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Emerging data from chimeric antigen receptor (CAR) T-cell trials in B-cell malignancies demonstrate that a common mechanism of resistance to this novel class of therapeutics is the emergence of tumors with loss or downregulation of the target antigen. Antigen loss or antigen-low escape is likely to emerge as an even greater barrier to success in solid tumors, which manifest greater heterogeneity in target antigen expression. Potential approaches to overcome this challenge include engineering CAR T cells to achieve multispecificity and to respond to lower levels of target antigen and more efficient induction of natural antitumor immune responses as a result of CAR-induced inflammation. In this article, we review the evidence to date for antigen escape and downregulation and discuss approaches currently under study to overcome these obstacles.

**Significance:** Antigen escape and downregulation have emerged as major issues impacting the durability of CAR T-cell therapy. Here, we explore their incidence and ways to overcome these obstacles in order to improve clinical outcomes. Cancer Discov; 8(10); 1219–26. © 2018 AACR.
Clinical trial from Seattle Children’s Research Institute (SCRI). In a CD19-negative or partially negative relapse (an additional six at least 15 of 61 (25%) complete responders went on to develop relapses characterized for CD19 expression, 15 were demonstrated to be CD19-negative. Therefore, with limited follow-up, at least 15 of 61 (25%) complete responders went on to develop CD19-negative or partially negative relapse (an additional six relapses were not analyzed for CD19 expression; ref. 17). In a clinical trial from Seattle Children’s Research Institute (SCRI) of a similar CD19-4-1BB-ζ CAR for B-ALL, 7 of 40 patients (18%) who achieved a CR later relapsed with loss of CD19 (10).

A trial of a CD19-CD28-ζ CAR at the NCI in pediatric patients was marked by shorter T-cell persistence and patients frequently underwent hematopoietic stem cell transplant (HSCT) after CAR therapy, but 2 of 12 patients who had achieved a minimal residual disease (MRD)–negative response also developed CD19-negative B-ALL (neither patient underwent HSCT; ref. 9). A follow-up report in abstract form that included expansion cohorts from the NCI study indicated that 5 of 28 patients (18%) who were MRD-negative after CAR eventually relapsed with diminished expression of CD19, including some patients who relapsed following HSCT (20).

Data from post-CAR relapses of adults with B-ALL are scant: The Fred Hutchinson Cancer Center (FHCC) phase I trial of a CD19-4-1BB-ζ CAR found CD19-negative relapses in 2 of 29 patients (7%) who achieved a CR (11). Similarly, a trial from Memorial Sloan Kettering Cancer Center (MSKCC) of a CD19-CD28-ζ CAR in adults with B-ALL saw CD19-negative leukemia in only 4 of 44 patients (9%) achieving a CR (21). The reason for the lower rates of CD19-negative leukemia in trials of adults versus children is unclear. The shorter persistence of the CD19-CD28-ζ CAR T cells employed by MSKCC may partially explain the low rate of CD19-negative relapse in their trial, as a shortened period of immune pressure due to the limited persistence of the CD19-CD28-ζ CAR could diminish the risk of antigen loss escape (21). Similarly, most post-CR relapses in the FHCC trial were CD19-positive relapses among patients who did not receive fludarabine as part of their conditioning regimen, which has been shown to limit the persistence of CAR T cells (11). A summary of the rate of CD19-negative relapse in CD19 CAR trials for B-ALL can be found in Table 1, although caution should be taken when comparing across studies given that they differ greatly in the period of follow-up. CD19-negative relapse of B-ALL is also observed after treatment with the bispecific T-cell engager blinatumomab (CD3 × CD19), occurring in 12% to 21% of complete responders, depending on the study (22–24).

In summary, although CD19-negative escape is a major cause of relapse following CD19-CAR therapy for B-ALL, the true incidence of this phenomenon has not been defined and factors that predict for an increased likelihood of CD19-negative relapse are poorly understood. Nonetheless, as the application of CD19-directed immunotherapy with both CD19 CARs and blinatumomab grows, it is clear that the clinical impact of CD19-negative B-ALL will increase as well.

CD19 CARs have also demonstrated impressive activity in high-grade, relapsed, refractory non-Hodgkin lymphoma (NHL), and both tisagenlecleucel and axicabtagene ciloleucel are FDA-approved for this indication (16). The role of CD19 antigen loss or downregulation is more poorly defined in lymphoma than in B-ALL. Unlike leukemia, biopsies in NHL are not always obtained at the time of relapse, and many trials have therefore not analyzed CD19 expression at relapse (12). In addition, the determination of CD19 expression is often made based on IHC, which is not reliable for distinguishing between intracellular versus membranous antigen expression and more difficult to quantify than flow cytometry, which is commonly employed for B-ALL. The unreliability of IHC for CD19 is illustrated by a recent clinical trial of CD19 CAR T cells for adults with NHL in which 6 of 8 patients who were noted to have CD19-negative disease by IHC prior to CAR infusion demonstrated an objective response to anti-CD19 CAR T cells (16).

In a combined analysis of phase I and phase II trials of axicabtagene ciloleucel for NHL, of the 11 patients who progressed after having a response to CD19 CAR and also had tissue available for analysis, 3 patients had biopsy proven CD19 loss by IHC (16). Notably, however, a very stringent cutoff (<1% of cells expressing CD19) was used for negativity in this assessment, and it remains possible that a higher frequency of patients could have experienced emergence of CD19 antigen loss that contributed to relapse. In a trial at the University of Pennsylvania of a CD19 CAR for NHL, 5 nonresponders underwent postinfusion biopsies and one demonstrated absent CD19 expression (25). Other cases of CD19-negative lymphoma following CD19-CAR therapy have been reported by CHOP and the NCI in pediatric patients (26, 27) and by the NCI in an adult (28). Together, the data demonstrate that emergence of antigen loss variants is the most common cause of relapse following CD19-CAR therapy for B-ALL, and emerging data provide evidence that CD19-negative relapses also occur in NHL, although the

### Table 1. A summary of antigen escape in CD19 CAR trials for ALL

| Trial Population CD19 CAR construct Relapse rate CD19-negative relapse rate References |
|---|---|---|---|---|---|
| Children’s Hospital of Philadelphia phase I Pediatric FMC63-4-1BB-ζ | 36% (20/55) | 24% (13/55) | 8, 19 |
| Novartis phase II (ELIANA) Pediatric FMC63-4-1BB-ζ | 33% (20/61) | 25% (15/61) | 17 |
| Seattle Children’s Research Institute phase I Pediatric FMC63-4-1BB-ζ | 45% (18/40) | 18% (7/40) | 33 |
| NCI phase I Pediatric FMC63-CD28-ζ | 29% (8/28) | 18% (5/28) | 9, 20 |
| Memorial Sloan Kettering phase I Adult SJ25C1-CD28-ζ | 57% (25/44) | 9% (4/44) | 21 |
| Fred Hutchinson Cancer Center phase I Adult FMC63-4-1BB-ζ | 31% (9/29) | 7% (2/29) | 11 |

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incidence of this phenomenon remains less clear. Furthermore, loss of target antigen has also been observed in a patient with multiple myeloma treated with a CAR targeting BCMA (29) as well as in patients with glioblastoma multiforme who were treated with CARs targeting either EGFRvIII (30) or IL13Rα2 (31).

Antigen Loss: Mechanisms
Given the large clinical experience of CD19 CARs in pediatric B-ALL, most data regarding the mechanism of antigen loss come from studies of patient samples in those trials (10, 32, 33). Thus far, the published data have demonstrated CD19 loss occurring via two distinct mechanisms: antigen escape or lineage switch (34). In antigen escape, after achieving a remission in response to CD19 CAR, patients relapse with a phenotypically similar disease that lacks surface expression of a CD19 molecule capable of binding the anti-CD19 antibodies incorporated into the CARs. Lineage switch occurs when a patient relapses with a genetically related but phenotypically different malignancy, most often acute myeloid leukemia (AML; Fig. 1).

The group at the CHOP described mechanisms responsible for at least some of the antigen escape seen in pediatric patients with B-ALL after CD19 CAR. Sotillo and colleagues found several CD19 splice variants expressed by B-ALL, including Δexon-2, which specifically lacks the exon containing the extracellular epitope of CD19 recognized by both the FMC63 (CHOP-University of Pennsylvania/Novartis, NCI/Kite, FHCC-SCRI/Juno-JCAR017) and SJ25C1 (MSKCC/Juno-JCAR015) anti-CD19 binders. In addition, they observed variants Δexon-5,6, which lack the transmembrane domain of CD19 and therefore lead to loss of surface expression. Immune pressure by the CD19 CAR results in selection of leukemia cells expressing higher proportions of these splice variants, leading to escape from detection by CD19 CAR T cells (32). Recent work suggests that patients with ALL already express CD19 splice variants at diagnosis and therefore anti-CD19 therapy may simply select for cells that express these alternative forms of CD19 (35). This mechanism does not account for all cases of CD19 loss in cells that retain a B-ALL phenotype, and other mechanisms merit exploration (36). For instance, one group reported a single patient with loss of CD81, a chaperone protein for CD19, as a mechanism for CD19 loss after blinatumomab (37).

Lineage switch is another mechanism for CD19 loss that has been observed in clinical trials (33). Most often seen in patients who harbor MLL rearrangements, such as infants with B-ALL, lineage switch occurs when the leukemic phenotype changes from lymphoid to myeloid in response to CD19-directed immunotherapy. The evolved leukemic population not only no longer expresses CD19, but also acquires other phenotypic characteristics of AML. This was observed in 2 pediatric patients with MLL-rearranged ALL treated with CD19 CAR on the SCRI trial (33) and one adult on the CD19 CAR trial at the FHCC (11). In addition, this phenomenon was modeled in a murine leukemia by Jacoby and colleagues, who demonstrated that CD19 CAR induces lineage switch in a murine ALL model that is dependent on the E2a:PBX transgene, which, like MLL rearrangement, can drive the development of either lymphoid or myeloid neoplasms (36). Lineage switch has also occurred in both MLL-rearranged and nonrearranged patients after CD19-directed therapy with blinatumomab (38–40). Similarly, a single case of CLL that transformed to a clonally related plasmablastic lymphoma after CD19 CAR treatment has been reported (41).

Figure 1. Mechanisms of tumor antigen escape. CAR T cells encounter adequate amounts of target surface antigen on cancer cells, activate, and kill the target cells. Tumor cells can escape killing by expressing alternative forms of the target antigen that lack the extracellular epitopes recognized by CAR T cells (“antigen escape”), by switching to a genetically related but phenotypically different disease (“lineage switch”), or by downregulating the surface target antigen to levels below those needed for CAR T-cell activation (“antigen downregulation”).

OVERCOMING ANTIGEN LOSS
Delineation of the multitude of mechanisms involved in CD19 antigen loss suggests that creating CARs to target alternative epitopes on CD19 may not prove effective, because many of the examples involve loss of CD19 surface expression. The data also suggest that neither CD19-CAR nor
blinatumomab-mediated T-cell killing drives meaningful induction of immune responses to coexisting immunogenic targets on B-ALL, sometimes referred to as “epitope spreading.” This may reflect the relative low tumor mutational burden in B-ALL, which likely limits inherent immunogenicity of this disease (34, 42). In addition, the use of lymphodepleting agents prior to adoptive transfer of T cells could blunt the native immune response as host T cells are depleted, similar to what has been observed in murine models. In one study, Pmel-1 T cells recognizing gp100 were adoptively transferred to lymphopenic versus lymphoretic mice bearing B16 melanomas. This therapy was found to be more effective in lymphoretic animals, largely due to enhanced epitope spreading (43).

Thus far, the degree to which epitope spreading is induced by CAR T cells has been incompletely studied. In a murine CAR model targeting EGFR, mice that were cured of EGFR+ tumors by EGFR CAR T cells later rejected EGFR+ tumors when rechallenged (44). This elegant model demonstrated that epitope spreading can be induced by CAR T cells, but it is unclear to what extent this occurs in human studies and whether the incidence of this phenomenon might be more common if effective CAR-based therapeutics were used to target tumors with higher inherent immunogenicity. One clinical trial of a CAR targeting mesothelin did find that patients who received CAR T cells also developed an antitumor antibody response (45). It is possible that combining CAR T cells with radiation (46), checkpoint inhibition (47–49), vaccines (50, 51), or other immune agonists (34, 52) will result in epitope spreading that could help counter immune escape, and we anticipate such immune agonists (34, 52) will result in epitope spreading that could help counter immune escape, and we anticipate such

Another approach to overcoming antigen loss following CAR T-cell therapy is to simultaneously target more than one antigen on cancer cells, an approach that is compelling for B-ALL, given that CD22 CAR T cells have also demonstrated substantial clinical efficacy (53). There are several ways to engineer a T-cell product for multispecificity (Fig. 2). T-cell products that are separately transduced for different CARs can be simultaneously or sequentially administered (“coadministration”; Fig. 2; ref. 54), or vectors for two CARs can be combined during cell production to achieve a mixed product with some cells that are positive for a single CAR and others that are positive for both CARs (“cotransduction”; Fig. 2). The disadvantages of these approaches are the high cost of producing multiple vectors and the heterogeneity of the infused product, which can complicate clinical analysis.

A CAR molecule itself can also be engineered to recognize multiple antigens. This can be accomplished by linking two binders on a single molecule (“tandem CAR”; Fig. 2), which appears, in some cases, to enhance the strength of the immune synapse. Hegde and colleagues developed a tandem CAR that can simultaneously target both HER2 and IL13Rα2. They demonstrated enhanced potency and antitumor activity in vivo when two CARs were expressed as a single molecule compared with expressing two separate CARs individually on each T cell or coinfusing two populations of cells, each expressing a monospecific receptor (55). In designing so-called tandem CARs, the position of the target antigen should determine how each binder is oriented relative to the membrane. For instance, in a study of a tandem CD19-CD20 bispecific CAR, the authors found that given the proximal location of CD20 to the cell membrane, the anti-CD20 ScFv needed to be in the distal position in the CAR molecule (56).

Alternatively, two or three separate CARs can be expressed on a single T cell using a single vector by taking advantage of ribosomal skip sequences or internal ribosomal entry sites (“bicistronic CAR”; Fig. 2). Recently, a trivalent vector encoding three independent CARs, each targeting a different antigen on glioblastoma, was described (57). It is likely that over the next several years, multiple methods for creating
multispecific CARs will be evaluated and compared for efficacy in both preclinical and clinical settings. Several clinical trials are under way testing multispecific CAR T cells. We recently reported on a tandem CAR targeting both CD19 and CD22 (53) that is now in clinical trials in children and adults (NCT03241940, NCT03233854, and NCT03448393), and other groups have generated tandem CARs targeting CD19 and CD20 (56, 58, 59), one of which is currently being tested in humans (NCT03019055). The mixed product approach is currently being explored in a clinical trial for pediatric B-ALL targeting CD19 and CD22 (NCT03330691). An abstract was recently presented for a bispecific CAR recognizing CD19, CD20, and CD22 (60). In addition, one group has reported a bispecific CD19 and CD123 CAR in the aims of overcoming both antigen escape and lineage switch as CD123 is expressed broadly in the hematopoietic compartment (61), although such an agent would be expected to induce substantial hematopoietic toxicity (62). We anticipate increasing numbers of trials testing CARs capable of simultaneously targeting two or more antigens in the near term.

**LOW ANTIGEN DENSITY: CLINICAL DATA**

In recognizing that single antigen targeting was unlikely to be successful in many cases of pediatric B-ALL, we developed a CAR targeting CD22, another B-cell antigen broadly expressed on lymphoblasts (63). In the first clinical trial of this CAR in B-ALL, we observed a high remission rate in patients with both CD19-negative and CD19-positive disease. However, 8 of 12 patients (67%) attaining a CR relapsed within 12 months after CD22-CAR infusion and at the time of relapse, CD22 expression was retained in 7 patients, albeit at lower levels than observed at the time of CD22-CAR therapy. The diminished expression of CD22 was not accompanied by any detected mutations at the genomic level or diminished expression of CD22 mRNA, suggesting that downregulation of CD22 expression occurs at a posttranscriptional level. In several patients, both CD22-low lymphoblasts and persistent anti-CD22-CAR T cells were found in the bone marrow, indicating that the CAR was unable to effectively eliminate CD22-low cells (53). This was further demonstrated by generating leukemia lines with variable CD22 expression levels, and directly demonstrating that the capacity for the CD22 CAR to produce cytokine and control tumor cells in xenograft models was exquisitely dependent upon surface expression levels of CD22. However, this mechanism of escape is consistent with substantial emerging data regarding CAR T-cell activation requirements. We and others have demonstrated the need for high target antigen density in order for CARs to fully activate and exert in vivo activity (64–66). This was recently described in detail in studies focused on a CAR targeting anaplastic lymphoma kinase (ALK), wherein cytotoxic production was highly dependent upon antigen expression levels, with sub-maximal levels observed below 10,000 molecules/cell. As a result, although cell lines engineered to express very high levels of ALK were readily eradicated in vivo, those expressing physiologic levels were not controlled by ALK-CAR T cells. Whether the activity of CD19-CAR T cells might be limited by insufficient CD19 expression remains unknown. This seems unlikely in B-ALL, because the pattern of CD19 expression in this disease appears to be homogeneously high. However, CD19 expression in CAR trials for NHL has not been systematically studied, as researchers have largely relied on IHC where quantification is unreliable (16).

The biological basis for the requirement for high target antigen levels for optimal CAR T-cell activity remains incompletely understood, but could reflect limitations in the nature of antigen recognition by CAR receptors (Fig. 1). Natural TCRs are capable of recognizing antigen at low density, making it tempting to speculate that the differences in antigen density requirements may emerge from the dramatic differences in structure between natural TCRs and CARs. CARs are elegant in their simplicity, but are also a crude imitation of the highly evolved system of TCR antigen recognition. Natural TCRs contain several signaling domains (gamma, delta, epsilon, zeta), whereas CARs typically incorporate TCRζ as the sole TCR signaling element. Although data suggest that signals downstream of TCRζ largely replicate that of the complete TCR signaling complex (6, 67), this matter remains incompletely investigated. Furthermore, during the course of antigen recognition, natural TCRs create a highly organized immune synapse that incorporates coreceptors to enable recognition of very low antigen density (68, 69). Emerging studies demonstrate that the immune synapse created when CARs recognize antigen is less organized than that of a natural TCR (70). Finally, the nature of antigen binding itself differs substantially between the TCR and the CAR receptor, because TCRs are low-affinity binders (Kd in the micromolar range), whereas scFvs incorporated into most CARs recognize antigen with very high affinity (Kd in nanomolar range; ref. 71). These distinctions are likely to significantly affect the quality of responses induced in T cells expressing CARs as compared with natural TCRs (72), but the full extent of the distinctions remains incompletely characterized.

The recognition that CARs require high antigen expression for significant activity has numerous implications for the future development of these therapeutics for solid tumors. Given that nearly all targets on solid tumors for which clinical trials of CAR T cells are planned are heterogeneously expressed (57, 73–76), it is likely that as nonspecific CARs targeting solid tumors become more potent, clinical successes will be limited by the rapid selection of antigen low variants. CARs for AML face similar hurdles (77). Alternatively, a CAR T-cell requirement for high antigen density also opens the possibility of a therapeutic window based upon differential target antigen density between malignant and nonmalignant tissues. The potential for CAR T cells to attack normal tissues based upon low levels of antigen has been debated at length as a result of a fatal event in a single patient treated with a CAR targeting HER2 at the NCI. This patient died of cardiovascular collapse following infusion of 10e10 Her2-28-41BB-ζ CAR T cells (78, 79). CAR T-cell infiltration was found in the patient’s lungs and the cause of death was initially attributed to on-target, off-tumor toxicity. However, additional insights regarding the pathophysiology of cytokine release syndrome (CRS) following CAR T-cell therapeutics suggest that cardiovascular collapse in this patient was more likely related to uncontrolled, systemic T-cell activation leading to fatal CRS (80). The cell dose administered to this patient (78) is one hundred times the maximum tolerated dose later found for CD19-CAR T cells (21). In addition, the patient received exogenous IL2, itself associated with high levels of toxicity (81).
Consistent with CRS were dramatic elevations in circulating IFNγ levels. Moreover, Ahmed and colleagues have since used escalating doses of HER2-CD28+ CAR T cells to treat patients with sarcomas and demonstrated both safety and initial signs of clinical efficacy, including a CR in a patient with rhabdomyosarcoma in his bone marrow (82, 83). Together, the clinical experience is most consistent with a model whereby differential expression of the HER2 target antigen between tumor and normal tissues provides a therapeutic window for safety, which is consistent with the emerging understanding of the need for high antigen density for optimal CAR activity in vivo.

OVERCOMING LOW ANTIGEN DENSITY

For appropriate targets where the differential expression of target antigen between tumor and normal tissue is high, we predict that the efficacy of CAR therapies would be enhanced by engineering CAR T cells to respond to lower antigen densities. One approach would be to treat patients with agents that increase expression of the target antigen. In preclinical studies, this approach has been taken using all-trans retinoic acid to increase expression of folate receptor beta in AML (84), and researchers at the NCI have found that bryostatin can increase expression of CD22 on leukemia cells, which could lead to increased efficacy of the CD22 CAR or prevention of outgrowth of CD22-low variants (85, 86).

Alternatively, CAR engineering could be used to enhance activity against lower antigen densities. The most commonly attempted modification has been to enhance the affinity of the ScFv for its target. For some CARs, it appears that altering the affinity can result in recognition of lower levels of target antigen. Two groups have demonstrated that altering the affinity of EGFR and/or HER2 CARs can result in T cells that are more or less likely to recognize lower levels of antigen as might be expressed on normal tissue (87, 88); however, it remains unclear whether the impact of enhancing scFv activity plateaus. This is suggested by the fact that increasing the affinity of two different CD22 CARs did not result in enhanced function (63, 85).

CONCLUSION

Clinical experience with B-cell malignancies has demonstrated that CAR T cells have the potential to alter the landscape of cancer immunotherapy. However, the emergence of antigen-negative and antigen-low tumor variants has shown that, like all anticancer agents, CARs are likely to require combinatorial approaches to bring about cures in a high fraction of patients. Whereas in hematologic malignancies, lineage-derived antigens are expressed at high levels and can be efficiently targeted by CARs, in solid tumors most viable antigens are expressed at lower levels and more heterogeneously. Reengineering CARs for multispecificity and activity at lower levels of antigen will be an area of important research as the community attempts to enhance the potency of CAR T cells and the breadth of diseases for which they can provide clinically meaningful effects.

Disclosure of Potential Conflicts of Interest

C.L. Mackall reports receiving commercial research grants from Bluebird Bio and Obsidian, has ownership interest (including stock, patents, etc.) in Juno Therapeutics, and is a consultant/advisory board member for Unum Therapeutics, Adaptimmune, GlaxoSmithKline, Vor Pharmaceuticals, Allogene, and NCarta Therapeutics. No potential conflicts of interest were disclosed by the other author.

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Overcoming Antigen Escape from CAR T-cell Therapy


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MINI REVIEW


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β-Catenin Activation Promotes Immune Escape and Resistance to Anti–PD-1 Therapy in Hepatocellular Carcinoma

Marina Ruiz de Galarreta¹²³, Erin Bresnahan¹²³, Pedro Molina-Sánchez¹²³, Katherine E. Lindblad¹²³, Barbara Maier¹³, Daniela Sia², Marc Pulgvehi²,5, Verónica Miguela¹²³, María Casanova-Acebes¹³, Maxime Dhainaut¹³, Carlos Villacorta-Martín², Aatur D. Singhi⁶⁷, Akshata Moghe⁶, Johann von Felden²,8, Lauren Tal Grinspan¹²³, Shuang Wang², Alice O. Kamphorst¹³,4, Satdarshan P. Monga⁶⁷, Brian D. Brown³,4, Augusto Villanueva²⁴, Josep M. Llovet²⁹¹⁰, Miriam Merad¹³,4, and Amaia Lujambio¹²³
PD-1 immune checkpoint inhibitors have produced encouraging results in patients with hepatocellular carcinoma (HCC). However, what determines resistance to anti-PD-1 therapies is unclear. We created a novel genetically engineered mouse model of HCC that enables interrogation of how different genetic alterations affect immune surveillance and response to immunotherapies. Expression of exogenous antigens in MYC;Trp53−/− HCCs led to T cell-mediated immune surveillance, which was accompanied by decreased tumor formation and increased survival. Some antigen-expressing MYC;Trp53−/− HCCs escaped the immune system by upregulating the β-catenin (CTNNB1) pathway. Accordingly, expression of exogenous antigens in MYC;CTNNB1 HCCs had no effect, demonstrating that β-catenin promoted immune escape, which involved defective recruitment of dendritic cells and consequently impaired T-cell activity. Expression of chemokine CCL5 in antigen-expressing MYC;CTNNB1 HCCs restored immune surveillance. Finally, β-catenin–driven tumors were resistant to anti-PD-1. In summary, β-catenin activation promotes immune escape and resistance to anti-PD-1 and could represent a novel biomarker for HCC patient exclusion.

SIGNIFICANCE: Determinants of response to anti–PD-1 immunotherapies in HCC are poorly understood. Using a novel mouse model of HCC, we show that β-catenin activation promotes immune evasion and resistance to anti–PD-1 therapy and could potentially represent a novel biomarker for HCC patient exclusion.

See related commentary by Berraondo et al., p. 1003.

INTRODUCTION

Hepatocellular carcinoma (HCC) represents a major health problem, causing more than 700,000 deaths annually worldwide (1). Although HCC treatment has greatly improved over the last decades, patients with HCC diagnosed at advanced stages are ineligible for curative ablative therapies such as liver resection or transplantation. Until recently, the only FDA-approved therapy for such patients was sorafenib (2), a multikinase inhibitor that provides a 3-month survival benefit on average. In the last two years, several other multikinase inhibitors have shown efficacy in patients with advanced HCC (3–5). Lenvatinib has been approved as a first-line therapy (3), and regorafenib, an inhibitor closely related to sorafenib, is approved in second line (4). Unfortunately, these multikinase inhibitors also confer limited survival benefits. More recently, nivolumab and pembrolizumab, two PD-1 immune checkpoint inhibitors, were granted accelerated approval by the FDA for HCC treatment in second line after obtaining promising outcomes in phase II clinical trials (6, 7). The results from the nivolumab and pembrolizumab trials showed that some patients with HCC achieve unprecedented responses (6, 7). However, not all patients are sensitive, indicating the existence of mechanisms that drive resistance to anti–PD-1 therapy and highlighting the urgent need to identify biomarkers for optimal patient selection.

Cancer immunotherapy is revolutionizing the clinical management of a variety of cancers (8). Among the different immunotherapy strategies, PD-1 pathway inhibitors have provided the best clinical outcomes (9–11). Unfortunately, the clinical efficacy of PD-1 pathway inhibition as monotherapy is limited to subsets of patients, with overall response rates of 20% or less (9). In other malignancies, response rates have been significantly improved through selection of patients presenting mismatch repair deficiency (12, 13) or the combination of PD-1 pathway inhibition with other therapeutic strategies, such as CTLA4 mAbs (10), strongly supporting efforts to identify biomarkers for patient selection and novel combinatorial therapies. The general consensus is that anti–PD-1 therapies are effective in tumors that are able to trigger some level of antitumor immunity, as evidenced by the existence of CD8+ T-cell infiltrates (9). Conversely, most tumors that disrupt
antitumor immunity lack CD8+ T-cell infiltration and tend to be resistant (9). Tumor-intrinsic properties, such as mutational load (14, 15), presentation of tumor antigens (16, 17), or specific oncogenic pathways (18, 19), can greatly influence antitumor immunity and response to anti-PD-1 therapies. In melanoma, activation of β-catenin (encoded by CTNNB1; ref. 19) or PTEN deletion (18) can lead to T-cell exclusion and resistance to anti-PD-1. In HCC, two recent studies in patients have shown that β-catenin activation correlates with T-cell exclusion (20) and resistance to anti-PD-1 therapy (21). However, the mechanistic link between β-catenin activation and immune resistance has not been provided, in part due to the relative delay of the clinical trials testing immunotherapies in HCC when compared with other malignancies [such as melanoma or non–small cell lung cancer (NSCLO)], and also due to the lack of appropriate models.

Several mouse models have been generated to gain insights into the mechanisms by which tumors may subvert immune responses, but each of these has critical limitations (22, 23). For example, transplantation of primary or cultured tumor cells is commonly used, but the ectopic introduction of fully developed tumor cells bypasses the initial steps of tumorigenesis and can lead to aberrant inflammatory responses (24, 25). Carcinogen-induced models lead to robust immune responses, but the presence of multiple and heterogeneous mutations hampers the understanding of the contribution of each mutated gene to the observed phenotypes (26). Genetically engineered mouse models (GEMM) of cancer accurately recapitulate both the genetic and histopathologic progression of human disease (27), but tumors tend to be nonimmunogenic and therefore fail to reproduce the interplay between tumor cells and the immune system that is characteristic of human tumors (23). Transgenic mouse models of cancer that develop tumors spontaneously and overexpress model antigens throughout targeted organs exist, but the widespread expression of the antigens tends to induce tolerance (28), failing to recapitulate the immune responses against human tumors. Recently, Tyler Jacks’s laboratory has addressed these limitations by combining a conditional GEMM (KrasG12D; Cre; Sin; Ova257-264) with the delivery of lentiviruses that simultaneously express Cre recombinase (which recombines the Lox sites, allowing the expression of mutant Kras and deletion of Trp53) and exogenous antigens (17, 29). The expression of exogenous antigens in mosaic tumor cells led to tumor delay as a result of tumor immune surveillance (17, 29) and formally demonstrated cancer immunoeediting in vivo (17). Although this strategy represents a technical and conceptual advancement from previous models, it is limited by the availability of existing conditional GEMMs.

In an effort to investigate the role that different genetic alterations have in HCC immune surveillance and response to immunotherapies, we have adopted a system to quickly induce autochthonous and mosaic liver tumors that harbor specific and customizable genetic alterations and varying levels of immunogenicity. The model is based on the hydrodynamic tail-vein delivery of genetic elements (30) to overexpress oncogenes (with transposon-based vectors), delete or mutate tumor suppressor genes (with CRISPR/Cas9 vectors), and modulate immunogenicity (with exogenous antigens) specifically in hepatocytes. This model, which is amenable to rapid genetic manipulation, is technically and conceptually innovative, as it will allow us to study how different tumor-intrinsic signaling pathways affect antitumor immunity. With this model, we have shown that β-catenin activation promotes immune escape in HCC. Mechanistically, β-catenin activation led to a defective recruitment of dendritic cells (DC) and antigen-specific T cells, and as a consequence, to an impaired antitumor immune response. Reexpression of chemokine (C-C motif) ligand 5 (CCL5), a chemokine found to be downregulated in both murine and human tumors driven by β-catenin activation, restored immune surveillance. Finally, β-catenin activation conferred resistance to anti-PD-1 therapy in our murine model. We have shown that our model can be used to identify mechanisms of immune escape and resistance to anti-PD-1 that are relevant to human disease and could provide the rationale for improved patient selection and personalized cancer immunotherapies.

RESULTS

Expression of Exogenous Antigens in Murine MYC;Trp53−/− HCCs Leads to a Delay in Tumor Development

Two of the most frequently altered genes in patients with HCC are the oncogene MYC (amplified in 17% of HCCs) and the tumor suppressor TP53 (deleted or mutated in 33% of HCCs). Their alterations frequently co-occur in patients with HCC (6.5%), suggesting cooperation (Fig. 1A). We previously showed that we can generate liver tumors resembling human HCC by performing hydrodynamic tail-vein injections of a transposon vector expressing MYC (pT3-EEF1a-MYC), a vector expressing SB13 transposase (CMV-SB13), which is required to integrate the transposon-based vector into the hepatocyte genomic DNA, and a CRISPR/Cas9 vector expressing a single-guide RNA (sgRNA) targeting Trp53 (pX330-sg-p53; ref. 31). Hydrodynamic tail-vein injections (30) allow the delivery of DNA specifically into the hepatocytes by creating an increase in blood pressure that redirects the flow of blood directly into the liver. To modulate the immunogenicity of the MYC;Trp53−/− liver tumors, we modified the transposon vector expressing MYC to also express luciferase (MYC-luc), which is mildly immunogenic (32), or a highly immunogenic version of luciferase (MYC-lucOS) that is linked to three model antigens: SIYRYYGL (SIY), SIINFEKL (SIN; OVA257-264), and OVA323-339 (Fig. 1B; ref. 29). Hydrodynamic injection of pX330-sg-p53 and CMV-SB13 in combination with MYC-luc or MYC-lucOS into 6-week-old C57BL/6 female mice led to equivalent luciferase expression in the livers measured by bioluminescence imaging at day 6, indicating similar injection efficiency and expression levels in both groups (Fig. 1C and D). Interestingly, 25 days after the injection there was a drastic reduction in luciferase signal in MYC-lucOS;sg-p53 mice, suggesting clearance of luciferase and antigen-expressing hepatocytes (Fig. 1C and D). Additional experiments demonstrated that the decrease in luciferase signal in MYC-lucOS;sg-p53 mice occurred by day 13 after the injection (Supplementary Fig. S1A). Accordingly, tumor formation was markedly delayed in MYC-lucOS;sg-p53 mice compared with MYC-lucOS;sg-p53 mice and was accompanied by a significant increase in survival (Fig. 1E and F). Similar effects were observed in female and male C57BL/6 mice, indicating that the phenomenon occurs
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A

**A**

TP53 (33%)
MYC (17%)

**B**

**C**

**D**

**E**

**F**

**G**

**Figure 1.** Expression of exogenous antigens in murine MYC;Trp53−/− HCCs leads to tumor delay. **A,** Oncoprint of TP53 and MYC alterations in 366 patients with HCC (TCGA, provisional, December 2018, cBioPortal; ref. 61). The percentage of patients harboring the alteration is shown. Amp, amplification; del, deletion; trun, truncating; mut, mutation; infr, in-frame; miss, missense. **B,** Schematic of vectors injected into mice. The transposon-based vector over-expressing MYC can also express luciferase (luc) or a luciferase fused to model antigens (lucOS). **C,** Bioluminescence imaging 6 and 25 days after injection of vectors into representative mice. The color code for the luciferase signal is shown. **D,** Quantification of normalized luciferase signal 6 and 25 days after injection of vectors (n = 5 per group). Mean and SD are shown. Mann–Whitney test. Survival curves in C57BL/6 WT females (**E**) and males (**F**). Number of mice per group is shown as well as median survival. Undef, undefined. Log-rank Mantel–Cox test. **G,** Pictures of representative livers from **E** and **F.** The number indicates the number of days from injection to death for that particular mouse. Scale bars, 1 cm. **,** P < 0.01; **,** P < 0.001; **,** P < 0.0001.

irrespective of sex (Fig. 1E and F). Most MYC-lucOS;sg-p53 mice did not develop any tumors within 4 months, whereas the majority of MYC-luc;sg-p53 mice presented gross liver tumors (Fig. 1G) that caused death, with a median survival of 35 to 44 days (Fig. 1E and F). Deep sequencing analysis of MYC-luc;sg-p53 livers 7 days after the injection detected 5.645% frameshift mutations at the sg-p53 target site, whereas only 0.457% indels were found in mice injected without sg-p53, and those indels were spread across the whole sequence, indicating background sequencing errors (Supplementary Fig. S1B and S1C). This confirms that px330-sg-p53 can directly generate mutations in Trp53 in the mouse liver (33). In established tumors, approximately 80% frameshift mutations and prevalence of two specific indels that produce truncated proteins were detected in tumors with sg-p53 (Supplementary Fig. S1D and S1E), suggesting selection from a single Trp53-mutated cell. In addition, transgenic MYC overexpression was confirmed in MYC;Trp53−/− HCCs when compared with normal
CD8+ T Cells Eliminate Antigen-Expressing MYC;Trp53−/− HCCs

To functionally interrogate the involvement of T cells in the elimination of antigen-expressing cancer cells, we performed hydrodynamic injection of px330-sg-p53 and CMV-SB13 in combination with MYC-luc or MYC-lucOS into 6-week-old B and T cell–deficient Rag2−/− mice in C57BL/6 background (Fig. 2A and B; Supplementary Fig. S2A–S2D). The survival benefit observed in wild-type (WT) mice harboring MYC-lucOS;sg-p53 tumors was abolished in Rag2−/− mice (Figs. 1E and F and 2A and B; Supplementary Fig. S2A and S2B), confirming the role of lymphocytes in eliminating antigen-expressing hepatocytes. In contrast, lack of B and T cells in Rag2−/− mice had no significant effect in the development of MYC-luc;sg-p53 tumors (Supplementary Fig. S2C and S2D), indicating that the expression of antigens is critical for an effective lymphocyte-mediated immune response. Both MYC-lucOS;sg-p53 and MYC-luc;sg-p53 Rag2−/− mice developed large macroscopic tumors (Fig. 2C) that caused death, with a median survival of 32 to 50 days (Fig. 2A and B; Supplementary Fig. S2A–S2D). Luciferase signal was similar between WT and immunodeficient mice three days after the
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hydrodynamic injection (Supplementary Fig. S2E), ruling out that the differences observed in tumorigenesis could be due to distinct hepatocyte transfection efficiency. Moreover, injection of anti-CD4 and anti-CD8 antibodies into MYC-lucOS;sg-p53 WT mice durably depleted CD4+ and CD8+ T cells (Supplementary Fig. S2F and S2G) and led to a decrease in survival when compared with mice treated with control antibodies (Fig. 2D–F), further confirming the role of T lymphocytes in eliminating antigen-expressing hepatocytes. Additional experiments demonstrated that mainly CD8+ (P = 0.0351) but also CD4+ T cells to some extent (P = 0.0730) were involved in the elimination of antigen-expressing MYC;Trp53−/− tumor cells (Fig. 2E and F; Supplementary Fig. S2G). Taken together, expression of exogenous antigens in the context of MYC overexpression and Trp53 loss in murine hepatocytes leads to immune surveillance mediated primarily by CD8+ T cells.

β-Catenin Signaling Is Activated in Immune-Escaped MYC;Trp53−/− HCC Tumors

The expression of antigens in the context of murine MYC;Trp53−/− HCC tumors leads to immune surveillance. However, the clearance of tumor cells was not complete in all mice, and some tumors eventually escaped the immune system (Fig. 1E and F). To identify the signaling pathways involved in the immune escape of MYC-lucOS;sg-p53 tumors, we performed RNA sequencing (RNA-seq) of bulk tumors from MYC-luc;sg-p53 and MYC-lucOS;sg-p53 female mice. Gene set enrichment analysis (GSEA; ref. 34) was used to evaluate functional enrichment of datasets related to different signaling pathways involved in HCC. Analysis of 188 oncogenic signatures available at MSigDB Collections (35) showed no significant differences between the two groups (Supplementary Table S1). Analysis of four HCC-specific gene signatures (36) demonstrated that a molecular class associated with CTNNB1 (β-catenin)-mutant human HCCs was significantly enriched in escaped MYC-lucOS;sg-p53 tumors, which was confirmed in a larger set of MYC-lucOS;sg-p53 and escaped MYC-lucOS;gtg-p53 tumors by qRT-PCR (Fig. 3C) and also by protein analysis (Supplementary Fig. S3B). Only one out of 22 MYC-luc;sg-p53 tumors presented Axin2 mRNA levels that were higher than the mean expression in escaped MYC-lucOS;sg-p53 tumors, whereas eight out of 23 MYC-lucOS;sg-p53 tumors had Axin2 levels higher than the mean (Fig. 3D).

To better understand mechanisms of immune escape in HCC, we performed RNA-seq of four additional escaped MYC-lucOS;sg-p53 tumors with low levels of Axin2 (hereafter referred to as low-Axin2–escaped tumors), which suggests they escaped through a different immune escape mechanism, and compared them to the escaped MYC-lucOS;sg-p53 tumors analyzed before (hereafter referred to as Axin2-escaped tumors; Fig. 3A and B). As expected, the molecular class associated with CTNNB1 (β-catenin)-mutant human HCCs (36) was enriched in Axin2-escaped tumors (Fig. 3E). Interestingly, a gene set related to adaptive immune response was significantly enriched in low-Axin2–escaped tumors (Fig. 3F), suggesting that there may be an association between β-catenin activation and the type of immune escape mechanism. CTNNB1 mRNA levels were unchanged (Supplementary Fig. S3C), which suggests that β-catenin activation, rather than CTNNB1 mRNA levels, is critical for its activity. To test whether β-catenin activation occurred in tumor cells, we separated tumor cells from immune cells in escaped MYC-lucOS;sg-p53 tumors by Percoll gradient centrifugation. Axin2 was found predominantly overexpressed in tumor cells in the escaped MYC-lucOS;sg-p53 tumors (Supplementary Fig. S3D). However, because stromal cells could not be separated from the bulk hepatocyte fraction, we cannot rule out that stromal cells may contribute to the increase in Axin2 levels and activation of the β-catenin pathway seen in MYC-lucOS;sg-p53 tumors. Interestingly, β-catenin activation promotes immune escape and resistance to anti–PD-1 therapy in HCC tumors (20) and resistance to anti–PD-1 therapy in patients with HCC (21), which together with our data suggests a role for β-catenin activation in HCC immune escape in a subset of HCC tumors.
that antigen expression in the context of MYC overexpression and β-catenin activation does not lead to immune surveillance and β-catenin activation promotes immune escape.

Luciferase signal was equivalent in MYC-luc;sg-p53, MYC-lucOS;sg-p53, MYC-luc;CTNNB1, and MYC-lucOS;CTNNB1 livers six days after the hydrodynamic injection (Supplementary Fig. S4D), excluding that differences in initial hepatocyte transfection could have an effect on the immune surveillance and immune escape observed in the MYC-lucOS;sg-p53 and MYC-lucOS;CTNNB1 mice, respectively. Furthermore, MYC-luc;sg-p53 and MYC-luc;CTNNB1 mice, which are not subjected to immune pressure, presented similar median survival [35 vs. 35.5 days in females (Figs. 1E and 4E); 44 vs. 42 days in males (Figs. 1F and 4F)], indicating that the tumor growth rate was similar in both models. As expected, MYC;CTNNB1 tumors overexpressed MYC, activated β-catenin, and displayed WT p53 (Supplementary Figs. S1E and S4E). To address whether β-catenin activation is truly driving immune escape of MYC-lucOS;CTNNB1 tumors and to rule out the

Figure 3. β-catenin signaling is activated in immune-escaped HCC tumors. A, GSEA of an HCC CTNNB1 gene signature in escaped MYC-lucOS;sg-p53 tumors (n = 3 per group). NES, normalized enrichment score. B, Volcano plot representing genes according to their fold change and P value in MYC-luc;sg-p53 escaped tumors when compared with MYC-lucOS;sg-p53 tumors. Axin2 is highlighted in red. C, Relative levels of Axin2 in MYC-luc;sg-p53 and escaped MYC-lucOS;sg-p53 tumors by qRT-PCR. Each dot represents one tumor coming from one independent mouse. Number of samples is shown. The samples used in the RNA-seq (A and B) are highlighted in blue. Mean and SD are shown. Mann–Whitney test. D, Number of cases with Axin2 levels higher (blue) or lower (black) than 2.5 (which is the mean value in MYC-lucOS;sg-p53 tumors in C). Number of samples is shown. Fisher exact test. GSEA of an HCC CTNNB1 gene signature (E) and an adaptive immune response signature (F) in Axin2-escaped MYC-lucOS;sg-p53 tumors (n = 3) or low-Axin2-escaped MYC-lucOS;sg-p53 tumors (n = 4) *. P < 0.05.
**Figure 4.** β-catenin signaling activation promotes immune escape in HCC. A, Oncoprint of CTNNB1 and MYC alterations in 366 patients with HCC (TCGA, provisional, December 2018, cBioPortal; ref. 61). The percentage of patients harboring the alteration is shown. Amp, amplification; del, deletion; trun, truncating; mut, mutation; infr, in-frame; miss, missense. B, Schematic of vectors injected into mice. The transposon-based vector overexpressing MYC can also express luciferase (luc) or a luciferase fused to model antigens (lucOS). C, Bioluminescence imaging 6 and 27 days after injection of vectors into representative mice. The color code for the luciferase signal is shown. D, Quantification of normalized luciferase signal 6 and 27 days after injection of vectors (n = 5 per group). Mean and SD are shown. Mann–Whitney test. Survival curves in C57BL/6 WT females (E) and males (F). Number of mice per group is shown as well as median survival. Log-rank Mantel–Cox test. G, Pictures of representative livers from E and F. The number indicates the number of days from injection to death for that particular mouse. Scale bars, 1 cm. *, P < 0.05.

potential involvement of WT p53, we assessed the effect of mutating p53 in the context of MYC-lucOS;CTNNB1 tumors. Tumor formation and survival were equivalent in MYC-lucOS;CTNNB1;sg-p53 and MYC-lucOS;CTNNB1 mice (Supplementary Fig. S4F and S4G), confirming the role of β-catenin activation in driving immune escape of MYC-lucOS;CTNNB1 tumors. These results also indicate that β-catenin activation can directly promote immune escape of MYC-lucOS;sg-p53 tumors, which otherwise undergo immune surveillance (Fig. 1).

**β-Catenin Activation Impairs DC Recruitment in the Context of HCC**

There are multiple mechanisms by which cancer cells escape the immune system, involving changes in cancer and/or immune cells (8). To identify the potential changes in cancer...
cells contributing to β-catenin activation–mediated immune escape, we performed RNA-seq of bulk tumors from MYC-lucOS;CTNNB1 and MYC-lucOS;CTNNB1 female mice. As expected, β-catenin–driven tumors were significantly enriched in the dataset representing CTNNB1-mutant human HCCs (36) when compared with MYC-lucOS;sg-p53 tumors (Supplementary Table S2). In addition, levels of Axin2, a direct target of β-catenin, were higher in the β-catenin–driven tumors when compared with MYC-lucOS;sg-p53 tumors by qRT-PCR in a larger subset of tumors (Supplementary Fig. S5A). Moreover, luciferase transcripts were present in both groups, at similar levels, whereas transcripts corresponding to the OS region of lucOS were present in only MYC-lucOS;CTNNB1 tumors, confirming that antigen expression was not lost in the immune-escaped tumors (Supplementary Fig. S5B). High PD-L1 (CD274) has been observed in metastatic CTNNB1-mutant HCC tumor cells (40).

In our murine tumors, Pdl1 expression was similar in MYC-lucOS;sg-p53 and β-catenin–driven tumors cells (Supplementary Fig. SSC), suggesting that the immune escape observed in β-catenin–driven tumors was not due to Pdl1 expression in tumor cells. Interestingly, transcriptional differences between MYC-lucOS;CTNNB1 and MYC-lucOS;CTNNB1 tumors were negligible (156 genes overexpressed and 183 genes downregulated in MYC-lucOS;CTNNB1 tumors compared with MYC-lucOS;CTNNB1 tumors). The lack of changes in the “lucOS-expressing” tumors suggested that MYC-lucOS;CTNNB1 tumors may not be subjected to immune pressure, unlike MYC-lucOS;sg-p53 tumors, and that β-catenin could drive a program that completely abolishes the antitumor immune response.

To identify mechanisms of immune escape related to changes in the immune cell compartment, we performed flow cytometry analysis of the livers two weeks after the injections, a time point that already shows a decrease in luciferase signal (Supplementary Fig. S1A), in control WT, MYC-lucOS;sg-p53, and MYC-lucOS;CTNNB1 mice. The lucOS transgene leads to the expression of the model antigens SIYRYYGL (SIY), SIINFKEL (SIIN; OVA257-264), and OVA323-339 (Supplementary Fig. S1A), in control WT, MYC-lucOS;CTNNB1 (without immune pressure) and low–β-catenin–driven tumors, which present abundant T cells at 2 weeks, compared with MYC-lucOS;sg-p53 tumors (Supplementary Table S3), we reasoned that Axin2–escaped MYC-lucOS;sg-p53 tumors may present an intermediate level of activation of β-catenin and therefore undergo an immune escape that is also phenotypically intermediate. In fact, transcripts related to DCS, such as Batf3, Isgg (Cd103), Irβ, and Thbd (Cd141), and to T cells, including Cd3d, Cd4, Cd8a, and Cd8b1, in general displayed intermediate levels compared with MYC-lucOS;sg-p53 (without immune pressure) and low-Axin2–escaped MYC-lucOS;sg-p53 tumors, which present abundant immune transcripts (Fig. S5G; Supplementary Table S3). MYC-lucOS;CTNNB1 tumors, with high activation of the β-catenin pathway, harbored significantly less immune-related transcripts, suggesting that they are immune-excluded (Fig. S5G; Supplementary Table S3). Similar results were obtained by immunofluorescence staining for the T-cell marker CD3 in MYC-lucOS;sg-p53 and MYC-lucOS;CTNNB1 livers at 2 weeks, recapitulating the flow cytometry results (Supplementary Fig. SSE and S5K), and, in established tumors, recapitulating the RNA-seq results (Fig. S5G; Supplementary Fig. S5K).

We also assessed the transcriptional profiles of 360 samples from patients with HCC [liver hepatocellular carcinoma (LIHC), available at The Cancer Genome Atlas (TCGA); ref. 37]. As expected, CTNNB1-mutant samples (97/360, 26.79%) were significantly enriched in the dataset representing CTNNB1-mutant HCCs (36) when compared with CTNNB1 WT samples (Supplementary Table S2). In fact, expression of AXIN2 and GLUL, two well-established targets of β-catenin, was significantly higher in CTNNB1-mutant samples (Fig. S5H; Supplementary Table S4). Most importantly, CTNNB1-mutant samples presented significantly reduced expression of DC markers (Batf3, Irf8, Thbd), T-cell markers (Cd3d, Cd3e, Cd4, Cd8a), and the exhaustion marker Pdcd1 (Pd-1; Fig. S5H; Supplementary Table S3), suggesting that CTNNB1-mutant HCCs exhibit immune exclusion. In fact, in a cohort of 59 HCC patient samples, nuclear staining of β-catenin was associated with significantly lower numbers of CD8+ T cells in the tumors (Fig. S1I and J), and in another cohort of 216...
Figure 5. β-catenin impairs DC recruitment in the context of HCC. Quantification of the percentage of SIINFEKL-specific CD8+ T cells (A) or number of DC1 dendritic cells (B) in the livers of the corresponding mice (n = 5–6 per group). Representative of three independent experiments. N, normal liver; sg-p53, MYC-lucOS;sg-p53; CTNNB1, MYC-lucOS,CTNNB1. Mean and SD are shown. ANOVA test. C, Survival curves in C57BL/6 WT or Batf3−/− females harboring MYC-lucOS;sg-p53, MYC-lucOS;CTNNB1 females. Mean and SD are shown. ANOVA test. Heat map showing the average expression values of different genes in murine tumors (G) and human TCGA tumors (H). Colors are adjusted for each row and for each group comparison from high (red) to low (blue). luc p53, MYC-lucOS;sg-p53 (n = 3); lucOS p53, MYC-lucOS;sg-p53 (n = 3); low Axin2, low-Axin2-escaped MYC-lucOS;sg-p53 (n = 4); luc CTNNB1, MYC-lucOS;CTNNB1 (n = 7); lucOS CTNNB1, MYC-lucOS;CTNNB1 (n = 5); CTNNB1 WT, wild-type (n = 263); (CTNNB1 mutant, (n = 97); CTNNB1 low (n = 120), CTNNB1 high (n = 120). Samples were stratified depending on CTNNB1 status as WT or mutant, or CTNNB1-mutant HCC gene signature enrichment levels (in tertiles). I, Number of CD8+ T cells in tumor and peritumor areas in HCC patient samples (n = 59), which were classified as having membrane (mb; n = 37) or nuclear (nuc; n = 22) staining for β-catenin protein. Mean and SD are shown. Mann–Whitney test. J, Representative pictures of the stainings for CD8 and β-catenin summarized in I. *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001.
patients with HCC, those with enrichment of CTNNB1-mutant HCC signature (36) were associated with a significant decrease in immune cell infiltration assessed by hematoxylin and eosin staining (Supplementary Fig. SSL). To test the importance of β-catenin pathway activation levels on the immune escape phenotype, we stratified the 360 patients with HCC according to their level of enrichment of the dataset representing CTNNB1-mutant HCCs (ref. 36; low, first tertile; intermediate, second tertile; high, third tertile). As observed in the murine tumors (Fig. S5G; Supplementary Table S3), samples from patients with HCC with intermediate and high activation of the β-catenin pathway presented less immune cell transcripts than samples in the low activation group, further suggesting that β-catenin pathway activation levels have an impact on the extent of immune exclusion. Taken together, the immune escape driven by β-catenin activation is mediated by a defect in DC recruitment, which in turn impairs the subsequent antitumor immune response, in both murine and human HCCs.

**CCL5 Expression Restores Immune Surveillance in β-Catenin–Driven HCCs**

To identify mechanisms explaining the defective DC activity in the context of β-catenin activation in HCC, we explored the expression of chemokines in MYC-lucOS;p53 tumors (control; not exposed to immune pressure) and β-catenin–driven tumors (MYC-lucOS;CTNNB1 and MYC-luc;CTNNB1). Six chemokines (CCL5, CXCL1, CCL20, CXCL12, CXCL17, and CXCL10) out of 34 chemokines quantified by RNA-seq were significantly downregulated in β-catenin–driven tumors (there were no chemokines upregulated; Fig. 6A; Supplementary Fig. S6A; Supplementary Table S5). Among these, CCL5, CXCL1, CCL20, CCL28, CCL17, and CXCL10 out of 34 chemokines quantified by RNA-seq were significantly downregulated in β-catenin–driven tumors (there were no chemokines upregulated; Fig. 6A; Supplementary Fig. S6A; Supplementary Table S5). Among these, CCL5, CXCL1, CCL20, CXCL28, CCL30, and CXCL11 were also downregulated in human CTNNB1-mutant HCC samples (Supplementary Fig. S6B; Supplementary Table S6). Because CCL5 has been shown to affect different immune cells, including DCs (42), we decided to focus on CCL5. We further confirmed the low levels of CCL5 in murine β-catenin–driven tumors by qRT-PCR of an independent subset of MYC-lucOS;p53 tumors (control; not exposed to immune pressure) and β-catenin–driven tumors (MYC-lucOS;CTNNB1 and MYC-luc;CTNNB1; Supplementary Fig. S6C). CCL5 levels were similar in MYC-lucOS;p53 and Axin2-escaped MYC-lucOS;p53 tumors. However, compared with low-Axin2-escaped MYC-lucOS;p53 tumors, CCL5 levels were slightly lower (although not statistically significant) in Axin2-escaped MYC-lucOS;p53 tumors (Supplementary Fig. S6D), which present intermediate activation of the β-catenin pathway (Fig. 5G). Similarly, in TCGA HCC patient samples, there was a graded decrease in CCL5 expression with increasing β-catenin activation: Tumors with the lowest activation of the β-catenin pathway showed higher CCL5 expression than tumors with intermediate activation of the β-catenin pathway (although again not statistically significant; Fig. 6B). However, tumors with high activation of the β-catenin pathway displayed significantly less CCL5 than tumors with low activation of β-catenin, further supporting the link between β-catenin and CCL5 expression (Fig. 6B). CCL5 was found to be expressed in both immune and tumor cells in MYC-lucOS;p53 mice (Supplementary Fig. S6E). Interestingly, CCL5 expression increased significantly in MYC-lucOS;p53 livers between 7 and 21 days (Supplementary Fig. S6F), replicating the timing of immune cell infiltration and suggesting that CCL5 may be a critical mediator of the antitumor immune response.

To test whether or not CCL5 overexpression in tumor cells could somehow elicit an antitumor immune response and revert the immune escape observed in β-catenin–driven tumors, we tested the effects of the expression of model antigens in the context of simultaneous β-catenin activation and CCL5 overexpression. For that, we cloned a cDNA encoding for Ccl5 in the same vector as CTNNB1-N90 (Fig. 6C). We then performed hydrodynamic tail-vein injections of this vector in combination with MYC-luc or MYC-lucOS and CMV-SB13 into 6-week-old C57BL/6 female mice. Tumor formation and survival were significantly delayed in MYC-lucOS;CTNNB1-Ccl5 mice when compared with MYC-luc;CTNNB1-Ccl5 mice, suggesting that CCL5 expression restores immune surveillance in the context of β-catenin activation and antigen expression (Fig. 6D and E). Mechanistically, expression of CCL5 in β-catenin–driven tumors led to a significant increase in the levels of DC1 (DAPI-CD45lin MHCIilCD11cCD24+CD103+CD11b−; Fig. 6F) and antigen-specific CD8+ T cells (Fig. 6G) compared with healthy livers, which could potentially explain the restoration of immune surveillance. As expected, injection of anti-CD4 and anti-CD8 antibodies into MYC-lucOS;CTNNB1-Ccl5 WT mice led to a decrease in survival when compared with mice treated with control antibodies (Fig. 6H and I). This is similar to the effects seen in DC-deficient Batf3−/− mice (Fig. 6J–L) and further confirms the role of CCL5 in mounting an antitumor immune response. In conclusion, CCL5 expression is downregulated in β-catenin–driven murine and human HCCs, and CCL5 reexpression leads to the restoration of the immune surveillance program.

**β-Catenin Signaling Activation Confers Resistance to Anti–PD-1 Therapy in HCC**

Nivolumab and pembrolizumab, two anti-PD-1 inhibitors, have recently been approved by the FDA for second-line therapy in patients with advanced HCC (6, 7). To test the therapeutic relevance of β-catenin–driven immune escape in response to anti-PD-1, we treated our novel mouse models of HCC with blocking mAbs against murine PD-1. Of note, because most MYC-lucOS;p53 mice do not develop tumors (Fig. 1E–G), a higher dose of vector DNA was used to force tumor formation. Mice harboring MYC-lucOS;p53 tumors were responsive to anti–PD-1 treatment (Fig. 7A). In contrast, mice harboring MYC-lucOS;p53 tumors did not respond (Fig. 7B), demonstrating that expression of tumor antigens is a requirement for responding to anti–PD-1 therapy. In the case of β-catenin–driven tumors, neither MYC-lucOS;CTNNB1 or MYC-luc;CTNNB1 models were responsive to anti–PD-1, proving that β-catenin activation promotes resistance to immunotherapy in our models (Fig. 7C and D).

The association between β-catenin activation and resistance to anti–PD-1 therapy has been observed in patients with HCC (21). None of the 10 patients with HCC with activating mutations in CTNNB1 had response to anti–PD-1 or anti–PD-L1 therapy whereas 50% of CTNNB1 WT patients had a response. To test this further, we collected tumor specimens from 15 patients with HCC treated with nivolumab at Mount Sinai Hospital. Overall, 6 (40%) patients responded to anti–PD-1 therapy, with a median survival time of 22.2 months,
Figure 6. CCL5 expression restores immune surveillance in β-catenin-driven HCCs. A, Venn diagram displaying the chemokines differentially expressed in mice or human liver tumors. The number of samples for each dataset is included. Red, significantly upregulated; blue, significantly downregulated. The intersection shows the chemokines dysregulated in both datasets. B, Expression of CCL5 in 360 human HCC samples (LIHC, liver hepatocellular carcinoma, from the TCGA). Box and whisker plot, with the central line representing the median, the ends of the box representing the upper and lower quartiles, and the whiskers extending to the highest and lowest observations. Mann–Whitney test. CTNNB1 signature low (n = 120), intermediate (inter; n = 120), and high (n = 120). C, Schematic of vectors injected into mice. The transposon-based vector overexpressing CTNNB1 also expresses Ccl5. D, Survival curves in C57BL/6 WT females. Number of mice per group is shown as well as median survival. Log-rank Mantel–Cox test. E, Pictures of representative livers from D. The number indicates the number of days from injection to death for that particular mouse. Scale bars, 1 cm. F and H, Number of DC1 dendritic cells (F) or percentage of SIINFEKL-specific CD8+ T cells (G) in the livers of the corresponding mice (n = 5–7 per group). N and N, normal liver; CTNNB1-Ccl5, Myc-lucOS;CTNNB1-Ccl5. Mean and SD are shown. Mann–Whitney test. H, Survival curves in C57BL/6 WT females with combined CD4+ and CD8+ T-cell depletion. Number of mice per group is shown as well as median survival. Undef, undefined. Log-rank Mantel–Cox test. J and K, Survival curves in C57BL/6 WT or Batf3−/− males (J) and females (K). Number of mice per group is shown as well as median survival. Undef, undefined. Log-rank Mantel–Cox test. L, Pictures of representative livers from K. The number indicates the number of days from injection to death for that particular mouse. Scale bars, 1 cm. *, P < 0.05; **, P < 0.01; ***, P < 0.001.
and 9 (60%) patients did not respond, with a median survival of 6.2 months (P = 0.034; Supplementary Fig. S7A). Of note, 3 patients harbored CTNNB1 mutations, two being nonresponders and one being a responder (Fig. 7E and F). Because of the small sample size (power calculation of sample size indicates that at least 89 patients would be needed), we were not able to establish statistical significance. Nevertheless, our data together with the previously published study (21) suggest a role for β-catenin in promoting resistance to anti-PD-1 therapy in HCC.

**DISCUSSION**

In this study, we have demonstrated that β-catenin activation in HCC tumor cells is an important mechanism of immune escape that confers resistance to anti-PD-1 therapies. The use of mAbs directed against inhibitory receptors on immune cells, known as immune checkpoint blockade, has aroused tremendous enthusiasm among clinicians, scientists, and patients (9). In particular, mAbs targeting PD-1/PD-L1 have shown remarkable antitumor activity in numerous malignancies (11, 43–45), leading to their regulatory approval (9). However, despite the unprecedented efficacy of these agents in some patients, the lack of response in the majority emphasizes the pressing need to identify biomarkers that can select the patients that are most likely to benefit from therapy. In other malignancies, mismatch repair deficiency (12, 13), mutations in the SWI/SNF chromatin remodeling complex (46–48), or ADAR1 mutations (49) sensitize tumors to respond to immunotherapies. In contrast, β-catenin activation (19), PTEN deletion (18), or JAK2 mutations (16) lead to resistance to immunotherapies.

Similar studies in HCC have been missing, in part due to the relative delay of the clinical trials testing immunotherapies when compared with other malignancies, such as melanoma or NSCLC. A comprehensive transcriptional...
analysis of HCC patient samples has previously found a correlation between CTNNB1 mutation and T-cell exclusion (20), suggesting that β-catenin activation could be involved in immune escape and resistance to immunotherapies in patients with HCC. This correlation between CTNNB1 mutation and T-cell exclusion has been validated across a large set of human cancers (50). Furthermore, in a small cohort of patients with HCC, alterations in the β-catenin pathway correlated with lack of response to anti–PD-1 or anti–PD-L1 therapies (21). Here, by using a novel mouse model of HCC, we have functionally shown that β-catenin activation leads to immune exclusion and resistance to anti–PD-1 therapy, which emphasizes the utility of our models to identify processes that are relevant to human disease. One limitation in HCC clinical research is that tumor biopsies are not recommended for patients with advanced HCC (51). A change in the clinical guidelines may be needed to enable liver biopsies in patients with advanced HCC to facilitate the identification of biomarkers of response and implement biomarker-guided therapies.

Mechanistically, we have demonstrated that β-catenin activation in HCC tumor cells impaired recruitment of CD103+ DCs, which are critical cells in mounting an effective antitumor immune response (52). This defective recruitment of DCs in turn impaired the presence of antigen-specific CD8+ T cells in the liver, further confirming the reduced immune surveillance. Interestingly, at an early time point, CTNNB1-mutant HCC tumors presented CD8+ T cells that were not antigen-specific and could be bystander T cells (53, 54). However, in murine and human CTNNB1-mutant established tumors, T cells were rare, consistent with an immune exclusion phenotype. Moreover, transcriptional analysis of HCC patient samples revealed that transcripts related to DCs and T cells were significantly downregulated in CTNNB1-mutant HCC tumors when compared with CTNNB1 WT tumors, extending our findings to the human setting. A similar observation has been made in melanoma, where β-catenin activation also leads to a defective recruitment of CD103+ DCs (19). In the melanoma study, the reduced recruitment of CD103+ DCs into the tumor microenvironment could partially be explained by a defective production of the chemokine CCL4. In our murine HCC model and in HCC patient samples, chemokine CCL4 expression levels were unchanged between CTNNB1-mutant and CTNNB1 WT tumor samples. Instead, we found a significant reduction in the levels of chemokines CXC1, CCL20, and CCL5 in CTNNB1-mutant tumors in both murine and human CTNNB1-mutant samples. Because CCL5 could potentially affect DCs (42), we decided to further pursue the effect of CCL5 on immune surveillance. Indeed, overexpression of chemokine CCL5 in β-catenin–driven HCC cells led to a higher recruitment of CD103+ DCs, antigen-specific CD8+ T cells, and restoration of immune surveillance, demonstrating its causal role. Restoration of intratumor DCs by intratumor injection of FLT3 ligand–induced bone marrow–derived DCs also had an anti-tumor effect in melanoma (19). It is striking that in different tumor types, the same signaling pathway, β-catenin activation, elicits a similar mechanism although mediated by different chemokines. It is also possible that additional chemokines and secreted molecules may be involved in the recruitment of DCs in both settings. Nevertheless, therapeutic strategies that promote DC recruitment (55) could improve the response of CTNNB1-mutant tumors to anti–PD-1 therapy.

In HCC, around one third of the patients present activating mutations in CTNNB1 and could potentially be resistant to anti–PD-1 therapies (56). So far, the clinical trials testing nivolumab (6) and pembrolizumab (7) have demonstrated that only around 15% to 20% of patients with HCC exhibit an objective response to these therapies. This suggests that other mechanisms of immune resistance beyond β-catenin activation exist. In fact, in our mouse model, less than 50% of the immune-escaped HCC tumors presented β-catenin activation (as measured by an increase in the expression levels of β-catenin target Axin2). Characterization of the remaining tumors has shown that escaped murine liver tumors, which present abundant immune-related transcripts, can escape through different mechanisms. In addition, the temporal study of MYC;LucOSS;tg p53 tumor cells by single-cell RNA-seq may shed light on the initial changes occurring in tumor cells subjected to immune pressure and undergoing immune surveillance. Single-cell RNA-seq may also enable better establishment of the changes occurring in different cell compartments and the contribution of each compartment to immune escape. Moreover, it is possible that mutations co-occurring with CTNNB1 mutation may modulate the effect that β-catenin activation has in antitumor immunity. In addition, the levels of activation of the β-catenin signaling pathway may affect the phenotype of immune escape. Additional studies will be needed to identify distinct mechanisms of resistance to immunotherapies and refine the set of mutations that cooperate with CTNNB1 mutation to confer resistance. To address this, it will be critical to combine mechanistic studies in mice with the analysis of HCC patient samples.

To understand the role that different genetic alterations in HCC tumor cells have in immune surveillance and response to immunotherapies, we have generated a novel mouse model of HCC. The model is based on the hydrodynamic tail vein delivery (30) of genetic elements encoding oncogenes, CRISPR targeting tumor suppressor genes, and exogenous antigens. A similar approach, comparing tumor formation in the absence or presence of exogenous antigen expression, was used to study immune surveillance in lung cancer (29) and to demonstrate immunoediting in the context of sarcoma (17). A recent study in HCC performed hydrodynamic injection of mutant NRAS, AKT, and exogenous antigens to demonstrate that antigen-specific T cells undergo exhaustion (57). The strength of our approach is that we can compare the effect that the expression of exogenous antigens has in the context of different genetic alterations, which, coupled with the use of both immunocompetent and immunodeficient mouse models, can lead to fundamental discoveries. For example, performing experiments in the absence or presence of antigens has enabled us to demonstrate that antigen expression in the context of MYC;Trp53+/− liver tumors leads to immune surveillance. This strong antitumor immune response is driven by antigen expression and not by the loss of p53, because elimination of immune cells in mice harboring MYC;Trp53+/− liver tumors that do not express antigens has no effect. Similarly, antigen expression is critical for responding to anti–PD-1 immunotherapy because mice harboring MYC;Trp53+/− liver tumors that do not express antigens are not responsive to...
the therapy. The fact that the model antigens are linked to luciferase allows monitoring of tumor growth and immune responses over time by simply performing bioluminescence imaging. However, because the antigens are genetically linked to the driving oncogene, MYC, it is likely that there is a selective pressure against the loss of the antigens. The advantage of this may be that the system allows the study of mechanisms of immune escape different from the loss of antigens. It will be interesting to study how HCC-specific tumor antigens, such as α-fetoprotein or glypican 3 (SB), instead of model antigens, affect mechanisms of immune surveillance in mice. The benefit of using model antigens, such as the ones used in our study, is that they have the potential to elicit strong immune responses that can be overcome only by bona fide immune escape mechanisms. The temporal control of the expression of the antigens by using inducible systems, uncoupled from the expression of the driving oncogene, may better recapitulate tumor evolution and immune responses. Finally, HCC arises in the context of underlying liver disease. It will be critical to test how different types of liver damage (viral, alcohol-mediated, dietary), which can be easily combined with our model, affect response to immunotherapies.

In conclusion, we provide a novel mouse model of HCC that can be used to identify mechanisms of immune escape and resistance to immunotherapies that are relevant to human disease. This model represents a paradigm of personalized mouse model of HCC that recapitulates immune surveillance and allows interrogation of the role of virtually any genetic alteration in antitumor immunity. With this model, we have found that β-catenin activation promotes immune escape and resistance to anti–PD-1 therapies in HCC. By dissecting the underlying biology, we also propose a mechanism to restore immune surveillance in β-catenin–driven tumors. Finally, our results suggest that CTNNB1 mutational status could be used as a biomarker for patient exclusion. The identification of additional tumor-intrinsic signaling pathways that disrupt antitumor immunity and affect response to anti–PD-1 by using our novel mouse model may help optimize patient selection.

**METHODS**

**Vector Design and Use**

To generate the pT3-EF1α-MYC-RES-luciferase (MYC-luc) vector, the pT3-EF1α-MYC plasmid was opened with *Pmel* restriction enzyme, the “IRES-luciferase” sequence was PCR-amplified from pMSCV-IRES-luciferase, and the cloning of the “IRES-luciferase” fragment into the linearized pT3-EF1α-MYC was performed by using the In-Fusion HD Cloning Plus ( Takara Bio). The pT3-EF1α-MYC-IRES-luciferase-OS (MYC-lucOS) vector was generated by In-Fusion cloning of the *Pmel*-linearized pT3-EF1α-MYC vector, the “IRES-luciferase” fragment, and the “OS” fragment, which was amplified from *Lenti-LucOS* vector by PCR. To generate the pT3-EF1α-CTNNB1-RES-CdS vector, the pT3-EF1α-NRAS-RES-GFP plasmid digested with *XbaI* and *EcoRV* restriction enzymes was used as a donor vector. The “CTNNB1,” “IRES,” and “CdS” sequences were PCR-amplified from the pT3-N90-CTNNB1, the pT3-EF1α-NRAS-RES-GFP, and the pMD-wtCdS plasmids, respectively, and the cloning of the three fragments into the donor vector was performed by In-Fusion cloning. The CMV-SBI3, pT3-EF1α-NRAS-RES-GFP, and pMSCV-IRES-luciferase plasmids were kindly provided by Dr. Scott Lowe (Memorial Sloan Kettering Cancer Center, New York, NY). The pT3-EF1α-MYC and pT3-N90-CTNNB1 (Addgene plasmid #31785) were a kind gift from Dr. Xin Chen (University of California, San Francisco, CA). Lent-LucOS (Addgene plasmid #22777) was a gift from Dr. Tyler Jacks. The full-length cDNA of CdS was obtained from Sino Biological (plasmid reference: MG50022-M). The px330-vgp53 was previously published and validated (31). The px330 vector was a gift from Feng Zhang (Addgene plasmid #42230). All constructs were verified by nucleotide sequencing and vector integrity was confirmed by restriction enzyme digestion. The new vectors will be made available through Addgene.

**Hydrodynamic Tail-Vein Injection**

A sterile 0.9% NaCl solution/plasmid mix was prepared containing DNA. We prepared 11.4 μg of pT3-EF1α-MYC-RES-luciferase (MYC-luc), 12 μg of pT3-EF1α-MYC-RES-luciferase-OS (MYC-lucOS), 10 μg of pT3-N90-CTNNB1 (CTNNB1), 27 μg of pT3-EF1α-CTNNB1-RES-CdS (CTNNB1-CdS), 10 μg of px330-vgp53 (vgp53), and a 4:1 ratio of transposon to SB13 transposase-encoding plasmid dissolved in 2 mL of 0.9% NaCl solution and injected 10% of the weight of each mouse in volume. Because two independent “hits” are required for tumor formation in C57BL/6 mice (30), only those hepatocytes that receive the three plasmids (transposon-based, transposase, and CRISPR-based) will have the potential to form tumors. We also show that a single hepatocyte can take up to four plasmids, as shown in MYC-lucOS,CTNNB1;vgp53 tumors. For the anti–PD-1 experiment, we used 13 μg of pT3-EF1α-MYC-RES-luciferase-OS, 13 μg of px330-vgp53 (vgp53), and a 4:1 ratio of transposon to SB13 transposase-encoding plasmid per 2 mL. Mice were injected with the 0.9% NaCl solution/plasmid mix into the lateral tail vein with a total volume corresponding to 10% of body weight in 5 to 7 seconds. Vectors for hydrodynamic delivery were produced using the QIAGEN plasmid Plus Mega kit (QIAGEN). Equivalent DNA concentration between different batches of DNA was confirmed to ensure reproducibility among experiments.

**Mice**

Different batches of WT C57BL/6 mice were purchased from Envigo and were used for the treatment experiments (with mAbs) or for flow cytometry experiments. Rag2−/− and Batf3−/− mice were in C57BL/6 background were obtained from Jackson Laboratories and bred at Icahn School of Medicine at Mount Sinai (ISMMS). Controls for Rag2−/− and Batf3−/− mice were WT C57BL/6 mice purchased from Jackson and bred at ISMMS. All mouse experiments were approved by the ISMMS Animal Care and Use Committee (protocol number IACUC-2014-0229). Mice were maintained under specific pathogen-free conditions, and food and water were provided *ad libitum*. All animals were examined prior to the initiation of the studies to ensure that they were healthy and acclimated to the laboratory environment. All experiments were performed with 6- to 8-week-old mice, and both males and females were used in most experiments (analyzed separately). Once the animals were sacrificed, livers were collected, formalin-fixed and paraffin-embedded, frozen, or embedded in OCT (Tissue Tek).

**Deep Sequencing of CRISPR-Modified Trp53 Locus**

The genomic region of Trp53 targeted by gvp53 was PCR amplified using Platinum SuperFi (Invitrogen) high-fidelity DNA polymerase and PCR purified. The primers used were 5′-AAGGCCATAGGGGTTTGTTTG-3′ (forward) and 5′-GATACAGGTTAGCGGGATG-3′ (reverse). Libraries were made from 500 ng of the PCR products using the Nextera protocol and sequenced on Illumina MiSeq (250 base pair paired-end). Data were processed according to standard Illumina sequencing analysis procedures. The raw illumina reads were checked for adapters and quality via FastQC. The raw illumina sequence reads were trimmed of their adapters and nucleotides with poor quality using Trimomatic v. 0.36. Paired sequence reads were then merged to form a single sequence if the forward and reverse reads were able...
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Treatments

Treatments were initiated one week after the hydrodynamic delivery of the plasmids, a time point that already presents malignant tumor cells. For the experiments with anti–PD-1 mAbs, three doses of either anti–PD-1 (200 μg, clone RMPI-14, BioXcell) or IgG (200 μg, IgG2b, clone LTF-2, BioXcell) were given intraperitoneally at days 7, 9, and 11. For the T-cell depletion experiments, mice were injected intraperitoneally with anti-CD4 (200 μg, clone GK1.5, BioXcell), anti-CD8 (200 μg, clone 2.43, BioXcell), or IgG (200 μg, IgG2b, clone LTF-2, BioXcell) at days 7, 9, 11, and 13, and then on once weekly until the end of the experiment. For the combined depletion of CD4+ and CD8+ T cells, 200 μg of each antibody were used and 400 μg of IgG.

Luciferase Detection

In vivo bioluminescence imaging was performed using an IVIS Spectrum system (Caliper LifeSciences, purchased with the support of NCRR S10-RRO26561-01) to quantify liver tumor burden before being evenly assigned to various treatment study cohorts. Mice were imaged 5 minutes after intraperitoneal injection with fresh α-luciferin (150 mg/kg; Thermo Fisher Scientific). Luciferase signal was quantified using Living Image software (Caliper LifeSciences). Normalized luciferase signal was calculated by subtracting the background signal. Each treatment cohort had equivalent average luciferase signal. Those mice with a luciferase signal a log of magnitude lower than the average signal were excluded from the study.

RNA-seq and Analysis

RNA was poly-A selected, and multiplexed RNA-seq libraries were prepared using the TruSeq RNA Sample Preparation kit (Illumina) according to the manufacturer’s instructions at the ISMMS Genomics Core. The libraries were quantified using the Qubit Broad Range kit (Thermo Fisher Scientific) and sequenced using the Illumina HiSeq 4000 system (SR100). The RNA-seq data was analyzed using Basepair software (www.basepairtech.com) with a pipeline that included the following steps. Reads were aligned to the transcriptome derived from UCSC genome assembly mm10 using TopHat2 with default parameters. Read counts for each transcript were measured using featureCounts (59). Differentially expressed genes were determined using DESeq2 (60) and a cutoff of 0.05 on adjusted P value (corrected for multiple hypotheses testing) was used for creating gene lists. GSEA was performed on normalized gene expression counts, using gene permutations for calculating P value, to characterize the molecular alterations enriched between different groups (34). GSEA, FDR value < 0.25, and P < 0.05 (as accepted). The files can be found at GEO (GSE125336).

Human HCC Sample Analysis

MYC, TP53, and CTNNB1 genomic alterations in patients with HCC (n = 566) were obtained from the eBioPortal (61) TCGA dataset. Gene expression profiling of a total of 360 human samples was extracted from the TCGA (December 2018). Samples were stratified depending on CTNNB1 status as WT or mutant, or CTNNB1-mutant HCC gene signature enrichment levels (in tertiles). Mann–Whitney test was performed to test for differences in gene expression values on the log scale.

Patient Cohort and Evaluation of Treatment Response

Patients receiving nivolumab at ISMMS were eligible to be enrolled in the study if they had a confirmed histologic diagnosis of HCC and viable tumor tissue (either biopsy or archival sample) prior to the start of immunotherapy. Once local Institutional Review Board (IRB) approval was granted, written informed consent for tumor profiling was obtained from each patient on a retrospective protocol (IRB number 17-01728) in accordance with the Belmont Report. Initial diagnosis of HCC was made following the clinical practice guidelines from the European Association for the Study of the Liver (62). All included patients presented an advanced (BCLC-C) or intermediate (BCLC-B) stage with prior progression to surgery and/or locoregional therapies at the moment of immunotherapy initiation. Nivolumab was administered at a dose of 240 mg every 2 weeks and was continued until toxicity, progression, or death, according to the treating physician. Assessment of response was conducted at least 3 months after treatment initiation and performed by mRECIST criteria (63). Treatment response was defined as follows: complete response (CR); disappearance of any intratumor arterial enhancement in all target lesions; partial response (PR); at least a 30% decrease in the sum of diameters of viable (enhancement in the arterial phase) target lesions, taking as reference the baseline sum of the diameters of target lesions; progressive disease (PD); an increase of at least 20% in the sum of the diameters of viable (enhancing) target lesions, taking as reference the smallest sum of the diameters of viable (enhancing) target lesions recorded since treatment started; stable disease (SD; any cases not qualifying for either PR or PD). The electronic medical records were reviewed to extract information on patient’s gender, age, race, etiology, date of diagnosis, specimen location (liver, local recurrence, or extrhepatic metastasis), extent of disease, treatment history, type, number and dates of systemic therapy with radiographic response, date of progression, and the last date of follow-up or date of death.

Statistical Analysis

Data are expressed as mean ± SD. Statistical significance was determined using Mann–Whitney U test (when n < 10 or nonnormal distribution) or Student t test (n > 10 and normal distribution). For comparisons of more than two groups, we used ANOVA test. For paired comparisons, we used the Wilcoxon test. For frequency comparisons, we utilized the χ2 test. Group size was determined on the basis of the results of preliminary experiments, and no statistical method was used to predetermined sample size. Group allocation for treatments was performed to ensure equivalent luciferase signal, and outcome assessment was not performed in a blinded manner. The differences in survival were calculated using the Kaplan–Meier test. GraphPad Prism 6 software was used to create the graphs and for the statistical analysis. Significance values were set at *, P < 0.05; **, P < 0.01; ***, P < 0.001.

The rest of materials and methods can be found in Supplementary Methods.

Disclosure of Potential Conflicts of Interest

A. Villanueva is a consultant/advisory board member for Guidepoint, Fujifilm, Exact Sciences, Nucleix, NGM Pharmaceuticals, and Exelixis. J.M. Llovet reports receiving commercial research grants from Bayer Healthcare Pharmaceuticals, Eisai Inc., Bristol-Myers Squibb, and IPSEN, and is a consultant/advisory board member for Eli Lilly, Bayer HealthCare Pharmaceuticals, Navigant, Leerink Swann LLC, Midatech Ltd., Fortress Biotech Inc., Spring Bank Pharmaceuticals, Nucleix, Can-Fite Biopharma, Bristol-Myers Squibb, Eisai Inc., Celonis, Exelixis, Merck, Blueprint, Ipsen, and Glycotest. A. Lujambio reports receiving commercial research grants from Pfizer and Genentech and has received speakers bureau honoraria from Exelixis. No potential conflicts of interest were disclosed by the other authors.

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REFERENCES


β-Catenin Promotes Immune Resistance in Liver Cancer


IL6/STAT3 Signaling Orchestrates Premetastatic Niche Formation and Immunosuppressive Traits in Lung
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ABSTRACT

Cancer cells that succeed in forming metastasis need to be reprogrammed to evade immune surveillance and survive in a new microenvironment. This is facilitated by metastatic niches that are either postformed through reciprocal signaling between tumor cells and local stromal cells or preformed as premetastatic niches before tumor cell arrival. IL6/STAT3 signaling is aberrantly activated in lung tumorigenesis and metastasis, however, the roles and mechanisms of action of IL6 remain controversial. Here, we showed that blockade of intrinsic STAT3 signaling in lung tumor cells suppressed lung metastasis in immune-competent syngeneic mice, but not in immune-deficient nude mice. Collectively, repression of STAT3 signaling in tumor cell nuclei made them susceptible to T-cell-mediated cytotoxicity. Thus, STAT3-mediated immunosuppression is crucial for metastasis. Noticeably, lung metastasis was greatly increased in Gprc5a-knockout (ko; Sa−/−) mice compared with wild-type mice, which correlated with upregulated IL6 in the tumor microenvironment. Depletion of IL6 via combined deletion of Il6 and Gprc5a genes almost completely eliminated lung metastasis in Gprc5a-ko/Il6-ko (Sa−/−;Il6−/−) mice. Mechanistically, dysregulated IL6 reprogrammed the STAT3 pathway in metastatic tumor cells, and induced recruitment of myeloid-derived suppressor cells and polarized macrophages to evade host immunity. Consistently, IHC staining showed that activated STAT3 correlated with repressed infiltration of CD8+ T cells in non–small cell lung cancer. Therefore, IL6/STAT3 signaling is crucial for orchestrating premetastatic niche formation and immunosuppression in lung.

Significance: IL6 plays important roles not only in cell autonomous propensity for metastasis, but also in establishing the metastatic niche.

Introduction

Tumor metastasis is a complex process consisting of multiple steps, involving dissemination of cancer cells to local and distant organ sites, and their adaptation to new environments (1, 2). The processes of metastasis are driven by the interplay between cancer cells and the metastasis-supportive microenvironment (3). On one hand, proinflammatory cytokines in microenvironment can induce cancer cells to program epithelial-to-mesenchymal transition (EMT), a key step of the metastatic process (4, 5); on other hand, cancer cells can release factors or cytokines to recruit immunosuppressive lymphocytes, which facilitate the metastatic niche formation (6). Cancer cells may thus be reprogrammed to become invasive and immunosuppressive during the process. Recently, cancer cells undergoing EMT process were found to acquire immunosuppressive functions during metastasis (7, 8). These observations provide a functional link between metastatic potential and immunosuppressive traits. Because most of studies on EMT and metastasis are based on the analyses in vitro and/or in immune-deficient nude mice, the interplay between EMT-like traits of tumor cells and host immunity in metastasis remains poorly characterized.

The metastasis-supportive microenvironment, or metastatic niches, can be either postformed by reciprocal signaling between the metastasized tumor and stromal cells or preformed as premetastatic niches in tissue microenvironment before tumor cells arrived. Emerging evidences suggest that inflammatory stimuli provoke premetastatic niche formation. For example, inflammatory response induced by endotoxin via Toll-like receptor 4 (TLR4) increases vascular permeability and leukocyte mobilization to the lungs (9). Tumor exosomal RNAs promote lung premetastatic niche formation by activating alveolar epithelial TLR3 via recruitment of neutrophils (10). These studies suggest that the proinflammatory stimuli, either via TLR4 or TLR3, facilitate lung premetastatic niche formation. Alternatively, the oncogenic program in the metastasized cells can be activated by the signaling network in target tissue microenvironment.

IL6, a cytokine of the chemokine family, is widely expressed in a variety of immune cells and malignant tumors including lung cancer (11–13). IL6 activates JAK/STAT3 signaling by binding its receptor, and subsequently dimerizes with its coreceptor gp130, followed by recruitment of cellular signaling proteins, including JAKs...
and STAT3 (14–16). Elevated levels of IL6 or activated STAT3 have been observed in chronic inflammatory tissues and many solid tumors (17). Importantly, elevated levels of systemic and pulmonary IL6 are associated with poor survival of patients with non–small cell lung cancer (NSCLC; refs. 18, 19). In parallel, activated STAT3 can also promote IL6 gene expression, resulting in a feed-forward autocrine feedback loop (20). Intrinsically, IL6 can act directly on tumor cells to induce the expression of STAT3 target genes, to drive proliferation (such as via cyclin D1; ref. 21), and survival (such as via BCL2-like protein 1 (BCL2-xl; ref. 22)). Extrinsically, IL6/STAT3 signaling has a profound effect on tumor-infiltrating immune cells in the microenvironment. STAT3 negatively regulates neutrophils, natural killer (NK) cells, effector T cells, and dendritic cells (DC); STAT3 positively regulates regulatory T (Treg) cells and myeloid-derived suppressor cell (MDSC) populations (23). Because metastasis is an outcome of tumor cell survival in a new tissue microenvironment under selection pressure, we reasoned that, the major oncogenic program of metastatic tumor cells should be compatible to the signaling network in the target tissue microenvironment. Consequently, the signaling network in the tissue microenvironment should have a great impact on the outcome of metastatic potential and immunosuppressive traits via reprogramming. Therefore, targeting the key player of the signaling network may greatly facilitate the inhibition of metastasis.

GPRC5A (G-protein–coupled receptor, family C, member 5A), also known as RAIG1 or RA13, is a retinoic acid–inducible gene and is predominately expressed in lung tissue (24). Gprc5a–knockout (ko) mice are prone to develop spontaneous and carcinogen-induced lung cancer, indicating that Gprc5a is a lung tumor suppressor gene (24–26). Importantly, lung tumor development in Gprc5a–ko mice is associated with chronic inflammation (25, 27). Of note, GPRC5A is repressed in tissues of most human NSCLC and all chronic obstructive pulmonary disease (COPD; refs. 25, 27, 28), supporting that GPRC5A repression contributes to lung cancer development. Thus, Gprc5a–ko mice provide a unique immune-competent mouse model with chronic inflammation in lung.

In this study, we investigated the roles and mechanisms of IL6/STAT3 signaling in lung metastasis in Gprc5a–ko mouse model. Our study shows that immunosuppression induced by IL6/STAT3 signaling is critical for lung metastasis. We found that IL6/STAT3 signaling facilitates lung metastasis by orchestrating the premetastatic niche formation and immunosuppressive traits.

Materials and Methods

Cell lines and cell culture

Primary mouse lung cancer cells (SJT1601) were obtained from Gprc5a–ko mouse lung tumor at the age of 14 months, and SJT1601-luc were obtained from the center of the spheres. Images of the spheroids were captured by Nikon camera.

Reagents and antibodies

Detailed information is provided in the Supporting Information, Table S1.

Migration and wound healing assay

Detailed information is provided in the Supplementary Experimental Procedures.

Sphere culture assay

A total of 5,000 cells were added to each well of a 96-well low-adherence culture plate and suspended in culture medium to simulate a three-dimensional culture environment. The medium was changed every 3 days. Spheroid size was monitored to avoid necrosis in the center of the spheres. Images of the spheroids were captured by Nikon camera.

Soft agar colony formation assay

Detailed information is provided in the Supporting Experimental Procedures.

Western blot analysis

Cells were lysed with RIPA lysis buffer (29). Experiments were performed as described previously (30). Briefly, whole cell lysates were separated by SDS-PAGE and transferred onto nitrocellulose membrane. The membranes were probed with indicated antibodies and protein expression was detected by chemiluminescence (GE Healthcare).

RNA interference and transfection

Pairs of complementary oligonucleotides against Gp130 or non-specific (NS) control shRNA were synthesized by Sangon Biotech, annealed, and ligated to the PGIPZ Lentiviral Vector (Clontech Laboratories, Inc.). The shRNA-carrying retroviruses produced in 293T cells were used to infect cells.

Flow cytometry analysis

Mice were sacrificed, and the lungs lavaged three times with ice-cold PBS. Then lung tissues were scissors-cut and digested with 150 µi/mL Collagenase IV (Sigma-Aldrich) and 0.001% DNase1 (Sigma-Aldrich) in PBS for 45 minutes at 37°C; single-cell suspensions were prepared by passing the cell digest through 70 µm and then 40 µm nylon mesh. Cells were incubated with Red Blood Cell Lysate (Sigma-Aldrich) for 5 minutes at room temperature and washed twice with PBS containing 10% FBS. Cells were stained with indicated fluorescently labeled antibodies on ice for 30 minutes. Then cells were analyzed by FACS (BD Biosciences), and the data were further analyzed by Flowjo7.6.1 software. Antibodies information is listed in Supporting Information, Table S2.

Mouse models

Gprc5a–ko mice were mixed backgrounds of 129sv C57BL/6 as described previously and cross-bred with C57BL/6j mice for more than 7 times (24). IL6–ko mice were obtained from Jackson laboratory (stock number 002650), and the background strain is C57BL/6j. Gprc5a–ko/IL6–ko mice were generated by cross-breeding Gprc5a–ko mice with IL6–ko mice. Mice were maintained accordingly to a protocol approved by Shanghai Jiao Tong University School of Medicine Animal Care and Use Committee [experimental animal use permission no.: SYXK (Shanghai) 2008-0050] in the specific pathogen-free animal facility in the university.

IL6 Promotes Lung Metastasis

Primary mouse lung cancer cells (SJT1601) were obtained from Gprc5a–ko mouse lung tumor at the age of 14 months, and SJT1601-luc were obtained from stable transfection of luciferase in SJT1601 cells, Lewis lung carcinoma (LLC) cell line was obtained from Zhejiang University (Hangzhou, China), and Calu-1 cells were obtained from ATCC. All the cells were cultured with DMEM (Hyclone) supplemented with 10% PBS (Gibco).

Reagents and antibodies

Detailed information is provided in the Supplementary Experimental Procedures.

qRT-PCR

tRNA was extracted from lung tissue using RNA Extract Kit (TIANGEN). Then, cDNA was obtained by Fast Quant Kit (TIANGEN). The qRT-PCR were performed using SuperReal Premix Plus SYBR Green Kit (TIANGEN). All experiments were performed according to the manufacturer’s instructions. Primers are listed in Supporting Information, Table S1.
Tumorigenicity and migration experiment in xenograft

For migration study, SJT-1601/SJT-1601-Luc and Lewis cells were injected into the tail vein (5 × 10^5/0.2 mL cells in PBS) of designated mice and were sacrificed after 3 weeks. In a separate study, SJT-1601-NS, SJT-1601-Gp130 were injected into the tail vein (5 × 10^5/0.2 mL cells in PBS) of C57BL/6 WT, Gprc5a-ko mice; and (1 × 10^5/0.2 mL cells in PBS) were injected into nude mice. These animals were sacrificed after 3 weeks. For tumorigenicity study, SJT-1601-NS, SJT-1601-Gp130, SJT-1601, SJT-1601-dnStat3 were injected subcutaneously (2 × 10^5/0.2 mL cells in PBS) in the left and right hind leg of mice in C57BL/6 and nude mice respectively. After the tumor was palpable (approximately 100 mm²), the tumor volume was measured twice a week. Tumor volume (cm³) was calculated by the formula: (a × b²)/2, where “a” is the long diameter and “b” is the short diameter (mm). For in vivo IL6 blockade assay, SJT-1601 cells were injected into the tail vein (5 × 10^5/0.2 mL cells in PBS) of Gprc5a-ko mice, 1 week later, 20 mg/kg dose of Tocilizumab (A2012, Selleck) or control IgG1 (Sigma-Aldrich) were intraperitoneally injected twice a week for 2 weeks; these animals were sacrificed and lung metastasis was assessed after 3 weeks.

Ex vivo experiment

Mouse lung tumor cells, SJT-1601-GFP cells (1.5 × 10^5/0.2 mL cells in PBS) were intravenously injected into WT, Gprc5a-ko, and Gprc5a-ko/+IL6-ko mice. Ten days later, single-cell suspensions from mouse lung tissues were obtained and then GFP+ cells were harvested via FACS-sorting. For immunofluorescence experiments, the study was done as described previously (25).

IHC

A tissue microarray composed of tumor and adjacent normal tissue from NSCLC samples were obtained from Shanghai Chest Hospital, Shanghai Jiao Tong University (Shanghai, China). The tissue microarray and fixed mouse lung tissues samples were processed for IHC; the detailed protocol and score method were performed as described previously (25). Shanghai Chest Hospital (Shanghai, China) approved the use of the NSCLC samples in this study.

T-cell–mediated tumor cell–killing assay (cytotoxic T-cell lymphocyte)

Detailed information is provided in the Supplementary Experimental Procedures.

Statistical analysis

Comparisons among groups were performed by the Student t test or Tukey–Kramer comparison test followed by analysis with GraphPad Prism Software (GraphPad Software). A P < 0.05 was considered significant (* P < 0.05; ** P < 0.01; *** P < 0.001; **** P < 0.0001).

Results

Aberrantly activated IL6/STAT3 signaling is highly correlated with tumorigenesis and metastasis in Gprc5a-ko mouse lungs

Tobacco carcinogen 4-(N-Methyl-N-nitrosamino)-1-(3-pyridyl)-1-butanol (NNK) induced lung tumorigenesis in Gprc5a-ko (5α/−/−) mice (100%), but not in wild-type (WT) mice (0%; Fig. 1A and B; ref. 25). IHC staining showed that active STAT3 (p-STAT3) was strongly positive in lung tumors of Gprc5a-ko mice, but not in WT lungs (Fig. 1B). This finding suggests that STAT3 signaling is involved in lung tumorigenesis in Gprc5a-ko mice. Of note, p-STAT3 was also positive in adjacent normal lung tissues of Gprc5a-ko-NNK-12m mice with lung tumors, especially in the small/terminal bronchioles region (S/TB; Fig. 1C). This observation implies a paracrine effect by cytokines from tumor microenvironment (TME). Further analysis via qRT-PCR and ELISA showed that the IL6 level was indeed significantly upregulated in the tumors from Gprc5a-ko mouse lungs compared with adjacent normal lungs and WT lungs (Fig. 1D and E). These data are consistent with the previous observation, in which Gprc5a-ko mouse lungs are sensitive to proinflammatory stimuli (27, 31). Because IL6/STAT3 signaling is involved in a variety of solid cancers, we questioned, whether the IL6–enriched inflammatory tissue microenvironment of Gprc5a-ko mouse lung facilitates metastasis.

To determine the impact of the tissue microenvironment on metastasis, we performed an experimental metastasis analysis. The mouse lung tumor cell line SJT-1601 (1601), derived from Gprc5a-ko mouse lung tumor, was intravenously injected into syngeneic C57BL/6 (WT) and Gprc5a-ko (5α/−/−) mice (Fig. 1F).

Lung metastatic nodules were assessed 3 weeks later. Hematoxylin and eosin (H&E) staining analysis showed that SJT-1601 cells formed significantly more metastatic nodules in Gprc5a-ko mouse lungs than in WT mouse lungs (Fig. 1G–I), suggesting that premetastatic niches were increased in Gprc5a-ko mouse lungs compared with those in WT ones. In addition, IHC staining showed that p-STAT3 staining was more intense in the metastatic tumor tissues of Gprc5a-ko mouse lungs than in WT ones (Fig. 1G and J), and ELISA analysis showed that the IL6 level was significantly higher in Gprc5a-ko mouse lungs than that in WT ones (Fig. 1K). Taken together, these results suggest that upregulated IL6/STAT3 signaling is associated with metastasis in Gprc5a-ko mouse lungs.

IL6/Gp130/STAT3 signaling is essential for the prometastatic traits of lung cancer cells

To determine the role of IL6/STAT3 signaling in the metastatic features of lung cancer cells, we knocked down Gp130 in SJT-1601, a mouse lung cancer cell line derived from Gprc5a-ko mouse lung adenocarcinoma and human lung cancer cell line Calu-1, and established the stable transfectants, 1601-shGp130 and Calu-1-shGp130 (Fig. 2A). Immunoblot analysis showed that the levels of mesenchymal markers, including N-cadherin and Twist, were reduced in 1601-shGp130 and Calu-1-shGp130 cells, and the level of epithelial marker E-cadherin was increased in 1601-shGp130, although there was no significant change in vimentin level (Fig. 2A and B). These findings suggest that IL6/Gp130 signaling is essential for the induction of the EMT-like features in these lung cancer cells. Next, we examined the effect of IL6 on cell migration and invasion activities in vitro using wound healing and transwell assays. Addition of exogenous IL6 significantly enhanced the migration (Fig. 2C and D) and invasion (Fig. 2E and F) of 1601-NS and Calu-1-NS cells, but not 1601-shGp130 and Calu-1-shGp130 cells (Fig. 2C–F), which indicates that IL6 signaling is essential for migration and invasion. Furthermore, we examined the role of the IL6/Gp130 pathway on lung metastasis in vivo. The results showed that metastasis of 1601-NS cells was significantly increased in Gprc5a-ko mice compared with WT mice (Fig. 2G–I). And, the number of metastatic nodules of 1601-shGp130 cells was significantly reduced compared with those of 1601-NS cells in both WT and Gprc5a-ko mouse lungs (Fig. 2G–I), suggesting that the intrinsic IL6/Gp130/STAT3 signaling pathway of lung tumor cells is essential for the metastatic potential. Interestingly, the numbers of metastatic nodules of 1601-NS and 1601-shGp130 in immune-
deficient nude mice were similar (Fig. 2J–L), suggesting that immune-evasion is a major mechanism of IL6/Gp130/STAT3-mediated metastatic potential. Thus, although the IL6/Gp130/STAT3 pathway alters many features of lung tumor cells in vitro, Gp130/STAT3-mediated immunosuppression is critical for lung metastasis in the immune-competent host in vivo. Gp130/STAT3 signaling is intrinsically linked to the stem-like and immunosuppressive traits of lung cancer cells

EMT-like features are functionally linked to stemness, metastatic potential, and immunosuppressive features of cancer cells (6). To determine the roles of IL6/Gp130 signaling in regulation of these features in lung tumor cells, we examined the stem-like markers in the...
Figure 2.
IL6/Gp130/STAT3 signaling is essential for induction of the EMT-like features of NSCLC cells. A and B, Western blot analysis of E-cadherin, N-cadherin, vimentin, Twist, and Gp130 protein levels in 1601-NS and 1601-shGp130 cells (A), and Calu-1-NS and Calu-1-shGp130 cells (B). C and D, Migration analysis of 1601-NS and 1601-shGp130 (C), and Calu-1-NS and Calu-1-shGp130 cells (D) with or without IL6 by wound healing. E, Transwell migration analysis of 1601-NS and 1601-shGp130 cells, and Calu-1-NS and Calu-1-shGp130 cells with or without IL6. F, Statistical analysis of E, G and H. 1601-NS or 1601-shGp130 cells were intravenously injected into WT and Gprc5a-ko mice; representative images of lung tissues with H&E (n = 5). I, Number of metastatic nodules in G and H. J and K, 1601-NS or 1601-shGp130 cells were intravenously injected into nude mice; representative images of lung tissues (J) and tissues stained with H&E (K; n = 5). L, Number of metastatic nodules in nude mice. *: P < 0.05; **: P < 0.01.
NS and shGp130 stable transfectants of 1601 and Calu-1 cells. Immunoblot showed that p-STAT3, the stem-like markers (Abcg2, CD44, Sox2), and immune checkpoint marker PD-L1 were significantly reduced in shGp130 transfectants compared with the NS control (Fig. 3A and B), whereas p-ERK and p-STAT3 of these cells remained at similar levels. This suggests that Gp130 knockdown mainly inhibits STAT3 signaling and stemness in these lung cancer cells. Consistently, three-dimensional sphere formation (Supplementary Fig. S1F), colony formation in soft agarose (Fig. 3A and B), and clonogenic assay (Supplementary Fig. S1A and S1B) were also greatly suppressed in 1601-shGp130 and Calu-1-shGp130 cells compared with their NS transfectants. Thus, Gp130/STAT3 signaling is intrinsically essential for the stem-like features of lung cancer cells in vitro. Next, we examined the tumorigenicity of these cells in vivo. Interestingly, 1601-NS and 1601-shGp130 cells generated tumors with similar efficiency in immune-deficient nude mice (Fig. 3G–I). However, the tumorigenicity of 1601-shGp130 cells was greatly suppressed compared with 1601-NS in immune-competent syngeneic WT/C57 mice (Fig. 3J–L). In addition, we examined the biological features of 1601 cells expressing dominant negative Stat3 transfectants (1601-dnStat3; Supplementary Fig. S1C). The tumorigenicity of 1601-dnStat3 was similar, as compared with 1601 cells, in immune-deficient nude mice (Supplementary Fig. S1D–S1F), but significantly suppressed in WT/C57 mice (Supplementary Fig. S1G–S1I). Taken together, these results suggest that the immunity of immune-competent C57 mice is responsible for suppression of the tumorigenicity of SJT-1601-shGp130 cells, which is similar to lung metastasis in these mice (Fig. 2G–I). Thus, the major impact of STAT3 signaling in tumorigenicity and lung metastasis is mainly through immunosuppression.

PD-L1 overexpression was reportedly associated with poor prognosis (32, 33). Immunoblot showed that PD-L1 levels in 1601-NS and 1601-shGp130 cells were significantly reduced compared with NS cells (Fig. 3A and B; Supplementary Fig. S1C). Importantly, 1601-shGp130 and 1601-dnStat3 cells were much more sensitive to T-cell–mediated cytotoxicity than 1601-NS cells in cytotoxic T-cell lymphocyte (CTL) assay (Fig. 3M). These results suggest that intrinsic STAT3 signaling, possibly through overexpression of immunosuppressive molecules like PD-L1, in tumor cells endows the resistance to cytotoxic T (Tc) cells. Thus, intrinsic IL6/Gp130/STAT3 signaling is critical for the immunosuppressive features of the lung cancer cells.

IL6 signaling is extrinsically crucial for premetastatic niche formation in Gprc5a-ko mouse lungs

We reasoned that STAT3 signaling in metastatic tumor cells was activated by IL6 in microenvironment. To determine the role of IL6 signaling in lung metastasis, we generated Gprc5a-ko/I6e-ko mice by cross-breeding Gprc5a-ko mice with I6e-ko mice (Fig. 4A). Next, we performed lung metastasis in these mice by intravenous injection of 1601-luc (luciferase) cells followed by in vivo imaging. Lung metastasis was dramatically increased in Gprc5a-ko mice compared with WT mice. Strikingly, lung metastases in Gprc5a-ko/I6e-ko mice were almost completely eliminated (Fig. 4B–D). IL6 appears to be the major cytokine responsible for STAT3 activation because the expression of other IL6 family cytokines remains unchanged, and upregulated IL6 was correlated with increased metastatic potential (Fig. 4E and F). These observations suggest that although tumor cells or “seeds” are the same, tissue microenvironment or “soil” has great impact on their metastatic potential. To determine the effect of tissue microenvironment on reprogramming the oncogenic signaling of the metastatic tumor cells in vivo, we examined the gene expression in the STAT3 and EMT pathways in lung tissues. Immunoblot showed that IL6, p-STAT3, N-cadherin, vimentin, PD-L1, and Twist were significantly upregulated, whereas E-cadherin expression was reduced in lung tissues from Gprc5a-ko mice injected with 1601 tumor cells compared with those from WT and Gprc5a-ko/I6e-ko mice (Fig. 4G, right). The difference of the gene expression in normal control lungs is negligible (Fig. 4G, left). Collectively, these results suggest that (i) IL6/STAT3 signaling in tumor-containing lung tissues is activated via the reciprocal interaction between the metastatic tumor cells and stromal cells in tissue microenvironment; and (ii) dysregulated IL6 is essential for the effects. To exclude the noise of stromal cells in background in detection and to specifically define the status of STAT3 signaling in the metastatic tumor cells from different tissue microenvironment, we performed an ex vivo experiment. Mouse lung tumor cells, 1601-GFP, were intravenously injected into WT, Gprc5a-ko, and Gprc5a-ko/I6e-ko mice. Ten days later, GFP+ cells were harvested from mouse lungs via FACS-sorting (Supplementary Fig. S2A and S2B). Immunofluorescent analysis showed that PD-L1–staining intensity in 1601-GFP cells isolated from Gprc5a-ko mouse lungs was significantly enhanced (red in Fig. 4H) as compared with those isolated from WT and Gprc5a-ko/I6e-ko mouse lungs (Fig. 4H and I). These results suggest that the metastatic tumor cells are reprogrammed to exhibit the active STAT3 pathway by tissue microenvironment of Gprc5a-ko mouse lungs, whereas IL6 is essential for the effect.

Consistently, IHC staining showed that Ki-67, p-STAT3, and PD-L1 were all increased while CD8 was decreased in the lung tumors from Gprc5a-ko mice compared with lung tissues from WT and Gprc5a-ko/I6e-ko mice (Fig. 4J; Supplementary Fig. S2C). These results suggest that activated STAT3 is associated with increased proliferation and PD-L1 upregulation. Metastasis is known to associate with angiogenesis and cell proliferation (34). qRT-PCR showed that the angiogenesis biomarkers, BV8 and ANGPT, but not VEGF, were significantly upregulated in Gprc5a-ko mouse lungs compared with WT and Gprc5a-ko/I6e-ko mice (Fig. 4K). These findings further support the role of IL6 in premetastatic niche formation.

To extend this observation, we repeated the metastasis experiments using LLC cells in these mice and observed similar results. Biologically, lung metastasis via LLC cells was greatly increased in Gprc5a-ko mice compared with WT and Gprc5a-ko/I6e-ko mice (Supplementary Fig. S3A–S3C). Biochemically, IL6, p-STAT3, PD-L1, N-cadherin, vimentin, and Twist were all increased while E-cadherin was decreased in Gprc5a-ko mouse lungs compared with WT and Gprc5a-ko/I6e-ko mice (Supplementary Fig. S3D). Taken together, lung metastasis, via either SJT-1601 cells or LLC cells, is greatly enhanced in Gprc5a-ko mouse lungs in which IL6/STAT3 signaling plays a critical role.

IL6 signaling mediates the inhibition of innate and adaptive immunity in Gprc5a-ko mouse lungs

MDSCs and tumor-associated macrophages (TAM) are important negative regulators of host innate immunity in tumor microenvironment. Upregulation of these cells is associated with metastasis (35, 36). MDSCs can be subclassified into granulocyte-like myeloid-derived suppressor cells (G-MDSC) and monocytic myeloid-derived suppressor cells (M-MDSC). To determine the extrinsic roles of IL6 on innate immunity, we assessed MDSC levels in mouse lungs from lung metastasis experiments via FACS analysis. G-MDSCs were significantly increased while M-MDSCs were decreased in the lung from Gprc5a-ko mouse lungs compared with WT mouse lungs (Fig. 5A and B). Of note, Il6 gene knockout in Gprc5a-ko/I6e-ko mice completely reversed the G-MDSC upregulation and M-MDSC downregulation.
in Gprc5a-ko mice (Fig. 5B). Consistently, G-MDSC–related markers, S100a8 and S100a9, were significantly upregulated in Gprc5a-ko mice compared with WT and Gprc5a-ko/Il6-ko mouse lungs (Fig. 5C). These data suggest that IL6 is essential for upregulated G-MDSCs in Gprc5a-ko mouse lungs. Next, we examined via qRT-PCR the expression of a panel of soluble factors that are potentially involved in MDSCs recruitment. IL6, G-CSF, and prostaglandin E synthase (Ptges) were highly upregulated (red), whereas IL10 was significantly downregulated (blue) in Gprc5a-ko mouse lungs compared with the WT, and all of these alterations were reversed in Gprc5a-ko/Il6-ko mouse lungs (Fig. 5D). These results suggest that IL6 is essential for recruitment of MDSCs in Gprc5a-ko mouse lungs.
IL6 signaling is essential for lung metastasis in Gprc5a-ko mice lung tissues. SJT-1601-luc cells were intravenously injected into WT, Gprc5a-ko, and Gprc5a-ko/Il6-ko mice. A, Model diagram of Gprc5a-ko/Il6-ko mice. B, Fluorescence images of excised lung tissues from mice. C, The quantitative value of fluorescence intensity of B. D, Lung weight of mice. E, The mRNA level of IL6 family cytokines in mice lung tissues. F, ELISA analysis of IL6 level in mice lung tissues. G, Western blot analysis of mouse lung tissues. H, Immunofluorescence staining for PD-L1 in 1601-GFP cells cultured in vitro and 1601-GFP cells sorted from indicated mouse lung tissues with tumors. I, Quantification of relative fluorescence intensity (PD-L1/DAPI) from H. J, IHC scores from lung metastatic nodules of Ki-67, p-STAT3, PD-L1, and CD8. K, The mRNA level of Bv8, Angpt2, and Vegf in lung tissues. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$. 

Figure 4.
Figure 5.
IL6 signaling mediates the inhibition on innate and adaptive immunity in Gprc5a-ko mouse lungs. SJT-1601-luc cells were intravenously injected into WT, Gprc5a-ko, and Gprc5a-ko/Il6-ko mice. A, Gating strategy for G-MDSCs and M-MDSCs staining in tumor lung tissues and representative FACS images. B, Quantitative analysis of G-MDSCs and M-MDSCs by FACS analysis. C, The mRNA level of S100a8 and S100a9 in tumor lung tissues. D, The mRNA level of inflammatory genes responsible for MDSCs recruitment in tumor lung tissues. E, Quantitative analysis of TAM by FACS analysis. F, The mRNA level of type I macrophage biomarkers: Ccl2, Nos2, and Cxcl10; and type II macrophage biomarkers: Arg, Fizzl, and Mrc in tumor lung tissues. G, Quantitative analysis of T cells by FACS analysis. H, The mRNA level of TNFα and Ccl5, which are responsible for T-cell activation and recruitment, and IFNγ, Gzmb, and Tbx21, which are associated with CD8⁺ T-cell cytotoxic activation in tumor lung tissues. I, T-cell-mediated cytotoxicity for 1601-shGp130 and 1601-dnStat3 cells with or without MDSCs. *, P < 0.05; **, P < 0.01; ***, P < 0.001.
M-MDSCs recruited to tumor tissues will rapidly differentiate to TAMs (37). Consistent with this pattern, we found that the infiltrated macrophages in tumors were significantly increased in Gprc5a-ko mouse lungs compared with WT and Gprc5a-ko/I6-ko ones (Fig. 5E, Supplementary Fig. 5A). This suggests that IL6 is responsible for increased macrophages in Gprc5a-ko mouse lungs. Further analysis by qRT-PCR showed that the markers of polarized type II macrophages, Arg, Fizz, and Mrc, were greatly increased, while the markers of type I macrophages, Cc12, Nos2, and Cxcl10, were greatly suppressed in Gprc5a-ko mouse lungs compared with WT and Gprc5a-ko/I6-ko mouse lungs (Fig. 5F). Thus, Gprc5a-ko mouse lungs are enriched with TAMs that exhibit significant polarization of type II macrophages.

G-MDSC has the potential to inhibit CD8+ T, DC, and NK cells (38, 39). To determine the effects of IL6 on adaptive immunity, we examined the T-cell composition and related gene expression. FACS analysis showed that both CD4+ and CD8+ T cells were significantly reduced in Gprc5a-ko mouse lungs compared with WT and Gprc5a-ko/I6-ko mouse lungs (Fig. 5G; Supplementary Fig. 5B). Consistently, qRT-PCR analysis showed that T-cell activation gene products, such as TNFα, chemokine ligand 15 (Ccl5), IFNγ (Ifng), Granzyme B (Gzb), and T-box transcription factor 21 (Tbx21), were all significantly suppressed in Gprc5a-ko mouse lungs compared with levels in WT and Gprc5a-ko/I6-ko lungs (Fig. 5H).

To evaluate the biological impact of dysregulated MDSCs on T-cell immunity, we performed CTL analysis. While 1601-shGp130 and 1601-dnStat3 cells were sensitive to T cells (T), addition of exogenous MDSCs (T+ MDSCs) greatly suppressed T-cell–mediated cytotoxicity (Fig. 5I). This observation indicates that MDSCs are the major component of immunosuppression to T-cell–mediated cytotoxicity. In addition, NK cells were reduced in Gprc5a-ko mice (Supplementary Fig. 5C and 5D), whereas DC cells were similar among these groups (Supplementary Fig. 5E and 5F). Consistent with the status of the immune cells, the immune organs including spleen and thymus exhibited obvious atrophy in Gprc5a-ko mice compared with WT tissues. However, these alterations were reversed in Gprc5a-ko/I6-ko mice (Supplementary Fig. 5G and 5H).

IL6-mediated regulation of innate and adaptive immunity was also observed in lung metastasis induced by LLC. LLC-induced metastasis was correlated with increased G-MDSC (Supplementary Fig. 5A and SSB) and TAM (Supplementary Fig. 5C and SSD), and suppressed CD8+ T cells (Supplementary Fig. 5E and 5F) and NK cells (Supplementary Fig. 5G and SSH). Collectively, these findings suggest that IL6 is crucial in inducing enhancement of MDSC and TAM, and suppression of T cells and NK cells in Gprc5a-ko mouse lung. Thus, IL6 is a promising target for restoration of host immunity.

**Targeting IL6 suppresses MDSCs, restores T-cell cytotoxicity, and inhibits lung metastasis in Gprc5a-ko mice**

Because IL6 plays a central role in the suppression of host immunity and enhancement of lung metastasis, we investigated the therapeutic effects of IL6 targeting on lung metastasis. SJT-1601 cells were intravenously injected into Gprc5a-ko mice. One week later, anti-IL6 antibody was intraperitoneally injected twice a week for 2 weeks. On day 21, mice were sacrificed for analysis of lung metastasis (Fig. 6A). We found that targeting IL6 significantly inhibited lung metastasis compared with control mouse lungs (Fig. 6B and C). Functionally, G-MDSCs were also significantly reduced following anti-IL6 treatment, although M-MDSCs remained at a similar level, as analyzed by FACS (Fig. 6D and E). TAMs were also significantly reduced following anti-IL6 treatment (Fig. 6F and G). In contrast, CD8+ T cells were significantly increased following anti-IL6 treatment, although CD4+ T cells remained at a similar level (Fig. 6H and I). Furthermore, NK cells were also upregulated significantly following anti-IL6 treatment, although NK-T cells remained unchanged (Fig. 6J and K). Taken together, these observations demonstrate that targeting IL6 restores the innate and adaptive immunity in Gprc5a-ko mouse lungs and inhibits lung metastasis. Thus, IL6 signaling in lung is crucial for premetastatic niche formation and immunosuppressive traits.

**Dysregulated IL6/STAT3 is correlated with reduced CD8+ T-lymphocyte infiltration in NSCLCs**

Next, we asked if the gene products of an active IL6/STAT3 axis can serve as biomarkers for the immunosuppressive features in human NSCLC. By IHC staining analysis, we examined the levels of IL6, p-STAT3, PD-L1, and CD8+ in 140 NSCLC samples. The expression levels of IL6, p-STAT3, and PD-L1 were significantly higher in tumors than in adjacent normal tissues, whereas CD8+ expression was higher in adjacent normal tissues than tumor tissues (Fig. 7A and B). Of note, areas of tumor tissues that were low in IL6/p-STAT3 were also low in PD-L1, but high in CD8+, whereas areas that were high in IL6/p-STAT3 were also high in PD-L1, but low in CD8+ (Fig. 7A). These observations support the notion that activated IL6/STAT3 induces PD-L1 expression and other immunosuppressive features that lead to the suppression of CD8+ T-cell infiltration. Statistical analysis showed that IL6 was highly correlated with p-STAT3 in NSCLC as expected (Fig. 7C). Similarly, IL6 and p-STAT3 were significantly correlated with PD-L1 (Fig. 7C). Importantly, p-STAT3 was inversely correlated with CD8+ in expression (Fig. 7C), which suggests that the activated IL6/STAT3 axis inhibits the infiltration of CD8+ T cells in tumors.

Finally, by The Cancer Genome Atlas analysis, we found that increased IL6 and increased PD-L1 predict poor overall survival and disease-free survival rates (Fig. 7D and E). These observations further support the model that activated IL6/STAT3 contributes to immunosuppression, including upregulation of PD-L1, and reduced infiltration of CD8+ T cells in NSCLCs.

**Discussion**

In this study, we showed first that STAT3-mediated immunosuppressive trait in tumor cells is mainly responsible for lung metastasis in vivo. Second, dysregulated IL6 reprogrammed the oncogenic signaling in the metastatic tumor cells for increased metastasis in Gprc5a-ko mouse lungs. Third, IL6 induces recruitment of MDSC and macrophage polarization, which inhibits host immunity. And fourth, the active IL6/STAT3 axis can be used as a biomarker for suppressed CD8+ T-lymphocyte infiltration in NSCLC samples. Thus, IL6 plays a key role in orchestrating premetastatic niche formation and immunosuppression in lung.

In this study, we showed that although 1601-shGp130 cells exhibited suppressed EMT-like features, still they formed metastasis and tumors in immune-deficient mice, but did not do so in immune-competent mice. This suggests that STAT3-induced immunosuppression is functionally critical for the metastatic potential in immune-competent mice. Indeed, 1601-shGp130 or -dnStat3 cells are susceptible to T-cell–mediated cytotoxicity. Previously, Snail-induced EMT and immunosuppression were functionally linked in melanoma metastasis model (40). Consistently, a strong correlation between EMT and expression of immunosuppressive genes was revealed by an integrated, global analysis of genomic and proteomic profiles (8). Thus, the EMT-like features are functionally linked to the immunosuppressive traits in cancer cells.
GPRC5A repression is prevalent in NSCLCs. On one hand, lungs from Gprc5a-ko mice are prone to a variety of inflammatory stimuli as well as to spontaneous and carcinogen-induced lung tumor-igenesis (27, 31). On the other hand, inflammation via NF-κB signaling inhibits GPRC5A expression. Thus, repressed GPRC5A is an indication of the inflammatory status in lung tissue such as in COPD (41). We showed that lung metastasis via the same tumor cells was greatly increased in Gprc5a-ko mouse lungs compared with lungs from WT mice. This finding suggests that although the tumor cells or “seeds” are the same, different tissue microenvironments or “soils” would have a great impact on lung metastasis. Thus, upregulated IL6 in Gprc5a-ko mouse lungs is essential for premetastatic niche formation as compared with those in WT and Gprc5a-ko/Il6-ko mice.

An inflammatory microenvironment is implicated as a contributory factor to tumor development and metastasis in many cases. For example, the incidence of lung cancer in patients with COPD is 6-fold higher than that in control group (42). Obesity, a chronic, low-grade inflammation was found to enhance breast cancer metastases to lung by altering lung myeloid cells (43). Smoking, an inflammatory stimulus, increases the incidence of pulmonary metastasis in colorectal cancer (44). And fatty liver facilitates liver metastases in patients with NSCLC (45). Although the normal lung

**Figure 6.** Targeting IL6 inhibits MDSCs and lung metastasis in Gprc5a-ko mice. SJT-1601 cells were intravenously injected into Gprc5a-ko mice and anti-IL6 antibody was intraperitoneally injected according to the prescribed method (n = 5). A, Model diagram of targeting IL6 with anti-IL6 antibody in the Gprc5a-ko mice lung metastasis model. B, Representative images of lung tissues stained with H&E. C, Number of metastatic nodules of B. D, Representative FACS images for G-MDSCs and M-MDSCs staining in tumor lung tissues. E, Statistical analysis of D. F, Representative FACS images for stained TAM in tumor lung tissues. G, Statistical analysis of F. H, Representative FACS images for stained T cells in tumor lung tissues. I, Statistical analysis of H. J, Representative FACS images for stained NK cells in tumor lung tissues. K, Statistical analysis of J. *P < 0.05.
serves as a defensive barrier against foreign pathogens and particulates, the microenvironment of lung tissue can be reprogrammed, such as by chronic inflammation or smoking, to a status that is protumorigenic or prometastatic. In fact, lung is an organ in which metastases are frequently formed from a variety of malignancies. In this study, inflammatory microenvironment of Gprc5a-ko mouse lungs was shown to enhance lung metastasis, supporting the notion.

Figure 7. Dysregulated IL6/p-STAT3 is correlated with suppressed CD8+ T lymphocytes in NSCLC. A, Representative images from a microarray of human lung tissue that includes tumor and adjacent normal tissues (n = 140) stained by IHC for IL6, p-STAT3, PD-L1, and CD8. B, IHC scores from human lung tissues chip of A. C, Correlation analysis for the expression of IL6, p-STAT3, PD-L1, and CD8 in the microarray of human lung tissue. D, IL6 and PD-L1 expression were negatively correlated with patient overall survival rate. E, IL6 and PD-L1 expression were negatively correlated with patient disease-free survival rate. *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001.
Upregulated IL6 is linked to increased metastasis in Gprc5a-ko mouse lungs, whereas IL6 gene deletion in Gprc5a-ko/I6-ko mice almost completely eliminated lung metastases. This observation strongly supports the impact of the extrinsic roles of IL6 in lung metastasis. Persistent STAT3 activation was previously shown to strongly support the impact of the extrinsic roles of IL6 in lung metastasis. Gprc5a features or biological characterization in immune-deficient mice in vivo. In this study, we hypothesize that the major oncogenic program in metastatic tumor cells should be compatible with the signaling network in tumor microenvironment. In support, the metastatic tumor cells are reprogrammed to an activated STAT3 status by tissue microenvironment in Gprc5a-ko mouse lung, whereas depletion of IL6 in Gprc5a-ko/I6-ko mice abrogated the effect. Thus, the compatibility between the oncogenic pathway in metastatic tumor cells and the extrinsic signaling in tissue microenvironment dictates the efficiency of metastasis. Similar in concept, a recent study showed that the intrinsic programs of cancer cells are dictated by tissue microenvironment (46). Thus, cancer cells that succeed in forming metastasis should be reprogrammed to evade immune surveillance, which is compatible to the target tissue microenvironment. Here, IL6 is shown to play a critical role in lung metastasis.

In this study, IL6 targeting therapy significantly inhibited lung metastasis in Gprc5a-ko mice. This suggests that the metastatic tumor cells are addicted to IL6 signaling, whereas neutralization of IL6 by antibody therapy disrupts the oncogenic program that is required for metastatic process. The IL6 targeting strategy was also effective in the treatment of a mouse model with IL6-expressing LLC cells, which caused cachexia (47). In the clinic, serum IL6 levels were reportedly higher in patients with cachexia than in those without. And a high IL6 serum level strongly correlated with short survival in patients with chemotherapy-resistant lung cancer (47). However, anti-IL6 mono-therapy in clinical trials appears to be tolerated and shows no clinical activity in several solid tumors, including colorectal, ovarian, and pancreatic cancers. It is possible that the early stages of tumor formation may be IL6-dependent, whereas late-stage disease may be not. Practically, human solid tumors are more complex than mouse model and multiple clones of cancer cells could be another factor for tolerance. Thus, IL6 inhibition alone may not be sufficient in treating advanced solid tumors (48). Interestingly, a recent study showed that treatment of ovarian cancer cells with neutralizing IL6 antibodies resulted in upregulated EGFR, whereas combination of neutralizing IL6 antibodies and the EGFR inhibitor gefitinib exhibited enhanced anticaner activity (49). Thus, it is expected that combination of IL6/STAT3-targeted therapy with other therapy, such as chemo- or immunotherapy, will be more efficient than monotherapy.

Generally, tumors can be subclassified into “hot” and “cold,” which reflects the status of T-lymphocyte infiltration and the ability to respond to immunotherapy. In this study, we showed that an active IL6/STAT3 signaling system is inversely correlated with CD8+ T-lymphocyte infiltration in NSCLC samples. This finding suggests that the IL6/STAT3 axis provides the candidate markers for “hot” and “cold” tumors, which is potentially used for therapeutic application. Taken together, we found that IL6/STAT3 signaling promotes lung metastasis by orchestrating premetastatic niche formation and immunosuppressive traits (Supplementary Fig. S6).

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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References


Monoclonal Antibody Targeting Sialyl-di-Lewisα-Containing Internalizing and Noninternalizing Glycoproteins with Cancer Immunotherapy Development Potential

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ABSTRACT

Tumor glycans constitute attractive targets for therapeutic antibodies. The sialylated glycoalyx plays a prominent role in cancer progression and immune evasion. Here, we describe the characterization of the mAb, FG129, which targets tumor-associated sialylated glycans, and demonstrate its potential for multimodal cancer therapy. FG129, obtained through BALB/c mouse immunizations with liposomes containing membrane glycan extracts from the colorectal cancer cell line LS180, is an IgG1 that targets sialyl-di-Lewisα-containing glycoproteins. FG129, as well as its chimeric human IgG1 variant, CH129, binds with nanomolar functional affinity to a range of colorectal, pancreatic, and gastric cancer cell lines. FG129 targets 74% (135/182) of pancreatic, 50% (46/92) of gastric, 36% (100/281) of colorectal, 27% (89/327) of ovarian, and 21% (42/201) of non–small cell lung cancers, by IHC. In our pancreatic cancer cohort, high FG129 glyco-epitope expression was significantly associated with poor prognosis (P = 0.004). Crucially, the glyco-epitope displays limited normal tissue distribution, with FG129 binding weakly to a small percentage of cells within gallbladder, ileum, liver, esophagus, pancreas, and thyroid tissues. Owing to glyco-epitope internalization, we validated payload delivery by CH129 through monomethyl auristatin E (MMAE) or maytansinoid (DM1 and DM4) conjugation. All three CH129 drug conjugates killed high-binding colorectal and pancreatic cancer cell lines with (sub)nanomolar potency, coinciding with significant in vivo xenograft tumor control by CH129-vcMMAE. CH129, with its restricted normal tissue distribution, avid tumor binding, and efficient payload delivery, is a promising candidate for the treatment of sialyl-di-Lewisα-expressing solid tumors, as an antibody–drug conjugate or as an alternative cancer immunotherapy modality.

Introduction

Recent advances in cancer cell glycomics have highlighted the differential glycan make-up of tumor cells versus their normal counterparts. During transformation, cancer cells alter their glycosylation profile because of genetic, epigenetic, and metabolic reprogramming of glycosyl transferase activity as well as the availability of nucleotide glycan donors (1–3). This results in glycan structures with altered branching (N-glycans), truncation due to incomplete synthesis, increased sialylation and fucosylation as well as higher density glycosylation (O-glycans), or altogether novel structures due to neosynthesis (4, 5). This altered glycome affects cancer cell biology, as over 50% of the cellular proteome and a significant fraction of membrane lipids are glycosylated that affects their folding, distribution, and activity (6). Specifically during cancer progression, altered glycosylation and sialylation of proteins and lipids have profound effects, ranging from immune evasion to increased cellular proliferation and the capacity to metastasize. Immune evasion stems from the interaction of tumor sialylglycans with immune inhibitory sialic acid–binding immunoglobulin-type lectins (siglecs) expressed by innate immune cells, thereby dampening immune cell activation or evading natural killer–mediated killing (7–11). Increased proliferation results from direct and indirect effects on growth factor signaling, and the interaction of sialylated glycans with activated endothelial cell selectins supports cancer cell metastasis (12–14). Oversialylation of a range of human gastrointestinal and pancreatic cancers as well as melanoma has consistently been associated with enhanced proliferative and metastatic potential (15–18).

The original carbohydrate antigen (CA)19.9 was identified via the CA19.9 mAb, obtained through immunizations with the human colon carcinoma cell line SW-1116. CA19.9 is a sialyl-Lewisα-containing monoganglioside glycolipid that is overexpressed in many gastrointestinal and pancreatic cancers (19, 20). In pancreatic and colon cancer, CA19.9 on secreted mucins is the most consolidated carbohydrate tumor marker, used for early diagnosis as well as for monitoring responses to therapy (21–24).

Here we describe the generation of FG129, a murine (m) IgG1 with kappa light chain, which predominantly targets sialyl-di-Lewisα–containing glycoproteins. We include extensive evaluation of the FG129-binding specificity as well as its glyco-epitope distribution. On the basis of the favorable FG129 glyco-epitope distribution, we report the creation of a chimeric human (h) IgG1 version, CH129, and demonstrate its potential for payload delivery to target-expressing solid tumors.
Materials and Methods

Materials, cells, and antibodies

All cancer cell lines—gastric (AGS and MKN45), colorectal (COLO205, HCT-15, HT29, LoVo, LS180, DLD1 and SW480), pancreatic (ASPC1 and BxPC3), lung (H69, DMS79 and EKVX), ovarian (OVCA3, OVCAR4, and OVCA433), breast (DU4475 and MCF-7), as well as the murine myeloma N50 cell line and normal human umbilical vein endothelial cells (HUVEC)—were purchased from ATCC. All cell lines were authenticated using short tandem repeat (STR) analysis. Normal cell lines were obtained from ATCC and passaged at the tissue culture facility. The HUVECs were purchased from Lonza. The murine myeloma NS0 cell line was purchased from ATCC. All cell lines were authenticated using STR analysis. All reagents were purchased from Sigma-Aldrich. Mouse antibodies were purchased from Abcam, the anti-HLA-ABC (clone W6/32) from eBioscience, the anti-CD40 mAb included as adjuvants. Five days after the final immunization, the splenocytes were harvested and fused with NS0 myeloma cells. Hybridoma supernatant was screened by ELISA and by flow cytometry for LS180 reactivity. Stable clones were established by repeated limiting dilutions and the FG129 mAb was purified from hybridoma supernatant using standard protein G affinity chromatography. mAb isotyping was performed using a standard isotyping kit (Mouse Monoclonal Antibody Isotyping Test Kit, Bio-Rad).

Generation of mAbs

Plasma membrane lipid extract from 5 x 10⁷ LS180 cells, harvested at day 7 posttransfection. The CH129 mAb was obtained from IsoSepAB.

FG129-variable regions’ cloning and generation of CH129 (hlgG1) expression construct

Total RNA was prepared from 5 x 10⁵ FG129 hybridoma cells using TRIzol (Invitrogen) following the manufacturer’s protocol. First-strand cDNA was prepared from 3 μg of total RNA using a First-Strand cDNA Synthesis Kit and AMV reverse transcriptase following the manufacturer’s protocol (Roche Diagnostics). PCR and sequencing of heavy- and light-chain variable regions was performed by Syd Labs, Inc and the variable region family usage analyzed using the IMGT database (25). FG129-variable regions were subsequently cloned into the hlgG1/kappa double expression vector pDCOrig-hlgG1 (26) and the sequence confirmed by sequencing.

CH129 HEK293 transfection and mAb purification

The CH129 mAb was obtained following transient transfection of Exp293F cells using the ExpiFectamine 293 Transfection kit (Gibco, Life Technologies). Briefly, HEK293 cells in suspension (100 mL, 2 x 10⁶/mL) were transfected with 100 ng DNA and conditioned medium harvested at day 7 posttransfection. The CH129 mAb was purified using standard protein G affinity chromatography and dia-lyzed against PBS.

Indirect immunofluorescence and flow cytometry

Cancer cells (1 x 10⁶) or whole blood (50 μL/well) were incubated with primary mAbs (at 33.3 nmol/L) for 1 hour at 4°C, as described previously (27); followed by 1-hour incubation at 4°C with anti-mouse FITC-labeled secondary antibody, lysis of red blood cells (for whole blood binding analysis; Cal-Lyse, Invitrogen), and fixing in 0.4% formaldehyde (Sigma). Cells were run on Beckman Coulter FC-500 and analyzed using WinMDI 2.9. Determination of specific antibody binding capacity (SABC), corresponding to the mean number of accessible antigenic sites per cell was performed using the QiFi kit (Dako) according to the manufacturer’s instructions.

Western blot analysis

Cancer cell lysates (from 1 x 10⁶ cells) and total lipid extracts (from 1 x 10⁶ cells) were subjected to SDS-PAGE (4%–12% Bis-Tris, NOVEX, Invitrogen) and transferred to Immobilon-FL PVDF membranes (EMD Millipore). Triplicate samples were loaded and membranes were blocked for 1 hour [5% (w/v) milk in PBS-Tween 20 (0.05% (v/v)] followed by incubation with primary mAbs (7.4 nmol/L) in 2% BSA-PBS-Tween 20 (0.05% (v/v)) overnight at 4°C. Negative control consisted of omission of primary antibody. Secondary antibodies were IRDye 800CW donkey anti-mouse or IRDye 800CW goat anti-human (LI-COR Biosciences), both used at 1:5,000 for 1 hour at room temperature. mAb binding was visualized using a LI-COR Odyssey scanner.

Lewis antigen and sandwich ELISA

ELISA plates were coated with 100 ng/well Lewis-HSA antigens (direct ELISA) or 200 ng/well FG129/CH129 (for sandwich ELISA), blocked with 2% (w/v) BSA-PBS, followed by incubation with primary mAb (direct ELISA) or sera from healthy donors, patients with pancreatic cancer, or mouse serum from a COLO205 xenograft model (sandwich ELISA). Bound antibody (direct ELISA) was detected using streptavidin-HRPO (Thermo Fisher Scientific; bound antigen (sandwich ELISA) was detected using in-house biotinylated-FG129 and streptavidin-HRPO. Plates were read at 450 nm on a Tecan Infinite F50.

Glycan array analysis

FG129 was screened by the Consortium for Functional Glycomics (CFG) for binding to ≥600 natural and synthetic glycans (core H group, version 5.1). Briefly, slides were incubated with 300 nmol/L FG129 for 1 hour, before detection with Alexa Fluor 488–conjugated secondary mAb.

Affinity determination

The kinetic parameters of FG129 and CH129 binding to sialyl-Lewis*-APD-HSA (sialyl-di-Lewis* is not commercially available) were determined by surface plasmon resonance (SPR; Biacore 3000, GE Healthcare). Increasing concentrations (0.3 nmol/L–200 nmol/L) of FG129 and CH129 were injected across a sialyl-Lewis*-APD-HSA–coupled CM5 chip and binding data fitted, using BIA evaluation 4.1. The chip contained four cells, two of which, HSA-coated (in-line reference cells), the other two were coated with low [30 response units (RU)] and high amounts (360 RU) of sialyl-Lewis*-APD-HSA, mimicking cell surfaces with low and high glyco-epitope expression.

IHC

Tumor and normal tissue binding was assessed on tissue microarrays (TMA) as described previously (27). In brief, after antigen retrieval and blocking of endogenous peroxidase activity and nonspecific binding sites, the slides were incubated with FG129 (7.4 nmol/L) at room temperature for 1 hour, followed by detection with a biotinylated secondary mAb (Vector Laboratories). The slides were subsequently incubated with preformed streptavidin/biotin-HRPO (Dako Ltd.) and 3,3’-diaminobenzidine and finally counterstained with hematoxylin. Staining was scored by two independent assessors, using New Viewer software 2010. The sections were given a semiquantitative histologic score (H-score, 0–300) based on the cellular staining.
intensity: negative (0), weak (1), moderate (2), and strong (3) and on the percentage of positive cells. Stratification cut-off points for the survival analysis (SPSS 13.0, SPSS Inc) were analyzed using X-Tile software (28). P values < 0.05 were considered significant.

**Patient cohorts**

The study populations included cohorts from the consecutive series of 462 archived colorectal cancer specimens (1994–2000; median follow up 42 months; censored December 2003; ref. 29); patients with lymph node–positive disease routinely received adjuvant chemotherapy with 5-fluorouracil/folinic acid, 350 ovarian cancer samples (1982–1997; median follow-up 192 months; censored November 2005; patients with stage II to IV disease received standard adjuvant chemotherapy, which in later years was platinum based; ref. 30), 142 gastric cancer samples (2001–2006; median follow up 66 months; censored January 2009; no chemotherapy; ref. 31), 68 pancreatic and 120 biliary/ampullary cancer samples (1993–2010; median 45 months; censored 2012; 25–46% of patients received adjuvant chemotherapy with 5-fluorouracil/folinic acid and gemcitabine; ref. 32), and 220 NSCLC (January 1996–July 2006: median follow up 36 months; censored May 2013; none of the patients received chemotherapy prior to surgery but 11 patients received radiotherapy and 9 patients received at least 1 cycle of adjuvant chemotherapy post-surgery) obtained from patients undergoing elective surgical resection of a histologically proven cancer at Nottingham University Hospital (Nottingham, United Kingdom) or Derby University Hospitals (Dery, United Kingdom). No cases were excluded, unless the relevant clinicopathologic material/data were unavailable. Studies involving human participants were approved by the Nottingham Research Ethics Committee and were in accordance with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Written consent was obtained from all participants.

**Competition flow cytometry–based cell binding assay**

AlexaFluor 488–labeled {according to the manufacturer’s protocol (Invitrogen) FG129, 66.7 nmol/L} was preincubated for 1 hour at 37°C with 100 μL of undiluted serum from patients with pancreatic cancer or healthy human volunteers. Subsequently, cell binding to prefixed (1% formaldehyde) HCT-15 (1 × 105 cells) was analyzed on a Beckman Coulter FC-500 (WinMDI 2.9).

**Confocal microscopy**

AlexaFluor 488 (495/519nm)-labeled FG129 was added to 1.5 × 105 cancer cells on glass coverslips and incubated for 1 hour at 37°C. During the last 30 minutes of the incubation, Hoechst 33258 (350/461 nm) nucleic acid stain (1 μg/mL), and LysoTracker deep red (647/668 nm) lysosomal stain (50 nmol/L), and CellMask Orange (554/567nm) plasma membrane stain were added to the cells (Invitrogen). Cells were imaged with a ZEISS LSM 510 ConfoCor II confocal microscope (63×1.4 NA oil objective) and LSM Image Browser was used for image processing.

**mAb internalization and payload delivery assay**

Payload delivery by FG129 was initially evaluated by measuring the cytotoxicity of immune-complexed FG129 with a saporin-conjugated anti-mouse or anti-human (33). Fab-ZAP secondary conjugate (Advanced Targeting Systems; ref. 33). Target cells were plated overnight in triplicate into 96-well plates (2 × 104 cells, 90 μL/well). After preincubation (30 minutes at ambient temperature) with a concentration range of FG129 and 50 ng of the Fab–ZAP conjugate, 10μL of conjugate or free FG129 were added to the wells and incubated for 72 hours. Control wells, consisted of cells incubated without conjugate, incubated with secondary Fab-ZAP without primary mAb and incubated with a control mAb preincubated with Fab-ZAP. Cell viability was measured by 3H-thymidine incorporation during the final 24 hours. Normalized results are expressed as a percentage of cells incubated with primary mAb only (control).

**Drug conjugation**

The following payload and linker chemistries were used for CH129: (i) auristatin monomethyl auristatin E (MMAE) linked via a cleavable dipeptide valine-citrulline (vc) linker, CH129-vc-MMAE, including a para-aminobenzylalcohol (PABA) self-immolative spacer; (ii) maytansinoid emtansine, DM1, via the noncleavable linker succinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate) (SMCC); and (iii) maytansinoid DM4 via the hindered disulfide linker succimidyl 3-(2-pyridyldithio)propionate (SPDP) CH129-DM4, which is immediately cleavable. Direct payload conjugation, via reduced interchain disulfides, was performed by ADC Biotechnology Ltd. Briefly, Tris (2-carboxy-ethyl)-phosphin-HC–reduced CH129 (formulated at 23 mg/mL in PBS) was pH-adjusted to approximately pH 7.5. The antibody was then conjugated to MMAE by the addition of five molar equivalents of vc MMAE (60 minutes). CH129 conjugation to DM1-MCC and DM4-SPDP occurred in a similar manner, at 10 molar equivalents relative to the mAb (2 hours). Conjugates were purified on a G25 desalting column. A matched set of control (non-targeting) rituximab (10 mg/mL in pH 6.5 citrate buffer)-based antibody–drug conjugate (ADC) constructs was also produced. The drug-to-antibody ratios ( DAR) were determined following size-exclusion chromatography (SEC) in the case of MMAE conjugates and by hydrophobic interaction chromatography (HIC) for the DM1 and DM4 conjugates (Supplementary Table S1).

**In vitro ADC cytotoxicity**

The cytotoxic effect of the CH129-ADC or control RTX-ADC constructs was assessed using the water-soluble tetrazolium salt WST-8 (CCK8 kit, Sigma-Aldrich). Briefly, after overnight plating of cancer cells (2 × 104 cells/well), the ADC constructs were added at different concentrations in a final volume of 10 μL/well, and the plates incubated at 37°C (5% CO2) for 72 hours. WST-8 reagent was added (10 μL/well) followed by a further 3-hour incubation after which the OD 450nm was determined (Tecan Infinite F50) and the surviving fraction calculated. EC50 values were determined using nonlinear regression (GraphPad Prism v 5).

**In vivo ADC tumor control in COLO205 xenograft model**

The study was conducted by CrownBio UK under a UK Home Office Licence in accordance with NCRI, LASA, and FELAS guidelines. Animal welfare for this study complies with the UK Animals Scientific Procedures Act 1986 (ASPA) in line with Directive 2010/63/EU of the European Parliament and the Council of September 22, 2010 on the protection of animals used for scientific purposes. Subcutaneous tumors of a human colorectal adenocarcinoma model of COLO205 were established in age-matched female BALB/c nude (Charles River) mice via injection of 5 × 106 viable cells in 0.1 mL serum-free RPMI. Matrigel (1:1) into the left flank of each mouse. Mice (n = 10) were randomly allocated to treatment groups based on their tumor volume (mean tumor volume per group at start of dosing: 63.2 mm3 ± 3.9 mm3; study day 7) and dosed intravenously (i.v.), biweekly, with 0.1 mg CH129-ADC or RTX-ADC (control) or vehicle (PBS, 100 μL) for a total of four doses. Tumor growth was monitored up until week 4. Body weight and tumor volume were assessed three times weekly and
reduction in tumor volume analyzed statistically using a two-way ANOVA test with Bonferroni posttest (interaction factors; GraphPad Prism v 7.4, GraphPad Inc).

**Results**

**FG129 as well as chimeric hIgG1, CH129, recognize their sialyl-di-Lewis* glyco-epitope on glycoproteins from a range of cancer cell lines**

FG129 is a mouse IgG1 with kappa light chain (Supplementary Fig. S6). Variable region sequencing demonstrated that the FG129 heavy chain belongs to the mouse heavy chain IGHV10-1’/IGHD1-1’/IGHJ4’ family with three mutations compared with the parental germline sequence. The FG129 light chain belongs to the mouse kappa chain IGKV8-19’/IGKJ4’ family with two mutations compared with the parental germline sequence.

Glycan array (CFG) analysis of the FG129 glyco-epitope binding profile revealed a high specificity of FG129 for sialyl-di-Lewis* (100%) as well as the two closely related glycans, sialyl-Lewis* (89%) and sialyl-Lewis* (89%), the latter only when presented on a long carbon spacer (eight carbons, sp8; Fig. 1A; Supplementary Table S2 and http://www.functionalglycomics.org/glycomics/HServlet?operation=view&sideMenu=no&psId=primscreen_6165). FG129 did not bind the closely related, dietary-derived, N-glycolybibranaminic acid (Neu5Gc)-Lewis*, nor did it bind unsialylated Lewis*, Lewis*, sialyl-Lewis*, or sialyl-Lewis*, indicating that its specificity is largely driven by terminal and accessible sialyl-Lewis* recognition. In addition, the recognition was specific for sialic acid in alpha 2–3 linkage as no binding to alpha 2–6 or alpha 2–8 sialylated glycoconjugates was detected.

The overall cancer cell surface binding by FG129 was analyzed using flow cytometry. FG129 showed strong cell surface binding [geometric mean (Gm) ≥1,000] to colorectal tumor cell lines such as HCT-15 and COLO205, somewhat lower to pancreatic lines such as ASPC1 and BxPC3; moderate binding (Gm 100–1,000) to LS180, DLD1, MKN45, DMS79, and H69 and low binding (Gm < 100) to HT29. FG129 displayed negligible binding to AGS, SW480, EKVX, MCF-7, LoVo, DU4475, OVCA14, and OVCA43 (Fig. 1B; Supplementary Fig. S1A). Quantitative analysis of the antibody-binding capacity (ABC) on a subset of the high- to moderate-binding cell lines revealed 6.2 × 10⁵ antigenic sites for COLO205 compared with 2.2 × 10⁵ for HCT-15 and approximately 1.2 × 10⁵ for both BxPC3 and
ASPC1. Crucially, FG129 did not bind granulocytes or lymphocytes from a representative normal blood donor (Supplementary Fig. S1B) nor did FG129 bind to normal HUVECs (Supplementary Fig. S1C). These results identified several high-binding colorectal as well as pancreatic cancer cell lines with which to assess FG129-based therapeutic modalities in vitro.

A clinically more relevant hIgG1 chimeric variant, CH129, was created via cloning of the FG129 heavy- and light-chain variable sequences in-frame with the hIgG1 constant regions. CH129 bound a similar range of cancer cell lines compared with FG129 (Fig. 1C), with strong binding observed on COLO205 and HCT-15, moderate binding to DLD1, BxPC3, ASPC1, and LS180 and low binding on DMS79.

On the cancer cell surface, glyco-epitopes can be present on glycoproteins as well as glycolipids. Consequently, we assessed recognition of biologically relevant glyco-targets by FG129 and CH129, in more detail, by Western blotting using total lipid cell extracts and whole-cell lysates from colorectal (COLO205 and HCT-15) and pancreatic (BxPC3) cell lines. FG129, as well as CH129, bound to a range of predominantly high-molecular weight (50–200 kDa) glycoproteins in all lysates, with more intense staining observed for COLO205 compared with the HCT-15 and BxPC3 lysates, potentially reflecting the differences in ABC (Fig. 1D). Interestingly, both the COLO205 and BxPC3 staining patterns displayed a band of very high molecular weight, not observed for the HCT-15 lysate, perhaps suggesting mucin binding. In comparison, the CA19.9 glycan as defined by mAbs 19-9 and 52a is a monosialoganglioside, a sialylated N-fucopentaose II (20, 34). Accordingly, Western blot analysis of the same cell lysates and total lipid extracts with CA19.9-recognizing SPM110 showed a prominent glycolipid band at the dye front, in all three total lipid extracts, as well as a band at the dye front, in all three total lipid extracts, as well as a

The tumor tissue binding of FG129 was assessed by IHC. FG129 bound to 74% (135/182) of pancreatic tumors, 50% (46/92) of gastric tumors, 36% (100/281) of colorectal tumors, 27% (89/327) of ovarian, and 21% (42/201) of NSCLC tumors (Table 2). Representative images of different staining levels of tumor tissues are shown in Fig. 2A. In the pancreatic cancer cohort, Kaplan–Meier analysis of disease-free survival of patients with pancreatic revealed a significantly lower mean survival time in the high FG129–binding group (low, mean survival: 90 months; n = 82; P = 0.004, log-rank test). On multivariate analysis using Cox regression, high FG129 antigen expression in pancreatic cancer was a marker for poor prognosis which was independent of perineural invasion and lymph node involvement (P = 0.012) which are key prognostic factors in pancreatic cancer. This suggests that in this cohort, patients with high-density glyco-epitope expression would benefit from FG129-based therapy.

The normal tissue distribution of FG129 was evaluated using two normal human TMAs: AMSBio, T8234708-5, covering 31 tissues, one normal human individual per tissue, in duplicate, and US Biomax, FDA999i, 32 types of normal organs from three individuals, single core per case. On the AMSBio array, FG129 displayed a very restricted binding pattern and did not bind most normal tissues, including vital tissues such as heart, brain, stomach, and kidney (Supplementary Table S3; Fig. 2C). Weak to moderate binding of a very small percentage of cells was seen in gallbladder, ileum, liver, esophagus, pancreas, and thyroid. In contrast, the CA19.9 mAb stained a subset of tissues: esophagus, liver, and pancreas with strong intensity (Supplementary Table S3). In addition, the majority of tissues on the US Biomax array were negative with low-to-moderate staining of a small fraction of cells on tonsils (1/3), thymus (2/3); salivary gland (1/3); esophagus (3/3); adjacent normal (1/3), and cancer adjacent uterine cervix tissue (2/3; Supplementary Fig. S3).
FG129 recognizes secreted glyco-antigen in the serum of patients with pancreatic cancer and a mouse COLO205 xenograft model, but not healthy donors

CA19.9 can be expressed on glycolipid (monosialoganglioside) as well as mucins and detection of this antigen in sera of pancreatic and colorectal cancer is used in clinical practice to monitor disease progression and response to therapy (16, 23, 35). This is currently the only FDA-approved test for pancreatic cancer. As FG129 detects terminal/accessible sialyl-Lewis^a^-containing glycoproteins, we analyzed a subset of patient sera from our pancreatic cancer cohort for the presence of glyco-antigen using sandwich ELISA. No secreted glyco-antigen was detected in 12 sera from healthy donors, whereas in 33% (7/21) pancreatic cancer sera, glyco-antigen levels were significantly raised (Fig. 3A). Similarly, serum from a mouse COLO205 xenograft model, included as a positive control, also contained significantly elevated secreted glyco-antigen (Fig. 3A). We next set out to investigate whether the presence of secreted glyco-antigen would impede tumor cell binding, using flow cytometry. FG129 was preincubated at 37°C with the relevant sera, after which cell binding to HCT-15 was evaluated (Fig. 3B). FG129 maintained HCT-15 binding after pre-incubation with patients’ sera, suggesting that its nanomolar cell surface functional affinity is sufficient to retain tumor cell targeting, even in the presence of secreted glyco-antigen.

Efficient cellular internalization of FG129 as well as CH129 and delivery of indirectly conjugated payload on high-binding cancer cells

As the murine and human 129 mAbs displayed avid glycotarget binding on high-binding colorectal and pancreatic cancer cell lines, we sought to analyze their internalization potential. Confocal microscopy of Alexa Fluor 488-labeled FG129 and CH129, incubated with COLO205 cells for 60 to 90 minutes, showed very efficient internalization of both mAbs with a significant proportion colocalizing with lysosomal compartments, as well as a proportion remaining on the cell surface (Fig. 4A). Similar results were obtained using HCT-15 and BxPc3 (Supplementary Fig. S4A and S4B, respectively), suggesting that high-level 129 mAb binding at the cell surface drives target internalization.

Lysosomal targeting by the 129 mAbs was further validated through assessing the toxicity of saporin (“ZAP”, IT-48, ATS Bio)-conjugated anti-mouse/human Fab immune complexes containing FG129 or CH129 on high-binding cancer cell lines. Internalization of Fab-ZAP-FG129 led to a dose-dependent decrease in cell viability (EC_{50} ~ 1 pmol/L) on high-binding cells COLO205 and HCT-15 but surprisingly, not on BxPc3 (Fig. 4B, i). MUC1 expression by BxPc3 has been linked to chemoresistance as a result of increased expression of multidrug resistance (MDR) genes (36). The antigen-low cell line LoVo was refractory to Fab-ZAP-FG129 with a modest inhibition of viability only at the highest concentrations. Fab-ZAP preincubated with isotype control showed negligible cytotoxicity. Importantly, this was corroborated for CH129, where the saporin-conjugated CH129 displayed dose-dependent toxicity with picomolar EC_{50} on the high-binding COLO205 and HCT-15 (Fig. 4B, ii). Together, the results indicate efficient internalization and payload delivery in high-binding cancer cell lines by FG129 and CH129. Advantageously, in addition to their efficient internalization, the 129 mAbs retained potent ADCC effector activity on the high-binding COLO205 (Fig. 4C), and HCT-15 (Supplementary Fig. S4C), with EC_{50} values in the subnanomolar range (~0.1 nmol/L), indicating that the glyco-proteins associated with mAb internalization are distinct from the ones targeted for effector functions and suggests multimodal development potential.

CH129-drug conjugate (ADC) shows high in vitro activity on high-binding cancer cell lines

To directly assess the ADC potential of CH129, we conjugated it to clinically validated linker/payload combinations (Fig. 5A), as described in the Materials and Methods Section (DAR; Supplementary Table S1) and compared their cytotoxicity to that of a nontargeting RTX-ADC control on the high-binding colorectal cancer cell lines COLO205 and HCT-15. In vitro cytotoxicity analysis of the CH129–ADC constructs on high-binding COLO205 showed potent toxicity of all three CH129–ADC constructs, CH129-vcE (65.8 pmol/L EC_{50}), CH129-SMCC-DM1 (0.2 nmol/L EC_{50}) and CH129-SPDP-DM4 (0.1 nmol/L EC_{50}), and 100% inhibition at the highest concentration tested (Fig. 5B; Supplementary Table S4). Activity of the CH129-ADC compounds on HCT-15 (Fig. 5C) was lower compared with COLO205, with nanomolar EC_{50} values for all three CH129-ADC constructs (Supplementary Table S4). HCT-15 was more resistant to killing by the CH129–ADC constructs, cell survival being about 30%–40% at the highest concentration tested, possibly due to the presence of MDR1 or other ATP-dependent efflux systems (37). On the pancreatic line BxPc3, EC_{50} values were in the low nanomolar range (Fig. 5D; Supplementary Table S4). On COLO205 and BxPc3, CH129-vcE, with the peptidase-cleavable linker, outperformed the noncleavable linker-containing ADCs. The nontargeting control RTX-vcE showed no cytotoxicity over the entire concentration range tested, whereas RTX-DM1 only showed significant activity at the highest concentration (100 nmol/L). RTX-DM4 displayed more potent nonspecific toxicity (Supplementary Table S4), potentially due to its slightly higher DAR compared with the other control ADCs (Supplementary Table S1).

Cytotoxicity assays on cell mixtures containing increasing amounts of glyco-antigen–negative cells (AGS) in combination with the high-binding COLO205 were performed to assess the potential for bystander killing by CH129–ADC conjugates with cleavable linkers, such as the CH129-vcE and CH129-SPDP-DM4, and thus the possibility of targeting heterogeneous tumors containing cells expressing lower amounts or no glyco-target, as well as tumor stromal cells. The two

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**Table 2. Binding of FG129 (7.4 nmol/L) by IHC to gastric, colorectal, pancreatic, ovarian, and lung TMAs, by staining intensity.**

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Gastric</th>
<th>Colorectal</th>
<th>Pancreatic/biliary/ampullary</th>
<th>Ovarian</th>
<th>Lung (adenocarcinoma)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Staining (%) (n/total)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>50 (46/92)</td>
<td>64 (18/281)</td>
<td>25 (45/180)</td>
<td>73 (238/327)</td>
<td>79 (159/201)</td>
</tr>
<tr>
<td>Weak</td>
<td>27 (25/92)</td>
<td>26 (72/281)</td>
<td>21 (37/180)</td>
<td>19 (65/327)</td>
<td>10 (21/201)</td>
</tr>
<tr>
<td>Moderate</td>
<td>11 (10/92)</td>
<td>9 (25/281)</td>
<td>34 (61/180)</td>
<td>6 (21/327)</td>
<td>4 (9/201)</td>
</tr>
<tr>
<td>Strong</td>
<td>12 (11/92)</td>
<td>1 (3/281)</td>
<td>21 (37/180)</td>
<td>2 (5/327)</td>
<td>6 (12/201)</td>
</tr>
</tbody>
</table>
CH129-ADC constructs with cleavable linkers and thus bystander potential comprised CH129-vcE and CH129-SPDP-DM4, whereas CH129-SMCC-DM1, with its uncleavable linker, was used as a control in these experiments. A fixed amount of antigen-positive COLO205 cells was mixed with increasing amounts of antigen-negative AGS cells and cell killing by the CH129-ADC compounds analyzed at 1 nmol/L. This concentration was chosen for maximum killing of COLO205 and at the same time absence of killing of antigen-negative AGS cells (Supplementary Fig. S5). Only CH129-DM4 maintained its COLO205 killing activity in the presence of increasing amounts of antigen-negative AGS cells, inducing over 50% killing at a ratio of 10:1 of AGS:COLO205 cells; CH129-vcE displayed intermediate bystander killing, showing a gradual decrease in killing activity with increasing amounts of AGS cells (Fig. 5E). Finally, CH129-DM1 with its
uncleavable linker required a near homogenous COLO205 population to induce cell killing. Combined, the results suggest linker cleavability dependent bystander killing by CH129-DM4 as well as CH129-vCE.

**CH129-vCEx conjugate potently controls tumor growth in vivo in a COLO205 xenograft model**

On the basis of the subnamolar in vitro killing potency (65.8 pmol/L EC½) as well as its intermediate bystander killing activity, we selected CH129-vCEx for evaluation of in vivo tumor control in a COLO205 xenograft model. Tumors were established and dosing started on day 7. In this model, biweekly administration of four doses of CH129-vCEx established a significant reduction in tumor volume compared with the control ADC, RTX-vCEx (two-way ANOVA, P < 0.0001; Fig. 6A). Impressively, 7 of 10 mice became tumor-free for the duration of the study. The compounds were well-tolerated, with no adverse effects on mean body weight (Fig. 6B).

**Discussion**

We have generated a mouse IgG1k anti-sialyl-di-Lewisα (Neu5Acα2-3Galβ1-3(Fucα1-4)GlcNAcβ1-3Galβ1-3(Fucα1-4)-GlcNAcβ3) mAb, as well as a chimeric hlgG1 variant. The mAb only bound mono-sialyl-Lewisα if presented on a long carbon spacer suggesting a preference for accessible (terminal) sialyl-Lewisα-containing glycan. Importantly, no cross-reactivity with the dietary-derived Neu5Gc-Lewisα was observed. Characterization of the cell surface glycotarget of our 129 mAb suggests it to be selectively expressed on glycoproteins; predominantly, but not exclusively, on colorectal, pancreatic, and gastric cancer cell lines. In contrast, the CA19.9 targeting mAb, SPM110, bound both glycolipids and glycoproteins upon Western analysis. This differential binding profile may be the result of differing binding affinities, combined with the distinctive glycan clustering patterns of glycoproteins compared with lipid-associated glycans. In addition, the fine-binding specificities of the two mAbs may contribute to their differing target binding profile. In this context, SPM110 has been shown to cross-react with Neu5Gc-Lewisα (CFG, primscreen_5461). In addition, a recent comparison of a selection of CA19.9 targeting mAbs, currently used in clinical diagnostics, demonstrated varied recognition of Neu5Ac- as well as Neu5Gc-conjugated glycans (38).

Both 129 mAbs (FG129 (mIgG1) as well as CH129 (chimeric hlgG1) displayed high functional sialyl Lewisα binding affinity: Kd ~0.2 nmol/L and 20–50 nmol/L on high- and low-density surfaces (SPR), respectively, likely reflecting the target density-dependent differential binding modes (bivalent versus monovalent). ELISA experiments yielded similar EC½ values compared with SPR, whereas cell surface binding functional affinity was slightly reduced, albeit still in the nanomolar range, presumably due to the more fluid nature of the cell membrane and the occurrence of other biological processes such as internalization (27). The findings may also reflect the different target antigen utilized (sialyl-Lewisα-APD-HSA in ELISA and SPR vs. complex glycoproteins on the cell surface). Taken together the apparent nanomolar functional affinity should enable robust tumor targeting (39).

The 129 mAb glyco-target displayed a wide tumor tissue distribution encompassing pancreatic, colorectal, and gastric cancers, as well as ovarian and NSCLC to a lesser degree. Increased sialylation is associated with enhanced proliferative and metastatic potential of both colorectal and pancreatic cancer, rendering sialyl-di-Lewisα targeting therapy an attractive option (22, 40–42). Earlier work has demonstrated the prognostic value of serum secreted sialyl-Lewisα in pancreatic adenocarcinoma (23, 43). Our study reveals that the FG129 glyco-epitope expression in pancreatic cancer is also an independent marker for poor prognosis, suggesting that therapy based on the 129 mAb would have the highest impact on the more aggressive tumors. In addition, we found that FG129 recognized secreted sialyl-di-Lewisα in around 30% of the pancreatic patients’ sera, whereas...

![Image](https://example.com/image.jpg)
negligible secreted target was detected in the serum from healthy individuals. This is a reduced frequency compared with the current diagnostic/prognostic CA19.9–based detection levels that frequently detect secreted antigen in 62%–68% of pancreatic cancer patients’ sera (38) and may reflect the different binding specificities as well as affinities of the mAbs. Critically, under conditions mimicking in vivo application, the FG129 maintained binding to its cell surface glyco-target in the presence of secreted antigen, suggesting that secreted circulating glyco-antigen may not be detrimental to 129 mAb tumor targeting. Normal human tissues displayed very restricted 129 glyco-epitope expression. Vital tissues such as heart, brain, lungs, and kidneys were all negative, as well as most other nonvital tissues. Weak to moderate FG129 binding was observed to normal gallbladder, ileum, liver, esophagus, pancreas, and thyroid, but only on a small fraction of cells. This differential tissue binding profile with strong tumor tissue reactivity and limited normal tissue cross-reactivity, renders the 129 mAb an attractive candidate for clinical development.

FG129 exhibited very efficient cellular internalization and lysosomal localization as demonstrated by two indirect approaches, confocal microscopy and targeted toxin delivery. Internalization of indirectly toxin-conjugated 129 mAbs led to killing of high-binding colorectal cancer cell lines with picomolar EC50. The internalization was directly dependent on the cell surface antigen density in cell lines, with high glyco-epitope–expressing cell lines internalizing better than low-density–expressing cell lines. On that basis, we directly conjugated CH129 with three clinically validated linkers and drugs [auristatin (vcMMAE, cleavable) as well as maytansinoids (SMCC-DM1, uncleavable and SPDP-DM4, moderately cleavable) thereby covering a range of linker chemistries, as well as bystander killing potential. All three CH129-ADCs exhibited subnanomolar killing of high-binding colorectal cancer lines and nanomolar killing on moderately binding colorectal cancer as well as pancreatic cell lines. CH129-vcMMAE consistently exhibited the most potent killing against uniform cells, whereas CH129-DM4 exerted the most efficient bystander killing in mixed (antigen-positive and negative cells) cultures. As such, CH129-vcMMAE exerted potent in vivo tumor control in a COLO205 xenograft model. Interestingly, our analysis indicated that the sera from mice with established tumors contained secreted glyco-antigen; however, this did not impede the significant in vivo activity of CH129-vcE. Alternative site-specific conjugation strategies, as well as more potent drugs, for example the very potent Pyrrolobenzodiazepine (PBD), that are currently being evaluated, would constitute attractive alternatives for conjugation to the 129 mAb (44, 45).

Importantly, in addition to drug conjugate potential, the 129 mAb maintains strong ADCC effector function, suggesting that alternative approaches with cancer targeting utility could be adopted. These include T-cell redirecting bispecific (TCB) formats in combination with an anti-CD3 arm, as well as the use of the 129 variable regions for reformatting for chimeric antigen receptor (CAR)-T approaches, both
of which rely on minimal normal tissue cross-reactivity (46). Recently, an attractive strategy for increased tumor-specific targeting in the TCB setting was proposed in the shape of an avidity-optimized HER2 TCB (47). This strategy hinged on the combination of low monovalent target affinity, precluding normal tissue (with low target expression) binding, with avidity-driven tumor targeting and this approach could be relevant for the 129 mAb. 

In this global immuno-oncology era, cancer-associated sialylated glycans constitute attractive targets for cancer immunotherapy. The immunologic consequences of aberrant cancer cell surface expression of sialic acid–containing glycoconjugates range from disabling the killing mechanisms of effector cells, increasing the production of immunosuppressive cytokines, complement regulation, as well as reducing the activation of antigen-presenting cells, many aspects of which are governed by the interaction with immune-inhibitory siglec receptors (9, 10, 13, 48–50). This was recently substantiated by data demonstrating that sialic acid blockade had a major impact on tumor immune cell composition and effector cell killing ability, creating an immune-permissive tumor microenvironment (51).

In conclusion, our sialyl-di-Lewis^a^-targeting 129 mAb with restricted normal tissue binding combined with a wide-ranging tumor distribution, favorable functional target affinity, as well as efficient internalization and potential multimodal application, constitutes an attractive candidate for cancer immunotherapy.

Figure 5.
A, Potent in vitro cytotoxicity of CH129–ADC compounds on colorectal and pancreatic cell lines. Schematic drawing of the three CH129-ADC compounds used in this study. *In vitro* cytotoxicity of activity of CH129-ADC compounds on COLO205 (B), HCT-15 (C), and BxPC3 (D). E, Bystander killing activity of CH129-ADC compounds at 1 nmol/L in mixed cultures of antigen-positive (COLO205) and antigen-negative (AGS) cells.
Figure 6.
Potent in vivo tumor control by CH129-vcE ADC in a COLO205 xenograft model. A, Significant (P < 0.0001) in vivo tumor control by 129-vcE compared with vehicle control and compared with the nontargeting RTX-vcE in a COLO205 xenograft model (Balb/c mice). B, No significant adverse effect was observed on mean body weight during the course of the study. Arrows indicate day of dosing. Significance was deduced from two-way ANOVA with Benferroni corrections for multiple comparisons.

Disclosure of Potential Conflicts of Interest
S.T. Tivadar reports receiving a commercial research grant from Scancell Ltd (during PhD studies), received a scholarship from Scancell Ltd covering 2 years of tuition fees and stipend). L.G. Durrant is a CSO, reports receiving a commercial research grant from, and has ownership interest (including patents) in Scancell Ltd. No potential conflicts of interest were disclosed by the other authors.

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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): S.T. Tivadar, R.S. McIntosh, A.M. Zaitoun, S. Madhusudan
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): S.T. Tivadar, R.S. McIntosh, A.M. Zaitoun, L.G. Durrant, M. Vankemmelbeke
Writing, review, and/or revision of the manuscript: T. Parsons, A.M. Zaitoun, S. Madhusudan, L.G. Durrant, M. Vankemmelbeke

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Study supervision: L.G. Durrant
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Reduced Neoantigen Expression Revealed by Longitudinal Multimomics as a Possible Immune Evasion Mechanism in Glioma

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Abstract

Immune-based therapies have shown limited efficacy in glioma thus far. This might be at least in part due to insufficient numbers of neoantigens, thought to be targets of immune attack. In addition, we hypothesized that dynamic genetic and epigenetic tumor evolution in gliomas might also affect the mutation.neoantigen landscape and contribute to treatment resistance through immune evasion. Here, we investigated changes in the neoantigen landscape and immunologic features during glioma progression using exome and RNA-seq of paired primary and recurrent tumor samples obtained from 25 WHO grade II–IV glioma patients (glioblastoma, IDH-wild-type, n = 8; grade II–III astrocytoma, IDH-mutant, n = 9; and grade II–III oligodendroglioma, IDH-mutant, 1p/19q-codeleted, n = 8). The number of missense mutations, predicted neoantigens, or expressed neoantigens was not significantly different between primary and recurrent tumors. However, we found that in individual patients the ratio of expressed neoantigens to predicted neoantigens, designated the "neoantigen expression ratio," decreased significantly at recurrence (P = 0.003). This phenomenon was particularly pronounced for "high-affinity," "clonal," and "passenger gene–derived" neoantigens. Gene expression and IHC analyses suggested that the decreased neoantigen expression ratio was associated with intact antigen presentation machinery, increased tumor infiltrating immune cells, and ongoing immune responses. Our findings imply that decreased expression of highly immunogenic neoantigens, possibly due to persistent immune selection pressure, might be one of the immune evasion mechanisms along with tumor clonal evolution in some gliomas.

Introduction

Gliomas are the most frequent primary brain tumors, classified as grade I–IV by the World Health Organization (WHO; ref. 1, 2). Of these, grade IV glioblastoma (GBM) is the most aggressive type; patients with GBM have a median survival of less than 2 years with the current standard therapy (3, 4). Grade II or III gliomas also have malignant potential to grow invasively, progress to higher grades, and become resistant to therapies (5). Although much effort has been expended in optimizing treatment regimens for glioma patients, no real progress has been made, and the clinical prognosis remains extremely poor (6). New treatment modalities, such as immunotherapy, are urgently required.

There have been major advances in cancer immunotherapy (7) and the development of immune-checkpoint inhibitors (ICI), which enhance endogenous antitumor immune responses, has been especially effective in achieving remarkable clinical benefits for some patients with several different types of cancer (8). In some cases, clinical outcome has been reported to be associated with the tumor mutation and neoantigen burden (9–11). Neoantigens derived from somatic mutations are recognized as "foreign"...
by the immune system, and are thus considered ideal targets for immune-based therapies, as well as in constitutive immune surveillance (12–17).

In gliomas, however, thus far there has been very limited success of immune-based therapies, not only ICI, but also vaccination and adoptive chimeric antigen receptor T-cell (CAR-T) treatment (18, 19). This may be at least partly due to the relatively low number of neoantigens on gliomas that can be targeted by the immune system, together with several immune resistance mechanisms that have been documented for this cancer type (20, 21). It remains unclear whether the small number of neoantigens that may be present are in fact recognized and edited by the immune system during glioma progression. We hypothesized that dynamic genetic and epigenetic evolution during disease progression (22, 23) might also affect the status of the mutation/neoantigen landscape and contribute to treatment resistance through immune evasion in gliomas. Accordingly, in the present study, we performed longitudinal multimomics analyses on paired primary and recurrent tumor samples from 25 glioma patients. We evaluated temporal changes of the mutation/neoantigen landscape and the involvement of host immunity to further define immune evasion mechanisms of gliomas.

Materials and Methods

Clinical samples

Clinical fresh frozen tumor samples and matched blood samples from a total of 36 glioma patients who underwent multiple glioma surgeries were retrospectively collected at The University of Tokyo Hospital, National Cancer Center Hospital, Kyorin University Hospital, Saitama Medical University International Medical Center, and Dokkyo Medical University Hospital. Of these patients, we excluded 10 who lacked either whole-exome sequencing (WES) or RNA-seq data for both primary and recurrent tumor samples. We also excluded a case in which the recurrent tumor was IDH-mutant, 1p/19q codeletion (IDH-O). Most of the patients had received standard therapy and grade II glioma surgeries were retrospectively collected at The University of Tokyo Hospital, National Cancer Center Hospital, Kyorin University Hospital, Saitama Medical University International Medical Center, and Dokkyo Medical University Hospital. Of these patients, we excluded 10 who lacked either whole-exome sequencing (WES) or RNA-seq data for both primary and recurrent tumor samples. We also excluded a case in which the recurrent tumor was found to be a hypermutator (Fig. 1A) because these are considered to have atypical immunologic features (24–26). The remaining 25 cases were analyzed in this study (Supplementary Table S1). The study cohort partly overlapped with cases from our previous studies (22, 27–29). Histopathologic diagnoses were made based on isocitrate dehydrogenase 1 or 2 gene (IDH1/2) mutation and chromosome 1p/19q status as well as histology according to the 2016 WHO guidelines (1). Based on the initial diagnoses, patients were classified into three histologic groups: glioblastoma, IDH-wild-type (GBM); grade II–III astrocytoma, IDH-mutant (IDH-A); and grade II–III oligodendroglioma, IDH-mutant, 1p/19q-codelleted (IDH-O). Most of the patients had received standard therapy between the first and second surgeries according to each institutional decision. No patients received any immune-based therapies prior to the second surgery (Supplementary Table S1).

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institution and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. This study was approved by the research ethics committees of the University of Tokyo (No. G10028) and other institutes. Informed written consent was obtained from all individual participants included in the study.

DNA and RNA extraction

DNA and RNA extraction, followed by WES and RNA-seq, was carried out as previously described (27, 28, 30). Briefly, genomic DNA was extracted from fresh-frozen tumor tissue and matched peripheral blood samples using the DNeasy Blood and Tissue kit (Qiagen). Total RNA was extracted from fresh-frozen tumor tissue using the RNeasy Mini kit (Qiagen).

WES

Sequencing libraries of genomic DNA from primary and recurrent paired tumors and matched blood samples were prepared using the SureSelect XT Human All Exon Kit v4 and v5 (Agilent Technologies) following the manufacturer’s protocols. The enriched libraries were sequenced as 100-bp paired-end reads using the HiSeq2000 (Illumina). The mean coverage of all protein-coding sequences (CDS) by WES was 125.3 ×; and 95.1% of CDS were covered by ≥20 independent reads. Sequencing data are summarized in Supplementary Table S2. Exome reads were independently mapped to the human genome (GRCh37/hg19) using Burrows–Wheeler Aligner (BWA; http://bio-bwa.sourceforge.net) and Novoalign software (Novocraft Technologies). After removal of PCR duplicates, bam files were then locally realigned with Short-Read Micro re-Aligner (https://sourceforge.net/projects/srma/). Normal–tumor pair bam files were processed using an in-house integrative genotypy karkinos (http://github.com/genome-rcast/karkinos) to detect somatic mutations and copy-number variations (31).

IDH status was assessed using WES and in some cases validated by Sanger sequencing and/or RNA-seq data. In cases of IDH-O, 1p/19q codelletion was examined using microsatellite analysis or multiplex ligation-dependent probe amplification and validated using WES data, as previously described (27).

RNA-seq

An RNA-seq library was prepared using the TruSeq Stranded mRNA LT Sample Prep Kit (Illumina) following the manufacturer’s protocols. Briefly, 1 mg of total RNA was purified using oligo-dT magnetic beads and poly A+ RNA was fragmented by heating at 94°C for 2 minutes. cDNA was synthesized using SuperScript II (Thermo Fisher Scientific), and adapter-ligated cDNA was amplified using 12 cycles of PCR. The enriched libraries were sequenced as 100-bp paired-end reads using the HiSeq2000 (Illumina). An average of 32.3 million passfilter reads with 100 base length per sample were obtained on each sample and mapped to a human transcriptome database (UCSC genes) and the reference genome (GRCh37/hg19) using BWA. Expression values were calculated as fragments per kilobase of exon per million fragments mapped (FPKM) using Cufflinks (http://cole-trapnell-lab.github.io/cufflinks/). Variant allele frequency (VAF) of each missense mutation in the RNA read was calculated as the proportion of variant read count per depth using bam-readcount (https://github.com/genome/bam-readcount).

HLA typing and MHC class I epitope binding prediction

In all 25 patients, HLA types were assigned from WES data of blood-derived genomic DNA using HLA typing software Omixon target HLA (Omixon; Supplementary Table S3). Mutated peptides derived from missense mutations from WES data of tumor were used for MHC class I binding prediction as previously reported (30, 32). In brief, binding affinities of 8-mer to 11-mer peptides containing the mutation or of wild-type counterparts to a specific HLA allele for each patient were predicted using the Immune Epitope Database analysis resource tool NetMHCpan v2.8 (33). Mutated peptides with IC50 value ≤ 500 nM/L were

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Figure 1.
Numbers of missense mutations, p-neoantigens, and e-neoantigens remain similar from primary to recurrent tumors. A, Flow chart of case selection. B, Pie charts showing the total number of nonsynonymous mutations for primary and recurrent tumors in 25 cases. C, The median value and range of missense mutations, predicted neoantigens (p-neoantigens; IC50 value ≤ 500 nmol/L) and expressed neoantigens (e-neoantigens; FPKM value ≥ 1 and VAF ≥ 0.04 in RNA-seq) in primary and recurrent tumors in 25 cases. D–F, The number of missense mutations (D), p-neoantigens (E), and e-neoantigens (F) shared between primary and recurrent tumors or unique to either are shown in different colors. G, Clinical and molecular information of each case is summarized on the right. H–M, Temporal changes of the number of missense mutations, p-neoantigens, and e-neoantigens in all 25 glioma cases (H, I, and J, respectively) and separately in GBM (n = 8), IDH-A (n = 9), and IDH-O (n = 8) cases (K, L, and M, respectively). In boxplots, the ends of the boxes and the middle line represent the lower and upper quartiles, and medians, respectively. Whiskers represent 1.5 times the interquartile range (IQR). RT, radiation treatment.
Immune Evasion by Reduced Neoantigen Expression in Glioma

considered as candidate neoepitopes (Supplementary Tables S3–S5). We defined a missense mutation capable of generating one or more HLA-A, -B, or -C-restricted neoepitopes as a "predicted" neoantigen (p-neoantigen).

Clonality analysis and assigning driver or passenger origin of neoantigens

We estimated the cancer cell fraction (CCF) of each p-neoantigen in each tumor sample using PyClone v0.13.0 (34). Based on reference and variant allele read counts, allele-specific copy-number information, and tumor purity provided by karkinos as described above. We ran PyClone with 10,000 iterations and a burn-in of 1,000. Neoantigens were considered as clonal if the upper limits of the 95% confidence interval (CI) of the estimated CCF exceeded 0.95 (Supplementary Fig. S1; ref. 35). In addition, we characterized each neoantigen derived from driver or passenger genes assigned from the intOGen database [GBM and lower grade glioma (LGG) separately; https://www.intogen.org; Supplementary Fig. S2].

Assessment of neoantigen expression

Among p-neoantigens, we specified “expressed” neoantigens (e-neoantigens) according to the criteria we previously reported that gene expression (FPKM) was ≥1 and VAF ≥ 0.04 (32). In addition, we calculated the ratio of e-neoantigen count to p-neoantigen count on each tumor sample, designated the "neoantigen expression ratio." For comparison, the expression ratio was also calculated for the other nonantigenic missense mutations (missense mutations with IC_{50} > 500 nmol/L). Furthermore, we obtained data from paired primary and recurrent tumor samples of 19 patients in TCGA-GBM and TCGA-LGG project (dbGaP study accession: phs000178.v1.p1) through gdc.bio-portal website (https://portal.gdc.cancer.gov) as of November 2018 (36, 37) and utilized for external validation.

Gene-expression analyses

In order to evaluate temporal changes of gene expression from primary to recurrent tumors, each gene’s fold change (FC) was calculated for each patient: log_{2}(FC) = log_{2}(FPKM_{primary} + 0.01)/[FPKM_{primary} + 0.01]). In a comparison of log_{2}(FC) between two groups, significantly upregulated genes at recurrence in each group were identified according to the following criteria: Welch t test P value < 0.05 and absolute difference of the mean log_{2}(FC) among two groups > 1 (Supplementary Table S6). Analysis and visualization of Gene Ontology (GO) terms associated with differentially expressed genes was performed using ClueGO 2.3.2 (with default parameters, except for Pathways = "BP, MF, CC, and ISP from GOA"; pV Correction = "Benjamini–Hochberg"; Show only Pathways with P < 0.05; ref. 38).

To test for differential gene expression across two time points (primary and recurrent) within each group, count data were obtained from RNA-seq bam files using the featureCounts function of Rsubread 1.26.1 [with default parameters except for annot.ext = TRUE (Homo_sapiens.GRCh37.87.chr.gff downloaded from Ensembl); isPairedEnd = TRUE; requireBothEndsMapped = FALSE; allowMultiOverlap = TRUE; countMultiMappingReads = TRUE; juncCounts = TRUE; http://bioconductor.org/packages/Rsubread/]. These data were utilized for pairwise comparison using the Wald statistical test DESeq2 1.16.1 (http://bioconductor.org/packages/DESeq2/). Generated mk files in which all evaluated protein-coding genes were reordered based on the Wald-test statistic in the output of DESeq2 were used as the input for the gene set enrichment analysis (GSEA; GseaPreranked software with default parameters except for scoring scheme = "classic," min gene set size = "5," https://genePattern.broadinstitute.org/gp; Supplementary Table S7). For the assessment of tumor-infiltrating leukocytes (TIL), we used the gene set of pan-cancer metagenes for 28 immune cell subpopulations consisting of 782 genes in total (39). For antigen presentation machinery (APM) assessment, we assembled the collection of 20 gene sets retrieved from C2 and C5 in the Molecular Signature Database (MSigDB) v6.1 (http://software.broadinstitute.org/gsea/msigdb/) with the search queries: "antigen" and "presentation" or "processing".

IHC

IHC staining was performed at Kyodo Byori Inc. Human CD8 mouse monoclonal antibody (clone 4B11, Leica) and human HLA class I-A, B, C mouse monoclonal antibody (clone D4M8R-5, Hokudo) were used to assess infiltrating cytotoxic T cells and APM on 4-μm-thick formalin-fixed paraffin-embedded tumor tissue sections. The slides were counterstained with hematoxylin–eosin. Images were captured with a fluorescence microscope, BZ-9000 (Keyence). The tumor-infiltrating CD8+ T cells were quantified digitally with BZ-II Analyzer image analysis software (Keyence) and divided into two classes as follows: if the positively stained cell density was less than the median of all examined tumor samples, it was judged as "sparse," and otherwise judged as "dense." According to the criteria that Torigoe and colleagues established (40). HLA class I expression was assessed by two independent examiners and defined as 2+ if the membrane was stained as strongly as endothelial cells in more than 75% of the tumor cells; 1+ if membrane staining was heterogeneous in 25% or more of the tumor cell constituents; and 0 if membrane staining was lost in more than 75% of the tumor cells. For verification, CD8 expression on IHC was compared with FPKM values of the CD8A gene in each tumor sample (Supplementary Fig. S3). Similarly, HLA class I expression was compared with geometric mean FPKM values of HLA-A, HLA-B, and HLA-C in each tumor sample.

Statistical analysis

For statistical analysis of paired comparisons between primary and recurrent tumor samples, the nonparametric two-sided Wilcoxon signed-rank test was applied. With respect to survival analyses, progression-free survival (PFS), overall survival (OS), and survival after second surgery (second OS) were defined as follows: the time interval between the two surgeries; the time from the first surgery to death or latest follow-up; and the time from the second surgery to death or latest follow-up, respectively. All survival analyses were carried out using the R package survival and survminer 0.4.0. Kaplan–Meier estimator of survival was used to construct the survival curves. Log-rank tests were used to compare survival between patients in different groups; and HR and 95% CIs are provided for comparison of two or more groups using univariate or multivariate Cox proportional hazard models, visualized as forest plots showing HR and 95% CIs using forest-model 0.4.3. P values < 0.05 were considered statistically significant unless otherwise specified. All statistical analyses were performed using R 3.4.1 (https://www.r-project.org/).

Data availability

The data sets generated and analyzed during the current study are available in the Japanese Genotype-Phenotype Archive.

Results
Patient characteristics
A total of 25 gliomas with complete WES and RNA-seq data sets from both primary and recurrent tumor samples were analyzed in this study (Fig. 1A). Their clinicopathologic characteristics are shown in Supplementary Table S1. The median age at first surgery was 39 (range, 24–76); 9 of the 25 were female. The study cohort consisted of 8 GBM, 9 IDH-A, and 8 IDH-O cases. Treatment prior to the second surgery included radiotherapy and/or chemotherapy [temozolomide (TMZ) or nimustine-based (ACNU)] or observation only. None of the patients received immune-based therapy including ICI.

Similar numbers of mutations and neoantigens between primary and recurrent tumors
Neoantigens derived from somatic mutations may be recognized as critical targets of antitumor immune responses, and studies have shown that some immunogenic neoantigens can be lost as a result of immune-based therapies in several cancer types (35, 41–43). To test whether the same may apply to glioma during standard therapy (not immune-based), we compared the number of mutations and neoantigens in paired primary and recurrent tumors from the same patients.

We identified 877 and 1,118 nonsynonymous mutations in total from primary and recurrent tumors, respectively (Fig. 1B). There were 746 and 895 missense mutations (85.1% and 80.1%), respectively, accounting for the majority of nonsynonymous mutations detected only at recurrence was relatively greater in IDH-A than in the other types, it was not associated with other factors such as histologic type (Supplementary Fig. S4; Supplementary Table S8).

We then investigated the second possibility. The ratio of e-neoantigen per p-neoantigen, here designated the “neoantigen expression ratio,” was compared between primary and recurrent tumors in each patient (Supplementary Table S8) and found to be significantly decreased at recurrence (median 0.37 and 0.28, P = 0.003; Fig. 2A). Similar results were obtained when calculated separately for the different histologic groups GBM, IDH-A, and IDH-O (P = 0.05, 0.20, and 0.04, respectively), although these were not all statistically significant because of the small numbers (Fig. 2B).

Next, to test whether this trend was specific to ”p-neoantigen” (IC50 ≤ 500 nmol/L), we also determined the expression ratio of mutations deemed unlikely to generated neoantigens (i.e., with an IC50 > 500 nmol/L). In this case, no differences between primary and recurrent tumors were found for any of the 25 cases (median 0.36 and 0.33, P = 0.41; Fig. 2C) or for each histologic group separately (P = 0.67, 0.83, and 0.68, respectively; Fig. 2D). This suggests that the significantly decreased expression ratio at recurrence was observed only for ”p-neoantigen.” In the TCGA cohort [n = 19: GBM (IDH-wild-type), 5; astrocytoma, IDH-wild-type, 2; IDH-A, 6; IDH-O, 6], we observed a similar decreasing trend from primary to recurrence with regard to the neoantigen expression ratio, though it did not reach statistical significance (Supplementary Fig. S5).

In addition, when we further stratified p-neoantigens according to the strength of binding affinity within the IC50 ≤ 500 nmol/L set, the ratio was mostly decreased in those with the highest binding affinity (IC50 ≤ 50 nmol/L; Fig. 2E). Furthermore, we examined the binding affinity index (IC50WT per IC50MT) and found that neoantigen expression ratio significantly decreased in recurrence in neoantigens with higher indices of IC50WT/IC50MT (Supplementary Fig. S6; ref. 44). These results suggest that the ”neoantigen expression ratio” decreased at recurrence due to immune selective pressure against the most highly immunogenic neoantigens.

We further assessed the association of decreased neoantigen expression ratios with several characteristics of the neoantigens. We first examined the temporal changes of the neoantigen expression ratio from the viewpoint of ”shared” or “unique” p-neoantigens described in Fig. 1E and F. Of 1,124 p-neoantigens in total from 50 samples, *shared* neoantigens included 218 pairs of p-neoantigens (436 p-neoantigens) and the “unique” neoantigens included 296 and 392 p-neoantigens in primary and recurrent tumors, respectively (Supplementary Fig. S7; Supplementary
Figure 2.
Neoantigen expression ratio decreases from primary to recurrent tumors. A and B, Temporal changes in neoantigen expression ratios (the ratio of e-neoantigens to p-neoantigens) from primary to recurrent tumors in 25 cases (A) and separately in GBM (n = 8), IDH-A (n = 9), and IDH-O (n = 8) cases (B). C and D, Expression ratios for nonneoantigens (defined here as IC50 value > 500 nmol/L) in 25 cases (C) and in separate groups (D). E, Violin plots showing the relationship between the strength of MHC class I binding affinity and decreased neoantigen expression ratio. F and G, Temporal changes in neoantigen expression ratios from primary to recurrent tumors are also shown separately for clonal or subclonal neoantigens (F) and for neoantigens derived from driver or passenger gene mutations (G).
There were no significant differences in the expression ratios between primary and recurrent tumors either for "shared" or "unique" p-neoantigens. Next, we considered clonal neoantigens (present in the majority of tumor cells) versus subclonal neoantigens (present only in a subset of tumor cells), as previously reported (ref. 45; Supplementary Fig. S1). Decreased expression ratio was observed only for clonal neoantigens (Fig. 2F). We finally considered whether neoantigens derived from "driver" or "passenger" mutations differed in this respect (ref. 46; Supplementary Fig. S2). We found that the expression ratio decreased only in neoantigens from passenger but not driver mutations (Fig. 2G). Taken together, these results suggest that the "neoantigen expression ratio" decreased particularly for clonal neoantigens and/or passenger mutation–derived neoantigens during glioma progression.

Figure 3.
Association of decreased neoantigen expression ratios and glioma prognosis. A, Waterfall plot showing differences of the neoantigen expression ratio from primary to recurrent tumors (Δ neoantigen expression ratio). Cases corresponding to the top (n = 8) and bottom tertiles (n = 8) of decreased neoantigen expression ratios are designated “group D” and “group N,” respectively. Bottom plots indicate molecular diagnosis and treatment prior to the recurrence of each patient. B, Changes in neoantigen expression ratios from primary to recurrent tumors in each individual case in groups D and N. C–E, Kaplan-Meier analyses of the two groups for PFS (C), OS (D), and second OS (E). F, Forest plot showed multivariate-adjusted HR with 95% CI for death after second surgery.

Table S4).
Changes of neoantigen expression and their implications for clinical prognosis

Reduced neoantigen expression during ICI therapy has been observed in patients who experience favorable clinical responses (35). Therefore, we next investigated whether a decrease of the neoantigen expression ratio from primary to recurrent tumors (Δneoantigen expression ratio) is associated with glioma prognosis even under standard therapy. For this, we extracted the top tertile of patients with decreased (8 cases, group D) and those with non-decreased (bottom tertile; 8 cases, group N) Δneoantigen expression ratios (Fig. 3A and B). We confirmed that the neoantigen expression ratio decreased at recurrence, especially for clonal neoantigens and/or passenger mutation–derived neoantigens in group D (Supplementary Fig. S8). Patients were distributed almost equally in both groups with respect to molecular diagnosis as well as treatment (Fig. 3A). Kaplan–Meier survival
analyses showed no significant difference between group D or N, either for PFS or for OS (log-rank, $P = 0.37$ and 0.22, respectively; Fig. 3C and D). There was a slightly better prognosis in group D for second OS, but this did not reach statistical significance ($P = 0.14$; Fig. 3E).

We next compared these results with other clinical parameters contributing to prognosis, using univariate and multivariate Cox regression analyses (Supplementary Table S9). Because there was a significant correlation of histology with IDH and 1p/19q status, which are established prognostic factors in glioma (1), in this cohort we used histology as the only covariate other than the D neoantigen expression ratio in the multivariate analysis. For second OS, histologic subtype became an independent prognostic factor, as expected (Fig. 3F). In contrast, the multivariate-adjusted HR for group D (with group N as a reference) was 0.32 (95% CI: 0.08–1.30, $P = 0.11$), indicating that the D neoantigen expression ratio remained only a weakly associated factor.

**Decreased neoantigen expression ratio associated with immune responses and immune cell infiltration**

We hypothesized that as the decreased neoantigen expression ratio was associated with a slightly more favorable prognosis in group D, this could be attributable to ongoing antitumor immune responses in the tumor microenvironment. Therefore, we tested whether immune-related genes were upregulated in recurrent relative to primary tumors in group D but not in group N. In accordance with our hypothesis, we found that 124 genes were exclusively upregulated at recurrence in group D (Fig. 4A; Supplementary Table S6) including many immune-related genes such as CXCL1, CXCR1, CTLA4, GZMB, etc. (Fig. 4B). GO analyses using ClueGO (38) yielded several immune-related GO terms such as “leukocyte migration involved in inflammatory response,” “CXCR chemokine receptor binding,” and “T-cell differentiation involved in immune response” (Fig. 4C; Supplementary Table S10). One hundred twenty-eight genes upregulated in group N but not in group D (Fig. 4A and B) yielded no GO terms. Collectively, these findings demonstrate a relationship between decreased neoantigen expression ratio and immune responses in the tumor microenvironment.

Because immune-related genes were upregulated in group D, we next investigated infiltration of immune cells by pairwise-designed GSEA using a “pan-cancer metagene” consisting of 28 gene sets for TILs (39). In group D, gene sets for “activated CD8$^{+}$ T cell” (act CD8) and “Neutrophil” (Neu), followed by “effector memory CD8 T cell” (Tem CD8) and “CD56 (dim) natural killer cell” (CD56dim) were significantly enriched at recurrence (Fig. 5A; Supplementary Table S11). In contrast, in group N, many gene sets including act CD8 and Tem CD8 were significantly depleted in recurrent tumors (Fig. 5B). IHC for CD8 in tumor tissues also supported the conclusion that the fraction of CD8$^{+}$ T cells was maintained from primary to recurrence in group D but depleted at recurrence in group N (Fig. 5C and D; Supplementary Figs. S3 and S9; Supplementary Table S12).
Retained neoantigen expression ratio is associated with impaired APM

Based on the findings that antitumor immune responses and immune infiltrates were decreased at recurrence in group N despite retention of the neoantigen expression ratio in recurrent relative to primary tumors, we hypothesized that T-cell recognition of these neoantigens was compromised by impairment of the APM. By pairwise-designed GSEA for APM-related gene sets retrieved from C2 and C5 of the MSigDB, we found that most of these gene sets were significantly depleted at recurrence in group N (but not in group D). In particular, the gene sets "GO antigen processing and presentation of peptide antigen," "GO antigen processing and presentation of exogenous peptide antigen via MHC class I," and "REACTOME cross presentation of soluble exogenous antigens endosomes" were greatly depleted in group N [normalized enrichment score (NES) < -2.77, -2.57, and -2.62, respectively; Fig. 6A and B; Supplementary Table S13]. These in silico analysis results were validated by IHC for HLA class I molecules on tumor tissues; thus, expression of HLA class I protein was reduced at recurrence in group N, but not in group D (Fig. 6C and D; Supplementary Figs. S3 and S9; Supplementary Table S12). These results suggest that immune selective pressure against neoantigen expression was weakened due to impaired APM, resulting in retention of their expression in group N.

Discussion

In this study, we compared WES and RNA-seq data from pairs of primary and recurrent tumors in 25 glioma patients, in order to assess changes in the mutation/neoantigen burden and immunologic features over time. We found no differences between primary and recurrent tumors in this cohort for the total number of mutations, or for "predicted" or "expressed" (p- and e-) neoantigens. However, we found that the ratio of e-neoantigens to p-neoantigens, here designated the "neoantigen expression ratio," significantly decreased at recurrence in each individual patient.

Neoantigens derived from somatic mutations are targets of immune responses. Compared with melanomas or non–small cell lung cancers (NSCLC), the mutation/neoantigen burden is generally not high in glioma, with the exception of certain "hypermutators" (25, 47, 48). Although therapy-induced clonal evolution following cytotoxic chemotherapy with alkylating agents such as TMZ (22, 23) may increase the number of mutations or neoantigens in gliomas (e.g., TMZ-induced hypermutator, which we had excluded the inclusion in this study), we found that the total number of mutations or neoantigens did not increase at recurrence.

It has been established that tumors may respond to selective immune pressure during TIL therapy or over the course of ICI by selective loss of immunogenic neoantigen expression (35, 41–43). In our cohort, the "neoantigen expression ratio" but not "neoantigen frequency" per se decreased at recurrence, suggesting that reduced neoantigen expression rather than the elimination of antigenic mutations was responsible for antigen loss during glioma progression. We hypothesized that the decrease of the neoantigen expression ratio was caused by immune selective pressure even under standard therapy. Consistent with this, the expression ratio greatly decreased for those mutations predicted to give rise to high-affinity binding sequences (IC50 <
50 nmol/L) but did not decrease at all for those with a predicted low affinity (IC50 > 500 nmol/L). Further support for this interpretation derived from the findings that the recurrent tumors of the top tertile patients with a decreased neoantigen expression ratio exhibited persistent immune cell infiltrates and responses suggestive of the intact function of APM. We therefore concluded that decreased neoantigen expression ratios occurred as a result of host immune selective pressure against highly immunogenic neoantigens, although we have not formally demonstrated the underlying mechanism responsible for decreased expression of neoantigen mRNA.

It has been shown that clonal neoantigens elicit T-cell immune responses and are expressed by tumors sensitive to immune-based therapies (35, 45). We determined that decreased neoantigen expression ratios were observed primarily for clonal rather than subclonal neoantigens, suggesting the superiority of the former as targets for the immune system even under standard nonimmune-based therapy. We further showed that decreased neoantigen expression ratios were preferentially seen for those neoantigens derived from passenger but not driver gene mutations (i.e., the expression of the latter was maintained in recurrent tumors). This might be because driver mutations are essential for cancer cell survival but give rise to poorly antigenic sequences allowing immune evasion. Otherwise, cancer cells expressing highly immunogenic neoantigens derived from driver gene mutations would have been targeted and eliminated by immunosurveillance during tumor formation (49). In contrast, highly immunogenic neoantigens derived from passenger genes, which are not necessary for cancer cell survival, may be more susceptible to immune elimination and immunoediting.

Other than the loss of the antigen itself, downregulation of HLA or β2-microglobulin chains is known to be a mechanism of tumor evasion (50). Reports have further indicated that HLA loss is closely related to elevated neoantigen burden and represents a major mechanism of immune evasion by cancer cells (49, 51). Moreover, strongly immunogenic neoantigens were found to bind more frequently to the lost HLA allele. These studies suggest that immunoediting would result in the expansion of tumor cell clones that had lost HLA alleles, and this would lead to a subsequent increase in the mutation/neoantigen rate in the expanded tumor cell populations. In our cohort, impaired APM was associated with retained neoantigen expression in recurrent tumors. Therefore, our current data also support these reports regarding the relationship between neoantigens and HLA in the mechanisms of immune evasion.

Figure 7. Model of tumor immune evasion by neoantigen “invisibility” through reduced neoantigen expression or impaired APM. With intact APM function, cells with neoantigens are continuously exposed to immune selection pressure; this causes their neoantigen expression to gradually decrease and finally become invisible to the immune system (left). As a result, tumor cells can evade immune surveillance and survive at recurrence, maintaining APM function. In contrast, APM dysfunction as a result of mutation or lower expression of genes such as HLA, STAT1, and TAP1/2 may occur at an earlier time, causing still-present neoantigens to become invisible to the immune system (right). As a result, tumor cells can evade antitumor immunity and survive while maintaining neoantigen mRNA expression. β2MG, β2-microglobulin; ER, endoplasmic reticulum; GC, Golgi complex; TAP, transporter associated with antigen processing; TCR, T-cell receptor.
The decrease of neoantigen expression and impaired APM share a common immune evasion feature in terms of antigen "invisibility" to immune system (Fig. 7). However, we assume that APM dysfunction occurs at a relatively early time point to render any potential mutation-derived neoantigens invisible. As a result, tumor cells can evade immune attack and survive, maintaining neoantigen expression. In contrast, in tumors with intact APM function, antigen-specific T cells continuously exert antitumor immune pressure against the neoantigens, resulting in their loss of expression and finally tumor evasion of immune attack.

In terms of prognosis, we found no differences in PFS between groups D and N. Therefore, the two different escape mechanisms (i.e., decreased neoantigen expression or impaired HLA expression) did not affect the length of time between the first surgery (primary) and the second surgery (recurrence). However, patients in group D had a tendency toward slightly better OS and especially OS after the second surgery compared with group N. This might be because immune responses/immune cell infiltrates were retained in recurrent tumors in group D compared with those in group N, despite decreased targeting of neoantigens.

There are several limitations to this study. First, although the clinical feature of gliomas requiring multiple surgeries enabled us to analyze longitudinal data, this cohort was relatively small but nonetheless heterogeneous, including three different histologic groups (GBM, IDH-A, and IDH-O). Moreover, treatments prior to the second surgery were variable, and they would be expected to influence OS after the second surgery compared with group N. This might be because decreased neoantigen expression and impaired APM share two separate but complementary mechanisms contributing to tumor cell resistance to immune selective pressure.

In conclusion, it is likely that neoantigens are targeted by the immune system in gliomas, even during standard nonimmunologic chemotherapy, expression of neoantigen mRNA was reduced over time. We also suggest that decreased neoantigen expression and impairment of APM function are two separate but complementary mechanisms contributing to tumor cell resistance to immune selective pressure. Therefore, we propose that there is a need to develop a strategy to overcome these immune evasion mechanisms in addition to performing ICI and/or neoantigen-based immunotherapy in glioma patients.

Disclosure of Potential Conflicts of Interest
Y. Narita reports receiving other commercial research support from AbbVie, Ono Pharmaceutical Co., Daiichi-Sankyo Pharmaceutical Co., Eisai, Dainippon-Sumitomo Pharmaceutical Co., and Byer and has received honoraria from speakers bureau of Chugai Pharmaceutical Co. No potential conflicts of interest were disclosed by the other authors.

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References


TFEB Mediates Immune Evasion and Resistance to mTOR Inhibition of Renal Cell Carcinoma via Induction of PD-L1

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Abstract

Purpose: Despite the FDA approval of mTOR inhibitors (mTORi) for the treatment of renal cell carcinoma (RCC), the benefits are relatively modest and the few responders usually develop resistance. We investigated whether the resistance to mTORi is due to upregulation of PD-L1 and the underlying molecular mechanism.

Experimental Design: The effects of transcription factor EB (TFEB) on RCC proliferation, apoptosis, and migration were evaluated. Correlation of TFEB with PD-L1 expression, as well as effects of mTOR inhibition on TFEB and PD-L1 expression, was assessed in human primary clear cell RCCs. The regulation of TFEB on PD-L1 was assessed by chromatin immunoprecipitation and luciferase reporter assay. The therapeutic efficacies of mTORi plus PD-L1 blockade were evaluated in a mouse model. The function of tumor-infiltrating CD8+ T cells was analyzed by flow cytometry.

Results: TFEB did not affect tumor cell proliferation, apoptosis, and migration. We found a positive correlation between TFEB and PD-L1 expression in RCC tumor tissues, primary tumor cells, and RCC cells. TFEB bound to PD-L1 promoter in RCCs and inhibition of mTOR led to enhanced TFEB nuclear translocation and PD-L1 expression. Simultaneous inhibition of mTOR and blockade of PD-L1 enhanced CD8+ cytolytic function and tumor suppression in a xenografted mouse model of RCC.

Conclusions: These data revealed that TFEB mediates resistance to mTOR inhibition via induction of PD-L1 in human primary RCC tumors, RCC cells, and murine xenograft model. Our data provide a strong rationale to target TFEB and PD-L1 jointly as a novel immunotherapeutic approach for RCC treatment.

Introduction

Renal cell carcinoma (RCC) encompasses a heterogeneous group of cancers derived from renal tubular epithelial cells (1). Patients with localized RCC after partial or radical nephrectomy often go on to develop metastatic disease, which requires systemic therapies that are rarely curative (2, 3). The mTOR is a serine/threonine kinase that forms two complexes mTORC1 and mTORC2 (4, 5). These sense the availability of nutrition, growth factors, and energy levels to regulate cell growth, proliferation, and differentiation. Dysregulation of mTOR pathways and mutations of mTOR pathway-related genes are often associated with tumor growth in a variety of cancers (6, 7). Inhibitors of mTOR, everolimus, and temsirolimus that are derived from rapamycin have been approved by the FDA to treat advanced metastatic renal cancers (8, 9). Despite initial excitement of mTOR inhibitors for the treatment of RCC, mTOR inhibitors rarely achieve complete responses and most patients ultimately develop resistance to mTOR inhibitor therapy (10, 11). However, the underlying mechanisms by which the RCC resists mTOR inhibition are elusive.

The transcription factor EB (TFEB) is a member of the microphthalmia family of basic helix-loop-helix-leucine-zipper (bHLH-Zip) transcription factors (MiT family), which binds to the coordinated lysosomal expression and regulation (CLEAR) consensus motif and plays important functions in regulation of lysosome biogenesis, autophagy, and metabolism (12, 13). TFEB...
Tumor growth in immune competent but not nude mice by despite no effect on tumor cell biology. Overexpression of a inhibition by upregulation of PD-L1 expression to promote cross-regulation of tumor cells and functions of immune cells, in regulated by many transcriptional factors including HIF-1

mTOR and PD-1/PD-L1 axis jointly as a novel approach for tumors. Simultaneously targeting mTOR and PD-L1 enhanced the efficacy in a mouse RCC xenograft model. Thus, our data provide rationale for a combinational strategy that targets mTOR and PD-1/PD-L1 axis jointly as a novel approach for patients with RCC.

Translational Relevance

Despite the significant progress achieved by targeting mTOR in renal cell carcinoma (RCC), the effects of mTOR inhibitors are modest and patients often develop resistance. The lack of understanding of cancer cell–intrinsic mTOR-mediated pathways remains a major hurdle for the development of effective therapies. Here, we uncovered that TFEB expression is positively correlated with PD-L1 expression in RCC cells. Furthermore, inhibition of mTOR in RCC enhances TFEB nuclear localization and expression that subsequently drives PD-L1 expression and immune evasion in RCC cell lines and primary tumors. Simultaneously targeting mTOR and PD-L1 enhanced the efficacy in a mouse RCC xenograft model. Thus, our data provide evidence and rationale to support the combination of mTORi and PD-L1 blockade as a potential therapeutic approach to treat RCC.

Materials and Methods

Cell culture

Cells were cultured at 37°C and 5% CO₂ in a humidified incubator. 786-O, ACHN, H1975, AS49, H2126, HCT116, LoVo, SW480, and Renca cells were from ATCC, 769-P, OS-RC-2, and Caki-1 cells were from and authenticated by Cell Repository, Chinese Academy of Sciences (Shanghai, China). 786-O, ACHN, OS-RC-2, 769-P, and Caki-1 cells were authenticated by STR profiling and authentication of other cell lines was not routinely performed. All of the cell lines were passaged less than 2 months after each thaw and tested to be free of mycoplasma contamination (D101, Vazyme).

Transient transfection and generation of stable cell lines

769-P, HEK 293T, and Renca cells were transfected with either pcDNA3.1 control plasmid (EV) or pcDNA3.1 encoding the constitutively active form of TFEB (S211A) (TFEB) using lipofectamine 2000 (11668019, Invitrogen) as described by the manufacturer. TFEB mutant of TFEB-S211A was generated by site-directed point mutagenesis using MutanBEST kit (D401, Takara). Transfected 769-P and Renca cells were selected with G418 (A2513, APEX BIO) for 3 weeks and subcloned by dilution at 1 cell/well in 96-well microliter plates. 786-O cells were transduced with control lentivirus expressing a scrambled shRNA or lentiviral particles encoding two TFEB short hairpin RNAs (shRNAs) as follows. ShTFEB-1 (forward): 5’-CCGG-CACACCTTGTTGCTAAATCCTGCGAGCTATTAGCACCAAGGACATCGTTT-3’; ShTFEB-1 (reverse): 5’-AAATCCAAAGAC-CATTTTGTTGCTAAATCCTGCGAGCTATTAGCACCAAGGACATCG-3’. ShTFEB-2 (forward): 5’-CCGGCCCATGTGTCCTTGCG-TACATCAACTCGAGTTGATGTAGCCAAGGACATCGTTTTTG-3’; ShTFEB-2 (reverse): 5’-AAATCCAAAGACCATGATCTCCGTG-ATCAACTCGAGTTGATGTAGCCAAGGACATCGTITTTGG-3’. Cells were selected with puromycin (HY-B1743, MCE) for 3 weeks and subcloned by dilution at 1 cell/well in 96-well microliter plates.

Immunoblotting

Immunoblotting was performed according to standard method with primary antibodies against TFEB (ab2636, Abcam), TFE3 (SAB4503154, Sigma), PD-L1 (17952-1-AP, ProteinTech), GAPDH (5174, Cell Signaling Technology), PCNA (AV03018, Sigma), BAX (2772, Cell Signaling Technology), phospho-S6 (4851, Cell Signaling Technology), phospho-4EBP1 (2855, Cell Signaling Technology), Histone H3 (17168-1-AP, ProteinTech), HIF-1α (14179S, Cell Signaling Technology), p65 (8242, Cell Signaling Technology), phospho-p65 (3033, Cell Signaling Technology), Histone H3 (17168-1-AP, ProteinTech), TFEB mutant of TFEB-S211A was generated by site-directed point mutagenesis using MutanBEST kit (D401, Takara). Transfected 769-P and Renca cells were selected with G418 (A2513, APEX BIO) for 3 weeks and subcloned by dilution at 1 cell/well in 96-well microliter plates.

Cell Counting Kit-8 and 5-ethynyl-2′-deoxyuridine assay for cell proliferation

The different experimental groups of 786-O and 769-P cells were plated in 96-well plates at 1 × 10⁵ cells per well and cultured
for 5 days or 7 days, respectively. Cell proliferation was determined by Cell Counting Kit-8 (CCK04, Dojindo) every 24 hours according to the manufacturer’s instructions. The incorporation of 5-ethynyl-2’-deoxyuridine (EdU) was stained with the EdU Cell Proliferation Assay Kit (Q10310-1, RiboBio) according to the manufacturer’s protocol. The percentage of EdU+ for each field of view captured was recorded and quantified.

Cell apoptosis determined by Annexin V-PI staining
786-O and 769-P cells were pretreated with DMSO or paclitaxel (10 µM) for 12 hours and then digested with 0.25% Trypsin (25200-114, Invitrogen) and washed twice with ice-cold PBS. Samples were stained with Annexin V-FTTC (640906, BioLegend) and propidium iodide (P4170, Sigma) in Annexin V binding buffer according to the manufacturer’s specifications. Samples were analyzed on a BD Versa Flow cytometer and data were analyzed using FlowJo software.

Transwell migration assay
The transwell migration assay was performed with standard method. In brief, the bottom chambers were filled with 600 µL of DMEM medium containing 10% FBS. In total, 2.5 x 10⁴ cells were suspended in 100 µL of DMEM containing 1% FBS and seeded in the top chamber. After 24 hours, nonmigrated cells were removed and migrated cells were fixed with 4% paraformaldehyde and stained with 1% crystal violet. Images were taken using Olympus model IX83 inverted fluorescence microscope and the percentages of migrated cell areas in the total areas were quantified.

Mice
Wild-type BALB/c and BALB/c nude mice (7–8 weeks of age, HuaFukang) were housed in a specific-pathogen-free facility at the Tongji Medical College, HUST. All experiments were performed according to the guidelines of the Institutional Animal Care and Use Committee of Tongji Medical College, HUST.

Xenograft mouse tumor models
In total, 1 x 10⁶ Renca cells that were stably transfected with either an empty vector or TFE3-S21A pCDNA3.1 plasmids were injected subcutaneously on the back of WT BALB/c mice. For the experiments performed with nude mice, 5 x 10⁴ (EV or TFE3) Renca cells were injected. For the combinatorial therapy, WT BALB/c mice were subcutaneously injected with 1 x 10⁸ Renca cells. Once tumor volumes reached 50 to 100 mm³, mice were injected intraperitoneally with either 10 mg/kg temsirolimus daily, 200-µg anti-mouse PD-L1 antibody (10F.9G2, BioXCell) every 3 days, combination of both, or vehicle together with control rat IgG2b (LTE-2, BioXCell). Tumor volumes were measured along major axis (a) and minor axis (b) daily and were calculated using the formula: V = ab²/2. Mice were sacrificed and tumors were excised and weighted.

Flow cytometry
For the analysis of PD-L1 and PD-L2 expression in human tumor cell lines or primary RCC cells, cells were harvested under normal condition or after administration of rapamycin (AB167, APExBIO), Torin-1 (A8312, APExBIO), and EBSS (E2888, Sigma) for the indicated times. Cells were stained with antibodies against phycocerythrin (PE)-conjugated anti-human PD-L1 antibody (329706, BioLegend) and APC-conjugated antihuman PD-L2 antibody (345507, BioLegend). Mouse RCC cells were stained with PE-conjugated antimouse PD-L1 antibody (124308, BioLegend) or FITC-conjugated antihuman/mouse Ki67 antibody (115639-82, Invitrogen). The antibodies used to stain tumor-infiltrating lymphocytes (TIL) were listed as followed: anti-CD45-APC/Cy7 (103115, BioLegend), anti-CD8-PerCP/Cy5.5 (100734, BioLegend), anti-CD107a-APC (121613, BioLegend), anti-GZMB-FTTC (515403, BioLegend), anti-IL2-BV421 (503825, BioLegend), anti-TNF-PE/Cy7 (557644, BD), and anti-IFN-APC (554413, BD Biosciences). Samples were collected on a BD Verse Flow cytometer and data were analyzed using FlowJo software.

Histology/IHC
RCC/tissue specimens were isolated after surgery, formalin fixed and paraffin embedded, and stained with hematoxylin and eosin. IHC was performed as standard protocol with antibodies against TFE3(ab2636, Abcam), PD-L1 (clone 22c3, Dako), and carbonic anhydrase IX (CAIX) (TA336805, ZSGB-BIO, Beijing). Slides containing both PD-L1 positive and negative areas were taken for analysis of TFE3 expression. Intensities of PD-L1 were determined according to the clinical score guideline for PD-L1 staining (clone 22c3, Dako). The 42 cases were divided into PD-L1− group (<1%), PD-L1− low group (1%–49%), and PD-L1− high group (≥50%) with (≥1+) PD-L1 cellular and membrane staining of viable tumor cells. The tumor grades were assigned to the highest (≥5%) within the tumors if the tumors were heterogeneous. IHC reactivity of cytoplasmic or nuclear TFE3 was scored as follows: multiplication of the intensity of immunostaining (1, weak; 2, moderate; and 3, strong) and the percentage of positive tumor cells, which resulted in a score of 0 to 300. The total TFE3 expression was evaluated as the mean of cytoplasmic and nuclear score. A score of less than 10 was considered as 0, a score of 10 to 40 was considered as 1+, 41 to 140 as 2+, and 141 to 300 as 3+. IHC data were evaluated by two independent pathologists.

Patients and specimens
Studies with human RCC specimens have been approved by the Ethics Committee of Tongji Hospital of HUST (Wuhan, China), and signed informed consents were obtained from all patients’ family. The demographic and clinical characteristics of the enrolled patients are presented in Supplementary Table S1.

Chromatin immunoprecipitation assay
Cells were harvested following cross-linking for 10 minutes with 1% (vol/vol) formaldehyde. Afterward, cells were lysed by sonication. The cell lysates were immunoprecipitated with anti-TFE3 (ab2636, Abcam) overnight at 4°C. After washing and elution, cross-links were reversed for 4 hours at 65°C. The eluted DNA was purified and analyzed by qPCR using a Bio-Rad SYBR Green intercalating fluorophore system with PD-L1 primers: (forward): 5’-AGTTATGTCGCGTTCATATC-3’; (reverse): 5’-GGAATTTGCTGTTCATATC-3’. The Cq value of each sample was normalized to corresponding input value.

Luciferase reporter assay
The PD-L1 promoter sequence (~281 bp to +43 bp) relative to the transcription start site was amplified by PCR from human peripheral blood mononuclear cells and inserted into the pGL3-basic vector (E1751, Promega). The primers used for cloning the PD-L1 promoter are: forward: 5’-CGCTTCGATGCGGGCAGATTTTTT-3’ and reverse: 5’-ATTCGAGGCGAGAGGTCTTTTTT-3’.
HEK 293T cell was cotransfected with pRL-TK (E2241, Promega), pGL3-PD-L1 or pGL3-basic, empty pcDNA3.1 vector, or TFEB-S211A pcDNA3.1 plasmids in 24-well plates with Lipofectamine 2000. After 48 hours, firefly and Renilla luciferase activities were measured using the Dual-Luciferase Reporter Assay Kit (E1901, Promega) with microplate reader (Synergy H1, Bio-Tek) and the ratio of firefly/Renilla luciferase was determined.

Isolation of primary tumor cells and TILs
Tumor specimens were gently minced into small pieces and then digested with 6 mL PBS containing 50 μL 25 mg/mL collagenase IV (17104019, Invitrogen) and 25 μL 10 mg/mL DNase I (10104159001, Roche) for 1 hour at 37°C. Cell suspensions were filtered twice and centrifuged at 1,500 rpm for 5 minutes. Tumor cells and TILs were enriched and harvested separately by Percoll gradient (17-0891-01, GE Healthcare) following the manufacturer's protocol.

Immunofluorescence
Primary RCC cells were seeded on glass slides and incubated overnight for proper attachment. Cells were stimulated with vehicle or Torin-1 (500 nmol/L) for 3 hours and then washed three times with PBS and fixed with 4% paraformaldehyde for 30 minutes, permeabilized with 0.05% Triton X-100 for 30 minutes, and blocked in 5% BSA for 1 hour. Cells were incubated with anti-TFEB (ab2636, Abcam) and anti-PD-L1 (clone 22c3, Dako) overnight at 4°C. Secondary antibodies labeled with FITC or Cy3 (Life Technologies or Jackson Laboratories) were added for 1 hour at room temperature, and DAPI was used for nuclear counterstaining for 10 minutes. Samples were imaged with an Leica TCS SP5 confocal microscope in 24 hours after mounting.

Statistical analysis
Statistical analysis was performed using Prism (GraphPad, San Diego) software. Statistical significance was determined by Student t test or for variances by ANOVA. P values less than 0.05 were considered significant.

Results
TFEB does not affect cell proliferation, apoptosis, and migratory potential of RCC cells
TFEB expression has been linked with both occurrence and a poor prognosis in RCC (19). To determine whether TFEB affects the proliferative, apoptotic, and metastatic capacities of RCCs, we took the advantage of the differential expression of TFEB in human 786-O and 769-P RCC cell lines (Fig. 1A). Knockdown of TFEB in 786-O cells did not affect expression of PCNA, a marker for cell proliferation, as well as proapoptotic protein BAX expression (Fig. 1B). Consistent with these, knockdown of TFEB expression did not affect cell proliferation (Fig. 1C). These results were further confirmed with EdU staining (Fig. 1D and E). Next, we looked at cell survival; knockdown of TFEB in 786-O cells had no effect on apoptosis in untreated cells or cells treated with paclitaxel (Fig. 1F and G). Finally, using an in vitro transwell assay, we found that downregulation of TFEB in 786-O cells had no impact on cell migration compared with cells transduced with scrambled shRNA lentivirus (Fig. 1H and I).

To confirm these conclusions, we overexpressed a constitutive active form of TFEB mutant, TFEBS211A, in 769-P cells. Consistent with the knockdown data, enhanced TFEB expression did not affect 769-P cell proliferation, apoptosis, and migration in vitro (Supplementary Fig. S1A–S1E).

TFEB mediates immune evasion of renal carcinoma cells
We next explored the function of TFEB in an in vitro model of RCC. To this end, we first generated a mouse-derived RCC Renca cell line that overexpressed TFEBS211A mutant (Fig. 2A). Then we hypodermically inoculated control Renca-EV cells or Renca-TFEB (S211A) cells to wild-type BALB/c mice. We found that mice that received cells overexpressing TFEBS211A showed significantly greater tumor burdens compared with control group (Fig. 2B). Greater tumor burdens were associated with significantly reduced frequencies of CD107α, GZMB, IL2, and TNFα-producing CD8^+ cytotoxic cells (CTL) within tumors from mice that received Renca cells overexpressing TFEB (Fig. 2C–F), compared with mice that received control cells. There was a similar reduction in the percentages of IFNγ-expressing CTL in TFEBS211A Renca tumors, but this was not significant (Supplementary Fig. S2A). In contrast, the percentages of CD107α^− NK cells and presence of tumor-associated macrophages and myeloid-derived suppressive cells within TFEBS211A Renca tumors were unaltered compared with control Renca tumors (data not shown).

To resolve our contrasting findings between in vitro and in vivo experiments, we repeated the same experiment with BALB/c nude mice, which lack T cells. In contrast with the experiments performed on wild-type BALB/c host animals, there was no significant difference in tumor growth (Supplementary Fig. S2B and S2C). Moreover, the percentage of Annexin V^+ as well as Ki-67^+ tumor cells in TFEBS211A groups isolated from nude mouse was comparable to the control group (Supplementary Fig. S2D and S2E), suggesting that the effect of TFEB is dependent on the presence of tumor-infiltrating T cells rather than intrinsic differences within the cells themselves.

This led us to investigate the effect of TFEB on expression of inhibitory coreceptor ligands. We found that forced expression of TFEB in Renca cells enhanced PD-L1 expression within tumor tissues (Fig. 2G and H). In addition, the PD-L1 expression levels within tumors were positively associated with tumor weights (Fig. 2I). Taken together, TFEB mediated immune evasion of RCC via suppressing the cytotoxic function of CD8^+ T cells.

TFEB positively regulates PD-L1 expression in RCCs
We next evaluated whether TFEB correlated with PD-L1 expression in primary RCC cells from patients. Within individual tumors, PD-L1 staining showed heterogeneous expression, which can be readily differentiated into PD-L1^+ and PD-L1^- areas. The PD-L1^- regions had higher expression and enhanced nuclear localizations of TFEB (Fig. 3A and B); the representative images of differential intensities of cytoplasmic or nuclear TFEB staining were shown in Supplementary Fig. S3A. To explore possible link between PD-L1 expression and tumor progression, we extended the study to a cohort of 42 patients with clear cell renal cell carcinoma (ccRCC). On the basis of the percentages of PD-L1 expression in viable tumor cells, patients are divided into three groups: PD-L1^+ (≤1%), PD-L1^low (1%–49%), and PD-L1^high groups (≥50%). PD-L1 high-expression patients had higher tumor grades and more advanced tumor stages (Fig. 3C and D). Next, we compared...
surface PD-L1 expression in a panel of human RCC cell lines. Although ACHN cells had the highest expression of PD-L1, which is followed by 786-O, Caki-1, and OS-RC-2 cells, 769-P cells had the lowest surface PD-L1 expression (Fig. 3E). We obtained similar results for PD-L1 expression using immuno-blotting analysis; furthermore, expression of TFEB was positively correlated with the levels of PD-L1 (Fig. 3F). In contrast, expression of TFE3, another member of MITF, was not associated with PD-L1 expression in RCC cell lines (Fig. 3F; Supplementary Fig. S3B).

Knockdown of TFEB expression led to reduced PD-L1 expression in 786-O cells (Fig. 3G and H), which was accompanied with reduced TFEB binding to the PD-L1 promoter (Fig. 3I). Conversely, overexpression of TFEB in the 769-P cells...
Figure 2.
RCC cells overexpressing TFEB suppress cytotoxic CD8⁺ T-cell function. **A**, Renca cells were transfected with either an empty vector (EV) or TFEB-S211A (TFEB) pcDNA3.1 plasmids to generate stable cell lines. TFEB expression was determined by immunoblot analysis. **B–I**, A total of 1 × 10⁶ Renca cells (EV or TFEB) were injected subcutaneously on the back of WT BALB/c mice. Tumor growths were shown and significances were determined by t test (**B**, n = 9). **C–F**, At day 23, mice were sacrificed, TILs were isolated, and cell surface was stained with antibodies against CD8 and CD107a (**C**), followed with intracellular staining with antibodies against Granzyme B (**D**), IL-2 (**E**), and TNFα (**F**; n = 5–9). **G**, IHC staining of TFEB and PD-L1 within tumor tissues. Representative images are shown. **H**, Single-cell suspensions were prepared from tumor tissues, PD-L1 expression was determined on gated CD45⁺ cells, and significance of MFI was determined by t test (n = 7). **I**, The correlation of tumor weights and PD-L1 staining scores was determined (mean ± SEM; *P* < 0.05; **P** < 0.01; ns, not significant).
significantly enhanced TFEB binding to the PD-L1 promoter and PD-L1 expression (Supplementary Fig. S3C and S3D). Furthermore, transient expression of TFEB significantly enhanced luciferase activity driven by the PD-L1 promoter (Fig. 3I). Together, these findings demonstrate that TFEB directly binds to the PD-L1 promoter and positively regulates PD-L1 expression.

mTOR inhibition enhances PD-L1 expression via activation of TFEB in RCC cells

Given that TFEB is a major target of mTORC1 (14), we asked whether inhibition of mTOR could lead to changed expression of PD-L1. Inhibition of mTOR pathway, either directly by rapamycin and Torin-1 or indirectly by cell starvation, significantly enhanced TFEB expression and its nuclear accumulation in 786-O cells.
mTOR inhibition enhances PD-L1 expression via TFEB in RCC cells. 786-O (A and B) and 769-P cells (C and D) were treated with 100 nmol/L rapamycin for 6 hours or 500 nmol/L Torin-1 for 3 hours or starved for 6 hours. PD-L1, S6 and 4-EBP1 phosphorylation, nuclear TFEB, and total TFEB expression in 786-O cells (A) or 769-P cells (C) were determined by immunoblot analysis. Cell surface PD-L1 expression in 786-O cells (B) or 769-P cells (D) was determined by flow cytometry. E, Cell lysates of 786-O or 769-P cells were prepared and chromatin was fragmented with sonication and precipitated with anti-TFEB. The immunoprecipitated DNAs were amplified for the PD-L1 promoter. F, 786-O cells were transduced with scramble shRNA and TFEB shRNA lentiviral particles and then treated with rapamycin, Torin-1, and starvation as described in A. The amounts of TFEB, PD-L1, and S6 phosphorylation were determined by immunoblot (F).

The significance of intensity changes of TFEB (G) and PD-L1 (H) was determined by two-way ANOVA. All experiments were repeated for three times (mean ± SEM; *P < 0.05; **P < 0.01; ***P < 0.001; ns, not significant).

(Fig. 4A). Furthermore, this was associated with enhanced PD-L1 expression (Fig. 4A and B). HIF-1α, STAT3, and p65 have all been implicated as regulators of PD-L1 expression. In our hands, inhibition of mTOR did not affect the phosphorylation of STAT3 and p65 but significantly reduced HIF-1α expression (Supplementary Fig. S4A and S4B). We found similar results in 769-P cells (Fig. 4C and D; Supplementary Fig. S4C and S4D). The negative regulation of mTOR on PD-L1 seemed to be RCC specific, as inhibition of mTOR resulted in reduced expression of PD-L1 in lung carcinoma H1975 cells (Supplementary Fig. S4E) and no change of expression in A549 and H2126 lung carcinoma cells, as well as HCT116, LoVo, and SW480 colon cancer cells (Supplementary Fig. S4E and S4F).

Torin-1 treatment enhanced TFEB binding to the PD-L1 promoter in both 786-O and 769-P cells, compared with control groups (Fig. 4E). To further substantiate that PD-L1 induction by mTOR inhibition was via activation of TFEB, we knocked down TFEB expression by shRNA in 786-O cells and looked at the effect of mTOR inhibition on PD-L1 expression. Knockdown of TFEB abolished the enhanced PD-L1 expression upon inhibition of mTOR by rapamycin, Torin-1, or starvation (Fig. 4F–H).

Together, these data demonstrate that inhibition of mTOR specifically promoted PD-L1 expression in RCC via TFEB activation.

mTOR inhibition enhances PD-L1 expression via TFEB activation in human primary RCC cells

Next, we asked if the inhibition of mTOR enhanced PD-L1 expression in human patients with renal cancer. To this end, we isolated primary renal cancer cells from freshly surgically removed
tumor tissues. IHC staining showed that tumor tissues had a CAIX positive staining, a transmembrane protein and marker for ccRCC (Fig. 5A). The isolated primary ccRCC cells from patients were almost all CAIX-positive, indicating the purity of primary cells (Fig. 5B). Torin-1 induced rapid drop in the cytoplasmic concentrations of TFEB at 30 minutes, followed by a slower Figure 5.

TFEB mediates enhanced PD-L1 expression upon mTOR inhibition in human primary renal cancer cells. A, H&E and IHC staining with anti-CAIX antibody on human ccRCC tissues. B, Immunofluorescence staining with anti-CAIX antibody on human primary ccRCC cells. C, Isolated human primary ccRCC cells were treated without or with 500 nmol/L Torin-1 for 30, 60, and 90 minutes. TFEB expression in fractions of cytoplasm and nuclear was determined. Significance of TFEB intensity changes was determined by one-way ANOVA. D–F, Human primary RCC cells were untreated or treated with 100 nmol/L rapamycin for 6 hours, 500 nmol/L Torin-1 for 3 hours, or 6-hour starvation. The amounts of TFEB, PD-L1, and S6 phosphorylation were determined by immunoblot analysis and significance of PD-L1 changes was determined (D). Cell surface PD-L1 (E) and PD-L2 (F) expression was determined by flow cytometry. G, Immunofluorescence staining with anti-TFEB and anti-PD-L1 antibodies on primary RCC cells treated with vehicle and 500 nmol/L Torin-1 for 3 hours. The experiments were repeated for three times (mean ± SEM; *, P < 0.05; **, P < 0.01; ***, P < 0.001; ns, not significant).
increase in the nuclear concentration of TFEB (Fig. 5C). In line with this, prolonged mTOR inhibition led to enhanced PD-L1 expression measured by immunoblot analysis and flow cytometry (Fig. 5D and E). In contrast, PD-L2 expression was not affected by mTOR inhibition (Fig. 5F). These results were confirmed using immunofluorescence staining of TFEB and PD-L1 (Fig. 5G). Together, these data demonstrate that mTOR inhibition induces PD-L1 expression via TFEB activation in human primary renal cancer cells.

Anti-PD-L1 immunotherapy enhances the response to mTOR inhibition in RCC

We then asked whether combination of antibody against PD-L1 could potentiate the efficacy of mTOR inhibition by

Figure 6. Simultaneously targeting PD-L1 significantly enhances mTOR efficacy in a mouse xenograft model. A–F, Wild-type BALB/c mice were subcutaneously injected with $1 \times 10^6$ Renca cells. Once tumor volumes reached 50 mm$^3$, mice were treated with vehicle and IgG, temsirolimus (TEM; 10 mg/kg), anti-PD-L1 (200 μg/mouse), or a combination of TEM, and anti-PD-L1 for 12 days ($n = 7$–9). Tumor volumes were recorded daily. Schematic representation of the treatment was shown (A). Comparison of Renca tumor growth in different groups (B) and the mice were necropsied at day 24 and tumors were shown (C). D, Phosphorylation of S6 within CD45-negative tumor cells was determined by flow cytometry. E, H&E and IHC staining of TFEB, PD-L1, and Ki67 within tumor tissues. F, TILs were isolated and stained with CD8, CD107a, and GZMB. Representative histograms shown on the left panel. Percentages of CD107a$^+$ CD8$^+$ or GZMB$^+$ CD8$^+$ are shown on the right panel (mean ± SEM; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ns, not significant).
temsirelimus on ccRCC growth in a xenograft mouse model. When tumor volume reached 50 mm³, mice were treated with either temsirolimus (10 mg/kg, i.p.) daily, anti-PD-L1 (200 µg per mouse, i.p.) four times over 12 days, combination of both, or vehicle plus control IgG (Fig. 6A). There was a reduction in tumor growth in mice treated with either anti-PD-L1 alone or temsirolimus alone inhibited tumor growth compared with the control group, but this was not significant (Fig. 6B and C). In contrast, the combination of temsirolimus and anti-PD-L1 therapy resulted in a significant reduction in tumor size compared with all other groups and complete disappearance of tumors in 3 mice after 23 days (Fig. 6B and C). Temsirolimus suppressed mTORC1 activation in tumor cells as measured by ribosome S6 phosphorylation and enhanced TEBF and PD-L1 expression within tumor tissues (Fig. 6D and E; Supplementary Fig. S5A), compared with control group, indicating the in vivo significance of TEBF–PD-L1 axis during tumor growth. Although temsirolimus did not significantly inhibit tumor cell proliferation assessed by Ki67 IHC staining, anti-PD-L1 led to a small but significant reduction in Ki67 staining and combination of PD-L1 and temsirolimus had a synergistic effect on suppression of Ki67 staining (Fig. 6E; Supplementary Fig. S5B).

Next, we tested the effect of mTOR and PD-L1 inhibition on cytotoxicity in tumor-infiltrating CD8⁺ T cells. Temsirolimus treatment suppressed CD107a and GZMB expression in CTL, anti-PD-L1 treatment had little effect but the combination of anti-PD-L1 and temsirolimus significantly enhanced their expression (Fig. 6F). The combination of the two significantly enhanced IFNγ expression compared with the CTL from untreated animals, although IFNγ was not inhibited by temsirolimus treatment alone and was enhanced by anti-PD-L1 alone (Supplementary Fig. SS). Together, these data demonstrate that only the combination of inhibiting mTOR and PD-L1 signaling significantly induced the expression of all three markers of T-cell cytotoxicity.

Discussion

Identifying the mechanism by which tumors are resistant to targeted mTOR inhibitors has largely focused on analysis of intracellular signaling pathways with limited focus on the immune microenvironment of the tumors (25, 26). In this study, we demonstrated that inhibition of mTOR in RCC cell lines and human primary RCC cells leads to enhanced translocation and expression of TEBF, which subsequently induces PD-L1 expression. Furthermore, combination of mTOR inhibition and anti-PD-L1 enhanced the cytotoxic functions of tumor-infiltrating CTL and therapeutic efficacy in a mouse RCC xenograft model.

TEBF has been linked with many aspects of cellular events including proliferation, metabolism, and autophagy (12, 13). Yet in our hands, alteration of TEBF expression has little intrinsic effects on in vitro RCC tumor biology including cell proliferation, survival, and migration. Only in the presence of T cells did we see the effect of TEBF. Identification of the TEBF target genes in RCC cells may provide better understanding of the functions of TEBF in regulation of RCC tumorigenesis and the interaction between RCC cells and the immune microenvironment.

PD-L1 expression in cancer cells is regulated by a variety of transcriptional factors including HIF-1α, NF-κB, STAT1, and STAT3 (24). Our studies revealed a strong association between PD-L1 protein and TEBF expression in RCC cells, in which TEBF directly binds to the PD-L1 promoter. Of note, the induction of PD-L1 in RCCs was irrespective of the VHL status, as the tested RCCs contain both VHL-negative and VHL-positive cells (27).

Consistent with this, PD-L1 expression was enhanced concurrent with enhanced TEBF expression, despite reduced HIF-1α expression in both 786-O and 769-P cells upon mTOR inhibition. Furthermore, knockdown of TEBF in RCC rendered the cells less responsive to PD-L1 induction upon mTOR inhibition, indicating a critical role of TEBF in regulation of PD-L1 expression in RCC cells.

The induction of TEBF and PD-L1 by mTOR inhibition seems to be RCC-specific, as PD-L1 expression in colon cancer and lung carcinoma cells was not enhanced by mTOR inhibitors. The activity of TEBF is tightly regulated by protein phosphorylation at multiple serine sites, which can be mTOR-dependent (S122) or -independent (S138 and S134; refs. 28, 29). It is conceivable that the activity of TEBF is independent of mTOR regulation in non-RCCs. In line with this, the phosphorylation and nuclear translocation of TEBF can be regulated by GSK3β in breast cancer cells (15). A PARP inhibitor that inactivated GSK3β in breast cancer cells can enhance PD-L1 expression and cancer-associated immunosuppression (30). It is tempting to speculate that PARPi induces PD-L1 expression via activation of TEBF.

In contrast to the positive role of PI3K-AKT-mTOR in the regulation of PD-L1 expression in lung carcinoma cells (31), mTOR inhibition led to enhanced PD-L1 expression in RCC cells in our hands, which is consistent with a previous study (32). PI3K-AKT-mTOR can regulate PD-L1 expression following growth factors or inflammatory stimuli in both an IFNγ-dependent and -independent manner in non-small cell lung cancer (NSCLC), glioma, breast cancer, and melanoma cells (24). Together, these data highlight the contextual roles of PI3K-AKT-mTOR in regulation of PD-L1 expression in tumor cells.

Although checkpoint and mTOR inhibitors have been successful as cancer therapies, as monotherapies these drugs seem to be insufficient to fully block cancer progression (33, 34). Consistent with our findings, targeting mTOR and PD-1/PD-L1 axis simultaneously has improved efficacies in treatment of oral cavity cancer and hepatocellular carcinoma (35, 36). Although the enhanced tumor control with combination of mTOR and PD-L1 targeting depends on CTL but not NK cells (35), some of the mechanisms may be different. mTOR inhibition leads to enhanced MHC-I expression in oral cavity tumors; in HCC, PD-L1 promotes tumor growth via enhancing the phosphorylation of 4-EBP1 and ribosomal protein S6 (36).

In summary, our data demonstrated that TEBF mediates PD-L1 upregulation by mTOR inhibitors, which can attenuate mTORI therapeutic efficacy via tumor-associated immune suppression. These data provide strong scientific rationale for the combination of mTOR-targeted therapy and anti-PD-L1 immunotherapy, which may benefit patients with RCC.
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References

Tumor Cell–Derived IL1β Promotes Desmoplasia and Immune Suppression in Pancreatic Cancer
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ABSTRACT

Pancreatic ductal adenocarcinoma (PDA) is an aggressive malignancy typified by a highly stromal and weakly immunogenic tumor microenvironment that promotes tumor evolution and contributes to therapeutic resistance. Here, we demonstrate that PDA tumor cell–derived proinflammatory cytokine IL1β is essential for the establishment of the protumorigenic PDA microenvironment. Tumor cell–derived IL1β promoted the activation and secretory phenotype of quiescent pancreatic stellate cells and established an immunosuppressive milieu mediated by M2 macrophages, myeloid-derived suppressor cells, CD14+CD5+ regulatory B cells, and Th17 cells. Loss of tumor cell–derived IL1 signaling in tumor stroma enabled intratumoral infiltration and activation of CD8+ cytotoxic T cells, attenuated growth of pancreatic neoplasia, and conferred survival advantage to PDA-bearing mice. Accordingly, antibody-mediated neutralization of IL1β significantly enhanced the antitumor activity of α-FD-1 and was accompanied by increased tumor infiltration of CD8+ T cells. Tumor cell expression of IL1β in vivo was driven by microbial-dependent activation of toll-like receptor 4 (TLR4) signaling and subsequent engagement of the NLRP3 inflammasome. Collectively, these findings identify a hitherto unappreciated role for tumor cell–derived IL1β in orchestrating an immune-modulatory program that supports pancreatic tumorigenesis.

Significance: These findings identify a new modality for immune evasion in PDA that depends on IL1β production by tumor cells through TLR4-NLRP3 inflammasome activation. Targeting this axis might provide an effective PDA therapeutic strategy.

Introduction

Pancreatic ductal adenocarcinoma (PDA) is a highly lethal malignancy with a mortality rate approaching the rate of incidence (1). In addition to lack of efficient early diagnosis methods, disease survival is compromised by resistance to conventional chemotherapy and immunotherapeutic strategies that are proving effective in the treatment of other cancers (2, 3). It is becoming increasingly recognized that this recalcitrance is largely attributable to an elaborate network of tumor–stromal interactions that are orchestrated by paracrine factors released by the tumor epithelium, activated fibroblasts, and immune cells (4, 5). Identification and functional characterization of such factors, and the processes they control, is therefore an essential prerequisite for rational development of strategies that can circumvent therapeutic barriers and improve immune responsiveness of PDA tumors.

The cytokine IL1β is an inflammatory mediator that is frequently upregulated in a variety of cancers and its production is associated with poor prognosis (6, 7). Upregulation of either IL1B expression or posttranslational processing in head and neck squamous carcinoma, breast cancer, lung cancer, and melanoma results in increased tumor infiltration of immunosuppressive macrophages and myeloid-derived suppressor cells (MDSC), thereby promoting immune evasion and tumor development (8–10). Other protumorigenic effects of IL1β have been attributed to the induction of neoangiogenesis (11) and the regulation of expression in stromal cells of soluble mediators that enhance tumor cell survival and metastasis (7). These effects are mediated by IL1β–dependent signaling cascades that under conditions of IL1β overabundance result in the sustained activation of NFκB and MAPK pathways (6).

Several lines of evidence suggest a role for IL1β in pancreatic cancer development and progression. Increased pancreatic levels of IL1β are observed in association with pancreatitis, a well-established PDA risk factor (12). High intratumoral and serum IL1β levels in patients with pancreatic cancer correlate with poor overall survival and increased chemoresistance (13–15). In mouse models of PDA, adipocyte-secreted IL1β is found to promote obesity-induced pancreatic carcinogenesis and drug resistance through recruitment of tumor-associated neutrophils (16). In addition, regulatory pathways that control IL1β production in PDA–associated myeloid cells have been reported to support tumor progression by promoting immune tolerance (17, 18). Overall, several lines of evidence suggest a heterotypic distribution of IL1β expression in PDA with implications in disease pathogenesis. Thus, in this study, we sought to elucidate the mechanisms underlying the regulation and function of IL1β in PDA, with an eye on assessing its potential as a therapeutic target.

Here, we identify the tumor cell compartment as a prominent source of IL1β production in human and mouse PDA through activation of the TLR4–NLRP3 inflammasome signaling pathway. Targeted depletion of IL1β in established mouse models demonstrates acute dependency of pancreatic cancer evolution on tumor cell–derived IL1β through protumorigenic modulation of the stroma and immune microenvironment. Overall, our study identifies IL1β as an attractive target that may improve PDA response to therapeutic strategies, including immunotherapy.
Materials and Methods

Animals

All mouse protocols were reviewed and approved by the Institutional Animal Care and Use Committee of the New York University (NYU) Grossman School of Medicine (New York, NY). The LSL-Kras\(^{G12D}\)/LoLo, LSL-Tp53\(^{R172H}\)/LoLo, and p53\(^{-}\)mice have been described previously (19, 20). Eight- to 10-week-old wild-type (WT) C57BL/6 (stock 027) mice were purchased from The Charles River Laboratories. Eight- to 10-week-old IL1\(^{−/−}\) (stock # 003245) mice were purchased from The Jackson Laboratories. All mice were on a C57BL/6 genetic background. Female mice were used for orthotopic injections of Kras\(^{G12D}\)/PDEC and KPC cells (21, 22). Briefly, mice were anesthetized using a ketamine (100 mg/kg)/Xylazine (10 mg/kg) cocktail administered via intraperitoneal injection. After making a small incision on the left abdominal wall, either 10\(^6\) Kras\(^{G12D}\)/PDEC cells or 5 \times 10\(^5\) KPC cells in ice-cold PBS mixed at 1:1 dilution with Matrigel (#354234, Corning) in a volume of 50 \(\mu\)L were injected into the tail of the pancreas using a 28-gauge needle. For pancreatic stellate cell (PSC) coimplantation experiments, 10\(^5\) Kras\(^{G12D}\)/PDEC cells and 10\(^5\) PSCs were mixed in 50 \(\mu\)L of ice-cold PBS: Matrigel (1:1) and injected into the tail of the pancreas using a 28-gauge needle. The incision was closed using 5-0 Vicryl RAPIDE sutures (Ethicon) for the body-wall and 4-0 PROLENE sutures (Ethicon) for the skin. All animals were given buprenorphine (0.1 mg/kg) for pain relief directly after surgery and once a day for three days postsurgery. Mice were euthanized by carbon dioxide–induced narcosis 2 weeks postimplantation of Kras\(^{G12D}\)/PDEC. For KPC cells, mice were euthanized 2 weeks and 4 weeks postimplantation for flow cytometry analysis and tumor volume assessment, respectively. KPC tumors were measured using digital caliper (VWR) at the endpoint and tumor volume was calculated using the formula \(V=\frac{4}{3}\pi r^3\).

Anti-PD-1, anti-IL1β, and CDB⁻ depletion

Mice were orthotopically injected into the pancreas with 5 \times 10\(^4\) KPC cells. On day 7 postinjection, mice were intraperitoneally administered either 10 mg/kg anti-PD-1 (Novartis), 10 mg/kg anti-IL1β (Novartis), or IgG control (Novartis) antibody diluted in 200 \(\mu\)L of sterile PBS. Thereafter, anti-PD-1 antibody was administered on days 9, 11, and 16 and anti-IL1β was administered every 2 days. For CDB\(^{−}\) T-cell depletion, 200 \(\mu\)g of anti-CD8 (BioXCell, clone 53-6.7) or an IgG isotype control antibody (BioXCell, clone 2A3) diluted in 200 \(\mu\)L of sterile PBS were administered intraperitoneally daily starting 3 days prior to tumor cell injection and every 5 days after tumor cell injection. Efficiency of CDB\(^{−}\) T-cell depletion was assessed by flow cytometry.

Murine bacterial depletion

Mouse gut microbiota depletion was performed as described previously (23). Briefly, 6-week-old WT mice were administered 0.2 mL of 0.1 mg/mL amphotericin-B (Sigma) by oral gavage every 12 hours for 3 days. Subsequently, water flasks were supplemented with 1g/L ampicillin (Fisher Bioreagents) and antibiotic cocktail containing 0.2 mL of 5 mg/mL vancomycin (Cayman Chemical Company), 10 mg/mL neomycin (Sigma), and 10 mg/mL metronidazole (Sigma) was administered by oral gavage once a day for 2 weeks presurgery and then for the duration of the experiment. Fresh antibiotic cocktail was mixed every day and ampicillin and water was renewed every seventh day. To assay for microbial depletion, fecal pellets were collected from mice at day 0 and day 14 of antibiotic treatment. DNA was isolated from fecal pellets with QIAamp DNA Stool Mini Kit (Qiagen) as per manufacturer’s instructions. Bacterial 16S DNA gene quantification was assessed by quantitative PCR as described previously (24).

Cell lines

Isolation, culture, and adenoviral infection of PDEC were carried out as described previously (21). The KPC cell line (line 4662) was a kind gift from Dr. R.H. Vonderheide. The immortalized PSC cell line was a kind gift from Dr. A.C. Kimmelman. Isolation and culture of primary PSCs was carried out as described previously (25). Cell lines were not authenticated and were tested for Mycoplasma contamination every 4 months. Scramble control and shRNAs against IL1β, Nlrp3, and Tlr4 were cloned into the lentiviral pLKO.1 hygro vector obtained from Addgene (#24150). shRNA sequences used were as follows—scramble: GGCAGATCCTCTATCCTGTTAGTA; IL1β-sh1: GTGGTCAGGACAATAATTGACTTC; IL1β-sh2: GGCAGACAATCAACAGAGCTTCA; NLRP3-sh2: AGCCCTGACCTGACTATA-GTCTTC; NLRP3-sh3: CTTGAGAATGGAGGGCTCAGAA; TLR4-sh1: GCCAATCCCTAGAACGTATA; TLR4-sh2: GCAATACTGGGGACACATGAAAG; TLR4-sh3: GCCCTACCTAGAACGTATA.

Lentiviral particles were generated by transfecting HEK-293T cells with the plKO.1 vector, the packaging construct (psPAX2), and the envelope plasmid (pMD2G). Supernatants containing viral particles were collected over a period of 48 hours and stored at 4°C. Following final collection, supernatants were filtered through a 0.45-μm syringe filter and concentrated using 100 MWCO Amicon Ultra centrifugal filters (Millipore). A multiplicity of infection of 15 was used for lentiviral infection of Kras\(^{G12D}\)/PDEC or KPC cells in the presence of 10 μg/mL polybrene (Chemicon) and infected cells were selected using 150 μg/mL hygromycin (Sigma).

Human data generation

130 human PDA tumor (\(n = 75\)) and adjacent normal (Adj Norm; \(n = 55\)) mRNA expression profiles generated on the same array (Affymetrix GeneChip Human Genome U133 Plus 2.0) were downloaded from GEO (https://www.ncbi.nlm.nih.gov/geo/; GSE15471, GSE16515). Adj Norm samples clustering with PDA tumors, PDA tumor profiles clustering with subsets of Adj Norm samples, and duplicates were discarded (as described previously; ref. 26), for a remainder of \(n = 74\) tissues (\(n = 50\) PDA tumor and \(n = 24\) Adj Norm). Raw data were processed and normalized in one batch using a GC-content background correction robust multi-array average (RMA) algorithm (GC-RMA), performed in: R: A language and environment for statistical computing. Unpaired Student \(t\) tests were generated in GraphPad Prism (GraphPad Software; www.graphpad.com).

Human pancreas specimens

For the purposes of analyzing IL1β, NLRP3, and TLR4 expression patterns, we examined 8–10 patient PDA lesions and corresponding adjacent normal tissue samples. Samples consisted of 5-mm sections that were cut from formalin-fixed, paraffin embedded blocks provided by the Center for Biospecimen Research and Development at NYU Langone Health. This study was conducted in accordance with the Declaration of Helsinki; all samples were anonymized prior to being transferred to the investigator’s laboratory and therefore met exempt human subject research criteria.

Histology and IHC

Mouse pancreata were fixed in 10% buffered formalin (Thermo Fisher Scientific) overnight and embedded in paraffin as described earlier (21). Trichrome staining was performed at NYU Grossman School of Medicine Histopathology Core Facility. For IHC, deparaffinized sections (6 μm) were rehydrated, quenched in 2%
hydrogen peroxide/methanol for 15 minutes, and antigen retrieval was performed in 10 mmol/L sodium citrate/0.05% Tween-20 (pH 6.0) for 15 minutes in a microwave oven. Blocking was done in 10% serum/1% BSA/0.5% Tween-20 for 1 hour at room temperature, followed by incubation with the primary antibodies diluted in 2% BSA overnight at 4°C. Primary antibodies are detailed in Supplementary Data. After incubating with secondary biotinylated antibodies and ABC solution (both from Vector Laboratories), sections were developed with DAB peroxidase substrate kit (Vector Laboratories) according to the manufacturer’s instructions. After counterstaining with Harris hematoxylin (Sigma), slides were subjected to an alcohol dehydration series and mounted with Permount (Thermo Fisher Scientific). Slides were examined on a Nikon Eclipse 80i microscope and images were analyzed to measure stained area using ImageJ software.

**Immunofluorescence**

Formalin-fixed, paraffin-embedded sections were deparaffinized and rehydrated, permeabilized with TBS/0.1% Tween-20, and washed in PBS. Citrate buffer antigen retrieval (10 mmol/L sodium citrate/0.05% Tween-20, pH 6.0) was performed in a microwave for 15 minutes. Blocking was performed in 10% serum/1% BSA/0.5% Tween-20/PBS for 1 hour at room temperature. Primary antibodies were diluted in 2% BSA/0.5% Tween-20/PBS and incubated on sections overnight at 4°C. Primary antibodies are detailed in Supplementary Data. Secondary antibodies (Alexa Fluor–labeled; 1:1,000, Invitrogen) were diluted in 2% BSA/PBS, and incubated on sections for 1 hour at room temperature. Sections were washed with PBS and stained with DAPI. Slides were examined and imaged on a Nikon Eclipse Ti2 microscope.

**Flow cytometry**

Single-cell suspensions were prepared from pancreas as described previously (27). For isolation of tumor-infiltrating lymphocytes, tumor tissue was minced into 1 to 2 mm pieces and digested with collagenase IV (1.25 mg/mL, Worthington) and 0.1% trypsin inhibitor from soybean (Sigma), in complete RPMI for 25 minutes at 37°C. For isolation and FACS analysis of epithelial and fibroblast compartments, minced tumor tissue was digested with Pronase (0.2 mg/mL, Roche), Collagenase P (0.5 mg/mL, Roche), and DNase I (0.5 mg/mL, Roche). Cells were suspended in 1%FBS/PBS, passed through a 70-μm strainer and treated with RBC lysis buffer (eBioscences). Single-cell suspensions were blocked with anti-CD16/CD32 antibody (Fc Block, BD Biosciences) for 5 minutes on ice and labeled with mAbs against mouse antigens as detailed in Supplementary Data. All samples were acquired on LSR II (BD Biosciences) at NYU Flow Cytometry Core Facility and analyzed by FlowJo version 10.2 (TreeStar, Inc.). Cell sorting using a BD FACS ARIA II sorter was performed to isolate Ep-CAM+ cells, CD140a+ fibroblasts and CD45+ cells, and >95% purity of sorted cells was achieved.

**Quantitative RT-PCR**

For RNA isolation from tumors, pancreata processed to single-cell suspension were stained for flow cytometry. CD45–CD34–CD140a+Ep-CAM– fibroblasts were FACS sorted using a 100-μm nozzle into the lysing reagent RLT and total RNA was extracted as per the manufacturer’s instructions (RNeasy Mini Kit, Qiagen). To check knockdown in KrasG12D−/−PDEC and KPC cells, 105 cells were lysed in 350 μL RLT reagent and total RNA was extracted as per the manufacturer’s instructions (RNeasy Mini Kit, Qiagen). Total RNA (1 μg) was reverse-transcribed using the Quantitect Reverse Transcription Kit (Qiagen). Subsequently, specific transcripts were amplified by SYBR Green PCR Master Mix (USB) using a Stratagene Mx 3005P thermocycler. Where fold expression is specified, comparative Ct method was used to quantify gene expression. Expression was normalized to GAPDH. Primers used for qPCR are detailed in Supplementary Data.

**Supernatant collection and cytokine analysis**

For cytokine analysis of mouse pancreata, the tissues were harvested, minced with a sterile razor blade, and incubated in 500 μL of complete media for 24 hours before supernatant collection. Mouse IL1β protein levels were determined by Mouse IL1β Quantikine ELISA Kit (R&D Systems) as per manufacturer’s instructions.

**Statistical analysis**

At least 7 to 15 mice were used in each group, and the experiments were repeated a minimum of two times to validate reproducibility. Group means were compared with Student t tests. Significance in variations between two groups was determined by an unpaired Student t test (two-tailed). Statistical analyses were performed using GraphPad Prism software (version 7.0d), and data are presented as mean ± SD. P < 0.05 was considered statistically significant.

**Results**

**Tumor cell-derived IL1β promotes pancreatic tumorigenesis**

To assess IL1β production in PDA, we first examined microarray data from 50 PDA patient tumors and 24 adjacent normal tissue samples. Our analysis revealed significant upregulation of IL1β expression in PDA tumors relative to normal adjacent pancreatic tissue (Fig. 1A). We next assessed the distribution pattern of IL1β by IHC staining of tumor sections from patient PDA samples. Consistent with previous reports documenting the expression of IL1β by innate immune cells and fibroblasts (15, 16, 18), robust IL1β staining was detected in the tumor stroma (Supplementary Fig. S1A). However, unexpectedly, we also observed significant IL1β staining in the ductal epithelium (Fig. 1B). Similarly, robust expression of IL1β in the CK8+ epithelial tumor cell compartment was revealed by immunofluorescence staining of pancreata from the slowly progressive KrasG12D; p48Cre (KC) mouse model of pancreatic neoplasia (19) and the KrasG12D; p53R172H; p48Cre (KPC) invasive PDA mouse model (Fig. 1C and D; Supplementary Fig. S1B; ref. 20). The relative levels of IL1β production by stromal (CD140a+ fibroblasts and CD45+ immune cells) and tumor cells (EpCAM+ epithelium) from KC mice was further analyzed by flow cytometry (Fig. 1E; Supplementary Fig. S1C). In agreement with the IHC data, the epithelial compartment displayed the highest levels of IL1β production (Fig. 1E).

Next we sought to investigate the functional significance of tumor cell–derived IL1β. Utilizing a RNAi strategy, two independent short hairpin (sh) sequences targeting IL1β were introduced into pancreatic ductal epithelial cells derived from either KrasG12D+ KrasG12D−/−PDEC or KrasG12D+Trp53R172H; p48Cre mouse cells (KPC, refs. 21, 22). Knockdown efficiency was ascertained by qPCR and immunofluorescence staining (Supplementary Fig. S1D–S1F). Orthotopic injection of IL1β−/sh KrasG12D+−/−PDEC into pancreata of syngeneic mice led to grafts that displayed a significant reduction of CK8+ pancreatic intraepithelial neoplasia (PanIN)-like lesions, relative to scramble control (Fig. 1F; Supplementary Fig. S1G). A role for tumor cell–derived IL1β was also evident in the context of more advanced
**Figure 1.**

Tumor cell–derived IL1β is required for pancreatic oncogenesis. A, mRNA transcript levels of IL1β in PDA compared with normal adjacent tissue from publicly available human transcriptomic data (74 patient samples, n = 50 PDA and n = 24 adjacent normal). Each data point indicates an individual tissue sample. Error bars, SD; P-values determined by the Student t test (two-tailed, unpaired). B, Representative IHC detection of IL1β expression in sections from human PDA and adjacent normal tissues (n = 10 patient samples). C, Representative immunofluorescence detection of IL1β expression on a section from a 4-month-old KC mouse pancreas (N = 12; CK8 red; IL1β green; DAPI blue). D, Representative immunofluorescence detection of IL1β expression on a section from a 2-month-old KPC mouse pancreas (N = 8 at 2–4 months; CK8 red; IL1β green; DAPI blue). E, Quantification of flow cytometric analysis of distribution of IL1β expression in epithelial (EpCAM⁺), fibroblast (CD140a⁺), and immune (CD45⁺) cells sorted from pancreata of 1– to 2-month-old KC mice (N = 7). Error bars, SD. F, Graph indicates quantification of percentage of CK8⁺ signal per lesion from IHC staining with CK8 antibody on sections of orthotopic pancreatic grafts 2 weeks after implantation of KRasG12D⁺POEC expressing scrambled shRNA (scr-sh) control or IL1β shRNAs (IL1β-sh1 and IL1β-sh2) in wild-type (WT) mice (N = 8–9). Error bars, SD; P values determined by the Student t test (two-tailed, unpaired). Data representative of three independent experiments. G, Representative tumors 4 weeks after orthotopic implantation of KPC cells expressing scrambled shRNA (scr-sh) or IL1β shRNA (IL1β-sh) in pancreata of WT mice. H, Graph represents quantification of G, indicating tumor volume (N = 15). Error bars, SD; P values determined by the Student t test (two-tailed, unpaired). Data representative of three independent experiments. I, Kaplan–Meier curve for survival analysis of mice bearing pancreatic tumors derived from orthotopically implanted scr-sh or IL1β-sh KPC cells (N = 8). P values determined by the Student t test (two-tailed, unpaired). Data representative of two independent experiments. *** P < 0.001; **** P < 0.0001.

**IL1β Promotes Immune Suppression in Pancreatic Cancer**
Figure 2.
Tumor cell–derived IL1β promotes immune suppression in the pancreatic tumor microenvironment. A–C, Representative flow cytometry plots (top) and quantification (bottom) from orthotopic pancreatic grafts of scr-sh or IL1β-sh KRasG12D-PDEC in WT mice 2 weeks postimplantation analyzing CD11b F4/80+ tumor–associated macrophages (A), CD11b Gr1+ MDSCs (B), and CD11b Ly6G+ tumor–associated neutrophil populations (C). For A–C, graphs indicate immune subpopulations as a percentage of CD45+ cells (N = 5–8). Error bars, SD; P values determined by the Student t test (two-tailed, unpaired). Data representative of two independent experiments. D–F, Representative flow cytometry plots (top) and quantification (bottom) from orthotopic pancreatic grafts of scr-sh or IL1β-sh KRasG12D-PDEC in WT mice 2 weeks postimplantation analyzing CD5+ regulatory B (Breg) cells measured as a percentage of total CD19+ CD1dhi B cells (N = 6; D) and RORγt+ T~H~1~7~ cells measured as a percentage of total CD4+ Th cells (N = 8–9; E, F, CD206+ M2-polarized macrophages measured as a percentage of total stromal macrophages (N = 5–6). Data representative of two (D and E) or three (F) independent experiments. For D–F, error bars, SD; P values determined by the Student t test (two-tailed, unpaired). G, IHC detection of CD8+ T cells on sections of scr-sh and IL1β-sh KRasG12D-PDEC grafts in WT mice, 2 weeks postorthotopic implantation. Representative images are shown. Graph depicts quantification of IHC, indicating the average percentage of CD8+ cells per field of view (FOV) of the implant (4–6 FOV per animal, N = 5). Error bars, SD; P values determined by the Student t test (two-tailed, unpaired). Data representative of two independent experiments. Representative flow cytometry plots and quantification (right) from orthotopic pancreatic grafts of scr-sh or IL1β-sh KRasG12D-PDEC in WT mice 2 weeks postimplantation analyzing activated cytotoxic CD8+ T cells (Tc) as measured by IFNγ (H) and granzyme B (GzmB) expression. Quantification of IFNγ+ and GzmB+ cells is represented as a percentage of total CD8+ Tc cells (N = 5–7). Error bars, SD; P values determined by the Student t test (two-tailed, unpaired). Data representative of two independent experiments. *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001.
lesions, with IL1β-depleted KPC cells forming significantly smaller tumors upon orthotopic implantation (Fig. 1G and H). Furthermore, IL1β knockdown increased the survival of mice bearing orthotopic KPC tumors (Fig. 1I). Overall, these results establish a prooncogenic role for tumor cell–derived IL1β in pancreatic cancer.

Tumor cell–derived IL1β induces a tolerogenic immune state in the PanIN microenvironment

Given the well-established role of IL1β as an inflammatory mediator, we tested whether tumor cell–derived IL1β promotes pancreatic oncogenesis through its interactions with the tumor microenvironment (TME) by implanting KrasG12D–PDEC or KPC cells into the pancreas of Il1r1 null mice (28). Absence of IL1β signaling in the pancreatic stroma phenocopied the depletion of tumor cell–derived IL1β, with reduced growth of orthotopic KrasG12D–PDEC grafts and KPC tumors in Il1r1-null mice relative to wild-type control (Supplementary Fig. S1H–S1K). In addition, the overall survival of KPC cell–implanted animals was extended in Il1r1-null mice, relative to wild-type control (Supplementary Fig. S1L). Notably, surface expression of IL1R1 is nearly undetectable in KrasG12D–PDEC cells (Supplementary Fig. S1M). These observations suggest a paracrine role for tumor cell–derived IL1β and therefore prompted us to investigate the fibro-inflammatory effects of tumor cell–derived IL1β on the TME.

Flow cytometric analysis of pancreatic grafts formed by IL1β-sh KrasG12D–PDEC revealed a pronounced alteration of the TME immune landscape, relative to the scramble control. Specifically, depletion of tumor cell–derived IL1β significantly decreased stromal...
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Figure 4. ILβ neutralization sensitizes PDA tumors to PD-1 checkpoint blockade. A, Schematic of anti-CD8 antibody treatment regimen. Anti-CD8 or control IgG antibody (red arrow) was administered every day for three days prior to orthotopic implantation of KrasG12D-PDECs and then every five days hence. B, Graph depicts quantification of IHC analysis of CD8 staining on sections of orthotopic pancreatic grafts 2 weeks postimplantation of scr-sh or IL1β-sh KrasG12D-PDEC and indicates percentage of CD8+ signal per lesion (N = 8). Error bars, SD; P values determined by the Student t test (two-tailed, unpaired). Data representative of two independent experiments. C, Schematic of anti-IL1β and anti-PD-1 antibody treatment regimen. Treatment was initiated 1 week post orthotopic implantation of KPC cells. Green and red arrows indicate anti-PD-1 and anti-IL1β antibody administration, respectively. D, Graph represents quantification of analysis in C, indicating tumor weight (N = 8). Error bars, SD; P values determined by the Student t test (two-tailed, unpaired). Data representative of two independent experiments. E, Representative flow cytometry plots (left) of KPC tumors treated with vehicle control, anti-PD-1 antibody alone, anti-IL1β antibody alone, or both anti-PD-1 and anti-IL1β antibody, indicating tumor-infiltrating CD8+ T cells. Graphs depict quantification of FACS analysis, represented as either percentage of CD8+ immune cells (top right; N = 8) or absolute number of CD8+ T cells relative to tumor weight (bottom right; N = 7). Error bars, SD; P values determined by the Student t test (two-tailed, unpaired). Data representative of two independent experiments. *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001.

The immune cell profile resulting from the suppression of IL1β production is consistent with a role for tumor cell–derived IL1β in constraining antitumor immunity in PDA. For instance, TAMs have been reported to downregulate T-cell infiltration in the PDA stroma (29). In addition, M2 TAMs, MDSCs, and CD1d+CD5+ Bregs are immunosuppressive cell populations that have been shown to inhibit the tumor lytic activity of CD8+ T cells (21, 27, 30). Indeed, loss of stromal immunosuppressive subpopulations in IL1β-depleted KrasG12D-PDEC grafts was accompanied by a significant increase in tumor infiltration (Fig. 2G) and activation of CD8+ Tc cells, as measured by IFNγ and granzyme B expression (Fig. 2H and I). Similar immune changes were also observed in tumors formed by orthotopic transplantation of IL1β-sh KPC cells (Supplementary Fig. S2D–S2L), indicating a role for IL1β in shaping the immune microenvironment in advanced disease as well.

Together, these observations implicate tumor cell–derived IL1β in promoting the establishment of an immunosuppressive microenvironment. Notably, we observed a reduction in IL1β-expressing CD45+ immune cells present in IL1β-sh KrasG12D-PDEC pancreatic grafts relative to the scramble control (Supplementary Fig. S2M), suggesting a feedforward mechanism wherein the tumor-derived IL1β could dictate the abundance of stromal-derived IL1β.
Pro-IL1β processing in pancreatic tumor cells is regulated by the NLRP3 inflammasome. A and B, IHC detection on sections of 2- to 4-month-old KC mouse pancreata of NLRP3 expression (N = 8; A) and cleaved caspase-1 expression (N = 8; B). Insets show respective isotype controls. Representative images are shown. C, Immunofluorescence detection of NLRP3 and phospho-ASC (Y144) colocalization in a 2-month-old KPC mouse pancreas (N = 8; NLRP3, red; p-Asc, green; DAPI, blue). Representative image is shown. D, Immunofluorescence detection of NLRP3 and phospho-Asc (Y144) colocalization on a section of human PDA tissue (N = 8; NLRP3, red; p-Asc, green; DAPI, blue). Representative image is shown. The 36% ± 3.42% ductal epithelium costained positively for NLRP3 and phospho-Asc, as measured using ImageJ (3-5 field of view (FOV)/sample, N = 8). E, IHC detection of NLRP3 and cleaved caspase-1 expression on sections of orthotopic pancreatic grafts 2 weeks postimplantation of KRasG12D-PDEC expressing scramble shRNA (scr-sh) control or NLRP3 shRNA (NLRP3-sh) in WT mice (N = 6). Representative images are shown. F, ELISA analysis for assessing IL1β protein production in scr-sh or NLRP3-sh KRasG12D-PDEC in orthotopic pancreatic grafts 2 weeks postimplantation in WT mice (N = 4). P values determined by the Student t test (two-tailed, unpaired). Data representative of two independent experiments. G, Graph depicts quantification of immunofluorescence detection of IL1β on orthotopic pancreatic graft sections, 2 weeks postimplantation of scr-sh or NLRP3-sh KRasG12D-PDEC in WT mice. Represented as percentage of IL1β-positive epithelium per FOV of the implant (4-6 FOV per animal, N = 8). Error bars, SD; P values determined by the Student t test (two-tailed, unpaired). Data representative of two independent experiments. H, Graph depicts quantification of IHC analysis of CK8 staining on sections of orthotopic pancreatic grafts 2 weeks postimplantation of scr-sh or NLRP3-sh KRasG12D-PDEC and indicates percentage of CK8 + signal per lesion (N = 8). Error bars, SD; P values determined by the Student t test (two-tailed, unpaired). Data representative of two independent experiments. I, Representative tumors 4 weeks after orthotopic implantation of KPC cells expressing scrambled shRNA (scr-sh) or NLRP3 shRNA (NLRP3-sh) in pancreata of WT mice. J, Graph represents quantification indicating tumor volume (N = 7; I). Error bars, SD; P values determined by the Student t test (two-tailed, unpaired). Data representative of two independent experiments. K, Kaplan–Meier curve for survival analysis of mice bearing orthotopically implanted pancreatic tumors derived from scr-sh or NLRP3-sh KPC cells (N = 7). P values determined by the Student t test (two-tailed, unpaired). Data representative of two independent experiments. **, P < 0.01; ***, P < 0.001; ****, P < 0.0001.
Figure 6.
The pancreatic microbiome drives IL1β expression in tumor cells through TLR4 signaling. A, mRNA transcript level of TLR4 in PDA compared with normal adjacent tissue from publicly available human transcriptomic data (74 patient samples, n = 50 PDA and n = 24 adjacent normal). Each data point indicates an individual tissue sample. Error bars, SD; P values determined by the Student t test (two-tailed, unpaired). B, IHC detection of TLR4 expression on a section from human PDA tissue (n = 8 patient samples). Representative image is shown. The 67% ± 8.94% ductal epithelium costained positively for TLR4, as measured using ImageJ (3–5 field of view (FOV)/sample, N = 8). C, IHC detection of TLR4 expression on a section from a 4-month-old KC mouse pancreas (N = 8). Representative image is shown. D, Immunofluorescence detection of IL1β expression on sections of orthotopic pancreatic grafts 2 weeks postimplantation of KRasG12D-PDEC expressing scramble shRNA (scr-sh) control or TLR4 shRNA (TLR4-sh) in WT mice (N = 7; CK8, red; IL1β, green; DAPI, blue). Representative images are shown. (Continued on the following page.)
Tumor cell–derived IL1β promotes immunosuppression, in part, by regulating activation and secretory phenotype of pancreatic stellate cells

In addition to the immune changes described above, we also observed a significant decrease in stromal fibrosis, as detected by collagen deposition, in IL1β-sh KRasG12D-PDEC pancreatic grafts (Supplementary Fig. S3A). Because the most prominent source of collagen deposition in the extracellular matrix are cancer-associated fibroblasts (CAF) that are generated by the activation of PSCs, we assessed the state of PSC activation in IL1β-sh pancreatic grafts by assaying for the activation marker αSMA (31). While prominent αSMA staining was observed in the microenvironment of scramble control KRasG12D-PDEC grafts and KPC tumors, the IL1β-sh KRasG12D-PDEC grafts and IL1β-sh KPC tumors were largely devoid of αSMA staining (Fig. 3A and B). In contrast, no change in the abundance of vimentin-positive fibroblasts was detected in the IL1β-sh KRasG12D-PDEC grafts (Fig. 3A), indicating that tumor cell–derived IL1β is required for PSC activation and not viability.

The potential relevance of this activation mechanism to our observations is supported by the findings that these cells display surface expression of IL1β receptor as determined by FACS analysis (Supplementary Fig. S3B).

To distinguish between direct and indirect effect of tumor-derived IL1β on PSC activation, we isolated primary PSCs from wild-type or Il1r1-null mice and coimplanted each with KRasG12D-PDECs in Il1r1-null mice. Under these conditions, all components of the host stroma are Il1r1 null and therefore by definition unresponsive to tumor cell–derived IL1β. We found that coimplanted wild-type PSCs successfully underwent activation, as detected by αSMA staining (Supplementary Fig. S3C). These results implicate tumor cell–derived IL1β as the primary driver of PSC activation. Consistent with this interpretation, the Il1r1-null PSCs derived from pancreata of Il1r1-null mice failed to undergo activation when coimplanted with tumor cells (Supplementary Fig. S3C). The functional significance of the tumor-derived IL1β/PSCs axis for pancreatic tumor growth is indicated by the observation that the growth defect of KRasG12D-PDEC pancreatic grafts in Il1r1-null mice could be rescued by coimplanted wild-type PSCs but not Il1r1-null PSCs (Supplementary Fig. S3D).

The protumorigenic effects of CAFs is well documented and is mediated by multiple paracrine mechanisms (31, 32). In addition to secreting extracellular matrix proteins and growth factors, the transition of PSCs from a quiescent to an activated state has been shown to be accompanied by the induction of an inflammatory program with upregulation of cytokines and chemokines such as IL6, CCL2, CCL5, and CCL8 (32). To investigate the effect of tumor cell–derived IL1β depletion on the inflammatory secreteome of CAFs, we sorted CAFs from scramble control and IL1β-sh KRasG12D-PDEC pancreatic grafts using the fibroblast marker CD140 (Supplementary Fig. S3E) and analyzed them for expression of previously characterized cytokines (33). Loss of tumor cell–derived IL1β significantly downregulated the expression of several inflammatory cytokines, relative to scramble control (Fig. 3C). Moreover, flow cytometric analysis of CD140+ CAFs derived from IL1β-sh KRasG12D-PDEC grafts showed a significant decrease in Ly6C expression, a surface glycoprotein that marks cytokine-producing CAFs (Supplementary Fig. S3F; ref. 34). Our results thus indicate that tumor cell–derived IL1β promotes the activation and shapes the secretory phenotype of CAFs.

Many of the cytokines produced by CAFs in response to IL1β are known modulators of immune cell function (35). We therefore postulated that tumor cell–derived IL1β-mediated PSC activation may, in turn, contribute to the establishment of an immune-suppressive TME. To test this hypothesis, we coimplanted immortalized CAFs isolated from KPC tumors (36) with IL1β-sh KRasG12D-PDEC in wild-type mice pancreata. Successful coimplantation was verified by restoration of stromal αSMA staining (Fig. 3D), increase in Ly6C+ CD140a+ cell population (Supplementary Fig. S3F) and increased collagen deposition (Supplementary Fig. S3G). Significantly, CAF coimplantation with IL1β-sh KRasG12D-PDEC specifically rescued the decrease in macrophage recruitment and M2 TAM polarization induced by loss of tumor cell–derived IL1β (Fig. 3E and F; Supplementary Fig. S3H–S3K) as well as restored the inactive state of CD8+ T cells and the decrease in CD8+ T cell infiltration (Fig. 3G and H). This phenotype is consistent with the observed IL1β-dependent production by CAFs of CCL2 and CCL5 (Fig. 3C), which promote macrophage infiltration and M2 polarization (37, 38), and CXCL12 (Fig. 3C), which is known to impede tumor infiltration of CD8+ T cells (39). To determine the functional significance of CD8+ T-cell exclusion in the protumorigenic role of tumor cell–derived IL1β, we depleted CD8+ T cells in mice prior to orthotopic implantation of scramble or IL1β-sh KRasG12D-PDECs (Supplementary Fig. S4A). CD8+ T-cell depletion completely rescued tumor growth defect of IL1β-sh KRasG12D-PDEC pancreatic grafts (Fig. 4A and B; Supplementary Fig. S4B), indicating that the oncogenic role of tumor cell–derived IL1β is mediated through immune suppression of CD8+ T-cell infiltration and activity.

IL1β neutralization sensitizes pancreatic tumors to anti-PD-1 checkpoint therapy

The poor response of pancreatic tumors to immune checkpoint blockade has been primarily attributed to its immunosuppressive microenvironment and poor CD8+ T-cell infiltration (3). Because depletion of tumor-derived IL1β significantly increases CD8+ T-cell infiltration and activity, we reasoned that IL1β neutralization may sensitize PDA tumors to PD-1 checkpoint blockade. To this end, orthotopic KPC tumor–bearing mice were treated with neutralizing antibodies against IL1β and PD-1 (Fig. 4C). Indeed, addition of (Continued).
α-IL1β treatment significantly enhanced the antitumor activity of α-PD-1 (Fig. 4D; Supplementary Fig. S4C). As predicted, combined treatment of α-IL1β and α-PD-1 resulted in increased tumor infiltration of CD8+ T cells, relative to vehicle control or α-PD-1 alone (Fig. 4E).

**IL1β production in pancreatic tumor cells is mediated by NLRP3 inflammasome**

Having established its importance in pancreatic tumorigenesis, we next wanted to dissect the molecular pathway regulating IL1β production in tumor cells. In innate immune cells, IL1β mRNA is translated to produce an inactive precursor pro-IL1β form, which is further processed to yield the mature secreted form of the cytokine by a multimeric protein complex called the inflammasome (6). The most well-characterized inflammasomes are comprised of a Nod-like receptor protein family pyrin-domain containing (NLRP) protein that serves as an activation sensor, which associates with apoptosis-associated speck-like proteins containing a CARD complex (ASC) (40). In complex with NLRP, ASC recruits procaspase-1 that autocatalyzes its cleavage to active caspase-1. Active caspase-1, in turn, cleaves pro-IL1β to produce the functional IL1β protein. Of the various NLRP proteins that can form inflammasomes, the NLRP3 inflammasome appears most relevant to our study because its activation was found to be necessary for induction of pancreatitis (41), a major risk factor for PDA development. In addition, NLRP3 inflammasome activity is associated with malignancies such as colon cancer and melanoma (42). We therefore analyzed the activation status of the NLRP3 inflammasome axis in the pancreatic tumor epithelium. A robust presence of NLRP3 was detected in the tumor epithelial compartment of KC mice pancreata (Fig. 5A). NLRP3 expression in these tumor cells strongly correlated with the expression of cleaved caspase-1 (Fig. 5B), a product of active inflammasomes. Moreover, NLRP3 was found to colocalize with phospho-ASC (Y223) in speck-like aggregates (43) in tumor cells of both KC and KPC mouse pancreata (Fig. 5C; Supplementary Fig. S5A) as well as in human PDA samples (Fig. 5D), further validating the presence of active NLRP3 inflammasomes in these cells.

To determine whether the NLRP3 inflammasome is the primary source of pro-IL1β–processing in tumor cells, we knocked down NLRP3 expression in KRASG12D-PDEC and KPC cells using two independent short hairpins (Supplementary Fig. S5B and S5C). Depletion of NLRP3 in the transformed ductal epithelia significantly reduced cleaved caspase-1 expression and IL1β production in IL1β-sh KRASG12D-PDEC pancreatic grafts, relative to scramble control (Fig. 5E–G; Supplementary Fig. S5D). This was accompanied by a decrease in growth of IL1β-sh KRASG12D-PDEC pancreatic grafts (Fig. 5H; Supplementary Fig. S5E) as well as decreased tumor growth and increased overall survival of orthotopic IL1β-sh KPC tumor-bearing mice (Fig. 5I–K). Together, these results implicate the NLRP3 inflammasome in the production of tumor cell–derived IL1β and define a tumor-supportive role for NLRP3 in pancreatic cancer.

**Tumor-derived IL1β expression is regulated by TLR4 and the pancreatic microbiome**

NLRP3 inflammasome assembly and the posttranslational processing of IL1β that ensues is predominantly regulated by Toll-like receptors (TLR) through induction of IL1β and NLRP3 expression in response to pathogens or cellular damage (44). Members of the TLR family are expressed in various cancers and have been shown to promote tumor growth (45). In pancreatic cancer, while most TLRs have been shown to be expressed largely in stromal cells, elevated TLR4 expression has been reported in the tumor cell compartment of patient PDA samples and shown to be correlated with reduced survival (46, 47). Consistent with these findings, analysis of a panel of patient PDA samples revealed significant upregulation of tumor-associated TLR4 expression, relative to adjacent normal tissue (Fig. 6A), and IHC analysis demonstrated robust TLR4 expression in tumor cells in human PDA as well as KC and KPC mouse pancreata (Fig. 6B and C; Supplementary Fig. S6A). To determine whether IL1β production in tumor cells is TLR4-driven, we employed RNAi to stably knockdown TLR4 expression in KRASG12D-PDEC (Supplementary Fig. S6B). Transformed ductal epithelium of TLR4-sh KRASG12D-PDEC pancreatic grafts had significantly reduced IL1β production, relative to scramble control (Fig. 6D and E). Moreover, TLR4 knockdown in KRASG12D-PDEC and KPC cells decreased growth of orthotopic KRASG12D-PDEC pancreatic grafts (Fig. 6F; Supplementary Fig. S6C) and KPC tumors, respectively (Fig. 6G and H; Supplementary Fig. S6D) and increased overall survival of TLR4-sh KC orthotopic tumor bearing mice, relative to scramble control (Fig. 6I). We conclude TLR4 thus serves as a critical regulator of tumor cell–derived IL1β production and pancreatic tumorigenesis.

Having identified TLR4 as the receptor that controls IL1β production in pancreatic tumor cells, we next searched for possible cues in the pancreatic microenvironment that can induce TLR4 signaling. Recent reports on the existence of a complex pancreatic microbiome (23) prompted us to hypothesize that microbial-derived ligands that are known to activate TLR4 signaling (17) could be responsible for inducing IL1β production in pancreatic tumor cells. To test this hypothesis, we treated wild-type mice with an antibiotic cocktail for 3 weeks to ablate their microbiome prior to implantation of KRASG12D-PDEC (Supplementary Fig. S6E). KRASG12D-PDEC grafts formed in antibiotic-treated mice indeed displayed a significant reduction in tumor cell IL1β expression without affecting TLR4 level, relative to vehicle-treated control mice (Fig. 6J and K; Supplementary Fig. S6F). Overall, our results indicate a role for the pancreatic microbiome in initiating a signaling cascade, likely through TLR4, to activate IL1β production in pancreatic tumor cells.

**Discussion**

In this study, we demonstrate a role for tumor cell–derived IL1β in promoting pancreatic oncogenesis by paracrine induction of heterotypic stromal interactions. Specifically, we show that tumor-derived IL1β is critical for shaping the tolerogenic immune landscape of PDA by promoting stromal accumulation of immunosuppressive cell populations. These include M2-polarized macrophages, tumor-associated neutrophils, IL17-producing Th17 cells, MDSCs, and CD1dhiCD5+ regulatory B cells. In addition, we report that tumor-derived IL1β regulates PDA-associated desmoplasia by promoting activation of quiescent PSCs.

IL1β is a member of the IL1 family of proinflammatory cytokines, which also includes the coagonizing member, IL1α (7). Both IL1α and IL1β are critical immune regulators that signal through a common cell surface receptor (IL1R1-IL1RACp) to activate two main pathways: IKK-1/2–NF-kB and/or MKK-MAPK/NFκB (6). Despite considerable functional homology, the two cytokines differ appreciably in several aspects. While IL1α is predominantly a cytokotic or membrane-bound protein constitutively expressed in epithelial, endothelial, and immune cells, IL1β...
is a secretory protein chiefly produced by immune cells only in response to inflammatory cues (7). In PDA, previous studies have predominantly categorized IL1β signaling into tumor cell production of IL1β (48) and stromal production of IL1β (15, 16, 18). In fact, IL1β protein is reportedly undetectable in PDA cell lines and organoids in vitro (33, 49). Consistent with these reports, we too did not detect IL1β production by KrasG12D/PDEC and KPC cells cultured ex vivo. We did, however, observe a robust in vivo production of the IL1β protein in the tumor cell compartment of human and murine PDA. This suggests the existence within the pancreatic tumor microenvironment of regulatory cues that can induce the activation of the toll-like receptor signaling pathway in tumor cells to drive IL1β expression and posttranslational processing. This conclusion is supported by our finding that the TLR4/NLRP3 inflammasome signaling axis is active in pancreatic tumor cells and is required for the production of IL1β by these cells.

The TLR4 receptor is a specific sensor of exogenous microbial ligands such as lipopolysaccharides (LPS) as well as endogenous ligands termed damage-associated molecular patterns (DAMP), derived from host tissue or cells (46). Significantly, the pancreatic microenvironment has been shown to be rich in such endogenous TLR4 ligands including HMGB-1 and S100A9 that can activate TLR4 signaling in tumor cells (17). Furthermore, the recently described PDA-associated microbiome has been shown to be rich in microbial ligands capable of activating the toll-like receptor pathway (23). Our finding that bacterial dysbiosis leads to induction of tumor cell–derived IL1β production indicates that the pancreatic microbiome plays a significant role in inducing IL1β production in transformed cells, likely through the TLR pathway. Moreover, it raises the possibility that the prooncogenic role of the microbiome in pancreatic cancer (23) could be, in part, mediated by the activation of TLR4-mediated IL1β production in the tumor cell compartment.

We have established that the posttranslational mechanisms that drive IL1β processing and maturation in the tumor cells require the NLRP3 inflammasome. The precise nature of the TLR4-induced signals that promote the assembly and activation of the NLRP3 inflammasome in pancreatic tumor cells remains to be determined. In monocytes, TLR4 ligation can induce ATP release, which, in turn, triggers NLRP3 inflammasome assembly via the ATP-gated ionotropic receptor P2X7 (P2RX7) (40). Interestingly, P2RX7 is highly expressed in pancreatic cancer cells (50), suggesting that a cooption by the tumor cells of this immune cell–specific signaling axis might be responsible for the NLRP3 inflammasome activation.

As indicated by our loss-of-function and rescue experiments, the secretion by pancreatic tumor cells of IL1β instigates sweeping changes in the fibroinflammatory pancreatic milieu, in part, by modulating PSC function. PSCs have been implicated in the regulation of a plethora of protumorigenic processes including tumor cell growth and metabolic adaptation and metastasis (31, 32). Recently, Öhnlund and colleagues have described the presence of two distinct intratumoral CAFs subpopulations in PDA: myofibroblastic cancer-associated fibroblasts (myCAF) with elevated αSMA expression and inflammatory cancer–associated fibroblasts CAF (iCAF) expressing an array of cytokines and chemokines (51). Our data indicate a role for IL1β in regulating the secretory phenotype of inflammatory CAFs. Specifically, we demonstrate the dependence of stromal CAFs on tumor-derived IL1β for the production of cytokines and chemokines with documented roles in subverting anti-tumor immunity. These include the chemokines CCL2 and CCL5 that regulate chemotaxis of monocytes, and, in the context of pancreatic cancer, have been found to regulate macrophage infiltration and M2 polarization (37, 38), as well as the chemokine CXCL12, which is known to inhibit intratumoral accumulation of CD8+ T cells (39). In accordance with this IL1β–dependent secretory profile, we found that upregulation of M2-TAMs and restriction of CD8+ Tc cell tumor infiltration is dependent on stromal PSCs. Overall, our study delineates epistatic interactions between tumor cell–derived IL1β and PSCs that are critical for the establishment of immune tolerance in pancreatic cancer.

The low immunogenicity of pancreatic cancer due to poor tumor infiltration of CD8+ Tc cells is considered a major factor responsible for the failure of checkpoint immunotherapy in PDA (3). As demonstrated by our studies, neutralizing IL1β promotes intratumoral CD8+ Tc infiltration and function and sensitizes PDA to checkpoint immunotherapy. Hence, therapeutic strategies that target IL1β may increase the efficacy of immune checkpoint inhibitors in pancreatic cancer. It is noteworthy that in a recent analysis of pancreatic cystic neoplasms (PCN) in patients, intracystic bacterial load as well as increased IL1β protein levels were detected in cystic precursors to pancreatic cancer, which are considered a major factor responsible for the establishment of immune tolerance in pancreatic cancer.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**Authors' Contributions**

Conception and design: S. Das, D. Bar-Sagi

Development of methodology: S. Das, D. Bar-Sagi

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): S. Das, D. Bar-Sagi, S. Vogt

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): S. Das, D. Bar-Sagi, E.A. Vucic

Writing, review, and/or revision of the manuscript: S. Das, E.A. Vucic, S. Vogt, D. Bar-Sagi

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): S. Das

Study supervision: D. Bar-Sagi

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