



## OPEN In-silico analysis unveiling the role of cancer stem cells in immunotherapy resistance of immune checkpoint-high pancreatic adenocarcinoma

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Although immune checkpoint (IC) inhibition is a major treatment modality in cancer-immunotherapy, multiple cancers show low response. Our in-silico exploration by mining cancer datasets using R2, available clinical trial data, and Kaplan–Meier analysis from GEPIA depicted that unlike low-responder (LR) cancers, high-responder (HR) cancers furnish higher IC expression, that upon lowering may provide better prognosis. Contrastingly, pancreatic adenocarcinoma (PAAD) demonstrated high IC expression but low immunotherapy-response. Infiltration scores from TIMER2.0 revealed higher pro-tumor immune subsets and cancer-associated fibroblasts (CAFs) while depicting lower anti-tumor immune subsets in PAAD as compared to HR lung adenocarcinoma (LUAD). Additionally, bioinformatic tool cBioportal showed lesser tumor mutational burden, mismatch repair deficiency and greater percent of driver mutations in TP53, KRAS and CDKN2A in PAAD, supporting its higher immunotherapy-resistance than LUAD. Our search for the 'key' immunotherapy response-deciding factor(s) revealed cancer stem cells (CSCs), the known contributors of therapy-resistance and immune-evasion, to be positively correlated with above-mentioned driver mutations, pro-tumor immune and CAF subsets; and that PAAD furnished higher expression of CSC genes than LUAD. UMAP/tSNE analyses revealed that high CSC signature is positively correlated with immunotherapy-resistance genes and pro-cancer immune cells, while negatively with cytotoxic-T cells in PAAD. Our in-silico study explains the low immunotherapy-response in high IC-expressing PAAD, wherein CSC plays a pivotal role. Further exploration portrayed correlation of CSCs with immunotherapy-resistance in other LR and HR cancers too, substantiating the need for personalized CSC evaluation and targeting for successful immunotherapy outcomes.

**Keywords** Adenocarcinoma, Cancer stem cells, Immune checkpoint, Immunotherapy, In-silico study, Pancreatic, Resistance

Cancer-immunotherapy has revolutionized cancer treatment and has achieved remarkably effective and durable responses in a subset of patients with different cancer types. However, most patients receiving these therapies, even in combination, do not derive clinical benefit<sup>1</sup>. In fact, around 20–40% of patients respond to immunotherapy<sup>2</sup>, among which immune checkpoint inhibitor (ICI) therapy has gained global attraction, leading upto 3 fold increase in number of clinical trials from 2017 to 2020<sup>3</sup>. Immune checkpoints (ICs) like, programmed cell death 1 (PD1), cytotoxic T lymphocyte antigen 4 (CTLA4), lymphocyte activating gene-3 (LAG3) and T cell immunoglobulin and ITIM domain (TIGIT), are expressed on T cells and upon interacting with their respective ligands, act as brakes to limit the cytotoxic activity of T cells and inhibit their expansion, thereby, leading to tumor growth<sup>4,5</sup>. Among these, PD1 is mainly expressed on tumor-infiltrating lymphocytes (TILs) along with other immune cells that binds to PD ligand 1 (PDL1)/PDL2 on cancer cells and immune cells. Such interaction of PD1 with its ligands cause immune-tolerance, exhausted T cells and, low inflammatory

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signals<sup>6,7</sup>. FDA-approved immunotherapies targeting PD1 pathway have demonstrated encouraging responses in lung cancer and melanoma among others<sup>8</sup>. Another crucial IC molecule, CTLA4, that is primarily expressed in activated and regulatory T cells, competes with CD28 to bind with CD80 and CD86, leading to inhibition of T cell functions<sup>9</sup>. CTLA4 ICI-therapy has resulted in increased patient survival in lung, melanoma and renal cancers<sup>9</sup>. In fact, according to a recent study, FDA-approved anti-CTLA4 (ipilimumab) and anti-PD1 (pembrolizumab, nivolumab) have shown notable outcomes in anti-tumor immunity<sup>10</sup>. LAG3, another transmembrane molecule, is expressed on different subsets of T cells as well as, natural killer (NK) cells<sup>10</sup>. It reportedly, interacts with MHC-II on antigen-presenting cells (APCs) and tumor cells, negatively regulating CD4<sup>+</sup> and CD8<sup>+</sup> TILs. Clinical trials in renal and breast cancer using LAG3 antibody have shown promise<sup>10</sup>. Recently identified TIGIT was found to be expressed in activated and regulatory TILs, that binds to poliovirus receptor (PVR) in APCs and tumor cells. Expression of TIGIT has been linked to mediate suppressive functions in multiple malignancies<sup>10</sup>.

Despite significant durable responses and overall survival achieved by the ICs in lung cancer, melanoma, breast cancer and renal cell carcinoma<sup>4,11–14</sup>; certain cancers like, pancreatic cancer, prostate cancer and glioblastoma demonstrated resistance<sup>4,15–17</sup>. Moreover, reports suggest that higher expression of IC molecules is not necessarily reliable as a predictive biomarker for ICI treatment<sup>18,19</sup>. Mechanisms pertaining to immunotherapy-resistance other than expression of IC molecules and the type of cancer, are determined by other factors such as, components of tumor microenvironment (TME) consisting of immune cells like, macrophages, lymphocytes, NK cells and dendritic cells, which can either aid or provide resistance to immunotherapy<sup>20</sup>. The relative infiltration of pro-inflammatory immune components like, M1 macrophages, dendritic cells, NK cells, and CD4<sup>+</sup> T cells determines an immunological ‘hot’ tumor which are susceptible to better immunotherapy-response<sup>21</sup>. On the other hand, a ‘cold’ tumor, marked by lower infiltration of effector T cells and higher presence of immunosuppressive cells, contributes to TME-mediated or extrinsic immunotherapy-resistance<sup>5,22</sup>. Intrinsic immunotherapy-resistance factors include tumor mutational burden (TMB), mismatch repair deficiency (MMRd), driver gene mutations and altered signaling pathways in the cancer cells<sup>20,23</sup>. Although immunotherapy-responder cancers expressing high levels of PDL1<sup>24</sup> still show response rate below 20%; therefore, identification of the intrinsic therapy-resistance genes and factors other than IC molecules is of utmost necessity for predicting immunotherapy-response<sup>25</sup>. A major mechanism at play may be driver mutations in key genes, which can upregulate immunotherapy-resistance pathways, help in immune escape, lower TMB and even control the TME compartment, ultimately generating poor immunotherapy clinical outcomes<sup>23,26</sup>.

Accumulating evidences establish the role of a tiny sub-population of cancer stem cells (CSCs) in the TME to initiate tumor, promote metastasis, disease progression, resist therapy and evade immune destruction<sup>27–29</sup>. In fact, CSCs escape immune-detection by expressing low levels of antigens, immune-stimulatory molecules and high levels of ICs, such as PDL1<sup>30</sup>. Additionally, cancer stemness influences extrinsic immunotherapy-resistance factors, as it is reported to be negatively correlated with anti-tumor immunity<sup>31</sup> and promote immunosuppressive environment<sup>27,32</sup>. In fact, genes identified for ICI resistance were observed to be correlated with cancer stemness<sup>25</sup>, thereby signifying the possible contribution of CSCs in ICI therapy failure.

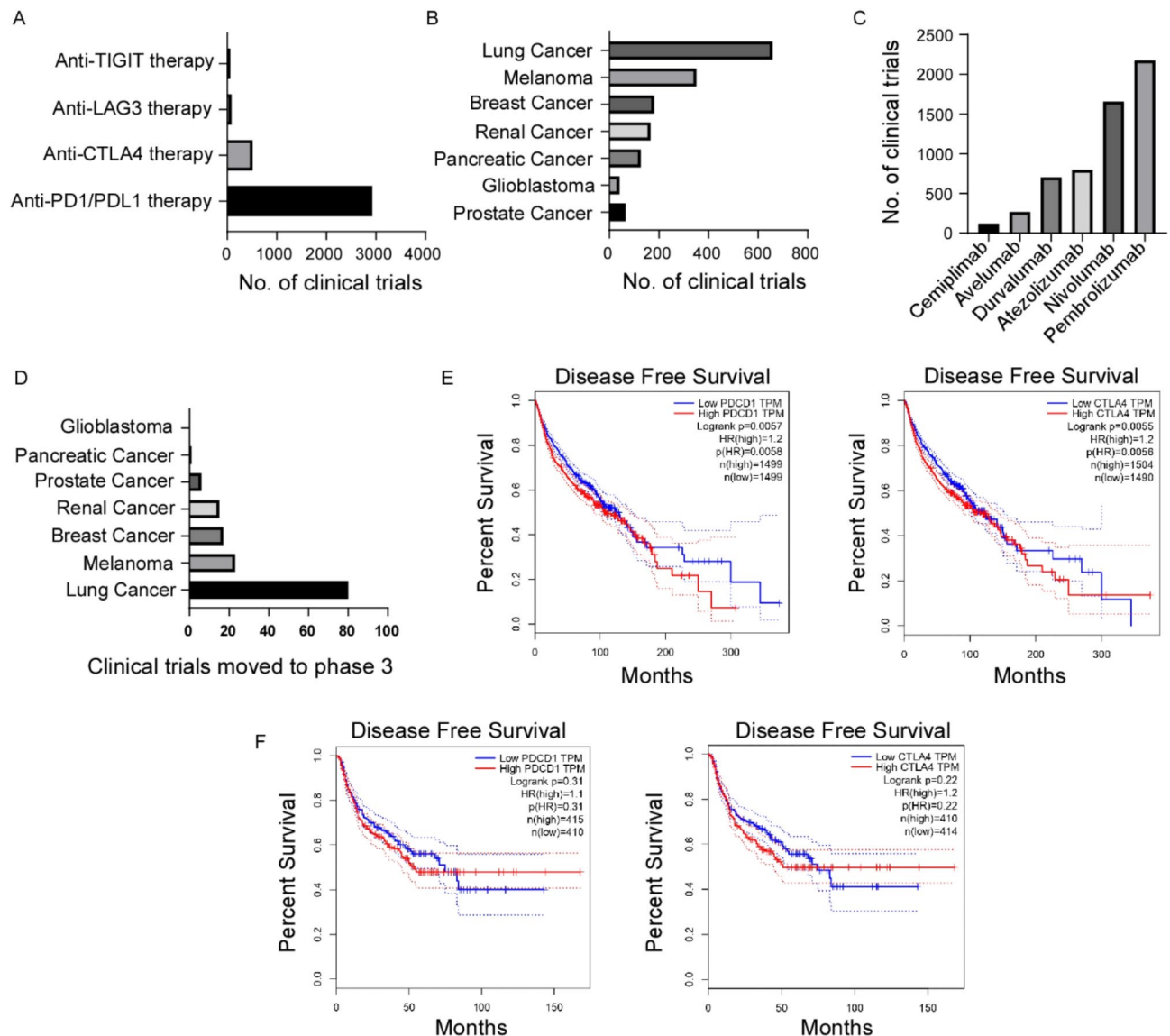
Here, employing in-silico analyses, we observed lower IC expression in low-responder (LR) cancers as compared to high-responder (HR) cancers. However, pancreatic adenocarcinoma (PAAD) expresses IC genes at a comparable level with HR cancers and even with the highest IC-expressing lung adenocarcinoma (LUAD). Although demonstrating high levels of IC, PAAD shows therapy-resistance. Our search for the factors responsible for such ‘contradictory’ behavior of PAAD revealed lower anti-tumor immune infiltration and higher pro-tumor TME subset incidence, in contrast to LUAD. Moreover, detailed patient data analyses showed lower mutational burden and higher incidence of driver mutations in key genes TP53, KRAS and CDKN2A in PAAD as compared to LUAD. Interestingly, these oncogenic mutations along with the pro-tumor TME subsets were also correlated with CSC-related genes in PAAD. Concurrently, PAAD furnished augmented expression of CSC markers than LUAD. Further analyses signified that CSC status of PAAD is directly associated with the expression of IC molecules, immunotherapy-resistance genes and pathways and even connected with the infiltration of extrinsic resistance immune subsets. The contribution of CSCs towards immunotherapy-resistance is not limited to LR cancers, but extended to HR LUAD as well, as immunotherapy-resistance genes were correlated with stemness. These results together bolster the profound role of CSCs towards deciding the fate of immunotherapy-response.

## Results

### Grouping of HR and LR cancers based on ICI clinical trial data

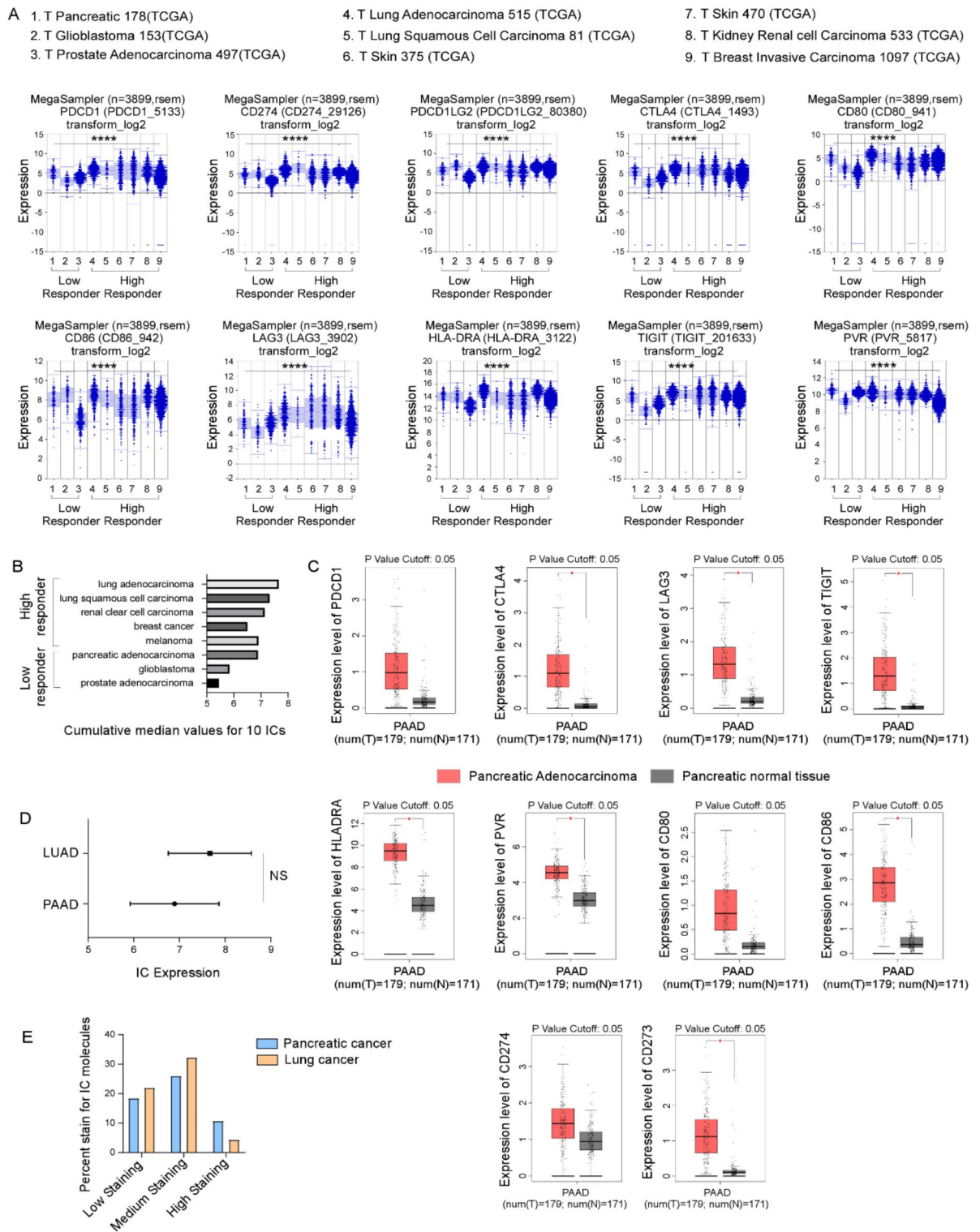
To evaluate different ICI treatment modalities, our search of the publicly available clinical trial database [clinicaltrials.gov](https://clinicaltrials.gov)<sup>33</sup> revealed 2929 clinical trials using PD1 intervention, 514 clinical trials in case of CTLA4, 88 for LAG3, and 62 for TIGIT-mediated intervention as well as in combination with other treatment (Fig. 1A), for study start date taken till 31st January, 2024. These results signify PD1 axis inhibition as the major ICI treatment option. However, recent studies highlighting that ICI therapy is not equally effective in all types of cancer, directed us to group the cancers according to their reaction towards immunotherapy as (i) HR cancers such as, lung cancer, melanoma, breast cancer and kidney cancer<sup>4,11–14</sup>; and (ii) LR cancers, such as, pancreatic cancer, prostate cancer and glioblastoma<sup>4,15–17</sup>. Consequently, a total of 658 PD1 intervention clinical trials were found in lung cancer, 351 in melanoma, 181 in breast cancer, 166 in renal cancer, 127 in pancreatic cancer, 41 in glioblastoma and 65 in prostate cancer, in line with reports categorizing them to HR or LR groups (Fig. 1B).

While validating the responsiveness of the aforementioned cancer types by using data provided by [clinicaltrials.gov](https://clinicaltrials.gov), Pembrolizumab (anti-PD1) was found to be highest used one considering the number of clinical trials among FDA-approved PD1/PDL1 axis inhibitors<sup>8</sup> when compared to five other PD1/PDL1 axis inhibitors (Fig. 1C). Next, the number of clinical trials using Pembrolizumab that successfully moved to Phase 3 studies after demonstrating positive response to previously conducted earlier phase studies<sup>34</sup> in different cancers, was evaluated. Clinical trial database [clinicaltrials.gov](https://clinicaltrials.gov) using Pembrolizumab depicted lung cancer (80 clinical



**Fig. 1.** Grouping of high-responder and low-responder cancers. **(A)** Clinical trial statistics from clinicaltrials.gov/ depicting the number of clinical trials by different ICI methods in cancer. **(B)** The number of clinical trials by PD1 intervention in different cancer types. **(C)** Number of clinical trials by the various FDA approved PD1/PDL1 axis inhibitors in cancer. **(D)** Number of clinical trials in phase 3 studies in different cancers by using Pembrolizumab. The clinical trial data numbers were taken till the study start date, 31st January, 2024. Disease free survival (DFS) plot obtained from GEPIA for **(E)** LUAD, LUSC, SKCM, KIRC and BRCA group and, **(F)** GBM, PAAD and PRAD group for immune checkpoint genes, PD1 (PDCD1) (left panel) and CTLA4 (right panel). LUAD: Lung adenocarcinoma, LUSC: Lung squamous cell carcinoma, SKCM: Skin cutaneous melanoma, KIRC: Kidney renal clear cell carcinoma, BRCA: Breast invasive carcinoma, GBM: Glioblastoma multiforme, PAAD: Pancreatic adenocarcinoma, PRAD: Prostate adenocarcinoma.

trials) as the highest, followed by melanoma (23 trials), breast cancer (17 trials), renal cancer (15 trials), prostate cancer (6 trials), pancreatic cancer (1 trial) and glioblastoma (0 trial) (Fig. 1D). Above results, validated our cancer grouping based on ICI therapy response, i.e., HR group (lung cancer, melanoma, renal cancer, breast cancer) and LR group (pancreatic cancer, glioblastoma and prostate cancer). Next, considering that targeting PD1 and CTLA4 in the tumor are the most widely-used ICI-therapies (Fig. 1A and C), supported by reports too<sup>35–37</sup> and, they demonstrate higher efficacies than targeting their ligands<sup>38,39</sup>, we have used PD1 and CTLA4 to study patient survival statistics in cancer. Consequently, Kaplan–Meier plot for Disease Free Survival (DFS) from GEPIA (<http://gepia.cancer-pku.cn/>)<sup>40</sup> showed that HR group comprising of LUAD, lung squamous cell carcinoma, skin cutaneous melanoma, kidney renal clear cell carcinoma and breast invasive carcinoma, was associated with high DFS corresponding to low PD1 ( $p < 0.01$ ) and CTLA4 ( $p < 0.01$ ) expression in patients (Fig. 1E), whereas, the LR group consisting of glioblastoma multiforme, PAAD and prostate adenocarcinoma demonstrated no significant association of DFS with the expression of PD1 and CTLA4 in patients (Fig. 1F).



Above observations based on literature studies, clinical trial data statistics and survival plots validate that lung, skin, kidney and breast cancers are in HR cancer group, wherein high patient survival depends on low IC expression. Therefore, cancers in this HR group are more likely to generate positive outcomes with ICI therapy, while pancreatic, glioblastoma and prostate cancers are in LR cancer group, show ICI therapy-resistance.

### LR cancers express low IC levels, whereas HR cancers have IC expression

Next, to comprehend the differential responsiveness of ICI therapy among HR cancers and LR cancers, we mined the expression of IC molecules and their respective ligands across total 9 cancer subsets, comprising of, HR (LUAD, lung squamous cell carcinoma, 2 datasets of skin cancer, kidney renal cell carcinoma, breast invasive carcinoma) and LR (glioblastoma, PAAD, prostate adenocarcinoma) groups using publicly available tumor datasets from 'R2: Genomics Analysis and Visualization Platform' and 'The Cancer Genome Atlas' (TCGA). The data obtained



◀ **Fig. 2.** Immune checkpoint (IC) expression for high- and low-responders. (A) Using Megasampler from 'R2: Genomics Analysis and Visualization Platform', expression ICs and their ligands, PD1 (PDCD1), PDL1 (CD274), PDL2 (PDCD1LG2), CTLA4, CD80, CD86, LAG3, HLA-DRA, TIGIT and PVR are represented for TCGA tumors, pancreatic adenocarcinoma, glioblastoma, prostate adenocarcinoma, lung adenocarcinoma, lung squamous cell carcinoma, skin cancer, kidney renal cell carcinoma and breast invasive carcinoma. Data were represented as box/dot plot (bands) and ANOVA was used to assess the data where  $*P < 0.05$ ,  $**P < 0.01$ ,  $***P < 0.001$ , and  $****P < 0.0001$ , as obtained from R2. (B) Mean of median values of all the 10 IC molecules from (A) was plotted in a bar graph for each of the cancer. (C) Expression of the 10 IC values in PAAD along with pancreatic normal tissue procured from GEPIA is depicted ( $p$  value cut off set at 0.05). (D) Each of the 10 IC median values from (A) were compared in a bar plot for PAAD and LUAD. ( $p = ns$ ; non-parametric Mann Whitney test). (E) Staining data from 'The Human Protein Atlas' showing low, medium or high staining of pancreatic and lung cancer tissues with the above-mentioned IC genes. PAAD: Pancreatic adenocarcinoma, LUAD: Lung adenocarcinoma.

revealed that among all the considered cancer groups, Glioblastoma (dataset: Tumor Glioblastoma-TCGA-153-rsem-tcgars;  $n = 153$ ) showed the lowest median expression of PD1 ( $p < 0.0001$ ), CTLA4 ( $p < 0.0001$ ), LAG3 ( $p < 0.0001$ ), TIGIT ( $p < 0.0001$ ) and PVR ( $p < 0.0001$ ), while prostate adenocarcinoma (dataset: Tumor Prostate Adenocarcinoma-TCGA-497-rsem-tcgars;  $n = 497$ ) demonstrated lowest median expression of PDL1 ( $p < 0.0001$ ), PDL2 ( $p < 0.0001$ ), CD80 ( $p < 0.0001$ ), CD86 ( $p < 0.0001$ ) and HLA-DRA ( $p < 0.0001$ ) among all the considered cancer groups (Fig. 2A). Therefore, LR Glioblastoma expressed lowest levels in 5 out of the 10 IC genes considered, whereas, LR prostate adenocarcinoma displayed lowest median levels in the rest 5 IC genes. In line with our grouping of cancers, all the cancers of HR group furnished high median IC expression (Fig. 2A). Among those, LUAD (dataset: Tumor Lung Adenocarcinoma-TCGA-515-rsem-tcgars;  $n = 515$ ) showed highest median expression in 5 checkpoint genes (PD1, CTLA4, CD80, CD86 and TIGIT) ( $p < 0.0001$ ); lung squamous cell carcinoma (dataset: Tumor Lung Squamous Cell Carcinoma-TCGA-81-rsem-tcgars;  $n = 81$ ) demonstrated highest median expression in PDL1 ( $p < 0.0001$ ); skin cancer (dataset: Tumor Skin Cutaneous Melanoma-TCGA-375-rsem-tcgars;  $n = 375$ ) displayed highest median expression in LAG3 ( $p < 0.0001$ ) and renal clear cell carcinoma (dataset: Tumor Kidney Renal Clear Cell Carcinoma-TCGA-533-rsem-tcgars;  $n = 533$ ) furnished highest median expression in HLA-DRA ( $p < 0.0001$ ), out of the 10 IC genes shown among the cancer datasets. Breast cancer (dataset: Tumor Breast Invasive Carcinoma-TCGA-1097-rsem-tcgars;  $n = 1097$ ) presented average median values in all the 10 IC molecules taken (Fig. 2A). Therefore, from Fig. 2A, LUAD depicted highest median expression in 5 out of 10 markers. Since among lung cancer subtypes, the most common one is non-small cell lung cancer (NSCLC), amid which adenocarcinomas are more prevalent ones<sup>41</sup>, the highest IC expression in LUAD seems justified from our results reflecting the highest ICI response in terms of clinical trial number (Fig. 1D).

These results signified that LR cancers have lower expression of IC genes, explaining the discouraging outcomes to ICI therapy, while HR cancers possess higher expression of IC genes, wherein, IC neutralisation-therapy can effectively block the IC molecules expressed in such cancers, bringing about positive response.

### LR cancer PAAD expresses high IC levels: an eye opener

Interestingly, LR cancer PAAD (dataset: Tumor Pancreatic adenocarcinoma - TCGA - 178 - rsem - tcgars;  $n = 178$ ), taken from R2 platform, demonstrated comparable median expression levels of these IC molecules with the HR cancers. It also showed highest median expression in PVR among all the considered cancer datasets (Fig. 2A). For better comprehension, mean was calculated of the median values for all the 10 ICs in different cancer types found from R2, which showed that while LR cancers, glioblastoma (mean: 5.84) and prostate adenocarcinoma (mean: 5.45) depicted lowest means among all the cancer subtypes, whereas, PAAD (mean: 6.89) furnished highest mean among the LR group. Not only this, PAAD depicted comparable IC mean with HR group melanoma (mean: 6.91), lung squamous cell carcinoma (mean: 7.32), renal clear cell carcinoma (mean: 7.14) and breast cancer (mean: 6.5). The highest IC mean was observed in HR LUAD (mean: 7.66) (Fig. 2B).

Reports showing high expression of PDL1 and CTLA4 in pancreatic tumors<sup>42,43</sup>, about 90% of which is PAAD<sup>44</sup>, therefore, strengthened our findings. In fact, upon mining expression data from GEPIA ( $p$  value cutoff at 0.05), levels of PD1, CTLA4, LAG3 and TIGIT along with their ligands were significantly higher in PAAD ( $n = 179$ ) than that of normal pancreatic tissue ( $n = 171$ ) (Fig. 2C). PAAD, although being lower in IC expression than LUAD (Fig. 2B), the median expression of IC values, as mined from R2 for PAAD (PDL1 4.67; PD1 5.01; PDL2 5.63; CTLA4 5.26; CD80 4.64; CD86 8.04; LAG3 5.53; HLA-DRA 13.96; TIGIT 5.7; PVR 10.5), when plotted against LUAD (PDL1 5.96; PD1 5.78; PDL2 6.48; CTLA4 6.06; CD80 5.46; CD86 8.47; LAG3 6.77; HLA-DRA 14.76; TIGIT 6.71; PVR 10.22), was not significantly different as observed by non-parametric Mann Whitney test ( $p = 0.105$ ; Fig. 2D).

To cross-validate our observation at protein level, data for lung and pancreatic cancer tissues furnished from another online resource 'The Human Protein Atlas'<sup>45</sup> was utilised and IC expression were evaluated based on percentage of samples presenting low, medium or high antibody staining. PD1 (Ab.: CAB038418), PDL1 (Ab.: CAB030018), PDL2 (Ab.: HPA013411), CD86 (Ab.: CAB004319), HLA-DRA (Ab.: HPA050162) and PVR (Ab.: HPA012568) data were used. However, staining of CTLA4, CD80, LAG3 and TIGIT were not found in this online resource. In lung cancer, 22% of the considered IC molecules showed low staining, 32.3% showed medium staining, whereas high staining was observed in 4.4% of total ICs (Fig. 2E). On the other hand, pancreatic cancer showed 18.5% low staining, 26% medium staining and 10.76% high staining of the cumulative IC markers considered (Fig. 2E). Fascinatingly, while pancreatic cancer demonstrated lesser percentage than lung cancer in

low and medium staining, it furnished higher percentage in high staining of cumulative IC molecules than lung cancer. Since PAAD and LUAD are the major subtypes of respective cancers, these observations showed that in spite having comparable expression of IC molecules, PAAD does not respond to immunotherapy<sup>46</sup>, while LUAD does<sup>47</sup>. Above findings signify that responsiveness to ICI therapy is not solely determined by IC expression, but by other factors, if any, as well. Consequently, a multipronged approach considering the contribution of both extrinsic and intrinsic factors was adopted.

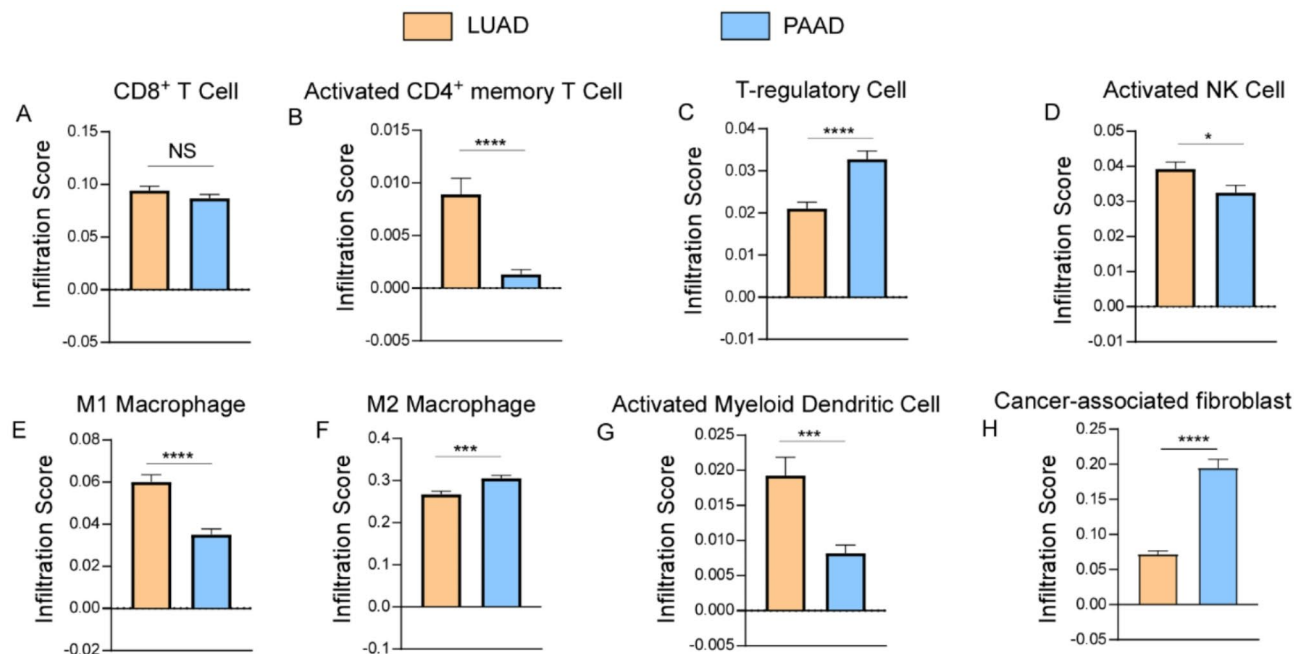
### PAAD shows higher infiltration of pro-tumor and lower infiltration of anti-tumor TME subsets than LUAD

To determine the influence of the extrinsic immune subsets in controlling the immunotherapeutic response, we evaluated the same from publicly available resource TIMER 2.0 (<http://timer.cistrome.org/>)<sup>48</sup> and obtained the CIBERSORT immune cell infiltration scores for TCGA tumors, PAAD ( $n = 179$ ) and LUAD ( $n = 208$ ) (Fig. 3). CIBERSORT scores for anti-tumor immune subsets<sup>20</sup>, activated CD4<sup>+</sup> memory T cell ( $p < 0.0001$ ) (Fig. 3B), NK activated cell ( $p < 0.05$ ) (Fig. 3D), M1 macrophage ( $p < 0.0001$ ) (Fig. 3E) and activated myeloid dendritic cell ( $p < 0.001$ ) (Fig. 3G) were significantly higher in LUAD than PAAD. Anti-tumor subset CD8<sup>+</sup> T cell showed no significant difference between PAAD and LUAD (Fig. 3A). On the other hand, pro-tumor immune subsets<sup>27,32</sup>, Tregs ( $p < 0.0001$ ) (Fig. 3C) and M2 macrophages ( $p < 0.001$ ) (Fig. 3F) furnished significantly higher CIBERSORT score in PAAD than LUAD, depicting higher immunosuppressive immune population and lower anti-tumor immune cell infiltration in PAAD, which may contribute to immunotherapy-failure<sup>23</sup>.

Additionally, other components of TME such as, cancer-associated fibroblasts (CAFs) have also been associated with immunotherapy-resistance in cancers<sup>49</sup>. To that end, we aimed at estimating their levels in PAAD and LUAD as well. Since CIBERSORT scores are only available for immune cells, therefore, EPIC infiltration scores were employed to determine CAF infiltration levels. Interestingly, much like the pro-tumor immune subsets, our results showed significantly higher ( $p < 0.0001$ ) CAFs in PAAD ( $n = 179$ ) than LUAD ( $n = 208$ ) (Fig. 3H), which might justify immunotherapy-resistance of PAAD.

### Intrinsic immunotherapy-resistance is higher in PAAD than LUAD

Next, among the intrinsic immunotherapy-resistance mechanisms, tumor mutational burden (TMB) was taken into account first. Higher TMB is associated with better immune recognition, immunotherapy-response and longer survival<sup>50</sup>. Using cBioportal (<http://cbiportal.org/>)<sup>51</sup>, a publicly accessible online platform, LUAD (luad\_tcga\_pan\_can\_atlas\_2018,  $n = 566$ ) showed a greater occurrence (194 out of 566 patients) of high-TMB defined by the presence of more than 10 mutations/Mb<sup>52</sup>, as compared to PAAD (paad\_tcga\_pan\_can\_atlas\_2018,



**Fig. 3.** Extrinsic immunotherapy-resistance contributing immune and CAF infiltration scores for PAAD and LUAD. TIMER2.0 database procured CIBERSORT scores for immune cell infiltration represented in the form of bar graph for TCGA tumors, PAAD ( $n = 179$ ) and LUAD ( $n = 208$ ) for (A) CD8<sup>+</sup> T cell, (B) Activated CD4<sup>+</sup> memory T cell, (C) T-regulatory cell, (D) Activated NK cell, (E) M1 macrophage, (F) M2 macrophage and (G) activated myeloid dendritic cell. (H) Bar graph representing EPIC score for CAF infiltration in PAAD ( $n = 179$ ) and LUAD ( $n = 208$ ). Data were represented as the mean  $\pm$  SEM and student's t-test (unpaired) was used to assess the data where \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , and \*\*\*\* $P < 0.0001$ . CAF: Cancer-associated fibroblast, PAAD: Pancreatic adenocarcinoma, LUAD: Lung adenocarcinoma.

$n = 184$ ) which furnished 0 out of 184 patients taken, using the dataset from TCGA Pan-Cancer Atlas Studies (Fig. 4A). The gene mutation count from the same datasets revealed a similar trend, wherein the highest number of LUAD patients demonstrated total gene mutation count above 280, and highest number of PAAD patients showed the count to be in the range from 20 to 40 (Fig. 4B), thereby, explaining the high ICI response in LUAD while failure of the same in PAAD.

It is acknowledged that the deficiency in DNA mismatch repair genes, MLH1, MSH2, MSH6 and PMS2, can generate mutations and antigenicity, effecting better response to ICI treatment<sup>53</sup>. In this regard too, LUAD (luad\_tcga\_pan\_can\_atlas\_2018,  $n = 566$ ) depicted higher mutation status in MLH1 (1.6%), MSH2 (1.9%), MSH6 (1.8%) and PMS2 (1.6%) than PAAD (paad\_tcga\_pan\_can\_atlas\_2018,  $n = 184$ ), which furnished 0.6% mutational status in all the mentioned mismatch repair genes (Fig. 4C), analysed by cBioportal.

Studies have shown that driver mutations also cause poor clinical outcomes for immunotherapy and multiple driver mutations are involved in immunotherapy-resistance<sup>23</sup>. In that respect, PAAD (paad\_tcga\_pan\_can\_atlas\_2018,  $n = 184$ ) presented higher mutation percentage in TP53 (59.8%), KRAS (65.4%), CDKN2A (19.6%) and SMAD4 (20.7%) than LUAD (luad\_tcga\_pan\_can\_atlas\_2018,  $n = 566$ ) depicting 52.1%, 29.7%, 4.4% and 4.2% mutation status, respectively, as visualised by cBioportal (Fig. 4D). Further analyses harnessing cBioportal also revealed a lower probability for overall survival in PAAD subset in case of altered TP53 ( $p < 0.05$ ), KRAS ( $p < 0.0001$ ) and CDKN2A ( $p < 0.0001$ ) patient group than the unaltered group (Fig. 4E). No significant difference was observed in the overall survival for altered and unaltered SMAD4 patient cohorts ( $p = 0.193$ ) in PAAD (Fig. 4E). On the other hand, when the similar survival analyses were conducted in LUAD, it was found that altered KRAS ( $p = 0.217$ ), CDKN2A ( $p = 0.055$ ) and SMAD4 ( $p = 0.377$ ) furnished no significant difference with unaltered groups towards overall survival of patients. However, altered TP53 in LUAD presented with significant ( $p < 0.05$ ) poor overall survival than unaltered set (Supplementary Fig. 1A).

Above results together depict that multiple intrinsic immunotherapy-resistance factors including driver mutations in KRAS, CDKN2A and TP53 are augmented in PAAD, and such driver mutations result to poor prognosis in patients.

### Driver mutations along with pro-tumor TME subsets are positively correlated with cancer stem cell status in PAAD

Recent reports have indicated the association between CSCs with multiple intrinsic immunotherapeutic resistance genes. Possible role of CSCs in conferring intrinsic immunotherapy-resistance and influencing extrinsic TME resistance has also been predicted<sup>54,55</sup>. Our literature search identified CD133, CD44, CXCR4, OCT4, c-MET, ALDH1A1, ABCB1 and ABCG2 as pancreatic CSC markers<sup>56–58</sup>. Next, by employing TIMER 2.0 database, we aimed at evaluating whether the driver mutation status affects intrinsic stemness genes in PAAD ( $n = 170$ ). Our results depicted that the expression levels of CD44 ( $p < 0.01$ ), c-MET ( $p < 0.0001$ ) and OCT4 ( $p < 0.0001$ ) were significantly higher in patient samples with KRAS mutation as compared to wild type KRAS patients in PAAD (Fig. 5A). In case of CDKN2A, expression levels of c-MET ( $p < 0.05$ ) and OCT4 ( $p < 0.05$ ) were upregulated in the mutated cohort of PAAD patients than the wild type ones (Fig. 5B).

Similarly, CD44 ( $p < 0.01$ ), c-MET ( $p < 0.001$ ) and OCT4 ( $p < 0.0001$ ) levels were augmented in TP53 mutated PAAD patients than the non-mutated ones (Fig. 5C). Reflecting our previous outcome in SMAD4 from Fig. 4E, no significant difference in expression levels of any of the CSC genes was found among the two groups (Fig. 5D).

Consequently, Kaplan–Meier plot from <https://kmplot.com/analysis/><sup>59</sup> bolstered the significance of mutation driven-CSC genes in pancreatic cancer by demonstrating lower overall survival for patients expressing higher CD44 ( $p < 0.01$ ;  $n = 1189$ ), c-MET ( $p < 0.0001$ ;  $n = 1237$ ) and OCT4 ( $p < 0.01$ ;  $n = 1189$ ) than the lower expressing group (Fig. 5E). Hence, these findings indicate a strong association of the driver mutations that are highly prevalent in PAAD, with the CSC-signature and, ultimately correlating to poor survival in such patients. Reports involving in-vivo models also validated our observations by showing higher expression of CSC-related genes in KRAS/TP53 mutated cells of pancreatic cancer<sup>60,61</sup>.

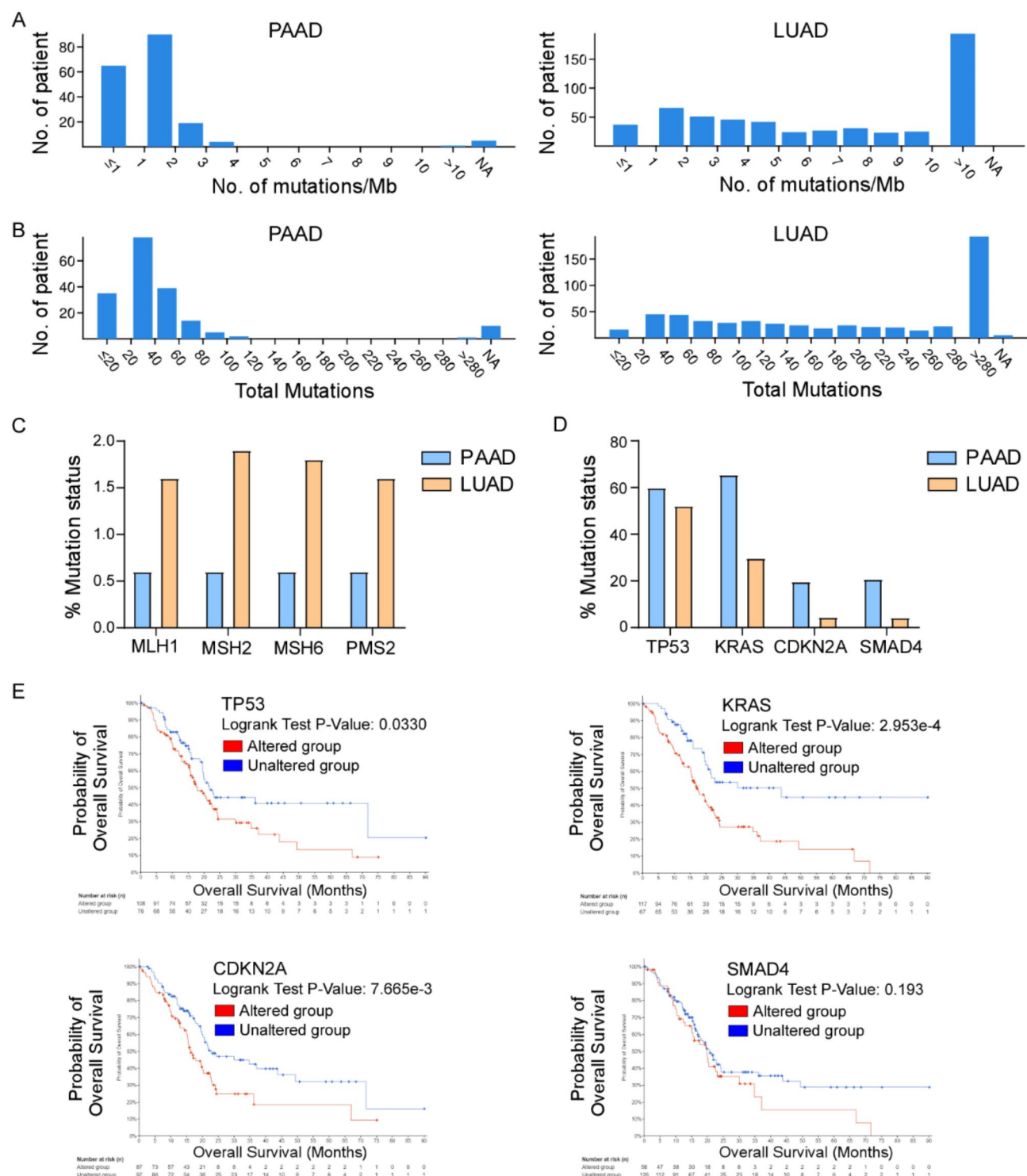
LUAD on the other hand, failed to show significant augmented expression in the mutated subset for any of the above-mentioned genes, except for c-MET in case of TP53 mutation, where it showed higher expression of this gene for the mutated patient group (Supplementary Fig. 1B).

Moreover, other factors such as pro-tumor TME components, which have already been observed to be higher in PAAD than LUAD (Fig. 3), may also contribute towards CSC status. For example, CAFs are reported for their role towards stemness gain in pancreatic cancer<sup>61</sup>. Consequently, we also observed a positive correlation of CAFs with CSC-associated genes in PAAD ( $n = 179$ ) (Fig. 5F). Furthermore, infiltration scores of pro-tumor immune cells, Treg and M2 macrophage, were also positively correlated with CSC-associated genes in PAAD ( $n = 179$ ) (Fig. 5F). Altogether, these findings point to the multi-faceted mechanisms involved, which probably work towards upregulating CSC nature in PAAD.

### PAAD demonstrates a greater CSC-signature than LUAD

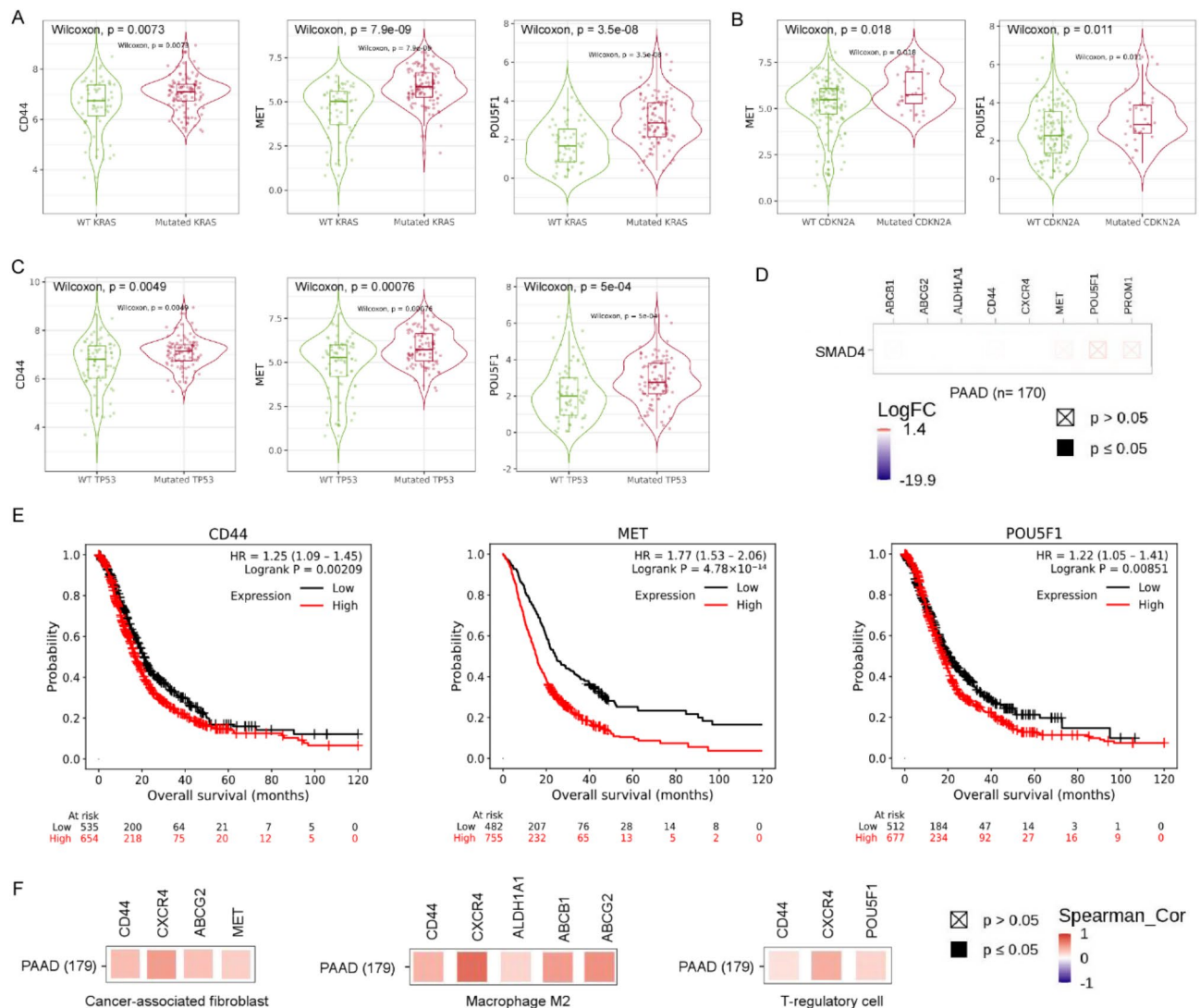
Since multiple factors such as, pro-tumor TME subsets and driver mutations are that higher in PAAD (Figs. 3 and 4D) showed a positive correlation with CSC-related genes in PAAD (Fig. 5A,C,F), we evaluated the relative expression levels of CSC-signatures in both the cancer types. To that end, CSC-related markers common to both pancreatic and lung cancers were identified<sup>56–58,62</sup> and their expression levels were checked from 'R2: Genomics Analysis and Visualization Platform'. We found that the expression levels of CD133, CD44, CXCR4, OCT4, ALDH1A1 and ABCB1 were higher in PAAD (dataset: Tumor Pancreatic adenocarcinoma - TCGA - 178 - rsem - tcgars;  $n = 178$ ) than lung adenocarcinoma (dataset: Tumor Lung Adenocarcinoma - TCGA - 515 - rsem - tcgars;  $n = 515$ ) (Fig. 6A).

Although the median value for ABCG2 in PAAD (6.18) was slightly higher than that of lung adenocarcinoma (6.05), the overall distribution of the same was not significantly different between two groups (Fig. 6A). Further,



**Fig. 4.** Intrinsic immunotherapy-resistance contributing factors in PAAD and LUAD. cBioportal database demonstrating (A) tumor mutational burden (TMB) depicted by total patients showing number of mutations/Mb, and (B) mutational count depicted by number of patients presenting total mutations for PAAD ( $n = 184$ ) (left panel) and LUAD ( $n = 566$ ) (right panel). Bar plots demonstrating percentage mutational status for (C) mismatch repair genes and, (D) key genes, TP53, KRAS, CDKN2A and SMAD4 for PAAD ( $n = 184$ ) and LUAD ( $n = 566$ ). (E) Kaplan Meier overall survival (OS) plot for PAAD patients ( $n = 184$ ) considering altered or unaltered cohorts for TP53, KRAS, CDKN2A and SMAD4 genes. PAAD: Pancreatic adenocarcinoma, LUAD: Lung adenocarcinoma.

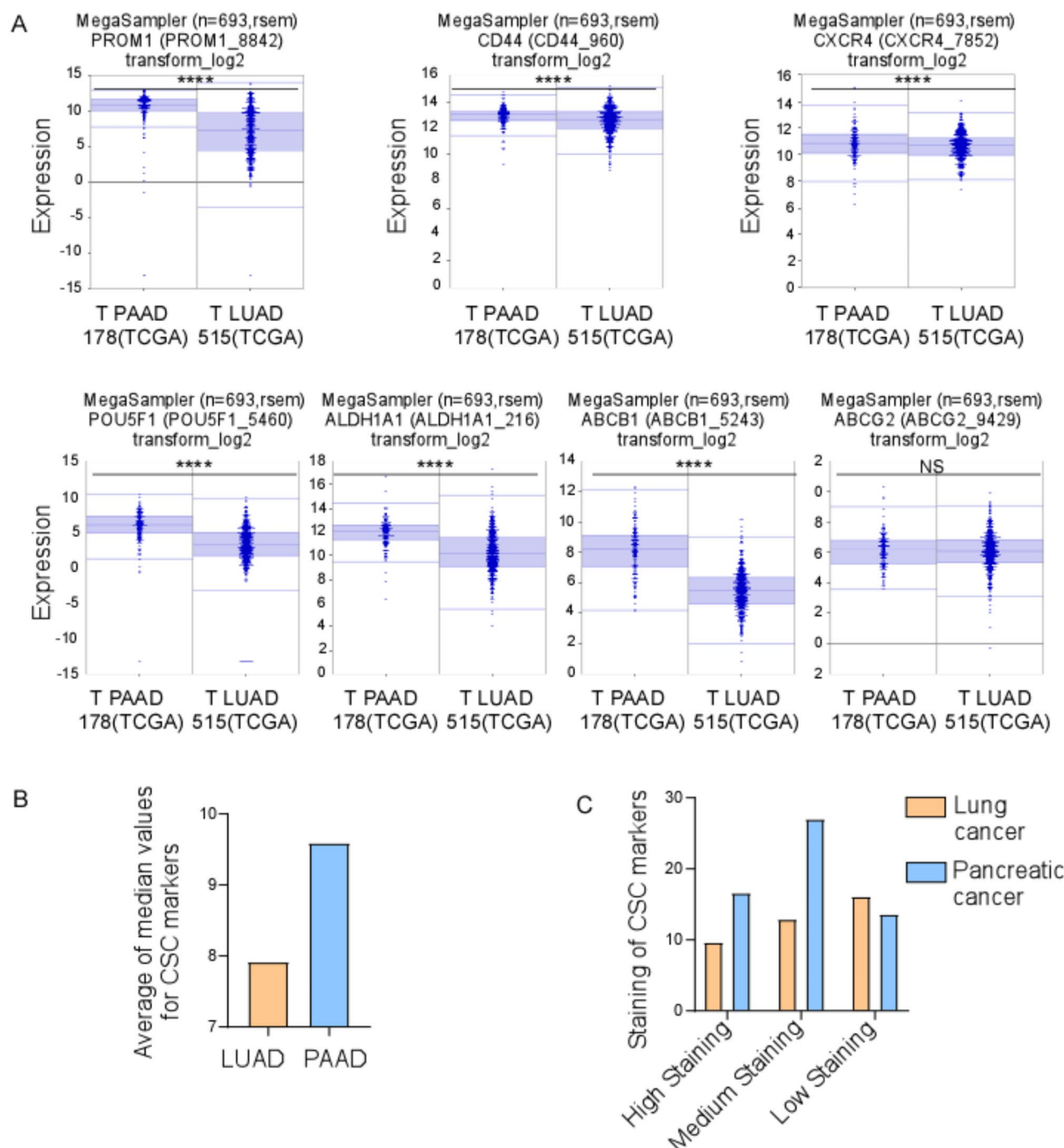




**Fig. 5.** Association of driver mutations with stemness markers in PAAD. Plots depicting log fold change expression for stemness markers, (A) CD44, MET and POU5F1(OCT4) in case of wild-type KRAS or mutated KRAS groups; (B) MET and POU5F1 in case of wild-type CDKN2A or mutated CDKN2A groups; and, (C) CD44, MET and POU5F1 in case of wild-type TP53 and mutated TP53 groups. (D) Log fold change expression for stemness genes in wild-type and mutated SMAD4 groups is shown by heatmap. (A), (B), (C) and (D) are taken for PAAD ( $n = 170$ ) from TIMER 2.0 database and Wilcoxon test was conducted to determine  $p$  value. (E) Kaplan Meier plot from kmplot.com showing overall survival probability in pancreatic cancer patients depending on low or high expression of CD44, MET and POU5F1 in them. (F) Heatmap spearman correlation depicting EPIC CAF infiltration scores with CSC genes as well as, CIBERSORT (ABS) Treg and M2 infiltration scores with CSC genes in PAAD ( $n = 179$ ) using TIMER2.0. PAAD: Pancreatic adenocarcinoma, CAF: Cancer-associated fibroblast.

mean was calculated from the median values for all the 7 CSC-related markers in PAAD and LUAD as obtained from R2, and the results showed higher cumulative mean in PAAD (9.59) than in LUAD (7.92) (Fig. 6B).

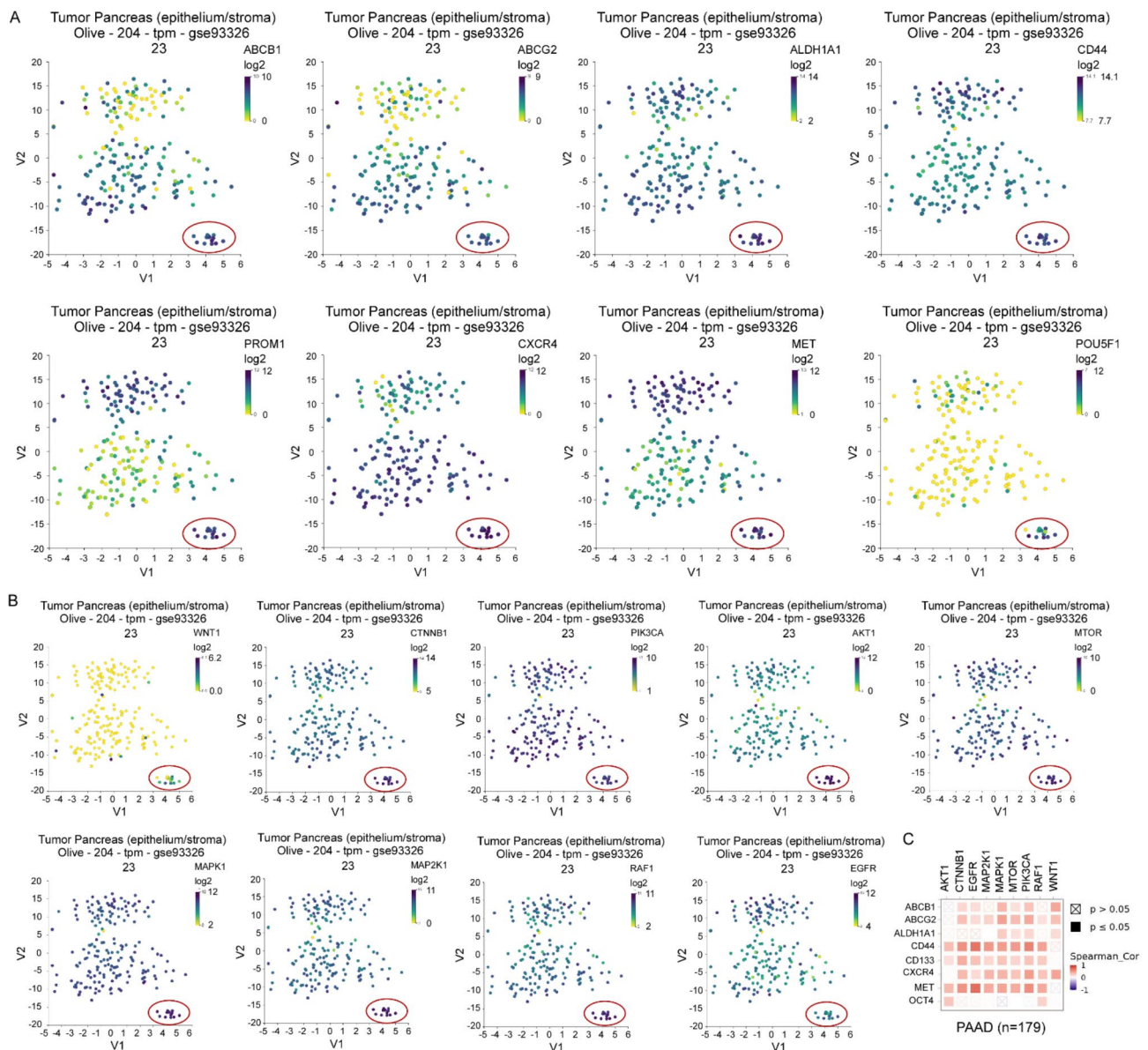
To cross-validate our observation, CSC markers in lung and pancreatic cancer tissues, as furnished by the data obtained from online resource 'The Human Protein Atlas'<sup>45</sup>, were evaluated based on percentage of samples presenting low, medium or high antibody staining. Data of CD133 (Ab.: HPA004922), CD44 (Ab.: HPA005785), OCT4 (Ab.: CAB026380), ALDH1A1 (Ab.: CAB020690), ABCB1 (Ab.: CAB001716) and ABCG2 (Ab.: HPA054719) were used. However, staining for CXCR4 was not found in this online resource. In pancreatic cancer, 16.6% of the cumulative CSC markers showed high staining, 27% showed medium staining, whereas low staining was observed in 13.6% of the considered genes. On the other hand, lung cancer showed 9.67% high staining, 12.9% medium staining and 16.12% low staining of CSC genes (Fig. 6C). This observation demonstrated greater percentages of high and medium staining of CSC-related factors in pancreatic cancer than lung cancer tissues, which strengthened our previous findings that PAAD displayed higher incidence of CSCs than LUAD, as these subsets are the major constituents of the mentioned cancers<sup>41,44</sup>.



**Fig. 6.** Expression of CSC markers in PAAD and LUAD. (A) Megasampler analyses from ‘R2: Genomics Analysis and Visualization Platform’ depicted by dot plots for PAAD ( $n = 178$ ) and LUAD ( $n = 515$ ) in case of CSC genes, PROM1 (CD133), CD44, CXCR4, POU5F1(OCT4), ALDH1A1, ABCB1 and ABCG2. Data were represented as box/dot plot (bands) and ANOVA was used to assess the data where, ns non-significant,  $*P < 0.05$ ,  $**P < 0.01$ ,  $***P < 0.001$ , and  $****P < 0.0001$ , as obtained from R2. (B) Mean of the cumulative median scores calculated from (A) is plotted for PAAD and LUAD. (C) Staining data from ‘The Human Protein Atlas’ showing low, medium or high staining of pancreatic and lung cancer tissues for the mentioned CSC genes in (A). PAAD: Pancreatic adenocarcinoma, LUAD: Lung adenocarcinoma, CSC: Cancer stem cell.

## Pancreatic CSCs are positively associated with immunotherapy resistance-related signaling pathways, pro-tumor immune subsets and immuno-modulatory factors

Next, to check whether the augmented CSC-signature is indeed responsible for immunotherapy-resistance in PAAD, we aimed at determining relationship if any, between the immunotherapy resistance-related signaling pathways and pancreatic CSCs. To that end, we employed UMAP/tSNE from R2 for the dataset 'Tumor Pancreas (epithelium/stroma) - Olive - 204 - tpm - gse93326' ( $n = 204$ ) and identified the patient cancer samples depicting high expression of the CSC-related genes<sup>56–58</sup> CD133, CD44, CXCR4, OCT4, ALDH1A1, ABCB1, ABCG2 and c-MET for PAAD (Fig. 7A), as visualised by heatmap in log2 expression. Higher expression of majority of these CSC markers in PAAD than LUAD has already been furnished in Fig. 6A. Reports also indicate up-regulation of signaling pathways like, WNT/ $\beta$  catenin, PI3K-AKT and MAPK, in immunotherapy-resistance in cancers<sup>20,23</sup>. Consequently, in our model, the selected pancreatic CSC-enriched samples from tSNE map also depicted high expression of the major genes from the mentioned signaling pathways, such as, WNT1, CTNNB1 ( $\beta$  catenin),



**Fig. 7.** Correlation of pancreatic CSC genes with immunotherapy-resistance pathway genes. **(A)** UMAP/tSNE plot of PAAD tumor dataset (Olive-204-tpm-gse93326; tSNE perplexity 23) from 'R2: Genomics Analysis and Visualization Platform' demonstrating samples (marked) with high expression of CSC markers, ABCB1, ABCG2, ALDH1A1, CD44, CD133 (PROM1), CXCR4, MET, and OCT4 (POU5F1). **(B)** The same group marked in **(A)** depicting high expression of immunotherapy-resistance associated signaling genes, WNT1, CTNNB1, PIK3CA, AKT1, MTOR, MAPK1, MAP2K1, RAF1, EGFR. **(C)** Correlation among the CSC genes from **(A)** and signaling genes taken in **(B)** were analyzed from TIMER database in PAAD ( $n = 179$ ) and represented as heatmap depending on values from Spearman correlation. PAAD: Pancreatic adenocarcinoma, CSC: Cancer stem cell.



PIK3CA (catalytic subunit of PI3K), AKT1, mTOR, MAPK1, MAP2K1 (MEK1), RAF1 and EGFR (Fig. 7B). Further verifying our observations in PAAD, TIMER database also presented a positive correlation of pancreatic stemness genes with the immunotherapy-resistance pathway genes (Fig. 7C) as seen in Fig. 7B.

Next, our aim of examining the relationship, if any, between the CSC status and the extrinsic factors that render immunotherapy-resistance revealed that pro-tumor immune subsets, i.e., Treg-signature gene FOXP3<sup>32</sup> and M2 macrophage-markers CD206, CD163 and CD200R<sup>63</sup>, were high in expression in the selected CSC-enriched subset from tSNE plot (Fig. 8A). Again, CSC genes, CD44, ABCG2, CXCR4 and ABCB1 were found to be positively correlated with the pro-tumor immune subset signature markers and ALDH1A1 was correlated with the M2 markers (Fig. 8B) as mentioned in Fig. 8A. Also, tumor immune dysfunction and exclusion (TIDE)<sup>64</sup> online database depicted a negative correlation of cytotoxic T cell levels with CSC markers, MET ( $p < 0.01$ ;  $r = -0.243$ ) and OCT4 ( $p < 0.05$ ;  $r = -0.193$ ) (Fig. 8C). Resultantly, pancreatic CSC-related genes not only influence the intrinsic resistance factors, but also manipulates the extrinsic factors towards rendering immunotherapy-resistance.

Our next approach demonstrated higher expression of immunotherapy resistance-related immuno-modulatory molecules, VEGF, TGF $\beta$ , IL10, IDO1 and TDO2 along with chemokines, such as CCL2, CCL7, CCL8, and CCL13<sup>20,23</sup>, in the selected pancreatic CSC-enriched group from tSNE map (Fig. 9A). Correlation analyses from TIMER in PAAD also furnished a positive correlation between most of these immune-modulatory factors with CSC-related genes, CD44, CXCR4, ABCB1 and ABCG2. c-MET was seen to be correlated with VEGFA, CCL7 and CCL13 whereas, OCT4 was correlated with TGF $\beta$ 1 and VEGFA (Fig. 9B). Other than being associated with extrinsic and intrinsic immunotherapy-resistance factors, CSC-related genes in PAAD were observed to be positively correlated with IC molecules expressed on tumor cells (Supplementary Fig. 1C). It would not be out of context to point here that, driver mutations (TP53, KRAS, CDKN2A) and IC expression were both highly expressed in PAAD (Figs. 2 and 4D). To that end, we found that among the considered driver mutations, IC molecules CD80 and CD86 were significantly upregulated in TP53-mutated cohort as compared to the wild type in PAAD (Supplementary Fig. 1D). However, no other significant changes could be observed in case of other IC molecules and driver mutations. While this observation hint towards factors other than driver mutations responsible for controlling high IC expression in PAAD, our result in Supplementary Fig. 1C bolster the significance of CSCs towards controlling such IC expression. Thereby, depicting CSC to be a probable 'other factor' in this case.

Taken together, our findings highlight high CSC incidence in PAAD, which is responsible for high IC levels leading to immune evasion, and provides immunotherapy-resistance, being positively associated with immunotherapy resistance-related signalling pathways, pro-tumor immune subsets and immuno-modulatory factors. This explains the probable reasons for low immunotherapy-response in high IC-expressing PAAD.

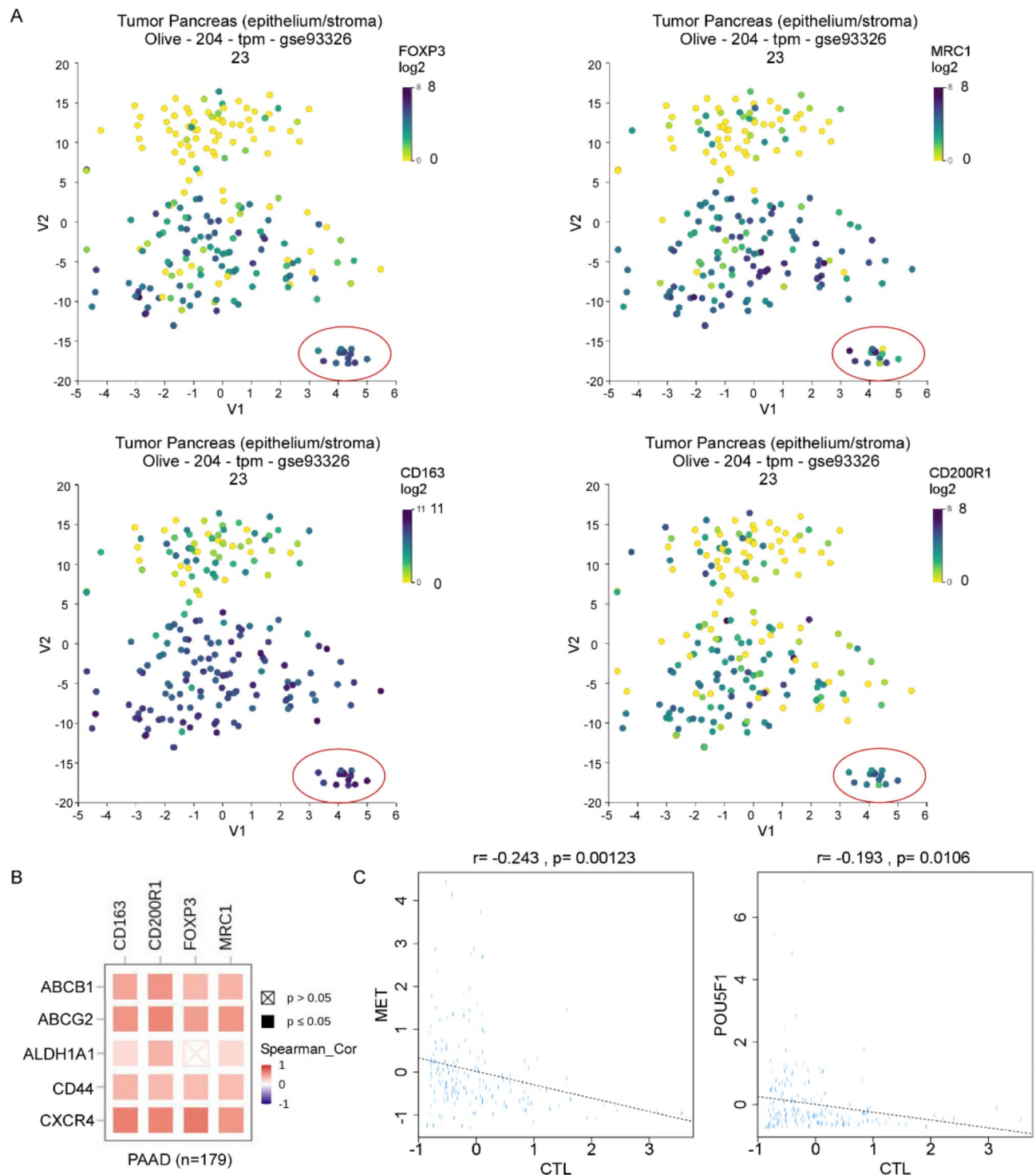
### Not only LR cancers but also HR ones fail to respond to immunotherapy depending on the stemness status of the cancer

To explore whether the contribution of CSCs in therapy resistance is limited to only PAAD or not, we took another LR cancer, i.e., prostate cancer. Our results, as obtained from the prostate adenocarcinoma dataset available from TIMER database, depicted positive correlation between the reported immunotherapy resistance-associated gene DKK1 in prostate cancer<sup>65</sup> with all the prostate cancer stem cell genes considered<sup>66</sup>, i.e., CD133, CD44, CD166, ALDH1A1, ABCG2 (Supplementary Fig. 2A). These results validate the role of CSCs in resisting immunotherapy in other LR cancers, as well.

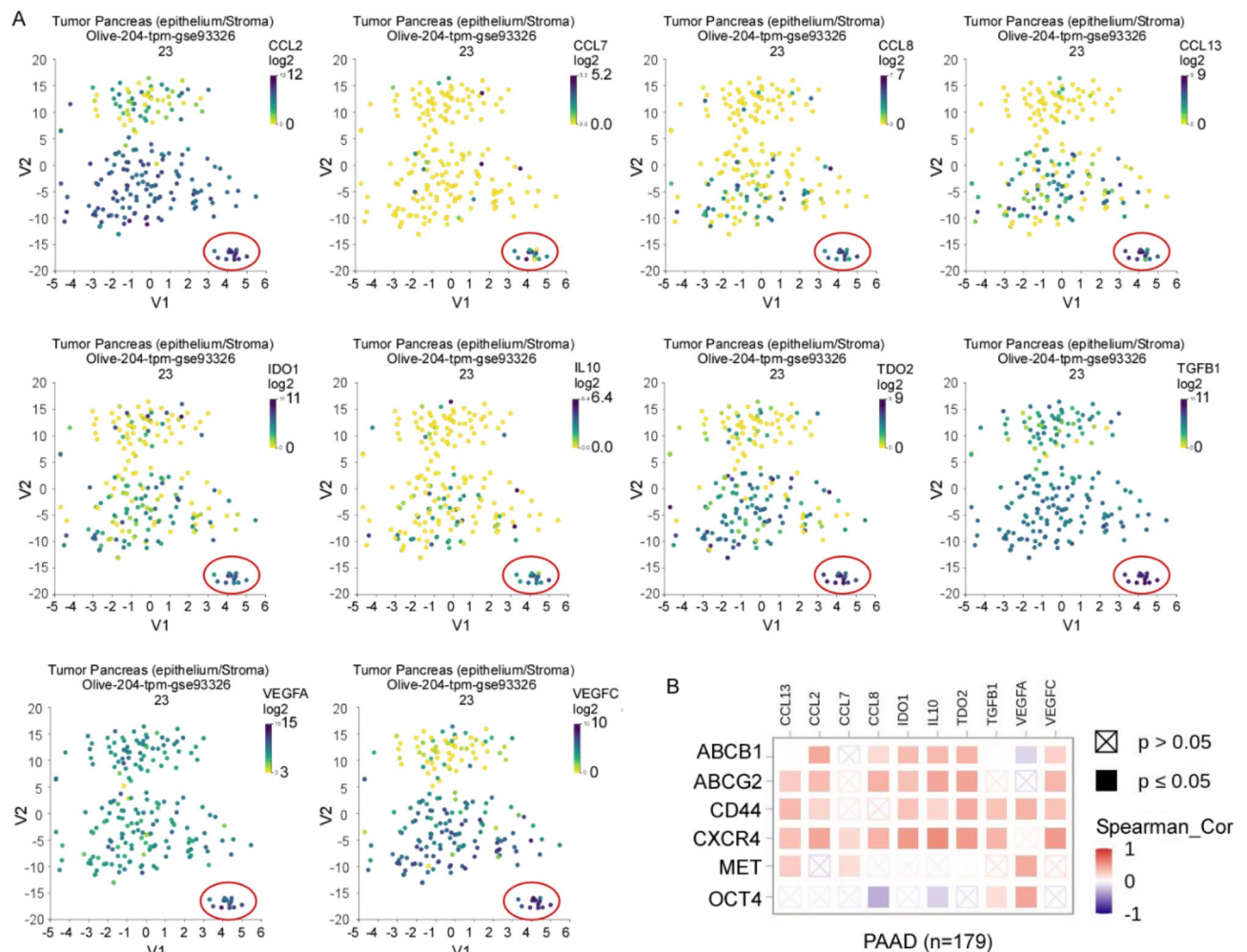
Next, we shifted our attention to another aspect of immunotherapy-failure. It is well accepted that not all patients of even high immunotherapy-responsive cancers, e.g., lung cancer, respond completely to ICI therapy<sup>54</sup>. In fact, a recent study<sup>54</sup> identified patient subsets correlating with immunotherapy-resistance based on WGCNA analysis in LUAD model. Furthermore, genes associated with immunotherapy resistance-related signaling pathways, such as, WNT, PI3K, MAPK, were identified in such subsets along with higher immune evasion and immunosuppressive markers<sup>54</sup>. Therefore, extending our line of thought about the plausible relation of resistance genes with stemness factors in one of the major HR cancers, i.e., LUAD, the identified resistance genes from the study, namely, THY1, COL5A1, COL1A2, COL3A1, COL1A1, COL5A2, COL6A3 were found to be indeed positively correlated with lung CSC markers, CD44, CXCR4 and ABCG2 (Supplementary Fig. 2B). The same study also indicated a positive correlation between THY1 and stemness factor, SOX9, thereby demonstrating that the failure of immunotherapy even in some subsets of HR LUAD is also associated with the stemness, and is independent of the expression of IC molecules in these resistant patients. Validating our hypothesis, expression levels of these therapy-resistance genes were found to be higher in PAAD than that of LUAD (Supplementary Fig. 2C) demonstrating the probable contribution of these additional factors towards higher resistance in PAAD too. Fascinatingly, pancreatic CSC genes CD44, CXCR4, ABCG2 and MET were also observed to be positively correlated with these resistance genes (Supplementary Fig. 2D). Our previous results showing that LR PAAD furnishes higher immunotherapy-resistance than LUAD due to the relatively higher incidence of CSCs that in turn generate high resistance factors, thus strengthening our notion. It would be noteworthy to mention that, analyses of UMAP/tSNE plots from PAAD sc-RNA seq dataset revealed high CSC nature observed in PAAD, exists as a sub-population of cancer cells expressing CSC-related markers (Supplementary Fig. 3), rather than the entire population.

All these results together led us to the hypothesis that irrespective of cancer type, immunotherapy responsiveness of any patient is decided not only by the IC levels but also by many other resistance-rendering factors, most of which depend on the magnitude of stemness present in the tumor of that patient. Therefore, future immunotherapeutic interventions might include evaluation of the magnitude of CSCs present in individual patient as a predictive biomarker, rather than administering treatment based only on cancer type or IC expression.





**Fig. 8.** Association of pancreatic CSC genes with extrinsic immunotherapy-resistance. **(A)** UMAP/tSNE plot of PAAD tumor dataset (Olive-204-tpm-gse93326; tSNE perplexity 23) from 'R2: Genomics Analysis and Visualization Platform' demonstrating the same marked samples from Fig. 7A, with high expression of Treg marker FOXP3, and M2 macrophage markers, MRC1, CD163 and CD200R1. **(B)** TIMER database showing spearman correlation between the immune cell markers from (A) and CSC genes, ABCB1, ABCG2, ALDH1A1, CD44, and CXCR4 in PAAD ( $n = 179$ ). **(C)** Tumor immune dysfunction and exclusion (TIDE) database depicting negative pearson correlation of CSC markers, MET and POU5F1 with cytotoxic T lymphocyte level in TCGA pancreatic cancer cohort. PAAD: Pancreatic adenocarcinoma, CSC: Cancer stem cell.



**Fig. 9.** Association of pancreatic CSC genes with immuno-modulatory molecules. **(A)** UMAP/tSNE plot of PAAD tumor dataset (Olive-204-tpm-gse93326; tSNE perplexity 23) from ‘R2: Genomics Analysis and Visualization Platform’ demonstrating the same marked samples from Fig. 7A, with high expression of immuno-modulatory molecules, CCL2, CCL7, CCL8, CCL13, IDO1, IL10, TDO2, TGFBI, VEGFA, and VEGFC. **(B)** TIMER database showing spearman correlation between the immuno-modulatory molecules from (A) and CSC genes, ABCB1, ABCG2, CD44, CXCR4, MET and POU5F1 in PAAD ( $n = 179$ ).

## Discussion

Immunotherapy, especially, ICI has emerged to be one of the leading methods and is used as an approved treatment modality in many cancers, including melanoma, lung, renal and breast cancer patients, due to its positive outcomes in these cancers<sup>4,11–14</sup>. While the mentioned cancers show durable responses to ICI treatment, cancers such as, pancreatic, glioblastoma and prostate cancers have not been quite responsive to such therapy measures<sup>4,15–17</sup>. Discrepancy in immunotherapy outcome is often associated with the IC expression, as conventionally, a cancer expressing higher IC molecules is more likely to respond to IC blockade mechanism and this line of thought has been supported by studies and clinical trials where the correlation between response and IC expression has been found<sup>67,68</sup>. However, it has also been observed that the presence of IC is not necessarily a reliable biomarker to immunotherapy-response<sup>18,19</sup>. Reports suggest that cancer with high IC expression can generate low response to immunotherapy<sup>42,43</sup>, while high IC expressing lung cancer show a response rate below 20%<sup>54</sup>. Among other factors, the cancer type and patient-to-patient response to immunotherapy also differ irrespective of IC expression. For example, as not all PDL1 positive patients show positive results, PDL1 negative subjects manage to furnish effective response to immunotherapy treatment<sup>68,69</sup>. In case of pancreatic cancer, although expression of PDL1 is considered as a prognostic marker for poor patient survival as they demonstrate high PDL1 levels<sup>42</sup>, anti-PDL1-based clinical trials in pancreatic cancer failed to furnish any objective response in patients<sup>70,71</sup>.

In fact, blocking IC impedes only one aspect of immunotherapy-resistance, but for generating therapy response, anti-tumor immune cells need to recognize and attack the tumor cells, which is otherwise limited by numerous components<sup>20</sup>. Therefore, upon trying to understand the other determining factors to immunotherapy-resistance, we came across multiple extrinsic and intrinsic mechanisms at play<sup>72</sup>. Extrinsic resistance to

immunotherapy is determined by relative pro-tumor or anti-tumor immune infiltration status that generates an immunological 'cold' or 'hot' tumor, respectively<sup>5</sup>. In addition to this, multiple intrinsic resistance mechanisms, determined by the cancer cells are also responsible for ineffectiveness of immunotherapy, such as low TMB and low mismatch repair deficiency<sup>20,23</sup>. Genetic alterations are involved in development of neoantigens, and thus better recognition by immune cells, and subsequently effective response to immunotherapy. HR cancers like, lung cancer and melanoma, demonstrate high TMB due to the frequent exposure to tobacco smoking and UV<sup>73</sup>. In fact, among the LR cancers, a small fraction of patients who responds to immunotherapy furnishes high-TMB and high mismatch repair deficiency<sup>74,75</sup>. Dysregulation of key genes in cancers often alters signaling pathways, increased intrinsic resistance mechanisms and even, extrinsic resistance and immune escape<sup>23,72,76</sup>. From analyzing publicly available patient data, we identified a LR cancer PAAD demonstrating high IC expression, but evading immunotherapy by furnishing higher CAF and pro-tumor immune infiltration along with, lower anti-tumor immune infiltration, lower TMB and mismatch repair deficiency genes. Alteration in key genes, KRAS, CDKN2A, TP53 and SMAD4 were also observed in this cancer, that has been associated with poor survival, as verified by reports<sup>23,77</sup>, except for SMAD4. While according to some studies, mutations in SMAD4 is related to tumor progression<sup>78,79</sup>, however, SMAD4 being directly involved in TGF $\beta$  signaling pathway<sup>80</sup>, mutations in SMAD4 might alter that pro-tumor pathway, thereby portraying differential effects.

CSCs, known for their tumor initiating, metastasis and relapse-causing potentials (24–26), are also involved in immune evasion, high IC expression, and immune-suppression<sup>27,32</sup>, thereby indicating their possible role in immunotherapy-resistance<sup>55</sup>. In cellular immunotherapy, lymphocytes are transferred to patients to effect better response<sup>81</sup>, however, previous finding from our laboratory demonstrated conversion of CD4 + T effector T cells to Tregs in the presence of CSCs, as low as in the ratio of 1:5 (CSC: T cell)<sup>32</sup>. Therefore, the incidence of CSC plays a pivotal role at evading immunotherapy. In our analyses from available patient statistics, LR PAAD showed higher CSC-signature than HR cancer, justifying immunotherapy-resistance in PAAD, even though it demonstrates IC expression like HR cancers. Higher CSC signature in PAAD can be due to presence of higher number of CSC sub-population in PAAD than LUAD, or due to higher CSC related-antigen expression in the CSC sub-population itself, in PAAD than that of LUAD. Pro-tumor CAFs, immune cells, and even, driver mutations in PAAD, were observed to collectively favor the CSC signature. Such CSCs harbor high IC levels, altered signaling pathways, and contributes to intrinsic and extrinsic immunotherapy-resistance<sup>55,82</sup>, as reflected in our observations as well. These findings also elucidate that, owing to the high CSC content in PAAD, tumor cell-associated ICs were highly correlated and expressed in this cancer. Such IC levels can hence be considered as markers for poor prognosis. However, targeting only these ICs did not yield positive response which might be due to the existence of CSCs, that modulate other mechanisms of immunotherapy-resistance. Besides, CSCs employ these resistance factors for other functions as well, for instance, VEGF for promoting neo-angiogenesis<sup>83</sup> and TGF $\beta$  for Treg polarization<sup>32</sup>.

The role of CSCs in immunotherapy-resistance is not limited to only one LR cancer but others as well. Another LR, i.e., prostate adenocarcinoma, has also shown positive relationship between CSCs and immunotherapy-resistance. In fact, EZH2 which is noted as a contributor to immunosuppressive action and consequently, therapy failure<sup>65</sup> is also marked as a prostate stem cell marker<sup>84</sup>. Similarly, in the case of LR glioblastoma, SOX2, another CSC marker (93), has been found to be linked with intrinsic resistance<sup>76</sup>, thus verifying our reported observation, that high and low expression of PD1 or CTLA4 had no distinct effect on the patient survival in LR cancers. Additionally, reflecting previous discussion, not all patients of HR cancers respond to therapy, and therefore, the resistance related-genes were also found to correlate with their stemness markers. Quite interestingly, subjection to immunotherapy has been linked to increasing CSC-signature in breast cancer<sup>85</sup>.

Overall, our detailed analyses of cancer patient statistics explain the role of CSCs towards determining immunotherapy-response in PAAD. Not only this, CSCs demonstrate their predictive role in immunotherapy-resistance in a multi-cancer scenario. ICI therapy based on IC expression profile, i.e., 'one size fits all', is therefore, not necessarily an efficient marker for effective response. Rather other factors such as, CSC incidence might also be strongly considered.

### Limitations and future prospectives

While our study underlines an important aspect of immunotherapy-resistance supported by in-silico patient data analyses, it needs to be further validated in in-vitro and in-vivo models for deeper understanding of the molecular mechanisms underlying the contribution of CSC in immunotherapy-resistance. Our study could also benefit by validating the findings in other datasets that consist of higher patient numbers, which might add to the prognostic value. Additionally, datasets demonstrating correlations do not fully portray the exact association and biological complexity in the system, but only serve as indications to a possible avenue for further research. To that end, experimental validations followed by combinatorial therapy aimed at sensitizing CSCs first, as seen in our previous lab reports<sup>29,86,87</sup>, can be considered in future, which might generate better outcomes when treated along with immunotherapy. Modern immunotherapy methods would be successful only if every contributing factor is taken into account and scored based on their presence, such as, cancer type, IC expression, CSC content, immune infiltration, TMB, among others.

### Methods

#### Collection of clinical trial data

For the purpose of obtaining statistics based on clinical trials in cancer by ICIs, clinicaltrials.gov<sup>33</sup>, an official database website of National Institutes of Health (NIH) was used and relevant disease terms, intervention type and study start date taken till 31st January, 2024, were supplied to filter our search.

## Collection of gene expression data

TCGA RNAseq gene expression data from 9 cancer datasets (Supplementary Table 1) comprising of 3899 patients were used from 'R2: Genomics Analysis and Visualization Platform'<sup>88</sup> and megasampler function from R2 was employed to compare the expression of IC genes. TCGA RNAseq expression of CSC markers and immunotherapy-resistance genes from LUAD and PAAD datasets consisting of 693 total patients, available from R2 were again compared using megasampler. log<sub>2</sub> transformation and *p* value analysis using ANOVA were carried out by the tool according to their pre-set protocols. UMAP/tSNE maps from R2 for the dataset 'Tumor Pancreas (epithelium/stroma)-Olive-204-tpm-gse93326' containing RNAseq data for 204 PAAD samples prepared with NuGEN and TruSeq methods, for analysing gene expression patterns. UMAP/tSNE plots for single cell-RNA dataset 'Mixed PDAC Treatment-naive - USER - 88031 - custom - scpp001; tumor cell subset (n = 43817)' was also used to evaluate CSC-related gene expression.

GEPIA (Gene Expression Profiling Interactive Analysis), a web based tool based on TCGA and GTEx data<sup>40,89</sup>, was used to find expression levels of IC molecules in PAAD for 179 patient samples and matched with 171 normal pancreatic patient samples. The differential analysis here is based on the selected datasets ("TCGA tumors vs. TCGA normal + GTEx normal"), with Log<sub>2</sub> fold change cutoff set to 1 and *p* value cut off set at 0.05. The fold change and *p* value analysis were carried out by the tool according to their pre-set protocols.

cBioportal developed by Memorial Sloan Keating Cancer Center, a publicly accessible online platform<sup>51</sup>, was used to access the mutational burden in LUAD (luad\_tcga\_pan\_can\_atlas\_2018) comprising of 566 patients and PAAD (paad\_tcga\_pan\_can\_atlas\_2018) comprising of 184 patients, from TCGA Pan-Cancer Atlas Studies, containing a total of 10,953 patients.

Another online resource, 'The Human Protein Atlas'<sup>45</sup> was used for determining protein expression data derived from antibody-based protein profiling using immunohistochemistry in lung and pancreatic cancer for IC molecule and CSC marker expressions.

## Correlation of gene expression

TIMER 2.0<sup>48</sup>, a publicly available online database hosted by Dana Farber Cancer Institute was used to find correlation between genes and cell infiltration scores using their correlation module and the data was represented as a heatmap based on purity adjusted-spearman correlation and *p* values conducted by the online tool based on their pre-set protocols. This database hosts data for about 40 cancer types, along with PAAD, LUAD and prostate adenocarcinoma. This database was also employed to find out differential gene expression between wild-type and mutation status of genes using their gene mutation module. The heatmap showed the log<sub>2</sub> fold changes of the differential expression of each gene for the cancer type.

## Evaluation of TME infiltration status

Infiltration estimation for TCGA tumors was obtained from TIMER 2.0<sup>48</sup> was used and CIBERSORT scores for different immune subsets and EPIC score for CAF subset were plotted for PAAD, comprising of 179 patients and LUAD comprising of 208 patients. TIDE (Tumor immune dysfunction and exclusion), a computational framework developed to evaluate the potential of tumor immune escape from the gene expression profiles of cancer samples<sup>64</sup> was used to evaluate cytotoxic T lymphocyte levels with CSC status in TCGA pancreatic cancer cohort. Correlation *r* value and *p* values were carried out by the tool according to their pre-set protocols.

## Survival analyses

GEPIA was used to evaluate disease free survival (DFS) for cancer groups based on gene expression. Log-rank test was used by the tool to determine hypothesis test. Cox proportional hazard ratio and the 95% confidence interval information were also included in the survival plot. cBioportal was used to determine Kaplan–meier plot based on altered and unaltered status of genes determining overall survival in PAADs and LUADs, from TCGA Pan-Cancer Atlas Studies. Log-rank test was used to determine the *p* value. Kaplan–Meier plot from kmplot.com<sup>59</sup> was used to evaluate the overall survival in pancreatic cancer with respect to, CSC genes by selecting auto best cutoff and significance was determined by log-rank test.

## Statistical analysis

For statistical analyses performed in immune infiltration estimation and differential IC median values in PAAD and LUAD, GraphPad Prism 9.3.1. was used. Data were analysed by unpaired student's *t* test and non-parametric Mann Whitney test respectively, and the resulting significant (*p* < 0.05) or non-significant (*p* > 0.05) *p* values have been furnished.

## Data availability

All data used in this study are publicly available. All datasets used in this study are obtained either from R2 platform (<https://hgserver1.amc.nl/cgi-bin/r2/main.cgi>), clinicaltrials.gov, GEPIA (<http://gepia.cancer-pku.cn/>), cBioportal (<http://cbioportal.org>), Human Protein Atlas (<https://www.proteinatlas.org/>), TIMER 2.0 (<http://timer.cistrome.org/>), TIDE (<http://tide.dfci.harvard.edu/>) or from <http://www.kmplot.com/analysis>. All data needed to evaluate the conclusions in the paper are present in the paper and/or the Supplementary Materials.

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## Author contributions

Conceptualization: TD, Methodology and Investigation: UB, Visualization: SM and SC, Supervision: TD, SGD, GS, Writing-original draft: UB, Writing-review and editing: TD. All authors reviewed the manuscript.

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## Declarations

## Competing interests

The authors declare no competing interests.

## Additional information

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