Resistance to durvalumab and durvalumab plus tremelimumab is associated with functional STK11 mutations in non-small-cell lung cancer patients 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 enrolled in three Phase 1/2 trials. *STK11* mutations were associated with resistance to the anti-PD-L1 antibody durvalumab (alone/with the anti-CTLA-4 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*mut NSCLC are less likely than *STK11*wt patients to respond to anti-PD-L1 ± anti-CTLA-4 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 *STK11*mut NSCLC.
INTRODUCTION

Monoclonal antibodies blocking the programmed cell death-1/programmed cell death ligand-1 (PD-1/PD-L1) pathway have become the standard of care for numerous oncology indications including non-small-cell lung cancer (NSCLC) (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 5–30% 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 impact the expression of genes affecting immune recognition (including the DNA sensor Stimulator of Interferon Genes [STING]) (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).

The current study explored the association between STK11 mutations in nonsquamous NSCLC and immunotherapy response in 3 independent trials of the anti-PD-L1 monoclonal antibody (mAb) durvalumab as monotherapy (Phase 1/2 Study 1108 [NCT01693562] and Phase 2 ATLANTIC [NCT02087423]), or in combination with the anti-cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) mAb tremelimumab (Phase 1b Study 006 [NCT02000947]) (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 (CPIs).
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 (Figure 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 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, STK11wt 32/164 (19.5%) vs STK11mut 1/17 (5.9%), $p = 0.166$; and Study 006, STK11wt 19/95 (20%) vs STK11mut 1/26 (3.8%), $p = 0.049$; Figures 1B and 1C). 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% CI, 1.64–4.89) (Figure 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 to those with STK11wt tumors (15.4 months), HR = 2.39 (95% CI, 1.34–4.25) (Figure 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 co-mutations vs single STK11mut or KRASmut NSCLC tumors (Supplementary Figure S1A and B). 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 3 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 STK11 (Supplementary Figure 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 STK11mut and STK11wt tumors in Study 006 (Supplementary Figure S3A). However, STK11mut tumors had relatively lower median PD-L1 expression in Study 1108 and Study 006 (Supplementary Figure S3B), consistent with previous results showing similar TMB but lower PD-L1 in STK11mut NSCLC (2). Furthermore, in Study 006, clinical activity with durvalumab plus tremelimumab occurring in patients with STK11mut vs STK11wt 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 STK11 was observed in tumor biopsies from patients with STK11 mutations in Study 1108 and Study 006 (Supplementary Figure S3C).

**Increased intratumoral and peripheral expression of cytokines associated with neutrophilic and Th17 contexture detected in patients with STK11 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 STK11mut vs STK11wt tumors (p ≤ 0.05; fold change ≥ 1.5). To further explore biologic associations with STK11 mutations, a functional annotation analysis was performed with the Ingenuity Pathways Analysis (IPA) on genes upregulated (n = 153) in STK11mut tumors. Genes upregulated in STK11mut 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) crosstalk between innate and adaptive immunity (Figure 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; Figure 2B). Additionally, protein levels of IL8 (CXCL8) and IL6 were significantly elevated in the serum of Study 1108 patients with STK11mut tumors (Figure 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-CTLA-4 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 immunohistochemical (IHC) assessment of primary tumors from Study 1108 and Study 006 (Supplementary Figure S3D).

**Whole blood immunophenotyping of patients with STK11mut vs STK11wt tumors**

To characterize the peripheral immune contexture of patients harboring STK11mut vs STK11wt 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 STK11wt and STK11mut patients including NK cells and CD4+ effector memory, CD4+ HLA-DR+, CD8+ effector memory and CD8+ HLA-DR+ T cells (Figure 3B-D). Baseline and post-treatment quantities of immune cells were observed to be statistically decreased in STK11mut tumors compared to STK11wt tumors (p < 0.05 by Wilcoxon rank-sum test).

**Overcoming CPI resistance in preclinical mouse models of STK11 mutation**

To understand the underlying mechanisms of CPI resistance mediated by inactivating STK11 mutation or loss, mouse syngeneic tumor models were generated with STK11 gene deletion. The KRASmut CT26 colorectal cancer cell line was subjected to CRISPR-mediated STK11 gene editing, and single-cell clones with loss of the STK11 gene and protein expression were selected (Supplementary Figure S4A). In each clone examined, loss of the STK11 gene resulted in a decrease in the ratio of phosphorylated AMPK to total AMPK protein levels, indicative of loss of STK11 kinase activity and phosphorylation of the downstream substrate AMPK. Ablation of STK11 did not substantially change the growth rate of CT26 cells in cell culture but did increase the in vivo tumor growth rate (Supplementary Figure S4B and S4C). The change in growth in vivo but not in vitro is consistent with decreased immune control of tumors in an immunocompetent host.
Increases in the percentage of neutrophil-like granulocytic cells in STK11 knockout (KO) tumors were seen by scRNA seq (Supplementary Figure S5A-F; Supplementary Table S4) and significant increases in intratumoral Ly6G+ granulocytes were observed by flow cytometry (Supplementary Figure S5G). Single cell RNA sequencing (scRNAseq) analysis revealed no change in the percentage of a combined monocyte/macrophage/dendritic cell (DC) cluster between STK11wt and STK11 KO clones; however, the proportion of mature M2-like macrophage clusters increased (Supplementary Figures S5E-F and S5H-L; Supplementary Table S5) and IHC staining showed a corresponding association with increased CD163+ tumor-associated macrophage (TAM) density; Supplementary Figure S5M). Examination of RNA expression in CD45 negative (CD45$^{\text{neg}}$) 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 Figure S5N), congruous with results observed in NSCLC patients. 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 STK11 loss-of-function and the silencing of STING (encoded by TMEM173) expression in tumor cells and reduced double-stranded DNA sensing and immune recognition (12, 13), we examined TMEM173 expression but did not find an association between STK11 loss and decreased TMEM173 in this model (Supplementary Figure S5N). However, consistent with the increase in potentially immunosuppressive cytokines, STK11 loss significantly decreased the intratumoral density of CD8+ T cells as determined by IHC (Supplementary Figure S6A-B) and resulted in a T-cell phenotype reflecting a reduced activation status as defined by scRNAseq (Supplementary Figure S6C-G; Supplementary Table S6). STK11 loss only moderately decreased the mean density of CD4+ conventional T cells and CD4+FoxP3+ regulatory T cells (Tregs) which did not reach statistical significance (Supplementary Figure S6H-I). In addition to reduced activated CD8+ T cell infiltration, lower levels of PD-L1 mRNA were observed in the CD45$^{\text{neg}}$ tumor cell and non-immune stromal fraction (Supplementary Figure S6J), which may reflect reduced levels of interferon gamma (IFNγ) 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 tumors, tumor sections were analyzed by IHC for phosphorylated STAT3 (pSTAT3). Results showed that CD45\textsuperscript{neg} tumor/non-immune stromal cells as well as CD45+ immune cells in STK11 KO clone 26C16 tumors had greater numbers of pSTAT3+ cells than either CT26 WT or CT26 26C16 STK11 KO tumors re-engineered to express STK11 (26C16 STK11 knock-in [KI]) (Supplementary Figure S7A). Higher numbers of CD163+ myeloid cells were also observed in STK11 KO tumors, as were CD163+ cells positive for phosphorylation of STAT3 (Supplementary Figure S7A).

To determine if 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 to STK11wt tumors (Supplementary Figure S7B). In further analyses using the TCGA database, we observed that STAT3 RNA expression was significantly higher in STK11mut vs STK11wt NSCLC samples (Supplementary Figure 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 labelling for pSTAT3 on human primary NSCLC patient-derived xenograft (PDX) tumors implanted in NSG mice. Human tumor cells were distinguished from murine immune and non-immune 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 vs STK11wt tumors (Supplementary Figure S7C-D), suggesting high tonic stimulation of STAT3 signaling in STK11mut tumors. Based on 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 3 clinical trials; these data are not discussed in the present 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-CTLA-4 therapy, whereas STK11wt CT26 tumors were sensitive (Figure 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) receptors OX40 or CD137 or of the inducible T cell co-stimulator (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 Figure S8A-B), 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 wild-type STK11 protein into STK11 KO clone 26C16 (26C16 STK11 KI) increased AMPK phosphorylation and restored sensitivity to CPIs (Supplementary Figure S4A; Figure 4E-G).

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 Lys6G+ granulocyte fraction compared to EMT6 WT tumors (Supplementary Figure S9A-B). 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 Figure S9C-E; 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-CTLA-4 therapy) in either CT26 WT or the STK11 KO clone 26C16. Surprisingly, this combination
did not show additive tumor growth control compared to AZD2014 monotherapy or anti-PD-L1 plus anti-CTLA-4 drug controls in the STK11 KO clone 26C16 but did show additive antitumor activity in mice engrafted with CT26 WT tumors (Supplementary Figure S10A-B).

Granulocytic myeloid-derived suppressor cells (gMDSCs) 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 therapeutics 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-CTLA-4 therapy (Supplementary Figure S10C-D). Likewise, the CXCR2 small molecule inhibitor AZD5069 (25) did not result in additional growth control when combined with anti-PD-L1 plus anti-CTLA-4 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 Figure S10E-H). An anti-IL6 mAb that neutralizes binding to the IL6 receptor (IL6R) showed moderate additive activity in combination with anti-PD-L1 plus anti-CTLA-4 therapy (Supplementary Figure S11A). Although responding mice showed growth inhibition compared to other treatment groups, no complete responses were observed, and the depth and duration of responses were limited. These results were similar in magnitude to the additive benefit observed in CT26 WT tumor-bearing mice (Supplementary Figure S11B).

In addition to CXCR2 and IL6R, receptors such as G-CSFR, IL10R, and vascular endothelial growth factor (VEGFR2) on immune cells utilize STAT3 signaling to mediate potentially immunosuppressive signaling. Therefore, multiple suppressive cell types and signaling 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 the current work, 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 (Figure 5A). Anti-PD-L1 or anti-PD-L1 plus anti-CTLA-4 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-CTLA-4. 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-CTLA-4 therapy, with many deep and durable responses that resulted in significantly prolonged survival in the combination groups (Figure 5B-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-CTLA-4. 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-CTLA-4 triple combination relative to the anti-PD-L1 + anti-CTLA-4 dual immunotherapy (Supplementary Figure S9F-G). This was true both in wild-type EMT6 cells and EMT6 cells harboring SKT11 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 (Figure 5A). The percentages of CD45+ immune cells and live Ly6G+ granulocytic cells within the tumor remained statistically indistinguishable in STAT3 ASO treatment vs control groups (Supplementary Figure S12A-B). 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 co-stimulatory potential of antigen-presenting cells (APCs) was altered by STAT3 ASO and combination therapy. For example, although the percentage of CD24+ CD64\textsuperscript{neg} major histocompatibility complex class II (MHCII)+ CD103+ conventional dendritic cells (cDC1) among the tumor immune cell population did not change significantly (Supplementary Figure 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-CTLA-4 showing the strongest effect (Figure 6A). Likewise, cell surface CD86 staining intensity (mean fluorescence intensity [MFI]) on the CD103+ cDC1 subset was highest in the triple combination group (Supplementary Figure S12D).

Within the tumor draining lymph nodes (TDLNs) of treated mice, increases in CD86 positivity were also observed in migratory CD24+ CD11b\textsuperscript{neg} CD103+ DCs (Supplementary Figure S12E-G). In this anatomical 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-CTLA-4, with the STAT3 ASO plus anti-PD-L1 plus anti-CTLA-4 triple combination group showing the highest levels. This suggests that CTLA-4 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 co-stimulation by activated DCs.

Similar to DCs, intratumoral CD24\textsuperscript{neg} CD64+ MHCII+ myeloid APCs showed no change as a percentage of CD45+ immune cells among treatment groups (Supplementary Figure 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 (Figure 6B, Supplementary Figure S12I). Examination of MHCII+ myeloid cells (CD11b+ F4/80+) that were CD206+ or CD163+ revealed a trend towards increased percentages among treatment groups (Supplementary Figure S12J-K). However, among M2-like MHCII\textsuperscript{neg} myeloid cells, statistically significant decreases in CD206+ and CD163+ cell percentages compared with
control were observed in groups treated with STAT3 ASO (Figure 6C-D), consistent with STAT3 knockdown reprogramming the balance of myeloid cells from a suppressive M2-like state to one less immunosuppressive (27).

An MDSC:T cell co-culture 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 SKT11 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 Figure 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 to mice administered isotype control antibodies or left untreated; however, the addition of an anti-CTLA-4 agent abrogated this effect (Supplementary Figure 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-CTLA-4 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 (Figure 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-CTLA-4 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-CTLA-4 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 the additive effects of anti-PD-L1 plus anti-CTLA-4 fully depended on these cells (Supplementary Figure S14A-C). In contrast, CD4+ cell depletion improved efficacy in each group that showed benefit in the absence of depletion.

**DISCUSSION**

Emerging data suggest poor outcomes with anti-PD-(L)1 agents in patients with STK11mut 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-CTLA-4 combination treatment in 3 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 CTLA-4 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 datasets 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. Additionally, 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 previously described (28, 29), heterozygosity is sufficient to demonstrate loss of function.

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 twofold 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 vs STK11wt tumors. Additionally, 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 contexture (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 NSCLC patients 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 non-immune 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 danvatirsen (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-CTLA-4 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 CSFR1, 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 neg 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 co-stimulatory potential of both CD103+ MHCII+ cDC1s and CD64+ MHCII+ 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 which 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. Since 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 MHCII. 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 impacts 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 the current 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 if 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 \textit{STK11} mutation. Likewise, genetically engineered mouse models of lung cancer with \textit{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 Cas9/CRISPR-mediated removal of \textit{STK11}. Removal of the gene mimics the lack of \textit{STK11} functional activity found in patients with predicted loss-of-function \textit{STK11} mutations (the criteria used to define \textit{STK11} mutant status in the current study). \textit{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 \textit{STK11}mut NSCLC. These findings suggest that such models are relevant to test hypotheses related to alterations commonly found in the microenvironment of human tumors harboring \textit{STK11} loss. The reversal of CPI resistance when combined with co-blockade of STAT3 signaling in the tumor microenvironment in these models supports the idea that this may be a combination option worth considering for NSCLC patients harboring \textit{STK11} mutations.

\textbf{METHODS}

\textbf{Study populations}

Patients with evaluable \textit{STK11} genomic status were selected from the full analysis sets of the 3 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 \textit{STK11} tumor mutational status (\textit{STK11}mut vs \textit{STK11}wt) and treatment response was measured as objective response rate (ORR) by Response Evaluation Criteria in Solid Tumors (RECIST) v1.1 and OS. \textit{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
The experimental approach to identifying patients with *STK11* mutations is shown in Figure 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* co-mutation 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 [TILs]/mm$^2$ of tumor using automated image analysis) was also assessed (42).

**mRNA sequencing**

RNAseq 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-sequencing 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 deletion (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-cdx). Full details of the protocol used are provided in the Supplementary Appendix.

**Flow cytometry-based immunophenotyping of whole blood from patients with STK11mut vs STK11wt tumors**

Flow cytometry-based immunophenotyping assays were used to quantify lymphocyte population absolute counts (cells/mm$^3$) 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 based on elevated surface expression levels of CD38 or HLA-DR compared to samples stained with isotype control antibodies. Memory T cell subsets were defined based on differential expression of CD197 and CD45RO, where naïve T cells were CD197$^{\text{neg}}$ and CD45RO$^{\text{neg}}$, central memory T cells were CD197$^{+}$ CD45RO$^{+}$ and effector memory T cells were CD197$^{\text{neg}}$ 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 bioanalytically 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 American Type Culture Collection (Manassas, VA; catalog #s CRL-2638 [RRID:CVCL_7256] and CRL2755 [RRID:CVCL_1923], respectively). Cells were authenticated through species-specific cell line authentication performed by IDEXX Bioanalytics (Westbrook, ME) using PCR-based testing. These parental cell lines and STK11 KO and KO/KI cells were tested for rodent viral 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 #4460623; ThermoFisher Scientific).
CT26 and EMT6 cells were cultured in RPMI complete media consisting of RPMI-1640 containing supplements (Life Technologies, Carlsbad, CA; catalog #A1049101) plus 10% v/v heat inactivated fetal bovine serum (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, Indianapolis, IN). Cells were suspended in complete media at 1x10⁶ 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, Coralville, IA). The following guide RNAs (gRNAs) were used: gRNA#1: AGCTTGGCGCGTTTGCGGCG; gRNA#2: CTTGACCGCCCTGCGGCATA; gRNA#3: ACTCCGAGACCTTATGCCGC. 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® RNAiMax Transfection reagent according to manufacturer’s protocol (Thermo Fisher Scientific, Waltham, MA). Twenty-four hours post-transfection, ATTO-550+ cells were sorted by fluorescence-activated cell sorting using an Aria Cell Sorter (BD Biosciences, Franklin Lakes, NJ) 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 (Danvers, MA). Finally, DNA sequencing was carried out to confirm the deletion of STK11 alleles in the respective clones.

Mouse STK11 (mSTK11) gene KI cell lines were developed from STK11 KO clones by stably infecting cells with adenovirus directing the expression of the mSTK11 gene. Briefly, CT26 or EMT6 STK11 KO clones were infected with adenovirus particles carrying the mSTK11 gene.
Transduced cells were enriched by puromycin (10 µg/mL) selection, and STK11 protein expression was confirmed by western blotting. Full methodological 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 phosphate-buffered saline (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 (mm$^3$) = (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 CTLA-4 mlgG1 clone 9D9 (anti-mouse CTLA-4 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, 0.1mg/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 MP5-20F3 (Bio-X-Cell, Lebanon, NH; 1 mg/kg twice weekly); AZD2014 mTORC1/2 inhibitor (AstraZeneca, daily oral gavage, 15mg/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 antisense oligonucleotide (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 2000 mm$^3$ or body weight decreased by > 20% post-treatment.

For immune cell depletion, mice received 8 mg/kg of anti-mouse CD8 clone 53-6.7, 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 (IF) 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.

**Single cell sequencing and data analysis on mouse tumor models**

scRNAseq of CD45+ immune cells isolated from tumors and RNAseq analysis of the CD45$^{\text{neg}}$, tumor/non-immune stromal cells from those same tumors was conducted. Full details of the protocol are in the Supplementary Appendix.
Myeloid-derived suppressor cell (MDSC) cell suppression assay

An MDSC:T cell co-culture 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 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 analysis of variance (ANOVA) followed by Dunnett’s correction for multiple comparisons using GraphPad Prism version 8.0.0 for Windows (GraphPad Software, San Diego, California USA; 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’s 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 Wilcoxon rank-sum test.
References


### Table 1

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Table Legends

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

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 in 3 independent studies evaluating (B) durvalumab or (C) durvalumab plus tremelimumab in nonsquamous NSCLC. Reduced overall survival in STK11mut patients treated with (D) durvalumab or (E) durvalumab plus tremelimumab.

Figure 2. Whole transcriptome analysis conducted in patients with STK11mut vs 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) Ingenuity Pathway Analysis (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 vs STK11wt tumors. (A) Increased baseline serum levels of IL6 and IL8 in patients with STK11mut vs STK11wt tumors. (B) In whole blood, at baseline, reduced circulating NK cells, (C) CD4+ effector memory and CD4+ HLA-DR+ cells and (D) CD8+ effector memory and CD8+ HLA-DR+ cells were observed in patients with STK11mut vs STK11wt tumors. Similar results were also observed following durvalumab treatment.

Figure 4. Effects of STK11 deficiency on CPI efficacy in the CT26 model. Survival of mice engrafted with (A) CT26 WT or the CT26 STK11 KO clones (B) 26C16 (C) 26C10 or (D) 26C4 and left untreated or treated with isotype control antibodies, anti-PD-L1, anti-CTLA-4, or dual checkpoint inhibitors as indicated. CT26 WT: *Isotype control vs anti-PD-L1, p = 0.0075; †Isotype control vs anti-PD-L1 + anti-CTLA-4, p = 0.0003; CT26 STK11 KO Clone 26C4: ‡Isotype control vs anti-PD-L1 + anti-CTLA-4, p = 0.026 using log rank (Mantel-Cox) survival analysis. (E-G) Independent experiment showing the survival of mice engrafted with (E) CT26 WT (F) STK11 KO clone 26C16 or (G) STK11 KO clone 26C16 re-expressing STK11 (26C16 STK11 KO/KI) and treated with isotype control or dual anti-PD-L1 plus anti-CTLA-4 mAbs as indicated. CT26 WT: §Isotype vs anti-PD-L1 + anti-CTLA-4, p = 0.0009; ‰CT26 STK11 KO clone 26C16: Isotype vs anti-PD-L1 + anti-CTLA-4, p > 0.99, non-significant; #26C16 STK11 KO/KI: Isotype vs anti-PD-L1 + anti-CTLA-4, p = 0.001 using log rank (Mantel-Cox) survival analysis.

Figure 5. STAT3 ASO and CPI combination efficacy and pharmacodynamic effects in STK11-deficient CT26. (A) Study schematic for STAT3 ASO studies including pharmacodynamic endpoints assessed independently from tumor growth inhibition. (B) Tumor growth curves for mice engrafted with STK11 KO clone 26C16 and treated with STAT3 ASO, anti-PD-L1, anti-PD-L1 plus anti-CTLA-4, or STAT3 ASO combinations as indicated. Graphing ends when mice are first removed from study groups. Experiment was conducted twice with similar results. (C) Survival curves for mice. *p < 0.0001 for all comparisons; ′p < 0.0001 for all comparisons except p = 0.047 vs STAT3 ASO; ″p < 0.0001 for all comparisons; ‡p < 0.0001 for all comparisons using log rank (Mantel-Cox) survival analyses, see also Supplementary Table S8.
Figure 6. Pharmacodynamic effects of STAT3 ASO and combinations in STK11-deficient CT26. (A) Percentage of intratumoral CD86-expressing CD103+ cDC1s. (B) Percentage of CD86-expressing intratumoral CD11b+ MHCII+ CD64+ myeloid cells. (C) Percentage of MHCII$^\text{neg}$ (M2-like) TAMs expressing CD206 or (D) CD163 in the TME as determined by flow cytometry. *p < 0.02; **p < 0.03, one-way ANOVA. (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 co-cultured with CD3/CD28-stimulated naive T cells from non-tumor bearing mice to induce T-cell proliferation ex vivo. Ratios indicate the MDSC:T cell ratio in co-culture 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.
Figure 1

A

- Study 1108/NCT01693562
  Non-randomized Phase 1/2; 1L+
  Durvalumab 10 mg/kg q2w (N = 118)

- ATLANTIC/NCT02087423
  Non-randomized Phase 2; 3L+
  Durvalumab 10 mg/kg q2w (N = 63)

- Study 006/NCT02000947
  Non-randomized Phase 1b; 2L+
  Durvalumab 20 mg/kg plus tremelimumab 1 mg/kg q4w (N = 121)

Subheading:
Circulating tumor DNA analysis (Guardant360, 70 gene cancer panel)

B

**Study 1108 + ATLANTIC**

- ORR (%)
  - STK11mut
  - STK11wt

p = 0.166

C

**Study 006**

- ORR (%)
  - STK11mut
  - STK11wt

p = 0.049

D

**Study 1108 + ATLANTIC**

- OS (%)
  - STK11mut (n = 17)
  - STK11wt (n = 164)

STK11mut/STK11wt
HR = 2.83 (95% CI, 1.64–4.89)

E

**Study 006**

- OS (%)
  - STK11mut (n = 26)
  - STK11wt (n = 95)

STK11mut/STK11wt
HR = 2.39 (95% CI, 1.34–4.25)
Figure 2

A

-Log(p-value)

<table>
<thead>
<tr>
<th>Pathway</th>
<th>-Log(p-value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Granulocyte Adhesion</td>
<td>3.5</td>
</tr>
<tr>
<td>Agranulocyte Adhesion</td>
<td>3.0</td>
</tr>
<tr>
<td>ERK/MAPK Signaling</td>
<td>2.5</td>
</tr>
<tr>
<td>Leukocyte Extravasation</td>
<td>2.0</td>
</tr>
<tr>
<td>Protein Kinase A Signaling</td>
<td>1.5</td>
</tr>
<tr>
<td>Cytokine production by M1 and T helper cells by IL17</td>
<td>1.0</td>
</tr>
<tr>
<td>Crosstalk between DC and NK</td>
<td>0.5</td>
</tr>
<tr>
<td>RhoDG1 Signaling</td>
<td>0.0</td>
</tr>
<tr>
<td>Neuroinflammation Signaling</td>
<td>0.0</td>
</tr>
</tbody>
</table>

B

**IL6**

Normalized expression (TMP)  

- **p = 0.009**

**KIR2DL4**

Normalized expression (TMP)  

- **p = 0.019**

**CSF3**

Normalized expression (TMP)  

- **p = 0.017**

**IL17REL**

Normalized expression (TMP)  

- **p = 0.012**

*STK11mut* | *STK11wt*
Figure 4

(A) CT26 WT

(B) CT26 STK11 KO Clone 26C16

(C) CT26 STK11 KO Clone 26C10

(D) CT26 STK11 KO Clone 26C4

(E) CT26 WT

(F) STK11 KO clone 26C16

(G) 26C16 STK11 KO/KI

Legend:
- Untreated
- Isotype Control
- Anti-PD-L1
- Anti-CTLA-4
- Anti-PD-L1 + Anti-CTLA-4

Survival curves show percent survival over time (days) for different treatments.
Figure 5

A

Pharmacodynamic endpoints

-STAT3 ASO
-STAT3 ASO
-STAT3 ASO

△ Anti-PD-L1 + Anti-CTLA-4 or Anti-PD-L1

B

Tumor Volume

- Untreated
- Isotype Control
- Anti-PD-L1
- Anti-PD-L1 + Anti-CTLA-4
- STAT3 ASO
- STAT3 ASO + Anti-PD-L1
- STAT3 ASO + Anti-PD-L1 + Anti-CTLA-4

Days after Cell Implantation

C

Survival

- Untreated
- Isotype Control
- Anti-PD-L1
- Anti-PD-L1 + Anti-CTLA-4
- STAT3 ASO
- STAT3 ASO + Anti-PD-L1
- STAT3 ASO + Anti-PD-L1 + Anti-CTLA-4

Percent survival

Time (days)
Figure 6
Resistance to durvalumab and durvalumab plus tremelimumab is associated with functional STK11 mutations in non-small-cell lung cancer patients and is reversed by STAT3 knockdown

Nabendu Pore, Song Wu, Nathan Standifer, et al.

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