Clinical and molecular features of acquired resistance to immunotherapy in non-small cell lung cancer

Graphical abstract

Highlights

- >60% of initial responders to ICBs with stage IV NSCLC acquire resistance (AR)
- AR tumors had upregulated or stable expression of IFNγ response genes
- An AR inflammatory phenotype can be recapitulated in multiple murine models
- Altered, persistent inflammation informs strategies to overcome AR to ICB in NSCLC

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In brief

Memon et al. demonstrate the relationship between persistent and upregulated interferon signaling and acquired resistance in tumors from patients with NSCLC who have developed acquired resistance to PD-(L)1 blockade. These findings inform approaches for overcoming acquired resistance, which occurs in >60% of patients with NSCLC who initially respond to PD-(L)1 blockade.
Clinical and molecular features of acquired resistance to immunotherapy in non-small cell lung cancer

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SUMMARY

Although immunotherapy with PD-(L)1 blockade is routine for lung cancer, little is known about acquired resistance. Among 1,201 patients with non-small cell lung cancer (NSCLC) treated with PD-(L)1 blockade, acquired resistance is common, occurring in >60% of initial responders. Acquired resistance shows differential expression of inflammation and interferon (IFN) signaling. Relapsed tumors can be separated by upregulated or stable expression of IFNγ response genes. Upregulation of IFNγ response genes is associated with putative routes of resistance characterized by signatures of persistent IFN signaling, immune dysfunction, and mutations in antigen presentation genes which can be recapitulated in multiple murine models of acquired resistance to PD-(L)1 blockade after in vitro IFNγ treatment. Acquired resistance to PD-(L)1 blockade in NSCLC is associated with an ongoing, but altered IFN response. The persistently inflamed, rather than excluded or deserted, tumor microenvironment of acquired resistance may inform therapeutic strategies to effectively reprogram and reverse acquired resistance.
INTRODUCTION

PD-(L)1 blockade can generate profound, durable responses in patients with lung cancer and has been rapidly incorporated into the treatment paradigm for most patients with advanced non-small cell lung cancer (NSCLC). 1,2 Unfortunately, even among those patients who initially respond to PD-(L)1 blockade, over half will eventually develop progression—termed acquired resistance (AR). 3 Alongside primary resistance (refractory to initial treatment), AR represents a significant and possibly under-appreciated clinical challenge. 4 Remarkably little is known about the molecular mediators of AR. Perhaps relatedly, effective therapies to circumvent or reverse AR largely remain elusive.

The landscape of immune AR to PD-(L)1 blockade is poorly understood. By contrast, several molecular mechanisms of AR to molecularly targeted therapies (e.g., EGFR and ALK-directed tyrosine kinase inhibitors) have been identified and led to significant therapeutic advances. 1,2 In patients with lung cancer treated with PD-(L)1 blockade, there have been a few published cases of AR. 5–10 Along with case reports in other diseases, these studies have identified that loss of key proteins associated with dysfunction and tumor resistance. 14–16 Improved understanding of the nature and biology underlying AR is imperative to develop more effective next-generation immunotherapies in the future.

To address the clinical and molecular landscape of AR to PD-(L)1 blockade in patients with NSCLCs, we examined a large clinical cohort (n = 118 out of 1,201 analyzed in the study) of AR to PD-(L)1 blockade in lung cancer paired with a systematic genomic and transcriptomic analysis in a subset of patients (n = 29) with available tissue samples. We then also examined several isogenically paired murine models of initially sensitive vs. late relapse derived resistant tumor lines to PD-(L)1 blockade to validate relationships identified in human samples.

RESULTS

AR to PD-1 blockade in NSCLC is common

Of 1,201 patients with NSCLC treated with PD-1 blockade at Memorial Sloan Kettering Cancer Center (MSK) between April 2011 through December 2017, 243 (20%) achieved initial response. Many patients who responded ultimately developed AR, with an estimated cumulative AR rate of 61% (95% CI 36%–85%) at 5 years of follow up using a competing risk model (Figure 1A). The onset of AR was variable (52% within 1 year, 39% 1–2 years, 11% > 2 years) (Figure 1B). The relative risk of developing AR decreased with longer duration of initial response (Figure 1C).

Although AR and primary resistance have not been directly compared previously, we hypothesize that these scenarios are distinct biologically and clinically. Consistent with this, we found that several baseline clinical features differed between patients with AR and primary resistance (Figure 1D). High tumor PD-L1 protein expression in baseline (pre-treatment) tissue, in particular, was enriched among patients with AR compared to primary resistance (55% vs. 28%, Fisher’s p = 0.02). The organ-specific pattern of progression also differed, with liver metastasis being a common site of progression at primary resistance but relatively uncommon in AR (31% vs. 7%, Odds Ratio 6.23, Fisher’s p < 0.0001, Figure 1E). Perhaps most notably, the post-progression overall survival was significantly longer in patients with AR compared to primary progression (median 18.9 months vs. 4.4 months, log rank p < 0.0001 Figure 1F), potentially suggestive of persistent, partially effective anti-tumor immune responses that permits prolonged survival even after the initial onset of AR. Overall, AR was largely characterized by distinct clinical features, suggesting AR may have underlying immunobiologic features that are distinct from primary resistance and in need of dedicated analysis.

Patient cohort for molecular profiling of AR to PD-1 blockade

To investigate the molecular mechanisms of AR to PD-1 blockade in patients with NSCLC, we generated microarray-based whole transcriptome expression data, and whole exome sequencing (WES) data from pre- and/or post-treatment tumors in a subset of patients. Patients with analyzed samples had similar baseline characteristics to those in the larger clinical cohort (Table S1). After quality control and sample prioritization, the primary analysis of the molecular data focused on 42 tumor samples (13 pre-treatment, 29 post-treatment) from 29 patients for expression data and 34 tumor samples (15 pre-treatment, 19 post-treatment) from 22 patients for exome data (Figure 2A and Table S2). Thirteen patients had expression data available from both pre- and post-treatment tissue; 12 patients had exome data available from both pre- and post-treatment tissue. All post-treatment samples were obtained following radiographic progression to PD-1 blockade (median time from progression to sample collection 3.7 weeks, interquartile range [IQR] 1.8–10.4) and prior to initiation of new systemic therapy (patients did not receive combination PD-1 blockade with chemotherapy; Figure 2B).

Our work and others 17 have shown that AR frequently occurs in an oligoprogressive pattern, highlighting the importance of assessing the lesion-level response in the analysis of AR. Therefore, we examined the lesion-level response (and resistance) from which each sample was collected to optimize that pre-treatment and post-treatment samples reliably represented the biology of responsive and AR tumors, respectively. Specifically, all post-treatment samples were derived from sites with lesion-specific radiologic rebound growth or de novo growth (Figures 2C, S1A, S1B, and S2).

AR to PD-1 blockade is associated with a distinct transcriptional landscape

Principal components analysis (PCA) of protein-coding gene expression profiles from whole transcriptome data of all 42 samples showed no major technical or clinical factors influenced clustering, including batch and site of sample collection (i.e., lung, lymph node, adrenal, etc) (Figures S3A and S3B). There was also no separation among lesions that were present pre-treatment and later grew compared to those that emerged de novo during treatment (Figure S3C). We summarized gene expression values to pathway-level scores using single sample gene set enrichment analysis (ssGSEA) 18 on hallmark 19 gene sets categorized into oncogenic, cellular stress, immune,
stromal and other processes as previously applied. PCA clustering of 26 paired samples using enrichment scores (ES) showed a separation of samples based on paired pre- and post-treatment timepoints, with the separation primarily driven by immune-related hallmark gene sets (Figures 3A and 3B). Differential expression analysis of paired samples for hallmark gene sets showed a significant upregulation of IFN alpha/gamma response, oxidative phosphorylation, and DNA repair pathways after treatment (false discovery rate [FDR] < 0.1, Figure 3C and Table S3). Clustering of paired samples based on computational deconvolution of immune cell estimates from bulk expression derived using CIBERSORT showed a separation of pre- and post-treatment samples particularly driven by infiltration of CD8+ T cells (Figures 3D and 3E). Significant increase in immune

Figure 1. Clinical features of acquired resistance to immunotherapy in lung cancer
(A) Cumulative incidence of developing acquired resistance among patients with NSCLC with initial response to PD-1 blockade therapy. (B) Time to onset of acquired resistance among responders (n = 243). (C) Estimated rate of developing acquired resistance defined by duration of initial response. (D) Rates of baseline clinical features among patients with primary (n = 346) and acquired resistance (n = 118). Asterisk represents significant comparisons of Fisher’s p < 0.05. (E) Common organ sites of progression at time of primary or acquired resistance. * represents significant comparisons of Fisher’s p < 0.05. (F) Post-progression overall survival in patients with primary or acquired resistance (log rank p < 0.0001). See also Table S1.
infiltration (Wilcoxon signed-rank test p < 0.05; Figure S3D) and specifically CD8+ T cells was also observed post-therapy from differential analysis of paired pre-treatment and post-treatment samples (FDR < 0.1, Figure 3F and Table S3).

Several clinical and pre-clinical studies have generated bulk or single-cell RNA-seq datasets to identify gene sets associated with immune checkpoint blockade (ICB) resistance and T cell dysfunction. We curated a non-redundant resource of these gene sets plus the hallmark gene sets (hereafter termed Hallmark and ICB resistance gene set, see STAR Methods for details) and compared differential changes among the paired samples (Tables S3 and S4). Among these, comparing post-treatment to pre-treatment samples, we found an increase in expression of AP pathway, IFNγ,22 CD8 T effectors,23 and proliferating exhausted CD8+ T cells,24 while genes belonging to WNT25 pathway showed modest reduction in expression (Figure 3G). Consistent with these gene sets associated with ongoing immune response to PD-1 blockade, expression of individual genes enriched in post-treatment tumors included GZMA, B2M, and CXCL9 (Figure 3H and Table S5).

Chronic and therapy-dependent increase in IFNγ response pathway as a potential route to AR to ICB

Given the variability in the time to progression in the patient cohort (Figure 2B), we next applied pseudotime analysis using Phenopath26 to model disease progression in a continuous “latent” space based on gene expression variability within the cohort (Figure 4A). Using the 500 genes with the largest gene expression variability in pre- and post-treatment samples and subject ID and treatment as covariates, pseudotime scores generally increased from pre- and post-treatment samples, in particular, for a group of patients with low pre-treatment pseudotime estimates (Figure 4B). To identify pathways that potentially are associated with AR, we performed correlation analysis between change in pseudotime and change in pathway ssGSEA ES and signatures using the Hallmark and ICB resistance gene set compendium (Figure 4C). Among the top 10 positively correlated pathways with pseudotime, several IFN type I and II (IFNγ) signatures were correlated significantly, including IFN-stimulated genes (ISGs) that comprise the IFNγ hallmark gene set (Figure 4D, FDR < 0.01). Notably, samples could be separated into two subsets, with about half of samples showing little to no increase pre- to post-treatment and the other half characterized by elevated expression in ISGs related to an IFNγ response. This led us to categorize the patients into an IFNγ response “stable” and an IFNγ response “increase” group (Figures 4D and 4E).

As sustained cancer-intrinsic IFN signaling has been linked to ICB resistance in pre-clinical mouse models of melanoma and other cancers, we tested whether the change in ISG signatures (IFNα and IFNγ hallmark gene sets) observed in our clinical cohort related to a resistance signature derived from an ICB-resistant mouse model of melanoma.15,27 We found a significant association between the mouse-derived ICB resistance signature and the treatment-induced change in IFNγ response (Spearman’s rank correlation r = 0.90; p = 2.2e-16; Figure 4F), which persisted after removing overlapping genes (r = 0.86; p = 0.0003), Separately, PCA of change in enrichment score of hallmark gene sets between paired lesions showed a separation of patients on the 1st principal components based on the extent of change of ISG signatures (Figures S4A–S4C). The correlation was significantly stronger for change in the IFNγ-related ISGs (r = 0.9; p < 2.2e-16) when compared to change in IFNα-related ISGs (r = 0.48; p = 0.09; Figures S4D and S4E).

Consistent with a differential change of IFNγ response genes in these patients, tumors with an increase in IFNγ-related ISGs

Figure 2. Overview of the patient cohort used for the exome and expression analyses

(A) Flow diagram depicting molecular profiling of samples from patients with NSCLC treated with PD-1 blockade who developed acquired resistance. Paired samples are those collected prior to treatment initiation with PD-1 inhibitor and at time of resistance from the same patient. Unpaired samples include single timepoints of collection; prior to treatment initiation or at time of resistance.

(B) Swimmer’s plot of when each patient was molecularly profiled. Course of treatment, progression-free survival, and time to tissue acquisition are depicted. Lines within circles identify the type of sequencing completed.

(C) Waterfall plot of Response Evaluation Criteria in Solid Tumors (RECIST) best overall response in patient (dark blue) and lesion (light blue). Dashed line represents 30% shrinkage. Asterisk represents new metastatic lesions that appeared during treatment and continued to grow consistent with a site of acquired resistance (de novo growth). See also Figures S1 and S2; Tables S1 and S2.
Figure 3. Resistant lesions show up-regulation of IFNγ response pathway and infiltration of CD8+ T cells

(A) Principal components analysis of paired samples using enrichment scores of hallmark gene sets derived from ssGSEA. Paired pre- and post-treatment lesions from the same patient are connected using a dashed line (n = 26). The light gray arrow indicates the average directionality of change for each pair.

(B) Principal components feature loadings of hallmark gene sets with both magnitude and direction. Biological processes in hallmark gene sets were categorized into sub-groups as previously described and colour-coded accordingly.

(C) Differential comparison of hallmark enrichment scores between pre- and post-treatment samples. Each point represents a hallmark gene set and point size indicates the number of genes in a gene set. The x axis indicates the change in hallmark enrichment scores for paired samples from each patient (Post vs. Pre) and the y axis is false discovery rate (FDR)-adjusted p value derived from the comparison of enrichment scores of hallmark gene sets using paired t-test. The black dashed line represents FDR cutoff to identify significant gene sets (FDR < 0.1).

(D) Principal components analysis of immune cell estimates derived using CIBERSORT immune cell deconvolution approach.
generally had an increase in inferred activity of individual transcription factors associated with activation of ISGs, STAT1 and IRF1, as well as immune signatures (estimated by hallmark and literature gene sets) associated with CD8+ T cell exhaustion across several studies. In addition to signatures of T cell exhaustion, an increase in regulatory T cells was also noted. FOXP3 was specifically upregulated in samples from the subgroup with an “increase” in IFNγ-related genes (paired t test \( p = 0.005; \) Figure S4F). In contrast, patients with “stable” expression of IFNγ-related genes were characterized by a lack of change in these immune-related pathways and genes (Table S6). Together these data suggest a recurrent pattern of AR to PD-1 blockade in NSCLC is associated with activation of an IFNγ transcriptional program in tumors, presumptive alteration in tumor-specific IFNγ signaling (given persistent tumor growth clinically), and a concomitant increase in exhaustion of CD8+ T cells in the microenvironment.

**Positive selection pressure for antigen presentation gene mutations in AR**

To examine somatic alterations and potential mechanisms of AR, we next evaluated the exome sequencing data pre- vs. post-treatment for 12 patients (24 samples with germline single nucleotide polymorphisms [SNPs] confirming paired samples belonged to the same patient; Figure S5A). NSCLC is characterized by a high mutation burden, a strong predictor of response to immunotherapy. Overall, there was no significant difference in tumor mutation burden (Wilcoxon signed-rank test \( p = 0.6; \) Table S7), known driver genes, neoantigen burden, fitness (Wilcoxon signed-rank test \( p = 0.74; \)), or tumor heterogeneity (Wilcoxon signed-rank test \( p = 0.37 \) before versus after immunotherapy treatment at a summary level (Figures S5A, S5B, and S5C). However, there was evidence of remodeling of clonal or sub-clonal structure in seven patient samples. For five of these patient samples, clonal mutations were retained while a subset of sub-clonal mutations were lost and/or new sub-clonal mutations were also acquired (Figures S5B and S5D). For two patients (AR_20 and AR_27), post-treatment lesions did not share any somatic mutations with their respective pre-treatment lesions indicative of emergence of a potentially new tumor or outgrowth of a rare (i.e., below the limit of detection by WES) pre-existing tumor clone (Figure S5E). Among the clonal mutations detected in the post-treatment lesion of AR_20 included a nonsense mutation in the STK11 gene consistent with previous observations of an association between mutations in STK11 and resistance to ICB in lung adenocarcinoma. Several mutational processes, including extrinsic factors, particularly smoking, can influence somatic molecular profile in NSCLC and can be detected as mutational signatures. The smoking signature was the dominant signature in pre-treatment lesions and these mutations persisted in post-treatment lesions. However, post therapy the clonal composition of these tumors had changed potentially shaped by different sets of factors indicated by depleted proportion of smoking related mutations (Figure S5F). Recent studies have shown an enrichment of APOBEC mutational signature in samples from patients who benefit from immunotherapy treatment. In two patients, AR_08 and AR_20, we observed a noticeable increase in fraction of private mutations contributing to APOBEC mutational signatures 2 and 13 in the post-treatment lesions (48.3% in AR_08 and 14.3% in AR_20 relative to those in the pre-treatment lesions (5.4% in AR_08 and 1.6% in AR_20). Given previous studies describing loss of B2M and other genes such as TAP1, TAP2, and TAPBP involved in AP pathway as a potential mechanism of immune escape in resistant tumors, we performed an unbiased analysis to evaluate positive selection pressure on individual genes before and after therapy. As expected, canonical driver mutations in lung cancer such as KRAS and TP53 were under strong positive selection pressure, and there were no recurrently altered driver genes with significant enrichment in post-treatment tumors compared to pre-treatment (Figures 5C and S5G). However, a nonsense mutation and a frameshift deletion in B2M were exclusively identified in post-treatment tumors of AR_14 and AR_19 respectively, and other immune-related genes such as IL21R, PDCD5, FKBP1A, and FNIP1 were indeed enriched post-therapy (Figures 5C, S5H, and S5I). No potential pathogenic mutations were observed in TAP1, TAP2, and TAPBP genes. Given the selective identification of mutations in B2M and other immune-related genes in the ICB-resistant tumor samples, we evaluated additional gene sets involved in AP pathways using the GSEA approach. Specifically, we asked whether there was evidence of an association between IFNγ selective pressure and dysregulation of AP pathways (Figure 5D). Overlapping mutational changes with IFNγ status for the cases with both expression and mutation data, we observed mutation enrichment in the AP pathway to be more common among patient samples that show an “increase” in IFNγ response in contrast to those with “stable” IFNγ response pathway. Notably, three out of four patients with significant change in clonal or sub-clonal architecture (AR_20, AR_27, AR_19) showed presence of new mutations in the AP pathway genes in their post-treatment lesions (Figure 5E). All four of these patients (AR_20, AR_27, AR_19, AR_26) also had available tissue for B2M and class 1 HLA protein expression testing on tumor cells and all were negative or decreased from baseline (Figures 5F and S5).

To explore expression patterns of AR in an independent cohort, we analyzed RNA-seq data from primary and metastatic tumor samples obtained prior to enrollment onto a phase 1b study of durvalumab in combination with tremelimumab in subjects with...
advanced NSCLC ("Study 06", NCT02000947). Patients enrolled in the Study 06 trial were either naive to ICB treatment or failed on a previous line of anti-PD-(L)1 monotherapy. Those who failed to respond were further categorized as ICB primary resistant or ICB AR (Figure 5H). Patients with primary resistance had documented radiographic disease progression ≤16 weeks from the start of treatment with no evidence of clinical benefit. Patients with AR had radiographic disease progression following initial
Figure 5. Genomic dynamics in acquired resistance to PD-1 blockade in lung cancer

(A) Summary of somatic mutations (missense and indels) in samples from our immune checkpoint blockade (ICB)-resistance cohort for known driver genes in non-small cell lung cancer (NSCLC). Pattern of mutations of recurrently mutated genes derived from a previous study.32 The heatmap also indicates the unique and shared mutations in each sample and the proportion of mutations associated with key somatic signatures (smoking and APOBEC) associated with lung cancer.

(B) Percentage loss or gain of clonal and sub-clonal mutations in paired samples (n = 24) from each patient.

(C) Comparison of global p value estimates for genes (n = 20,091) derived from dN/dS analysis of missense, truncations and indels to evaluate gene-level selection pressure in pre- and post-treatment samples estimated using dndscv method.36

(D) Comparison of global p value estimates genes to identify gene sets under positive selection in pre- and post-treatment samples. The change in gene level global p value between pre- and post-treatment samples (shown in (C)) was used to order genes and estimate GSEA normalized enrichment score and p value for each gene set.

(E) Summary of key changes in expression and mutations in nine patients with pre- and post-treatment measurements for both expression and exome. The change in gene level NES between ICB naive (n = 58) and patients with AR (n = 27), had progressed without initial objective response (primary resistant), or progressed after an initial objective response (acquired resistant) on a previous line of anti-PD-(L)1 monotherapy.

(F and G) Immunohistochemistry based quantification of (F) HLA/MHC-I and (G) B2M. The pre-treatment lesion of patient AR_19 did not have enough tissue for private mutations in post-treatment lesions of patients in genes part of antigen presentation pathway (KEGG or REACTOME) are shown.

(H) Schematics of samples obtained from patients prior enrollment on Study 06—a phase 1b study in advanced NSCLC where patients were treated with durvalumab (Durva) and tremelimumab (Treme) as a second line therapy. Patients enrolled in the Study 06 trial were either naive to immunotherapy (IO) treatment, had progressed without initial objective response (primary resistant), or progressed after an initial objective response (acquired resistant) on a previous line of anti-PD-(L)1 monotherapy.

(I) Violin plot of ssGSEA enrichment scores for the hallmark IFN-γ response gene set for samples from the three patient groups. ssGSEA enrichment scores of samples from patients with acquired resistance and patients who were ICB naive were compared using Wilcoxon rank-sum test. See also Figure S5, Tables S7 and S8.
significant enrichment of myeloid cells, T cells, and IFN-γ-related ISGs compared to treatment naive samples (FDR < 0.05, Figures 5I, S5J, and S5K; Table S8).

AR is associated with elevated ISGs and alterations in tumor IFN-γ signaling

To further explore the transcriptional features that are associated with AR to ICB in our clinical cohort, we also examined cancer cell intrinsic transcriptional programs using a pre-clinical murine model system of AR to ICB inhibitors. Similar to PD-1-responsive human lung cancer, the CT26 murine model is carcinogen-induced, has high tumor mutation burden, and is highly sensitive to immunotherapy treatment, and is therefore a well suited pre-clinical analogue for interrogating AR. As expected, subcutaneous CT26 tumors showed significant reduction in tumor volume over 3 weeks of anti-PD-1 treatment (Figure 6A). To model AR, persistent viable cells following anti-PD-1 treatment were excised, cultured in vitro, and reimplanted in mice. This process was repeated for several passages until CT26 tumors were no longer responsive to anti-PD-1 antibody therapy (Figure 6B). Bulk RNA-seq was performed on the ICB-resistant cancer cell lines derived from tumors from the 2nd round (n = 2) and 4th round (n = 4) of in vivo passage and compared against the ICB-sensitive parental cell line (n = 3; Figure 6C). PCA of whole transcriptome data did not show any clear trend (Figure S6A); however, PCA of hallmark gene sets showed the parental and 2nd round samples tend to cluster separately from the 4th round samples with the separation mainly driven by IFN alpha/gamma response.

Figure 6. Cell lines derived from mouse CT26 tumors with acquired resistance to PD-1 show dysfunctional IFN-γ signaling

(A) Tumor volume over time after treatment with anti-PD-1 therapy or control (Vehicle) for parental (CT26 parental) and resistant cells (CT26 anti-PD-1 Res.) (n = 9 per group).

(B) Percentage of mice that resisted anti-PD-1 treatment.

(C) Experimental design for development of ICB-resistance model from anti-PD-1 treatment of CT26-derived tumors in mice. Cell lines were derived from tumors and subjected to RNA sequencing.

(D) Principal component analysis (PCA) of IFN-γ-untreated samples i.e., parental (sensitive), 2nd round and 4th round ICB-resistant cells based on enrichment scores of hallmark gene sets.

(E) Principal components feature loadings of hallmark gene sets with both magnitude and direction. Biological processes in hallmark gene sets were categorized into sub-groups as previously described and the vectors were color-coded accordingly.

(F–I) Enrichment scores in parental, 2nd and 4th round cells for the following genesets: (F) IFN-γ response pathway, (G) STAT1, (H) IRF1, and (I) antigen processing machinery.

(J) Comparison of significance of change in enrichment score between IFN-γ stimulated (IFN-γs) and IFN-γ untreated (IFN-γu) 2nd round and significance of change in enrichment score between IFN-γs and IFN-γu parental cells.

(K) Comparison of significance of change in enrichment score between IFN-γs and IFN-γu 4th round and significance of change in enrichment score between IFN-γs and IFN-γu parental cells.

(L–O) Comparison of enrichment scores between IFN-γu vs. IFN-γs (parental or 2nd or 4th) cells for the following genesets: (L) IFN-γ response pathway, (M) IRF1, (N) STAT1, and (O) antigen processing machinery. Statistical comparisons in (F), (G), (H), (I), (L), (M), (N), and (O) were performed using unpaired t test. For all panels, error bars are the standard error from the mean. See also Figure S6 and Table S9.
pathway (Figures 6D and 6E). Systematic comparison of 4th-round samples with parental samples showed a significant upregulation of IFNα and IFNγ response pathway genes (Figures 6F and Table S9). An increase in genes for other biological processes including TNFalpha signaling (FDR ≤ 0.1) and the AP pathway (Figure 6I) were also evident in cells from 4th-round resistant tumors. In contrast, no significant change in gene sets was observed from the comparison of the 2nd-round and parental cell lines (Figures S6B and S6C). Similar to the human data, these findings indicate that anti-PD-1 resistance was associated with elevated baseline expression of IFNγ-related ISGs.

Alongside the increase in baseline IFNγ response pathway genes in the 4th round cells, we also observed an increase in the activity of IFN-related transcription factors STAT1 and IRF1 inferred from their regulons (Figures 6G and 6H). Consistent with this increase in transcription factor activity and with our previous findings linking elevated ISGs to epigenetic changes, we further examined the chromatin accessibility by ATAC-Seq showed enrichment of the JAK-STAT signaling pathway in 4th-generation anti-PD-1 resistant cells relative to parental cells (q-value < 0.1; Figure S6G) or in vivo passaged control cells (q-value < 0.1; Figure S6H). Moreover, an unbiased search for motif sequences in the peaks of promoter regions showed significant enrichment of transcription factor binding motifs for IRF1 in the resistant cells (Figure S6B) but not in parental cells. These findings suggest that enhanced activity of IFN-related transcription factors may contribute to the elevated baseline ISG expression associated with resistant cancer cells.

To explore if ICB resistant cancer cells with elevated ISGs can further induce ISGs after IFNγ stimulation, cell lines were stimulated with IFNγ for 24 h and compared to unstimulated controls. While the parental and 2nd-round cell lines showed an increased expression of genes involved in IFN signaling after IFNγ stimulation, the 4th-round cell line did not show an overall induction of ISGs at the transcriptional level (Figures 6J–6L, S6D–S6F, and Table S9). In addition, transcription factors downstream of IFNγ signaling, such as STAT1 and IRF1, showed the same pattern with no statistically significant differences in expression between IFNγ stimulated vs. control in the 4th-round cell line (Figures 6M and 6N). As IFNγ signaling is known to upregulate AP machinery pathway genes, we also specifically investigated the effect of IFNγ on these genes, which further supported the observations and showed no additional induction to IFNγ stimulation in 4th-round cells (Figure 6O).

To examine ISG expression and IFNγ response after AR in another well-established immunogenic tumor model, we also analyzed MC38 colorectal carcinoma tumors. Consistent with reports that MC38 is highly responsive to anti-PD-1 treatment with better immunogenecity than the CT26 model11 (Figure S6J) indeed, subcutaneous MC38 tumors showed significant reduction in tumor volume after three weeks of anti-PD-1 treatment (Figure S6K). Similarly to CT26, we also derived serially progressive MC38 tumors no longer responsive to anti-PD-1 after the 5th round (n = 4) of in vivo passage. Resistant MC38 cancers showed significantly higher levels of multiple ISGs, including Stat1, Stat2, Mirtm2, and Mirtm3 when compared to parental cells (Figures S6L–S6P). Stimulation with IFNγ for 24 h further increased expression; however, compared to parental controls, resistant cells had significantly reduced transcriptional induction for several of these ISGs (Figures S6Q–S6U). Thus, like CT26 tumors, MC38 tumors that relapse after anti-PD-1 are associated with elevated baseline ISGs and develop an altered response to IFNγ stimulation. This altered IFNγ response could be either a broad insensitivity or a blunted induction for a subset of ISGs.

**Chronic IFNγ stimulation of lung cancer cells promotes elevated ISGs, resistance, and immune dysfunction**

We previously demonstrated that chronic stimulation of cancer cells with IFNγ can be sufficient to render cells resistant to ICB. Moreover, like with the CT26 and MC38 tumors that AR to anti-PD-1, these chronically stimulated cells increased their baseline expression and chromatin accessibility for a subset of ISGs. In order to determine if chronic IFN stimulation is sufficient to render NSCLCs resistant to ICB and also promote T cell dysfunction, we utilized two syngeneic mouse lung cancer models: the Krasloxp/stop-lox(lsl)-G12D/+; Tp53lox/lox (KP) genetically engineered mouse model and the Lewis lung carcinoma (LLC1) (Figures 7A and 7B). With both models, tumors that spontaneously relapsed after ICB were compared to tumors derived from cancer cells that were treated with low levels of IFNγ for 3–4 weeks in vivo prior to implantation into mice (Figures 7B and 7D). Like KP and LLC1 tumors derived from cells that persist after ICB (ResResKP, ResResKP, ResResPlate, and ResResLLC1), KP and LLC1 tumors from cells exposed to chronic IFNγ (γKP and γLLC1) were more resistant to ICB and showed increased expression of IFNγ-related ISGs, as assessed from cancer cells sorted from untreated tumors (Figure S7C). Tumors associated with chronic IFN signaling either by chronic

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**Figure 7.** Acquired resistance to immune checkpoint blockade (ICB) associates with induction of terminally exhausted CD8+ T cells in the LLC1 syngeneic lung cancer mouse model

(A) Experimental design of anti-PD-1 + anti-CTLA-4 therapy treatment schedule in the LLC1 mouse model following implantation of parental (LLC1), 3–4 weeks IFNγ stimulated (γLLC1) and ICB-resistant LLC1 cell lines.

(B) Comparison of tumor weights harvested on Day 16 in parental (LLC1), respective tumor types following ICB demonstrated that tumors with chronic IFN expression (γLLC1 and ResResLLC1) do not respond to ICB.

(C) Representative flow cytometric plots to show the expression of PD-1 and TIM-3 on CD8+ T cells from non-treated and ICB-treated tumors.

(D) Comparison of percentage of PD-1+ TIM-3+ terminally exhausted CD8 T cells in tumors.

(E) Unsupervised clustering of CD8 T cell population based on expression of T cell focused immune profiling.

(F) A heatmap to indicate the levels of the different T cell related markers across the 12 meta-clusters, defined of the unsupervised clustering of CD8 T cells.

(G and H) Comparison of frequency percentage of meta-clusters 5 (MC05) and 6 (MC06) within the CD8 T cell population between non-treated and ICB-treated LLC1, γLLC1 and ResResLLC1 tumors. The boxplots in (B), (D), (G), and (H) indicate median and the lower and upper hinges correspond to the first and third quartiles. The upper whisker extends from the hinge to the largest value no further than 1.5 * IQR (inter-quartile range) from the hinge. The lower whisker extends from the hinge to the smallest value at most 1.5 * IQR of the hinge. Statistical comparisons in (B), (D), (G), and (H) were performed using Wilcoxon rank-sum test. See also Figure S7.
IFNγ stimulation in vitro for 3–4 weeks (γLLC1) or a late relapse-derived tumor cell line (ResResLLC1) showed a diminished response to double ICB, a combination of anti-PD-1 + anti-CTLA-4 (Figure 7B). These pre-clinical data confirm our previous finding that high ISGs are associated with progression after ICB in murine tumor models. The diminished ICB efficacy in the LLC1 models harboring chronic IFN signaling (γLLC1) or late relapse-derived LLC1 tumor cell line (ResResLLC1) are characterized by the accumulation of dysfunctional PD-1+TIM-3+ exhausted T cells in the tumor post-ICB (Figures 7C and 7D). We then systematically characterized the immune infiltrate in the LLC1 tumors (Figures 7E and 7F). Tumor-infiltrating immune cells were categorized into twelve meta-clusters using unsupervised clustering. Interestingly, meta-clusters 5 and 6, that were dominant for T cell exhaustion markers (PD-1+, TIM-3+, etc.), showed an increase in frequency post-ICB in the chronically IFN stimulated or late relapsed tumor samples, but not in the initially sensitive tumor samples (Figures 7G, 7H, and S7D).

**DISCUSSION**

Although PD-1 blockade has been transformative in the treatment of patients with NSCLC, AR is common and understanding of the molecular mechanisms of resistance remains quite limited. Before embarking on this report, we had hypothesized that “non-inflamed” or “cold” tumors, characterized by exclusionary immunologic barriers or an absence of T cell infiltration, would significantly contribute to resistance.10 Previously, neoantigen loss and tumor-mediated immunsuppression have been associated with primary resistance to immunotherapy.4,42,43 In contrast, we found that neoantigen depletion does not appear to be a dominant mediator of AR. In fact, most tumors have retained or increased inflammatory characteristics, rather than immune excluded or desert phenotype, with significant upregulation of IFNγ suggestive of persistent, but evidently insufficient anti-tumor immune response. The persistent, if incomplete, anti-tumor immune response may also manifest in the clinical observation that some patients who develop AR can still have durable survival for many years following initial emergence of resistance. In addition to associations with chronic upregulation of the IFNγ response pathway, we also observed strong upregulation of oxidative phosphorylation and DNA repair pathway genes which are consistent with a recent report14 which proposes acquisition of a hypermetabolic state with high expression of glycolytic and oxidative phosphorylation pathway genes as a potential escape mechanism in ICB-resistant melanoma cells.

The inflammatory phenotypes we identify have implications for future rational development of new immunotherapy strategies for patients with AR. Most notably, immune recruitment and infiltration did not appear to be the primary biologic challenges, which provide credence to strategies aimed to reprogram and rescue native anti-tumor immunity. Delivery of de novo anti-tumor immunity via engineered antigen-specific cellular or T cell receptor-based therapies45–48 also appears well-suited to exploit the lack of barriers to immune trafficking and persistent tumor antigen expression. Supporting this hypothesis, we have reported notable responses among patients in this population.10,50 We are also exploring strategies to interrupt persistent IFNγ signaling to reinvigorate immune function and immune checkpoint inhibitor activity in tumors resistant to ICB.51 While we did observe a few instances of sub-clonal/clonal neoantigen loss, these changes were relatively uncommon and mutation burden was generally unchanged pre- vs. post-treatment.

One potential limitation of our clinical cohort is that it relies on bulk exome and transcriptome data which are prone to be affected by tumor purity. Due to constraints associated with sample purity, we were unable to accurately analyze copy number or loss of heterozygosity. Furthermore, IFNγ groupings were only possible for patients with paired pre- and post-progression samples because they were determined relative to each individual. Interestingly, recent analyses utilizing single-cell multi-omics have demonstrated enrichment of CD8+ T cell exhaustion in the setting of AR to PD-1 blockade in samples from two patients, similar to the exhausted CD8+ T cells we found in our lung cancer mouse model.52 Future efforts incorporating single cell multi-omics will be important to parse cancer cell-intrinsic vs. immune or stromally related mechanisms of resistance. Our analysis is limited to correlational observations and focused on PD-1 immunotherapy and not on PD-1 and chemotherapy combination. Although this is now a routine treatment regimen, it is complicated by the uncertainty of contributions of components both to response and to resistance, so for the moment, we have excluded analyses of samples from these patients.53 Further, heterogeneity in the duration and depth of response to PD-1 blockade in the clinical cohort could not be explored deeper in our molecular cohort due to small sample sizes. Future work is needed to both address heterogeneity in response to mono-therapy with ICB as well as resistance to combinations of chemotherapy and immunotherapy regimens.

Our work informs and builds upon the prior pre-clinical and translational data supporting the intricate role of IFNγ in sensitivity and resistance to immunotherapy. Whereas initial IFNγ exposure may be fundamental to T cell activation and a hallmark of immune response, persistent IFNγ related effects and upregulation may potentially signal immune dysfunction14,15 and IFNγ insensitivity.10,16,54 In contrast to previous reports linking IFNγ insensitivity to mutations in the JAK-STAT pathway, we did not identify specific defects in the IFNγ signaling pathway to explain the dysfunctional nature of IFNγ response observed in a subset of patient samples. While we found some evidence of B2M and other AP alterations, these changes were predominantly sub-clonal and generally co-occurred in tumors with upregulation of IFNγ potentially suggesting they may be an evolutionary consequence rather than an initiating cause of resistance. We have previously shown that chronic IFNγ signaling may trigger a cascade of epigenetic modifications in tumor cells including enhanced ISGs and ultimately generate a feedback loop of innate and adaptive immune exhaustion and dysfunction.14,15 Across multiple murine models of acquired resistance presented here, we recapitulate how acquired resistance is associated with upregulated cancer-intrinsic IFNγ response and ultimately tumor insensitivity to effective anti-tumor immunity. Separately, we also show that pre-treatment exposure with IFNγ in vitro leads to resistance in vivo to ICB treatment. Furthermore, we preliminarily observe that in vivo generated acquired resistant cell lines in general have altered ISG response as IFNγ stimulation in vitro
is associated with relatively lower activation of ISGs compared to parental cells stimulated with IFNγ. Further work is needed to identify the specific mechanistic deficits in response to the dynamics of IFNγ signaling in both immune cells and tumor cells. Overall, these data can further guide more rationally guided therapeutic strategies to prevent, overcome, and reverse AR to PD-1 blockade for patients with lung cancer.

**STAR METHODS**

Detailed methods are provided in the online version of this paper and include the following:

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**SUPPLEMENTAL INFORMATION**


**ACKNOWLEDGMENTS**

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**AUTHOR CONTRIBUTIONS**


**DECLARATION OF INTERESTS**

A.J.S. reports consulting/advising role to J&J, KSQ therapeutics, BMS, Merck, Enara Bio, Perceptive Advisors, Oppenheimer and Co, Umoja Biopharma, Legend Biotech, Iovance Biotherapeutics, Prelude Therapeutics, Immuno-core, Lyell Immunopharma, Amgen and Heat Biologics. Research funding: GSK (Inst), PACT Pharma (Inst), Iovance Biotherapeutics (Inst), Achilles therapeutics (Inst), Merck (Inst), BMS (Inst), Harpoon Therapeutics (Inst) and Amgen (Inst). MDH reports research grant from BMS; personal fees from Achilles; Arecus; AstraZeneca; Blueprint; BMS; Genentech/Roche; Genzyme/Sanoﬁ, Immunai; Instil Bio; Janssen; Merck; Mirati; Natera; Nektar; Pact Pharma; Regeneron; Shattuck Labs; Syndax; as well as equity options from Arcus, Factoral, Immunai, and Shattuck Labs. A patent filed by Memorial Sloan Kettering related to the use of tumor mutational burden to predict response to immunotherapy (PCT/US2015/062208) is pending and licensed by PGDx. J.L. has received honoraria from Targeted Oncology and Physicians’ Education Resource. D.M. is an employee of M.M Bio Limited. D.M. reports consulting role to Shattuck Labs and Corbus Pharma. T.M. is a consultant for Daiichi Sankyo Co, Leap Therapeutics, Immunos Therapeutics, and Pfizer, and co-founder of Imvaiq Therapeutics. T.M. has equity in Imvaiq therapeutics. T.M. reports grants from Bristol Myers Squibb, Surface Oncology, Kyn Therapeutics, Infinity Pharmaceuticals, Peregrine Pharmaceuticals, Adaptive Biotechnologies, Leap Therapeutics, and Aprea. T.M. is an inventor on patent applications related to work on oncolytic viral therapy, aliphasivirus-based vaccines, neo-antigen modeling, CD40, GITR, OX40, PD-1, and CTLA-4. B.D.G. has received honoraria for speaking engagements from Merck, Bristol Meyers Squibb, and Chugai Pharmaceuticals; has received research funding from Bristol Meyers Squibb and Merck; and has been a compensated consultant for Darwin Health, Merck, PMV Pharma, Shennon Biotechnologies, and Rome Therapeutics of which he is a co-founder. B.D.G. is part of a patent related to neoantigen prediction (WO2018136664A1, PCT/US2023/011643). G.F. and T.H.S. are employees and stockholders of Shattuck Labs, Inc. M.L.M. has received honorarium from GSK, H.R., X.Z., M.R.K., I.A., R.S., J.C.B., M.L.M., and M.D.H. are current employees and stockholders of AstraZeneca.
REFERENCES


### STAR METHODS

#### KEY RESOURCES TABLE

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#### Biological samples

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| CT26 DNA samples for ATACseq | Azenta/Genewiz | – |
| MC38 RNA samples for qPCR | Shattuck Labs | – |
| KP RNA samples for RNAseq | University of Pennsylvania | – |
| LLC1 RNA samples for RNAseq | University of Pennsylvania | – |

#### Chemicals, peptides, and recombinant proteins

| Interferon Gamma | R&D Systems | 485-MI-100 |
| Collagenase | StemCell Technologies | 07902 |
| Venor GeM Mycoplasma Detection Kit | Sigma | MP0025 |

#### Critical commercial assays

| First Strand cDNA Synthesis Kit | Origene | NP100042 |
| RNase Kit | Qiagen | 75144 |
| QIAshredder | Qiagen | 79656 |
| RNase-Free DNase Set | Qiagen | 79254 |
| SsoAdvanced Universal SYBR Green Supermix | Bio-Rad | 1725270 |

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RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Matthew Hellmann (matt.hellmann@gmail.com).

Materials availability
This study did not generate new unique reagents.

Data and code availability
Microarray expression data from MSK Study Cohort has been deposited in GEO database (GSE248249). Exome sequencing data from MSK Study Cohort has been deposited in dbGaP database (phs002834.v1.p1): https://www.ncbi.nlm.nih.gov/projects/gap/cgi-bin/study.cgi?study_id=phs002834.v1.p1. RNA-seq and ATAC-Seq data from CT26 mouse model and RNA-seq data from LLC1 and KP mouse models have been deposited in GEO database (GSE249000; GSE249001; GSE246922) and are publicly available. This paper does not report original code. Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Clinical cohort
MSK study
Following MSK institutional review board approval, patients with advanced NSCLC treated with PD-(L)1 based therapy between April 2011 and December 2017 were identified. Response Evaluation Criteria in Solid Tumors (RECIST) version 1.1 was used to assess objective response outcomes (n = 1201). Patients with primary resistance were defined as those with progressive disease (PD) at their first on-treatment scan evaluation. Pseudoprogression was excluded from the non-responder group. Patients with AR were defined as those with partial or complete response (PR/CR) followed by isolated or systemic progression on or before the date of their last scan (median follow-up 33.6 months). Post-progression overall survival was calculated from the date of progression on PD-(L)1 inhibitor. Patients who did not die were censored at the date of last contact. A cumulative incidence function with death as a competing risk was used to estimate the proportion of AR over time. Overall survival was estimated using the Kaplan-Meier method. Tumor

Continued
tissue samples were obtained with informed consent from patients under protocol #06-107 and #12-245 approved by MSK. This study was approved by the MSK Institutional Review Board and was conducted in accordance with the US Common Rule.

**Study 06**

NCT02000947 was a global, non-randomised, open-label, phase 1b study investigating the safety and tolerability of escalating doses of durvalumab in combination with tremelimumab. Subjects were 18 years of age or older, with histologically or cytologically confirmed NSCLC and must have failed to respond to, relapsed following, been ineligible for, or failed to tolerate any line of standard treatment. Patients had to have at least one measurable lesion, with adequate organ and marrow function, and Eastern Cooperative Oncology Group (ECOG) performance status of 0–1. During the initial dose-escalation phase, subjects must not have had prior exposure to immunotherapy. Once the maximum tolerated dose (MTD) or highest protocol-defined dose for each agent in the absence of exceeding the MTD was determined for the immunotherapy-naïve cohort, subjects who had prior exposure to immunotherapy were evaluated. The study was undertaken in accordance with the ethical principles of the Declaration of Helsinki and the International Council on Harmonization guidelines on Good Clinical Practice. The study protocol was reviewed and approved by the Institutional Review Board or Independent Ethics Committee at all participating centers and written informed consent was obtained from all patients. Patients were treated for up to 12 months or until progression or discontinuation due to toxicity. Patients who achieved disease control within the initial 12-month period entered follow-up, and upon evidence of progressive disease were eligible for readministration of treatment provided they had not received additional treatment and continued to meet the protocol defined eligibility criteria.

**Animal models**

For CT26 and MC38 models, female inbred BALB/cJ (RRID:IMSR_JAX000651) or C57BL/6J mice (RRID: IMSR_JAX:000664), respectively, between 8–12 weeks of age were purchased from The Jackson Laboratory. Mice were housed and acclimated in groups within the internal vivarium for approximately 5–10 days before beginning tumor inoculations. Mice were given a standard diet, allowed free access to water, and were housed on 12-hour light/dark cycles. Animal protocols were approved by an internal Institutional Animal Care and Use Committee (IACUC) and licensed veterinarian. Mice were humanely euthanized through CO₂ asphyxiation followed by cervical dislocation if their tumor volume exceeded 1800mm³, there was evidence of severe ulceration at the tumor cell injection site, or if any other significant distress was noted.

All animal experiments for the LLC1 and KP models were performed according to protocols approved by the IACUC of the University of Pennsylvania. Five- to seven-week-old female C57BL/6 (stock# 027) were obtained from Charles River Laboratory. Mice were maintained under specific pathogen free conditions and randomly assigned to each experimental group. Mice were acclimated for 7 days in the vivarium before tumor inoculation. Mice were given a standard diet, allowed free access to water, and were housed on 12-hour light/dark cycles. When tumors reached 15mm in any dimension the mice were euthanized through CO2 asphyxiation followed by cervical dislocation.

**Cell lines**

CT26 colon adenocarcinoma cells (of female origin, RRID:CVCL_7256) were purchased from ATCC. CT26 cells were cultured in RPMI-1640 media containing 10% fetal bovine serum, 2mM glutamine, 10mM HEPES, 1mM sodium pyruvate, 4500 mg/L glucose, 1500 mg/L sodium bicarbonate, and penicillin/streptomycin. Cells were grown at 37°C in a humidified incubator and cell lines in culture were tested monthly using the Venor GeM Mycoplasma Detection Kit (Sigma). MC38 cells were cultured in Dulbecco’s modified MEM with 10% fetal bovine serum, 2mM glutamine, 0.1mM nonessential amino acids, 1mM sodium pyruvate, 10mM HEPES, and penicillin/streptomycin.

LLC1 cells were purchased from ATCC, and KP (Kras mutant P53 mutant) lung cancer cells were a gift from the laboratory of David Feldser. Cells are cultured in GlutaMAX DMEM (DMEM, high glucose, GlutaMAX Supplement, pyruvate, Catalog number: 10569010) in 10% FBS with 100U/mL pen/strep. Cells were passaged every two days and maintained in a 37°C incubator. Chronic IFN cell lines (KP, LLC1) were chronically stimulated for 3.5 weeks with 10ng/ul of IFNγ (peprotech Catalog Number:315-05) and media replenished every 2 days during splitting, then IFNγ washed off and passed for an additional week before implanting into mice or freezing cell stocks. Rest[istant] cell lines were generated by harvesting late relapse tumors from parental cell lines implanted into mice and treated with immune checkpoint blockade.

**METHOD DETAILS**

**Generation of the molecular dataset from MSK cohort**

Tumor tissue samples from pre- and post-treatment timepoints were obtained from a subset of patients with NSCLC treated with PD-1 blockade (n = 29). All samples were processed as formalin-fixed paraffin-embedded (FFPE). Sixteen samples were obtained prior to initiation of therapy (pre-treatment) and 37 samples were obtained at time of AR. Most patients had a best overall response of CR or PR per RECIST criteria (n = 22, 76%), with a small subgroup with stable disease (SD, less than 10% tumor shrinkage). Lesion-level response was obtained for all samples (Figure S2). Pre-treatment lesions were those that had at least a 30% reduction in size on treatment or were resected prior to initiating therapy (n = 4). In patients with resected lesion samples, lesion-level response could not be obtained so overall patient-level must have been CR or PR per RECIST. Of note, consistent with prior work demonstrating
Gene expression profiling from MSK cohort

RNA was extracted from FFPE samples using the RecoverAll Total Nucleic Acid Isolation from Thermo Fisher Scientific (Catalog Number: AM1975). Global RNA expression was measured using the human Affymetrix Clariom D Pico assay. The RNA samples quantification on Affymetrix Arrays was performed in two separate batches. Samples from each batch were processed independently using Affymetrix Expression Console Software. Initially the samples were normalized using the SST-RMA algorithm and outlier samples were excluded. Samples from the two batches were then combined into a single dataset and subjected to batch normalization using ComBat. Finally, all the samples were further normalized together using LOESS normalization. For genes with multiple measurements, we selected the measurement with the highest coefficient of variation. The data analysis was focussed on 14,668 annotated protein-coding genes with expression measurements in the arrays. The expression dataset was thoroughly evaluated for technical artifacts such as batch effects (Figure S3A). Differential expression analysis was performed using the limma package in R. Normalized expression data of protein-coding genes were fitted to a linear model using lmFit function and subject to empirical Bayes (eBayes) moderated t-statistics test to identify differentially expressed genes in paired lesions.

Estimation of gene set enrichment scores from expression data

Enrichment scores were calculated for gene sets from the normalized expression matrix of protein-coding genes using the GSVA package in R with default parameters except for method = ‘ssgsea’ and norm = ‘TRUE’. This approach was used to estimate enrichment scores for the hallmark gene sets (msigdb v6.1 database) and non-redundant cancer and immune-related gene sets in literature. Although clinical data for AR to immunotherapy is fairly limited, recent studies have investigated the impact of chronic ICB treatment in in vitro cell lines and in vivo settings using mouse models generating either bulk or single-cell RNA-Seq datasets. We manually collated gene sets reported in many of these studies to build an extensive resource of biological processes and gene sets associated with cancer and immune pathways and more specifically ICB resistance (Table S4). In order to select for non-redundant gene sets from this resource, jaccard similarity coefficient was calculated between gene sets based on the number of shared genes and used this metric to perform hierarchical clustering of gene sets and construct a dendrogram with similar gene sets clustering together. Clusters of gene sets were obtained by cutting the dendrogram at a particular level using cutree function in R (h = 1.1). A non-redundant list of gene sets was created by selecting one gene set per cluster (Table S4).

Patient samples were classified into ‘increase’ and ‘stable’ sub-groups based on the difference in the enrichment of IFNγ response pathway between the paired pre- and post-treatment samples. The ‘increase’ subgroup consisted of samples from patients with differences in scaled enrichment score of IFNγ pathway >0.025 while ‘stable’ subgroup was defined by minimal change in enrichment score of IFNγ pathway (<=0.025 and > –0.025). Overlap coefficients were calculated between IFNγ gene set and other gene sets to make sure correlation in enrichment score across samples was not driven by shared genes. Change in enrichment score in paired samples was calculated by first scaling the signed enrichment scores values using min-max normalization and then taking the difference in the scaled enrichment scores for paired post and pre-treatment samples for each patient. Enrichment scores were also calculated for transcription factors using the same approach as for other gene sets using previously published regulons of each of the 164 transcription factors. Deconvolution of immune cells from bulk microarray expression data was performed using CIBERSORT tool with default parameters and normalized protein-coding gene expression matrix as input. Significance of change in enrichment score or immune cell estimates for paired samples was calculated using either paired t-test, welch t-test, or wilcoxon signed-rank test depending on the evaluation of equality (Bartlett’s test or Levene’s test) of variance and normality assumptions. All pairwise correlations between gene sets based on change in enrichment scores were performed using spearman’s rank-order correlation method.

Pseudotime analysis

To decode temporal information that traces the underlying biological process from a cross-sectional cohort of individuals, we applied pseudotime analysis to 13 patients with both pre- and post-treatment gene expression data by using the R package PhenoPath. This analysis assumes that individuals in the cross-sectional cohort behave asynchronously and each is at a different stage of progression. Furthermore, we assumed that the ICB treatment reverses the stage of progression for each individual. Specifically, we used the 500 most variable genes among pre-treatment and 500 most variable genes among post-treatment tumor samples for the pseudotime analysis. Since each subject contributed two gene expression profiles, two covariates (subject and treatment status) were specified in the regression model. To identify the pathways that were associated with pseudotime, we assessed the Spearman
correlations between change in pseudotime and change in ssGSEA enrichment scores of 131 pathways of the hallmark and ICB resistance gene set. To account for multiple testing, we calculated false discovery rate (FDR).

**Whole exome sequencing of MSK cohort**

Whole exome sequencing was performed using the Illumina protocol at the Broad Institute of MIT and Harvard, Cambridge, MA, USA using the same protocol as described in Jimenez-Sanchez et al. 2017.57 Exome samples were aligned to human reference genome (hg19) using bwa aligner (v0.7.17)36 and the aligned BAM files were subjected to deduplication and base recalibration methods in GATK (v4.0.2.1).59 These processed BAM files were used for all subsequent analyses. Mutation calling for SNPs and Indels was performed for each tumor-normal (serum) pair using GATK-Mutect2 (v.4.0.2.1) with default parameters and additional filters to remove germline mutations including SNPs detected in gnomAD (Genome Aggregation Database)60 and mutations in PoN (panel of normal) formed for each tumor-normal (serum) pair using GATK-Mutect2 (v.4.0.2.1) with default parameters and additional filters to remove germline mutations including SNPs detected in gnomAD (Genome Aggregation Database)60 and mutations in PoN (panel of normal) samples obtained from combining all normal samples in the cohort. Since exome samples were generated in multiple batches with different capture kits (Illumina’s Rapid Capture Exome Kit (38Mb target territory), Agilent SureSelect Human All Exon V2 (44Mb target territory), Agilent SureSelect Human All exon V4 (51MB target territory)), mutations were only called on common regions captured by the three different kits. The mutation calls were annotated using the Oncotator v161 tool.

**Tumor heterogeneity and clonality**

Tumor heterogeneity was evaluated using the Mutant-Allele Tumor Heterogeneity (MATH) score derived from the variant allele frequencies of somatic mutations as described previously.62 The clonal population structure of somatic mutations in tumor samples was inferred using Pyclone-VI.65 This method uses a Bayesian statistical approach to estimate cellular prevalence of mutations after accounting for purity of samples. The tumor purity estimates for Pyclone-VI were obtained from FACETS64 and manually corrected for each sample based on the distribution of variant allele frequency. The mean cellular prevalence (MCP) estimates from Pyclone-VI were used to classify somatic mutations as clonal (MCP >0.6), sub-clonal (MCP ≤ 0.6) or absent (MCP <0.02).

**Estimation of somatic signatures in tumor exome data**

Mutational signatures were estimated using the Sigfit65 package in R. For each exome sample, the proportion of mutations associated with each of the 30 mutational signatures in the COSMIC66 database were estimated. Signature 4 corresponds to smoking signature while Signature 13 corresponds to APOBEC signature.

**Analysis of selection pressure in mutation data**

Gene-level selection pressure was quantified for pre-treatment and post-treatment samples using the dNdScv36 package in R. The dNdScv approach quantifies dN/dS ratios based on missense, truncations (nonsense and essential splice site) and indel mutations in a group of samples and identifies genes under positive selection in cancer based on the global p values derived from likelihood tests. Selection pressure was calculated for each gene for pre-treatment and post-treatment samples separately and the difference in selection pressure between the two groups was used to identify potential biologically important genes associated with AR to ICB treatment. Gene sets with significant change in selection pressure between pre- and post-treatment samples were identified via the GSEA approach using the clusterProfiler68 package in R with the difference in the -log10(global p values) between pre- and post-treatment samples, used as a metric to rank genes.

**Phylogeny tree reconstruction**

For 12 patients with both pre- and post-treatment whole exome sequencing available, mutations were filtered based on the following criteria: 1) total coverage for tumor ≥ 10, 2) variant allele frequency (VAF) for tumor ≥ 4%, 3) number of reads with alternative allele ≥ 9 for tumor, 4) total coverage for normal ≥ 7, and 5) VAF for normal ≤ 1% at a given mutation. These filters applied to all mutations except for mutations in the KRAS gene. Then pre- and post-therapy mutations were aggregated per patient. PhyloWGS69 software package (https://github.com/morrislab/phylowgs) was used to infer the clonal structures and estimate clone sizes.

**Neoantigen prediction and fitness score**

Filtered mutations were annotated with snpEff.v4.3t software70 with options set as “-noStats -strict -hgvs1LetterAa -hgvs -canon -fastaProt [fasta file name]”. All wild-type (WT) and mutant genomic sequences corresponding to coding mutations were translated to an amino acid sequence consistent with the GRCh37 reference genome (GRCh37.75). Only annotations without “WARNING” or “ERROR” were kept and the most deleterious missense mutation was prioritized in mapping a genomic mutation to a gene. The mutant amino acid from a missense mutation was centered in a 17 amino acids long peptide. Then 9-mers were extracted in a left-to-right sliding fashion. Each mutant 9-mer contained the mutant amino acid on one of the nine positions. In essence, one missense mutation produced up to nine 9-mer peptides. Predictions of MHC class-I binding for both wildtype peptide (PWT) and mutant peptide (PNeo) were estimated using the NetMHC 3.471 software with patient-specific HLA-I type. All PNeo’s with predicted IC50 affinities <500 nM to a patient-specific HLA-I type were defined as neoantigens. Filtered neoantigens were aligned to the known positive epitopes in the Immune Epitope Database72 (IEDB, http://www.iedb.org) for all human infectious disease, class-I restricted targets with positive immune assays using blastp73 software (https://blast.ncbi.nlm.nih.gov/Blast.cgi). We then calculate the alignment scores with the Biopython Bio.pairwise2 package (http://biopython.org) for all identified alignments.
Clonal structure, MHC class-I affinities, and epitope alignment scores were put together into the fitness modeling framework in Luksza et al.24 Neoantigens were mapped to the clonal structure based on the underlying genomic mutations. Then fitness score was calculated for each clone, and the scores were averaged over all the clones in a sample after weighting on clonal sizes.

**Study 06 sample cohort and transcriptomic analysis**

Patients enrolled into the Study 06 trial (https://clinicaltrials.gov/ct2/show/NCT02000947) were either naive to ICB treatment or failed on a previous line of anti-PD-(L)1 monotherapy. Those who failed to respond were further categorised as ICB refractory (i.e. primary resistant) or ICB relapsed (i.e. acquired resistant). Patients who were refractory to ICB had documented radiographic disease progression ≤16 weeks from the start of treatment with no evidence of clinical benefit. Patients who relapsed had radiographic disease progression following initial clinical benefit (i.e., CR, PR, or SD on any scan).

Transcript-per-million (TPM)-normalised RNA-Seq data was available for 26,334 genes in 113 samples from 111 unique patients from Study 06: 58 samples from patients who were ICB-naïve, 28 post-ICB samples from patients who relapsed, and 27 post-ICB samples from patients with refractory disease. The TPM matrix was used as input for ssGSEA as implemented in GSVA R package version 1.42.0 to compute hallmark IFNγ signature enrichment scores (ES) in individual samples. ssGSEA ES were compared between samples from patients who relapsed and samples from patients who were ICB naïve or refractory using wilcoxon rank-sum test.

Gene set enrichment analysis (GSEA) from GSEA_R package version 1.2 was performed on 50 hallmark as well as ConsensusTME immune cell gene sets, using the TPM matrix as input. Briefly, t-tests were run for all 26,334 genes to compare TPM distributions between samples from patients who relapsed and who were ICB naïve or refractory. Genes were then ranked based on t-statistics and GSEA was run on the ranked gene list with 1000 permutations to compute normalised enrichment scores and associated FDR for each gene set. The analysis was run separately for hallmark and ConsensusTME gene sets and volcano plots show combined output of these separate runs.

**Generation of anti-PD-1 resistant CT26 tumors**

BALB/C mice were acquired from The Jackson Laboratory, after several days of acclimation, mice were inoculated with 500k CT26 on the rear flank. When the average tumor volume reached 80–100 mm³ (indicating day 0), mice were given a series of intraperitoneal injections of anti-PD-1 (clone RMP1-14; BioXcell), consisting of 100 μg each on days 0, 3, and 6. Tumors were excised from mice that did not respond to anti-PD-1 therapy, approximately 10–14 days following the initial treatment. Tumors were dissociated using collagenase (Stemcell Technologies), washed in 1X PBS, and plated in IMDM culture media supplemented in 10% fetal bovine serum, 1% GLUTIMAX, and 1% Antibiotic-Antimycotic (all GIBCO). Cells were passaged at least 5 times and then inoculated into new recipient mice according to the same protocol as above. Again, when tumors reached 80–100 mm³, another treatment course of anti-PD-1 began. This process was repeated for a total of four rounds, at which point none of the treated mice responded to anti-PD-1 therapy. The cell lines generated after two rounds of anti-PD-1 selection are referred to throughout this manuscript as ‘2nd round’, ‘2nd generation’ or ‘F2 generation’, and the cell line generated after four rounds of anti-PD-1 selection are referred to as ‘4th round’, ‘4th generation’, or ‘F4 generation’.

**Transcriptomic and ATAC-Seq profiling of anti-PD-1 resistant CT26 cell lines**

Three distinct vials of parental CT26 cells (ATCC; ‘experimental replicates’), two independently isolated tumors from ‘2nd round’ mice, and four independently isolated tumors from ‘4th round’ mice (both ‘biological replicates’), were cultured +/- 20 ng/ml of mouse IFNγ (Biolegend) for 24 hours at 37°C/5%CO2. The following day, RNA was isolated from cells using Qiagen RNaseasy reagents according to manufacturer’s instructions, including QiaShredder homogenization and on-column DNase I digestion. Isolated RNA was sent to Genewiz (www.genewiz.com) for library generation, RNA-sequencing, and data processing. The NEBNext Ultra II RNA Library Prep Kit for Illumina & NEBNext Poly(A) mRNA Magnetic Isolation Module (New England Biolabs, Ipswich, MA, USA), including clustering and sequencing reagents, was utilized according to the manufacturer’s recommendations. Briefly, mRNAs were enriched with Oligod(T) beads. Enriched mRNAs were fragmented for 15 minutes at 94°C. First strand and second strand cDNA were subsequently synthesized. cDNA fragments were end repaired and adenylated at 3’ ends, and universal adapters were ligated to cDNA fragments, followed by index addition and library enrichment by PCR with limited cycles. Briefly, sequencing libraries were generated and sequenced on an Illumina HiSeq (2x150 paired end reads), targeting >20x10⁶ reads per sample. Sequences were trimmed using Trimmomatic v.0.3675 and mapped to Mus musculus GRCm38 reference genome using STAR aligner v.2.5.2b.76 Unique gene hits were calculated by using featureCounts from the Subread package v.1.5.2.77 Only unique reads that fell in exonic regions were counted. The TPM values were obtained for each protein-coding gene and subsequently log-transformed (log2(TPM +1)) for downstream analysis. Mouse orthologs of genes in hallmark gene sets and antigen processing machinery78 and regulons of IRF1 and STAT1 were identified using Ensembl v8779 and ssGSEA was performed in a similar fashion as described for the clinical cohort.

For ATAC-Seq, CT26 cell pellets from two distinct vials of parental CT26 cells, five independently isolated tumors from mice, and five independently isolated tumors from ‘4th round’ mice were collected according to the same procedure as described above for RNA isolation, and pellets were sent to Genewiz (www.genewiz.com) for nuclei isolation, library generation, and sequencing. Nuclei were treated with Tn5 enzyme (Illumina, Cat. #20034197) for 30 minutes at 37°C and purified with Minelute PCR Purification Kit (Qiagen, Cat. #28004) to produce tagmented DNA samples. Tagmented DNA was barcoded with Nextera Index Kit v2 (Illumina,
Cell suspensions were prepared after RBC lysis with ACK Lysis Buffer (Life Technologies). Tumors were weighed prior to enzymatic digestion. Tumors, spleens, and draining lymph node (DLN) were harvested at day 16 post tumor implantation. For spleens and DLNs, single-cell suspensions were created. Tumor cells were isolated using a series of genes, in triplicate, and SYBR Green signal was assessed on the BioRad CFX Opus 96 and CFX Touch 96. Mouse validated gene primer sequences from Origene were used, and included mouse house-keeping control. Fold-change in gene expression at baseline was calculated using the \( 2^{-\Delta CT} \) method where each IFN\( \gamma \) treated tumor samples was normalized to untreated samples.

**Generation of anti-PD-1 resistant MC38 tumors**

Wild-type (WT) MC38 (colonorectal carcinoma) cells were acquired by license from the NCI, and were cultured in IMDM media, with 10% FBS, antibiotic/antimycotic, and gentamycin (all Gibco), and were cultured in an incubator at 37°C with 5% CO\(_2\). Cell lines in active culture are tested monthly using the Venor GeM Mycoplasma Detection Kit (Sigma). MC38 cells were inoculated (500,000 cells) on the hind flank of C57BL/6 mice (Jackson Laboratories), and when tumors became palpable, mice where either treated with vehicle (PBS) or anti-PD-1 (100 \( \mu \)g of clone RMP1-14 on days 0, 3, and 6 via intraperitoneal injection (IP); BioXCel). Tumor growth was measured over time and after approximately 20 days following the first treatment, tumors were isolated (indicating round 1), dissociated using collagenase (StemCell Technologies), washed in 1X PBS, and plated in culture media. Cells were passaged a minimum of two times and were then used to inoculate new C57BL/6 mice. Again, another course of vehicle or anti-PD-1 was given to the animals, tumor measurements were taken over time, and tumors were isolated approximately 20 days after treatment, from non-responding animals (indicating round 2). This in vivo anti-PD-1 selective pressure was performed for a total of five rounds until none of the mice responded to anti-PD-1 therapy. These isolated tumors are referred to throughout as ‘5th round’ and represent the tumor cells used to characterize the MC38 acquired resistance model (MC38/AR).

**Gene expression profiling of anti-PD-1 resistant MC38 cell lines**

Cells from four WT (or vehicle treated mice) and four 5th round AR tumors were treated +/- 20 ng/mL mouse IFN\( \gamma \) (R&D Systems) for three hours. After three hours, cell culture supernatant was removed and RLT lysis buffer (Qiagen) prepared with 5% 2-mercaptoethanol was added directly to the cells. Following lysis, lysates were homogenized with Qiagen QIAshredder and RNA was harvested using Qiagen RNeasy columns including on-column DNase I digestion. Then, 1 \( \mu \)g of RNA was reverse transcribed using Origene First Strand cDNA synthesis reagents. cDNA was diluted further with nuclease-free water and qPCR was performed at a series of genes, in triplicate, and SYBR Green signal was assessed on the BioRad CFX Opus 96 and CFX Touch 96. Mouse validated gene primer sequences from Origene were used, and included mouse Stat1, Stat2, Tap1, Ifitm2, Ifitm3, and the house-keeping control Rps18. Fold-change in gene expression at baseline was calculated using the \( 2^{-\Delta CT} \) method where the first WT tumor sample was set to 1. Each additional gene was compared to this sample and the Rps18 house-keeping control. Fold-change in IFN\( \gamma \) responsiveness was also calculated using the \( 2^{-\Delta CT} \) method where each IFN\( \gamma \) treated tumor samples was normalized to its representative IFN\( \gamma \) untreated samples.

**Generation of LLC1 and KP tumor cell lines**

In vivo mouse lymphocyte studies in LLC1 mouse model

Tumors, spleens, and draining lymph node (DLN) were harvested at day 16 post tumor implantation. For spleens and DLNs, single-cell suspensions were prepared after RBC lysis with ACK Lysis Buffer (Life Technologies). Tumors were weighed prior to enzymatic
digestion with Type 4 collagenase and DNase I at 1mg/mL. After enzymatic digestion or ACK Lysis, all tissues were filtered through 100-micron filters. Cells were stained with Fc Block and Zombie Live/Dead stain for 10 minutes prior to surface stain. Surface stain was done for 30 minutes at room temperature. Samples were fixed and permeabilized by incubating in 100 μl of Fix/Perm buffer at room temperature for 30 minutes and washed in Perm Buffer. Intracellular stains were performed overnight at 4°C. Cell counting beads were spiked into each sample prior to data acquisition. Data acquisition was done on a FACSsymphony A5. See key resources table for list of antibodies and buffers.

**Flow cytometry feature clustering**

For quantification and statistical analysis of flow cytometry data, both OMIQ and custom R scripts were utilized. Manual gating was used to define total CD8+ T cells and subsets of exhausted CD8+ T cells among the TILs. Furthermore, 15K CD8+ T cells were equally sampled from each FCS file and projected into opt-SNE space using OMIQ. FlowSOM clustering identified 12 clusters which was biologically defined based on geometric mean fluorescence intensity levels detailed in the heatmap.86,87

**QUANTIFICATION AND STATISTICAL ANALYSIS**

All statistical tests were performed in R. Statistical details of individual experiments can be found in the relevant figure legend and results section. For all analyses, p values <0.05 were considered significant.