Title
Mutations Associated with Acquired Resistance to PD-1 Blockade in Melanoma.

Permalink
https://escholarship.org/uc/item/4157s0ms

Journal
The New England journal of medicine, 375(9)

ISSN
0028-4793

Authors
Zaretsky, Jesse M
Garcia-Diaz, Angel
Shin, Daniel S
et al.

Publication Date
2016-09-01

DOI
10.1056/NEJMoa1604958

Peer reviewed
Manuscript Information

Journal name: New England journal of medicine (Overseas ed.)
NIHMS ID: NIHMS817075
Manuscript Title: Mutations Associated with Acquired Resistance to PD-1 Blockade in Melanoma
Submitter: New England Journal Of Medicine (pmiller@nejm.org)

Manuscript Files

<table>
<thead>
<tr>
<th>Type</th>
<th>Fig/Table #</th>
<th>Filename</th>
<th>Size</th>
<th>Uploaded</th>
</tr>
</thead>
<tbody>
<tr>
<td>manuscript</td>
<td></td>
<td>nejmoa1604958.pdf</td>
<td>1171006</td>
<td>2016-09-15 14:24:45</td>
</tr>
<tr>
<td>supplement</td>
<td>suppapp</td>
<td>nejmoa1604958_appendix.pdf</td>
<td>16866898</td>
<td>2016-09-15 14:24:53</td>
</tr>
</tbody>
</table>

This PDF receipt will only be used as the basis for generating PubMed Central (PMC) documents. PMC documents will be made available for review after conversion. Any corrections that need to be made will be done at that time. No materials will be released to PMC without the approval of an author. Only the PMC documents will appear on PubMed Central -- this PDF Receipt will not appear on PubMed Central.
Mutations Associated with Acquired Resistance to PD-1 Blockade in Melanoma


BACKGROUND
Approximately 75% of objective responses to anti–programmed death 1 (PD-1) therapy in patients with melanoma are durable, lasting for years, but delayed relapses have been noted long after initial objective tumor regression despite continuous therapy. Mechanisms of immune escape in this context are unknown.

METHODS
We analyzed biopsy samples from paired baseline and relapsing lesions in four patients with metastatic melanoma who had had an initial objective tumor regression in response to anti–PD-1 therapy (pembrolizumab) followed by disease progression months to years later.

RESULTS
Whole-exome sequencing detected clonal selection and outgrowth of the acquired resistant tumors and, in two of the four patients, revealed resistance-associated loss-of-function mutations in the genes encoding interferon-receptor–associated Janus kinase 1 (JAK1) or Janus kinase 2 (JAK2), concurrent with deletion of the wild-type allele. A truncating mutation in the gene encoding the antigen-presenting protein beta-2-microglobulin (B2M) was identified in a third patient. JAK1 and JAK2 truncating mutations resulted in a lack of response to interferon gamma, including insensitivity to its antiproliferative effects on cancer cells. The B2M truncating mutation led to loss of surface expression of major histocompatibility complex class I.

CONCLUSIONS
In this study, acquired resistance to PD-1 blockade immunotherapy in patients with melanoma was associated with defects in the pathways involved in interferon-receptor signaling and in antigen presentation. (Funded by the National Institutes of Health and others.)
Durable responses in metastatic cancers have been achieved with a variety of immunotherapies such as interleukin-2, adoptive cell transfer of tumor-infiltrating lymphocytes, antibodies that block cytotoxic T-lymphocyte–associated antigen 4 (CTLA4),1-5 and antibodies that block programmed death 1 (PD-1).6-10 However, in a recent study, approximately 25% of patients with melanoma who had had an objective response to PD-1 blockade therapy had disease progression at a median follow-up of 21 months.11

The mechanisms of immune-resistant cancer progression are mostly unknown. Previous studies involving humans examined the loss of beta-2-microglobulin as a mechanism of acquired resistance to several forms of cancer immunotherapy.12-14 In preclinical models, defects in the interferon signaling pathway have been proposed as a potential mechanism of cancer escape (insensitivity) to immunotherapy.15,16 In the current study, we assessed the effect of anti–PD-1 therapy on cancer genomic evolution, including acquired mutations in the genes affecting the interferon pathway and antigen-presentation pathway, in an effort to determine genetic mechanisms of acquired resistance to PD-1 blockade therapy.

**METHODS**

**PATIENTS, RESPONSE ASSESSMENT, AND TUMOR BIOPSIES**

Of 78 patients with metastatic melanoma who were treated with the anti–PD-1 antibody pembrolizumab at the University of California, Los Angeles (UCLA), 42 had an objective response, of whom 15 went on to have disease progression. Four of these 15 patients met all three selection criteria for this analysis. First, they must have had an objective tumor response while participating in a clinical trial with single-agent pembrolizumab.6,7,10,11 Tumor responses were evaluated at 12 weeks and confirmed 4 weeks later, and patients were assessed by imaging every 12 weeks thereafter with the use of both the Response Evaluation Criteria in Solid Tumors17 and the immune-related response criteria.18 Second, patients had to have late acquired resistance, defined as in situ recurrence or new lesion development, despite continuous dosing, after more than 6 months of tumor response. Third, patients had to have adequate biopsy material for whole-exome sequencing at two time points: before the initiation of pembrolizumab therapy and after disease progression. We processed tumor biopsy samples as described previously to perform pathological analyses, obtain DNA and RNA, and attempt to establish cell lines.19,20

**IMMUNOHISTOCHEMICAL, IMMUNOFLOURESCENCE, WESTERN BLOT, AND FLOW-CYTOMETRIC ANALYSES**

Immunohistochemical and immunofluorescence analyses19 as well as Western blot and flow-cytometric analyses21 were performed and analyzed as described previously. Full methods are included in the Supplementary Appendix, available with the full text of this article at NEJM.org.

**GENETIC AND TRANSCRIPTIONAL-PROFILING ANALYSES**

Whole-exome sequencing was performed at the UCLA Clinical Microarray Core with the use of the NimbleGen SeqCap EZ Human Exome Library, version 3.0 (Roche). Mutation calling was performed as described previously.22 Selected gene-expression profiling on interferon exposure was performed with the use of nCounter (NanoString Technologies). Whole-exome sequencing data have been deposited in the National Center for Biotechnology Information Sequence Read Archive under the accession number SRP076315.

**FUNCTIONAL STUDIES**

Patient-derived and previously established human melanoma cell lines were used to analyze recognition by T-cell receptor transgenic T cells23 with the use of in vitro coculture assays that detect antigen-induced release of interferon-γ assessed by enzyme-linked immunosorbent assay. Cell-proliferation and growth-inhibition assays were performed with the use of an automated live-cell imaging system (IncuCyte, Essen BioScience) with or without exposure to interferons. Full methods are described in the Supplementary Appendix.

**STUDY OVERSIGHT**

Data generated and collected by the study investigators were analyzed by the last author, who vouches for the completeness and accuracy of the data, analyses, and reported results. Summaries of the clinical protocol have been reported by Hamid et al.6 and Robert et al.10
Acquired Resistance to PD-1 Blockade

**Statistical Analysis**

Student’s t-test and a two-way analysis of variance were used for cell-culture experiments, with Dunnett’s correction applied for multiple comparisons with untreated controls.

**RESULTS**

**Clinical Course and Immune Infiltrates**

We analyzed paired tumor samples from four (nonconsecutive) selected patients with metastatic melanoma who had had a relapse while receiving PD-1–inhibition therapy with pembrolizumab (Tables S1 and S2 in the Supplementary Appendix). All four patients met objective criteria for a partial response, though with slightly different kinetics (Fig. 1, and Figs. S1, S2, and S3 in the Supplementary Appendix). The mean time to relapse was 624 days (range, 419 to 888). The baseline biopsy samples were obtained just before the initiation of pembrolizumab therapy in Patients 2, 3, and 4, whereas for Patient 1, the only available baseline biopsy sample was obtained before an earlier course of therapy with the BRAF inhibitor vemurafenib. The baseline biopsy samples from Patients 1, 2, and 3 showed preexisting CD8 T-cell infiltrates at the invasive margin that colocalized with programmed death ligand 1 (PD-L1) expression at baseline and again at the time of relapse at the tumor margin in Panels B and C, respectively. In the immunofluorescence images, red indicates PD-L1, yellow CD8 T cells, light blue the melanoma marker S100 (cytoplasmic staining), and dark blue the melanoma marker Sox10 (nuclear staining).

Figure 1. Clinical Pattern of Acquired Resistance to Anti–Programmed Death 1 (PD-1) Therapy in Patient 1.

In Panel A, computed tomographic images show a melanoma small-bowel metastasis at baseline, at the time of maximum response, and at the time of an in situ relapse after a year of minimal residual disease; the red dots in the graph on the right indicate these three time points. Immunohistochemical staining and multiplexed immunofluorescence analysis showed abundant CD8 T-cell infiltrates and programmed death ligand 1 (PD-L1) expression at baseline and again at the time of relapse at the tumor margin in Panels B and C, respectively. In the immunofluorescence images, red indicates PD-L1, yellow CD8 T cells, light blue the melanoma marker S100 (cytoplasmic staining), and dark blue the melanoma marker Sox10 (nuclear staining).
filtrates (Figs. S1C, S2C, and S3C and Table S3 in the Supplementary Appendix; no biopsy sample during therapy was available for Patient 1). At the time of relapse, all four biopsy samples showed CD8 T-cell infiltration and PD-L1 expression concentrated at the tumor margins again (Fig. 1C, and Figs. S1D, S2D, and S3D in the Supplementary Appendix). Multiplex immunofluorescence assays revealed that melanoma cells at the time of relapse in Patients 1 and 2 were negative for PD-L1 even when directly adjacent to T cells, whereas macrophages and stromal cells were positive for PD-L1.

**GENETIC CHANGES IN RELAPSE BIOPSY SAMPLES**

The pattern of a strong initial response, long dormancy, and rapid late progression led us to hypothesize that relapse in these patients resulted from immune-mediated clonal selection and tumor outgrowth. To identify mutations that might confer immune resistance, we extracted DNA from bulk-tumor biopsy samples or early-passage primary cell lines (Table S2 in the Supplementary Appendix) and performed whole-exome sequencing to compare baseline and matched relapsed tissues. We achieved a median coverage of 149x, and the percent of tumor cells (as compared with stromal cells) was more than 40% in all samples (Table S2 in the Supplementary Appendix). Nonsynonymous mutations for all samples are shown in Table S4 in the Supplementary Appendix.

**JAK MUTATIONS WITH CONCURRENT LOSS OF HETEROZYGOSITY AT RELAPSE**

We found strong evidence that the relapsed tumors were closely genetically related to their baseline counterparts, despite up to 2 years between biopsies. In the case of Patients 1 and 2, of 1173 and 240 nonsynonymous mutations, respectively, originally identified in the baseline sample, 92.5% and 95.8% were also seen in the resistant tumor (Fig. 2A, and Fig. S4 in the Supplementary Appendix). The relapsing tumors also contained the same chromosomal loss-of-heterozygosity events as the baseline tumors, and all differences were due to further loss in the relapse samples. In the relapse biopsy samples from both patients, we identified new homozygous loss-of-function mutations in the kinases associated with the interferon-receptor pathway, with a Q503* nonsense mutation in the gene encoding Janus kinase 1 (JAK1) in Patient 1 (Fig. 2A and 2B) and a F547 splice-site mutation in the gene encoding Janus kinase 2 (JAK2) in Patient 2 (Fig. S4 in the Supplementary Appendix). RNA sequencing showed that the JAK2 splice-site mutation caused intron inclusion, producing an in-frame stop codon 10 bp after exon 12 (Fig. S5 in the Supplementary Appendix). Therefore, both mutations were upstream of the kinase domains and probably truncated the protein or caused nonsense-mediated decay. Neither mutation was seen at baseline in the exome sequencing reads, by Sanger sequencing, or by targeted amplicon resequencing (Fig. S6 in the Supplementary Appendix).

The JAK2 mutation was the only homozygous mutation (adjusted variant allele frequency, >85%) of 76 new nonsynonymous mutations in Patient 2, and the JAK1 mutation was 1 of only 3 homozygous mutations among 53 new mutations in Patient 1 (Table S5 in the Supplementary Appendix). To become homozygous, both JAK mutations were acquired in the context of a copy-number–neutral nondisjunction event, resulting in loss of the wild-type chromosome and duplication of the mutated allele. This is seen clearly in Patient 1: at relapse, chromosome 1p (containing JAK1) showed a decrease in minor-allele frequencies for germline single-nucleotide polymorphisms relative to baseline (Fig. S7 in the

---

**Figure 2 (facing page). Acquired JAK1 Loss-of-Function Mutation at Relapse, with Accompanying Loss of Heterozygosity.**

In Panel A, a Circos plot of Patient 1 shows differences in whole-exome sequencing between the pre-pembrolizumab and post-relapse biopsies. The red circle highlights a new, high-allele-frequency, relapse-specific mutation in the gene encoding Janus kinase 1 (JAK1) in the context of chromosomal loss of heterozygosity (asterisk). Each wedge represents a chromosome. In the outer track (black background), each point represents a nonsynonymous mutation, with most detected in both biopsy samples (gray) rather than at relapse only (red) or baseline only (green). The y-axis position shows copy-number status for the baseline and relapse biopsy, respectively; dark green in the subtrack indicates loss of heterozygosity. In Panel B, Integrative Genomics Viewer (IGV) plots (top) show that the JAK1 Q503* nonsense mutation is relapse-specific, and the cBioPortal diagram (bottom) shows that the JAK1 mutation is upstream of the kinase domains.
ACQUIRED RESISTANCE TO PD-1 BLOCKADE

A Genetic Changes between Baseline Tumor and Relapse Tumor

Mutations (Nonsynonymous)

Chromosomal Copy Number

VAF

0.5

1.0

Baseline

Relapse

Mutations

Relapse only
Baseline only
Baseline and relapse

Copy Number

Loss of heterozygosity

B IGV Plots and cBioPortal Diagram

Baseline (whole tumor)

Relapse (whole tumor)

bp 65,321,320 65,321,330 65,321,340

No. of Mutations

JAK1 Q503E

Amino Acid

No. of Mutations

P kinase_Tyr

P kinase_Tyr

bp 65,321,320 65,321,330 65,321,340

57 bp

57 bp
observed To assess the functional consequences of the 

Functional Effects of

JAK
tumor and that acquisition of the 

nous population derived directly from the base-

line. A similar loss-of-heterozygosity event oc-

urred for chromosome 9 in Patient 2 (Fig. S8 

and Table S5 in the Supplementary Appendix). 

Together, these data suggest that the tumors 

resistant to anti–PD-1 are a relatively homoge-

nous population derived directly from the base-

line tumor and that acquisition of the 

JAK mutations was an early founder event before clonal 

selection and relapse despite the fact that the 

mutation was not detected in pretreatment tumor 

tissue.

FUNCTIONAL EFFECTS OF JAK2 MUTATION

To assess the functional consequences of the observed JAK mutations, we focused on the JAK2 mutation from Patient 2 using two cell lines es-

established at baseline (M420, wild-type JAK2) and 

at the time of relapse (M464, JAK2 F547 splice-

site mutation). Whole-exome sequencing con-

firmed that the original bulk tumor was well 

represented by M464 (Fig. S9 in the Supplemen-

tary Appendix). Western blot analysis showed 

that the baseline cell line responded to interferon 

alpha, beta, and gamma with the expected signal 

transduction, including an increase in signal trans-

ducer and activator of transcription 1 (STAT1) and 

interferon regulatory factor (IRF) expression, 

STAT1 phosphorylation (pSTAT1), and the pro-

duction of downstream interferon targets such as 

PD-L1, transporter associated with antigen pro-

cessing 1 (TAP1), and major histocompatibility 

complex (MHC) class I (Fig. 3A). However, the 

cell line from the progressing lesion showed a 

total loss of JAK2 protein (Fig. 3A), resulting in 

a lack of response to interferon gamma and are 

consistent with JAK2 being required for signal-

ing through the interferon-γ receptor, as op-

posed to the interferon-αβ receptor, which uses 

TYK2 and JAK1.27-29

LOSS OF INTERFERON GAMMA–INDUCED GROWTH 

ARREST THROUGH ACQUIRED JAK MUTATIONS

We hypothesized that inactivating JAK mutations may result in a functional advantage for the pro-

gressive tumors because the lack of interferon 

signaling either decreased antigen presentation 

or allowed escape from interferon-induced inhibi-

tion of growth. In addition to using M420 and 

M464, we engineered the human melanoma cell 

line M407 by means of the CRISPR (clau-

sely interspaced short palindromic repeats)— 

Cas9 approach to create sublines without expres-

sion of JAK1 or JAK2 (Figs. S10 and S11 in the Supplemen-

tary Appendix). These created truncat-

ing mutations analogous to those from Patients 1 

and 2, and M407 is positive for HLA-A*02:01 and 

expresses the cancer–testis antigen NY-ESO-1, 

which allowed us to model T-cell recognition 

using cells genetically modified to express an 

NY-ESO-1–specific T-cell receptor.23 M407 and 

both JAK-loss sublines were equally recognized by 

NY-ESO-1–specific T cells, leading to high levels 

of interferon-γ production (Fig. 4A).

When cultured in recombinant interferon alpha, 

beta, or gamma, the M420 and M407 parental cell 

lines showed interferon-induced growth inhibi-

tion in a dose-dependent manner (Fig. S12 in the 

Supplementary Appendix). However, both the 

JAK2-deficient M464 cell line (from Patient 2 at 

relapse) and the M407 JAK2-knockout subline 

were insensitive specifically to interferon gamma– 

induced growth arrest, yet remained sensitive to 

type 1 interferons alpha and beta; in contrast, the 

M407 JAK1-mutated subline was resistant to all 

three interferons (Fig. 4B). This is again consist-

tent with the specific association of JAK2 with 

the interferon-γ receptor and the common use of 

JAK1 by all three interferon receptors.27-29 As an 

orthogonal test of these effects, we treated our 

cell lines with 2′3′-cGAMP (cyclic guanosine 

monophosphate–adenosine monophosphate); this 

dinucleotide, which is produced in response to
Figure 3. Loss of Interferon Gamma–Induced Signaling and Gene-Expression Changes through Acquired JAK2 Mutation.

In Panel A, Western blot analysis of lysates from cell lines M420 (Patient 2, baseline) and M464 (Patient 2, relapse) shows Janus kinase (JAK)–signal transducer and activator of transcription (STAT) signaling events and downstream target induction after either 30 minutes (m) or 18 hours (h) of exposure to interferon (IFN) alfa, beta, or gamma (C indicates untreated control). Janus kinase 2 (JAK2) protein expression was absent in the relapse cell line (asterisk), and M464 failed to phosphorylate intermediate signaling components STAT1 and STAT3 or to up-regulate interferon-response targets TAP1, PD-L1, and major histocompatibility complex (MHC) class I after treatment specifically with interferon gamma (red box), as compared with intact signaling in M420 (blue box). There was no change in response to interferon alfa or beta. As shown in Panel B, a lack of response to interferon gamma exposure was also seen in surface staining for PD-L1 and MHC class I by flow cytometry. Each point represents an independent experiment, T bars represent standard deviations, and P values are for a two-way analysis of variance with Dunnett’s correction. MFI denotes mean fluorescent intensity, and NS not significant.

Panel C shows log₂ RNA counts of expression for 790 immune-related genes on exposure to interferon gamma or vehicle control. The baseline cell line M420 (top) showed up-regulation of many interferon-stimulated genes (line represents an increase by a factor of 4), whereas the JAK2 mutated progression cell line M464 (bottom) lacked a similar response.
A Interferon-γ Production

![Graph showing Interferon-γ production](image)

**Effector:Target Ratio**
- 10:1
- 2:1:1
- 1:1
- 0.25:1
- 0

B Treatment with Interferon Alfa, Beta, or Gamma

![Graph showing treatment effects](image)

C Treatment with 2′,3′-cGAMP

![Graph showing cGAMP treatment effects](image)
In Patient 3, whole-exome sequencing of the M407 parental cell line as well as the M407 JAK1-knockout and JAK2-knockout sublines were recognized by NY-ESO-1–specific, HLA-A*02:01–restricted T cells, as assessed by interferon-γ production after 24 hours of in vitro coculture. M420 is negative for HLA-A*02:01 and served as a negative control. In Panel B, cell lines M420 and M407 showed growth inhibition in response to direct in vitro treatment with interferon alpha, beta, or gamma (left), whereas the JAK2-deficient counterpart M464 and the M407 JAK2 knockout were insensitive specifically to interferon gamma (middle). The M407 JAK1 knockout was insensitive to all three interferons (right). In Panel C, treatment with 2′3′-cGAMP (cyclic guanosine monophosphate–adenosine monophosphate), a direct cytosolic agonist of the stimulator of interferon genes (STING), was able to produce growth arrest in all cell lines, regardless of JAK2 status, yet had no effect in M407 with JAK1 knockout. Growth curves represent the percent change in the number of melanoma cells over time as measured by IncuCyte continuous live-cell imaging in one of three independent experiments. Bars in Panels A, B, and C indicate standard deviations for three replicate wells. Three asterisks indicate P<0.001 for the percent change in growth with the treatment shown at the 72-hour end point as compared with the untreated control, with Dunnett’s multiple-comparison correction applied in Panel B. NS denotes not significant.

**Figure 4 (facing page). Loss of Interferon Gamma–Induced Growth Arrest through Acquired JAK Mutations.**

In Panel A, the M407 parental cell line as well as the M407 JAK1-knockout and JAK2-knockout sublines were recognized by NY-ESO-1–specific, HLA-A*02:01–restricted T cells, as assessed by interferon-γ production after 24 hours of in vitro coculture. M420 is negative for HLA-A*02:01 and served as a negative control. In Panel B, cell lines M420 and M407 showed growth inhibition in response to direct in vitro treatment with interferon alpha, beta, or gamma (left), whereas the JAK2-deficient counterpart M464 and the M407 JAK2 knockout were insensitive specifically to interferon gamma (middle). The M407 JAK1 knockout was insensitive to all three interferons (right). In Panel C, treatment with 2′3′-cGAMP (cyclic guanosine monophosphate–adenosine monophosphate), a direct cytosolic agonist of the stimulator of interferon genes (STING), was able to produce growth arrest in all cell lines, regardless of JAK2 status, yet had no effect in M407 with JAK1 knockout. Growth curves represent the percent change in the number of melanoma cells over time as measured by IncuCyte continuous live-cell imaging in one of three independent experiments. Bars in Panels A, B, and C indicate standard deviations for three replicate wells. Three asterisks indicate P<0.001 for the percent change in growth with the treatment shown at the 72-hour end point as compared with the untreated control, with Dunnett’s multiple-comparison correction applied in Panel B. NS denotes not significant.

With the approval of PD-1 checkpoint-blockade agents for the treatment of patients with melanoma, lung cancer, and other cancers, it is anticipated that cases of late relapse after initial response will increase. Understanding the molecular mechanisms of acquired resistance by focused comparison of biopsy samples from paired baseline and relapsing lesions may open options for the rational design of salvage combination therapies or preventive interventions and may guide mechanistic biomarker studies for the selection of patients, before the initiation of treatment, who are unlikely to have a response.

Tumor-infiltrating T cells are the effectors that kill cancer cells during PD-1 blockade therapy. We found it striking that after intratumoral CD8 T-cell infiltration during active response, CD8 T cells were usually still present and abundant at the time of relapse, though they were restricted to the tumor margin. This observation suggested to us that the T cells were no longer able to exert their cytotoxic activity, because of either a lack of tumor antigen recognition and activation or a loss of sensitivity to their effector molecules by the cancer cells. The gen-

**Diagram**: Loss of Interferon Gamma–Induced Growth Arrest through Acquired JAK Mutations.

In Panel A, the M407 parental cell line as well as the M407 JAK1-knockout and JAK2-knockout sublines were recognized by NY-ESO-1–specific, HLA-A*02:01–restricted T cells, as assessed by interferon-γ production after 24 hours of in vitro coculture. M420 is negative for HLA-A*02:01 and served as a negative control. In Panel B, cell lines M420 and M407 showed growth inhibition in response to direct in vitro treatment with interferon alpha, beta, or gamma (left), whereas the JAK2-deficient counterpart M464 and the M407 JAK2 knockout were insensitive specifically to interferon gamma (middle). The M407 JAK1 knockout was insensitive to all three interferons (right). In Panel C, treatment with 2′3′-cGAMP (cyclic guanosine monophosphate–adenosine monophosphate), a direct cytosolic agonist of the stimulator of interferon genes (STING), was able to produce growth arrest in all cell lines, regardless of JAK2 status, yet had no effect in M407 with JAK1 knockout. Growth curves represent the percent change in the number of melanoma cells over time as measured by IncuCyte continuous live-cell imaging in one of three independent experiments. Bars in Panels A, B, and C indicate standard deviations for three replicate wells. Three asterisks indicate P<0.001 for the percent change in growth with the treatment shown at the 72-hour end point as compared with the untreated control, with Dunnett’s multiple-comparison correction applied in Panel B. NS denotes not significant.

**Discussion**

With the approval of PD-1 checkpoint-blockade agents for the treatment of patients with melanoma, lung cancer, and other cancers, it is anticipated that cases of late relapse after initial response will increase. Understanding the molecular mechanisms of acquired resistance by focused comparison of biopsy samples from paired baseline and relapsing lesions may open options for the rational design of salvage combination therapies or preventive interventions and may guide mechanistic biomarker studies for the selection of patients, before the initiation of treatment, who are unlikely to have a response.

Tumor-infiltrating T cells are the effectors that kill cancer cells during PD-1 blockade therapy. We found it striking that after intratumoral CD8 T-cell infiltration during active response, CD8 T cells were usually still present and abundant at the time of relapse, though they were restricted to the tumor margin. This observation suggested to us that the T cells were no longer able to exert their cytotoxic activity, because of either a lack of tumor antigen recognition and activation or a loss of sensitivity to their effector molecules by the cancer cells. The gen-

**Diagram**: Loss of Interferon Gamma–Induced Growth Arrest through Acquired JAK Mutations.

In Panel A, the M407 parental cell line as well as the M407 JAK1-knockout and JAK2-knockout sublines were recognized by NY-ESO-1–specific, HLA-A*02:01–restricted T cells, as assessed by interferon-γ production after 24 hours of in vitro coculture. M420 is negative for HLA-A*02:01 and served as a negative control. In Panel B, cell lines M420 and M407 showed growth inhibition in response to direct in vitro treatment with interferon alpha, beta, or gamma (left), whereas the JAK2-deficient counterpart M464 and the M407 JAK2 knockout were insensitive specifically to interferon gamma (middle). The M407 JAK1 knockout was insensitive to all three interferons (right). In Panel C, treatment with 2′3′-cGAMP (cyclic guanosine monophosphate–adenosine monophosphate), a direct cytosolic agonist of the stimulator of interferon genes (STING), was able to produce growth arrest in all cell lines, regardless of JAK2 status, yet had no effect in M407 with JAK1 knockout. Growth curves represent the percent change in the number of melanoma cells over time as measured by IncuCyte continuous live-cell imaging in one of three independent experiments. Bars in Panels A, B, and C indicate standard deviations for three replicate wells. Three asterisks indicate P<0.001 for the percent change in growth with the treatment shown at the 72-hour end point as compared with the untreated control, with Dunnett’s multiple-comparison correction applied in Panel B. NS denotes not significant.

**Discussion**

With the approval of PD-1 checkpoint-blockade agents for the treatment of patients with melanoma, lung cancer, and other cancers, it is anticipated that cases of late relapse after initial response will increase. Understanding the molecular mechanisms of acquired resistance by focused comparison of biopsy samples from paired baseline and relapsing lesions may open options for the rational design of salvage combination therapies or preventive interventions and may guide mechanistic biomarker studies for the selection of patients, before the initiation of treatment, who are unlikely to have a response.

Tumor-infiltrating T cells are the effectors that kill cancer cells during PD-1 blockade therapy. We found it striking that after intratumoral CD8 T-cell infiltration during active response, CD8 T cells were usually still present and abundant at the time of relapse, though they were restricted to the tumor margin. This observation suggested to us that the T cells were no longer able to exert their cytotoxic activity, because of either a lack of tumor antigen recognition and activation or a loss of sensitivity to their effector molecules by the cancer cells. The gen-
eral possibilities are loss of mutational or shared tumor antigens that are recognized by T cells, loss of antigen-presenting machinery components (e.g., beta-2-microglobulin and HLA),12-14 tumor-cell–induced or myeloid-cell–induced inactivation of T-cell signaling,32,33 or insensitivity to the proapoptotic effects of toxic granules (e.g., perforin and granzymes), death receptors (e.g., Fas and tumor necrosis factor–related apoptosis-inducing ligand [TRAIL]), or interferons.14 Any of these escape mechanisms would be hypothesized to be fostered by the selective pressure of CD8 attack, which would be particularly active during the new round of immunoediting15 that is unleashed after PD-1 blockade.

The inactivation of JAK1 or JAK2, as noted in two of the patients, may be particularly advantageous to cancer cells in the context of anti–PD-1 therapy as compared with other immunotherapies. The interferon-induced adaptive expression of PD-L1, which allows the cancer to inactivate adjacent CD8 T cells,16 would be of no use after the PD-1–PD-L1 interaction is blocked by therapeutic antibodies. We propose that without this benefit, the advantage for cancer cells tilts toward abolishing interferon signaling in order to avoid the detrimental increase in antigen presentation and direct antiproliferative effects.27 Although we identified inactivating mutations in JAK1 and JAK2, which are receptor-level signaling bottlenecks, interferon insensitivity through other means — such as epigenetic silencing of interferon-signaling components as previously documented in lung-cancer and prostate-cancer cell lines15,16 or increased expression of negative regulators37 — might lead to the same end. We also documented one case of beta-2-microglobulin inactivation, which corroborates a previously described mechanism of acquired resistance to cancer immunotherapy in humans through loss of this shared component of all human MHC class I molecules that is required for CD8 T-cell recognition.12,14

In conclusion, the nearly identical mechanism of acquisition, functional consequence, and evidence of clonal selection for JAK1 or JAK2 mutations in two independent cases with a similar clinical course of acquired resistance suggests that resistance to interferon gamma contributes to immune resistance and escape. This genetic alteration of immune resistance joins the previously described loss of B2M in decreasing immune-cell recognition of cancer cells, leading to acquired resistance to cancer immunotherapy. Although we have identified four cases and worked out a potential mechanism of resistance in three of them, additional cases will need to be closely examined to assess the generalizability of these findings.

REFERENCES


Acquired Resistance to PD-1 Blockade


Copyright © 2016 Massachusetts Medical Society.
The NIHMS has received the file 'nejmoa1604958_appendix.pdf' as supplementary data. The file will not appear in this PDF Receipt, but it will be linked to the web version of your manuscript.