Resistance to Durvalumab and Durvalumab plus Tremelimumab Is Associated with Functional STK11 Mutations in Patients with Non–Small Cell Lung Cancer and Is Reversed by STAT3 Knockdown

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ABSTRACT
Mutations in the STK11 (LKB1) gene regulate resistance to PD-1/PD-L1 blockade. This study evaluated this association in patients with nonsquamous non–small cell lung cancer (NSCLC) enrolled in three phase I/II trials. STK11 mutations were associated with resistance to the anti–PD-L1 antibody durvalumab (alone/with the anti-CTLA4 antibody tremelimumab) independently of KRAS mutational status, highlighting STK11 as a potential driver of resistance to checkpoint blockade. Retrospective assessments of tumor tissue, whole blood, and serum revealed a unique immune phenotype in patients with STK11 mutations, with increased expression of markers associated with neutrophils (i.e., CXCL2, IL6), Th17 contexture (i.e., IL17A), and immune checkpoints. Associated changes were observed in the periphery. Reduction of STAT3 in the tumor microenvironment using an antisense oligonucleotide reversed immunotherapy resistance in preclinical STK11 knockout models. These results suggest that STK11 mutations may hinder response to checkpoint blockade through mechanisms including suppressive myeloid cell biology, which could be reversed by STAT3-targeted therapy.

SIGNIFICANCE: Patients with nonsquamous STK11-mutant (STK11mut) NSCLC are less likely than STK11 wild-type (STK11wt) patients to respond to anti–PD-L1 ± anti-CTLA4 immunotherapies, and their tumors show increased expression of genes and cytokines that activate STAT3 signaling. Preclinically, STAT3 modulation reverses this resistance, suggesting STAT3-targeted agents as potential combination partners for immunotherapies in STK11mut NSCLC.

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mAbs blocking the PD-1/PD-L1 pathway have become the standard of care for numerous oncology indications including non–small cell lung cancer (NSCLC; ref. 1). Most patients, however, do not respond to therapy, creating a need to determine mechanisms associated with treatment resistance. Mutations in the STK11/LKB1 gene have recently been identified as an important regulator of resistance to PD-1/PD-L1 blockade (2, 3). These alterations, found in approximately 5% to 30% of patients with NSCLC (4–8), have been associated with lack of response to nivolumab, both as monotherapy or in combination with ipilimumab (2, 4), and lack of benefit from the addition of pembrolizumab to platinum doublet chemotherapy (9). STK11 is a serine/threonine kinase that is a critical regulator of cellular metabolism and energy sensing through activation of AMP kinase (AMPK) and AMPK-related family members (10, 11). Loss of STK11 increases serine utilization and synthesis of S-adenosyl methionine (SAM), a substrate of DNMT1, EZH2, and other epigenetic silencing enzymes that may affect the expression of genes affecting immune recognition [including the DNA sensor Stimulator of Interferon Genes (STING); refs. 10, 12, 13]. STK11 mutations are associated with T cell–excluded tumors, which are characterized by low or absent PD-L1 levels, low T-cell densities, high levels of granulocyte colony stimulating factor (G-CSF; CSF3) and IL8 family cytokines and high density of neutrophil-like cells, and production of myeloid cell-recruiting chemokines such as IL6 (2, 14–16).

This study explored the association between STK11 mutations in nonsquamous NSCLC and immunotherapy response in three independent trials of the anti–PD-L1 mAb durvalumab as monotherapy [phase I/II study 1108 (NCT01693562) and phase II ATLANTIC (NCT02087423)], or in combination with the anti-CTLA4 mAb tremelimumab [phase Ib study 006 (NCT02000947); refs. 17–19]. In-depth translational evaluations were conducted to elucidate the biological phenomena associated with STK11 mutations. Finally, therapeutic interventions in preclinical models were explored to identify strategies to overcome immunotherapy resistance mediated by STK11 loss and to enhance the sensitivity to checkpoint inhibitors (CPI).
Figure 1. Study outline and association between STK11 mutations and immunotherapy response. A, Experimental approach conducted at AstraZeneca to identify patients with STK11 mutations in clinical trials. Somatic STK11 mutations correlated with poor objective response rate (ORR) in three independent studies evaluating durvalumab (B) or durvalumab plus tremelimumab (C) in nonsquamous NSCLC. Reduced overall survival in STK11 mut patients treated with durvalumab (D) or durvalumab plus tremelimumab (E).

RESULTS
Functional STK11 Mutations Correlated with Poor Clinical Benefit
A total of 118 and 63 patients in Study 1108 and ATLANTIC (durvalumab) and 121 patients in Study 006 (durvalumab plus tremelimumab) had evaluable samples for STK11 tumor mutational status (Fig. 1A). Patient demographics and best overall response are shown in Table 1. In an analysis of pooled data from Study 1108 and ATLANTIC and an analysis of data from Study 006, the objective response rate (ORR) of patients with confirmed STK11-mutant (STK11mut) status trended lower or was significantly less than that of patients with STK11 wild-type (STK11wt) tumors: Study 1108 + ATLANTIC,

ORR (%) OS (%)

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<thead>
<tr>
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<th>ORR (%)</th>
<th>OS (%)</th>
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<tr>
<td>STK11mut</td>
<td>100</td>
<td>100</td>
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<tr>
<td>STK11wt</td>
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HR = 2.39 (95% CI, 1.34–4.25)
CPI Resistance in STK11mut NSCLC Is Reversed by STAT3 Knockdown

Table 1. Characteristics of patients enrolled in Study 1108, ATLANTIC, and Study 006 who were evaluated for STK11 mutational status

<table>
<thead>
<tr>
<th>Study 1108</th>
<th>STK11wt (n=105)</th>
<th>STK11mut (n=13)</th>
<th>ATLANTIC</th>
<th>STK11wt (n=59)</th>
<th>STK11mut (n=4)</th>
<th>Study 006</th>
<th>STK11wt (n=95)</th>
<th>STK11mut (n=26)</th>
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<tbody>
<tr>
<td>Median age, years (range)</td>
<td>64 (44–79)</td>
<td>56 (51–69)</td>
<td>62 (48–75)</td>
<td>60 (59–65)</td>
<td>61 (42–76)</td>
<td>62 (50–76)</td>
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<tr>
<td>Male, n (%)</td>
<td>53 (50)</td>
<td>4 (31)</td>
<td>34 (58)</td>
<td>3 (7)</td>
<td>54 (57)</td>
<td>18 (69)</td>
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<tr>
<td>Race, n (%)</td>
<td>White</td>
<td>90 (86)</td>
<td>12 (92)</td>
<td>30 (51)</td>
<td>1 (25)</td>
<td>59 (62)</td>
<td>90 (86)</td>
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<tr>
<td></td>
<td>Asian</td>
<td>11 (10)</td>
<td>0 (0)</td>
<td>29 (49)</td>
<td>3 (75)</td>
<td>28 (29)</td>
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<td></td>
<td>African American</td>
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<td>4 (4)</td>
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<td>ECOG status, n (%)</td>
<td>0</td>
<td>27 (26)</td>
<td>2 (15)</td>
<td>23 (39)</td>
<td>0 (0)</td>
<td>28 (29)</td>
<td>5 (19)</td>
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<td></td>
<td>1</td>
<td>77 (73)</td>
<td>11 (85)</td>
<td>36 (61)</td>
<td>4 (100)</td>
<td>67 (71)</td>
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<td>0 (0)</td>
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<td>Smoking history, n (%)</td>
<td>Never smoked</td>
<td>24 (23)</td>
<td>0 (0)</td>
<td>12 (20)</td>
<td>0 (0)</td>
<td>21 (22)</td>
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<tr>
<td></td>
<td>Former/current smoker</td>
<td>81 (77)</td>
<td>13 (100)</td>
<td>47 (80)</td>
<td>4 (100)</td>
<td>74 (78)</td>
<td>24 (92)</td>
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<tr>
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<td>III</td>
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<td>0 (0)</td>
<td>9 (15)</td>
<td>2 (50)</td>
<td>4 (4)</td>
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<td>91 (96)</td>
<td>22 (85)</td>
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<td>KRAS mutant</td>
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<td>8 (62)</td>
<td>17 (29)</td>
<td>2 (50)</td>
<td>24 (25)</td>
<td>14 (54)</td>
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<tr>
<td>EGFR status, n (%)</td>
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<td>0 (0)</td>
<td>3 (5)</td>
<td>0 (0)</td>
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<td>2 (3)</td>
<td>0 (0)</td>
<td>1 (1)</td>
<td>0 (0)</td>
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<tr>
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<td>Partial response</td>
<td>17 (16)</td>
<td>1 (8)</td>
<td>14 (24)</td>
<td>0 (0)</td>
<td>18 (19)</td>
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<td>Stable disease</td>
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<td>1 (8)</td>
<td>23 (39)</td>
<td>3 (75)</td>
<td>35 (37)</td>
<td>7 (27)</td>
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<td>Progressive disease</td>
<td>41 (39)</td>
<td>5 (38)</td>
<td>20 (34)</td>
<td>1 (25)</td>
<td>35 (37)</td>
<td>12 (46)</td>
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Abbreviations: ECOG, Eastern Cooperative Oncology Group; wt, wild-type.

STK11wt 32/164 (19.5%) vs. STK11mut 1/17 (5.9%), P = 0.166; and Study 006, STK11wt 19/95 (20%) versus STK11mut 1/26 (3.8%), P = 0.049 (Fig. 1B and C). Furthermore, median overall survival (mOS) was reduced for STK11mut patients. In the durvalumab monotherapy studies, mOS in STK11mut patients was 3.3 months compared with 13.6 months in STK11wt patients [HR, 2.83; 95% confidence interval (CI), 1.64–4.89; Fig. 1D], with the OS curves diverging within the initial months of durvalumab treatment. Likewise, mOS was significantly reduced in Study 006 patients with STK11mut tumors treated with durvalumab plus tremelimumab (7.5 months) compared with those with STK11wt tumors (15.4 months; HR, 2.39; 95% CI, 1.34–4.25; Fig. 1E).

To evaluate whether the observed detrimental effect of STK11 mutations on response to CPIs was associated with the presence of KRAS mutations, the mOS data from monotherapy and combination CPI treatment were then segregated by the presence of STK11/KRAS tumor comutations versus single STK11mut or KRASmut NSCLC tumors (Supplementary Fig. S1A and S1B). While the results confirmed that STK11 mutations were associated with poorer mOS, the number of patients with STK11mut/KRASwt tumors in this dataset was too small for definitive conclusions to be drawn regarding the potential effect of KRAS mutations in this setting.

Further exploration of this concept is currently underway in larger ongoing phase III clinical trials, data for which will be released upon study finalization. Genomic assessments of patients with NSCLC in The Cancer Genome Atlas (TCGA) showed STK11 mutations were not associated with worse prognosis than wild-type (WT) STK11 (Supplementary Fig. S2). To characterize the correlation of STK11 mutations with tumor mutational burden (TMB) and PD-L1 expression, these variables were assessed in screening tumor samples from Study 1108 and Study 006. There was no significant
difference in TMB between \textit{STK1I}mut and \textit{STK1I}wt tumors in Study 006 (Supplementary Fig. S3A). However, \textit{STK1I}mut tumors had relatively lower median PD-L1 expression in Study 1108 and Study 006 (Supplementary Fig. S3B), consistent with previous results showing similar TMB but lower PD-L1 in \textit{STK1I}mut NSCLC (2). Furthermore, in Study 006, clinical activity with durvalumab plus tremelimumab occurring in patients with \textit{STK1I}mut versus \textit{STK1I}wt tumors was also evaluated in subgroups defined by tumor cell PD-L1 expression and TMB (Supplementary Table S1). Unfortunately, these data must be interpreted with caution due to the limited number of patient samples available. Decreased baseline expression of \textit{STK1I} was observed in tumor biopsies from patients with \textit{STK1I} mutations in Study 1108 and Study 006 (Supplementary Fig. S3C).

**Increased Intratumoral and Peripheral Expression of Cytokines Associated with Neutrophilic and Th17 Contexture Detected in Patients with \textit{STK1I} Mutations**

Whole gene expression profiling was performed on tumor specimens collected at baseline from patients enrolled in Study 1108 and Study 006. By doing so, a subset of 414 genes were found to be differentially expressed (Supplementary Table S2) in \textit{STK1I}mut versus \textit{STK1I}wt tumors (\(P \leq 0.05\); fold change \(\geq 1.5\)). To further explore biological associations with \textit{STK1I} mutations, a functional annotation analysis was performed with the Ingenuity Pathway Analysis (IPA) on genes upregulated (\(n = 153\)) in \textit{STK1I}mut tumors. Genes upregulated in \textit{STK1I}mut tumors were found to be mainly involved in (i) granulocyte signaling, (ii) regulation of cytokine production by macrophages/neutrophils and Th17 cells through IL17 signaling, and (iii) cross-talk between innate and adaptive immunity (Fig. 2A). In particular, increased expression of markers associated with neutrophil infiltration and myeloid immunosuppression (i.e., IL6, CSF3) and Th17 cells (i.e., IL17REL) was found at the transcript level in the tumor microenvironment (TME; Fig. 2B). In addition, protein levels of IL8 (CXCL8) and IL6 were significantly elevated in the serum of Study 1108 patients with \textit{STK1I}mut tumors (Fig. 3A; Supplementary Table S3). This suggests that a macrophage/neutrophilic and Th17 immune contexture may play a contributing role in driving resistance to anti-PD-L1 and anti-CTLA4 therapy in patients with NSCLC. Interestingly, no differences were observed in the mRNA expression of CD8, LAG3, or CXCL9, markers generally associated with increased immune activation and better clinical response to durvalumab (20). These results were confirmed by IHC assessment of primary tumors from Study 1108 and Study 006 (Supplementary Fig. S3–S3D).

**Whole Blood Immunophenotyping of Patients with \textit{STK1I}mut versus \textit{STK1I}wt Tumors**

To characterize the peripheral immune contexture of patients harboring \textit{STK1I}mut versus \textit{STK1I}wt tumors, whole blood derived from patients enrolled in Study 1108 was assessed at baseline for circulating quantities of T, B, or natural killer (NK) cells and activated or memory T-cell subsets using bioanalytically validated, flow cytometry–based immunophenotyping assays. Five lymphocyte populations were identified that exhibited 2-fold or greater differences in median quantities between \textit{STK1I}wt and \textit{STK1I}mut patients including NK cells and CD4+ effector memory, CD4+ HLA-DR+, CD8+ effector memory and CD8+ HLA-DR+ T cells (Fig. 3B–D). Baseline and posttreatment quantities of immune cells were observed to be statistically decreased in \textit{STK1I}mut tumors compared with \textit{STK1I}wt tumors (\(P < 0.05\) with Wilcoxon rank-sum test).

**Overcoming CPI Resistance in Preclinical Mouse Models of \textit{STK1I} Mutation**

To understand the underlying mechanisms of CPI resistance mediated by inactivating \textit{STK1I} mutation or loss, mouse syngeneic tumor models were generated with \textit{STK1I} gene deletion. The \textit{Kras}mut CT26 colorectal cancer cell line was subjected to CRISPR-mediated \textit{STK1I} gene editing, and single-cell clones with loss of the \textit{STK1I} gene and protein expression were selected (Supplementary Fig. S4A). In each clone examined, loss of the \textit{STK1I} gene resulted in a decrease in the ratio of phosphorylated AMPK to total AMPK protein levels, indicative of loss of \textit{STK1I} kinase activity and phosphorylation of the downstream substrate AMPK. Ablation of \textit{STK1I} did not substantially change the growth rate of CT26 cells in cell culture but did increase the \textit{in vivo} tumor growth rate (Supplementary Fig. S4B and S4C). The change in growth \textit{in vivo} but not \textit{in vitro} is consistent with decreased immune control of tumors in an immunocompetent host. Increases in the percentage of neutrophil-like granulocytic cells in \textit{STK1I} knockout (KO) tumors were seen by single-cell RNA sequencing (scRNA-seq; Supplementary Fig. S5A–S5F; Supplementary Table S4) and significant increases in intratumoral Ly6G+ granulocytes were observed by flow cytometry (Supplementary Fig. S5G). scRNA-seq analysis revealed no change in the percentage of a combined monocyte/macroage/dendritic cell (DC) cluster between \textit{STK1I}wt and \textit{STK1I} KO clones; however, the proportion of mature M2-like macrophage clusters increased (Supplementary Fig. S5E and S5F; Supplementary Table S5) and IHC staining showed a corresponding association with increased CD163+ tumor-associated macrophage (TAM) density; Supplementary Fig. S5H). Examination of RNA expression in CD45-negative (CD45−) cells from dissociated tumors showed significant upregulation of cytokines and chemokines involved in myeloid cell recruitment and immunosuppression, including IL6 and CXCL3 (GRO-γ/MIP-2b) which bind the STAT3-activating receptors IL6R and CXCR2, respectively (Supplementary Fig. S5I), congruous with results observed in patients with NSCLC. Evaluation of the change in cytokine RNA levels was considered exploratory, as statistical analyses were not corrected for multiple comparisons. Given the reported association between \textit{STK1I} loss of function and the silencing of \textit{STING} (encoded \textit{TMEM173} by) expression in tumor cells and reduced double-stranded DNA sensing and immune recognition (12, 13), we examined \textit{TMEM173} expression but did not find an association between \textit{STK1I} loss and decreased \textit{TMEM173} in this model (Supplementary Fig. S5J). However, consistent with the increase in potentially immunosuppressive cytokines, \textit{STK1I} loss significantly decreased the intratumoral density
Figure 2. Whole transcriptome analysis conducted in patients with STK11mut versus STK11wt NSCLC. Analysis of differentially expressed genes (N = 414) satisfying the criteria of t test P ≤ 0.05 and fold change magnitude ≥ 1.5. A, IPA of genes upregulated (n = 153) in STK11mut tumors. B, Expression of markers associated with neutrophil infiltration and myeloid immunosuppression.
Figure 3. Peripheral contexture of patients from Study 1108 with STK11mut versus STK11wt tumors. A, Increased baseline serum levels of IL6 and IL8 in patients with STK11mut versus STK11wt tumors. B, In whole blood, at baseline, reduced circulating NK cells, CD4$^+$ effector memory and CD4$^+$ HLA-DR$^+$ cells, and D, CD8$^+$ effector memory and CD8$^+$ HLA-DR$^+$ cells were observed in patients with STK11mut versus STK11wt tumors. Similar results were also observed following durvalumab treatment.

of CD8$^+$ T cells as determined by IHC (Supplementary Fig. S6A and S6B) and resulted in a T-cell phenotype reflecting a reduced activation status as defined by scRNA-seq (Supplementary Fig. S6C–S6G; Supplementary Table S6). STK11 loss only moderately decreased the mean density of CD4$^+$ conventional T cells and CD4$^+$FoxP3$^+$ regulatory T cells (Treg), which did not reach statistical significance (Supplementary Fig. S6H and S6I). In addition to reduced activated CD8$^+$ T-cell infiltration, lower levels of PD-L1 mRNA were observed in the CD45$^+$ tumor cell and nonimmune stromal fraction (Supplementary Fig. S6J), which may reflect reduced levels of IFN$\gamma$ and other inflammatory cytokines in the TME. These findings are similar to those found in human STK11mut NSCLC specimens as described above and elsewhere (2, 15).

To test whether STAT3-activating cytokines and chemokines may activate STAT3-responsive pathways in CT26 STK11 KO
CPI Resistance in STK11mut NSCLC Is Reversed by STAT3 Knockdown

To determine whether the observed increase in STAT3 signaling (pSTAT3) may also be associated with STK11 mutation in primary human NSCLC tumors, we evaluated levels of pSTAT3 using reverse-phase protein array (RPPA) proteomics data from the TCGA database. Levels of pSTAT3 were significantly increased in STK11mut compared with STK11wt tumors (Supplementary Fig. S7B). In further analyses using the TCGA database, we observed that STK13 RNA expression was significantly higher in STK11mut versus STK11wt NSCLC samples (Supplementary Fig. S7B). As it is not possible to distinguish which cell type the pSTAT3 or STAT3 RNA signal originates from using these methods, it is difficult to ascertain whether the increased STAT3 signaling was associated with tumor cells or immune cells. To shed further light on this, we performed IHC labeling for pSTAT3 on human primary NSCLC patient-derived xenograft (PDX) tumors implanted in NSG mice. Human tumor cells were distinguished from murine immune and nonimmune stromal cells using IHC for a human-specific mitochondrial marker. These PDX tumors do not contain human immune cells, which are eliminated upon repeated passage of the PDX tumors in mice. The results showed increased percentages of pSTAT3+ human tumor cells in STK11mut versus STK11wt tumors (Supplementary Fig. S7C and S7D), suggesting high tonic stimulation of STAT3 signaling in STK11mut tumors. On the basis of the collective results from our CT26 STK11 KO model and these results from primary human NSCLC tumors, it is reasonable to hypothesize that STAT3 signaling may play an immunosuppressive role in tumors that have lost STK11 function. This hypothesis is currently under investigation from a translational science perspective using NSCLC patient samples obtained from larger phase III clinical trials; these data are not discussed in this work.

Next, we examined the sensitivity of our CT26 STK11 KO model to immunotherapy. As with human lung cancers harboring STK11 mutation/loss, STK11 KO CT26 tumors were resistant to anti–PD-L1 monotherapy and anti–PD-L1 plus anti–CTLA4 therapy, whereas STK11wt CT26 tumors were sensitive (Fig. 4A–D). Because the CRISPR KO clones were similar to one another in terms of STK11 knockdown, immune phenotype in the TME, and CPI resistance, clone 26C16 was taken forward as a representative CT26 clone with STK11 gene deletion for further in vivo studies. In addition to inherent resistance to CPIs, we tested whether antitumor efficacy in the CT26 model observed upon agonism of TNF receptor superfamily (TNFRSF) receptorsOX40 or CD137 or of the inducible T-cell costimulator (ICOS) was abrogated by STK11 loss. CT26 STK11 KO clone 26C16 tumors were resistant to anti–OX40, anti–CD137, and anti–ICOS agonist antibodies, whereas CT26 STK11wt tumors were highly sensitive (Supplementary Fig. S8A and S8B), indicating that the resistance phenotype extended beyond CPIs. The CPI resistance phenotype was directly due to STK11 gene loss in this model, since reintroduction of WT STK11 protein into STK11 KO clone 26C16 (26C16 STK11 KI) increased AMPK phosphorylation and restored sensitivity to CPIs (Fig. 4E–G; Supplementary Fig. S4A).

Immunotherapy resistance was not limited to the CT26 syngeneic model. CRISPR KO of STK11 in the KrasWt EMT6 mouse mammary tumor model resulted in reduced phosphorylated AMPK and an increased Ly6G+ granulocyte fraction compared with EMT6 WT tumors (Supplementary Fig. S9A and S9B). Like the CT26 STK11 KO model, EMT6 STK11 KO tumors showed resistance to combined CPI therapy that was also reversed by reintroduction of STK11 (Supplementary Fig. S9C–S9E; Supplementary Table S7).

Intracellular signaling downstream of STK11 regulates the mTORC1 complex to modulate protein synthesis and cellular proliferation (10). To test the hypothesis that mTOR pathway activation downstream of LKB1/AMPK signaling was responsible for CPI resistance, the dual mTORC1/2 inhibitor AZD2014 (21, 22) was tested in combination with anti–PD-L1 plus anti–CTLA4 therapy in either CT26 WT or the STK11 KO clone 26C16. Surprisingly, this combination did not show additive tumor growth control compared with AZD2014 monotherapy or anti–PD-L1 plus anti–CTLA4 drug controls in the STK11 KO clone 26C16 but did show additive antitumor activity in mice engrafted with CT26 WT tumors (Supplementary Fig. S10A and S10B).

Granulocytic myeloid-derived suppressor cells (gMDSC) have a role in promoting a suppressive TME (e.g., by reducing T-cell activity and antigen presentation), and may limit the activity of CPIs (23, 24). Given the influx of gMDSCs in the STK11 KO clone, we tested whether therapies targeting gMDSC migration and/or function could restore sensitivity to CPIs. The anti-Ly6G mAb clone 1A8 administered at a dose that depletes gMDSCs within the CT26 TME did not show additive activity in combination with anti–PD-L1 plus anti–CTLA4 therapy (Supplementary Fig. S10C and S10D). Likewise, the CXCR2 small-molecule inhibitor AZD5069 (25) did not result in additional growth control when combined with anti–PD-L1 plus anti–CTLA4 therapy, nor did the CSF1R inhibitor AZD7507 (26) when used either alone or in combination with AZD5069, despite activity demonstrated by both inhibitors in mice engrafted with CT26 WT tumors (Supplementary Fig. S10E–S10H). An anti-IL6 mAb that neutralizes binding to the IL6 receptor (IL6R) demonstrated by both inhibitors in mice engrafted with CT26 WT tumors (Supplementary Fig. S10C and S10D).
Figure 4. Effects of STK11 deficiency on CPI efficacy in the CT26 model. Survival of mice engrafted with CT26 WT (A) or the CT26 STK11 KO clones 26C16 (B), 26C10 (C), or 26C4 (D) and left untreated or treated with isotype control antibodies, anti–PD-L1, anti-CTLA4, or dual checkpoint inhibitors as indicated. CT26 WT: *, Isotype control versus anti–PD-L1, \( P = 0.0075 \); †, Isotype control versus anti–PD-L1 + anti-CTLA4, \( P = 0.0003 \); CT26 STK11 KO Clone 26C4: ‡, Isotype control versus anti–PD-L1 + anti-CTLA4, \( P = 0.026 \) using log rank (Mantel–Cox) survival analysis.

Independent experiment showing the survival of mice engrafted with CT26 WT (E), STK11 KO clone 26C16 (F), or STK11 KO clone 26C16 (G) reexpressing STK11 and treated with isotype control or dual anti–PD-L1 plus anti-CTLA4 mAbs as indicated. CT26 WT: §, Isotype control versus anti–PD-L1 + anti-CTLA4, \( P = 0.0009 \); ¶, CT26 STK11 KO clone 26C16: isotype versus anti–PD-L1 + anti-CTLA4, \( P > 0.99 \), nonsignificant; *, 26C16 STK11 KO/KI: Isotype versus anti–PD-L1 + anti-CTLA4, \( P = 0.001 \) using log rank (Mantel–Cox) survival analysis.
pathways may be involved in maintaining the CPI resistance phenotype.

A murine-reactive STAT3 antisense oligonucleotide (ASO) was used as a strategy to inhibit multiple, STAT3-mediated immunosuppressive signaling pathways simultaneously. The physicochemical properties of this drug have been described previously (27), and enable efficient STAT3 ASO transduction and STAT3 RNA knockdown in stromal cells within the TME, particularly macrophages and DCs, CD4+ Tregs, fibroblasts, and endothelial cells, with relative sparing of tumor cells and other lymphocytes. The pharmacodynamic effects of the STAT3 ASO within the STK11wt CT26 TME in these previous studies included reductions in immunosuppressive CD206+ myeloid cells and increases in immunoreactive MHCII+ TAMs. This remodeling of the TME was associated with CD8+ T-cell-dependent antitumor activity when STAT3 ASO was administered as monotherapy, which was augmented by combination with an anti–PD-L1 agent (27). In this study, the ASO was administered to STK11 KO clone 26C16 tumor-bearing mice 2 days after implantation for 3 cycles of a 5-day-on/2-day-off schedule (Fig. 5A). Anti–PD-L1 or anti–PD-L1 plus anti–CTLA4 immunotherapies were started when STAT3 ASO–treated tumors reached a median size of 110 mm³, upon which animals were randomized to receive additional doses of STAT3 ASO, anti–PD-L1, or anti–PD-L1 plus anti–CTLA4. Monotherapy STAT3 ASO administration early after tumor implantation resulted in tumor growth inhibition and showed additive effects when combined with anti–PD-L1 or anti–PD-L1 plus anti–CTLA4 therapy, with many deep and durable responses that resulted in significantly prolonged survival in the combination groups (Fig. 5B and C; Supplementary Table S8). The most potent activity was observed in mice receiving the triple combination of STAT3 ASO with anti–PD-L1 and anti–CTLA4. The antitumor activity of STAT3 ASO was also observed in the EMT6 model where tumor growth delay and enhanced survival were observed in the STAT3 ASO monotherapy group relative to the control Ab–treated group, as well as the STAT3 ASO + anti–PD-L1 + anti–CTLA4 triple combination relative to the anti–PD-L1 + anti–CTLA4 dual immunotherapy (Supplementary Fig. S9F and S9G). This was true in both WT EMT6 cells and EMT6 cells harboring STK11 loss.

To better understand the cellular mechanisms underlying these findings, both cellular immunophenotyping by flow cytometry and an ex vivo MDSC suppression assay were conducted in the CT26 STK11 KO clone 26C16 model. Immune cells were isolated from tumors 14 days after implantation when animals had received STAT3 ASO for 2 cycles and a second dose of immunotherapy (Fig. 5A). The percentages of CD45+ immune cells and live Ly6G+ granulocytic cells within the tumor remained statistically indistinguishable in STAT3 ASO treatment versus control groups (Supplementary Fig. S12A and S12B). This suggested that changes in overall immune infiltrate or decreases in granulocytic cell number were not associated with response. However, the relative activation state and costimulatory potential of antigen-presenting cells (APC) was altered by STAT3 ASO and combination therapy. For example, although the percentage of CD24+ CD64+ MHC class II (MHCII+) CD103+ conventional dendritic cells (cDC1) among the tumor immune cell population did not change significantly (Supplementary Fig. S12C), an increase in the percentage of CD86+ cDC1s was observed with STAT3 ASO and anti–PD-L1 agents (both as monotherapy and in combination), with the triple combination of STAT3 ASO plus anti–PD-L1 plus anti–CTLA4 showing the strongest effect (Fig. 6A). Likewise, cell surface CD86 staining intensity [mean fluorescence intensity (MFI)] on the CD103+ cDC1 subset was highest in the triple combination group (Supplementary Fig. S12D).

Within the tumor draining lymph nodes (TDLN) of treated mice, increases in CD86 positivity were also observed in migratory CD24+ CD11b+ CD103+ DCs (Supplementary Fig. S12E–S12G). In this anatomic location, however, STAT3 ASO and anti–PD-L1 mAb monotherapy treatment groups did not show increased percentages of CD86+ DCs, but significant increases in the percentage of CD86+ migratory DCs and median CD86 MFI were observed in combination groups containing anti–CTLA4, with the STAT3 ASO plus anti–PD-L1 plus anti–CTLA4 triple combination group showing the highest levels. This suggests that CTLA4 contributes to modulation of the effects of the STAT3 ASO on DCs within the draining lymph node rather than within the tumor itself. Collectively, these results suggest that one mechanism by which STAT3 ASO enhances the immune effects of checkpoint blockade is through increased costimulation by activated DCs.

Similar to DCs, intratumoral CD24+ CD64+ MHCII+ myeloid APCs showed no change as a percentage of CD45+ immune cells among treatment groups (Supplementary Fig. S12H), but showed a significantly higher percentage of CD86+ cells and CD86 MFI in STAT3 ASO treatment relative to control groups, with the greatest increase observed in the triple combination group (Fig. 6B; Supplementary Fig. S12I). Examination of MHCII+ myeloid cells (CD11b+ F4/80+) that were CD206+ or CD163+ revealed a trend toward increased percentages among treatment groups (Supplementary Fig. S12J and S12K). However, among M2-like MHCII+ myeloid cells, statistically significant decreases in CD206+ and CD163+ cell percentages compared with control were observed in groups treated with STAT3 ASO (Fig. 6C and D), consistent with STAT3 knockout reprogramming the balance of myeloid cells from a suppressive M2-like state to one less immunosuppressive (27).

An MDSC:T-cell coculture assay was used to characterize the immunosuppressive activity of these cells on T-cell proliferation. First, we isolated Gr1+ MDSCs from either CT26 STK11wt or CT26 STK11 KO clone 26C16 tumors and compared the relative suppression of T-cell proliferation. MDSCs isolated from STK11 KO tumors were moderately more suppressive than those from STK11wt tumors at intermediate MDSC:T-cell ratios, although this did not reach statistical significance (Supplementary Fig. S13A). In a study using MDSCs isolated from STK11wt tumors, a modest reversal of the immunosuppressive effects of MDSCs was observed in mice treated with anti–PD-L1 therapy compared with mice administered isotype control antibodies or left untreated; however, the addition of an anti–CTLA4 agent abrogated this effect (Supplementary Fig. S13B). Administration of STAT3 ASO as monotherapy completely reversed MDSC immunosuppression at all MDSC:T-cell ratios tested, and this effect was also observed when STAT3 ASO was combined with anti–PD-L1 or anti–PD-L1 plus anti–CTLA4 therapy.
Similar to the observed results with CT26 STK11wt tumors, the STAT3 ASO also appeared to reverse MDSC immunosuppression in the CT26 STK11 KO model. In this model, T-cell proliferation was significantly impaired at an effector-to-target (E:T) ratio of 0.5:1 using MDSCs isolated from untreated and isotype control Ab–treated mice (Fig. 6E), and no change was observed with anti–PD-L1 immunotherapy. In contrast, STAT3 ASO monotherapy completely reversed the inhibitory potential of MDSCs at an E:T cell ratio of 0.5:1, while anti–PD-L1 plus anti-CTLA4 therapy only moderately reversed suppression at this ratio. Combination treatment of mice with STAT3 ASO and...
anti–PD-L1 further reduced the suppressive activity of MDSCs such that full proliferation was observed at an E:T ratio of 1:1, whereas MDSCs from the STAT3 ASO plus anti–PD-L1 plus anti-CTLA4 treatment group showed the same level of suppression as observed in the STAT3 ASO monotherapy group. These results suggest that STAT3 ASO treatment may enhance the activity of checkpoint blockade in the CT26 STK11 KO setting partly through the reversal of MDSC suppression in the TME.

To determine the dependency of the efficacy of STAT3 ASO monotherapy and STAT3 ASO combination treatment on CD8+ or CD4+ cells, these cells were depleted from mice prior to treatment. The efficacy of STAT3 ASO monotherapy depended partially upon CD8+ effector T cells, and
Figure 6. (Continued) E, Effects of CPI, STAT3 ASO, or combinations on the immunosuppressive function of Gr1+ MDSCs assessed ex vivo. MDSCs were isolated from mice engrafted with STK11 KO clone 26C16 tumors and treated with the indicated therapies, and then cocultured with CD3/CD28-stimulated naïve T cells from non–tumor-bearing mice to induce T-cell proliferation ex vivo. Ratios indicate the MDSC:T-cell ratio in coculture at fixed T-cell numbers. Dotted line indicates proliferation of T cells stimulated with anti-CD3/CD28 in the absence of MDSCs. Gray boxes highlight MDSC:T-cell ratios with the greatest differences in therapeutic effects.

**DISCUSSION**

Emerging data suggest poor outcomes with anti–PD-(L)1 agents in patients with STK11 mut tumors (2). In this study, we evaluated genomic alterations in STK11 as a determinant of primary resistance to anti–PD-L1 monotherapy and anti–PD-L1 plus anti-CTLA4 combination treatment in three independent retrospective cohorts of patients with nonsquamous NSCLC. Mutations in STK11 were shown to correlate with resistance to durvalumab monotherapy and durvalumab plus tremelimumab combination therapy independently of KRAS mutational status, highlighting STK11 as a potential driver of immune escape and innate resistance to PD-L1 and CTLA4 blockade. Of note, plasma testing for mutations using the Guardant360 panel is likely less sensitive than tumor DNA testing using the FoundationOne CDx panel, thus potentially leading to a reduced representation of STK11 mutations in the data sets from Study 1108 and ATLANTIC and to an even greater corresponding underrepresentation of the resulting downstream effects (immune evasion/innate resistance) in relation to durvalumab monotherapy. In addition, while tissue assessment by next-generation sequencing (NGS) using the FoundationOne CDx panel captures both homozygosity and heterozygosity, plasma assessment by targeted NGS of circulating tumor DNA (ctDNA) using the Guardant360 CDx panel is not able to capture homozygous deletions. However, as described previously (28, 29), heterozygosity is sufficient to demonstrate loss of function.

the additive effects of anti–PD-L1 plus anti-CTLA4 fully depended on these cells (Supplementary Fig. S14A–S14C). In contrast, CD4+ cell depletion improved efficacy in each group that showed benefit in the absence of depletion.
To elucidate the biology associated with STK11 mutations leading to poor clinical outcome following durvalumab ± tremelimumab treatment, in-depth translational evaluations were performed. In the periphery, a greater than 2-fold reduction in the median number of NK cells and CD4+ effector memory, CD4+ HLA-DR+, CD8+ effector memory, and CD8+ HLA-DR+ T cells was observed at baseline and following durvalumab treatments in patients with STK11mut versus STK11wt tumors. In addition, increased baseline levels of IL6 and the neutrophil-attracting cytokine IL8 were found in the serum of patients with STK11mut tumors. Likewise, in the TME, significantly increased baseline expression (P < 0.05; fold change > 2) of markers associated with neutrophils, (i.e., CXCL2, IL6, CSF3), Th17 contextue (i.e., IL17A), and immune checkpoints (i.e., killer Ig-like receptors) was found in STK11mut vs. STK11wt tumors. These data suggest that poor outcomes to immunotherapy observed in patients with NSCLC with STK11mut tumors may be determined by a compromised peripheral and intratumoral immune phenotype characterized by enrichment of immunosuppressive mechanisms.

Therapeutic interventions to reverse this immunosuppressive phenotype, in particular blockade of STAT3 signaling, were explored in preclinical models to identify strategies to overcome immunotherapy resistance mediated by STK11 loss and enhance sensitivity to CPIs.

STAT3 represents a central transcriptional node that integrates immunosuppressive signals downstream of cytokine and chemokine receptors such as CXCR2, G-CSFR, IL6R, IL10R, and VEGFR expressed by lymphoid and innate immune cells as well as nonimmune stromal cells such as endothelial cells and fibroblasts (30, 31). STAT3 plays a key role in myeloid cell–driven inflammation and tumor immune surveillance (32, 33). Given that the expression of ligands for these receptors (e.g., IL6 and CXCL3) was upregulated in CT26 tumors after STK11 loss, we surmised that STAT3-mediated immunosuppression may play a key role in CPI resistance in this model. The finding of increased pSTAT3 in immune cells and tumor cells further strengthened this hypothesis.

To modulate STAT3 function in the TME, we employed an antisense approach using a murine-reactive STAT3 ASO. This ASO, like danvatisen (formerly AZD9150), which targets human STAT3, primarily reduces murine STAT3 protein levels in immune cells, fibroblasts, and endothelial cells within the TME, with relative sparing of tumor cells (27). Administration of this drug as monotherapy showed activity in the CT26 STK11 KO model and dramatically enhanced sensitivity to anti-PD-L1 monotherapy and anti-PD-L1 plus anti-CTLA4 therapy. STAT3 ASO treatment of CT26 STK11wt tumors also enhanced CPI responses, suggesting that this signaling node in the TME generally opposes immune-mediated tumor control. However, the fact that other therapeutics such as inhibitors of TORC1/2, CXCR2, and CSF1R, granulocytic cell depletion, and anti-IL6 failed to combine effectively with checkpoint inhibition in the CT26 STK11 KO model suggests that STAT3 signaling may play a special role in mediating immunotherapy resistance resulting from STK11 loss.

STAT3 ASO administration in the CT26 STK11 KO model resulted in less T-cell suppression by Gr1+ MDSCs and shifted the balance of CD206+ macrophages from an MHCII+ M2-like phenotype to an MHCII+ one. The CSF1R inhibitor would be expected to reduce macrophage recruitment and the CXCR2 inhibitor and clone 1A8 antibody to prevent migration of gMDSCs to the TME or deplete them outright, respectively. The lack of activity with these therapies suggests that depleting myeloid and granulocytic cells in this model was not effective. Instead, reprogramming of these cells through STAT3 modulation may lead to better APC function and increased CPI efficacy.

In addition to lowering MDSC immunosuppression, STAT3 ASO treatment enhanced the T-cell costimulatory potential of both CD103+ MHCI+ cDC1s and CD64+ MHCI+ macrophages (as measured by CD86 upregulation). Activation of these APCs would be expected to enhance antitumor T-cell activity through priming of naive T cells or reactivation of tumor antigen-specific T cells in the TDLN and tumor, respectively. Importantly, CD103+ cDC1s are potent tumor antigen cross-presenting cells that are required for CPI efficacy in mouse models (34) and may be an important inducer of antitumor CD8+ activity in this setting. It is noteworthy that one of the defining features of human STK11mut NSCLC is loss of DC-LAMP+ dendritic cells in the TME (16), which likely represents loss of cDC1s and cDC2s. Because ablation of CD8+ T cells limited the efficacy of both STAT3 ASO monotherapy and STAT3 ASO plus combined CPI therapy, it is likely that STAT3 modulation of innate immunity ultimately acts through antitumor CD8+ T cells recognizing tumor-associated antigens presented by MHC1. We did not identify phenotypic changes or differences in the number of infiltrating CD8+ T cells in groups treated with STAT3 ASO, but this may reflect the timing of tumor preparation for this analysis and is an area of current investigation.

Given the immunosuppressive effects of the IL6, IL10, and VEGF receptors in the TME, it is tempting to speculate that STAT3 signaling downstream of these receptors in immune cells mediates STK11-driven CPI resistance. STAT3-mediated IL6R signaling in macrophages and DCs inhibits cell maturation and impairs antigen presentation (32, 35). In the CT26 STK11 KO model, blockade of IL6 using a neutralizing mAb partially reversed the resistance phenotype, as previously observed in a KRASmut/LKB1−/− genetically engineered mouse model of lung cancer (15). However, the relatively modest activity may be explained by the activity of other IL6 family members [such as IL11 or leukemia inhibitory factor (LIF)] that also interact with IL6R, as well as by the activity of additional immunosuppressive cytokines in the TME. For example, VEGFA acting through VEGFR2 promotes the expansion and suppressive effects of immature myeloid cells (MDSCs) as well as preventing activated T-cell adhesion and migration through tumor vasculature (36, 37). Likewise, IL10 potently suppresses antigen presentation by DCs, and IL10 KO or conditional STAT3 deletion enhances DC cytokine production, antigen presentation, and induction of antigen-specific T-cell proliferation (38–40). Although immune cell STAT3 activation may be a key mediator of immunosuppression caused by STK11 loss, it is possible that the effects of STAT3 ASO on endothelial biology and angiogenesis or on cancer-associated fibroblasts could also contribute to therapeutic activity. Formally demonstrating what cell types and biology are essential for drug activity is a daunting task given the number of cell types in which STAT3 signaling affects...
tumor progression; this is a topic for further exploration. However, given the relative lack of uptake and STAT3 knockdown by the ASO in tumor cells (27), it appears unlikely to be mediated by tumor cell-intrinsic effects.

An objective of this work was to identify therapeutic modalities with a mechanistic rationale to treat STK11mut NSCLC. We explored the use of murine syngeneic lung tumor cell lines as models for STK11-mediated immunotherapy resistance. However, the murine LL2 Lewis lung carcinoma and KLN205 lung tumor models available at our institution were resistant to immunotherapies at baseline (without STK11 loss), meaning they could not be used to determine whether STK11 mutation contributed to a resistant phenotype. Confirmation of the role of STK11 mutation in lung cancer immunotherapy resistance and reversal by STAT3 blockade in the TME would benefit from additional studies in lung syngeneic cancer models sensitive to immunotherapy and converted to a resistant phenotype by STK11 mutation. Likewise, genetically engineered mouse models of lung cancer with STK11 loss could be used to confirm results related to STAT3 blockade and reversal of immunotherapy resistance found in syngeneic mouse tumor models. In our preclinical studies, we used immunotherapy-sensitive mouse syngeneic colorectal and breast tumor models that were converted to a resistant state by CRISPR/Cas9-mediated removal of STK11. Removal of the gene mimics the lack of STK11 functional activity found in patients with predicted loss-of-function STK11 mutations (the criteria used to define STK11-mutant status in this study). STK11 loss in the EMT6 and/or CT26 models was accompanied by reductions in PD-L1 expression and CD8+ T-cell infiltration as well as relatively high levels of granulocytic cell infiltration, characteristics commonly found in STK11mut NSCLC. These findings suggest that such models are relevant to test hypotheses related to alterations commonly found in the microenvironment of human tumors harboring STK11 loss. The reversal of CPI resistance when combined with coblockade of STAT3 signaling in the tumor microenvironment in these models supports the idea that this may be a combination option worth considering for patients with NSCLC harboring STK11 mutations.

METHODS

Study Populations

Patients with evaluable STK11 genomic status were selected from the full analysis sets of the three studies. The studies were conducted in accordance with Good Clinical Practice and the Declaration of Helsinki and were approved by each Institution’s Ethical Review Board. Patients provided written informed consent. Additional details on patient characteristics are in Table 1.

The relationship between STK11 tumor mutational status (STK11mut vs. STK11wt) and treatment response was measured as ORR by Response Evaluation Criteria in Solid Tumors (RECIST) v1.1 and OS. STK11 mutational status was evaluated by targeted NGS of ctdNA (Guardant360 panel, Guardant Health) of pretreatment plasma samples from patients in Study 1108 and ATLANTIC and by NGS (FoundationOne CDx panel, Foundation Medicine, Inc.) of pretreatment tumors of sufficient quality from patients in Study 006. The experimental approach to identifying patients with STK11 mutations is shown in Fig. 1. The association between mutational status and OS was also assessed according to STK11 functional mutational status alone, KRAS mutational status alone, and STK11/KRAS mutational phenotypes. The prognostic role of STK11 mutation was evaluated in patients in TCGA database (https://www.cancer.gov/tcga).

IHC in Tumor Biopsies

Fresh or archival tissue biopsies of sufficient quality for IHC staining for PD-L1, CXCL9, LAG3, and CD8 were available for 120 patients. Tumor tissue samples were collected, processed, and analyzed for cellular expression of PD-L1 as described previously (41), using the analytically validated Ventana SP263 assay (RRID:AB_2819099) and automated BenchMark ULTRA platform (Ventana Medical Systems). Tumoral CD8+, LAG3+, and CXCL9+ cell density (tumor-infiltrating lymphocytes/mm² of tumor using automated image analysis) was also assessed (42).

mRNA Sequencing

RNA sequencing (RNA-seq) was conducted on frozen biopsies using the Illumina NextSeq instrument (Atlantic Lab Equipment) and sequencing protocols as described previously (43). Technicians were blind to clinical data. Full details of the protocol are provided in the Supplementary Appendix. RNA-seq data from Study 1108 have been deposited into the Gene Expression Omnibus (GEO) repository (ID number GSE110390).

Proteomic Evaluation

Selected serum proteins (N = 66) were measured by Myriad RBM multiplexed immunoassays for patients in Study 1108.

DNA Sequencing and TMB Analysis

DNA sequencing and TMB analysis were performed on archival or fresh formalin-fixed, paraffin-embedded tumor samples (N = 121, Study 006) using the validated assay FoundationOne CDx, which employs NGS to detect substitutions, insertions and deletions (indels), and copy-number alterations in 324 genes and select gene rearrangements. TMB was also analyzed by this targeted NGS methodology, and was calculated according to previously defined methods (https://www.foundationmedicine.com/genomic-testing/foundation-one-cdxs). Full details of the protocol used are provided in the Supplementary Appendix.

Flow Cytometry–based Immunophenotyping of Whole Blood from Patients with STK11mut versus STK11wt Tumors

Flow cytometry–based immunophenotyping assays were used to quantify lymphocyte population absolute counts (cells/mm³) in fresh whole blood specimens from patients enrolled in Study 1108. The assays were designed to quantify T, B, and NK cells and memory or activated T-cell subsets. Activated T-cell subsets were defined on the basis of elevated surface expression levels of CD38 or HLA-DR compared with samples stained with isotype control antibodies. Memory T-cell subsets were defined on the basis of differential expression of CD197 and CD45RO, where naïve T cells were CD197− and CD45RO−, central memory T cells were CD197+ CD45RO− and effector memory T cells were CD197− CD45RO+. Specific details of assay design and staining methodology are shown in the Supplementary Appendix.

Assays were performed on fresh whole blood samples, collected in either acid citrate dextrose-B (memory or activated T-cell assays) or CytoChex-BCT tubes (T, B, and NK assay). Each assay was bio-analytically validated for imprecision and stability levels based on context-of-use.

Cell Lines and Culture Conditions

CT26 WT and EMT6 WT cells were obtained from the ATCC [catalog nos. CRL-2638 (RRID:CVCL_7256) and CRL2755 (RRID:CVCL_1923),
respectively]. Cells were authenticated through species-specific cell line authentication performed by IDEXX Bioanalytics using PCR-based testing. These parental cell lines and STK11 KO and KO/KI cells were tested for rotoviral pathogens using the IMPACT II mouse PCR-based pathogen testing from IDEXX Bioanalytics. All cell lines were tested for Mycoplasma infection at AstraZeneca by a PCR-based method using the MycoSEQ Mycoplasma detection kit (catalog no. 4460862: Thermo Fisher Scientific).

CT26 and EMT6 cells were cultured in RPMI complete media consisting of RPMI 1640-containing supplements (Life Technologies; catalog #A1049101) plus 10% v/v heat-inactivated FBS and 1% v/v (1X) penicillin/streptomycin antibiotics (Life Technologies) in a humidified tissue culture incubator at 37°C and 5% CO2.

To measure in vitro cell proliferation, cells were removed from tissue culture plastic using 0.05% trypsin, washed in complete RPMI 1640 media, and counted on a Vi cell counter (Beckman Coulter). Cells were suspended in complete media at 1 × 10^6 viable cells per mL, and 100,000 cells added to 6-well plates in complete media. At the indicated time points, cells were trypsinized and counted on a Vi cell counter to determine the number of cells per well.

**CRISPR-Mediated Gene Editing of STK11 in CT26 and EMT6 Cell Lines**

Details of cell lines and culture conditions are located in the Supplementary Appendix. Mouse syngeneic STK11 CRISPR KO tumor cells (CT26 and EMT6) were developed by using the Alt-R CRISPR/Cas9 system from Integrated DNA Technologies (IDT). The following guide RNAs (gRNA) were used: gRNA#1: AGCTTGCGCGTGCGGCG; gRNA#2: CTTGACCGCCCTGCGGCATA; gRNA#3: ACTCCTGAGACCTTATGCGC; gRNA#4: AGCTTGCGCGTGCGGCG; gRNA#5: CTTGGACCCCTTGCCGATA; gRNA#6: ACTCCTGAGACCTTATGCGC. Briefly, ribonucleotide protein complex (RNP) comprised of CRISPR RNA, gRNA (labeled with ATTO-550 dye), and Cas9 protein were transfected into the respective cell lines using Lipofectamine RNAMax Transfection reagent according to manufacturer’s protocol (Thermo Fisher Scientific). Twenty-four hours posttransfection, ATTO-550+ cells were sorted by FACS using an Aria Cell Sorter (BD Biosciences) into single-cell clones and expanded. Individual clones were screened for STK11 editing efficiency and inhibition of relevant downstream signaling pathways, such as phosphorylation of AMPK, by Western blotting using antibodies from Cell Signaling Technology. Finally, DNA sequencing was carried out to confirm the deletion of STK11 alleles in the respective clones.

Mouse STK11 (mSTK11) gene KO cell lines were developed from STK11 KO clones by stably infecting cells with adenovirus directing mSTK11 out to confirm the deletion of STK11 alleles in the respective clones. mSTK11 transduced cells were enriched by puromycin (10 μg/mL) selection, and mSTK11 protein expression was confirmed by Western blotting. Full methodologic details are in the Supplementary Appendix.

**RNA Extraction, Library Preparation, and Sequencing of Mouse Tumor Models**

RNA extractions were performed using the Qiagen RNeasy Mini Kit, following the manufacturer’s suggested protocol to include the optional on-column DNase digestion treatment. To elucidate the underlying mechanisms that may drive resistance to CPI therapy in STK11mut patients, whole transcriptional gene expression of STK11mut and STK11wt tumors was performed and gene expression compared. The methodology is described in the Supplementary Appendix.

**Mouse Tumor Models and Treatments**

All mice were humanely treated and housed according to Institutional Animal Care and Use Committee approved protocols in the Laboratory Animal Resources facility at AstraZeneca, an Association for Animal Accreditation of Laboratory Animal Care and United States Department of Agriculture-licensed facility. Prior to cell engraftment, CT26 or engineered CT26 cell lines were trypsinized to remove them from the cell culture plastic and washed in complete media to remove excess trypsin. Cells of >95% viability were suspended in sterile PBS and engrafted onto the flanks of BALB/c mice by subcutaneous injection. Tumor dimensions were measured using a caliper at the indicated time points and tumor volumes were calculated using the following formula: V (mm³) = (length (mm) × width (mm) × width (mm))/2. Drug treatments included: anti-mouse PD-L1 mlgG1 D265A clone 80 (anti-mouse PD-L1 lacking effector function, produced by AstraZeneca, 10 mg/kg twice weekly); anti-mouse CTLA4 mlgG1 clone 9D9 (anti-mouse CTLA4 lacking effector function, produced by AstraZeneca, 10 mg/kg twice weekly); anti-mouse OX40 clone OX86 mlgG2a (anti-mouse OX40 mAb with effector function, produced by AstraZeneca, 0.1 mg/kg, one dose at randomization); anti-mouse CD137 mlgG1 (produced by AstraZeneca, 0.1 mg/kg, one dose at randomization); anti-mouse ICOS mlgG2a (anti-mouse ICOS with effector function, produced by AstraZeneca, 0.1 mg/kg, one dose at randomization); anti-mouse IL6 rat IgG1 mAb clone MPS-20F3 (Bio-X-Cell; 1 mg/kg twice weekly); AZD2140 mTORC1/2 inhibitor (AstraZeneca, daily oral gavage, 15 mg/kg, 2 days on 5 days off treatment); AZD5069 CXCR2 inhibitor (AstraZeneca, twice-daily oral gavage, 100 mg/kg); AZD7507 CSF1R inhibitor (AstraZeneca, twice-daily oral gavage, 50 mg/kg); anti-mouse STAT3 ASO (Ionis Pharmaceuticals, Inc. and AstraZeneca, once-daily subcutaneous injection, 50 mg/kg, 5 days on 2 days off treatment). Mice were monitored for body weight changes and signs of drug- or tumor-induced morbidity. Animals were sacrificed at a humane endpoint once tumor measurements exceeded 2,000 mm³ or body weight decreased by >20% posttreatment.

For immune cell depletion, mice received 8 mg/kg of anti-mouse CD8 clone 53–67, 8 mg/kg anti-mouse CD4 clone GK1.5, or 12.5 mg/kg anti-mouse NK clone NK1.1 on day 1, 4, 8, 11, 15 and 18 after tumor implantation for NK depletion and days 2, 6, 10, 14, and 18 for CD4+ or CD8+ cell depletion.

**Flow Cytometry of Mouse Tumor Models**

Immunophenotyping by flow cytometry was conducted on single-cell suspensions of mouse tumor, spleen, and inguinal TDLNs. The methodology is described in the Supplementary Appendix.

**IHC and Immunofluorescence Assay on Mouse Tumor Models**

Mouse model tumor tissue samples were collected for IHC or IF staining for CD4, CD8, CD45, CD163, FOXP3, and pSTAT3. Details of the methodology are described in the Supplementary Appendix.

**scRNA-seq and Data Analysis on Mouse Tumor Models**

scRNA-seq of CD45+ immune cells isolated from tumors and RNA-seq analysis of the CD45+ tumor/nonimmune stromal cells from those same tumors was conducted. Full details of the protocol are in the Supplementary Appendix.

**MDSC Cell Suppression Assay**

An MDSC-T-cell coculture assay was performed to determine the effects of STAT3 ASO and other treatments on MDSC-suppressive function. In this assay, Gr1+ myeloid cells (MDSCs) were isolated from tumors and co-cultured at various MDSC:E:T cell ratios with carboxyfluorescein succinimidyl ester (CFSE)-labeled resting T cells from non–tumor-bearing mice, activated using anti-CD3 and anti-CD28 mAbs, to stimulate T-cell proliferation (measured by CFSE dilution). Full details of the protocol used are in the Supplementary Appendix.

**Statistical Analyses**

Statistical significance of differences in pharmacodynamic changes observed in the CT26 STK11 KO preclinical model was calculated by...
one-way ANOVA followed by Dunnet correction for multiple comparisons using GraphPad Prism version 8.0.0 for Windows (GraphPad Software; RRID:SCR_002798).

Kaplan–Meier (KM) survival analysis in GraphPad Prism was used to analyze the animal survival data. Log rank (Mantel-Cox) test was applied to compare survival among different treatment groups. For the survival analysis of STAT3 ASO and CPI combination treatment in STK11 KO CT26, a total of 8 comparisons were conducted. P-values were not adjusted for multiple comparisons.

Concordance analyses between STK11 mutational status and response was performed with Fisher exact test. Differential gene expression analysis was done using R package limma (RRID:SCR_010943). KM survival analysis was done using a Cox proportional-hazards model in the R survival package (RRID:SCR_021137).

Exploratory analyses of TMB, PD-L1, and other protein biomarkers according to STK11 mutational status were conducted by Wilkinson rank-sum test.

Authors’ Disclosures

N. Pore reports other support from AstraZeneca outside the submitted work. S. Wu is an employee of AstraZeneca and owns AstraZeneca stock. N. Standifer reports other support from AstraZeneca during the conduct of the study; other support from AstraZeneca outside the submitted work. M. de los Reyes reports other support from AstraZeneca outside the submitted work. Y. Shrestha is an employee of AstraZeneca. R. Halpin is an employee of AstraZeneca. R. Rothstein is an employee of AstraZeneca and currently owns stock in the company. P. Martin reports other support from AstraZeneca (Medimmune) during the conduct of the study; other support from AstraZeneca (Medimmune) outside the submitted work. M. L. Ascierto reports other support from AstraZeneca outside the submitted work. I. Bisha reports personal fees from AstraZeneca outside the submitted work. T. A. Proia reports other support from AstraZeneca outside the submitted work. J. Jure-Kunkel reports other support from AstraZeneca outside the submitted work. A. Gupta reports other support from AstraZeneca outside the submitted work; in addition, A. Gupta has a patent for PD-1 in cancer issued. S. E. Abdullah reports other support from AstraZeneca outside the conduct of the study; other support from Immunocore Ltd. outside the submitted work. R. Raja reports being employed by and owning stocks of AstraZeneca. M. M. Frigault reports other support from AstraZeneca outside the submitted work. J. Barrett reports other support from AstraZeneca outside the submitted work. J. Meekin is an employee of AstraZeneca in Gaithersburg, MD. I. Bisha reports personal fees from AstraZeneca outside the submitted work. T. A. Proia reports other support from AstraZeneca outside the submitted work. R. J. Miragaya reports to be a shareholder of AstraZeneca. R. Herbst reports other support from AstraZeneca outside the submitted work. A. Gupta reports other support from AstraZeneca outside the submitted work; in addition, A. Gupta has a patent for PD-1 in cancer issued. S. E. Abdullah reports other support from AstraZeneca outside the conduct of the study; other support from Immunocore Ltd. outside the submitted work. R. Raja reports being employed by and owning stocks of AstraZeneca. M. M. Frigault reports other support from AstraZeneca outside the submitted work. J. Barrett reports other support from AstraZeneca outside the submitted work. M. L. Ascierto reports other support from AstraZeneca during the conduct of the study. M. D. Oberst reports other support from AstraZeneca outside the submitted work and owns AstraZeneca stock.

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Resistance to Durvalumab and Durvalumab plus Tremelimumab Is Associated with Functional STK11 Mutations in Patients with Non–Small Cell Lung Cancer and Is Reversed by STAT3 Knockdown

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