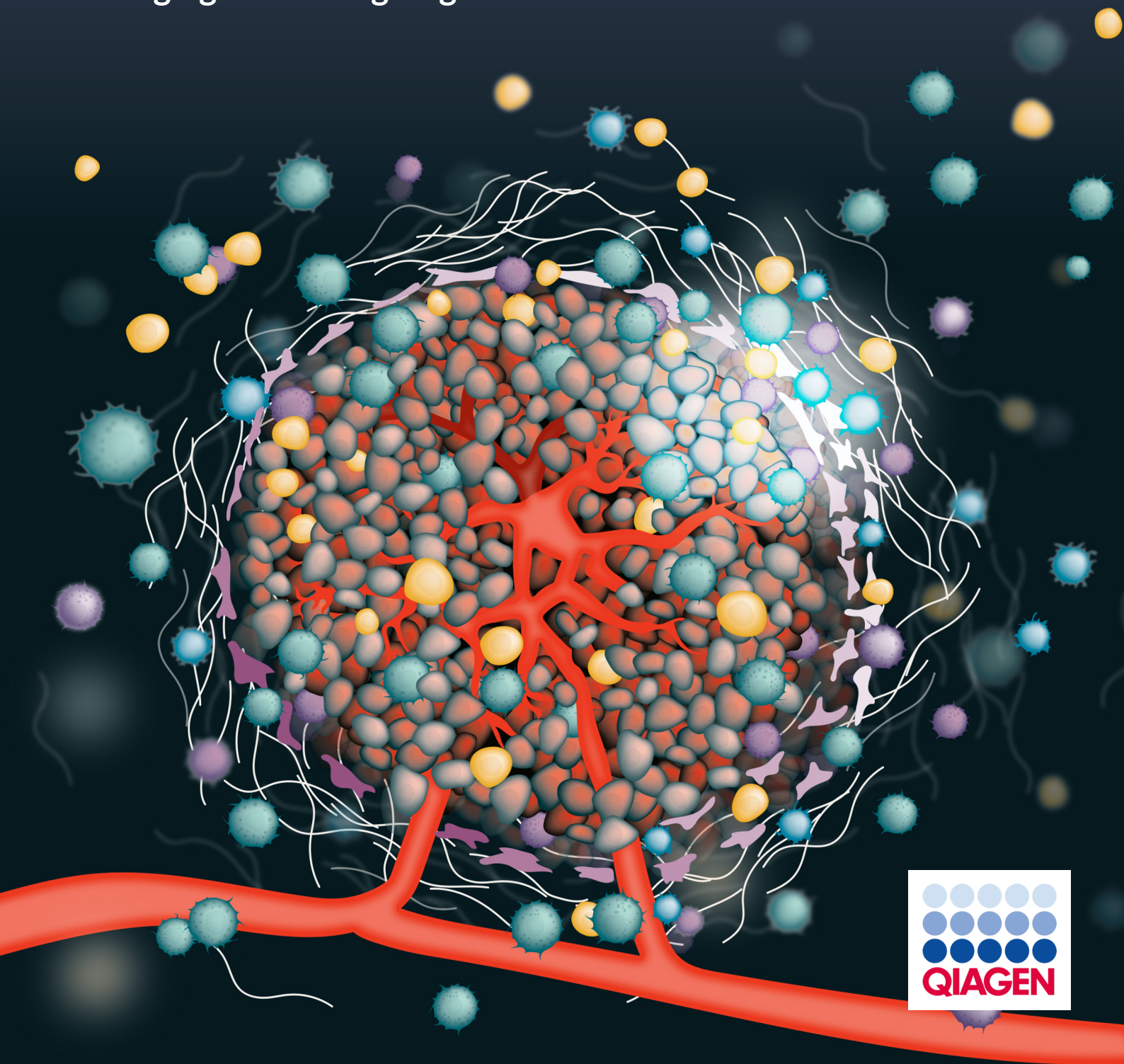
October 2022 Volume 3, Issue 9

IMMUNO-ONCOLOGY INSIGHTS

SPOTLIGHT ON:

Leveraging the cutting-edge TME toolkit



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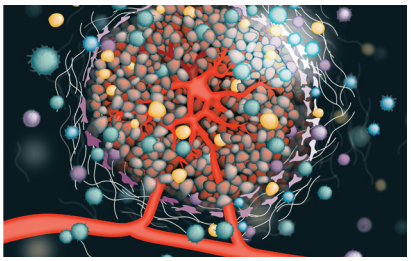


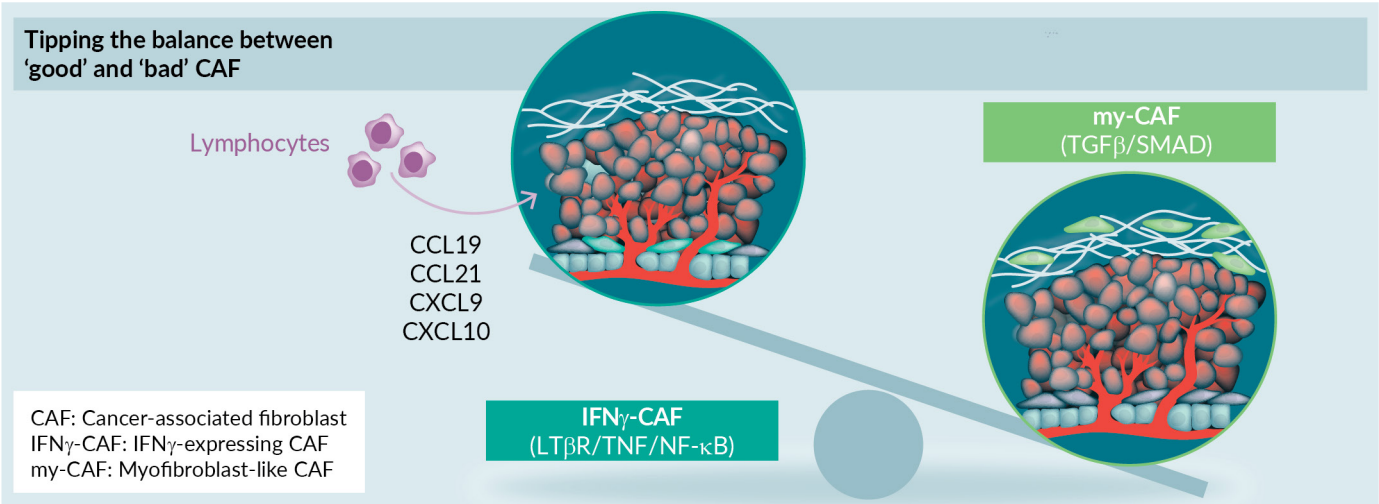
Leveraging the cutting-edge TME toolkit

REVIEW

Emerging characterization of the tumor-restraining subset of CAFs

Jamie Bates

Resistance to immunotherapy is driven by tumor intrinsic as well as tumor extrinsic factors, such as an immunosuppressive tumor microenvironment. Comparisons of bulk RNAseq signatures obtained from patient biopsies of immunotherapy responders versus non-responders suggests that TGFβ-driven activated myofibroblasts, called cancer associated fibroblasts (CAFs), contribute to immunotherapy resistance. However, attempts to deplete CAFs have inadvertently worsened survival and increased metastasis preclinically. The advent of single cell RNAseq has revealed considerable heterogeneity among fibroblasts found within and around the tumor that may allow selective targeting of pathogenic CAFs and raise the possibility of promoting a tumor-restraining CAF. This review will focus on the potential contributions of cancer associated fibroblasts to the immunosuppressive tumor microenvironment, the dichotomous tumor-promoting and tumor-restraining capabilities of CAFs, and the emerging tools allowing interrogation of this biology.



Immuno-Oncology Insights 2022; 3(9), 423–440

DOI: 10.18609/ioi.2022.046

INTRODUCTION

CAFs & resistance to immunotherapy

Immune checkpoint blockade (ICB) has revolutionized oncology, affording durable responses in a subset of patients. However, only roughly 30% of patients respond, making evaluation of resistance mechanisms to ICB a priority. A number of mechanisms of resistance to checkpoint blockade exist such as reduced tumor mutational burden and lack of genomic instability [1–3], lack of immune infiltration and/or prior T cell activation against the tumor [4], or accumulation of immunosuppressive myeloid cells [5], reviewed extensively in [6]. Recently, bulk RNAseq analyses have revealed that signatures associated with cancer associated fibroblasts (CAFs) with pronounced TGF-β-driven expression of extracellular matrix components point to another potential cause of lack of response to ICB [7–9]. Indeed, FAP expression, a marker highly expressed on CAFs, has been shown to predict lack of response to immunotherapy in NSCLC [10].

Overview of CAF development & function

Fibroblasts are thought to derive from mesenchymal cells that differentiate into tissue-specific fibroblasts by responding to tissue-specific cues. They perform multiple homeostatic functions including providing tissue architecture through production of extracellular matrix (ECM), promoting wound healing by becoming contractile through upregulation of α-smooth muscle actin (α-SMA), promoting angiogenesis by producing vascular endothelial growth factor A (VEGFA), as well as generating secondary lymphoid organs by recruiting immune cells along collagen networks when differentiated into fibroblastic reticular cells, (FRCs) [11,12]. Within the tumor microenvironment, they derive cues from the tumor cells such as TGF-β, as well as hypoxia and low pH, which drives them to produce increased ECM and collagen crosslinking enzymes, which in turn stiffens the microenvironment, providing activating signals through tension sensing mechanisms which can then increase the ability to retrieve more TGF-β via integrin-mediated TGF-β release [13–15]. This "activated" state is the hallmark of pathogenic CAFs. The advent of single cell RNAseq (scRNAseq) analysis has led to the discovery that CAFs exist as a heterogeneous pool of cells, potentially from different origins and performing varied functions within the TME [16]. While there are no individual markers that can label all fibroblasts, or even all CAFs, the defining feature of the pathological CAF is increased signaling through TGF-β which drives the SMAD transcriptional program, resulting in elevated secretion of extracellular matrix. Attempts to reduce the pathogenic CAF through TGF-β inhibition, CAF depletion, promoting a quiescent CAF phenotype and matrix reduction have yielded no clinical success to date, though these approaches continue to be explored [17]. Recent scRNAseq studies have highlighted the previously underappreciated heterogeneity of CAFs, which could be used to disentangle the pro-tumoral functions from the tumor-restraining capacity for some subsets of CAF. Future strategies may benefit from selectively targeting the pathogenic CAF, while sparing potentially tumor-restraining CAFs.

PAST THERAPEUTIC ATTEMPTS TO TARGET CAFs

Inhibition of TGF-β

The association between TGF-β signatures and resistance to ICB has prompted investigation into the combination of TGF-β inhibition and

checkpoint blockade [18]. TGF-β inhibition is known to reduce the activation of CAFs, but also to be broadly immunosuppressive in multiple immune cell types [19–23]. TGF-β inhibition boosted response to immunotherapy preclinically [24], however known toxicities of TGF-β inhibitors have led to efforts to improve therapeutic index by targeting TGF-β inhibition to the tumor [25], targeting directly to immune cells via TGF-β presentation molecules [26], inhibiting only specific isoforms (namely TGF-β1), [27], or blocking selective integrin-release of TGF-β ligands [28,29]. TGFBR2 inhibition with galunisertib was tested in many clinical trials but significant efficacy has not yet been reported, possibly a result of dosing limitations due to preclinical cardiac toxicology findings [30]. Fresolimumab (aka GC1008), an antibody blocking TGF-β ligands from binding to the TGF-β receptor, did not cause cardiotoxicity preclinically and no DLTs were observed clinically [31]. However, this antibody binds weakly to the TGF-β ligands compared to the high affinity the ligands have for the receptor, and no significant efficacy has been reported [32]. Later, more potent antibodies against TGF-β ligands demonstrated similar preclinical cardiac findings in mice and monkeys as the TGFBR2 inhibitors, strengthening the idea that full suppression of TGF-β systemically is associated with cardiac toxicity [33]. Several trials testing bintrafusp alfa, which targets a recombinant TGFBR2 that sequesters TGF-β ligands (referred to as a TGF-β-ligand TRAP) to the tumor via PD-L1, while demonstrating similar safety profiles to fresolimomab, have been discontinued due to lack of efficacy. While TGF-β ligands were completely reduced in the blood at doses used in the trials [34], it is possible that lack of enrichment for the TRAP in the tumor, or lack of proximity of PD-L1 to TGFBR on the cell surface could contribute to the lack of observed efficacy. Additionally, while inhibition of TGF-β signaling should suppress matrix deposition by CAFs and relieve immunosuppression of immune cells, recent data revealed an unexpected role for TGF-β signaling within the tumor. TGF-β has long been known to restrict tumor growth intrinsically; indeed, tumor-intrinsic TGFBR loss can drive neoplastic transformation [35]. Recently, tumor-intrinsic lack of TGFBR2 was shown to counterintuitively activate tumor-adjacent CAFs, stimulating increased extracellular matrix deposition and inducing T cell exclusion in a $KRAS^{G12D}$/$TP53^{-/-}$ (KP) GEMM model of lung cancer [36]. This suggests that targeting of the TGF-β-TRAP to the tumor could be detrimental, and that rather targeting this inhibition selectively to stromal or immune cells might be preferred. Understanding whether targeting of TGF-β inhibition to CAFs or to immune cells more effectively reduces immunosuppression could help guide future TGF-β-inhibitor targeting strategies.

CAF depletion

Because the TGF-β signature in ICB-resistant patient populations is thought to be CAF-derived, CAF depleting approaches have been explored to potentiate ICB. However, prior work depleting CAFs with the goal of reducing the extracellular matrix and increasing drug penetrance to the tumor raises concern about this approach. The CAF-derived fibrotic matrix was hypothesized to form a physical barrier blocking perfusion of chemotherapeutics, thereby reducing their activity in indications such as pancreatic ductal adenocarcinoma (PDAC), where matrix was abundant and few therapies effective [37]. The majority of fibroblasts in the human tumor microenvironment are known to express α-SMA and platelet-derived growth factor receptor β (PDGFRβ) [38]. Mice expressing the fibroblast-expressed promoters of α-SMA and PDGFRβ driving the viral thymidine kinase (tk) gene allowed for depletion of total CAFs by 80% and 60%, respectively, upon treatment with ganciclovir [39,40]. In spite of successful reduction in CAF content and extracellular matrix, deletion of α-SMA or PDGFRβ-expressing CAFs in combination with gemcitabine led to reduced survival and increased metastasis. This could have

been due to reduced vasculature exemplified by reduced NG2- or CD31- positivity, possibly a result of a deleterious effect on perivascular fibroblasts, promoting the ability of cancer cells to leave the tumor and enter the bloodstream. These authors concluded that the CAF-derived dense extracellular matrix in PDAC models played a role in controlling tumor metastasis. However, interestingly, combination of α-SMA$^+$ CAF-depletion with anti-CTLA-4 treatment, which causes Treg depletion and CD8 T cell activation, resulted in increased survival and tumor growth inhibition [39]. These results could be viewed as an interesting foreshadowing of our current understanding of the potential for CAF-induced immunosuppression and ICB resistance.

Interestingly, deletion of Collagen 1 (Col1) in α-SMA$^+$ fibroblasts accelerated disease in a KP pancreatic GEMM model, resulting in more undifferentiated and invasive PDAC, without impacting blood vessel density, pericyte coverage, or vascular integrity. α-SMA$^+$ cells were shown to be the major producer of collagen 1 in this model, and collagen was significantly reduced in α-SMA-driven Col1-deleted mice. This loss of Col1 was accompanied by an influx of suppressive myeloid cells, but also a lack of B and activated T cell infiltrate, and reduced type I IFN signaling as measured by RNAseq, suggesting that α-SMA$^+$ CAF-derived collagen could promote recruitment and activation of T cells. Further, lower Col1 correlated with fewer T cells in human PDAC [39]. Consistent with this observation, α-SMA expression was associated with increased survival in human PDAC samples [40]. These data point to the tumor-restraining potential of collagen produced by α-SMA$^+$ CAFs in some contexts.

In contrast, FAP expression was associated with worse survival in human PDAC [41]. Depletion of FAP$^+$ CAFs improved vascularity, reduced extracellular matrix, and improved tumor growth inhibition while reducing metastasis [41,42]. The impact of FAP$^+$ CAF depletion via diptheria toxin on tumor growth in the LL2/OVA model was dependent on the adaptive immune system, as the benefit was not observed in RAG-deficient animals, and tumor shrinkage depended upon expression of IFNγ and TNFα, suggesting that FAP$^+$ CAFs suppressed immunological responses to these cytokines [43]. Conditional deletion of FAP$^+$ cells using a FAP-driven diptheria toxin (DTx) sensitized a KPC-derived pancreatic tumor model to anti-PD-L1 and anti-CTLA-4 immunotherapies [44]. Further, FAP$^+$ cell-depletion via vaccination against FAP, in a B16F10 syngeneic mouse model, was accompanied by skewing of the myeloid compartment towards a more inflammatory phenotype, and augmented antigen-specific T cell responses [42]. FAP$^+$ cell-depletion also extended survival in the higher-bar, KP pancreatic GEMM model [41], and immunosuppression by FAP$^+$ CAFs has since been demonstrated by many groups [45]. However clinically, as a monotherapy, targeting of FAP$^+$ cells with anti-FAP antibodies did not meet the primary endpoint of improved ORR in colorectal cancer (CRC) [46], although results have yet to be described for subsequent, fourth generation FAP-directed CAR-T trials, or immunotherapy combinations incorporating FAP$^+$ cell-targeting. Also, there is an outlying question about the safety of highly efficient FAP$^+$ cell-targeting, due to known expression of FAP in bone marrow and skeletal muscle fibroblasts, which could limit the therapeutic index of such approaches [47].

Stroma/matrix modulation

Several attempts to target CAF activation and modulate stroma have resulted in failed clinical trials. One approach included blocking epithelial – fibroblast cross talk by inhibiting hedgehog signaling. Hedgehog inhibition resulted in increased survival and reduced metastasis, accompanied by improved vascularity of the tumors, when combined with chemotherapy in short term preclinical models [37]. However, hedgehog signaling was subsequently shown to drive stomal-dependent inhibition of tumor angiogenesis, where reduced α-SMA-positive cells were

DOI: 10.18609/ioi.2022.046

concomitant with less differentiated, more aggressive tumors in autochthonous models. Stromal cells were the predominant cell type responsive to hedgehog signaling, as demonstrated with a Gli reporter, although 43% of cells with active Gli were α-SMA-negative, suggesting broad activity in stromal cells as well as myeloid cells. How the loss of hedgehog signaling in the stroma led to this phenotype is not well understood, however this suggests that hedgehog signaling may drive an indirect tumor-restraining phenotype of the stroma via suppressing angiogenesis in longer term models [48], which may in part explain the worsening of disease observed clinically with hedgehog inhibitors in combination with chemotherapy and immunotherapy [49,50].

A recombinant PEGylated human hyaluronidase, PEGPH20, also improved efficacy and vascularity in combination with chemotherapy preclinically [51,52]. The efficacy was modest and resistance occurred in the animal models, which may explain the improved overall response rate and progression-free survival, but lack of improvement in overall survival in the Phase 3 clinical trial treating metastatic pancreatic patients with PEGPH20 in combination with nab-paclitaxel and gemcitabine [13].

LOXL2 inhibitors modulate matrix composition by blocking collagen crosslinking. LOXL2 inhibition with ellagic acid, a natural product inhibitor of LOXL2, as a single agent effectively depleted collagen crosslinking and reduced extracellular matrix as measured by Mason's Trichrome staining, and significantly reduced metastasis in a KP pancreatic GEMM-derived subcutaneous model [53]. This was concomitant with increased effector ($CD8^+CD3^+$) T cells and decreased exhausted ($PD1^+TIM3^+CD8^+$) T cells in the tumor. When combined with an anti-PD-L1 antibody, primary tumor volume and the number of metastatic nodules were significantly reduced, while effector T cells in the tumor were significantly increased, relative to anti-PD-L1 alone. An antibody targeting LOXL2 failed clinically in several trials, even in combination with immune checkpoint blockade [54,55]. However, no data were provided demonstrating the extent or duration of target coverage in the clinic, so whether the failure was due to a lack of effect of LOXL2 inhibition, or to a lack of target inhibition by the drug, remains unknown.

Andecaliximab was developed to inhibit MMP9, a metalloprotease thought to cause collagen degradation that could increase metastasis. Full inhibition of MMP9 was demonstrated by clearance of the protease from serum [56], however andecaliximab in combination with mFOLFOX6 failed to improve overall survival in a Phase III study in gastric cancer [57].

These data together suggest that fibroblasts may have opposing roles within the TME that differentially impact tumor growth, and that matrix modulation as opposed to overt matrix reduction might afford the best tumor-restraint. To this end, several groups have contributed to our evolving understanding of the relationship between the extracellular matrix components and the immune system, including the impact of matrikines on dendritic cell activation [58], the impact of collagen alignment on T cell exclusion [59] and direct immune cell-collagen interactions on T cell exhaustion and myeloid polarization [60], that could lead to future therapeutic approaches.

These examples suggest that a refined approach, targeting select subsets of fibroblasts, or particular aspects of CAF or ECM function, may be necessary to effectively reduce immunosuppression induced by these cellular and physical components of the TME. Understanding the heterogeneity within the CAF population will be necessary to dissect the multiple functions of CAFs.

RECENT FINDINGS ON CAF HETEROGENEITY & THERAPEUTIC IMPLICATIONS

Human scRNAseq datasets

The pathogenic phenotype of CAFs was thought to largely stem from their ability to

produce large quantities of matrix. Several approaches to reduce matrix production in CAFs in the setting of tumor fibrosis are being tested clinically, which is reviewed extensively elsewhere [61]. While robust matrix production does appear to be a hallmark of pathogenic CAFs, single cell analyses are deepening our understanding of immunosuppressive vs immune-activating CAF cell subsets within the tumor microenvironment, which could provide more nuance with which to strategically reduce their immunosuppressive capabilities (reviewed in [16]).

Single cell analysis including sufficient numbers of human fibroblast subsets have revealed dramatic heterogeneity amongst CAFs in multiple indications including HNSCC [62], NSCLC [63], PDAC [60,64], and Breast Cancer [65,66]. Some have tried to correlate enrichment of these CAF subsets with survival outcomes for patients. In one such example, single cell analysis of tumor samples from 16 pancreatic cancer patients identified three subsets of CAFs. By assessing enrichment for the signatures from each subset of CAFs in bulk RNAseq data obtained from patients for whom overall survival outcome was known, they were able to show that enrichment for one CAF subset was associated with increased probability of survival, while enrichment for another subset was associated with decreased probability of survival [64]. More relevant for this purpose, although less publicly available, are datasets for which there exists not only matched survival, but matched response to immunotherapy. In one such example, Keiffer *et al.* derived signatures from subsets of CAFs from breast cancer patient biopsies and applied these to bulk RNAseq data derived from separate patient cohorts from melanoma and lung immunotherapy trials [65]. They showed that three of six subsets of fibroblasts were significantly associated with lack of response (TGF-β-, ecm- and wound-myCAFs), while the other three fibroblast subsets (IL-, detox-, and IFNγ- iCAFs) lacked such prognostic associations. Among the three subsets lacking enrichment in non-responders was a subset expressing high levels of IFNγ-driven genes, which suggests an association with an immune-permissive tumor microenvironment. Whether this CAF subset correlates with immune activation is hard to disentangle from bulk RNAseq since many of the genes in the scRNAseq signature overlap with IFNγ-inducible genes from immune cells.

Even more relevant than using scRNA-seq-generated signatures to query bulk RNA-seq with associated survival data, is the emergence of single cell analysis performed on the same patients with associated ICB response data. In one such study, response to atezolizumab alone, or in combination with paclitaxel, was associated with the presence of T cells with high CXCL13 expression [67]. However, this exemplifies many datasets for which fibroblasts are not included in the results, which speaks to the difficulty of extracting sufficient numbers of fibroblasts from tumor biopsies, leading to low retrieval and exclusion from downstream analyses. Some datasets do happen to contain CAFs isolated by their standard protocols, while others have addressed this issue by developing tailored disaggregation protocols that enrich for stromal cells [68].

Because response to immunotherapy data is not always available, a surrogate for response to immunotherapy can be obtained by quantifying T cell clonality, which represents an early response to successful anti-PD1 treatment. One study used such an approach to compare 29 patient samples, from which they identified nine patients as being 'expanders', defined by the number of shared TCR sequences relative to total number of T cells, and 20 patients being "non-expanders", not meeting the threshold of TCR clonality [66]. Comparison of single cell analysis of cell types evaluated pre-treatment from expanders vs non-expanders revealed that the only two cell types that were predictive of response were T cells (enriched in expanders) and fibroblasts (enriched in non-expanders). Interestingly there was no difference between the groups in tumor mutation burden or genomic instability. The same analysis performed on samples obtained from the same patients while

 DOI: 10.18609/ioi.2022.046

on immune checkpoint blockade treatment, maintained these significant differences in T cells and fibroblasts, and additionally demonstrated differences in plasmacytoid (pDCs), which were significantly enriched in expanders, and cancer cells, which were significantly reduced in expanders, the latter strengthening the use of TCR clonality to predict response to ICB. Such analysis further suggests that CAFs, or at least an abundant subset of CAFs, play a role in resistance to ICB. Unfortunately, subset analysis of fibroblasts was not performed in this study.

In order to understand potential crosstalk between CAF and immune cell subsets, some have used algorithms designed to assess expression of receptor-ligand pairs shared by cell subsets from the scRNAseq analysis. Wu *et al* [69]. used this approach to understand the differences in signaling between fibroblast subsets with immune cells. They identified 4 subsets of CAFs; myCAFs, iCAFs, and two sets of perivascular cells, one immature and one differentiated. Their receptor ligand analysis predicted that myCAFs were signaling to myeloid cells via TGF-β1, while iCAFs did so via TGF-β2. It also suggested that iCAFs could recruit B cells via CXCL13, while perivascular cell subsets could recruit CD4 T cells via CCL21. Interestingly, quantification of total ligand production revealed that CAFs are the dominant producer of ligands over all other cell types evaluated, suggesting a prominent role for CAFs in influencing signaling within the TME.

The natural extension of such receptor-ligand paired analysis is confirmation that these cell types are interacting spatially. Early attempts to define CAF subsets histologically relied on known fibroblast markers such as dual expression of FAP and α-SMA to label myCAFs in pancreatic cancer samples, which were found to encircle the tumor [70]. Wu *et al.* used a combination of multiplex IF and morphology to distinguish the perivascular, my- and iCAF subsets in breast cancer samples, described above. They also found that α-SMA^{high} CAFs were peri-tumoral, while CD34^{+} iCAFs were more distal to the tumor and in close proximity to immune cells [69]. Most recently, additional markers were used to distinguish more subsets of CAFs defined by scRNAseq obtained using aggregation protocols enriching for stroma. They observed MYH11^{+} α-SMA^{+}, TGFβ-expressing myCAFs in close proximity to the tumor, and anti-correlated with CD3 positivity [63]. They also described an ADHB1^{+}CCL19^{+} CAF that co-localized to T cell zones of tertiary lymphoid structures (TLS). These studies further the notion that different subsets of CAFs can have opposing interactions with immune cells.

Mouse scRNAseq datasets

In mouse pancreatic cancer models, two types of CAFs have been reproducibly identified: the iCAF and the myCAF [9,70–73], where myCAFs are characterized by high levels of TGF-β, and are generally high expressors of extracellular matrix components, while iCAFs are thought to exhibit immunosuppressive behavior via secretion of cytokines such as IL-6. Transcriptional analysis of iCAFs and myCAFs determined iCAFs to be driven primarily by IL-1β and TNF-α signaling via NF-κB-driven transcriptional programs and characterized by expression of Ly6c and Dpp4, while myCAFs were driven by TGF-β signaling via SMAD-driven transcriptional programs, characterized by expression of Eng [9]. Elyada *et al.* identified a third type of CAF called "apCAF", so named for the defining expression of CD74, the MHCII invariant chain involved in antigen presentation [71]. However, Dominguez *et al.* found CD74 and HLA-DR to be expressed globally in human CAFs. In mouse, the apCAF population aligned best with the mesothelial lineage from normal pancreas described in Dominguez *et al* [9]. Grauel *et al.* identified a fourth CAF subset in mice they called the "IFNγ- licensed" CAF which was induced specifically in response to TGF-β inhibition and potentiated when TGF-β inhibition was combined with anti-PD-1 treatment in the 4T1 mouse model [74]. scRNAseq prior

to treatment confirmed the presence of the previously described iCAF and myCAF, but scRNAseq after TGF-β inhibition revealed a loss of the myCAF subset of CAFs and the emergence of a unique fibroblast subset characterized by high IFNγ signature expression, expression of antigen presentation molecules and the T cell chemo-attractants CXCL9, 10, and 11 [74]. These cells, like myCAFs and iCAFs, expressed PDPN, FAP, and Thy1, but also highly expressed Nt5e, the protein encoded by CD73. TGF-β inhibition in 4T1 and MC38 implanted subcutaneously induced similar IFNγ-licensed CAF populations, while also increasing T cell infiltration, slowing tumor growth, and potentiating response to anti-PD1. These data suggest that skewing CAFs towards the IFNγ-licensed CAF is either cause or consequence of successful induction of an immunogenic response.

EXPERIMENTAL APPROACHES TO ELUCIDATE THE FUNCTION OF CAF SUBSETS

Evaluation of CAF heterogeneity *in vivo*

The effect of CAFs on tumor growth and response to immunotherapy *in vivo* has been evaluated by co-injection of CAFs with tumor cells. Co-injection of TGF-β-activated fibroblasts significantly increases the α-SMA content of TC1, MC38, and 4T1 models, results in increased growth of tumors, and resistance to vaccine and anti-PD1 therapies [75]. One caveat of this approach to studying the impact of CAFs on response to immunotherapy is the known ability of activated fibroblasts to directly increase the growth kinetics of tumor cells, which could confound evaluation of response to immunotherapy. However, activated fibroblast co-injection also results in increased macrophage infiltration into the tumor, and decreased T cell infiltration into the center of the tumor, suggesting fibroblast crosstalk with immune cells. Knocking out NOX4, a TGF-β-inducible gene associated with intracellular ROS generation, reduces fibroblast α-SMA expression and contractility *in vitro* [71]. Co-injection of activated fibroblasts transduced with shRNA against NOX4 resulted in decreased tumor growth and reversed the T cell exclusion phenotype relative to the same tumor model co-injected with fibroblasts transduced with control shRNA constructs [75,76]. Co-injection with NOX4 knocked down-fibroblasts, or treatment with a NOX4 inhibitor, also sensitized the tumors to tumor-antigen vaccination and anti-PD1 treatment, respectively [75]. Another group found that co-injection of CAFs, but not normal mouse mammary gland fibroblasts promoted tumor growth and T cell exclusion [77]. In this case, co-injection of CAFs harboring CRISPR-mediated deletion of the gene Endo1, aka Mrc2, a TGF-β-inducible gene with a role in collagen endocytosis, also reversed WT CAF-induced T cell exclusion, and modestly sensitized to anti-CTLA-4 and anti-PD-L1 treatment. More recently, DTR-driven deletion of LRRC15^{+} CAFs resulted in loss of TGF-β-driven CAFs, and an emergence of a "universal fibroblast" CAF driven by NF-κB, TNF, and JAK/STAT pathways which resulted in slowed tumor growth and sensitization to anti-PD-L1 treatment in a KPP pancreatic GEMM-derived model [78]. These data suggest that skewing of the CAFs away from a TGF-β-driven myCAF state can positively influence response to immunotherapy.

Causal evidence that such CAFs exist that possess anti-tumorigenic function comes from a recent study where two subsets of CAFs, defined by protein expression of CD105, confer opposing phenotypes when subcutaneously co-injected with mouse KPC-derived tumor cells into immune-competent syngeneic hosts; CD105^{neg} CAFs inhibited tumor growth, while CD105^{pos} CAFs did not [79]. The observed inhibition was dependent on the adaptive immune system, as the same cells injected into NSG, or RAG null mice lacked any tumor inhibition, and tumor inhibition was greatly blunted when the same cells were injected into Batf3$^{-/-}$ mice, which lack cDC1 cells. Importantly,

DOI: 10.18609/ioi.2022.046

the CD105^{neg} CAF phenotype was dominant over the CD105^{pos} CAF when co-injected together, suggesting that this is an active induction of immune activation, rather than a removal of otherwise inhibitory cells. Another group identified dual MEK and STAT3 inhibition as potent inducers of CAF remodeling, and showed a reduction in both myCAF and iCAF subsets, with replacement by a mesenchymal-derived progenitor cell type concomitant to sensitization to anti-PD1 anti-CTLA-4 dual therapy [80]. The change in CAFs correlated with reduced α-SMA and PSR staining, increased CD31 staining, myeloid skewing towards an M1 phenotype, and a dramatic increase in T cell infiltrate. They confirmed the CAF-intrinsic effect on the immune cell infiltrate by recapitulating the effect on the TME by co-injecting KP cells and CAFs orthotopically, where the CAF harboring dual knock out of MEK1 and STAT3 increased immune infiltrate relative to co-injection of WT CAFs [80].

Interestingly, parallels exist between the pro-tumorigenic, CD105^{pos} fibroblasts and anti-tumorigenic CD105^{neg} fibroblasts with the myCAF identified in the Dominguez *et al.* and Elyada *et al.* reports, and the IFNγ-licensed CAF described by Grauel *et al.*, respectively. CD105 is the protein encoded by Eng (the gene that defined the TGF-β-driven CAF identified in Dominguez *et al.*), suggesting a similar TGF-β-driven myCAF phenotype. On the other hand, the CD105^{neg} cells express high levels of CD73 (aka. Nt5e, the gene defining the IFNγ-licensed CAFs in Grauel *et al.*), and mesothelial cell markers, some of which overlapped with the mesothelial signature found in both Dominguez *et al.* including Msln, Krt8 and Upk1b or Upk3b in Hutton *et al.* [9,71,74]. CD105^{neg} CAFs also expressed higher levels of MHCII genes suggesting similarities to the "apCAF" from Elyada *et al.* Ingenuity pathway analysis identified upstream regulators associated with the CD105^{pos} or CD105^{neg} fibroblasts, respectively; the CD105^{pos} pro-tumorigenic CAF signatures were predicted to be driven by TGF-β, while the CD105^{neg}, anti-tumorigenic fibroblast signatures were predicted to be driven by LTBR, TNF, and STING pathways. RNAseq differential gene expression analysis of these tumors upon co-injection of these CAFs suggested increased infiltration of T and dendritic cells, suggesting chemoattractive crosstalk between these CAFs and the immune infiltrate.

Importantly, many of the genes used to define fibroblasts were expressed to similar extents in the CD105^{pos} and CD105^{neg} fibroblasts, including α-SMA, PDGFRβ, LRRC15; even iCAF and myCAF signatures appear to co-exist in this subset of fibroblasts. While FAP is more highly expressed in CD105^{pos} fibroblasts, it is still expressed in CD105^{neg} fibroblasts. These data present the possibility that depleting the pathogenic, tumor-promoting CAFs by approaches using these canonical CAF-expressing genes such as α-SMA, PDGFRβ, LRRC15, and even FAP, could inadvertently deplete the anti-tumorigenic, T cell infiltrate-promoting, subset of CAFs. Perhaps these data provide a framework around which we can build strategies to skew, rather than deplete, the fibroblast niche.

Evaluation of CAF function *in vitro*

Attempts to directly measure crosstalk between CAFs and immune cells *in vitro* have shown dynamic plasticity of CAFs in culture, and most demonstrate immunosuppressive behavior of CAFs. Pancreatic stellate cells could be induced to form both iCAFs and myCAFs by co-culture with cancer cells *in vitro* [70], and conversely KPC tumor-derived iCAFs and myCAFs both promote tumor cell growth *in vitro* [9]. In culture, iCAFs could be robustly induced in a paracrine manner by co-culture in transwell with tumor cells [70]. Culturing in 2D converted iCAFs into myCAFs, which was preventable by culturing on a soft matrix demonstrating the plasticity of these CAF subsets and the impact of a stiff surface on fibroblast activation [70]. CD105^{neg} and CD105^{pos} cells

were shown to confer opposing phenotypes on tumor growth when co-injected with tumor cells into the flanks of mice [79]. Isolated CD105^{neg} and CD105^{pos} cells maintained differential protein expression of CD105 in culture but displayed some differences in response to stimuli. The tumor-restraining CD105^{neg} CAFs had higher NF-κB signaling in response to IL-1 α/ and β, and, while CD105^{pos} cells responded to IL-6 with greater STAT3 signaling and displayed more robust changes in gene expression in response to TGF-β. So, although they remain plastic, CAF subsets may preferentially respond to differing stimuli [79].

Reciprocal interactions between fibroblasts and myeloid cells, particularly macrophage, have long been appreciated [81]. Fibroblasts are known to secrete CCL2, which attracts myeloid cells via CXCR2, CSF-1 which drives macrophage differentiation via CSF1R, as well as TGF-β which can induce immunosuppression in myeloid cells. Reciprocally, myeloid cells can provide PDGFs and TGF-β, stimulating myCAF differentiation in CAFs [81]. Co-culturing monocytes with CAFs has been shown to promote an M2 macrophage phenotype, exemplified by increased expression of typical M2 markers such as CD163 [82,83]. Others validated the immunosuppressive behavior of the macrophage, or monocyte-derived immunosuppressive myeloid-derived suppressor cells (MDSCs), derived from fibroblast co-culture *in vitro* by assessing their ability to inhibit T cell proliferation, activation, and cytotoxicity, which may be the most definitive way to assess and dissect the impact this crosstalk has on immune activation [83–85]. In another example, co-culture of FAP^{+}CD29^{+} CAF with monocyte-derived macrophage drove a lipid-associated macrophage (LAM) phenotype with increased TREM2 expression, the resulting cells of which conferred a suppressive effect on T cell proliferation [86]. Macrophage phagocytosis of CAF-generated matrix has been shown to induce an immunosuppressive phenotype [87]. Reciprocally, media derived from collagen-engulfing macrophage promoted the pro-fibrotic phenotype of CAF, suggesting a feedforward mechanism between myCAF and immunosuppressive macrophage. Receptor ligand pair-interactions predicted CXCL12-CXCR4 mediated recruitment of macrophage to CAF, which was validated *in vitro* and consistent with prior reports [83,87]. Inhibition of CXCR4 with AMD3100 was shown to increase activated immune cell infiltrate, presenting the possibility of combining with immune checkpoint blockade. However, a Phase 2 clinical trial investigating the efficacy and safety of the combination of AMD3100, a CXCR4 inhibitor, in combination with Pembrolizumab in head and neck squamous cell carcinoma, was withdrawn without publication of results (NCT04058145).

Costa *et al.* validated a causal relationship between the observed correlation between a CAF subset and Treg cells by flow cytometry by testing their interactions *in vitro* [88]. They isolated four CAF subsets, called CAF-S1-4 based on expression of six canonical CAF markers: CD29 (integrin β1), FAP, FSP1, α-SMA, PDGRFβ, and CAV1. They compared two subsets *in vitro* that differed most significantly in their expression of FAP, with CAF-S1 expressing high FAP, and CAF-S4 being FAP-negative. They showed that the FAP-high subset, CAF-S1, preferentially increased migration of PBMC-derived CD4^{+}CD25^{+} T cells in a transwell assay towards the CAFs in a CXCL12-dependent manner. The CAF-S1- CD4 T cell interactions lasted longer than 14 h, the persistence of which was dependent on OX40L, PD-L2, and the adhesion molecule, JAM2, expression in the CAF. The same cells could promote the differentiation of Treg cells from CD4^{+}CD25^{+} cells and promote their ability to functionally block CD4 effector T cell proliferation. Interestingly, this same CAF-S1 subset was subsequently shown to comprise six subsets of myCAFs by scRNAseq analysis, of which signatures of three (TGF-β-, ecm- and wound-CAFs) were correlated with lack of response to immunotherapy in a separate bulk RNAseq

DOI: 10.18609/ioi.2022.046

dataset, and three of which were not (IL-, detox-, and IFNγ- iCAFs) [65]. They were able to generate two flavors of CAF *in vitro* by a) culturing tumors on plastic and recovering spreading, outgrowing fibroblasts, which generated a pool of CAFs resembling a mix of the TGF-β- wound- and ecm-myCAFs, referred to as ecm-myCAFs or b) by flow sorting $FAP^{hi}CD29^{med}$ cells, which they called iCAFs, that resembled a mix of IL- and detox- CAFs based on their transcriptional profiles. Unfortunately, no CAF resembling the IFNγ CAF described by the scRNAseq data were recovered by either of these methods. When culturing these iCAF and ecm-myCAF cultures with T cells, they found that ecm-myCAFs had a higher propensity to produce $FOXP3^+$ CD4 Treg cells than iCAFs. Conversely, culturing Tregs with either iCAFs or myCAFs induced expression of myCAF genes, suggesting that Tregs and myCAFs provide reciprocal, feed-forward signaling that promotes each other's differentiation whereas iCAFs were less potent participants in this feedback [65]. In addition to these examples of indirect repression of T cell activation, others have demonstrated a direct anti-proliferative impact of CAFs on T cells [89,90].

In most of the above-mentioned co-culture systems, CAFs demonstrate immunosuppressive behavior. The ability to recruit $CD4^+CD25^+$ T cells is a notable exception; however, the same CAFs could also induce differentiation of these cells into $FOXP3^+$ Treg cells. The propensity for myCAFs to emerge from 2D culture is a well-established phenomenon [70], as the stiffness of tissue culture plates has long been understood to induce fibroblast activation toward a TGF-β-signaling, matrix secreting phenotype, while culturing on Matrigel allows preservation of a more quiescent fibroblast phenotype [91]. Therefore, culturing on softer substrates may be necessary to prevent other subsets of fibroblasts from acquiring a myCAF phenotype, and to evaluate any propensity for promoting immune infiltration and/or activation.

CONCLUSION

Comparisons across datasets

Whether the CAFs that were increased with the presence of immune infiltrate and correlated with increased sensitization to immunotherapy across the three mouse models possess similar signaling capabilities remains to be determined. Many groups compare scRNAseq datasets in order to validate identification of CAF subsets. Dominguez *et al.* showed concordance between their iCAF and myCAF subsets with those of Elyada *et al.* and showed that the "apCAF" identified in the latter clustered with what they defined as 'normal' fibroblasts found in healthy pancreas. Likewise, Datta *et al.* identified four CAF subsets of which three clustered with the previously identified iCAF, myCAF, and apCAF subsets. However, whether the potentially tumor-restraining IFNγ-CAFs of Grauel *et al.*, the $CD105^{neg}$ fibroblasts of Hutton *et al.*, and the mesenchymal progenitor CAFs identified in Datta *et al.* contain similar flavors of fibroblasts, or whether any of them are similar to the human IFNγ-CAF subset identified in Keiffer *et al.*, remains to be determined. But consistently, the increased abundance of the IFNγ-licensed CAFs in tumors described in Grauel *et al.*, the co-injected $CD105^{neg}$ CAFs described in Hutton *et al.*, and the increase in the mesenchymal-progenitor like CAFs described in Datta *et al.*, were all functionally similar in their increased immune cell infiltrate. The increased immune infiltrate could be due to the chemoattractive potential suggested by predicted CAF-T cell signaling implicated by receptor ligand analysis in Wu *et al.*, or demonstrated in the *in vitro* transwell assays showing migration of T cells towards CAFs, and durable interactions between CAFs and T cells in Costa *et al.* Table 1 summarizes the key findings from these scRNAseq datasets. In order to extract any therapeutic benefit from this biology, the potential for CAFs to promote T cell infiltration into the tumor would need to be disentangled from their

TABLE 1

Summary of key findings from scRNAseq datasets.

First author	Tissue/model	Significant findings
Kieffer *et al.* 2020 [65]	Human Breast	Identified 6 CAF subsets; three predict lack of ICB response (TGF-β-my-CAF, ecm-myCAF, wound-myCAF) and 3 do not: (IL-iCAF, detox-iCAF, and IFNγ-iCAF)
Lin *et al.* 2020 [64]	Human PDAC	Identified fibroblast subsets with differing prognoses; cluster 0 (myCAF), associated with poor prognosis, expressing POSTN and MMP11 and cluster 2 (normal CAF) associated with good prognosis, expressing RGS5, NOTCH3, CSRP2
Wu *et al.* 2020 [69]	Human Breast	Identified two CAF subsets: myCAF & iCAF, and two subsets of perivascular cells, either differentiated or not; receptor ligand interaction analysis identifie several fibroblast-immune cell-interactions
Grout *et al.* 2022 [63]	Human NSCLC	Identified two myCAF populations associated with T cell exclusion (FAP^{+}α-SMA^{+} and MYH11^{+}α-SMA^{+}) as well as an ADHB1^{+} population associated with early-stage tumors, a subset of which expresses CCL19 and resides in TLS
Elyada *et al.* 2019 [71]	Mouse pancreatic KPC (*Kras$^{+/LSL\text{-}G12D}$;Trp53$^{+/LSL\text{-}R172H}$;Pdx1-Cre*)	Identified myCAF, iCAF, and apCAF, expressing MHC class II genes and Cd74, encoding the invariant chain. VIPER protein activation algorithm identified IFNγ activation in apCAF
Dominguez *et al.* 2020 [9]	Mouse pancreatic KPP (*Pdx1$^{cre/wt}$;LSL-KRAS$^{G12D/+}$; p16/p19$^{fl/fl}$*)	Identified myCAF-like: TGF-β/SMAD3-driven subset exemplified by Eng expression (encoding CD105 protein); iCAF-like: TNFα, IL-1β, NF-κB-driven—expressing CXCL9/10 and CXCL1; suggest apCAF from Elyada *et al.* most resemble normal mesothelial cells
Hutton *et al.* 2021 [79]	Mouse pancreatic KPC (*Pdx1-Cre;Kras$^{LSL\text{-}G12D/+}$; Trp53$^{LSL\text{-}R172H/+}$*)	Performed scRNAseq on two dichotomous CAF subsets: CD105^{pos} vs CD105^{neg}. Subcutaneous co-injection of CD105^{pos} cells with tumor cells derived from KPC mice promote tumor growth, while CD105^{neg} co-injection slows tumor growth. CD105 encodes Eng, a TGF-β-driven gene; ingenuity pathway analysis shows CD105^{pos} cells are driven by TGF-β/SMAD signaling while CD105^{neg} cells are driven by LTBR, TNFα, NF-κB, IL6, JAK2, and STING; a subset of CD105^{neg} cells express mesothelial genes: Wt1, Msln, Krt8/18, Upk3b, Ezr. Eng KO resulted in upregulation of mesothelial genes
Grauel *et al.* 2020 [74]	4T1 and MC38 mouse syngeneic models	Identified an IFNγ-licensed CAF expressing CXCL9, 10, and 11 that emerged upon treatment with TGF-β inhibitor
Datta *et al.* 2022 [80]	Mouse pancreatic PKT (Ptf1a$^{cre/+}$;LSL-Kras$^{G12D/+}$; Tgfbr2$^{fl/fl}$)	Demonstrated that dual inhibition of MEK and STAT3 resulted in reduced iCAF (expressing IL-6 and Cxcl1), reduced myCAF (expressing Lrrc15), and an increase in a mesenchymal population (Ly6a, Cd34, and Meflin) with concomitant sensitization to anti-PD1
Krishnamurty *et al.* 2022 [78]	Mouse pancreatic KPR (*Pdx.1Cre;KRAS$^{LSL.G12D}$; p16/p19$^{fl/wt}$;p53$^{LSL.R270H/wt}$*)	Demonstrated that deletion of LRRC15^{+} cells via diptheria toxin induced loss of TGF-β/hypoxia/WNT-driven CAF and replacement with a "universal CAF" subset that expressed ADH1B and was driven by NF-κB, TNF, and JAK-STAT pathways, which slowed tumor growth, and sensitized tumors to anti-PDL1 treatment

potential to induce immunosuppression in the cells they recruit.

Interestingly, the functional ability of CAFs to recruit immune cells is reminiscent of another homeostatic fibroblast; the fibroblastic reticular cell (FRC). FRCs derive from mesenchymal precursors which are driven to become lymphoid tissue organizer (LTo) cells. LTo cells are thought to be stimulated by CXCL13-recruited immune cells called lymphoid tissue inducer cells (LTi), that secrete lymphotoxin which signals through lymphotoxin β receptor (LTBR) on the LTos [12]. This drives differentiation into FRCs, which provide the structural architecture, and secrete chemokines necessary, to form secondary and tertiary lymphoid structures in healthy and diseased states, respectively. (Intriguingly, reminiscent of the LTBR signaling in the FRCs, pathway analysis identified LTBR as one of the upstream regulators of the CD105^{neg}, tumor-restraining, CAF population identified by Hutton *et al.*) In secondary lymphoid organs,

DOI: 10.18609/ioi.2022.046

these FRCs, can recruit T cells via CCL19, CCL21, CXCL12 and CXCL13 protein secretion which promotes fibroblast-T cell interactions that allow a platform for DCs to activate antigen-specific T cells. Tertiary lymphoid structures (TLS), which form within diseased tissues, confer a poor prognosis in inflammatory diseases, but in the context of the tumor microenvironment, are prognostic for response to ICB [92]. In fact, one of the first single cell analyses from human patient tumors revealed a counter-intuitive correlation of CAFs expressing CCL19 with T cell abundance in the tumor microenvironment [93]. While this correlation was intriguing, it was not accompanied with outcome data, and the impact on the TME isn't known. Intriguingly, increased CXCL13-expressing T cells were associated with response to immune checkpoint in scRNAseq data with paired immunotherapy response, [67], and in another dataset, CXCL13-expressing T cells associated with formation of TLS in [94], lending credence to the idea that inducing a TLS-like reaction by coaxing fibroblasts into a more FRC-like differentiation state could promote response to checkpoint blockade. These data suggest a feedforward mechanism between CXCL13-expressing immune cells and FRC-like fibroblasts could be leveraged to potentiate response to immunotherapy. Perhaps the ideal strategy would be to simultaneously inhibit the TGF-β-driven, ECM-secreting, immunosuppressive fibroblast while potentiating this immune infiltrate-attracting fibroblast functionality in combination with ICB.

Forward looking statement

To further advance the field, single cell RNAseq should be performed on patient samples for which response to immunotherapy is available, using disaggregation protocols that allow for characterization of stromal cells as well as immune cells. Predictions of cell-cell interactions from correlating abundance of cell subsets with each other and with immunotherapy response, along with receptor-ligand pair analysis, should be validated spatially either by IHC or emerging technology of spatial transcriptomics, which could allow signatures to be derived from spatially clustered CAFs. Identifying markers with which to spatially track CAF subsets identified with scRNAseq, as well as sort them from tumors, will greatly improve our capacity to study these cells. Single cell RNAseq datasets should be exhaustively compared to existing datasets, and ideally some universal nomenclature decided upon, to the extent that this is possible, both within and between species (ie. comparisons between mouse models and human tumors). To this end, there has been much success in aligning TGF-β-driven CAF signatures between datasets, but much less so for other alternative CAF subtypes. Perhaps these subsets are disease or model-specific. *In vitro* assays functionally validating these interactions should include evaluation of fibroblasts on a soft matrix to prevent stiff matrix-imposed activation. Rather than relying on markers of activation or immunosuppression to define the impact of CAFs on other immune cells, functional assays should be performed to demonstrate the impact of CAFs and CAF-skewed myeloid cells on T cell migration, proliferation, and activation. Finally, *in vivo* validation of therapeutics should be performed in syngeneic models with sufficient stroma such as KP GEMM-derived models, or CAF-co-injection models, where CAFs may play a more prominent role in immunosuppression. Given the potential for CAFs to possess tumor-restraining functions, screens identifying T cell chemo-attracting therapeutics should be performed, with counter screens for co-culture induced immunosuppressive traits. Ultimately, the hope is that the combination of therapies affording the repression of TGF-β-driven CAF immunosuppressive function, along with those promoting the T cell-attracting function, might be used in combination with immunotherapy to elicit robust reversal of the immunosuppressive tumor microenvironment.

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Contributions: *The named author takes responsibility for the integrity of the work as a whole, and has given their approval for this version to be published.*

Acknowledgements: *None.*

Disclosure and potential conflicts of interest: *The author has no conflicts of interest.*

Funding declaration: *The author received no financial support for the research, authorship and/or publication of this article.*

ARTICLE & COPYRIGHT INFORMATION

Article source: *Invited; externally peer reviewed.*

Submitted for peer review: *Sep 7 2022*; ***Revised manuscript received:*** *Oct 13 2022*; ***Publication date:*** *Oct 27 2022.*

IMMUNO-ONCOLOGY INSIGHTS

LEVERAGING THE CUTTING-EDGE TME TOOLKIT

SPOTLIGHT

INNOVATOR INSIGHT

Predicting patient response to immune-targeted therapies via interrogation of the tumor microenvironment

Mark Uhlik & Caroline Fong

More accurately predicting which patients will benefit from immune-targeted therapies, including checkpoint inhibitors, remains a crucial goal for the immuno–oncology field. This article will provide an overview of an RNA-based investigational tumor microenvironment panel to better interrogate tumor biology and support multiple oncology therapeutics – particularly immune checkpoint inhibitors and anti-angiogenic agents – across a range of tumor types. New translational data on predicting the benefit from maintenance durvalumab after first-line chemotherapy in oesophagogastric adenocarcinoma will be discussed, along with the potential for companion diagnostic development in support of clinical development.

Immuno-Oncology Insights 2022; 3(9), 513–525

DOI: 10.18609/ioi.2022.052

PREDICTIVE BIOMARKERS IN ONCOLOGY PRECISION MEDICINE

A number of challenges remain in oncology precision medicine, despite tremendous inroads towards predictive biomarkers. Most predictive biomarkers that support oncology therapies are DNA-based, and it is estimated that ~20% of cancer patients benefit from genomic precision medicine. The Xerna TME Panel is uniquely positioned to provide some additional solutions. The panel is RNA-based and can accommodate the complexity of biology that is relevant to ~80% of patients. The

Xerna TME Panel utilizes a machine learning algorithm including ~100 genes to capture a wide degree of complexity within the tumor microenvironment (TME). Analytics are used to perform a computational assessment of the biomarker to give robust and binary-like biomarker designations. Pre-clinical work that utilized an adenoviral construct expressing vascular endothelial growth factor (VEGF), a major driver of pathological angiogenesis, was the jumping off point for the later development of a model for understanding of gene signatures in the absence of tumor, whilst providing pathological microenvironment biology. This allowed the adaption of the model to translate to human biology in a way that would be broadly applicable across all tumors.

There are 46 FDA approved and cleared companion diagnostics in oncology as of October 2021. However, 41 of these measure single analytes which are mostly DNA or immunohistochemistry (IHC)-based. RNA expression signatures hold great potential within precision medicine even though there are yet to be any companion diagnostics (CDx) based upon RNA expression signatures. Gene signatures are particularly good at describing biological complexity and already use well-validated technical platforms that are currently in use as regulated tests. The computational and validation aspects remain to be overcome, which could be enabled by the Xerna TME Panel.

DEVELOPMENT OF THE XERNA TME PANEL

The TME is the focus of the Xerna TME Panel; two key areas it could help support are angiogenesis and immune biology. There is a significant unmet need to support anti- angiogenic agents with a biomarker. Immu- notherapies have been contributing greatly to patients over the past decade, with many successes employing biomarkers to support these therapies. Biomarkers such as PD–L1 support various checkpoint inhibitors such as pembrolizumab and nivolumab. However, there is not a single biomarker that is broadly applicable in solid tumor indications that has been successfully translated to different immuno-oncology therapies or across tumor types. There is still significant improvement to be made in the support of immune checkpoint inhibitors (ICIs) and other immune therapeutic modalities. Angiogenesis and immune biology are also highly interrelated and interconnected biologies, as there is a complex interaction between angiogenic factors and immune biology.

The Xerna TME Panel is a ~100-gene panel looking at RNA expression within the TME. It has been extensively trained and tested on more than 2000 cancer patient samples, and has been licensed to Exact Sciences for incorporation into their Oncomap ExTra platform and to QIAGEN for the development of their QIAseq NGS workflow and as a CDx for navicixizumab, OncXerna's anti-angiogenic agent in clinical development.

The panel uses the delineation of solid TMEs into one of four subtypes, the A, the IS, IA, or ID groups, as described in Figure 1. These subtypes correspond to discrete biologies within the TME (Figure 2). One of the key defining features and strengths of the Xerna TME Panel is its ability to identify the highly interconnected biologies of pathological angiogenesis and tumor immune biology.

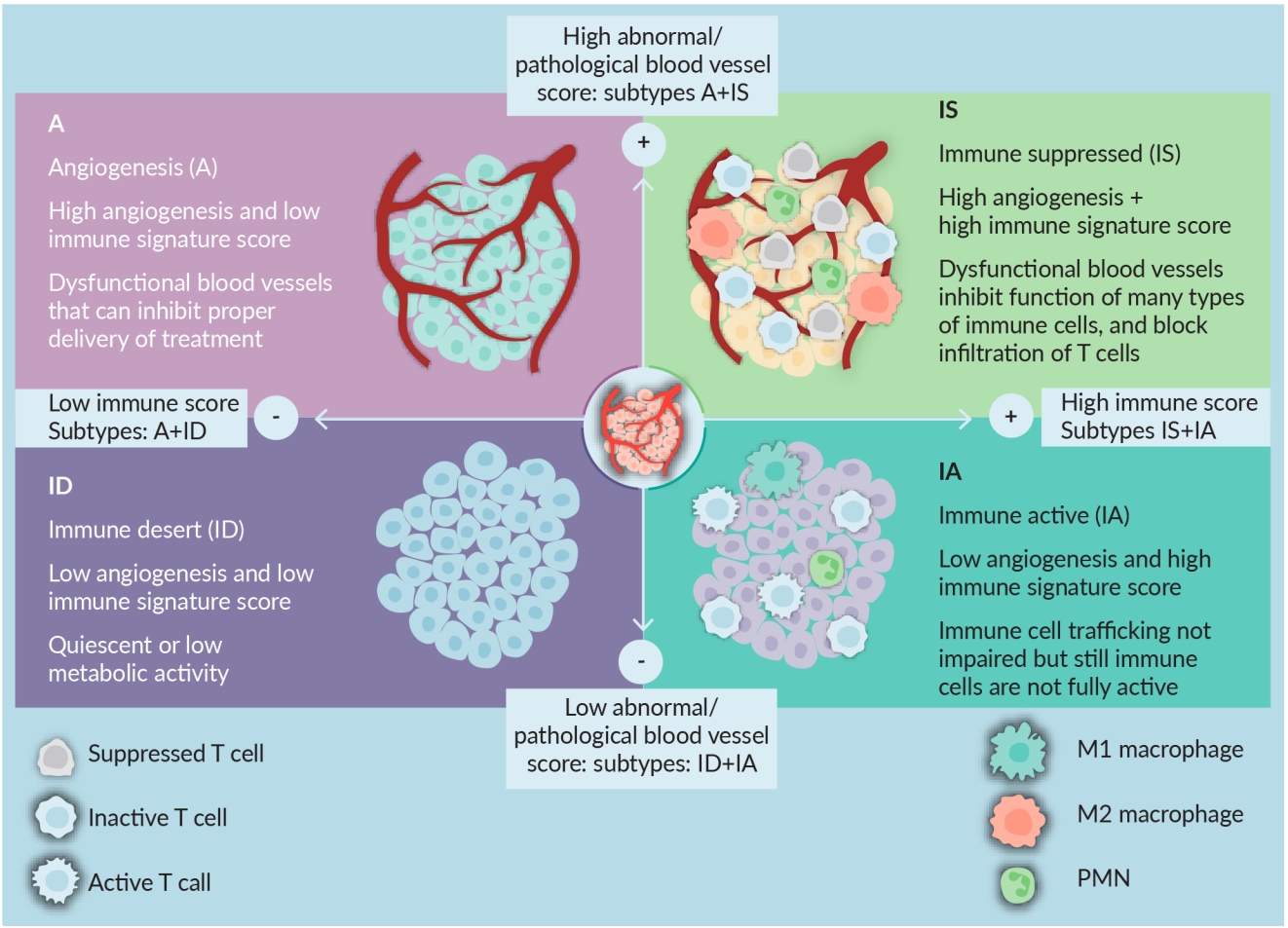
Extensive training and testing of the Xerna TME Panel has been completed across multiple patient tumor samples and clinical cohorts, with a focus on gastric cancer and other solid tumor types, to ensure the panel was tumor agnostic. There are correlations to support the biology in each of these four subtypes (Figure 2). There is both prognostic data and a therapeutic hypothesis appended to each subtype. For instance, in the IA subtype, there is the expression of genes for inflammatory response including PD–L1, IFN-γ and TNF-α. In general, this subtype tends to have the best prognosis across multiple tumor types and patient cohorts.

The workflow of the panel begins with a formalin-fixed, paraffin-embedded (FFPE) tissue sample. Total RNA sequencing is the preferred protocol, but gene array or whole

DOI: 10.18609/ioi.2022.052

FIGURE 1

The Xerna TME Panel identifies the dominant biology of the TME and assigns into therapeutically actionable tumor subtypes defined by angiogenesis and immune gene expression.



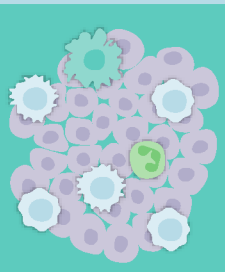
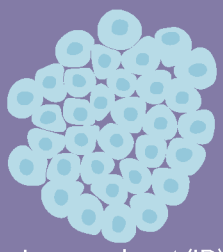
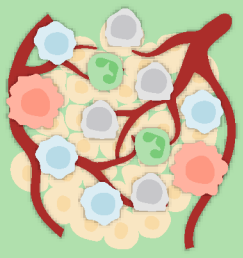
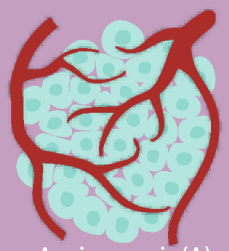
exome sequencing can also be performed. RNA expression data is run on the Xerna TME algorithm which is composed of two nodes, roughly corresponding to immune and angiogenesis signatures. There is a high degree of interconnectedness between these nodes, and an understanding of this was developed primarily through a machine learning approach. The combination of scoring on these nodes then yields an output into one of the four subtypes. Each of those subtypes is given a probability, and the highest probability is the subtype that is identified for that tumor sample. These probabilities in a cohort can be plotted on a latent space plot with contours associated with confidence values. Most current biomarker assays result in near-normal output distributions; thus many samples reside near the separation threshold. However, the Xerna TME Panel generates binary-like, clear-cut biomarker outputs.

CASE STUDY: ANALYZING THE PREDICTIVE POTENTIAL OF THE XERNA TME PANEL

The predictive potential of the Xerna TME Panel was tested in a gastric patient cohort treated with ICI monotherapy; either pembrolizumab or nivolumab (Figure 3). Tumor biopsies were collected prior to initiating ICI therapy. RNA was extracted from FFPE samples and run on RNAseq, followed by analysis using the Xerna TME Panel. TME immune subgroups IA and IS were hypothesized to derive the most clinical benefit, as read out by overall response rate (ORR). PD-L1 combined positive score

FIGURE 2

The biology of the TME subtypes is supported by correlations, with each being linked to a different prognosis and therapeutic hypothesis [1–3].

Xerna TME subtype	Correlations that support biology	Prognosis	Therapeutic hypothesis
 Immune active (IA)	• Expression of gene for inflammatory response and immune activation (i.e., PD-L1, IFNγ, TNFα) • Histology showing tumor infiltration by myeloid/lymphoid cells) • Correlates with MSI-high subtypes	Best	Immune checkpoint inhibitors
 Immune desert (ID)	• Lack of immune or angiogenesis gene signatures • Histology marked by low vessel density and low immune cell filtration	Moderate – good	Tumor vaccines
 Immune suppressed (IS)	• Angiogenesis gene and protein expression profile (i.e., VEGFR2, ACVRL1, Tie2, PDGFRβ) • Gene expression inflammation, M2 macrophage biology and Treg signatures (i.e., TIM3, IL-10 and TGFβ) • Histology marked by dense, pathological vessels as well as infiltration of myeloid and lymphoid cells	Moderate – poor	Combination immune therapies
 Angiogenesis (A)	• Angiogenesis gene and protein expression profile (i.e., VEGFR2, ACVRL1, Tie2, PDGFRβ) • Histology marked by dense, dysfunctional vessels	Worst	Anti-angiogenic agents

Pathological angiogenesis interferes with multiple aspects of immune function consistent with a decreased immune activity (e.g., Tregs, TGFβ etc.) in the IS group compared to the IA. This can also explain a worse prognosis of IS compared with IA.

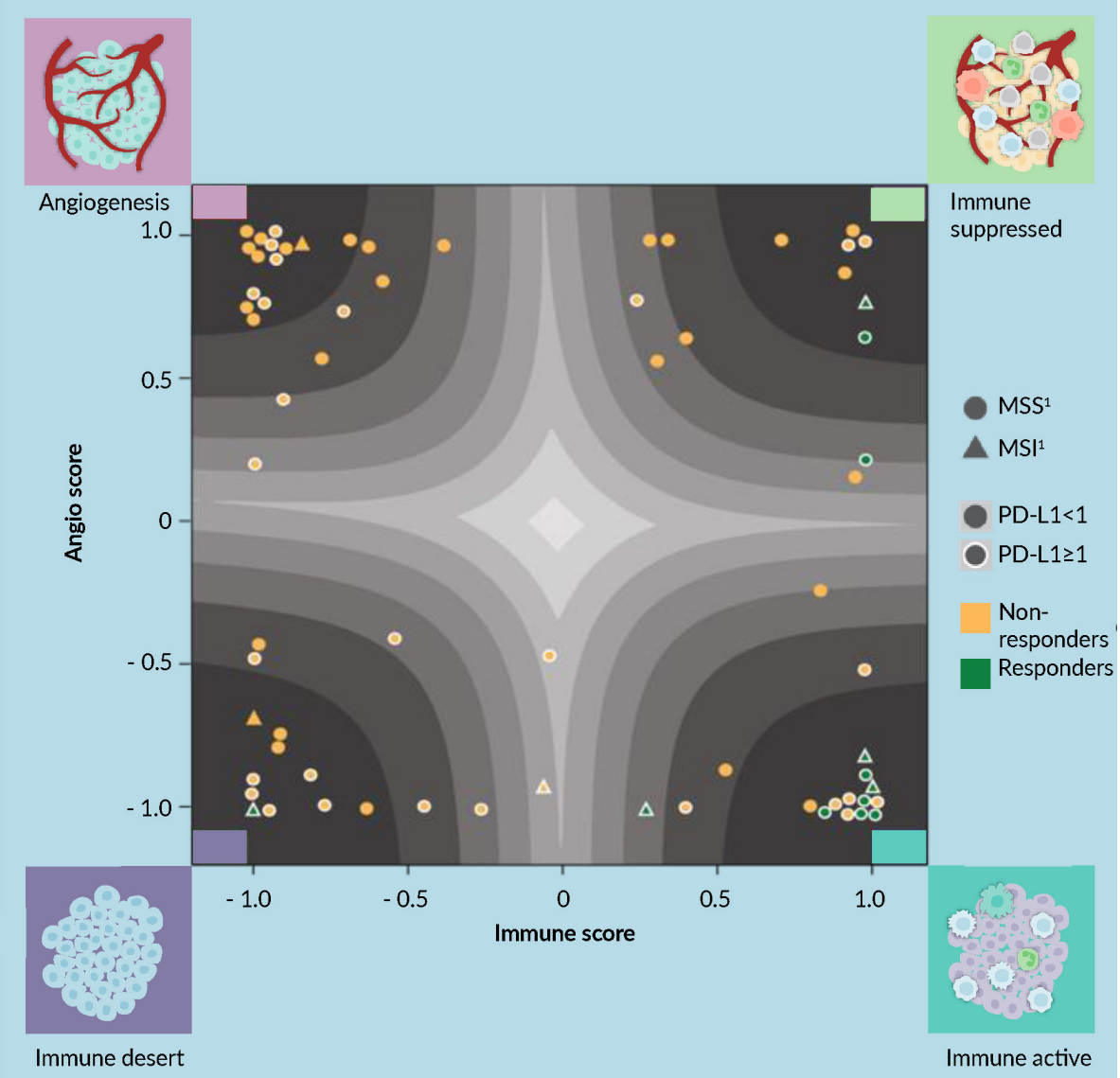
(CPS), which includes staining on immune as well as tumor cells, was also available for a majority of these patient samples.

The Xerna TME data clusters into high confidence areas indicating that robust calls with a high degree of separation were achieved. As expected, the majority (63%) in the microsatellite-high (MSI-H) group are responders and are also PD-L1 positive by CPS>1. A response rate of 58% was achieved

DOI: 10.18609/ioi.2022.052

FIGURE 3

Latent space plot of clinical validation results achieved using the Xerna TME algorithm.



[1]) MSI-H: Microsatellite instability); MSS:Microsatellite stable; PD-L1: Programmed death-ligand 1. Four MSS patients did not have a PD-L1 score available.

in the high probability IA group, with a high prediction of response (33%) also observed in the microsatellite stable (MSS) group in IA. The TME Panel enriched for responses in immune score high subtypes (IA+IS) that are PD-L1 positive (44% ORR), but also importantly identified non-responders in the immune score low (A+ID) subtypes within PD-L1 positive group (0% ORR). The panel was also able to enrich for response rates in Xerna immune score high (100%) versus immune score low (25%) subtypes in MSI-H.

The Xerna TME algorithm provides clear, binary-like cut points, in contrast to historical non-machine learning-based approaches, and results in strong analytical performance.

OVERVIEW OF OESOPHAGOGASTRIC CANCERS

Most patients are diagnosed with oesophagogastric (OG) cancers at an advanced stage where treatment is limited to palliative

chemotherapy. Over the past two decades, platinum-fluoropyrimidines have formed the cornerstone of chemotherapy options for patients newly diagnosed with advanced OG cancers but associated with an overall survival (OS) of more than 12 months. One exception to this is for HER2-positive cancers where the monoclonal antibody, trastuzumab, can improve survival up to 14 months. However, it can only be used in 15–20% of patients with advanced OG cancers.

Data from the global CheckMate 649 study showed that the OS of HER2-negative patients can be extended by adding nivolumab to platinum-fluoropyrimidine chemotherapy. Although this survival benefit was most pronounced in the PD–L1 CPS ≥5 population, where the addition of nivolumab improved OS from 11.1 to 14.4 months, a significant improvement in survival was also seen in PD-L1 CPS ≥1 and in all patients recruited into the study albeit at decremental margins of benefit. Based on this, chemotherapy with nivolumab has been FDA approved for use as first-line treatment irrespective of PD–L1 expression. In contrast, European guidelines stipulate that a CPS of ≥5 is required to determine eligibility for using Nivolumab together with chemotherapy in the first line. As the largest margin of benefit is seen in CPS ≥5 population, it reiterates the value of biomarker-driven therapy in OG cancers is crucial to improving patient outcomes. Nevertheless, the ability to better identify which patients will benefit from the available treatments is needed.

PD–L1 is a key predictive biomarker for the use of ICIs in advanced OG cancers. From a clinician's perspective, PD–L1 in OG adenocarcinoma has been a clinical conundrum. While it is clinically recognized that a CPS of 5 can identify a subset of patients who will benefit most from ICI, , there are many different antibody assays available with little robust data to demonstrate reliable intra-assay concordance. There has also been evidence to show there is discordance in PD–L1 expression between primary and metastatic sites, which from a clinician's perspective can add difficulty in interpreting a PD–L1 reading.

INTRODUCTION TO THE PLATFORM STUDY

The PLATFORM study is a Royal Marsden Hospital-sponsored, multi-center, randomized, and adaptive Phase 2 study assessing maintenance therapies in advanced OG adenocarcinoma. It began in 2015 and recruits from around 25 centers across the UK. This collaboration has previously generated practice-changing data in advanced OG adenocarcinoma, such as MAGIC and REAL-2. The overarching hypothesis of this study is that maintenance therapy after completion of first-line chemotherapy will prolong progression-free survival (PFS) in patients.

Recruitment into the study is via a two-step process. Firstly, patients are registered as potential candidates for the trial before or during first-line chemotherapy. At this point, archival diagnostic biopsies are donated, and these treatment-naïve samples are used to diagnose advanced OG adenocarcinoma. Once the patient has completed 18 weeks of chemotherapy, they need to demonstrate radiological disease control or response before they are randomized into the study according to their HER2 status. In the HER2 negative cohort, the trial initially opened with three arms: active surveillance, which is the current standard of care in the UK after first-line chemotherapy and also forms the control arm, capecitabine and durvalumab.

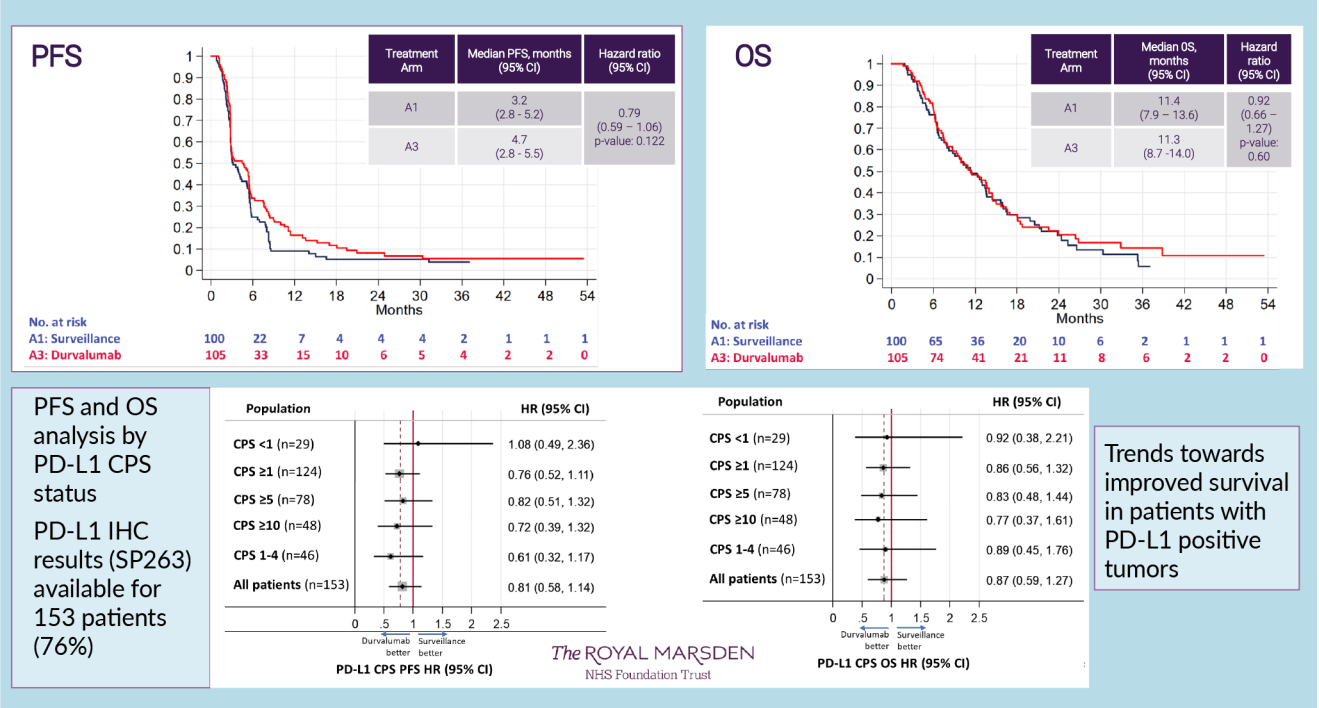
XERNA TME PANEL ANALYSIS OF PLATFORM PH2 STUDY

It was found that maintenance durvalumab does not prolong PFS or OS compared to active surveillance (Figure 4). An analysis of PD–L1 results from 75% of the total trial population was performed at varying thresholds. No significant difference in survival according to PD-L1 expression was found, but a trend towards improved survival with increasing PD–L1 threshold was observed. However, this analysis was limited by the relatively small sample size.

DOI: 10.18609/ioi.2022.052

FIGURE 4

PLATFORM: maintenance durvalumab does not prolong progression-free or overall survival compared to active surveillance.



CI: Confidence interval; CPS: combined positive score; HR: Hazard ratio ; OS: Overall survival; PD-L1: Programmed death-ligand 1; PFS: Progression free.

The Xerna TME Panel was assessed for its capability to distinguish patients who had gained a survival benefit from maintenance durvalumab from those who had not. It was hypothesized that patients with a high immune score who were IA or IS-derived would experience the most clinical benefit from maintenance durvalumab. PFS and OS analyses for the following biomarker-defined subgroups were compared using the Kaplan – Meier method (Figure 5 & Table 1):

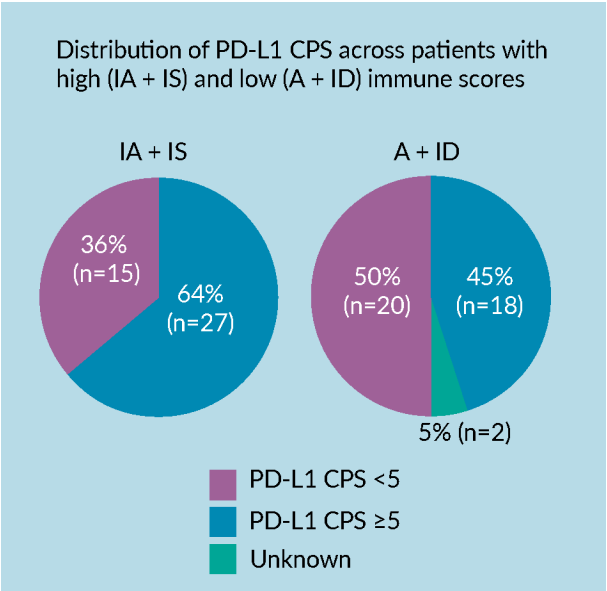
- IA + IS versus A + ID
- PD-L1 CPS <5 versus PD-L1 CPS ≥5
- Combinations of each TME and PD-L1 CPS subgroup

High PD-L1 Scores were found in the immune-positive subtypes (IA+IS; 64%) compared to immune-negative subtypes (A+ID; 45%).

The Xerna TME panel accurately predicted better PFS and OS in patients with

FIGURE 5

Xerna TME panel analysis of patient characteristics and biomarker status.



A: Angiogenesis; CPS: combined positive score; IA: Immune active; ID: Immune desert; IS: Immune suppressed; PD-L1: Programmed death-ligand 1.

TABLE 1

Xerna TME panel analysis of patient characteristics and biomarker status.

Patient characteristic	Action surveillance (n=38)		Durvalumab (n=44)	
	n	%	n	%
Median age (years)	66	-	66	-
Gender				
Male	30	79	34	77
Primary tumor site				
Esophageal	16	42	19	43
GOJ	10	26	12	27
Stomach	12	32	13	30
Disease extent				
Locally advanced	3	8	6	14
Metastatic	35	92	38	86
TME RNA status				
IA + IS	20	53	22	50
A + ID	18	47	22	50
PD-L1 CPS				
>5	18	47	17	39
≥5	19	50	26	59
Unknown	1	3	1	2
MMR status				
Proficient	34	89	40	91
Unknown	4	11	4	9

immune-positive biomarker status for maintenance ICI therapy (Table 2).

IA + IS patients in surveillance had 6- and 12-month PFS and 24 month OS rates suggestive of a poorer prognosis than A + ID patients. However, IA + IS patients had numerically higher 6- and 12-month PFS and 24-month OS rates than A + ID when treated with durvalumab. In contrast, survival function estimates at all time points for PFS and OS were similar in A + ID patients across both treatment arms.

Numerically higher survival rates were observed in PD-L1 CPS <5 patients randomized to surveillance. Survival benefit with durvalumab was limited to 12-month PFS and OS rates in PD-L1 CPS ≥5 compared to CPS <5.

In summary, Xerna TME biomarker positive patients were provided with benefit from durvalumab, whereas no differences were seen in the surveillance group. The Xerna TME Panel more accurately predict which patients will benefit from checkpoint inhibitors than PD-L1 CPS scores. Better 24-month survival was achieved when durvalumab was used in OG adenocarcinoma patients with 'high' Xerna TME Panel immune scores, despite these patients having a worse prognosis.

INSIGHT

IA + IS phenotypes (biomarker-positive) were identified as having improved survival with maintenance durvalumab. The Xerna TME Panel may identify HER2-negative OG patients who benefit from ICIs more consistently than PD-L1 CPS ≥5. In contrast, no survival differences were observed between the durvalumab and active surveillance in the A + ID (biomarker-negative) groups. Amongst CPS ≥5 patients, the Xerna TME Panel may further distinguish a subgroup of patients who derive the most durable survival benefit from ICIs. A + ID and/or CPS <5 may be prognostic in HER2-negative OGA. The predictive and prognostic capabilities of the Xerna Panel should be assessed in larger cohorts.

The Xerna TME Panel is continuing clinical development and commercialization. Engagement across numerous organizations is enabling the development of new biomarker approaches, utilizing machine learning. The Xerna TME Panel is a biomarker that can be applied across broad indications to support

TABLE 2

Xerna TME biomarker enriches for clinical benefit in durvalumab-treated gastric cancer patients.

Gastric cancer regimen	Xerna output (prevalence)	12 month PFS (%)	24 month OS (%)
Durvalumab	Biomarker positive (51%)	25	35
Surveillance		0	9
Durvalumab	Biomarker negative (49%)	5	23
Surveillance		6	24

DOI: 10.18609/ioi.2022.052

multiple therapies. This machine-learning artificial neural net model using RNA sequencing technology and RNA expression-based technology from FFPE tissue assesses the dominant biology of the TME. It leads to binary-like distributions with high confidence, providing prognostic and predictive potential.

ASK THE EXPERTS

Roisin Mcguigan, Editor, Immuno-Oncology Insights speaks to (pictured left to right) **Mark Uhlik, Vice President, Head of Research in Biomarkers Discovery, OncXerna Therapeutics** and **Caroline Fong, Specialty Registrar in Medical Oncology, Royal Marsden NHS Foundation Trust**

Q What are acceptable sample types and minimum inputs for the Xerna TME Panel, and can you analyze previously extracted RNA or liquid biopsy samples?

MU: The sample inputs we have focused on are FFPE tissues, which are the key primary substrate that we have worked on. We can analyze data from any tissue substrate that is amenable for RNA extraction, including frozen tumors and previously extracted RNA, provided they have sufficient quality and input for running on RNA sequencing-based platforms or gene expression profiling. In terms of liquid biopsies, they are not necessarily amenable to the Xerna TME Panel because we are focused on solid TMEs, which liquid biopsies do not have intact. This is unlikely to lead to any conclusive results concerning the biology of the microenvironment.

Q Do you have any other data available for other predictive biomarkers for immunotherapy for the PLATFORM cohort, such as tumor mutational burden (TMB)?

CF: When we extracted RNA for this project, we also extracted DNA simultaneously. We do not have whole exome data for the entirety of this cohort, but when we carried out an interim analysis a few years ago, we did collect whole exome sequencing data. There is some data to suggest that even in gastric cancers, TMB can predict response immunotherapy. The advantage of this TME panel is that it is not looking at a tumor-specific characteristic itself, but rather the TME in general.

Q The tissue used for the PLATFORM work was obtained prior to first-line chemotherapy. Could that chemotherapy have remodeled the TME and how would that affect the results of the panel?

CF: One would imagine that there is some TME remodeling in response to first-line chemotherapy. In the clinical setting, getting serial biopsies from patients who are frequently symptomatic from their cancer can be challenging. Realistically, baseline biopsies are going to be the most accessible in terms of determining predictive biomarkers in these patients.

There is some emerging data supporting TME remodeling in response to chemotherapy. A paper published in Cancer Discovery earlier this year observed 12 patients who had serial biopsies before and after chemotherapy suggested there was a change in T cell infiltration and antigen presentation after chemotherapy. This seems to be associated with a response to immunotherapy thereafter. However, the Xerna Panel indicates the potential to respond to ICI based on the dominant features of the TME, rather than active gene expression which can be dynamic.

Q Solid tumors may have niche-specific microenvironments depending on histology and location. How does the Xerna TME Panel account for these variations that exist between the microenvironments of different solid tumor histologies?

MU: Due to the way this panel was developed initially from an *in vivo* pre-clinical model, without a tumor present, we were looking at conserved dominant biology features of the TME that were not specific to any one tumor type. The applicability of this panel translates across many different tumor types. In almost every solid tumor type we have looked at, we can see evidence of some of these four subtypes as dominant biologies.

In development, we were directed towards ensuring the genes we selected that comprise this panel were not tissue-specific genes concerning any certain tumor type. We performed screening ahead of time to ensure they were represented at sufficient levels across the multiple different tumor types. It was at the beginning of development that we decided that this could be a tumor-agnostic panel, utilizing conserved features of the microenvironment common in all solid tumors.

Q How much heterogeneity is seen in the RNA panel analysis from single patients when biopsies from multiple tumor sites are compared?

MU: We do know that tissue heterogeneity is a problem that all biomarker assays have to be able to overcome. Tumor heterogeneity exists and we specify that we prefer to have multiple cores from the same tumor, and multiple regions when receiving our

 DOI: 10.18609/ioi.2022.052

tissues. Typically, we ask for three or five cores across a tumor to ensure that we are capturing as representative regions as possible.

In terms of looking at primary versus metastatic tumor sites, we have performed only a limited analysis on that. We would like to do more but what we have seen so far is that there are differences in those micro-environments. It is well known that a metastatic site is going to act differently from a primary site, and we can see that they represent different TMEs. The metastatic sites are the ones that typically are most prognostic for a patient's survival, so we lend more credence to treating the dominant biology of the metastatic site than the primary tumor. We have at times seen both consistency and differences between primary and metastatic sites, which is true of many biomarker assays.

BIOGRAPHIES

MARK UHLIK is a seasoned leader in translational oncology and drug discovery/development with over 18 years of experience in the pharmaceutical industry. Mark earned his BSc in Microbiology from Colorado State University and his PhD in Microbiology and Immunology at Pennsylvania State University College of Medicine. He performed post-doctoral work at University of Colorado Health Sciences Center and at University of North Carolina School of Medicine. Mark has authored over 35 peer-reviewed publications in addition to book chapters and patents.

CAROLINE FONG is a Medical Oncology Registrar at the Royal Marsden Hospital in London. She has recently completed a four-year Clinical Research Fellowship with the Gastrointestinal and Lymphoma Unit at the Royal Marsden, where she was the Trial Physician and a Co-Investigator of the PLATFORM study, an academic, multi-center, randomized Phase 2 clinical trial assessing the role of maintenance therapies in advanced oesophago-gastric cancers. She is currently working towards a thesis centred on predictive biomarkers in oesophago-gastric cancers.

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Contributions: All named authors take responsibility for the integrity of the work as a whole, and have given their approval for this version to be published.

Acknowledgements: None.

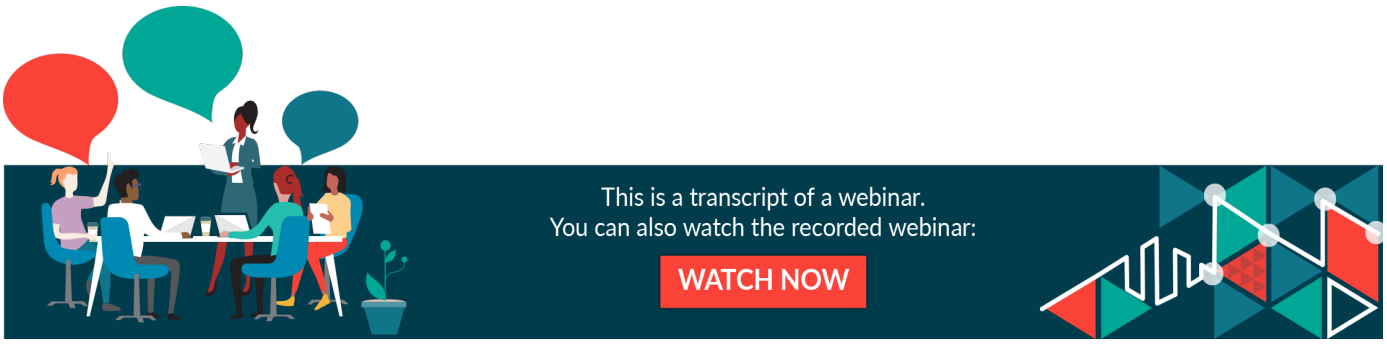
Disclosure and potential conflicts of interest: Uhlik M discloses he has patents planned, issued or pending from OncXerna Therapeutics, Inc. He also has stocks/stock options from OncXerna Therapeutics, Inc., Hibercell, Inc. (previously Biothera Pharmaceuticals, Inc.) Rigel. Fong C discloses she recieves payment or honoraria for lectures, presentations, speakers bureaus, manuscript writing or educational events from Bristol Myers Squibb.

Funding declaration: The authors received no additional funding for the research, authorship and/or publication of this article.

ARTICLE & COPYRIGHT INFORMATION

Article source: This article is a transcript of a webinar, which can be found here.

Webinar recorded: Oct 12 2022; **Revised manuscript received:** Nov 22 2022; **Publication date:** Dec 19 2022.



DOI: 10.18609/ioi.2022.052

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Sample to Insight

INTERVIEW

Studying the tumor microenvironment under pressure

Roisin McGuigan, Editor, *Immuno-Oncology Insights*, **speaks to Meenal Datta,** Assistant Professor, Department of Aerospace and Mechanical Engineering, University of Notre Dame

MEENAL DATTA is an assistant professor in the Department of Aerospace and Mechanical Engineering at the University of Notre Dame. Prof Datta received her PhD in Chemical and Biological Engineering from Tufts University in 2018, after which she completed a postdoctoral fellowship at Harvard Medical School and Massachusetts General Hospital. Her research focuses on deciphering the atypical tumor microenvironment that drives disease progression and treatment resistance in incurable cancers. By understanding and overcoming the biological, chemical, electrical, and mechanical abnormalities found in solid tumors, new therapeutic approaches can be discovered. Prof. Datta specializes in multidisciplinary and mechanism-based preclinical research that has the potential to be rapidly translated to improve treatment approaches in the clinic. She has spent her time as a researcher deciphering and reprogramming abnormal tissue microenvironments that present in a variety of diseases ranging from virulent tuberculosis to benign schwannoma to deadly glioblastoma that, surprisingly, share unifying features: abnormal blood vessels, abundant extracellular matrix, immunosuppression, and mechanopathologies. During her PhD, Dr Datta normalized the aberrant blood vasculature found in pulmonary tuberculosis granulomas to improve drug delivery. In her postdoctoral training, Dr Datta re-engineered the immunosuppressive brain tumor microenvironment to improve glioblastoma response to immunotherapy. As the director of the Tumor Immune Microenvironment & Mechanics Lab at Notre Dame (the TIME lab). Prof Datta's research group is applying engineering fundamentals and problem-solving approaches to explore mechano-immunological phenomena in the tumor microenvironment and discover novel biophysical targets of interest.

Immuno-Oncology Insights 2022; 3(9), 367–372

DOI: 10.18609/ioi.2022.039

Mechano-immunology is the study of how tissue mechanical properties and forces impact immune cells – including those in and around tumor microenvironments (TME). We spoke to Meenal Datta, an engineer by training, about how these forces can affect immunotherapy outcomes, her work in brain cancers including glioblastoma, and the unique perspective engineers can bring to cancer research.

Q Can you tell me a bit about what attracted you to studying the tumor microenvironment (TME)?

MD: I am a chemical and biological engineer by training, and I'm starting my second year as an assistant professor of Aerospace and Mechanical Engineering at the University of Notre Dame. It is an interesting department because you might not immediately think of aerospace and mechanical engineering when you think of cancer, but my department houses a bioengineering graduate and research program. That's where my research fits in. I started the Tumor Immune Microenvironment and Mechanics Laboratory (the TIME Lab) to study the mechanical microenvironment of tumors. Our work relies on the fundamental theory that the tumor microenvironment is abnormal in many ways: biologically, chemically, electrically, and mechanically.

In terms of what attracted me to studying the TME, I owe my current line of study to my PhD and postdoctoral mentor, Dr Rakesh K Jain at Massachusetts General Hospital and Harvard Medical School, one of the founders of the TME field. Dr Jain is also an engineer by training, and he found his way into this field because he was interested in the "biological conduits" (a.k.a., blood vessels) that bring drugs to tumors. As an up-and-coming graduate student, it was the first time I'd heard of an engineer having such a profound impact on cancer research – not just in terms of basic science and preclinical studies, but also from a translational and clinical angle, with real impact for patients. It was inspiring for me to have a mentor like that and this is when I became interested not just in the TME but in tissue microenvironments in general. I quickly realized that tissue microenvironmental abnormalities are not restricted to cancer alone.

For my PhD research, I studied the microenvironment of pulmonary tuberculosis granulomas and considered them much in the same way that we do cancerous tumor microenvironments. I applied the basic understanding of altered bio transport that we have learned from cancer to the field of Tuberculosis (TB). Through my exposure to cancer research during my PhD studies, I became interested in this area for my post-doctoral work, and this helped to inspire my current position as a cancer researcher and professor of engineering.

Q As an engineer, what unique perspectives do you bring to the immuno-oncology space?

MD: In the most simplistic terms, engineers tend to think about biological systems in the way that we would any other system, whether it's a manufacturing plant or a catalyst particle. We tend to take a higher order, systems-based approach to whatever it is we're studying. The advantage of this is that rather than getting bogged down in the complex biology of a single molecule, pathway, or cell type, we are able to apply universal concepts based in fundamentals of engineering to basic yet unanswered questions in physiology and pathophysiology. Bio transport is a great example of applying an engineering concept to cancer – for example, how drugs are delivered to tumors, and how this delivery can be thwarted within the TME because of the abnormal architecture of tumor blood vessels. As engineers we can bring unique and important perspectives, especially to incurable diseases like cancer. Cross-disciplinary approaches could mean the difference between treatment success and failure.

"...the problem is that in tumors every single facet can be, and often is, abnormal. We need to overcome as many of those abnormalities as we can in order to improve outcomes – not just in immunotherapy, but in any therapy."

Q One particular focus of your research is glioblastoma – when it comes to the solid tumor space specifically, what do you see as the key obstacles posed by the TME that need to be overcome?

MD: I've often heard researchers describe the TME as an organ, and I think that's an apt comparison. The tumor can contain all the same components as our normal tissues and organs, but the problem is that in tumors every single facet can be, and often is, abnormal. We need to overcome as many of those abnormalities as we can in order to improve outcomes – not just in immunotherapy, but in any therapy.

There are some facets of the TME that are very challenging – including the abnormal vasculature which includes poorly-functioning blood vessels and non-functional lymphatic vessels, tumor-supporting stromal components including cancer-associated fibroblasts, and dense extracellular matrix molecules such as collagen. There are also abnormal chemical conditions such as low oxygen and pH; all of these factors combined can hinder not only the delivery of drugs but their functionality as well.

Glioblastoma and other primary brain tumors are interesting because they tend to lack a lot of the same components that we might see in extracranial tumors such as cancer-associated fibroblasts, or large amounts of collagen. They pose their own unique set of challenges, including an abundance of hyaluronic acid, a disrupted blood–brain barrier, and a highly immunosuppressive TME.

Q Another theme of your work is mechano-immunology. What impact do tissue mechanics have on resistance to therapy, and

how can addressing mechano-immunological pathologies improve immunotherapy outcomes?

MD: Glioblastoma, like many other tumors, can exert physical forces both within the tumor and on the surrounding tissue. These forces can be fluid-based; in the case of glioblastomas this is referred to as oedema and it has been well-described. These forces can also come from the solid components of tissues – cells and the matrix that they produce. We refer to that physical force as solid stress, and that's a particular area of interest for me. It is a major mechanical pathology, because it can compress blood vessels and induce invasive behavior in cancer cells.

Together, these effects can promote treatment failure – both due to drug delivery issues and tumor progression. What we found in the brain is that solid stress actually impacts the surrounding normal brain tissue as well, to an alarming extent. It can compress blood vessels in the surrounding brain tissue, and it can damage or kill neurons. In my lab I am now interested in understanding how physical forces like solid stress impact immune cells in and around the TME, and also vice versa; how these cells potentially contribute to solid or fluid forces.

This is the basis for what I call "mechano-immunology." It links the fields of mechanics and immunology together in order to understand the potential crosstalk, or even reciprocal regulation, between some of these phenomena. In the case of glioblastoma, an interesting example is macrophages. They are under scrutiny in immuno-oncology from both targeting and repurposing angles, as they can be polarized to either support or fight the tumor. With the support of funding from the National Institutes of Health (NIH), we are now investigating if macrophages, in addition to being major mediators of immunosuppression, are involved in mechano-immunology in the glioblastoma TME as well. Our central question is: Can we reprogram the biophysical behaviors of immune cells like macrophages to improve immunotherapy outcomes?

Q Turning to enabling tools and technologies, what is the current state of the art, for you?

MD: As an engineer tools and technologies are my wheelhouse; they are probably what I like to talk about the most! But let's focus on one that many immunologists and tumor immunotherapists are thinking about right now: single cell technologies.

> "...solid stress [is] a particular area of interest for me. It is a major mechanical pathology, because it can compress blood vessels and induce invasive behavior in cancer cells."

Single cell RNA sequencing and even spatial transcriptomics are rapidly becoming standard practice in cancer research, including at the preclinical level. In collaboration with computer science engineers and bioinformaticians, my lab is currently exploring

DOI: 10.18609/ioi.2022.039

how multi-scale and multi-modal omics-based data – whether it's protein, DNA, RNA, or even epigenetic information – can be integrated in order to provide a more comprehensive map of the TME. Not just in terms of treatment-naïve samples, but also in response to therapies like immunotherapy. Integrating single cell multi-omics will help to reveal dynamic responses in multiple dimensions of time and space, and will also hopefully reveal targetable mechanisms that could be the difference between response and resistance. One of the main questions in immuno-oncology at the moment is: what determines whether a tumor is responsive, refractory, or resistant to immunotherapy? Leveraging machine learning to integrate multi-omics at the single cell level will allow us to understand some of these differences, and achieve more targeted and personalized approaches to immunotherapy.

Q How is our understanding of the TME and mechanisms of tumor resistance to immunotherapy evolving?

MD: In the last decade we have seen a tighter integration of tumor immunology with other aspects of the tumor microenvironment, including consideration of vascular biology, biochemistry, or mechanobiology, in order to understand how these other processes affect immunotherapy outcomes, and how they can be targeted, exploited, or reprogrammed to improve outcomes.

For example, my former mentor and others have shown that vascular normalization can be an effective approach to improve immunotherapy outcomes. This combinatorial strategy has been approved for over half a dozen cancers. In my lab I am building on co-targeting both TME abnormalities and immunosuppression. Targeting abnormal physical forces in glioblastoma and other incurable cancers may improve the outcome of standard immunotherapies like checkpoint blockade antibodies. We're also interested in repurposing Food and Drug Administration (FDA)-approved drugs that might have off-target effects that reprogram the TME, in addition to small molecule immunotherapeutics, which is another area of great interest in the field.

Q What are your own goals for the next few years?

MD: I hope to open up this new field of mechano-immunology in the context of TME. The focus in the short term will be on basic science and molecular and cellular mechanisms; understanding the fundamental interplay between physical forces and immune cells. In the long term, the hope is to further explore translational approaches that can improve outcomes of antibody-based, cell-based, and small molecule-based immunotherapy in preclinical studies. The ultimate goal is to reveal new insights and treatment targets that can be rapidly translated to the clinic.

In addition to the science, as an academic I am excited by the mentoring and teaching side of my job. It is a major focus of my career and also a personal joy. In particular, I am committed to justice, diversity, equity, and inclusion initiatives. It is my duty as a Principal

Investigator (PI) to provide a safe and supportive environment for people from any background, especially from under-represented groups, to learn about and perform interesting researchers in science, technology, engineering, and mathematics (STEM) fields if that's where their interest lies. I am training the next generation of cancer researchers and I am privileged to be in a position to arm them with the tools necessary to move forward on their own and accomplish impactful work. Our field can only benefit from welcoming unique perspectives, such as integrating under-represented engineers into cancer immunology research.

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Contributions: *All named authors take responsibility for the integrity of the work as a whole, and have given their approval for this version to be published.*

Acknowledgements: *None.*

Disclosure and potential conflicts of interest: *The author declares that they have no conflicts of interest.*

Funding declaration: *The author receives funding from: NIH/NCI K22 CA258410.*

Article source: *Invited.*

Interview conducted: *Jul 20 2022;* ***Publication date:*** *Aug 31 2022.*

DOI: 10.18609/ioi.2022.039

IMMUNO-ONCOLOGY INSIGHTS

LEVERAGING THE CUTTING-EDGE TME TOOLKIT

SPOTLIGHT

Immune modulatory vaccines: the safe way to inflame the tumor microenvironment & guide anti-cancer immunotherapies

Mads Hald Andersen

"Mapping the cell types and molecules present in the inimical tumor milieu will support the development of more effective IMVs and teach us how best to combine the current available options."

VIEWPOINT

Immuno-Oncology Insights 2022; 3(9), 401–407

DOI: 10.18609/ioi.2022.043

The tumor microenvironment (TME) is a highly heterogeneous milieu that consists of many different cell types, including tumor cells, stromal cells, and immune cells. In general, the differentiation, maturation, and function of immune cells are regulated by cytokines, chemical factors, and interactions between receptors and related ligands. In cancer, these factors comprise a TME that promotes tumor formation, progression, and metastasis, and at the same time, it hampers anti-tumor immunity. This hostile environment is created by the involvement of many different regulatory cell types, including tumor-associated macrophages (TAMs), tumor-associated neutrophils (TANs), myeloid-derived suppressor cells (MDSCs), cancer-associated fibroblasts (CAFs), and regulatory T cells (Tregs) [1–4]. The modulation of immune regulatory mechanisms was first achieved with immune checkpoint blockers (ICBs), which block the activation of immunosuppressive receptors on T-cells, either directly or indirectly through their ligands [5]. Currently, many therapeutic strategies focus on how best to regulate or diminish the effects of immunosuppressive cells. Some strategies aim to deplete or reprogram these cells, and other strategies target the functional mediators secreted by these cells [1,6].

Anti-regulatory T cells (anti-Tregs) are defined as T cells that specifically react to regulatory immune cells, including CAFs, Tregs, MDSCs, and TAMs. Anti-Tregs restrict the range of immunosuppressive signals mediated by these cells [7,8]. Anti-Tregs have been shown to recognize the HLA-restricted epitopes of proteins, including metabolic enzymes, like indoleamine 2,3-dioxygenase (IDO) [9–11] and arginase (ARG) [12–14], checkpoint molecules, like programmed death-ligand-1 (PD-L1) and PD-L2 [15-18], cytokines, like TGFβ [19, 20] and CCL22 [21, 22], and transcription factors, like FoxP3 [23]. Under normal conditions, the immune-regulatory system employs anti-Tregs to maintain immune homeostasis [24]. PD-L1- and IDO specific T cells have been shown to expand as a counter-response to inflammation [11,25]. Activated anti-Tregs can revert the microenvironment to a pro-inflammatory immune state by inhibiting the effects of suppressor cells [11,12,16,17,26]. In the context of cancer, the activation of anti-Tregs leads to both a direct attack on tumor cells and the modulation of the TME, rendering it immunocompetent and tumor-hostile. Immune modulatory vaccines (IMVs) are a novel therapeutic strategy that aims to activate anti-Tregs. Therefore, in contrast to other clinical strategies, which target the immunosuppressive environment, the effects of IMVs include both the depletion of suppressive cells (through direct killing by activating cytotoxic T cells) and the reprogramming of suppressive cells (by stimulating the release of pro-inflammatory cytokines). The latter approach includes stimulating the conversion of M2-like into M1-like macrophages and the conversion of immunosuppressive CAFs into immunocompetent fibroblasts. Hence, considering the highly immunosuppressive roles of fibroblasts and other stromal cells [2,27], IMVs also offer a means of targeting the functions of these cells.

The first clinical testing of an IMV was in patients with non-small-cell lung carcinoma (NSCLC) who were vaccinated with an IDO-based vaccine [28]. Though this was a small, phase I trial, vaccinated patients had a significantly longer median overall survival (26 months) compared to untreated control patients (8 months; P=0.03). Additionally, two patients showed long-term responses, without any other treatment, for 7 years after the first vaccination [29]. Even more encouraging, in a recent phase II trial, the combination of an IDO- and PD-L1-based IMV with an anti-PD1 antibody showed remarkable clinical effects as a first-line treatment in patients with metastatic melanoma. In that study, the objective response rate was 80%, and the complete response rate was 43% [30]. Over the years, many activating immunotherapies have failed to show clinical benefit, likely because we previously lacked understanding of immunosuppression in patients with cancer. Although ICB can sometimes effectively release the TME suppression

DOI: 10.18609/ioi.2022.043

of T-cell activities, ICB therapy relies on de novo T-cell activation. Thus, ICBs are known to work best in inflamed (or 'hot') tumors [31]. Due to the dual mechanisms of action of IMVs, combinations of immune-activating IMVs and ICBs are an appealing approach. IMVs can induce novel T cell activation and additionally target immunosuppressive cells in the TME. Therefore, the immune-modulatory effects of IMVs in combination with ICBs could increase the number of patients that respond to treatment. In addition, the combination of IMVs with other immunotherapies, like adoptive cell therapies or traditional cancer vaccines, is also an attractive approach. In this regard, the activation of anti-Tregs has been shown to increase immunity to cancer antigens in cancer-vaccine models, both *in vitro* [16,32] and *in vivo* [33].

In the above-mentioned melanoma trial, the vaccine activated both CD8 and CD4 responses. Generally, this important feature of IMVs is not found in traditional cancer vaccines, which mainly focus on activating CD8 cells. Both *in vivo* and *in vitro* studies have demonstrated that activating cytotoxic $CD8^+$ anti-Tregs could lead to the direct elimination of target cells, including melanoma and myeloid cells [11,16–18,21,26,34–39]. However, $CD4^+$ anti-Tregs are more potent in the release of pro-inflammatory cytokines. This feature may be important for IMV effectiveness because it provides a means to reprogram the TME to favor tumor rejection by supporting anti-tumor Tcell responses and stimulating antigen presentation. Many immune regulatory cells can be reverted into immune effector cells in a pro-inflammatory microenvironment; e.g., M2 macrophages (TAMs) were shown to revert to M1 macrophages [40]. Indeed, the melanoma trial illustrated that the induction of a pro-inflammatory TME was correlated with the re-polarization of innate immune cells, measured as an increase in class II HLA expression [30]. The importance of combining CD4 and CD8 T-cell epitopes in IMVs has also been illustrated *in vivo*, in animal models of cancer [26, 41]. Another advantage of IMVs, compared to traditional cancer vaccines, is that they can target non-transformed cells that have consistent human leukocyte antigen (HLA) expression. Inflammation often induces HLA expression in malignant cells; consequently, inflammation induced by IMVs should increase surface HLA expression on tumors. Hence, in contrast to traditional cancer vaccines, IMVs may have an impact on tumors with low HLA expression. Furthermore, IMVs may have therapeutic effects, regardless of whether the tumor cells express the cognate target antigens. For example, in a CT26-cancer animal model, an IDO-based vaccination inhibited tumor growth, even though only the myeloid cells in the TME expressed IDO [39]. Similarly, in a small clinical trial, patients treated with a PD-L1-based IMV showed regression in PD-L1-negative basal cell carcinoma cells [42]. The expression of both IDO and PD-L1 are known to increase in response to inflammatory stimuli, like type 1 and 2 interferons. This upregulation subsequently increases their recognition by IDO- and PD-L1–specific T cells [11,18]. This phenomenon should be considered when utilizing IMVs. The TME is often characterized by the presence of regulatory cells that express proteins, like ARG or TGFβ, which inhibit infiltration by T cells [27,43]. This type of environment may be altered by pro-inflammatory ARG- or TGFβ-specific anti-Tregs, which change the environment by exposing cells that are susceptible to further T-cell attack by, e.g., IDO- and PD-L1–specific T cells. Thus, combining different antigens in IMVs is an attractive therapeutic approach that might yield synergistic effects [44].

Importantly, to date, IMVs have been well tolerated in patients; they have not been associated with grade III or IV toxicity. As mentioned above, in the first clinical trial of an IDO vaccination in patients with non-small cell lung cancer [28], two patients continued vaccinations every 4 weeks for 5 years without any toxicity, and the presence of IDO-specific T cells was confirmed during treatment [29]. Similarly, in another study, ten patients with multiple myeloma

received, over the course of a year, 15 vaccinations with a PD-L1–derived peptide vaccine that induced a PD-L1–specific immune responses in all patients [45]. All adverse reactions to the PD-L1 vaccine were below grade III toxicity, according to common criteria, and most were grades I-II injection-site reactions [45]. Even when combined with anti-PD-1 antibody therapy, the systemic toxicity profile of a combination IDO- and PD-L1–based vaccination was comparable to that of anti-PD-1 antibody monotherapy [30]. The safety of IMVs has also been confirmed in several *in vivo* models of cancer [12,26]. In fact, anti-Tregs have been found spontaneously in the periphery of healthy individuals, without any associated toxicity [13,18,20,34,36,46]. Therefore, IMVs that rebalance the microenvironment seem to represent a safe means of increasing the effects of T-cell–enhancing immunotherapies. In conclusion, IMVs offer an attractive, novel approach for targeting the TME, when designing new immunotherapies and new treatment protocols for patients with cancer. In contrast to traditional cancer vaccines, which activate T cells that target the tumor directly, IMVs target the entire TME. IMVs kill tumor cells, both directly and indirectly, by modulating the TME and/or effector immune cells. Mapping the cell types and molecules present in the inimical tumor milieu will support the development of more effective IMVs and teach us how best to combine the current available options.

BIOGRAPHY

MADS HALD ANDERSEN is Director and Professor at the National Center for Cancer Immune Therapy (CCIT-dk) at Copenhagen University Hospital at Herlev. He has achieved doctoral degrees from both Copenhagen University as well as The Technical University of Denmark. He co-founded CCTT-dk in 2006. Professor Andersen has considerable pharmaceutical experience, and his research has laid the foundation for several biotech companies including Survac, and most recently IO Biotech. The latter is listed on Nasdaq, New York. He has been honored with several awards during his career. He has an extensive publication record, authoring more than 200 in peer reviewed journals, more than 20 patents as well as several book chapters. His research has been focusing on the characterization of the natural immune responses in cancer. He has identified several different T-cell antigens including survivin, the Bcl-2 family and RhoC. Professor Andersen has pioneered the field of immune modulating vaccines in cancer. He described the existence of circulating effector T cells that specifically target normal, self-proteins, e.g. IDO, PD-L1, Arginase, and TGFβ, that are expressed by regulatory immune cells. He defined these as 'anti-regulatory T cells' (anti-Tregs) since they are able to inhibit the effects of suppressor cells. Several clinical vaccination trials are currently running based on this research of Professor Andersen.

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DOI: 10.18609/ioi.2022.043

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DOI: 10.18609/ioi.2022.043

AUTHORSHIP & CONFLICT OF INTEREST

Contributions: *All named authors take responsibility for the integrity of the work as a whole, and have given their approval for this version to be published.*

Acknowledgements: *The author would like to thank all current and previous students, colleagues, and collaborators involved in the discovery and characterization of anti-regulatory T cells. This work was supported by Herlev Hospital.*

Disclosure and potential conflicts of interest*: Andersen MH discloses he is a shareholder and scientific advisor of IO Biotech ApS. He also discloses that they are an author of several patents and filed patent applications based on the use of activating anti-regulatory T cells byt vaccination. The rights of the patent applications have been transferred to Copenhagen University Hospital, Herlev, according to the Danish Law of Public Inventions at Public Research Institutions. The capital region has licensed some of these patents to the company IO Biotech.*

Funding declaration: *The author received financial support for the research, authorship and/or publication of this article from Herlev Hospital. The author has grants from Danish Cancer Society, Novo Nordisk Foundation, Vissing Foundation, The Independent Research Fund Denmark, Innovation Fund Denmark, the Danish Health Authority and Copenhagen University.*

ARTICLE & COPYRIGHT INFORMATION

Article source: *Invited; externally peer reviewed.*

Submitted for peer review: *Sep 19 2022*; ***Revised manuscript received:*** *Sep 19 2022*; ***Publication date:*** *Sep 29 2022.*

IMMUNO-ONCOLOGY INSIGHTS

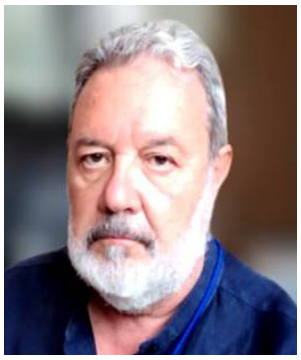
LEVERAGING THE CUTTING-EDGE TME TOOLKIT

SPOTLIGHT

INTERVIEW

Exploring the epigenetics of the TME

Roisin McGuigan, Editor, *Immuno-Oncology Insights*, **speaks to Jesús M Paramio,** Head of the Molecular and Translational Oncology Division CIEMAT & Head of the Cell and Molecular Oncology Group, Institute of Biomedical Investigation University Hospital "12 de octubre" Madrid, Spain



DR JESÚS M PARAMIO studied Biology at the University Autonoma of Madrid and specialized in Biochemistry and Molecular Biology (1979-84). He got a PhD in Biochemistry and Molecular Biology at the same University in 1992. He then moved to University of Dundee to work in the group of Prof EB Lane in close collaboration with Prof DP Lane and returned to CIEMAT in 1996, where he started new projects on epidermal differentiation, carcinogenesis and cell cycle using transgenic mouse models. In 2000, Jesús went to NIDCR/NIH to study signal transduction processes in epidermal differentiation and carcinogenesis. He then returned to CIEMAT to establish his own group focused on molecular and translational oncology with particular focus on transgenic mouse models and genomic analyses. In 2011, he also started a collaboration with a group of oncologists, pathologists, and urologist to study genitourinary (mainly bladder) cancer, including patient samples in his own studies. In 2014, formed a joint group between CIEMAT and University Hospital 12 de Octubre located at the Hospital and started collaboration with some Pharma companies. His current work is focused on molecular and translational characterization of bladder cancer, including some academic or industry supported clinical trials, although still does some work on other cancer types (head and neck, lung, kidney, ovarian, etc.). Most of his work is focused on aspects of epigenetic plasticity, immunotherapy and personalized medicine approaches.

Immuno-Oncology Insights 2022; 3(9), 417–422

DOI: 10.18609/ioi.2022.045

What role do epigenetic modifications play in creating an immunosuppressive tumor microenvironment (TME), and how can further understanding of these epigenetic modifications help to better predict or enhance response to immuno-oncology (I–O) agents? In this interview, Jesús M Paramio, Head of the Molecular and Translational Oncology Division, CIEMAT, discusses his work in bladder cancer, the importance of interdisciplinary research, and the complex epigenetic factors at play within the TME.

Q How long have you been working in cancer research?

JMP: I am a molecular biologist, and I have been working on different aspects of cancer, mostly on basic aspects, for more than 30 years. Most of my time has been spent working on mouse models of cancer trying to reproduce the molecular patterns of human disease; generating gene deletions or activity in genes that are the same as in patients. We are following different approaches for that.

The other aspect is validating the models – as there are obvious differences between humans and mice, we have to develop new approaches. We started around 10 or 15 years ago with functional transcriptomics, trying to figure out whether the disease in the animal fully recapitulates what happens in humans in terms of differential gene expression.

We then decided to pursue more translational research. This involves a joint group of medical oncologists, pathologists and urologists. Since 2010 we have mostly been working on genitourinary cancer, particularly bladder cancer. We have started a new hospital-based lab that provides the opportunity to work with more realistic problems in terms of the clinic, which we are really enjoying.

I have always looked at medicine as a very attractive field – I never wanted to treat patients, but rather wanted to understand the disease. With these present models, we are working in different preclinical settings and characterizing patient samples using functional genomics. We are developing new clinical trials, focusing on aspects of bladder cancer, and also other related areas like liquid biopsy.

Epigenetics plays a crucial role in bladder cancer, as many genes affecting chromatin remodeling are mutated or display altered expression. Treating these proteins is a very attractive approach. Immunotherapy ties into this too because when we analyze the epigenetics of bladder cancer, and manipulate the chromatin remodeling system, we find that many of the genes that are differentially expressed are related to the immune system. So, it was obvious to go there.

Q Why is working as part of a multidisciplinary team so important?

JMP: It is not possible to understand anything related to a certain disease if you don't have the opportunity to talk with medical doctors that treat it. They have the patient in front of them, and they provide you with crucial questions. We, as molecular biologists, may have the ability to provide answers to some of those questions in terms of genomics, modelling the disease, finding the best drug, and then moderating the disease and

DOI: 10.18609/ioi.2022.045

analyzing the effect of the drug. Ultimately what we all (basic researchers and clinicians) want to do is to cure people, or at least to make their lives more comfortable. Therefore, incorporating all possible points of view is strictly required.

Q What role do epigenetic modifications play in creating an immunosuppressive environment?

JMP: In all types of cancer, you have to bear in mind that it is a very complex system. When you have tumor cells you also have different stromal cells, endothelial, supporting fibroblasts, and many other types of adaptive and innate immune cells.

At the same time, likely through the signals that emerge from the tumor, all the cells change their behavior. They have very plastic activities, changing to favor the growth of the tumor. Even if the body is sending cells that will kill the lesion, there will also be crosstalk in this sense. If you change the behavior of the cells and the pattern of gene expression – in most cases without altering the gene content of the cells, i.e., the genomic structure – this means that epigenetic changes are modifying this behavior and countering this crosstalk.

Therefore, you need to understand the types of cells and how they are behaving in the context of cancer – or in the context of cancer and therapy, which is even more complex. Many people are focused solely on immune cells and cancer cells and how they crosstalk, but you also have to consider how other cells, like fibroblasts, modify this behavior. For instance, they become cancer-associated fibroblasts, and in many circumstances can produce cytokines like TGF-β which reduce immune cell activity.

This level of complexity makes it difficult to study. Most of our studies are done in bulk. Even if we have tools like deconvolution approaches to see the specific population of cells at the end, we still need to analyze different types of cells by themselves, their position and look at how they are changing. The second part is that even if you can do that, you only have a picture of that process. You need to add more frames to create the whole movie. This poses both technical and cost issues that make it difficult in the lab.

Q How can further understanding of these epigenetic modifications help to better predict or enhance response to I–O therapies such as checkpoint inhibitors?

JMP: We need to understand the behavior of different cells, how they change, and then we can envisage what types of modification or what drivers can cause this modification in each type of cell. From that point it's much easier to

"In all types of cancer, you have to bear in mind that it is a very complex system. When you have tumor cells you also have different stromal cells, endothelial, supporting fibroblasts, and many other types of adaptive and innate immune cells."

design the drug that you want to have. Artificial intelligence approaches such as neural networks and deep learning can be of value for these purposes.

In terms of our own work, we have developed mouse models in which we can modify tumor cells or other tumor microenvironment (TME) cells. We can try drugs that are currently in the clinic, or close to the clinic, and look at how the cells in the tumors change their behavior in the presence of the drug. From that, you can infer what will happen in humans. In human patients, it's difficult to get this type of analysis, such as single cell multiomics, but you may be able to perform bulk and/or spatial analysis before and after treatment. You can then try to see whether what you observe is similar to what you would expect from the mouse system.

I'm very optimistic in terms of being able to identify potential biomarkers. I don't know if we have to go to the tumor or if we have to go to the circulating tumor cells, or elsewhere, but I'm very optimistic about achieving that.

Q What for you is the current state-of-the-art in terms of the tools and technologies you are using to interrogate the TME?

JMP: Single-cell analysis is crucial. The problem with single-cell is that you can go for ATAC-Seq, RNA-Seq, or methylation to analyze different aspects, but in many cases, you cannot do it in the same cell.

We are trying to do single-cell multiomics which includes ATAC-Seq and RNA-Seq in the same cells, because you can purify nuclei, for example. The second main approach is spatial transcriptomics – looking at not only what changes in the cells, but also where the cells are located, which is crucial for the interpretation of the data and for the validation of therapies. We have learned from developmental Biology how relevant position of a signaling cell is in the context of a tissue. We are also trying to include metabolomics studies in the same context, but we are just starting this. These technologies are key. We also need the support of expert bioinformatics, which represent a major bottleneck. Funding is also a problem, but it's a problem that you can solve. Another is finding people trained in this field, who can solve these complex problems. In many circumstances supercomputing approaches are valuable tools.

In this regard, we are also looking at many other things like digital pathology, artificial intelligence, and computer-learning neural networks, and of course, all of this needs to be brought together.

We would like to carry out single-cell characterization in clinical specimens, but it is challenging from a logistic point of view. It is difficult to get fresh samples of tumor for performing this type of analysis. In contrast, running spatial transcriptomic analysis is very simple to do with clinical specimens.

Q ... and what do you predict for the future in this area?

 DOI: 10.18609/ioi.2022.045

JMP: It is hard to predict – the technologies in genomics are moving so fast that it is almost impossible to keep up. If you look at the journals and at the different software approaches that people are using, learning to do it all at the same time as pursuing your own research would prove very difficult.

I think that new fields like deep learning are exciting – the possibility that computers may help us in looking for things that we currently cannot look at properly. We have also started a collaboration with a physicist who works in high energy physics to try to identify patterns in digital pathology specimens. I also want to include in that all the genomic data and put together all of this information to predict whether the patient will respond to a specific therapy. This is beautiful work, but very challenging.

The last aspect is liquid biopsy; not only in terms of looking for the circulating tumor cells or DNA – which in itself is extremely interesting, because in some cases this can come from the fusion of tumor cells with other cells in the TME – but also to look for specific biomolecules or even metabolites that can be easily analyzed in fluids. For instance, in the case of bladder cancer, it's easy to look at urine. There are many more possibilities for detecting or knowing in a predictive manner whether the patient has a tumor or will respond to a particular therapy. There are some clinically oriented tests, but their applicability is still under development as they may have some relevant caveats.

Q How and where do you see better understanding of epigenetic factors impacting the I–O space? And what about the potential of I–O agents in combination with epidrugs?

JMP: Understanding and integrating epigenetics in the context of the spatial distribution of cells, behavior of cells, and changes that occur during tumor progression will provide a huge source of data for the development of new therapies.

With epidrugs, I think innovation in these proteins will open up a really interesting new field in the near future, but this may also have another side. The same protein can be playing different roles in different tissues. If you keep one protein in a tissue to kill cancer cells, that would be great. But you could be inhibiting the same protein in other cells where it is playing a role as a tumor suppressor. So, it is very attractive but may prove difficult in practice, in some cases. We would like to see the epidrugs that we have already tried in the lab go into the clinic, but this is something that we are not actively working on because there are regulatory aspects that are not covered in the lab.

We also think that advanced cell therapies used for the treatment of tumors using modified T or NK cells can be combined with epidrugs. For example, to avoid the exhaustion of CAR-T cells by using specific compounds. Many aspects of T cell exhaustion, even from the point of view of the metabolic lactate, or proton pumps, are also governed by epigenetic mechanisms. Everything is playing a part in the same game.

Q What will be your own goals and chief priorities over the next few years?

JMP: I would like to implement all of the approaches I discussed as soon as possible in the lab – but these are very expensive experiments, so cost does pose an issue.

Among other goals in bladder cancer, either non-muscle invasive or muscle invasive disease, one of my main goals is to understand the metastatic dissemination process in bladder cancer and try to avoid that and try to cure metastatic disease and/or provide new approaches for it, as at present it is incurable.

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Contributions: *All named authors take responsibility for the integrity of the work as a whole, and have given their approval for this version to be published.*

Acknowledgements: *None.*

Disclosure and potential conflicts of interest: *The author declares that they have no conflicts of interest.*

Funding declaration: *The author received no financial support for the research, authorship and/or publication of this article.*

Article source: *This article is based on a Interview which took place on Sep 15 2022*

Interview conducted: *Sep 15 2022*; ***Revised manuscript received:*** *Oct 5 2022*; ***Publication date:*** *Oct 7 2022.*

DOI: 10.18609/ioi.2022.045

LEVERAGING THE CUTTING-EDGE TME TOOLKIT

SPOTLIGHT

INTERVIEW

Exploring approaches for tackling the complexity of the tumor microenvironment: multi-parametric methods, new biomarkers & serial testing

Roisin McGuigan, Editor, Immuno-*oncology Insights*, speaks to **OncXerna Therapeutics' Laura Benjamin,** Founder and CEO and **Mark Uhlik,** Vice President, Head of Research and Biomarker Discovery about their work on enabling tools and technologies for exploring and understanding the tumor microenvironment, and OnXerna's clinical pipeline.

LAURA BENJAMIN received a PhD in Molecular Biology at the University of Pennsylvania in 1995, and BA in Biology from Barnard College, Columbia University in 1987. For her PhD thesis, she characterized a pediatric cancer-driving fusion protein and methodologies to extract and amplify that chromosomal fusion from paraffin-embedded tissues, an early foray into molecular pathology. Following a 20+ year career in academic research and pharmaceutical R&D, Laura followed her mission to driving change in cancer drug development and was the sole founder of OncXerna Therapeutics, a global precision medicine company with headquarters in Boston. The company is aiming to deliver next-generation precision medicine strategies for cancer patients currently underserved by today's approaches.

MARK UHLIK is a seasoned leader in translational oncology and drug discovery/development with over 15 years of experience in the pharmaceutical industry. Mark earned his BSc in Microbiology from Colorado State University and his PhD in Microbiology and Immunology at Pennsylvania State University College of Medicine. He performed post-doctoral work at University of Colorado Health Sciences Center and at University of North Carolina School of Medicine. Mark has authored over 35 peer-reviewed publications in addition to book chapters and patents.

Immuno-Oncology Insights 2022; 3(9), 441–447

DOI: 10.18609/ioi.2022.047

Q Can you tell me a bit about your current roles?

MU: I am Vice President of Research and Biomarker Discovery. I have been with OncXerna for a year and a half, and my current role is focused on the Xerna biomarker Platform; supporting it from both discovery and validation as well as working closely with our collaborators and licensees. We have numerous academic collaborators and investigators who are providing us with data sets, as well as agreements with both QIAGEN and Exact Sciences for commercial development of the Xerna TME Panel.

LB: I am the founder and CEO of OncXerna. I led the initial strategy and work to build the Xerna Platform, and we also have two clinical assets that we are currently advancing.

Q In your view, how is our understanding of the TME and mechanisms of tumor resistance to immunotherapy current evolving?

MU: With the increased utilization of immune checkpoint inhibitors (ICIs) across a wide variety of solid tumor indications, there has been an explosion of patients that have been exposed to and progressed on ICIs. ICIs are being used in earlier and earlier lines as well as in combinations with chemotherapies and targeted therapies. As a result, we have a large and heterogeneous population of patients with ICI-experienced cancers, and this has led to the need for next-generation immunotherapies. We are still in the early stages of understanding ICI-resistance and being able to predict which follow-on therapies patients should be receiving post-ICIs. Next-generation sequencing technologies are being employed to aid in understanding the molecular mechanisms of ICI resistance, as are other emerging technologies such as digital pathology and digital spatial imaging. The field is trying to assemble as complete a picture as we can of the tumor constituents, immune cellular phenotypes,

and activation states, to understand what progression on ICIs actually looks like. A major obstacle, in particular with regard to immune cells, is that the tumor microenvironment (TME) is a moving target – it dynamically changes in response to treatment . Thus, an important feature to consider is that the patient's TME biology after progressing on therapy is likely to be quite different than how it looked before that therapy. This necessitates serial testing of a patient's tumor during their treatment to ensure the 'current' biology is being assessed and properly treated. While biopsies are becoming more commonplace, this is still a challenge for both investigators and patients.

"We imagine the Xerna Panel will be used in helping to identify which patients might be responsive to ICI treatments, but who are not receiving those treatments today, and patients who would benefit from combinations in the immuno-oncology space." - Laura Benjamin

As our knowledge of the exceedingly complex TME evolves, we believe the field needs multi-parametric methods to interrogate the biology of the TME to a sufficient degree. One of the strengths of the Xerna platform and the Xerna TME Panel is picking up on that complexity. Right now, most of the landscape is dominated by single-analyte biomarkers, even in immune therapeutics where PD–L1 is dominating.

LB: We imagine the Xerna Panel will be used in helping to identify which patients might be responsive to ICI treatments, but who are not receiving those treatments today, and patients who would benefit from combinations in the immuno-oncology space. In the context of resistance, we see that the TME can change in response to checkpoint inhibitor treatment in ways that we think correlate with the emergence of resistance. This plays into the idea of restoring sensitivity. In the resistance space, there are also mutations and other factors that can lead to the loss of the antigen. The Xerna is not necessarily going to pick up changes like this, but it does have utility in helping direct how to treat each patient best, not only in the setting of resistance but also from the beginning of treatment.

Q How and where do you see this understanding impacting the clinical development of immune-oncology agents on an ongoing basis – for instance, in terms of innovation in clinical biomarkers or trial design?

MU: Utilization of biomarkers is becoming essential. With ICIs, we have seen variations in the biomarkers utilized as well as potential thresholds employed depending on the indication and mechanism of action of the drug. Many ICIs rely on PD–L1 testing, and they each have slightly varying thresholds and utilizations. There needs to be some degree of synching up on biomarker understanding and how they relate to mechanisms to get a true

appreciation of which patients are going to benefit from this class of drugs. Combinations of biomarkers may be useful – for example, combining PD–L1 testing with something like the Xerna TME Panel may provide additional information that could help segment the population that is going to receive the most clinical benefit.

One of the most important aspects of biology that we can understand within the field of immunotherapy is the nature of resistance to ICIs. It is likely that in addition to previously identified molecular mechanisms of resistance, such as mutations in antigen presentation and JAK/STAT/IFN signaling, there will be a continued understanding of cellular mechanisms involved. Understanding of the biology of immune suppression and the cellular components involved is still in its relative infancy. We have focused on the interplay of angiogenesis and immune cell biologies with our TME Panel because we believe these are highly interconnected, and dynamically regulate each other. Especially with ICI resistance, it is likely the TME is in a state of immune suppression and additional immune checkpoints or regulation is in place. This necessitates new immune-directed agents, such as myeloid targeting agents that may re-polarize to immune active states, aid in increasing antigen presentation, or prevent T cell anergy.

Our understanding of the TME and its relationship to immunotherapy dictates that we need as contemporary a view of the patient's disease state as we can. This includes molecular and pathological testing of a patient's tumor after each treatment failure and before starting each new therapy, and biopsies for biomarker testing to inform follow-on therapies. This will lead to trial designs and clinical practice implementing more collection of biopsies and biomarker testing between therapies, rather than relying on archival biopsy samples for guiding treatment. The Xerna TME Panel focuses on the biology of the TME at the tumor site, which we believe is the most important location to assess the dominant biology of the patient's disease. Emerging technologies, such as liquid biopsies, may only show limited utility in guiding which follow-on therapies a patient should receive. However, time will tell if liquid biopsies can provide important information, such as T cell clonality, tumor mutational burden, or even activation/repression states of immune cells, that may ultimately guide some future therapeutics.

LB: We can use the Xerna TME Panel to identify patients that will benefit even when they are PD–L1 negative. On the other hand, we can see that there is an orthogonal power that seems to be additive. Seeing a patient that is PD–L1 positive and in our immune active quadrant of the Xerna TME Panel is more powerful than either alone. There is still much learning to do through clinical trials, but the idea is that this is an assay that has already been analytically validated, so could be easily used for patient selection in a clinical trial. It is based on RNA-sequencing of formalin-fixed paraffin-embedded tissue to look at ~100 genes expressed in the TME, that were carefully curated to comprise the panel.

The machine learning algorithm employed utilizes all those genes to capture dominant biological phenotypes. We assign four phenotypes as the output of this algorithm. One of the phenotypes represents an ongoing activated immune response to the tumor. These are the patients who are still on the tipping point of getting benefits from their immune system, and who get the most impact from a checkpoint inhibitor like a PD1 or PD–L1 inhibitor. On the other hand, we have a group of patients with an ongoing immune response, but one which is more predominantly immunosuppressive, including regulatory T cells,

 DOI: 10.18609/ioi.2022.047

macrophages, myeloid suppressor cells, cytokines, and TGF-beta. These immunosuppressive pathways get in the way of checkpoint inhibitor impact. It does not mean that we do not see any responses in those groups, but we feel that it would be better to eliminate the immunosuppressive phenotype. This is where combinations with ICIs are going to find their home in the future, such as those that include innate immune modulators.

Post-progression on a checkpoint inhibitor, we see a prevalence shift. Admittedly, we are looking at relatively small cohorts, but we see that there is an enrichment in this immunosuppressive group, or a loss of the immune signal altogether. We see a reduction in those immune active patients when we look in a cohort of patients that are now refractory to ICIs. This is part of how the tumor might be evading or getting acquired resistance.

The third phenotype in this panel has a pathological angiogenesis signature. There have been principal studies showing the combinations of angiogenesis and immune therapy can be beneficial, this may be predominantly in patients with an angiogenesis-driven tumor microenvironment.

Finally, there is a group of patients that are described as immune desert. These patients do not have a strong signal either for an active immune response or immune suppression, nor do they have much of a pathological vascular signal. These patients anecdotally seem to have hallmarks of tumor proliferation, perhaps more pronounced, and will maybe benefit more from chemotherapies for example. In the context of immune therapy for these patients, you might think about CAR-Ts or vaccines.

Q What do you see on the horizon in terms of enabling tools and technologies with the potential to further increase understanding of the TME, for you?

LB: It's clear that often a single biomarker, whether based on DNA, RNA or protein does not perform as robustly as we would like, probably due the complexity of tumor biology. There are advances into more complex tests using multiple analytes. For example, multi-analyte immunohistochemistry (IHC) is a technology that we see increasingly used. We also see groups working on proteomic approaches using multiple analytes. Those have a way to go before they can be widely implemented globally to support clinical trials, in terms of throughput and technical challenges. We feel that it was the right time to focus on multi-gene panels using RNA sequencing. DNA sequencing is now well in hand, and RNA sequencing is becoming more accessible and reliable when using formalin-fixed tissues. We felt like the technical components that are needed to underly this are ready now. However, as time goes on, looking at protein is going to be helpful as well. Ultimately, the combined information from different biomarker modalities may be complementary and provide the most powerful means of patient tailoring.

MU: Historically archival biopsies have been used for biomarker analysis, and these may be sufficient in many cases for things like DNA mutations which might not change as readily through the course of therapy. The Xerna TME panel had been

used successfully on archival tissues, particularly in informing the nature of the angiogenic biology axis, which appears to be a bit more stable through treatments. But we have seen that the biology of the microenvironment can change upon successive therapies. The tumor immune microenvironment appears to be especially dynamic through a patient's treatment journey, so getting tissues to inform the next stage of treatment is important, particularly for guiding immune-directed therapies. Successive biopsies are becoming more and more commonplace in treatment. This is the nature of precision medicine and where it's going.

" Successive biopsies are becoming more and more commonplace in treatment. This is the nature of precision medicine and where it's going."
- Mark Uhlik

Q What will be your own goals and priorities in the next few years?

LB: We have a clinical pipeline that we are advancing. We built this panel to support our portfolio, and then recognized how broadly useful it could be to the whole field. With respect to the Xerna Panel, we enjoy that our contribution is helpful to support partnering and collaborations. We are open to academic collaborations to help broaden our understanding of how the panel works, how the microenvironment works, and how different drugs interact with the microenvironment.

We think that the approach that we have taken using RNA to capture biology and using biology as opposed to genetics to direct therapies is one that will enable us to advance the field of precision oncology. There is going to come a day when we get diminishing returns from only focusing on DNA mutations. We are on the third and fourth, and sometimes fifth generations of looking at mutations in the same genes, with ever smaller segments of the population. However, there are big swathes of the population that need precision medicine tools, and new therapeutic agents that are not targeting mutations. There is still a lot of room for the development of this approach, which falls into the Xerna platform, and building additional panels and thinking more broadly about other biological drivers of cancer is of interest to us. Focusing on the Xerna Platform, we have ideas for some next steps in terms of other key biological drivers of oncology and tumor progression. That would be an exciting place to go. We would like to see more uptake in general from other therapeutic classes, diving deeper into those biologies, because those are going to be emerging therapies.

MU: Over the next few years we have the goal of furthering the development of our clinical assets navicixizumab and bavituximab. Navicixizumab is our most mature asset, and it is a bispecific agent targeting DLL4 and VEGF that acts as an anti-angiogenic agent. Bavituximab is a monoclonal antibody that targets phosphatidylserine, which is involved in immune suppression. Both are attractive therapies to combine with ICIs. Each agent may address different mechanism of ICI resistance supporting evaluation in post-ICI failures.

DOI: 10.18609/ioi.2022.047

We also see tremendous potential for the utilization of the Xerna TME Panel to support additional immunotherapies (and anti-angiogenic and other TME-directed therapies) beyond our own existing pipeline assets. We would be excited to bring in additional assets or partner on the development of therapies that capitalize on the biomarker potential of our Xerna TME Panel.

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AUTHORSHIP & CONFLICT OF INTEREST

Contributions: *All named authors take responsibility for the integrity of the work as a whole, and have given their approval for this version to be published.*

Acknowledgements: *None.*

Disclosure and potential conflicts of interest: *Uhlik M discloses he has patents planned, issued or pending from OncXerna Therapeutics, Inc. He also has stocks/stock options from OncXerna Therapeutics, Inc., Hibercell, Inc. (previously Biothera Pharmaceuticals, Inc.) Rigel. Benjamin L discloses she has patents planned, issued or pending from OncXerna who have filed patents related to the subject of the article. She also discloses she has stocks from OncXerna Therapeutics, Inc.*

Funding declaration: *Uhlik M and Benjamin L received financial support for the research, authorship and/or publication of this article from OncXerna Therapeutics, Inc.*

Article source: *Invited.*

Interview conducted: *Sep 8 2022;* ***Publication date:*** *Nov 1 2022*

IMMUNO-ONCOLOGY INSIGHTS

LEVERAGING THE CUTTING-EDGE TME TOOLKIT

SPOTLIGHT

INNOVATOR INSIGHT

Modulation of both tumor & T cell apoptosis to enhance CAR-T immunotherapy

Marco Ruella

CAR-T cell immunotherapy is leading to outstanding clinical results, but only one-third of patients have long-term responses in mature lymphomas. Resistance to apoptosis in cancer cells is a key feature of CAR-T immunotherapy failure, and strategies to enhance tumor apoptosis during CAR-T therapy lead to better tumor control. This article will highlight the importance of apoptosis in both cancer cells and CAR-T cells in driving response to CAR-T immunotherapy, and describes potential strategies to overcome resistance.

Immuno-Oncology Insights 2022; 3(9), 527–539

DOI: 10.18609/ioi.2022.053

BUILDING UPON THE SUCCESS OF CAR-T THERAPY

CAR-T cell therapy has now seen tremendous success in the clinic for certain cancers. Anti-CD19 CAR-Ts have been particularly successful against B cell leukemia and lymphoma, with promising results leading to the approval of the first CART19 product, tisagenlecleucel, by the FDA. Registrational trials for tisagenlecleucel show that the outcomes are excellent for many patients with relapsed/refractory disease [1,2]. However, many patients do not see long-term benefit due to either relapse or lack of response. An important focus for researchers is therefore to understand CAR-T cell treatment pitfalls and to build upon current therapies via novel approaches.

Broadly, etiologies of CAR-T cell therapeutic failures can be broken down into pre-infusion barriers (e.g. lymphocyte collection failure, manufacturing failure, early-disease

progression, access, and cost) and post-infusion factors (e.g. toxicity or CAR T-cell biological failure). Biological factors contributing to CAR-T cell failure consist of three main categories: so-called CAR dysfunction, immunosuppressive tumor microenvironment, other host factors, and tumor intrinsic mechanisms.

RATIONALE FOR TARGETING APOPTOSIS TO IMPROVE RESPONSE

One strategy to identify key tumor genes involved in CART19 resistance is to utilize a functional genomics screen. To do this, the Brunello CRISPR library was used to engineer the $CD19^+$ B cell acute lymphoblastic leukemia (B-ALL) cell line, NALM6, allowing for single gene knockout (KO) per discrete leukemia cell. This Brunello lentiviral library transduced cell line was enriched using puromycin, purifying the leukemic pool to ensure each cell represented one of ~18,000 gene KOs. The gene-edited cells were then incubated with CART19 for short- and long-term co-cultures. The surviving leukemic cells were harvested to define the significantly enriched or depleted gene KOs.

Enriched gene KOs in leukemic cells were universally involved in the extrinsic apoptotic pathway, including CASP8, BID, FADD, and TNFRSF10B. Depleted gene KOs were of interest as they are negative regulators of apoptosis – so if depleted, would lead to CAR-T-sensitized apoptosis.

These results suggested that extrinsic apoptosis may be a key mechanism for resistance to CAR-T cells. The next step was to prove this in the laboratory and correlate in the clinical setting.

IN VIVO & *IN VITRO* EXPERIMENTS

In vivo and *in vitro* experiments were carried out, with a focus on BID and FADD KO. As shown in Figure 1, FADD KO in leukemia resulted in greater CART19 apoptosis resistance with earlier tumor progression compared to controls. Similarly, using a BID KO, tumor progression again occurs much faster than controls, and in both cases there is a clear difference in overall survival – the KO mice die earlier than the controls.

However, it is not clear why other mechanisms available to the T cells, that in theory should still induce tumor apoptosis, fail to overcome these defects in extrinsic apoptosis. For example, the perforin and granzyme axis does not rely on extrinsic pathway apoptosis signaling, but rather on an intrinsic mediated apoptosis.

Understanding how T cells function while interacting with tumor cells that are intrinsically apoptosis resistant is important for characterizing CAR T-cell dysfunction. CAR-T cells co-cultured with tumor cells that fail to die become progressively dysfunctional, and no longer proliferate (Figure 2). At the same time, they also ceased production of perforin and show limited production of granzyme. When RNA sequencing analysis of these T cells was carried out, it was observed that a number of exhaustion and dysfunction factors were enriched such as BTLA, TIGIT, and CTLA4.

CLINICAL VALIDATION

Following promising results in the laboratory, clinical validation was sought via studying the RNA expression in leukemic blasts of CART19 treated patients enrolled in ELIANA, a registrational trial for pediatric B-ALL. As seen in Figure 3A, there is a clear trend – patients without complete response at day 28 have diminished expression of the pro-apoptotic factors in the extrinsic pathway. This is similar to what was observed in the model discussed above.

Utilizing a scoring system based on RNA expression of the extrinsic pathway signaling (death receptor signature), the patients who have low expression (low score) of pro-apoptotic factors have a very poor prognosis (Figure 3B). As previously noted, CAR-T cells tend to develop exhaustion when they are

DOI: 10.18609/ioi.2022.053

FIGURE 1

Loss of death receptor signaling enables resistance to CART19.

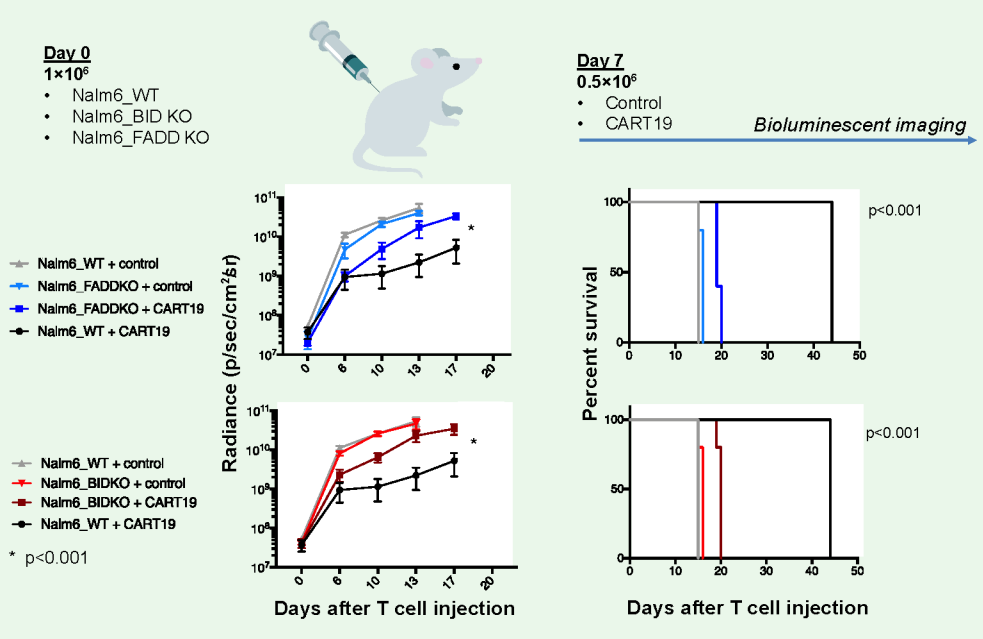

Figure reproduced from [3].

exposed to tumor cells that cannot undergo apoptosis. This was demonstrated in the clinic; CAR-T cell expansion in patients with a low score was much lower as compared to controls. A similar result was seen for persistence, indicating that these CAR-T cells do not perform as well as ones encountering a tumor that can be killed. To confirm this,

FIGURE 2

BID or FADD KO leukemia leads to CAR-T dysfunction.

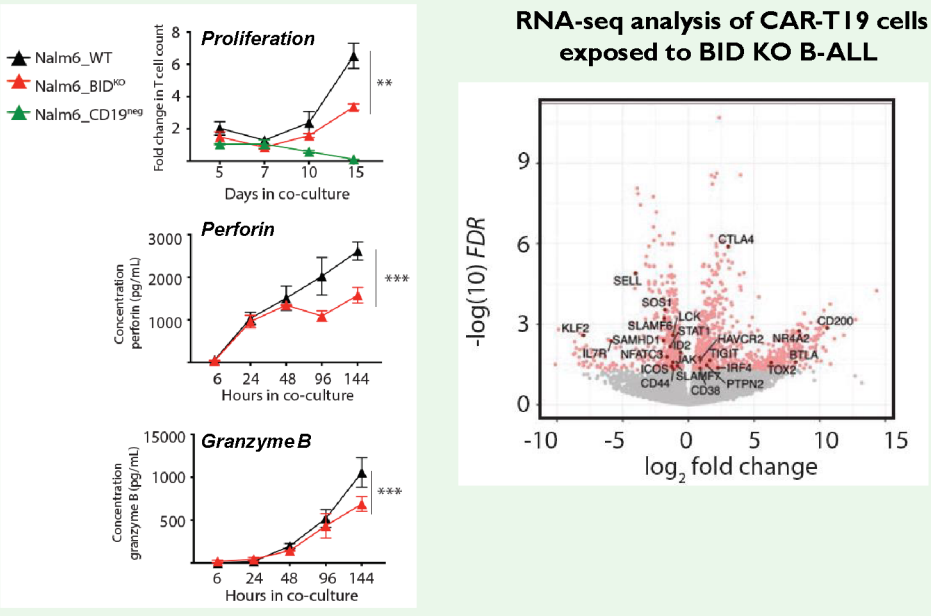

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FIGURE 3

Extrinsic apoptosis and CAR-T outcomes in B-ALL.

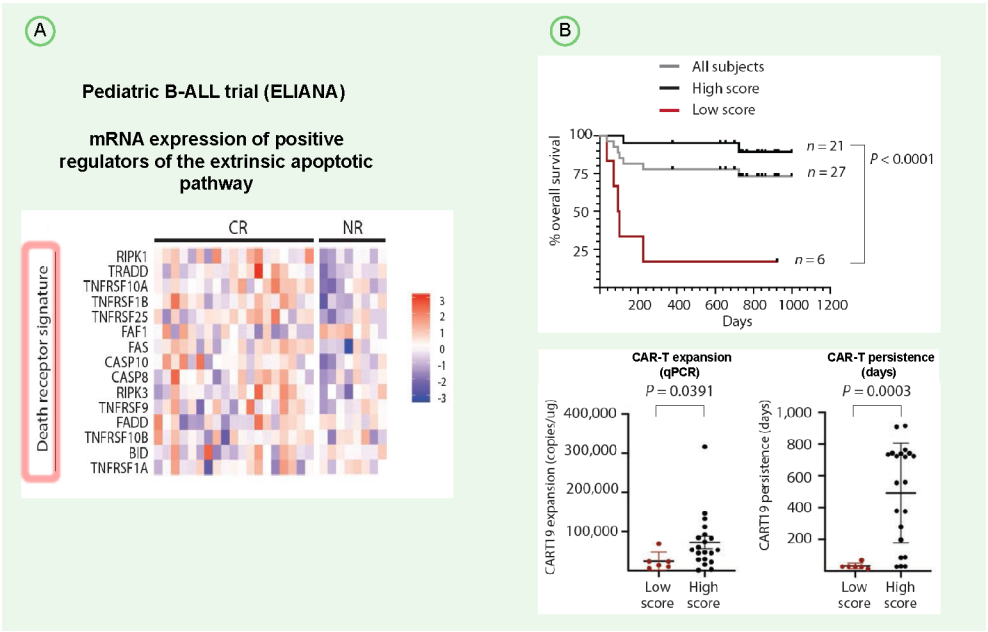


Figure reproduced from [3].

single-cell RNA sequencing was performed and a scoring system used to quantify dysfunction of the T cell. In patients who were non-responders, this revealed high numbers of exhausted T cells [Data not shown].

Taken together, these findings led to the hypothesis that these issues can be overcome using small molecules that can stimulate or sensitize apoptosis in cancer cells.

SMALL MOLECULE SCREENING TO ENHANCE CAR-T IMMUNOTHERAPY

To identify small molecules that could lower the apoptotic threshold during a T-cell-tumor interaction, a library of pro-apoptotic small molecules was screened with co-cultured CART19 and NALM6. Inhibitors of apoptosis proteins (IAP) inhibitors and BCL-2 inhibitors are two categories of small molecules found to enhance apoptosis in tumor cells under CART attack. IAP inhibitors, also termed SMAC mimetics due to SMAC's natural inhibition of IAPs, demonstrated the highest degree of synergy with CART. The lead compound, birinapant, has been tested in the clinical setting as an independent standalone therapy, and in combination. The lead BCL-2 antagonist, which is FDA-approved, is venetoclax. Both compounds were investigated to explore their potential for enhancing CAR-T immunotherapy.

SMAC mimetics

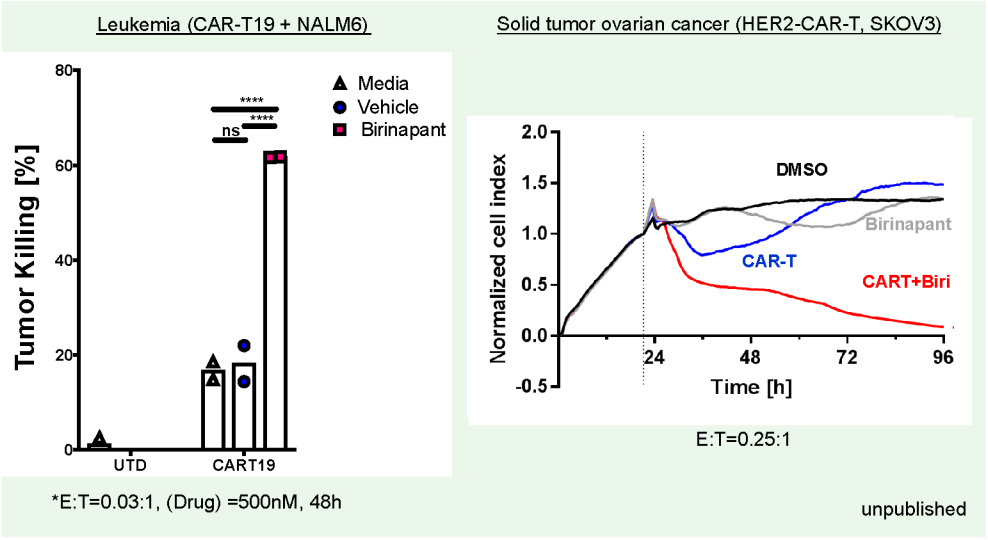
When tested *in vitro*, birinipant significantly enhanced CAR-T killing of cancer cells from 20% (vehicle control) to 60% (Figure 4). In a solid tumor ovarian cancer model using a HER2 CAR-T a similar trend is observed.

However when tested *in vivo*, despite an early trend of improved efficacy, the combination of birinapant and CAR-T showed progression compared to tumor controls treated with the CAR-T cell alone. To understand this disappointing result, CAR-T cell expansion was analyzed in peripheral blood, identifying a significant decrease in CAR-T cells when treated with birinapant compared to vehicle, indicating toxicity to the T cells. Further work is being carried out with the aim of overcoming this issue.

DOI: 10.18609/ioi.2022.053

FIGURE 4

SMAC mimetics enhance CAR-T cell killing *in vitro*.



BCL-2 inhibition

Venetoclax is an FDA-approved agent used for a variety of indications in the clinic including leukemias and lymphomas. Immune deficient mice engrafted with three human cell lines (OCI-Ly18, MINO and NALM6) were treated with CART19 in combination with either a vehicle or venetoclax, administered five times a week via oral gavage. An untransduced T cell (UTD) control was also performed.

Starting with the venetoclax-sensitive model (OCI-Ly18), results were promising with the combination of CART19 and venetoclax

FIGURE 5

BCL-2 inhibition and CAR-T in venetoclax-sensitive lymphomas.

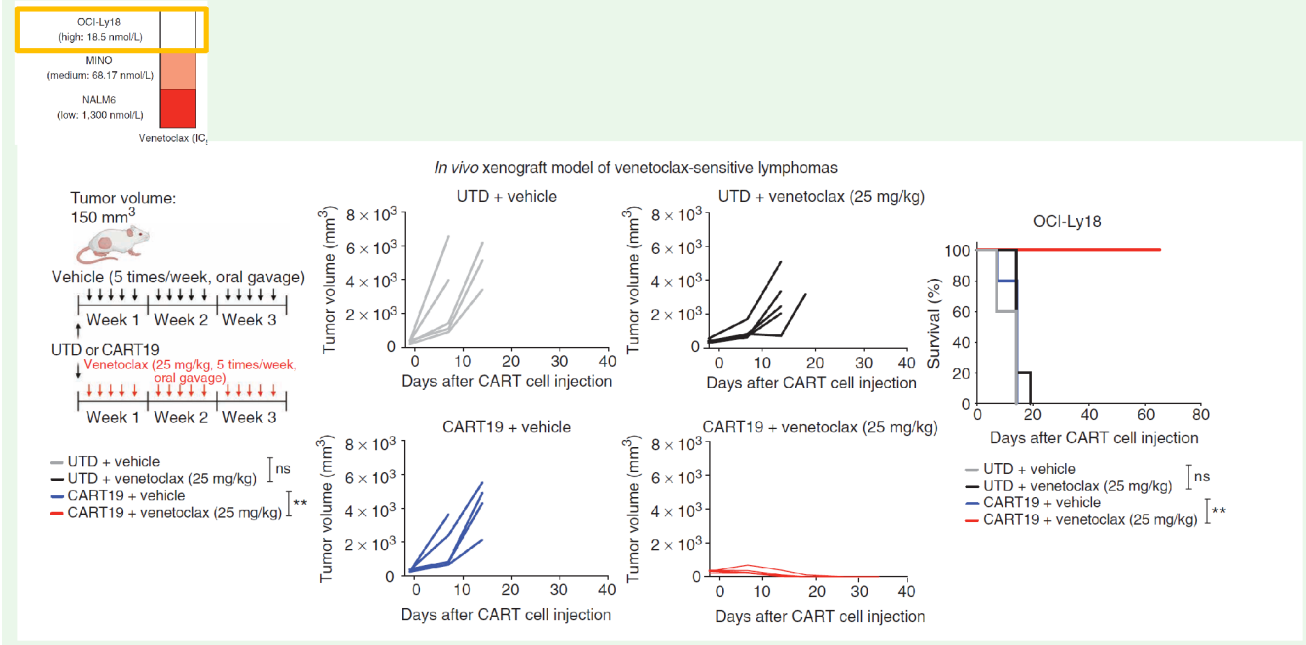


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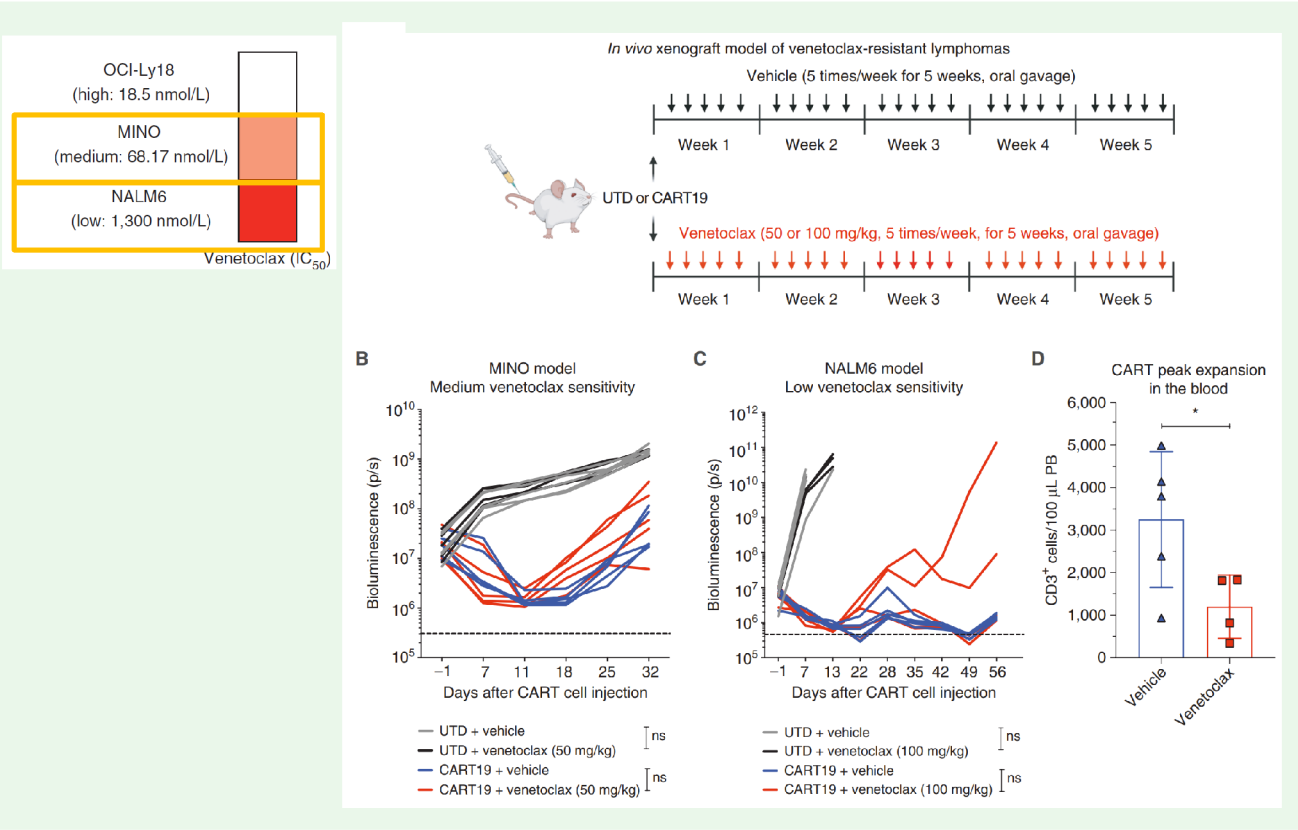
FIGURE 6

BCL-2 inhibition and CAR-T in venetoclax-resistant lymphomas.



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at 25 mg/kg – all mice were in complete remission and this translated to an advantage in order of survival (Figure 5).

For the models that are more resistant to venetoclax (MINO and NALM6), a similar experimental design was used, with higher doses of venetoclax administered to account for this resistance. When using a higher dose, toxicity was observed, and single agent CART19 performed better than

FIGURE 7

Potent synergy of CAR-T19-BCL2F104L with venetoclax.

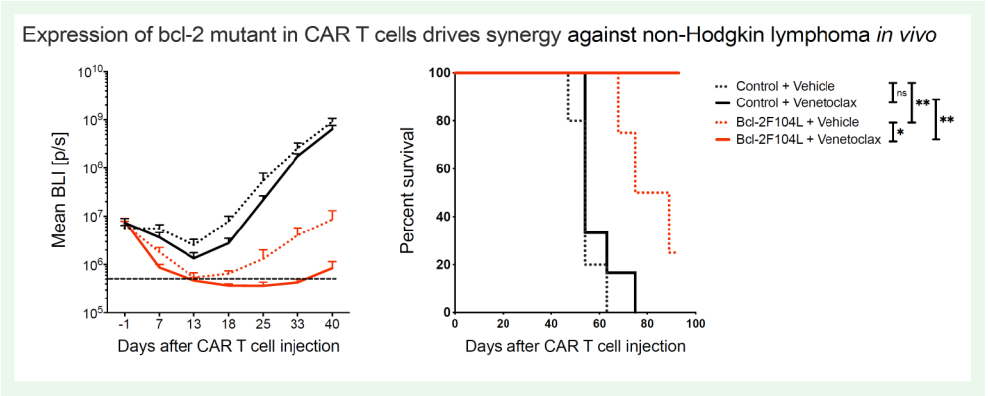

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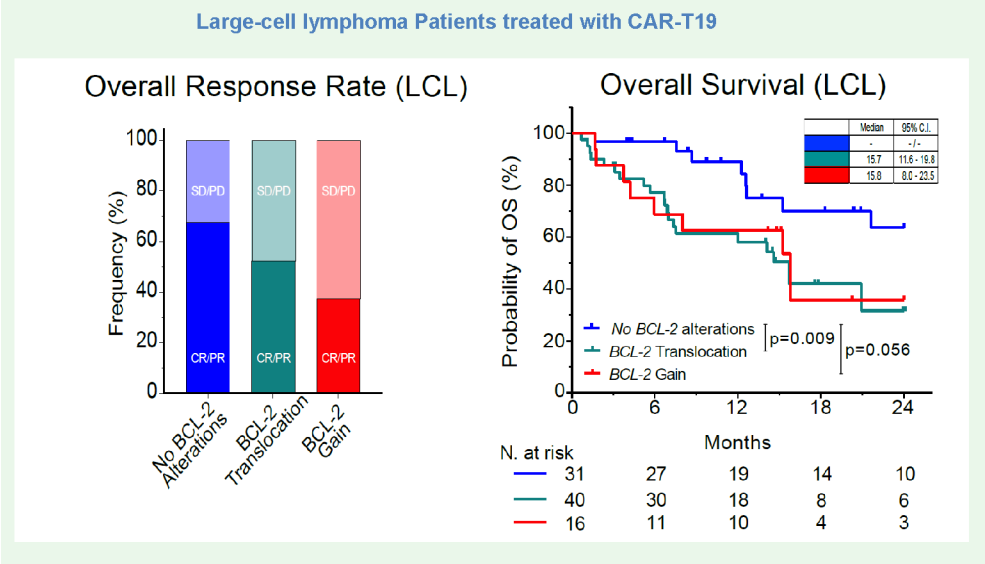
FIGURE 8

BCL-2 inhibition and CAR-T in lymphoma.



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the combination (Figure 6). As with birinapant, mice that were treated with CART19 plus a pro-apoptotic agent showed defects in CART19 expansion assessed in peripheral blood.

Based on these results, our aim was to widen an otherwise narrow therapeutic window in which these two agents could be combined efficaciously. It was hypothesized that apoptotic-resistant CAR-T cells could be developed in order to combine them with pro-apoptotic agents.

To resist venetoclax, cancers acquire and enrich for certain mutations, such as the mutant form of BCL-2 (F104L), that might also allow for resistance in CAR-T cells. This concept was then moved to *in vivo* models and a synergistic effect of CART19 combined with venetoclax was observed, along with a significant effect on overall survival (Figure 7).

Finally, in a large cohort of patients with large cell lymphoma with translocation or gain of BCL-2 showed reduced complete responses as compared to patients with non-BCL-2 alteration, and these differences in overall response rate correlate with a very clear difference in overall survival (Figure 8).

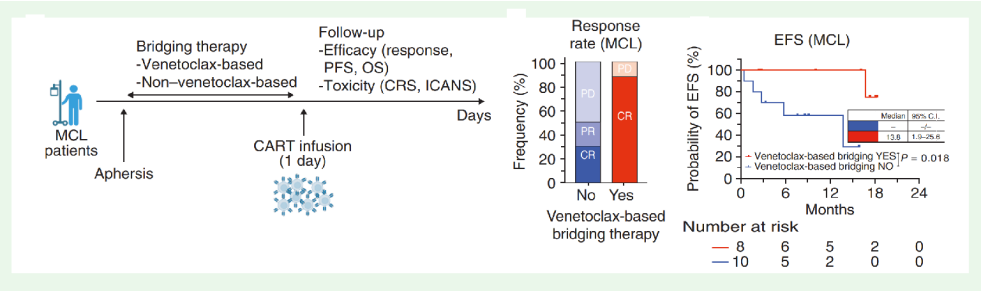
VENETOCLAX AS A BRIDGING THERAPY & OVER-EXPRESSION OF BCL-2

To find further evidence supporting the potential synergy of venetoclax and CAR-T, a good clinical scenario is mantle cell lymphoma patients who are treated with venetoclax as a bridging therapy prior to CAR-T cell therapy. Venetoclax-based bridging therapy was compared to non-venetoclax-based bridging therapy to explore if priming the tumor with venetoclax might lead to a differential effect. Results from a small group of 18 patients showed that patients receiving venetoclax-based bridging therapy had a very high rate of complete response as compared to those who did not receive venetoclax. These differences in response rates also translated to a difference in event-free survival (Figure 9).

Wild-type over-expression of BCL-2 in CAR-T cells also led to an improvement of CAR-T cell function, albeit not as significantly as with the mutation in combination with venetoclax [Data not shown]. This beneficial effect was explored further via a proliferation assay *in vitro* that demonstrated a significant increase in the persistence of CAR-T cells over-expressing wilde-type BCL-2.

FIGURE 9

Venetoclax bridging, response and event-free survival.



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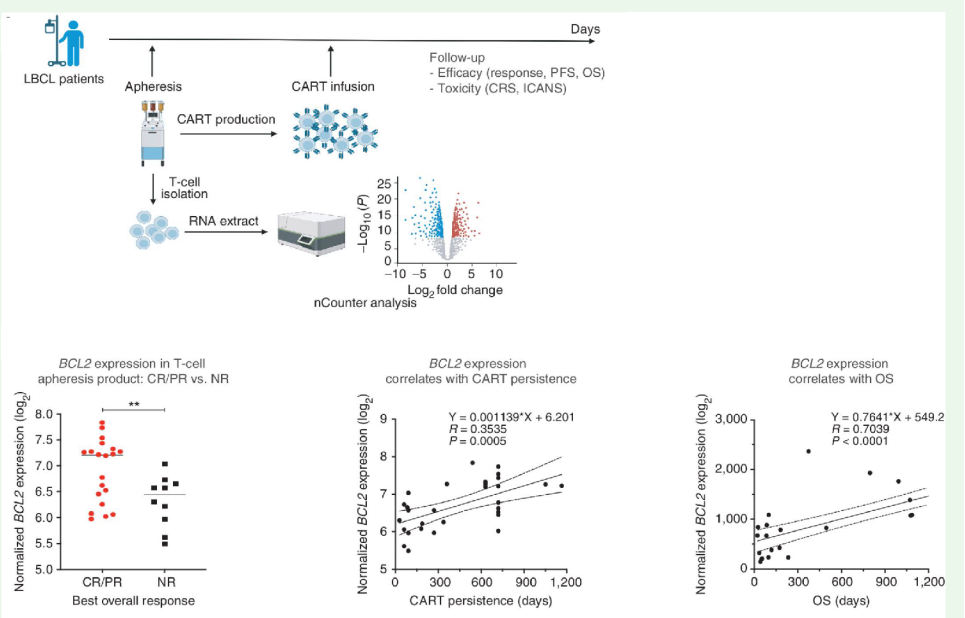
To validate this observation with a clinical-correlate, we utilized a biobank of CART19 apheresis products to ask the question "can we see a differential expression of BCL-2 in these products, and do these differences correlate with outcomes?" This was done via a collaboration with Nanostring in which T cells were isolated from the apheresis product, RNA was extracted, and then studied using the nCounter® CAR-T Characterization Panel run on the NanoString nCounter® Analysis System. One of the top genes that was expressed in patients with complete response as compared to lack of response was BCL-2. BCL-2 expression in complete responders and partial responders as compared to non-responders, with higher expression in the complete and partial responses can be seen in Figure 10. In addition, BCL-2 expression correlated with CAR-T cell persistence, showing a clear and direct correlation between the levels of BCL-2 in the T-cells and both their persistence in patients, and overall survival.

FIGURE 10

BCL-2 expression in CART19 apheresis and outcomes.



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DOI: 10.18609/ioi.2022.053

TRANSLATION INSIGHT

To summarize:

- Using unbiased screening assays, apoptosis was identified as a key mechanism for cancer resistance to CAR-T immunotherapy
- In particular, reduction of pro-apoptotic factors in the extrinsic pathway leads to resistance in B-ALL, while in NHL the BCL-2 pathway plays a major role
- Small molecules against IAPs or BCL-2 lead to enhanced killing at short term but drive CAR-T cell apoptosis over time
- A strategy was devised to make CAR-T resistant to BCL-2 inhibition and lead to synergy when combined with venetoclax

In terms of future directions, we plan to extend this approach to solid cancer and further test its safety.

Q&A

Róisin McGuigan, Editor, BioInsights speaks to (pictured) **Marco Ruella, Assistant Professor of Medicine, Scientific Director Lymphoma Program, Division of Hematology and Oncology and Center for Cellular Immunotherapies, University of Pennsylvania**

Q What is the most promising combination of pro-apoptotic small molecule and CAR-T?

MR: The challenge with combining small molecules with CAR-T cell therapy is that the small molecule can be toxic to the CAR-T cells. We showed that with both SMAC, mimetics such as birinapant, and BCL-2 antagonists, such as venetoclax. Based on our data, a safe way to combine apoptotic small molecules with CAR-T cells would have to include a modification of the CAR-T cell to make them resistant to the toxicity of the small molecule. In that regard, I do think the combination with venetoclax is the most promising one, as venetoclax is an FDA-approved agent with clear activity in both lymphoid and myeloid malignancies. But again we need protection for the CAR-T cells to ensure they don't die when they are administered with small molecules.

Q Can you expand on the possible clinical translation of the findings you outlined?

MR: There are two things we are investigating. Firstly, I showed the results in eighteen patients who were treated with Venetoclax as a bridging therapy before CAR-T cell

therapy. In this case you don't have overlap between the administration of the small molecule and the CAR-T cells. There were some signals that the bridging therapy as a way of priming the tumor with venetoclax would be beneficial for those patients. We would like to expand this retrospective core to see if the pre-exposure before CAR-T of patients with a small molecule can prime the tumor for better anti-tumor effect of the CAR-T cell. That is a possible clinical translation that is essentially short, because CAR-T cells are available and venetoclax is available, it's just about using it as a bridging strategy.

The second option would be more of an experimental one where we would need to use a product that would express the construct that we describe – BCL-2 mutation – and I've shown that the BCL-2 over-expression gives a stronger function to the T-cells. We could get a dual effect, the first one would be that the CAR-T cell *per se* would be better because they over-expressed BCL-2, it doesn't matter whether mutated or un-mutated, and the second advantage would be that you could combine that with venetoclax. This is something that we are working on, and we want to get more data on the safety of such an approach before moving forward.

Q Have you tested these combinations in solid cancers, and if not, what would be the challenges?

MR: We think that apoptosis is obviously also very relevant or the killing in solid cancer. We are still trying to figure out what pathway is the predominant one. We know that second mitochondrial activator of caspases (SMAC) mimetics do work in solid cancers. There are some initial clinical signals, and in our hands SMAC mimetics can enhance the killing of solid cancer with CAR-T cells, but again we would need to define a strategy that allows the CAR-T cell to be protected from these drugs. We are testing several ones, and it is somewhat complicated.

With the venetoclax approach we are a little bit behind from the point of view in solid cancer. I think there could be room for it. Although venetoclax doesn't have a single agent activity in solid cancer, it might be able to enhance the killing of CAR-T cells. So this is to be determined, but I think this is an option that will be effective in solid cancer. It's just a case of identifying the right small molecule for the right type of cancer.

Q What is the possible clinical toxicity of this approach?

MR: One issue could be that you are now using a small molecule with a CAR-T cell, so you can have the toxicity of the CAR-T cell, which we are very aware of, and the toxicity of the small molecule. The main toxicity of venetoclax is the cytopenias, and so cytopenias are a possibility. This should be fine if it's limited in time, and then venetoclax is stopped.

With this type of approach venetoclax doesn't need to be given for too long because you want to have the presence of venetoclax during the main action of the CAR-T cell as an anti-tumor effect. However, if you are thinking about using our construct of over-expressing

DOI: 10.18609/ioi.2022.053

BCL-2 in a T cell, that definitely changes the ability of the CAR-T cell to survive. We might have some increased cytokine release syndrome (CRS), we might have some increased neurotoxicity, but again, these possible side-effects need to be weighed against the benefits we might get with a stronger product.

The last comment is more about the role of using a construct that leads to over-expression of a mutant BCL-2. BCL-2 is often over-expressed in lymphomas, and so obviously the risk of transformation of the B cells is something that patients would need to be closely monitored. That's something I would be probably thinking about that as a possible toxicity resulting from the gene editing.

Q Can this approach be translated to other combinations?

MR: I believe it can. We described for the first time this idea of modifying the T cells to allow them to be combined with small molecules that would be otherwise toxic to them. Thinking of any small molecule that also has an activity against T cells, for example BRAF inhibitors with vemurafenib, if you want to combine that with an adoptive T cell therapy we will need to come up with a strategy to make the T cell persistent. So I do think that this is applicable to other combinations in the future.

Q Can you comment a bit more on the challenges when using CAR-T for solid tumors?'

MR: This is an important topic, because of course there are strong efforts from the scientific community in both academia and pharma to develop CAR-T cells for solid tumors.

There are several challenges related to this. We don't have optimal targets for solid cancers as most of the targets available are also expressed in healthy normal tissues that cannot really be spared. In addition the expression of the target we see in the tumor is always heterogenous, it's really rare to have 100% of the cells being highly positive for the antigen in question. The antigen issue is a major one.

Then there is the issue that the tumor microenvironment in solid cancer is particularly immunosuppressive, so any T cell or CAR-T cell that is able to get to the tumor site will need to overcome the strong immunosuppression that we see in our patients. Lastly there is an intrinsic lack of co-stimulation with solid cancer cells, where the interaction between T cells and the solid cancer cell might be more challenging as compared to interaction between a CAR-T cell in a lymphoma or leukemic cell.

However, there are a few publications, especially with brain tumors and localized regional administration of CARs, that show some responses. The field is progressing well and there will be better results in this setting, potentially using strategies that also take advantage of the stimulation of the native immune system, or some adaptive responses including tumor infiltrating lymphocytes, and so on. There is more progress to come in the next few years.

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Contributions: The named author takes responsibility for the integrity of the work as a whole, and have given his approval for this version to be published.

Acknowledgements: None.

Disclosure and potential conflicts of interest: The author declares that they have no conflicts of interest.

Funding declaration: The author has received funding from R00CA212302; Mark Foundation ASPIRE award; grants from AbClon; Beckman-Coulter; viTToria Biotherapeutics; ONI; Orflo; and consulting fees from nanoString; Bayer; and GLG. He has patents related to CART technologies, Univ. of Pennsylvania, partly licensed to Novartis, Tmunity, and viTToria biotherapeutics. He is on boards for AbClon; Sana Therapeutics; BMS; GLG; GSK; viTToria Biotherapeutics and has a leadership role on the board of viTToria biotherapeutics (Scientific Founder). He has also received materials from ONI Bio; Beckman-Coulter.

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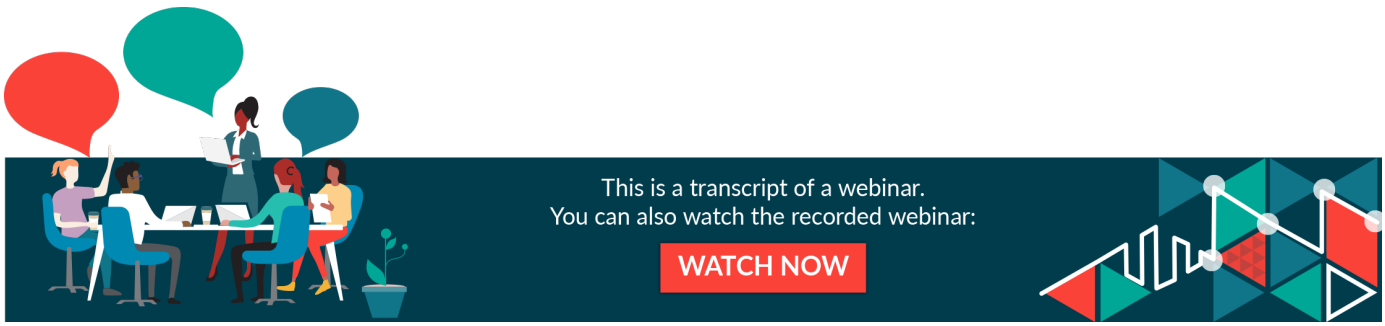
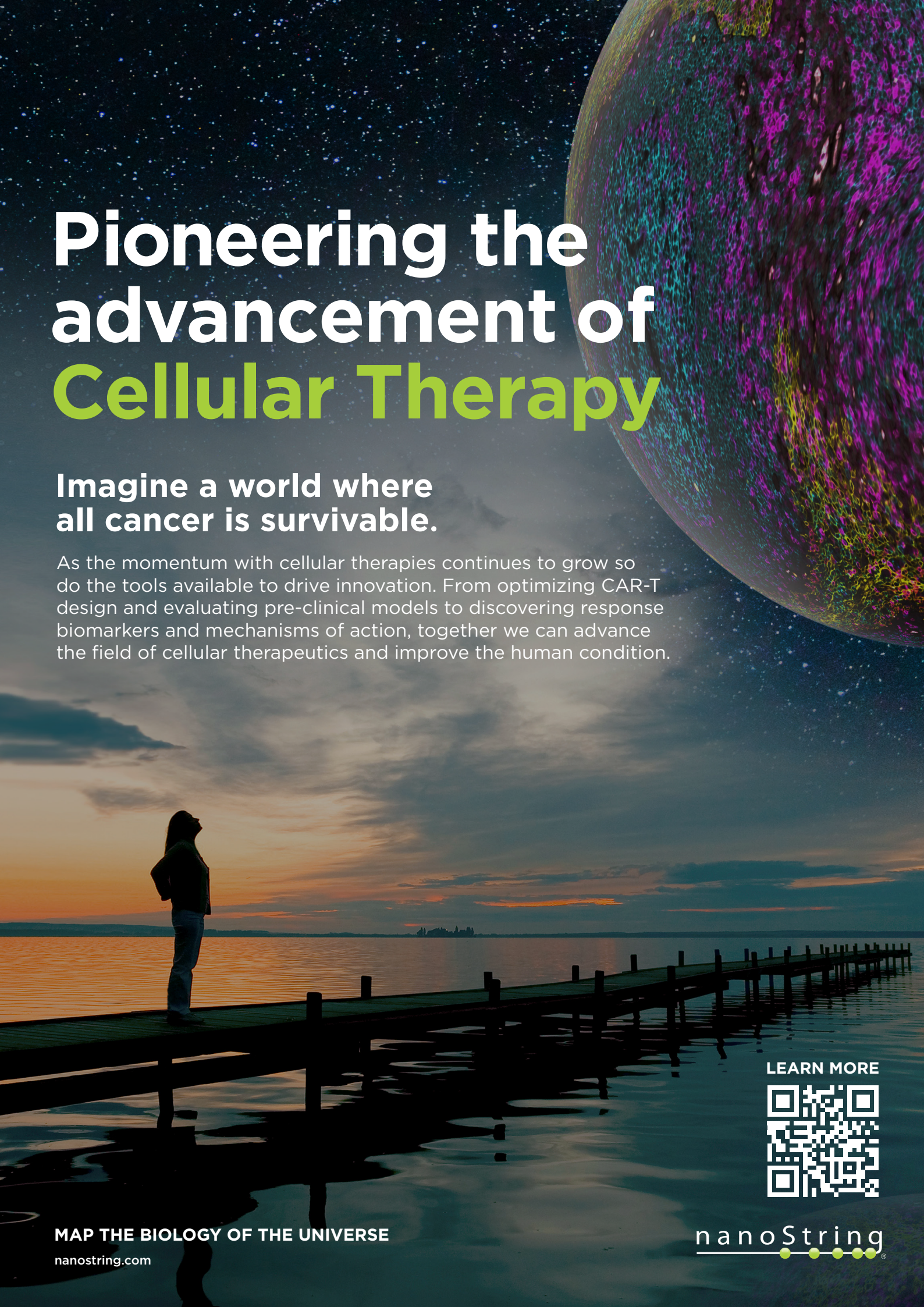
Article source: This article is a transcript of a webinar, which can be found here.

Webinar recorded: Oct 26 2022; **Revised manuscript received:** Dec 6 2022; **Publication date:** Dec 19 2022.



DOI: 10.18609/ioi.2022.053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