responses to peptides RLSNRLLLR (TAG-derived) and CLPHHILEKK ($LRCH1_{E>K}$-derived; wild type peptide CLPHHILEEK) were confirmed with >90% pure peptides in intracellular staining experiments, assessing T cells coexpressing TNF and IFNγ. T-cell cultures with high specificity for defined antigens (>95% specific, confirmed with p-MHC multimer staining, data not shown) were obtained by sorting relevant $CD8^+$ T cells within minimally expanded TILs with tetramers conjugated with two different colors, and then performing two sequential REP (14) of 8–10 + 7–9 days. Standard REP media were supplemented with 2 ng/mL of IL15 (PeproTech).

In all other cases, evaluation of T-cell responses was performed as described previously (11) with tumor reactivity evaluated by assessing the amount of $CD8^+$ T cells coexpressing TNF and IFNγ. FACS antibodies were obtained from BD Biosciences, unless indicated otherwise. Where indicated, tumor cells were pretreated with 100 IU/mL of recombinant human IFNγ (PeproTech) for 72 hours. The chromium-51 cytotoxicity assay was performed as previously described (15).

Gene and protein expression of tumor cells

Analysis of mRNA and proteins (Western blot analysis) on selected APM components and putative target antigens were

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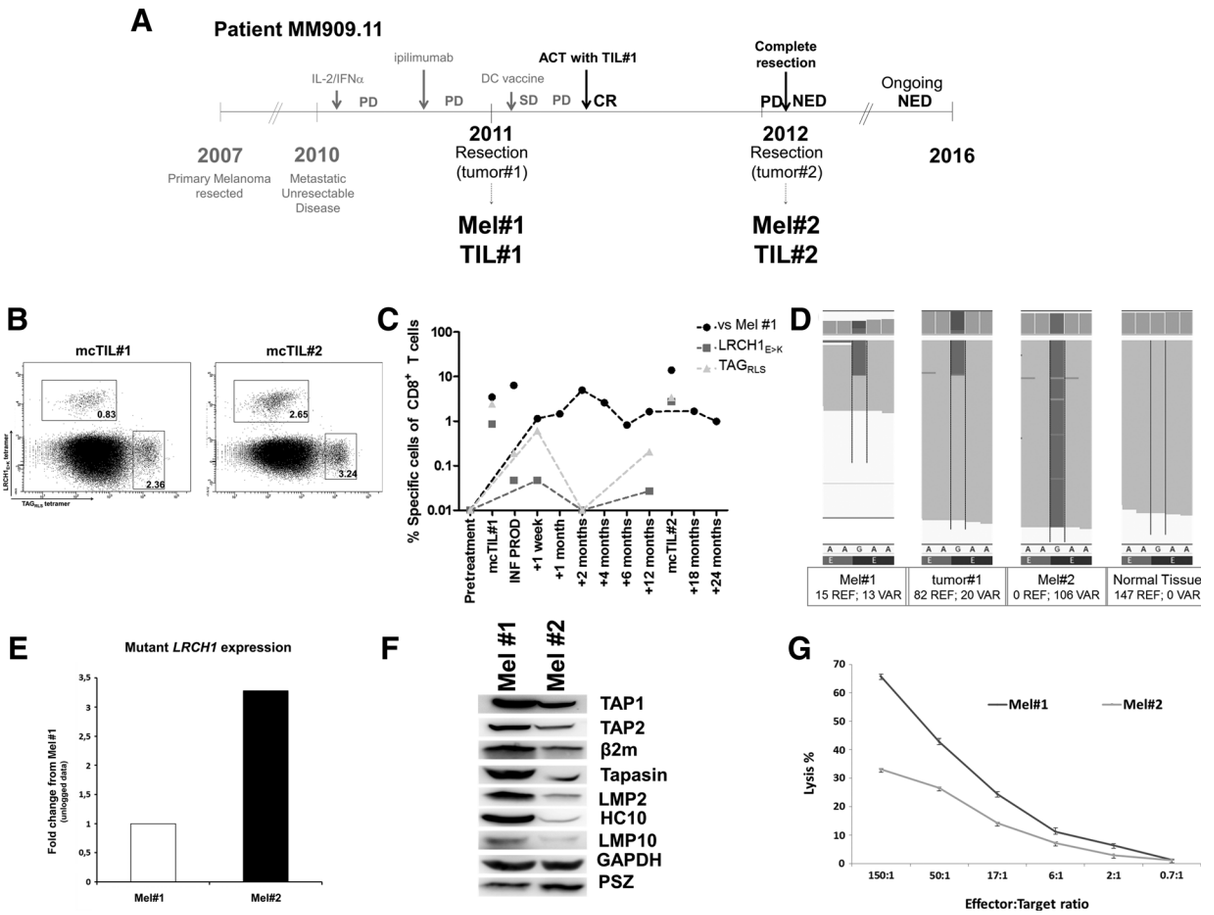

Figure 1.

T-cell immune recognition and immune resistance of melanoma in patient MM909.11. **A,** Clinical course, tissue collection with TILs, and tumor cell line generation. CR, complete response; PD, progressive disease; SD, stable disease; NED, no evidence of disease. **B,** Staining with $LRCH1_{E>K}$ and TAG_{RLS} p-MHC multimers of minimally cultured TIL#1 and TIL#2 detects specific T cells in both TILs (plots are gated on live $CD8^+$ T cells). One representative experiment is shown. **C,** The frequency of $CD8^+$ T cells recognizing Mel#1 (simultaneous production of TNF and IFNγ after coculture), or that binds $LRCH1_{E>K}$ or TAG_{RLS} fluorochrome-conjugated is shown in the graph. The percentage of tumor-antigen–specific $CD8^+$ T cells increases from undetectable before infusion of TILs to detectable variable levels. Tumor antigen–specific $CD8^+$ T cells are still detectable in the blood at the time of recurrence (around 12 months after primary immunotherapy) and in mcTIL#2, indicating ability to infiltrate the recurrent tumor. **D,** Identification of LRCH1 p.E672K mutation by whole-exome sequencing. In light gray, nucleotide positions correspond to the reference sequence. Mutations are displayed in dark gray. Collapsed sequencing reads are displayed. The mutation is present in a fraction of reads in Mel#1 and respective tumor sample (tumor#1), whereas in Mel#2 it occupies 100% of reads. The mutation is truly somatic as it is absent from the matched normal tissue. **E,** Fold change in mutant RNA expression of *LRCH1* in Mel#2 relative to Mel#1 showing increased expression of *LRCH1* in Mel#2. **F,** Multiple deficiencies in protein expression of selected components of the class I APM of Mel#2, compared with Mel#1. **G,** Chromium-51 release assay of Mel#1 or Mel#2 after coincubation with TIL#1 at different effector:target ratios. The graph shows reduced sensitivity of Mel#2.

performed as recently described (16). Briefly, for mRNA analysis the cDNA was synthesized using the Revert Aid H Minus First Strand cDNA Synthesis Kit (Fermentas) before qPCR was performed with APM-specific primers using the Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen) for 40 cycles, 95°C, 15 s, 60°C, 30s. Relative mRNA levels for specific APM components were normalized to β-actin. For protein analysis by Western blot analysis, 30 μg of protein/lane was separated on SDS-PAGE, transferred onto nitrocellulose filters and subsequently stained with APM component–specific mAbs. The staining was detected with horseradish peroxidase (HRP)–conjugated secondary antibodies (Dako). The membranes were washed and protein bands were visualized with a Lumilite (Roche Diagnostics) and exposed to a CCD camera (Eastman Kodak).

Results and Discussion

The initial complete response following infusion of autologous TILs generated from an initially resected tumor metastases (TIL#1 and tumor#1) is reported in Ellebaek and colleagues (9). Recurrent metastatic disease (tumor#2) was diagnosed about 1 year after primary treatment (summary of the clinical history is shown in Fig. 1A).

$CD8^+$ T cells recognizing one neoantigen derived from the mutation LRCH1 p.E672K ($LRCH1_{E>K}$) and the cancer-testis

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antigen (epitope TAG_{RLS}; Fig. 1B, left) were identified in minimally cultured (mc) TIL#1. About 6% of $CD8^+$ T cells in the infusion product, derived from TIL#1, recognized the unedited melanoma (Mel#1) *in vitro*, including T cells recognizing $LRCH1_{E>K}$ and TAG_{RLS}, which alone corresponded to up to about 60% of the total tumor reactivity of peripheral $CD8^+$ T cells one week after infusion (Fig. 1C). Therapy-induced tumor-reactive/specific $CD8^+$ T cells detected peripherally persisted beyond tumor regrowth (Fig. 1C). mcTIL#2, isolated from the recurrent tumors (tumor#2, from which the cell line Mel#2 was generated), contained at least comparable frequencies of tumor-reactive/specific T cells, including 3-fold higher percentages of $CD8^+$ T cells recognizing $LRCH1_{E>K}$ (Fig. 1B and C). In mcTIL#2, $CD8^+$ T cells recognizing TAG_{RLS} or $LRCH1_{E>K}$ did not express higher levels of coinhibitory molecules such as PD-1, LAG-3, or TIM-3 (Supplementary Fig. S1A and S1B). Thus, tumor regrowth occurred despite effective induction of a primary complete response, continuous T-cell persistence and effective infiltration of the recurrent edited tumor.

Over 80% of nonsynonymous and synonymous mutations detected were identical in tumor#1, Mel#1, and Mel#2 (Supplementary Table S1), including the $LRCH1_{E>K}$ mutation (Fig. 1D). However, while tumor#1 and Mel#1 expressed both the mutant and wild-type alleles, Mel#2 only harbored (DNA, Fig. 1D) and expressed (RNA, data not shown) the mutant allele, and at a slightly higher level as compared with Mel#1 (Fig. 1E). No dramatic reduced expression of the other known target antigen TAG (Supplementary Fig. S2A) as well as additional putative target antigens, such as common melanocyte differentiation antigens and cancer-testis antigens with the exception of MAGE-4 (Supplementary Fig. S2B), were found. However, it is important to highlight that no responses to other putative antigens were detected with the screening methods used (see Materials and Methods/analysis of T-cell responses). Although Mel#1 harbored multiple putative neoantigens that were absent in Mel#2, further focused screening efforts did neither identify $CD8^+$ T cells in TIL#1 and TIL#2 recognizing these candidate neoantigens (data not shown). No increased expression of the immunosuppressive PD-L1 was observed in Mel#2 (Supplementary Fig. S2C).

Importantly, Mel#2 displayed multiple newly emerged defects in the MHC class I APM (Fig. 1F for protein and Supplementary Fig. S2D for mRNA in qPCR), resulting in reduced surface expression of MHC class I antigens (HLA-ABC, Supplementary Fig. S2E). IFNγ could induce overexpression of the major APM components and other IFNγ-inducible molecules, demonstrating that Mel#2 maintained sensitivity to IFNγ (Supplementary Fig. S2D–S2F). Loss of heterozygosity (LOH) in chromosome 6 (HLA genes) and/or chromosome 15 (β2Microglobulin-*B2M*) were previously associated with immune resistance of relapsed melanoma lesions and impaired MHC class I APM (17). Here, both Mel#1 and Mel#2 had a LOH at *HLA-A* and *HLA-B* locus, that is, LOH of MHC I alleles was already present before treatment (Supplementary Fig. S3A and S3B). Thus, LOH in chromosome 6 happening at early stages of tumor development affected antigen presentation equally in Mel#1 and Mel#2.

Gene expression of HLA-B and HLA-C were strongly downregulated in Mel#2 (Supplementary Fig. S3C), whereas only minor changes of HLA-A (Supplementary Fig. S3C) and B2M (Supplementary Figs. S2D and S3C) were found. Because B2M protein is an essential part of the MHC complex, the decrease in the B2M protein level observed in Mel#2 (Fig. 1F) may be related to the strongly impaired expression of HLA-B and C. Chromosome 15, where *B2M* is located, shows no evidence of LOH in both Mel#1 and Mel#2 (Supplementary Fig. S3D). Overall, a specific genetic or epigenetic event to explain the observed immunoresistant phenotype could not be identified either at DNA (Supplementary Table S1) or RNA (Supplementary Fig. S4) level.

In parallel, recognition of Mel#2 by unselected TILs (Supplementary Fig. S5A and S5B for cytokine production; TIL#1 in Fig. 1G for killing), $CD8^+$ PBMCs obtained at time of recurrence (Supplementary Fig. S5C) or at other later time points (+15 months or + 18 months, data not shown), and TAG_{RLS}-specific $CD8^+$ T cells (Supplementary Fig. S5D) were significantly reduced, which could be partially rescued by preexposure of tumor cells to IFNγ (Supplementary Fig. S5B and S5D). In contrast, recognition of Mel#2 by $CD8^+$ T cells reactive to $LRCH1_{E>K}$ was increased in Mel#2 and further upregulated with IFNγ (Supplementary Fig. S5E), in parallel to increased expression of the $LRCH1_{E>K}$ mutation in Mel#2 (Fig. 1E).

On a different note, Mel#2 displayed different morphology with only spindle cells, in contrast to a biphasic population comprising of spindle and epithelioid cells in Mel#1, and accelerated growth compared with Mel#1 both *in vitro* (estimated doubling time ~35 vs. 68 hours; 35% vs. 10% Ki-67^+) and *in vivo* (tumor size of 50 mm^2 reached in less than 30 vs. over 70 days, Supplementary Fig. S2F). These immune-independent changes in the biology of tumor cells may have contributed to tip of the balance from tumor elimination/dormancy to radiologically evident tumor growth.

Despite obvious limitations of a single case in which a mechanistic explanation for the observed impaired MHC class I APM could not be identified, we report one instructive clinical example offering insight into the mechanisms driving disease recurrence after radiologic complete regression following cellular immunotherapy of cancer. Radiologic complete regression may leave behind microscopic cancer cell aggregates. Thus, immunologic pressure may promote the outgrowth of immunoresistant tumor cells with MHC class I APM deficiencies that can drive tumor recurrence even with apparently effective surveillance of the tumor mutanome and in the absence of antigen loss or defective IFNγ signaling. Loss of target antigens may be not essential in acquired resistance to cancer immunotherapies inducing rapid complete regression of large tumor masses with multitarget T-cell responses, as tumor cells should lose several target antigens at once for an efficient immune escape.

MHC class I APM-deficient tumors can be hardly targeted effectively by immunotherapies stimulating $CD8^+$ T-cell responses (18). Impaired MHC class I APM may represent a universal mechanism of resistance to $CD8^+$ T-cell responses targeting any tumor antigen (19), and a dichothomy with higher MHC class I APM expression in regressing compared with progressing metastases was observed in other studies as well (20). To prevent or treat tumor recurrences with acquired MHC class I APM deficiency, we suggest that alternative strategies such as restoring APM via targeted-delivery of APM-inducers, or non–T-cell–based immune strategies should be tested. In sum, these data together with recent works by Zaretsky and colleagues (8) highlight the need of comprehensive studies of the immunologic interactions within the

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individual patient to uncover molecular mechanisms leading to acquired resistance to cancer immunotherapy on a personalized basis.

Disclosure of Potential Conflicts of Interest

P. Kvistborg is a consultant/advisory board member for Personalis and Neon Therapeutics. H. Schmidt is a consultant/advisory board for BMS, Novartis, and Merck. No potential conflicts of interest were disclosed by the other authors.

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): M. Donia, K. Harbst, M.V. Buuren, M.F. Lindberg, R. Andersen, M. Idorn, A. Mueller, M. Lauss, S.R. Hadrup, B. Seliger, G. Jönsson

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