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Dynamic single-cell RNA sequencing identifies immunotherapy persister cells following PD-1 blockade

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CPP and DAB are inventors on a pending patent related on manipulating, culturing, and evaluating tumor spheroids. DAB is a consultant for N of One and Tango Therapeutics, has received honoraria from Loxo Oncology, Merck, Esai/H3 Biomedicine, and Madalon Consulting, research grants from BMS, Novartis, Lilly, and Gilead Sciences, and is co-founder and on scientific advisory board of Xsphera Biosciences Inc. CPP has received honoraria from Bio-Rad and AstraZeneca, is on scientific advisory board of DropWorks, and is co-founder and on scientific advisory board of Xsphera Biosciences. RWJ has financial interest in XSphere Biosciences. MG receives research funding from Bristol-Myers Squibb and Merck. MP-O, WDH, SG and PSH are employees and shareholders of Novartis.
Abstract

Resistance to oncogene-targeted therapies involves discrete drug-tolerant persister cells, originally discovered through in vitro assays. Whether a similar phenomenon limits efficacy of programmed death (PD)-1 blockade is poorly understood. Here, we performed dynamic single-cell RNA sequencing of murine organotypic tumor spheroids undergoing PD-1 blockade, identifying a discrete sub-population of immunotherapy persister cells (IPCs) that resisted CD8 T-cell mediated killing. These cells expressed Snail and stem cell antigen-1 (Sca-1), and exhibited hybrid epithelial-mesenchymal features characteristic of a stem cell-like state. IPCs were expanded by interleukin-6 (IL-6) but were vulnerable to tumor necrosis factor-alpha (TNF-α)-induced cytotoxicity, relying on Birc2 and Birc3 as survival factors. Combining PD-1 blockade with Birc2/3 antagonism in mice reduced IPCs and enhanced tumor cell killing in vivo, resulting in durable responsiveness that matched TNF cytotoxicity thresholds in vitro. Together, these data demonstrate the power of high-resolution functional ex vivo profiling to uncover fundamental mechanisms of immune escape from durable anti-PD-1 responses, while identifying IPCs as a cancer cell subpopulation targetable by specific therapeutic combinations.
Introduction

Drug-tolerant persister cells (DTPCs) mediate resistance to oncogene-targeted cancer therapies by adopting a quiescent and apoptosis-resistant state (1-3). Analogous to the concept of “antibiotic-tolerant” bacterial persister cells, these pre-existing sub-populations of cancer cells evade drug killing by slowing cell growth and then recovering upon drug withdrawal (4). Furthermore, DTPCs commonly avoid drug induced cell death by adopting features of the epithelial-mesenchymal transition (EMT) (5) and activating pro-survival cytokine signaling pathways such as interleukin-6 (IL-6) (6, 7).

Discovery of similar subpopulations that evade immune checkpoint blockade (ICB) has been hampered by a lack of functional models that recapitulate the tumor immune microenvironment (TIME), since characterization of persister cells in vivo is challenging and requires accurate repeat biopsies (8, 9). However, emerging evidence suggests that EMT may be linked to an immunosuppressive microenvironment that confers resistance to ICB. For example, breast carcinoma models with high expression of the transcription factor Snail and activation of a mesenchymal transcriptional program promote an immune suppressive microenvironment and resistance to CTLA4 blockade (10). Similarly, activation of Snail in murine KRAS-driven lung cancer cells fosters infiltration of neutrophils, which can promote resistance to PD-1 blockade (11, 12). Yet, it remains unknown whether pre-existing cancer cell populations avoid cancer immunotherapy response by adopting this mesenchymal cell state.

Features of EMT have also been linked with a tumor-initiating, stem cell-like state in cancer (13, 14). Increasing evidence suggests that activation of EMT promoting transcription factors can maintain cells in a hybrid state that is intermediate between a full epithelial or mesenchymal program, promoting cancer stem cell-like behavior (15), which could also be
involved in immune evasion. Indeed, tissue stem cells from multiple origins can adopt a quiescent cell state that avoids immune recognition and is likely an evolutionary adaptation to protect these critical cells (16).

Emerging technologies such as single-cell RNA sequencing and enhanced primary tumor culture methods provide a new opportunity to probe cancer biology in a high resolution and dynamic manner (17, 18). A major challenge for technologies such as organoid culture has been to maintain original features of the tumor immune microenvironment during the time needed to expand cancer cells. Recently, we demonstrated the feasibility of assessing ex vivo response to ICB using short-term microfluidic culture of patient- or murine-derived organotypic tumor spheroids (PDOTS/MDOTS) embedded in a three-dimensional collagen matrix (19). Detailed characterization of these spheroids, from both patients with multiple different tumor types and mice with a variety of syngeneic tumor backgrounds, reveals that they retain many of the key components of the TIME, including PD-1 expressing T cells, multiple myeloid cell populations and dendritic cells, as well as fibroblasts and endothelial cells. While we observed specific CD8 T cell-dependent killing of MDOTS derived from well-established syngeneic tumor models such as MC38, phenotypic analyses were limited to live/dead cell staining and cytokine profiling of conditioned media. Here, we explored the potential of bulk and single cell RNA-sequencing (RNA-seq) of the dynamic response to PD-1 blockade in this system to uncover potentially more fundamental biology of immune escape following CD8+ T cell activation.
Results

**Bulk RNA-sequencing analysis of MDOTS following PD-1 blockade uncovers a distinct transcriptional state**

To examine dynamic cellular transcriptomic changes following ex vivo culture and immune stimulation of well-defined MC38 MDOTS, we isolated bulk RNA from collagen embedded spheroids after 6 days (Figure 1A). MC38 MDOTS were treated during this time period either with isotype IgG control, interferon-gamma (IFN-γ), anti-PD-1 (αPD-1), or αPD-1 + CD8 neutralizing antibody (αCD8). As expected, this resulted in specific αPD-1 induced cell death that was CD8+ T cell dependent (Figure 1, B and C). Bulk RNA-seq analysis of IFN-γ treated MDOTS revealed highly significant upregulation of genes associated with interferon response, such as Gbp2, Gpb3, Irf1 and Cxcl10, compared to IgG (Figure 1D). Indeed, gene set enrichment analysis (GSEA) identified both IFN-γ and IFN-α signatures as the top two Hallmark pathways induced by IFN-γ treatment (Figure 1D and Supplemental Figure 1A), capturing the expected transcriptomic response and confirming the utility of this approach.

We next analyzed bulk RNA-seq data generated following αPD-1 treatment. Unsupervised gene-expression clustering revealed that the bulk transcriptome of cells that survived ex vivo PD-1 blockade was highly distinct compared with IFN-γ treated cells (Supplemental Figure 1B). Furthermore, these transcriptomic changes were completely abolished by αPD-1 + αCD8 treatment, which clustered most closely with IgG control treatment (Supplemental Figure 1B). Specifically, we observed that αPD-1 treated cells uniquely upregulated multiple genes involved in IL-6 and mesenchymal stem-like pathways, such as Socs3, Lif, Mmp2, and Snai1 (Figure 1E and Supplemental Table 1). We confirmed these results using qPCR (Supplemental Figure 1C) and also observed increased levels of IL-6 and LIF in conditioned media in response to PD-1
blockade (Supplemental Figure 1, D-F). GSEA of Hallmark gene sets identified TNF-α/NF-κB and epithelial-mesenchymal (E-M) transition signatures as among the top pathways expressed in these cells (Figure 1E, Supplemental Figure 1G, and Supplemental Table 2 and 3). Evaluation of this ex vivo RNA-sequencing pipeline using CT26, another colorectal cancer model with relative αPD-1 resistance (19), again demonstrated EMT among the top pathways enriched after PD-1 blockade, as well as substantial overlap with multiple other MC38 enriched pathways (Supplemental Figure 2 and Supplemental Table 4 and 5). These data suggested that tumors contain specific cellular subpopulations that persist despite effective PD-1 blockade by activating programs similar to those engaged by DTPCs. However, we recognized that this transcriptional program could be activated in residual tumor cells or in other cells within the tumor immune microenvironment of MDOTS, and therefore pursued single cell RNA-seq (scRNA-seq) to assess this response at higher resolution.

Single-cell RNA sequencing identifies unique cancer cell clusters resistant to anti-PD-1 therapy

We first sought to validate these results in vivo by treating syngeneic mice with established MC38 tumors with αPD-1 therapy (10 mg/kg IV), or negative control (isotype IgG) and then performing scRNA-seq. To focus on cancer cell subpopulations from residual MC38 tumors after treatment, we sorted CD45/CD90 double negative cells (enriched for tumor cells) and compared them with CD45/CD90 double positive immune cells (Figure 1F). We utilized the specific Hallmark pathway EMT genes that were upregulated by bulk RNA-seq analysis in αPD-1 treated MC38 MDOTS to generate a more specific signature (E-M+) for this resistant cell state (Supplemental Table 6). Similar to our findings in MDOTS, we observed strong enrichment of this E-M+ signature uniquely in αPD-1 treated cancer cell subpopulations (Figure 1F). To isolate the nature
of these cell subpopulations further, we took advantage of the fact that MC38 MDOTS contain a minority of CD90+ mesenchymal cells by day 6, and that digesting MDOTS from collagen substantially depletes CD45+ immune cells (Supplemental Figure 3, A and B). We therefore extracted collagen embedded residual cells from MC38 MDOTS at the end of treatment with IgG or αPD-1 and performed scRNA-seq from this ex vivo model (Supplemental Figure 3B). Unsupervised clustering of 2,543 IgG and 2,626 αPD-1 treated cells confirmed robust and distinct αPD-1 specific transcriptional states, including 4 unique clusters, with the majority of αPD-1 treated cells appearing in clusters 1 and 2 (Figure 2A). We next applied GSEA of Hallmark pathways to these clusters, which revealed that the two dominant αPD-1 resistant clusters 1 and 2 both shared prominent downregulation of IFN-γ and IFN-α signatures (Figure 2, B and D, and Supplemental Figure 4A). In contrast, clusters 1 and 2 were distinguished by highly divergent changes in cell-cycle gene expression, with upregulation of E2F targets and G2M associated genes in cluster 1, versus strong negative enrichment of these genes in cluster 2 suggestive of quiescence (Figure 2, C and D, and Supplemental Figure 4A). Clusters 1 and 2 shared activation of oxidative phosphorylation pathway genes and notably were also distinguished by upregulation of the EMT Hallmark signature in cluster 2 (Figure 2D). Taken together, these findings suggested that a specific subset of MC38 cells evade αPD-1 therapy by adopting a quiescent, mesenchymal phenotype.

Snail1 and Ly6a (Sca-1) mark a subpopulation of αPD-1 immunotherapy persister cells

We next compared expression differences in E-M+ signature high versus low cells to identify specific markers of cells expressing this feature. In consonance with the Hallmark pathway analysis, cells within the quiescence-associated cluster 2 most strongly expressed the top genes
from this E-M+ signature (Figure 3A). Additionally, as expected, this identified increased expression of genes included in the signature itself, such as Sdc1, Mmp2 and Dcn1 (Figure 3B and Supplemental Table 7). This analysis, also uncovered Snail and Ly6a (encoding stem cell antigen-1 or Sca-1) as among the most significantly upregulated transcripts in these E-M+ cells, using a stringent statistical threshold of Log2 fold change of >0.5 and -Log10 adjusted p value >50. These genes were selected for further evaluation due to their biological plausibility as IPC markers. As discussed above, Snail1 has been linked to a similar resistant hybrid E-M state that is a feature of tumor-initiating cells (20). Similarly, Sca-1 is also a well-established marker of hematopoietic, tumor initiating, and tissue stem cells, which can evade immune surveillance (21, 22). Together, these findings further suggested the potential existence of a specific Snail1+ Sca-1+ anti-PD-1 immunotherapy persister cell (IPC) population that escapes CD8 T cell-mediated killing following effective PD-1 blockade.

To further validate enrichment of this IPC signature in vivo after PD-1 blockade by an orthogonal method, we evaluated Snail expression in MC38 tumors using mRNA in situ hybridization (ISH) 1 week after αPD-1 therapy or negative control (isotype IgG + vehicle) to match ex vivo profiling studies. Consistent with our scRNA-seq profiling results, clusters of tumor cells expressing high levels of Snail mRNA signal were observed and significantly enriched in the peri-necrotic areas of all three αPD-1 treated MC38 tumors compared to isotype IgG-treated control tumors (Figure 3C). Evaluation of non-necrotic areas did not show increased Snail mRNA signal.

Similar to syngeneic murine models such as MC38, microsatellite instability-high (MSI-H) status in patient tumors is a robust predictive biomarker for response to anti-PD-1 therapy in the clinic (23). Recently, genomic and transcriptional profiling of an MSI-H colorectal cancer
patient tumor that was resistant to αPD-1 therapy identified biallelic loss for β2-microglobulin as a potential mechanism for resistance to immune-activation (24). In consonance with our findings, scRNA-seq analysis also identified the presence of SNAI1+ cancer cell subpopulations pre-existent within this αPD-1 resistant patient tumor (Figure 3D). Moreover, standardized SNAI1 expression from bulk RNA-seq of this patient’s pre-treatment tumor as compared with available samples of colorectal cancer (CRC) patients in The Cancer Genome Atlas (TCGA) was in the top 4.9th percentile of all samples and 8.2nd percentile of MSI-H colorectal tumor samples (Figure 3E), consistent with the intrinsic resistance to PD-1 blockade compared to typical MSI-H patients and our findings from the MC38 model. We also examined SNAI1 expression in pre-treatment melanoma tumors and found that high (top 10th percentile) SNAI1 expression significantly enriched for lack of durable clinical benefit from treatment with nivolumab (Figure 3F and Table 1). In contrast, pre-treatment expression of genes related to the TNF-α/NF-κB signature such as Birc2 and Birc3 were not predictive (Supplemental Figure 4B). However, the paucity of publicly available large-scale paired pre- and post-progression RNA-sequencing datasets limits robust analysis of on-treatment enrichment for each of these markers.

IPCs that resist CD8+ T cell killing pre-exist in syngeneic cancer cell models

In contrast to Snail, Ly6a (Sca-1) is a mouse-specific gene, but its utility as a cell surface marker for hematopoietic and tissue stem cells has been well documented (21, 22, 25). We therefore used this marker to investigate the pre-existence of IPCs in syngeneic murine cancer cell lines and their resistance to T cell killing. First, we generated ovalbumin (ova)-expressing MC38 cells (MC38-ova), co-cultured them with ova-specific cytotoxic OT-1 CD8+ T lymphocytes, and measured whether Sca-1+ cells emerge under immune selective pressure in this model system (26). As
expected, increasing effector: target (E:T) ratios of OT-I T cells to MC38-ova cancer cells led to decreased viability of target cancer cells (Figure 4A). Notably, we observed that MC38-ova cancer cells indeed contain a small proportion of Sca-1+ cells that were highly enriched among the surviving cells at higher E:T ratios, becoming the dominant population after immune selection pressure. These data are consistent with the persistence of this MC38 tumor cell subpopulation following ex vivo PD-1 blockade and confirms their relative resistance to cytotoxic T cell killing.

Next, to characterize this pre-existent Sca-1+ cell subpopulation further, we utilized flow cytometry to quantify their presence in colorectal (MC38 and CT26) and other syngeneic cancer cell models. Notably, both colorectal cancer lines were predominantly CD44+, and contained a small sub-population of Sca-1+ cells averaging ~1% in MC38 and ~3% in CT26 (Figure 4B and Supplemental Figure 5, A-C). Ras mutant lung cancer models LLC and CMT167 had substantially higher proportions of Sca-1+ CD44+ cells (Supplemental Figure 5, B and C). Whole exome sequencing of MC38 Sca-1+ and Sca-1- populations, isolated by flow sorting, confirmed that they shared known MC38 cancer gene variants, but uncovered broader genomic heterogeneity across independently sorted samples, consistent with stochastic acquisition of this cell state (Figure 4C and Supplemental Figure 5, D and E). Of note, there were no significant difference in surface MHC class I H-2Kb expression between Sca-1+ and Sca-1- cells in culture (Supplemental Figure 6A), indicating that resistance in the co-culture assay to cytotoxic T cells was not simply driven by impaired antigen presentation.

We next characterized the behavior of these Sca-1+ IPCs over time in culture. In the absence of immune cell pressure, purified MC38 Sca-1+ cells quickly reverted to Sca-1- cells, returning close to baseline proportion within 96 hours, whereas Sca-1+ cells from the more αPD-1 resistant CT26 and LLC models persisted over time (Figure 4, D and E and Supplemental
Furthermore, Sca-1 depleted fractions also acquired Sca-1+ cells at a proportion commensurate with their baseline positivity in bulk cultures (Supplemental Figure 6C). These data further reinforce the stochastic nature and plasticity of this cell state. We wondered whether differences in the proportion of MC38 and CT26 Sca-1+ cells could be related to certain growth factors or cytokines that support their expansion. Both Hgf and Fgf7 were upregulated in bulk RNA-seq data following αPD-1 treatment and have been implicated in resistance to oncogene-targeted therapies (27, 28). However, culture of Sca-1+ sorted MC38 cells in the presence of recombinant HGF or FGF7 could not rescue their depletion over time (Supplemental Figure 6D). We therefore explored differences in autocrine cytokine/chemokines in conditioned media (CM) from sorted MC38 versus CT26 Sca-1+ cells, as a more unbiased screen to identify factors that could explain the relatively greater persistence of Sca-1+ cells in CT26. Notably, among 32 potential candidates, IL-6 was consistently the most upregulated cytokine in CT26 Sca-1+ CM versus MC38 CM (Figure 4F and Supplemental Figure 7A). IL-6 was also found at higher levels in the CM of Sca-1+ cells versus Sca-1- cells for both MC38 and CT26 (Supplemental Figure 7, B and C).

Sca-1+ IPCs expand in response to IL-6 and show differential thresholds to TNF cytotoxicity

We next performed a focused in vitro screen using sorted MC38 Sca-1+ cells to identify which among the top upregulated cytokines and growth factors could significantly expand these cells over time. In addition to IL-6, HGF and FGF7, we included LIF, which was secreted by αPD-1 treated MC38 MDOTS and is known to support stem cell growth (29), as well as G-CSF, GM-CSF, M-CSF and VEGF, which were also higher in CT26 Sca-1+ CM (Figure 4F). IFN-γ treatment was utilized as a positive control inducer of Sca-1 surface expression (21, 30). Notably,
only IL-6 was capable increasing the proportion of MC38 Sca-1+ cells, similar to positive control IFN-γ treatment (Figure 5A and Supplemental Figure 8A). However, in contrast to IFN-γ, IL-6 failed to upregulate MHC class I expression in Sca-1+ cells (Figure 5B). We further confirmed that IL-6 expanded Sca-1+ cells in MC38 MDOTS (Figure 5C). Similar to MC38, IL-6 boosted Sca-1+ cells in vitro in other models, including CT26, LLC, and CMT167 (Figure 5D). IL-6 also promoted expansion of Sca-1+ cells in Sca-1 depleted MC38 and CT26 cells, confirming a robust role for IL-6/STAT3 signaling in expanding this cell population (Supplemental Figure 8B). Thus, IL-6 signaling supports this cell state, while limiting interferon response and tumor antigenicity.

We next compared IL-6 stimulation of MC38 Sca-1+ cells with TNF-α, since TNF-α/NF-κB signaling was also among the top αPD-1 enriched signature in MC38 MDOTS, but TNF-α was secreted at comparatively low levels in the CM (Figure 1E and Supplemental Figure 9A). In contrast to IL-6, exogenous TNF-α exposure potently inhibited MC38 Sca-1+ cell growth in culture and abrogated the ability of IL-6 or IFN-γ to expand these cells, consistent with its known dual pro-and anti-apoptotic functions (Figure 5E, Supplemental Figure 9B and Supplemental Figure 10A) (31, 32). Indeed, TNF-α treatment of Sca-1+ cells induced expression of multiple pro-apoptotic genes, including Fas and Trafl, countered by survival genes identified in the αPD-1 induced TNF-α/NF-κB signature including Birc2 and Birc3 (Figure 5F, Supplemental Table 2 and Supplemental Figure 9C).

Evaluation of other syngeneic models in culture further revealed differential sensitivity of Sca-1+ cells to TNF-α. While LLC Sca-1+ cells were especially sensitive, CT26 Sca-1+ cells were only modestly sensitive and CMT167 Sca-1+ cells were comparatively resistant (Figure 5E, Supplemental Figure 9D and Supplemental Figure 10, A and B). Furthermore, in contrast to MC38, IL-6 supplementation was able to rescue Sca-1+ cells at least partially in these three
models. Finally, consistent with these in vitro culture results, MC38 MDOTS showed significant reduction in live cells after culture for 6 days in presence of TNF-α in a concentration dependent manner, while less marked (and non-significant) effects were seen in CT26 MDOTS (Supplemental Figure 9E). These results support the presence of differential tumor TNF cytotoxicity thresholds within these stem-like populations.

Birc2/3 degradation by LCL161 further sensitizes IPCs to TNF-α and promotes durable αPD-1 response

Since Birc2 and Birc3 were induced in Sca-1+ IPCs, and have been previously implicated in CD8+ T cell TNF cytotoxicity thresholds and αPD-1 sensitivity (33, 34), we explored whether inhibition of Birc2/3 function could exploit this TNF-α mediated vulnerability of Sca-1+ IPCs. Indeed, addition of the second mitochondria-derived activator of caspase (SMAC) mimetic, LCL161 to TNF-α treatment completely eradicated MC38 Sca-1+ cells, irrespective of supplementation with IL-6 (Figure 6A and Supplemental Figure 10A). Treatment with LCL161 also reduced enrichment of MC38 Sca-1+ cells under immune selection pressure with co-culture of MC38-ova and OT-1 CD8+ T lymphocytes (Supplemental Figure 11A). Moreover, growth of comparatively TNF-α resistant CT26 Sca-1+ cells was also potently inhibited by co-treatment with LCL161, though still partially rescued by IL-6 (Figure 6A and Supplemental Figure 10A). These differential sensitivities and combinatorial activity of TNF-α and LCL161 were further recapitulated in MC38 and CT26 MDOTS (Supplemental Figure 11, B-D). Further evaluation in lung syngeneic cancer models also confirmed the potent activity of co-treatment with TNF-α and LCL161 in eradicating Sca-1+ IPCs in culture, especially in CMT167, which was resistant to TNF-α alone (Supplemental Figure 10B).
To determine the potential therapeutic relevance of these findings, we next evaluated the efficacy of αPD-1 ± LCL161 treatment in vivo. Syngeneic mice with established MC38 and CT26 tumors were treated with αPD-1 therapy (10 mg/kg IV weekly), LCL161 (100 mg/kg weekly orally in two split doses), combination therapy with αPD-1 and LCL161 or negative controls (isotype IgG + vehicle). Histopathologic evaluation one week after first treatment dose suggested enhancement of αPD-1 immune-mediated MC38 tumor cell killing by co-treatment with LCL161, with more complete eradication of tumor cells (Figure 6B). Indeed, over time, LCL161 and αPD-1 combination therapy yielded significant improvement in survival and complete responses compared to each monotherapy alone or isotype control in mice bearing MC38 tumors (Figure 6C and Supplemental Figure 12, A and B). These results were confirmed in two independent sets of experiments, with complete responses observed more frequently in the combination therapy group. Furthermore, consistent with the differential IPC TNF cytotoxicity thresholds observed in vitro and ex vivo, numerically higher but not statistically significant rates of complete response and durable survival were seen with combination therapy in mice bearing CT26 tumors compared to monotherapy with either αPD-1 or LCL161 (Figure 6D and Supplemental Figure 12, C and D). Taken together, these data reveal that Birc2/3 antagonism can co-opt this TNF-mediated vulnerability of IPCs and improve durable responsiveness to PD-1 blockade.
Discussion

Here, we utilize dynamic ex vivo single-cell RNA sequencing of organotypic tumor spheroids and identify a specific transcriptional program engaged by cells that persist despite effective PD-1 blockade. We further confirm that these immunotherapy persister cells represent pre-existing subpopulations of cancer cells with a stem-like phenotype that resist αPD-1-mediated CD8+ T-cell re-invigoration. The specific features of these cells are highly analogous to the DTPCs that emerge following oncogene-directed targeted therapies, sharing properties of quiescence, apoptosis resistance and epithelial-mesenchymal transition with tissue and cancer stem cells (13-16). In addition, IPCs downregulate IFN response, which has been associated with resistance to ICB in multiple studies (33, 35-37). Despite this ability to escape T cell killing, these cells remain vulnerable given their reliance on activation of TNF-associated NF-κB survival signaling. By enhancing the TNF-α pro-death response that these cells exhibit and lowering their TNF cytotoxicity thresholds, for example by interfering with the key survival factors Birc2/3, it is possible to kill these cells and improve the ability of PD-1 blockade to induce durable responsiveness in vivo.

IPCs are characterized by expression of Snai1 and Ly6a (Sca-1). Snai1 is a master transcription factor which is known to drive a hybrid epithelial-mesenchymal state (20). EMT induction in the MCF10 epithelial mammary cell-line by stably overexpressing SNAI1 resulted in increased activation of NF-κB/MAPK signaling and induction of IL-6/IL-8 on IL-1β stimulation, and was associated with chemoresistance (38). Snai1 has also been linked with resistance to CTLA-4 blockade in murine breast carcinoma models (10). Knockdown of SNAI1 by shRNA in high grade serous ovarian cancer models was associated with decreased expression of Nanog and Lin28, increased let-7 expression and decreased self-renewal capacity reflected in tumor burden
in orthotopic xenografts (39). Moreover, the role of metabolic pathways in promoting lung cancer metastasis has also been ultimately linked to enhanced stability of SNAIL mRNA via depletion of UDP-glucose by UDP-glucose 6-dehydrogenase on EGFR activation (40). Stem cell antigen-1, on the other hand, has been directly linked to both hematopoietic and tissue stem cells (21, 22, 25, 41). For example, in lung, Sca-1 expression marks bronchioalveolar stem cells that, upon transformation, give rise to adenocarcinoma (22). Besides normal stem cells, Sca-1 and other Ly6 family members have been linked with cancer stem cells (41). Of note, the tumor expression of human homologs of Sca-1, Ly6K and Ly6E has been associated with poor overall survival in breast cancer with a mechanistic link to TGFβ signaling (42). These data lend support to our findings identifying stem cell-like sub-populations of cancer cells that persist despite immune activation and may ultimately drive tumor progression and/or relapses. Similar to the DTPC concept, these primitive populations provide a reservoir that can escape effective T cell killing and contribute to anti-PD-1 immune evasion.

The discovery that NF-κB survival signaling pathways and IL-6 in particular are integrally involved in expansion and survival of IPCs is further reminiscent of activation of these pathways in DTPCs (6, 7, 43). The identification of IL-6 as an IPC growth factor is also consistent with the role of IL-6 signaling in promoting EMT (44, 45) and supporting cancer stem cell expansion (46, 47). IL-6 has been shown to enrich for a stem cell phenotype after treatment with platinum-based chemotherapy in ovarian cancer (48) and non-small cell lung cancer (49). Specific induction of IL-6 in DTPCs has been observed in multiple oncogene-addicted lung cancer models and is sensitive to transcriptional inhibitors such as THZ1 (6). Activation of NF-κB signaling following EGFR tyrosine kinase inhibitor (TKI) treatment in lung adenocarcinoma induces formation of an EGFR-TRAF2-RIP1-IKK complex (43) and is associated with endoplasmic reticulum (ER) stress
and STING activation (7), which promote DTPC survival. Thus, activation of NF-κB and induction of IL-6 may play a more general role in fostering expansion of these stem-like cancer cell subpopulations that resist not only chemo- and targeted therapies, but also anti-PD-1 immune checkpoint blockade.

Anti-apoptotic adaptation by increased expression of specific regulators of mitochondrial membrane permeability are often employed by cancer cells in response to therapeutic stress (50). For example, inhibition of Bcl2 or Bcl-XL is capable of overcoming these pro-survival signals and triggering apoptosis in EGFR TKI DTPCs (7, 51). In the case of IPCs, we have identified Birc2 and Birc3 as alternate anti-apoptotic nodes that are activated downstream of TNF-induced NF-κB signaling, providing a unique pharmacologic target by which to trigger their apoptosis. This is in agreement with genetic identification of Birc2 in two independent genome-wide CRISPR/Cas9 loss-of-function screens as a target that augments activity of immunotherapy (33, 34). Furthermore, these findings suggest variation in anti-apoptotic defense mechanisms adopted by cancer persister cells to resist targeted therapies versus anti-PD-1 therapies, potentially due to preferential induction of Birc2/3 dependency by TNF-α in the microenvironment in response to immune activating therapies.

There remains an unmet clinical need to develop predictive biomarkers to guide precision medicine efforts involving PD-1 blockade and anti-apoptotic drugs. There are at least 3 ongoing clinical trials which are evaluating combinations of SMAC mimetics/IAP antagonists with anti-PD-1 (NCT02890069 in advanced solid malignancies and NCT03111992 in multiple myeloma) or anti-PD-L1 (NCT03270176 in advanced solid malignancies) therapies. By uncovering IPCs, our study provides potential predictive biomarkers such as Snail1 expression that could enhance the precision of these trials. Furthermore, differential TNF cytotoxicity thresholds noted among
the syngeneic colorectal and lung cancer models in our study may potentially explain the variability in response anticipated in these clinical trials. IPCs with relatively lower thresholds (exemplified by MC38 and LLC models) were exquisitely sensitive to IAP antagonism in presence of TNF-α, whereas those with higher TNF cytotoxicity thresholds (exemplified by CT26 and CMT167 models) showed comparative resistance. Given the role of IL-6/STAT3 activation in expanding IPCs, evidence of IL-6 signaling could also represent a biomarker in pre- and/or on-treatment tumor biopsies which would enhance robustness of predicting tumor response or resistance in patients. Finally, ex vivo modeling of therapeutic response and resistance in patient-derived organotypic tumor spheroids may provide a direct avenue to study these dynamic changes further without the burden of repeated in vivo biopsies.

Discovery of IPCs, and their unique TNF cytotoxicity thresholds and anti-apoptotic dependencies uncovers multiple additional potential therapeutic opportunities. While our data demonstrates the feasibility of targeting one of these defense mechanisms to enhance durable anti-PD-1 responses, higher order combinations with PD-1 blockade may be necessary to completely eradicate IPCs in vivo, especially in those with high TNF cytotoxicity thresholds. More generally, this study highlights the power of dynamic high-resolution single-cell RNA sequencing using functional models of immunotherapy to understand more fundamental mechanisms of immune evasion, which could also be applied to dissect resistance to additional modes of treatment or combination immunotherapies.
Methods

Cell Lines and Primary Cultures

MC38 murine colon adenocarcinoma cells were provided by Dr. Gordon Freeman (Dana-Farber Cancer Center, DFCI) in 2015 under a material transfer agreement (MTA) from Dr. Jeffrey Schlom of NCI (Bethesda, MD). CT26, LLC, and B16 cells were purchased from ATCC in 2015, while 4T1 cells were purchased in 2016. ID8 and CMT167 cells were purchased from Millipore Sigma in 2016 and 2017, respectively. MC38 cells for in vivo experiments performed at Novartis Institute for Biomedical Research (Novartis) were received from the National Cancer Institute (Rockville, MD) under MTA# 38699-15. All cells were routinely tested for mycoplasma and found to be free of contamination.

Murine in vivo experiments

Thawed syngeneic cancer cells were cultured for three passages in DMEM (MC38) or RPMI (CT26) supplemented with 10% fetal bovine serum (FBS, Gemini Bioproducts). A cell aliquot was stained with acridine orange/propidium iodide (Nexelcom) and cell counts were performed prior to implantation using the Nexcelom Cellometer K2 image cytometer. For the MDOTS studies, MC38 or CT26 cells (5x10^5 cells/mouse in 100 µL), re-suspended in FBS-free media, were injected subcutaneously in the upper right dorsal flank of 8-12-weeks-old female C57BL/6 albino mice (The Jackson Laboratory, RRID:IMSR_JAX:000058) or BALB/c mice (The Jackson Laboratory, RRID:IMSR_JAX:000651), respectively. After the first week, post-injection tumor volumes were measured 3-4 times per week. Tumors were collected 2 to 3 weeks after implantation before they reached 600 mm^3 (MC38) or 350 mm^3 (CT26), or for humane reasons per IACUC regulations.
For in vivo therapeutic studies at Novartis, MC38 cells were thawed and cultured for one week prior to implant in DMEM medium supplemented with 10% heat-inactivated FBS; 10 mM HEPES; 1 mM Sodium pyruvate; and 1X NEAA, during which they were split approximately 2-3 times. 1x10^6 MC38 cells/mouse were implanted subcutaneously on the upper right dorsal flank of 5-7 weeks old female C57BL/6 mice (Charles River, RRID:MGI:5658459). When tumor volume reached approximately 100 mm³, mice were randomized by a computer using a Novartis program to control (isotype + vehicle) or treatment groups (monotherapy with anti-mouse PD-1, LCL161 or combination therapy with anti-PD-1 and LCL161). 17 mice were randomized per group. The vehicle control (30% 0.1N HCL, 70% NaAcetate, pH 4.63) and LCL161 (50 mg/kg) were administered orally once a week in 2 split doses (7 hours apart). Isotype control (mIgG1, 10 mg/kg, clone MOPC-21), and anti-mouse PD1 (10 mg/kg, clone 1D2) were administered intravenously once a week. The group with combination regimen received anti-PD-1 therapy and LCL161 concurrently. The treatment was continued for four consecutive weeks; except for 6 mice in control, anti-PD-1 and combination groups (7 mice in LCL161 group), which were sacrificed on days 7-8 to study treatment-related tumor changes. For the CT26 therapeutic study performed at DFCI, 0.25x10^6 CT26 cells/mouse were implanted subcutaneously on the right flank of 8-week-old female BALB/cJ mice (The Jackson Laboratory). On day 9 post-implantation, animals were randomized into the 4 treatment groups with n=7-8 mice/group, based on tumor volume using Studylog software (South San Francisco, CA) as: vehicle control (30% 0.1N HCL, 70% NaAcetate, pH 4.63 + Rat IgG2a, clone 2A3 (BioXCell)), LCL161 (50 mg/kg orally once a week in 2 split doses), anti-mouse PD-1 (10 mg/kg IV once a week, clone RMP1-14, BioXCell) or the combination of LCL161 and anti-PD-1 for 3 weekly treatments.
In therapeutic studies, the tumor volume was measured 2-3 times per week (length x width) with a digital caliper. The tumor volume was determined using the formula: \((l \times w^2) \times 3.14159)/6\). Body weight was recorded at the same time. Survival endpoint was defined as either tumor volume exceeding 1000 mm\(^3\) or if the tumors became necrotic and mice were humanely euthanized. Mice with no measurable tumors, defined as complete responders, were followed for 55 days (MC38 model) or 71 days CT26 model).

**Spheroid preparation and microfluidic culture**

MDOTS preparation and microfluidic culture was performed as described previously (19, 52). Briefly, fresh tumor specimens were first minced using sterile forceps and scalpel, followed by resuspension in DMEM (MC38) or RPMI (CT26) media containing 100 U/mL collagenase type IV (Life Technologies). Samples were resuspended in fresh media and strained sequentially through 100 \(\mu\)M and 40 \(\mu\)M filters to obtain S2 (40-100 \(\mu\)M) spheroid fraction. Finally, the mixture of S2 with type 1 rat tail collagen (Corning) was cultured in 3D microfluidic culture devices in presence of the media. Treatments were performed by diluting drugs in full media and adding 300 \(\mu\)L to each well of the device. The treatments consisted of \(\alpha\)PD-1 (10 \(\mu\)g/mL, clone RMP1-14, BioXCell), IgG2a (10 \(\mu\)g/mL, clone 2A3, BioXCell), \(\alpha\)CD8a (10 \(\mu\)g/mL, clone 53-6.7, BioXCell), \(\alpha\)IFN-\(\gamma\) (10 \(\mu\)g/mL, clone R4-6A2, BioXCell), IL-6 (100 ng/mL, cat#406-ML, R&D), TNF-\(\alpha\) (10-100 ng/mL, cat#410-MT, R&D) and/or LCL161 (500 nM, Novartis).

**Live/dead staining and quantification**

Viability staining was performed by loading microfluidic devices with Hoechst33342 and Propidium Iodide (Invitrogen) diluted in full media to the final concentrations of 10 \(\mu\)g/mL and 1
µg/mL respectively. The dyes were incubated in the microfluidic device for 30 minutes in a 37°C incubator with 5% CO₂. Images were captured on a Nikon Eclipse 80i fluorescence microscope equipped with ProScan III-controlled Z-stack and linear stage (Prior), and Zyla 5.5 sCMOS camera (Andor). Image capture and analysis were performed using NIS-Elements AR software package.

Whole well area was acquired using stitching of 4 fields of view captured with 4x objective. Three planes of focus 50 µm apart were acquired and an extended depth of focus image was generated.

Live/Dead measurements were done by quantifying both total Hoechst-stained and dead Propidium Iodide-stained cell areas.

Immunofluorescence staining and cytokine analysis of microfluidic devices

For immunofluorescence studies, MC38 MDOTS in triplicate wells at day 6 of the culture with either IgG, αPD-1 or IL-6 were washed directly in the devices with PBS and blocked with 1% Bovine Serum Albumin (BSA) in PBS for 15 minutes at room temperature. Directly conjugated antibodies were anti-mouse CD45-AlexaFluor 647 (clone 30-F11, BioLegend, shown in green for better visualization), anti-mouse CD90.2-PE (clone 30-H12, BioLegend) and anti-mouse Ly6a/e (Sca-1)-PE (clone D7, BioLegend). Antibodies were diluted 1:50 in 10 µg/mL solution of Hoechst 33342 in PBS and loaded into microfluidic devices for a 45-minute incubation at room temperature in the dark. Spheroids were washed twice with PBS with 0.1% Tween20 followed by PBS. Images were captured and whole well image acquisition was performed as described in the main methods section. For Sca-1 fluorescence quantification of tumor cells, we manually placed Regions of Interest (ROI) frames in the areas containing only CD45- cells. In these areas, mean Sca-1 fluorescence intensity was measured in all ROIs. Total number of ROIs was greater than 1000 for each treatment condition. Cytokine analysis of conditioned media from MDOTS at the end of
experiment was performed using Bio-Plex Pro Mouse Cytokine 23-plex Assay (BioRad), MILLIPLEX MAP Mouse Cytokine/Chemokine Magnetic Bead Panel - Premixed 32 Plex (Millipore Sigma) or V-PLEX Mouse TNF-α Kit (MSD).

**Bulk RNA-sequencing from microfluidic devices**

After treatment in the microfluidic devices, cells were lysed directly from the devices and RNA isolated using the Agencourt RNAdvance Tissue isolation kit (with DNase). RNA libraries were prepared from 250 ng total RNA using the Illumina Exome Capture kit per manufacturer’s instructions. RNA-seq was performed per the standard protocols at the Dana-Farber Molecular Biology Core Facilities (Illumina NextSeq 500). Sample-to-sample correlation heatmap was generated using VIPER (Visualization Pipeline for RNA-seq analysis) and displayed hierarchical clustering of Spearman rank correlations across samples. Differential expression analysis of bulk RNA-sequencing files was then performed using the R package, DESeq2 (53). Volcano plots were generated using R with a log2 Fold Change cutoff of 1.5 and an adjusted p-value cutoff of 1e-10. Pathway analysis of bulk RNA-sequencing was performed using the DEseq2 results and run through the R package, ClusterProfiler, using the Hallmark GeneSets from MSigDB (Broad Institute) (54). Plots of running enrichment score were generated with R.

**Single-cell RNA-sequencing of tumors treated in microfluidic devices and in vivo**

MC38 MDOTS were isolated from the microfluidic device on day 6 of treatment after a 15-minute collagenase treatment. The spheroids were then treated with trypsin in a 37°C incubator for 5 minutes to obtain single cell suspensions. The viable tumor cells were isolated via FACS, washed and assessed for viability. For residual MC38 tumors after in vivo treatment, an additional step
with FACS for CD45/CD90 double positive and double negative populations was performed. The cells from MDOTS or residual in vivo MC38 tumors were loaded onto a 10X Chromium instrument (10X Genomics) per the manufacturer’s instructions. Single-cell RNA libraries were generated using the Single Cell 3’ Reagent Kit (10X Genomics) per user guide. Quality control of the completed libraries was performed using Bioanalyzer High Sensitivity DNA Kit (Agilent) and then sequenced using the Illumina NextSeq 500 platform.

The raw sequencing reads were processed using the 10X Genomics CellRanger bioinformatics pipeline v3.0.2. The assembled matrix was then fed into the standard workflow of the R package, Seurat v2.1.0. Genes that were expressed in at least 3 cells, and only cells that expressed at least 200 genes, were kept for downstream processing. Additionally, cells expressing more than 7000 genes, and cells with more than 18% of UMIs mapping to mitochondrial genes, were removed from the analysis. All the samples were prepared and sequenced together on the same platform.

The filtered matrix was log-normalized using global scaling in Seurat. UMI and mitochondrial transcript content were used as regression parameters. The normalized matrix was scaled and centered gene-wise, and then underwent dimensionality reduction using principal component analysis (PCA) on the highly varying genes. After visual inspection of the PCA elbow plot, the top 18 PCs were chosen for further analysis. Clustering was performed on the chosen PCs using the shared nearest neighbor algorithm in Seurat, with default parameters. A t-SNE map was computed and plotted using the RunTSNE and TSNEPlot modules of Seurat. Cluster differential expression analysis was performed in Seurat using the FindMarkers command using the Wilcoxon Rank Sum test without thresholds. To further characterize these clusters, pathway analysis was carried out using the ClusterProfiler R package. FDR correction was done using Benjamini-
Hochberg method. To estimate pathway scores for single cells, the z-normalized expression values for pathway genes in each cell were summed. Scores using this method correlated highly with those generated using the AUCell R package (data not shown). Pathway scores were then added to the MetaData slot of Seurat objects (55).

E-M+ signature was generated using specific Hallmark pathway EMT genes that were upregulated by bulk RNA-seq analysis in αPD-1 treated MC38 MDOTS. We utilized the statistical threshold of Log2 fold change of >0.5 or <-0.5 and -Log10 Adjusted p value >50 for evaluation of differentially enriched genes in E-M+ cells. Candidate genes for further evaluation were selected based on biological plausibility and pre-test probability.

**Flow cytometry and FACS**

Syngeneic cancer cells in culture were examined for surface expression of Sca-1 positive cells by flow cytometry with BD LSRFortessa and following antibodies: Ly6a/e (Sca-1)-FITC (3:100, clone D7, Thermo Fisher Scientific) and CD44-APC (3:100, clone IM7, BioLegend). MHC class I expression was measured by evaluating surface expression of H-2Kb molecule using the Pacific Blue labeled antibody (2:100, clone AF6-88.5, BioLegend). CD45 expression was determined on pre-FACS and post-FACS tumor specimens by AF488 (1:100, clone 30-F11, BioLegend) and APC-Cy7 (1:100, clone 30-F11, BioLegend) antibodies respectively. FACS was performed with BD FACSMelody device to isolate Sca-1+ purified and Sca-1 depleted (Sca-1-) fractions by labeling MC38, CT26, LLC and CMT167 cells with the above-mentioned Sca-1 antibody and CD44-PE-Cy7 (3:100, clone IM7, BioLegend). FlowJo v10 was used to perform analysis of flow cytometry raw data. Gating was based on forward and side-scatter areas; positive and negative staining cell populations were determined using negative controls (rat IgG2a kappa-FITC, clone...
eBR2a, Thermo Fisher Scientific; rat IgG2b kappa-APC, clone RTK4530, BioLegend; rat IgG2b-PE-Cy7, clone RTK4530, BioLegend).

**Co-culture assay**

The MC38 cell line was transduced to stably express OVA antigen from pLVX-lucOS-IRES-Neo lentiviral vector (26). CD8+ T cells were isolated from 8-12 weeks old C57BL/6-Tg(TcraTcrb)1100Mjb/J OT-I mice (stock#003831, The Jackson Laboratory) using magnetic separation and LS columns per manufacturer's protocol (kit#130-049-401, Miltenyi Biotec). OT-I T cells were activated with Dynabeads Mouse T-Activator CD3/CD28 beads (Life Technologies) for 24 hours before co-culture. MC38-ova cells (target) were seeded in 24 well plates at a density of 50,000 cells per well in 1 mL of media and co-cultured with OT-I CD8+ T cells (effector) at effector to target (E:T) ratios of 0, 0.5, 1, and 2 for 48 hours. In some experiments, LCL161 (500 nM) or DMSO control were added concurrently with OT-I T cells for 48 hours. Flow cytometry experiments were performed as described above. Viability was assessed with Live/Dead Fixable Zombie NIR (BioLegend). Dead cells were gated out before Sca-1 assessment to eliminate possibility of non-specific staining.

**Bulk and single-cell sequencing for patient sample**

The details of the MSI-high colorectal cancer patient’s case and RNA sequencing studies have been described previously (24). The comparison with other colorectal cancer patients (with both MSI-high and microsatellite-stable cancers) was performed using data from previously published data and The Cancer Genome Atlas Program (56).
**Human anti-PD-1 RNA-sequencing dataset**

Bulk RNAseq data from pre-treatment samples were obtained from the Riaz cohort (57). Patients were grouped into durable clinical benefit (DCB) and non-durable clinical benefit (NCB) response groups using a combination of RECIST and survival criteria. DCB patients included those who experienced complete response, partial response, or stable disease with an overall survival greater than one year. NCB patients included those who experienced progressive disease, stable disease with an overall survival less than one year, or patients with death prior to disease assessment. Gene expression data represent upper quartile normalized TPM counts. Fischer’s exact test was performed to evaluate enrichment in the top 10th percentile of expressers for analyzed genes of interest.

**In vitro syngeneic cell cultures**

Sca-1+ purified and Sca-1 depleted fractions were loaded at same numbers and maintained in parallel in vitro cultures at 37°C in media in 6-well plates for 24 and 96 hours. Flow cytometry and cytokine/chemokine evaluation of conditioned media were performed at the end of respective culture time points. Mouse Cytokine/Chemokine Magnetic Bead Panel (MCYTMAG-70K-PX32, Millipore Sigma) was used to perform Luminex analysis of conditioned media. The role of cytokine/chemokine supplementation on expansion of Sca-1+ population was evaluated by addition of the following cytokines at the final concentration of 100 ng/mL individually to parallel in vitro cultures on the day they were established using same number of cells under identical conditions (except test cytokine): mouse IFN-γ (cat#485-MI/CF), IL-6, LIF (cat#8878-LF), VEGF (cat#493-MV), G-CSF (cat#414-CS), M-CSF (cat#416-ML), GM-CSF (cat#415-ML), FGF-7 (cat#5028-KG), HGF (cat#2207-HG) and TNF-α (all R&D Systems). In additional experiments,
LCL161 drug was added at the final concentration of 500 nM to MC38, CT26, LLC and CMT167 cultures in presence of TNF-α, IL-6 or both. Flow cytometry was performed after 96 hours of culture using Sca-1, CD44 and H-2Kb targeted fluorescently-labeled antibodies as described above.

**Whole exome sequencing**

DNA was isolated from MC38 Sca-1+ purified cells and Sca-1- cells immediately after FACS, using DNeasy Blood & Tissue Kit (Qiagen) per manufacturer’s instructions. Whole exome sequencing was performed by GeneWiz (Illumina HiSeq 2X150PE). On average 39 million, 150 bp paired-end reads were generated for each sample which first checked for quality control and then the adapters were trimmed. Then reads were first mapped to the mouse genome (MGSCv37). The GATK best practices workflow was utilized for variant calling followed by hard filtering on raw variants. Filtered SNP variants detected were annotated using SnpEff v.4.3i. Additional variant filtering was performed to remove known common variants. This was performed using the SnipSift v.4.3i package against dbSNP (v150), ExAC, and dbNSFP_3.5c databases. For 38,549 SNPs, the coverage of reads calculated for each SNP and those SNPs with coverage smaller than 10X were filtered out. For the remaining SNPs, additional filtering was performed for removing putative germline variants by comparing to C57BL_6NJ_R SNPs provided by Welcome Sanger Institute (58). As a result, 2,260 SNPs were selected for calculating the correlation between samples using BAMixChecker (59). Additionally, the previously characterized 7 exclusive somatic variations of MC38 cell line were examined in all the samples (60).

**Quantitative PCR with reverse transcription (RT-qPCR)**
MC38 Sca-1+ purified cells were cultured for 6 hours and subsequently stimulated with mouse TNF-α (100 ng/mL) at 37°C for 2 or 6 hours in parallel. The cells were washed with PBS and then extracted from 6-well plates using RLT Buffer supplemented with β-mercaptoethanol (Qiagen RNEasy Mini Kit) and cell lifter (Corning). RNA isolation was performed per manufacturer’s instructions. RT² Profiler PCR array Mouse Apoptosis Kit (Qiagen) was used to profile differential expression of 84 genes simultaneously in MC38 Sca-1+ cells on stimulation with TNF-α compared to no stimulation (control media). RT-qPCR was performed using Applied Biosystems StepOne Plus Real-Time PCR system and data analyzed in GeneGlobe Data Analysis Center. The results for Birc2 (Forward Primer, Fwd: 5′-AGTAGATTTGCACTATTCGACC-3′; Reverse primer, Rev: 5′-AGAATTAAGAGGGCTAGAGCACA-3′) and Birc3 (Fwd: 5′-TGAAGAGTGTGACACCTTTG-3′; Rev: 5′-GGAAAAGCTGAATACGTGGACAA-3′) gene expression were validated subsequently by repeating the experiments twice. Additionally, the results of bulk RNA-seq from MC38 MDOTS were validated by evaluating expression of Snail (Fwd: 5′-CACACGCTGCTTTGCTCC-3′; Rev: 5′-GGTCAGCAAAAGACGATGGT-3′), Socs3 (Fwd: 5′-ATGGTCACCCCACAGCAAGTTT-3′; Rev: 5′-TCCAGTGAATCCGCTCTCCT-3′), Mmp2 (Fwd: 5′-CAAGTCCCGGCGATGTC-3′; Rev: 5′-TTCTGGTGCAAAGGTCACCTGTCC-3′) and Lif (Fwd: 5′-ATTGTGCCCTTACTGCTGCTG-3′; Rev: 5′-GCCAGTTGATCTTGTCTG-3′) genes among αPD-1 versus IgG treated MC38 MDOTS by RT-qPCR analysis, as described before (19). The relative level of gene expression was normalized using the level of mouse 36B4 (Fwd: 5′-AGATTCGGATATGCTGTTG-3′; Rev: 5′-CGGGTCTCAGACGAGTCTG-3′).

**Immunofluorescent staining of in vivo tumors**
5 μM FFPE MC38 tumor slides were deparaffinized and placed in a pressure cooker in Antigen Retrieval Citrate (pH 6.0) buffer for 30 minutes at 115°C, followed by 15 minutes at 90°C. Slides were cooled for 30 minutes at room temperature and were then placed in Shandon Sequenza (Thermo Scientific) slide rack for immunostaining. Slides were first blocked with 1% BSA for 30 minutes. Anti-mouse Ly-6A/E (1:50, clone D7, BioLegend) was added to the cover plates and incubated overnight at 4°C. The slides were then washed 3 times with PBS with 0.1% Tween20, incubated with Alexa Fluor 568 goat anti-rat IgG (1:100, cat#A-11077, Invitrogen) for 30 minutes in the dark and washed again 3 times as described above. Directly conjugated anti-mouse CD45-Alexa Fluor 647 (1:50, clone 30-F11, BioLegend) was subsequently added to the cover plates. After a 1-hour incubation in the dark, the slides were washed 4 times as described above, removed from the cover plates, rinsed in ddH2O, and dried before Vectashield mounting medium with DAPI (Vector Laboratories) was applied. Images were captured on a Nikon Eclipse 80i fluorescence microscope as described above. Five-six areas (2560 x 2160 px) were captured per slide using a 40X objective. Percent of tumor and immune cells were evaluated based on CD45 staining and nuclear morphology by a pathologist (N.R.M.) who was blinded to the treatment groups.

**mRNA in situ hybridization of in vivo tumors**

mRNA in situ hybridization (ISH) was performed on freshly cut 5μm FFPE tumor sections using RNAscope 2.5 HD Duplex Assay (Advanced Cell Diagnostics) with Snai1 probe (Red) and control Huwe1 probe (Green). ISH scores were generated from peri-necrotic and non-necrotic regions by a board-certified pathologist (N.R.M.) at ×400 magnification (2-5 fields of view per tumor). RNAscope analysis was performed as modified H-score with the following signal intensity grades:
0 = no staining or <1 dot/10 cells; 1 = 1-3 dots per tumor cell; 2 = 4-9 dots per tumor cell, none or very few dot clusters; 3 = more than 10 dots per cell; and 4 = presence of dot clusters. Snail H-score was calculated by multiplying the percent of Snail-expressing tumor cells by signal intensity grade (61).

Statistical methods and data analysis

All graphs and scatter plots depict mean ± the standard error of the mean (SEM) unless otherwise indicated. Graphs were generated and statistical analysis was performed using GraphPad/Prism (v8.0) or Microsoft Excel. Statistical tests have been mentioned in figure legends where applicable. P value was considered significant at <0.05 (* <0.05, ** <0.01, *** <0.001 and **** <0.0001).

Study approvals

Informed written consent to participate in Dana-Farber/Harvard Cancer Center institutional review board (IRB)-approved research protocols was obtained from the human subject (24). The study was conducted per WMA Declaration of Helsinki and IRB-approved protocols. All animal experiments at DFCI were performed in compliance with established ethical regulations in AAALAC accredited vivarium and were approved by the Institutional Animal Care and Use Committee (IACUC). In vivo therapeutic studies at Novartis were conducted in accordance with Novartis IACUC regulations and guidelines.

Data deposition statement

Bulk and single-cell RNA sequencing datasets have been deposited at the Gene expression omnibus under accession numbers GSE160228 and GSE160400.
Table 1. Association of clinical benefit from nivolumab with Snail1 expression in pretreatment melanoma tumor samples in the Riaz cohort. Data are presented in Figure 3F and analyzed by Fisher’s exact test.

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<th>High Snail1 expression (&gt;90th percentile)</th>
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Author Contributions

KS, AP, PHL, EVI, JJM, CPP and DAB initiated the project, and designed and supervised research plan. AP, EVI, MN, RWJ and CJL performed MDOTS experiments under supervision of CPP and DAB. JRG and AV performed computational analysis of RNA sequencing and whole exome sequencing data. LJT and TyT performed mouse experiments at DFCI under supervision of PTK and PCG. KS, SK and TT performed in vitro culture experiments under supervision of DAB. PHL performed co-culture assay under supervision of DAB. CG and DeL performed computational analysis of patient RNA sequencing data under supervision of MG and DL. KS and TCT performed cytokine/chemokine analysis and RT-qPCR. MP-O, WDH, SG and PSH performed in vivo experiments at Novartis. EVI performed immunofluorescence/mRNA ISH and NRM performed blinded histopathological evaluation and immunofluorescence/mRNA ISH grading. KS, AP and EVI performed statistical analysis. KS, AP, CPP, and DAB wrote the manuscript. The order of first authorship was determined by contribution to manuscript writing. All authors edited and approved the manuscript.
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References


Figure Legends

A

Organotypic Tumor Spheroids

+ IFN-γ / aPD-1

6 Days

Profiling by RNA sequencing

C

Percent Cell Viability

* ***

Live

Dead

IgG

IFN

aPD-1

CD8

D

MC38 IFN-γ vs. IgG Bulk RNA-seq

Top Hallmark pathways:

- IFN-γ Response
- IFN-α Response
- Allograft Rejection
- Inflammatory Response
- IL-6 Jak stat3 Signaling

E

MC38 aPD-1 vs. IgG Bulk RNA-seq

Top Hallmark pathways:

- TNF-α signaling via NFκB
- Epithelial-Mesenchymal Transition
- Myc targets V1
- Hypoxia
- Mtorc1 Signaling

F

MC38 in vivo scRNA-seq

Bulk EMT Signature Overlay
Figure 1. Bulk RNA sequencing of MDOTS reveals a unique transcriptomic response to immune checkpoint blockade.

(A) Schematic of the workflow to profile surviving cells after treatment. αPD-1 = anti-PD-1 therapy, IFN-γ = interferon-gamma.

(B) Representative immunofluorescent microscopy images of live (AO)/dead (PI) staining of MC38 MDOTS in the microfluidic devices after 6 days of treatment (scale bar, 200 µm). αCD8 = CD8 neutralizing antibody [n =3].

(C) Quantitative measurement of tumor cell viability. Data are mean ± SEM and were analyzed by multiple t-tests with Bonferroni correction [n =3].

(D) Volcano plot of genes differentially expressed by MC38 MDOTS after 6 days of IFN-γ treatment compared to IgG isotype control. Genes with a log₂(fold change) > or < 0.5 are shown in red. The top 5 most significant Hallmark gene sets are shown.

(E) Volcano plot of genes differentially expressed by MC38 MDOTS after 6 days of αPD-1 treatment compared to IgG isotype control. Genes with a log₂(fold change) > or < 0.5 are shown in red. The top 5 most significant Hallmark gene sets are shown.

(F) Two-dimensional t-distributed stochastic neighbor embedding (tSNE) plots of single-cell RNA sequencing (scRNA-seq) performed on CD45/CD90+ and CD45/CD90- cells isolated from MC38 tumors at the end of in vivo treatment with either αPD-1 or IgG isotype control. tSNE plots are colored based on treatment condition (Left) and type of cells (Middle). Right, tSNE plot showing projection of bulk EMT signature (E-M) onto scRNA-seq-sequencing clusters.

* p value <0.05, ** p value <0.01, *** p value <0.001.
Figure 2. Single-cell RNA sequencing identifies unique cell clusters resistant to anti-PD-1 therapy.

(A) Two-dimensional tSNE plots of single-cell RNA sequencing (scRNA-seq) performed on MC38 MDOTS after 6 days of treatment. tSNE plots are colored based on treatment condition.
(Left) and unsupervised clustering distribution (Middle). Right, Bar graphs showing the number of cells in each cluster from αPD-1 treated (grey) or isotype IgG (black) groups.

(B, C) tSNE plots showing relative expression of hallmark IFN-γ response (B), and E2F targets (C) signature on single-cell RNA-sequencing clusters of MC38 MDOTS treated with αPD-1 (clusters 1-4, 5) versus isotype IgG control (clusters 5, 6-10).

(D) Cluster Pathway analysis using the Hallmark gene sets of the genes differentially expressed for single-cell RNA sequencing clusters 1 and 2 of MC38 MDOTS, with their normalized enrichment scores.
Figure 3. Snai1 and Ly6a (Sca-1) mark a subpopulation of αPD-1 immunotherapy persister cells.

(A) Two-dimensional t-distributed stochastic neighbor embedding (tSNE) plot showing projection of bulk EMT signature (E-M) onto single-cell RNA-sequencing (scRNA-seq) clusters of MC38 MDOTS.

(B) Volcano plot of E-M+ (z-based score > 0.25 for bulk EMT signature) versus E-M- cells in single-cell RNA sequencing clusters of MC38 MDOTS.

(C) Left, Representative mRNA in situ hybridization (ISH) images showing Snai1 expression in peri-necrotic regions at scale of 50 µm in hematoxylin-stained tumor specimens from MC38 mice 1 week after treatment with either negative control (isotype control IgG + vehicle) or αPD-1 monotherapy [n = 3 each]. Right, Summary of quantification of H score of Snai1 in the non-necrotic and peri-necrotic regions of tumors. Data are box and whiskers graphs with box representing inter-quartile range, solid line representing median and all the points ranging from minimum to maximum, and were analyzed by one-way ANOVA followed by Bonferroni correction.

(D) tSNE plot showing relative SNAI1 expression (log2 expression scale) localized to carcinoma cells from single-cell transcriptome analysis of an intrinsically αPD-1 resistant microsatellite instability-high (MSI-H) colorectal cancer (CRC) patient tumor. Gray color represents cells in which Snai1 reads were not detected.

(E) Normalized bulk RNA-seq SNAI1 expression scores for this αPD-1 resistant MSI-H CRC patient tumor compared to those of all CRC patient samples (n = 557) or MSI-high CRC patient samples (n = 73) available in The Cancer Genome Atlas (TCGA).
Normalized bulk RNA-seq $SNAI1$ expression scores on pre-treatment melanoma tumor specimens from patients who achieved versus did not achieve durable clinical benefit (see methods for definition) from treatment with nivolumab. Dotted red line represents top 10th percentile. Data are number of patients and were analyzed by Fisher’s exact test ($n = 51$, shown in Table 1).
Figure 4. IPCs that resist CD8+ T cell killing pre-exist in murine syngeneic cancer cell models.

(A) Representative graph of two independent experiments (each done in triplicates) of co-culture assay of target MC38 cells expressing ovalbumin antigen (MC38-ova) (T) and effector OT-I CD8+ T cells (E), showing viability of MC38-ova and percentage of Sca-1+ cells at increasing E:T ratios.
(B) Representative flow cytometry plots and summary of experiments evaluating percentage of pre-existing Sca-1+ CD44+ cells (hereafter Sca-1+ cells) in MC38 and CT26 cells [n = 5].

(C) Cluster dendrogram evaluating correlation between whole exome sequencing (WES) results for the two independently sorted MC38 Sca-1+ purified and Sca-1- samples.

(D) Schema of the following experiments conducted with Sca-1+ purified fraction of syngeneic cancer cells isolated by fluorescent-activated cell sorting (FACS).

(E) Representative flow cytometry plots and summary of experiments evaluating for persistence of Sca-1+ cells in culture of Sca-1+ purified fractions of MC38 and CT26 at 24 and 94 hours (H) [n = 3].

(F) Heat map showing log2 fold change (log2F) of differentially produced cytokines in conditioned media (CM) of Sca-1+ purified cell culture of MC38 versus CT26 cells at 24 and 96 H [n = 2, each was run in duplicates]. Triangle symbol represents above the level of detection.

(A, B, E) Data are mean ± SEM and were analyzed by multiple t-tests with Bonferroni correction (A) or two-tailed Student’s t test (B, E).

* p value <0.05, ** p value <0.01, *** p value <0.001.
Figure 5. Sca-1+ IPCs expand in response to IL-6 and show differential thresholds to TNF cytotoxicity.

(A) Summary of experiments showing proportion of Sca-1+ cells in 96 hours (H) cultures of Sca-1+ purified MC38 cells supplemented with growth factors (100ng/mL) compared to media (negative control) and IFN-γ (positive control) [n=3].

(B) Left, Representative histogram of mean fluorescent intensity (MFI) of H-2Kb of MC38 Sca-1+ cells in 96 H culture of Sca-1+ purified fractions (media alone vs IFN-γ vs IL-6 stimulation (100ng/mL)). Right, Summary of experiments [n=3].

(C) Representative immunofluorescence images and summary of experiments showing Sca-1 expression in MC38 MDOTS after treatment with IL-6 versus control (IgG) (scale bar, 200µm). Bottom, data are median (solid line) with first and third quartiles (dashed lines) and were analyzed by Wilcoxon rank-sum test.

(D) Representative flow cytometry plots showing effect of stimulation by IL-6 on expansion of Sca-1+ cells in 96H cultures of Sca-1+ purified fractions. Right, Summary of experiments [n=4 for MC38/ CT26, n=3 for LLC/CMT167].

(E) Summary of experiments showing effect of stimulation by IL-6, TNF-α or IL-6+TNF-α (100ng/mL) on fold change of proportion of Sca-1+ cells in 96H cultures of Sca-1+ purified fractions of MC38 and CT26 cells compared to media alone (negative control) [n=3].

(F) Heat map showing log2 fold change (log2F) of expression of genes involved in apoptosis pathway in MC38 Sca-1+ cells stimulated with TNF-α (100ng/mL) for 2 and 6H compared to media alone. Asterisks denote genes upregulated on RNA sequencing analysis of MC38 MDOTS after anti-PD-1 therapy.
(A, B, D, E) Data are mean ± SEM and were analyzed by multiple t-tests with Bonferroni correction (A, E), one-way ANOVA with Bonferroni correction (B), or two-tailed Student’s t test (D).

* p value <0.05, ** p value <0.01, *** p value <0.001, **** p value <0.0001.
Figure 6. Birc2/3 degradation by LCL161 further sensitizes IPCs to TNF-α and promotes durable αPD-1 response.

(A) Representative flow cytometry plots showing effects on proportion of Sca-1+ cells in culture of Sca-1+ purified MC38 and CT26 cells at 96 hours (H) with or without treatment with Birc2/3 antagonist LCL161 in presence of TNF-α, IL-6, TNF-α + IL-6 (both at 100ng/mL) or media alone [n=3].

(B) Representative immunofluorescence microscopic images and quantification of CD45 and DAPI-stained tumor specimens from MC38 tumor-containing mice 1 week after treatment with negative controls (isotype control IgG + vehicle); αPD-1 monotherapy, LCL161 monotherapy, or αPD-1 + LCL161 combination therapy [n=6 mice per group, except 4 for IgG + vehicle] (scale bar, 100µm). Data are box and whiskers graphs with box representing inter-quartile range, solid line representing median and all points ranging from minimum to maximum, and were analyzed by Kruskal-Wallis test.

(C, D) Summary of in vivo experiments with Kaplan-Meier curves showing tumor volumes (mm³) over time (days) in MC38 (C) or CT26 (D) tumor-containing mice treated weekly with negative controls (vehicle + IgG); αPD-1, LCL161, or αPD-1 + LCL161 combination therapy. Box with shaded region represents duration of treatment. Mice with complete response (CR)/ total number of mice tested are presented for each group. (C) For MC38, n = 11 mice, except 10 in LCL161 monotherapy group. (D) For CT26, n = 8 mice, except 7 in LCL161 monotherapy group. Some mice were euthanized due to tumor necrosis before they reached threshold for progression (2 mice in vehicle + IgG, 5 in αPD-1, 6 in LCL161, and 2 in combination therapy groups). 1 mouse in combination therapy group which achieved complete response had accidental death on Day 15.

* p value <0.05, ** p value <0.01, *** p value <0.001.