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Acid-base homeostasis orchestrated by NHE1 defines pancreatic stellate cell phenotype in pancreatic cancer

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Conflict-of-interest statement

The authors have declared that no conflict of interest exists.
Abstract

Pancreatic ductal adenocarcinoma (PDAC) progresses in an organ with a unique pH landscape, where the stroma acidifies after each meal. We hypothesized that disrupting this pH landscape during PDAC progression triggers pancreatic stellate cells (PSCs) and cancer-associated fibroblasts (CAFs) to induce PDAC fibrosis. We revealed that alkaline environmental pH is sufficient to induce PSC differentiation to a myofibroblastic phenotype. We then mechanistically dissected this finding focusing on the involvement of the Na\(^+\)/H\(^+\) exchanger NHE1. Perturbing cellular pH homeostasis by inhibiting NHE1 with cariporide partially alters the myofibroblastic PSC phenotype. To show the relevance of this finding in vivo, we targeted NHE1 in murine PDAC (KPfC). Indeed, tumor fibrosis decreases when mice receive the NHE1-inhibitor cariporide in addition to gemcitabine treatment. Moreover, the tumor immune infiltrate shifts from granulocyte-rich to more lymphocytic. Taken together, our study provides mechanistic evidence on how the pancreatic pH landscape shapes pancreatic cancer through tuning PSC differentiation.

Brief summary:

Adjuvant pancreatic cancer therapy with a Na\(^+\)/H\(^+\) exchanger NHE1 inhibitor targets the pancreatic pH landscape and reduces tumor fibrosis.
Pancreatic ductal adenocarcinoma (PDAC) has a detrimental prognosis, with an overall 5-year survival rate of 12% even with state-of-the-art therapy (1). The main limiting factors of PDAC therapy are early metastases and ineffective radio- and chemotherapy. The latter is caused, among others, by the predominant presence of stromal cells and extracellular components within the tumor mass (2). The stiff fibrotic extracellular matrix (ECM) of the tumor stroma, produced mainly by pancreatic stellate cells (PSCs), limits the diffusion of nutrients, rendering the PDAC stroma hypoxic and acidic compared to the healthy pancreas (3). Moreover, fibrosis hampers the anti-tumor immune response, as the cytotoxic CD8+ lymphocytes are substituted by neutrophil granulocytes and myeloid-derived suppressor cells (MDSCs) (reviewed in (4, 5)). In the healthy pancreas, PSCs are localized near acinar and ductal cells and are in a so-called quiescent state (6). In PDAC, PSCs are a heterogeneous cell group mainly functioning as pro-tumor cancer-associated fibroblasts (CAFs) (7). On the one hand, PSCs differentiate into myofibroblastic CAFs (myCAFs) upon noxious stimuli such as inflammation, hypoxia, or mechanical stress. They express activation markers such as alpha-smooth muscle actin (αSMA), migrate to the site of injury, proliferate, and remodel the extracellular matrix (ECM). On the other hand, PSCs can differentiate into immunomodulatory CAFs (iCAFs), attracting a wide range of tumor-promoting and anti-tumor immune cells. However, it is important to note that CAFs are a heterogeneous group of cells derived from different lineages and do not only originate from PSCs. Multiple therapeutic strategies – e.g., cellular reprogramming with vitamin D target tumor-promoting CAFs in pancreatic cancer (8, 9). However, none of these therapies exploits the unique pancreatic pH landscape, detailed below.

Acid-base homeostasis is a fundamental physiological function of every cell. The intracellular pH (pHi) is usually slightly more acidic (ΔpHi ~ 0.2) than the extracellular pH (pHe) (10). Numerous pH-sensory and pH-regulatory molecules maintain pHi. Perturbed pHe can be directly sensed by G-protein coupled receptors and other pH-sensing membrane proteins, e.g., ion channels (11). Cells use efficient acid extruders in case of intracellular acidification, such as the Na+/H+ exchanger NHE1 (Slc9a1), the Na+/HCO3− cotransporter.
NBC1 (Slc4a4), or the V-type H⁺-ATPase (Atp6v0) (10). From these transporters, NHE1 is particularly well-druggable: multiple validated inhibitors are available, from which cariporide has also been tested in large clinical trials (12, 13). In cancer cells, pH homeostasis is consistently dysregulated. Their pHᵢ is usually higher than pHₑ (14) because of increased expression of pH-regulating transporters such as NHE1 (15–18). However, how pH regulation is altered in PSCs during PDAC is unknown.

The healthy pancreas has a unique pH landscape (19). Upon each meal, ductal cells secrete up to ~150 mM HCO₃⁻ into the ductal lumen (20). This, in turn, leads inevitably to an equimolar efflux of H⁺ across the basolateral membrane into the stroma. Otherwise, the ductal epithelial cells would be unable to maintain their intracellular pH homeostasis. Consequently, all cells residing in the pancreatic stroma, such as pancreatic stellate cells, are intermittently exposed to a substantial acid load, detected in proof-of-principle measurements with pH microelectrode (21). However, in our view, the reported values are an underestimation of the postprandial interstitial acidosis of the pancreas. How PSCs react to such an environmental acidosis has not been investigated to date. It is plausible that tumorous transformation of the ductal cells in the early stages of pancreatic tumorigenesis (in pancreatic intraepithelial neoplasia (PanIN)) impairs ductal cell polarity, thus apical HCO₃⁻ secretion into the ductal lumen (19). Less apical HCO₃⁻ secretion results in reduced H⁺ extrusion into the pancreatic stroma and hence, in attenuated acidification or relative alkalinization of the interstitial pH. We hypothesize that this prolonged decrease in intermittent acidity is a major determinant of the PSC phenotype and leads to tissue fibrosis in PDAC. We tested this hypothesis by combining in vitro and in vivo experiments focusing on pH regulation and PSC and CAF phenotype, with the aim of better understanding desmoplasia in PDAC and identifying a potential therapeutic target for its disruption.
RESULTS

Altered environmental pH triggers a PSC phenotype switch in vitro

First, we aimed to illustrate the impact of environmental pH on PSC phenotype in the healthy and cancerous pancreas (Fig. 1A). We performed an unbiased screening using RNAseq of primary murine PSCs cultured at pH_e 6.6 and pH_e 7.4 (GSE223205). This pH range can plausibly occur locally in the pancreas stroma and along the course of PDAC (3). The replicates in each group were homogeneous according to principal component analysis (Supplementary Fig. 1A). We found a total of 1769 genes differentially regulated between the two groups (log_2 FC>0.58, p<0.05), with 804 genes being upregulated at pH_e 7.4 and 965 genes being upregulated at pH_e 6.6 (Supplementary Fig. 1B, Supplementary Table 1). A gene set enrichment analysis (Fig. 1B, Supplementary Figure 1C and D) revealed that cell proliferation, communication, adhesion, and cell cycle pathways are markedly upregulated in PSCs cultured at pH_e 7.4. In contrast, immune response-related pathways are upregulated at pH_e 6.6. Next, we compared our results with gene expression signatures of published PSC-derived CAF subpopulations (7, 22) (Fig. 1C). Genes from immunomodulatory CAFs (iCAFs) are highly expressed at pH_e 6.6, and PSCs cultured at pH_e 7.4 highly express myofibroblastic CAF (myCAF) marker genes (values detailed in Supplementary Table 3). These findings suggest that PSCs isolated from the healthy pancreas with a physiological history of intermittent acidification have an immunomodulatory phenotype when kept in an acidic environment. However, they acquire a myofibroblastic phenotype upon removal of the extracellular acidity when kept at pH_e 7.0 and 7.4. Therefore, we will designate them as iPSCs and myPSCs, respectively.

To confirm the pH_e-dependent phenotype switch of PSCs, we cultured freshly isolated PSCs from wild-type mice in media with a pH range from pH_e 6.0 - pH_e 7.4 for 72 h and then quantified αSMA expression as a derivative of the myofibroblastic phenotype. For a more robust readout of myofibroblastic PSC phenotype and αSMA quantity, we multiplied cell area with αSMA intensity. As expected from the RNAseq data, the myCAF phenotype heavily relies on the environmental pH (Figs. 1D and 1E). The highest expression of αSMA is found at pH_e 7.4: pH_e 6.0: 1166±146 a.u., pH_e 6.6: 8090±1137 a.u., pH_e 7.0:
140,365±17,508 a.u., pH\textsubscript{e}7.4: 323,756±30,015 a.u., (n≥142 cells from N=3 mice). Western blot experiments comparing αSMA normalized to vimentin protein levels in PSCs cultured at pH\textsubscript{e}6.6 versus pH\textsubscript{e}7.4 recapitulate the immunocytochemistry data (αSMA/vimentin protein expression: pH\textsubscript{e}6.6: 0.11±0.08 a.u., pH\textsubscript{e}7.4: 1.23±0.18 a.u., p=0.015, n=4 samples from N=4 mice) (Supplementary Fig. 2A-B). The percentage of viable cells in the population is reduced at pH\textsubscript{e}6.0 (12±2%) and pH\textsubscript{e}6.3 (31±5%), while it is hardly affected at pH\textsubscript{e} > 6.5 (pH\textsubscript{e}6.6: 83±2%; pH\textsubscript{e}7.0: 96±1%; pH\textsubscript{e}7.4: 94±2%, cells from n=5 fields of view of N=5 mice, p<0.0001) (see Supplementary Fig. 2C-D). Increased cell proliferation of myPSCs is underlined by the fact that p53 protein is more expressed at pH\textsubscript{e}6.6 than at pH\textsubscript{e}7.4 (Fig. 1F, rel. p53 expression to GAPDH: pH\textsubscript{e}6.6: 0.04±0.01; pH\textsubscript{e}7.4: 0.01±0.01 (lysates from N=3 mice each, p=0.038). Cell cycle progression is also diminished at pH\textsubscript{e}6.6 compared to pH\textsubscript{e}7.4 (Figs. 1G and 1H; % PSCs in G\textsubscript{0}/G\textsubscript{1} after 72h: pH\textsubscript{e}6.6: 93±1%; pH\textsubscript{e}7.4: 80±2% (n≥3 measurements from N=3 mice, p=0.017)). Taken together, environmental pH is crucial in tuning the differentiation of PSCs. They acquire iPSC and myPSC phenotypes at pH\textsubscript{e}6.6 and pH\textsubscript{e}7.4, respectively.

NHE1 is a key regulator of PSC pH homeostasis

As the myofibroblastic phenotype switch occurs in PSCs at pH\textsubscript{e}7.4 but not at pH\textsubscript{e}6.6, we hypothesized that pH sensory and regulatory ion transporters are involved in the process. Thus, we evaluated our RNAseq data regarding the respective gene ontology term (GO:0015075, Fig. 2A). We found 43 transporter genes differentially upregulated at pH\textsubscript{e}7.4 and 44 at pH\textsubscript{e}6.6 as well as a number of genes that are highly expressed in both groups (Supplementary Table 3). From these, we selected and further validated the expression of 5 transporter genes (Fig. 2B) and 10 ion channel genes (Supplementary Fig. 3) and compared them to the housekeeper genes Ywhaz and 18s rRNA by means of RT-qPCR. We used freshly isolated PSCs as well as PSCs cultivated at pH\textsubscript{e}7.4 or pH\textsubscript{e}6.6 for 5 days (Fig. 2B, n=6 from N=3 mice). Notably, the Na\textsuperscript{+}/H\textsuperscript{+} exchanger NHE1 (encoded by SLC9A1) has more mRNA expression in PSCs than most other pH-regulatory proteins under all investigated conditions. Because of its
pathophysiological relevance in cancer (23) and good druggability with small molecules such as cariporide (24) we decided to further investigate this protein.

To explore the subcellular localization of NHE1 in vitro, we performed immunocytochemistry. Freshly isolated PSCs derived from healthy wild-type mice, i.e., quiescent PSCs, express NHE1 predominantly intracellularly (Fig. 2C). However, NHE1 translocates to the plasma membrane of PSCs from healthy WT mice already after 24h of culturing in either pH_{i}=6.6 (iPSC) or pH_{i}=7.4 (myPSC). Similarly, CAFs freshly isolated from murine PDAC (KPfC) express NHE1 also mainly in their membrane.

Western blot analysis confirms our immunocytochemistry results. NHE1 is highly expressed in both freshly isolated and cultured PSCs as well as in PDAC-derived CAFs (Fig. 2D). Yet, there are distinct differences: freshly isolated PSCs express the small (~80kDa) intracellular, non-glycosylated NHE1 protein, whereas PSCs cultured for 120h, and tumor-derived CAFs express the larger (~100kDa) glycosylated, plasma membrane-residing NHE1.

We next focused on NHE1 functionality. We revealed its activity by measuring the intracellular pH recovery following an NH_{4}^{+} prepulse (25) (Fig. 2E). When PSCs from wild-type mice are cultured in vitro for 72h, the pH\_i closely follows the pH\_e (Fig. 2F). PSCs kept at pH_{i}=6.6 have a pH\_i of pH\_i=6.60±0.01 (n cells /N mice=74/3). In contrast, pH\_i of PSCs cultured at pH\_e=7.4 is pH\_i=7.10±0.02 (n cells / N mice=113/3).

Similarly, the intracellular pH of CAFs freshly isolated from KPfC-mice (cultured at pH\_e=7.4) is pH\_i=7.11±0.01 (n cells / N mice=41/3). The NH_{4}^{+} prepulse protocol revealed almost no Na^{+}-independent pH-recovery in any of these cells (Fig. 2G) (pH\_i=6.6: 0.040±0.004 pH unit/min, n cells /N mice=156/5; pH\_e=7.4: 0.060±0.003 pH unit/min, n cells /N mice=135/5, PDAC-CAF: 0.015±0.005 pH unit/min, n cells /N mice=41/3). In contrast, pH\_i immediately recovers upon readdition of extracellular Na^{+}. The rate of pH\_i recovery is faster in PSCs cultured at pH\_e=7.4 than in PSCs cultured at pH\_i=6.6 (Fig. 2H) (pH\_e=7.4: 0.38±0.05 pH unit/min, n cells /N mice=45/3; pH\_i=6.6: 0.11±0.01 pH unit/min, n cells /N mice=35/3; p<0.0001). The pH-recovery is primarily due to the action of NHE1, as inhibition of NHE1 with cariporide (10µM) almost completely abolishes pH\_i recovery (pH\_i=6.6: 0.019±0.003 pH unit/min, n cells /N mice=39/3; pH\_e=7.4: 0.061±0.003 pH unit/min, n cells /N mice=68/3). CAFs freshly isolated from tumor-bearing KPfC-mice
also express a highly active NHE1, with the resting pH, and the rate of NHE1-dependent recovery being similar to PSCs cultured at pH 7.4 (vehicle-treated KPfC-mice: pH recovery: 0.44±0.09 pH unit/min, n cells /N mice=11/3). This also applies to CAFs isolated from another PDAC mouse model that is driven by κB-Ras1 deficiency (26) (Supplementary Figs. 4A and 4B, n$_{pH6.6}$=111, n$_{pH7.4}$=94 cells from N=5 mice).

RNAseq and RT-qPCR analyses indicate that the Na$^+$-HCO$_3^-$ cotransporter NBC1 (Slc4a4) and the monocarboxylate transporter 4 (Slc16a3) and numerous other transporters are also expressed in PSCs which could complement and/or compensate for blocked NHE1 activity. To rule out this possibility, we performed intracellular pH measurements in CAFs derived from vehicle-treated KPfC-mice in a CO$_2$/HCO$_3^-$ buffered environment where HCO$_3^-$ transporters are active (Supplementary Fig. 4C). We found that Na$^+$-dependent pH-recovery is primarily due to the action of NHE1 (Supplementary Fig. 4D and E; pH recovery: 0.68±0.04 pH unit/min, n cells /N mice=30/3). In summary, NHE1 acts as the major acid extruder in PSCs and CAFs, and its inhibition with cariporide leads to intracellular acidification of in vitro cultured PSCs and ex vivo PDAC-derived CAFs.

Lack of acidity facilitates YAP-1-mediated mechanotransduction and myofibroblastic PSC differentiation

Next, we aimed to get mechanistic insight into how cellular pH alkalinization results in the PSC phenotype switch. It is known that PSCs are quite susceptible to mechanical stimuli and express αSMA and thus exhibit a myofibroblastic phenotype predominantly on a rigid substrate (27, 28). We hypothesized that this major pathway of myPSC differentiation may be influenced by environmental pH. Therefore, we plated freshly isolated WT murine PSCs onto hydrogels with varying stiffness (Fig. 3A) and cultured them at pH 7.4 or pH 6.6 for 72h. Cells are larger and have more αSMA (derived from cell area * αSMA intensity) when plated on the 1 GPa glass substrate than on the 11 kPa hydrogel surface (Fig. 3B) (1 GPa: 335,493±28,248 a.u., n cells /N mice=170/3; 11 kPa: 88,304±12,031 a.u., n cells /N mice=65/3; p<0.0001). This difference is not seen when culturing PSCs at pH 6.6 (1 GPa: 35,722±7,685 a.u., n cells /N
From this, we concluded that the myPSC but not the iPSC phenotype (PSCs cultured at pH\textsubscript{e} 7.4 and pH\textsubscript{e} 6.6, respectively) relies on substrate stiffness at least with respect to cell size and αSMA positivity.

We then investigated whether pH\textsubscript{e} influences mechanosignaling via YAP1, a well-characterized transcription factor in PSCs (29). Immunocytochemistry (Fig. 3D) revealed that the nuclear-to-cytosolic ratio of YAP1 is higher in pH\textsubscript{e} 7.4 than in pH\textsubscript{e} 6.6, indicating increased YAP1-mediated transcription in myPSCs as compared to iPSCs (Fig. 3D) (1 GPa, pH\textsubscript{e} 7.4: 1.5±0.1, n cells /N mice=63/3; pH\textsubscript{e} 6.6: 1.1±0.1, n cells /N mice=43/3; p=0.018). Overall, these data point out that the YAP1-mediated mechanosignaling is pH-dependent and not utilized in iPSCs kept in an acidic environment.

**Acidic pH\textsubscript{e} partially alters myPSC phenotype only in the presence of cariporide**

PSC phenotypes depend on the given stimulus (7, 30). Our results above indicate that environmental pH is crucial in PSC differentiation. However, in our initial experimental setting, we investigated a multitude of different pH-independent pathways by seeding cells from their native tissue environment onto plastic and into a cell culture medium. To test whether the sole removal of acidity is sufficient to induce differentiation of iPSC to myPSCs (e.g. through YAP-1 mediated mechanosignaling as shown above), we changed the medium pH of pH\textsubscript{e} 6.6-cultured iPSCs, either to pH\textsubscript{e} 6.6 (“Resting”) or to pH\textsubscript{e} 7.4 (“PanIN-like”) after 72 h (Fig. 4A). Indeed, after just 3 days of culture at pH\textsubscript{e} 7.4, PSCs differentiate from the iPSC to the myPSC phenotype as indicated by the drastic rise of αSMA expression (Fig. 4B) (pH\textsubscript{e} 6.6 → pH\textsubscript{e} 6.6: 35,722±7685 a.u., n cells /N mice= 100/3; pH\textsubscript{e} 6.6 → pH\textsubscript{e} 7.4: 1,414,669±222,374 a.u., n cells /N mice=64/3; p<0.0001).

Hence, we conclude that iPSCs cultured at pH\textsubscript{e} 6.6 can further differentiate into myPSCs, with extracellular alkalization (= relief of extracellular acidity) being a sufficient stimulus.

We next tested whether PSCs (“PanIN-like”) can be reprogrammed from the myPSC to the iPSC phenotype by simply changing back the environmental pH to pH\textsubscript{e} 6.6 (“PDAC-like”). Briefly, this is not the case (Fig.
PSCs retain their myofibroblastic phenotype since αSMA positivity and cell size do not change (Fig. 4B) (pH 7.4 → pH 6.6: 1,421,502±146,424 a.u., n cells /N mice=94/3; p=0.45). We reasoned that myofibroblastic PSCs retain their phenotype because of their pronounced NHE1 activity: reacidifying the environment alone would not alter pH, anymore because cells can get rid of excess H⁺ rapidly through NHE1. To test this hypothesis, we applied cariporide together with environmental reacidification (Fig. 4C). In this “PDAC-like” pH-shift, application of cariporide indeed affects the myPSC phenotype (Fig. 4D) (“PDAC-like+CARI”: 2,575,631±282,664 a.u., n cells /N mice=74/3; “PDAC-like”: 5,421,677±557,953 a.u., n cells /N mice=74/3; p<0.0001). This interpretation is supported by intracellular pH measurements (Fig. 4E). NHE1-mediated pH-recovery also occurs at a high rate in PSCs whose culture medium was re-acidified after activation (pH 7.4 → pH 6.6) (Fig. 4F) (Na⁺-dependent pH-recovery: control: 0.56±0.07 pH unit/min, n cells /N mice=35/3; cariporide: 0.14±0.01 pH unit/min, n cells /N mice=79/3; p<0.0001). These findings indicate that the NHE1 function maintains the myofibroblastic PSC phenotype in a harsh acidic tumor environment, while NHE1 inhibition partially disrupts it (Fig. 4G).

Adjuvant PDAC therapy with an NHE1-inhibitor reduces desmoplasia

If the inhibition of NHE1 decreases the myofibroblastic nature of PSCs, this will become evident by a reduced fibrosis in pancreatic cancer. We tested this idea in pancreatic tumor-bearing KPfC mice that harbor heterozygous loss of p53 and conditionally express mutant K-Ras (genotype Kras<sup>wt/LSL-G12D</sup> Tp53<sup>fl/+</sup> PDCl-Cre<sup>+</sup>) (31, 32). Cariporide was given as an adjuvant drug complementing the standard therapy with gemcitabine (28,29). To mimic the clinical situation of PDAC patients who usually suffer from a late diagnosis and therapy initiation, we started to apply cariporide at week 20. At that time point, most KPfC mice have already developed manifest PDAC (33). We treated the mice with 3mg/kg cariporide i.p. daily for one month and initiated chemotherapy with gemcitabine at the end of this period (Fig. 5A).

The macroscopic total volume of the pancreata (Fig. 5B) and microscopic relative area of tumor lesions normalized to total tissue area (Fig. 5C) do not differ between the gemcitabine+cariporide double treatment
group and the vehicle-treated group (volume: gemcitabine+cariporide: 0.4ml, 95%-CI: 0.3–0.7ml; p=0.84, vehicle: 0.4ml, 95%-CI: 0.3–0.7ml; relative tumor area: gemcitabine+cariporide: 35%, 95%-CI: 32–50%, N=12 mice; vehicle: 41%, 95%-CI: 34–54%, N=10 mice; p=0.78). However, the histochemical analysis of fibrosis by means of Sirius Red staining revealed a clear difference (Fig. 5D). The total area of fibrosis in tumors relative to total tumor area is reduced by ~ 30% in mice treated with gemcitabine+cariporide compared to vehicle treatment (Fig. 5E; gemcitabine+cariporide: 31±4%, N=12 mice; vehicle: 45±4%, N=11 mice; p=0.03). To ensure that statistics are not biased by a few individual tumor nodes that are very large in size, we investigated the extent of fibrosis in each individual tumor node in every tissue section (Fig. 5F). Thereby, we confirmed the antifibrotic effect of cariporide: Cariporide decreases fibrosis when compared to vehicle treatment (cariporide: 34%, 95%-CI: 31–37%, n individual tumor nodes /N mice=279/11; vehicle: 40%, 95%-CI: 37–42%, n individual tumor nodes /N mice=400/11, p=0.0005). Moreover, gemcitabine+cariporide treatment decreases fibrosis more than gemcitabine alone (gemcitabine+cariporide: 31%, 95%-CI: 27–34%, n individual tumor nodes /N mice=476/12; gemcitabine: 38%, 95%-CI: 35–41%, n individual tumor nodes /N mice=239/9 p=0.0001). These findings point out that NHE1 inhibition is an effective adjuvant therapeutic strategy to counter excess fibrosis in PDAC. The following sections delineate whether the decrease in fibrosis is due to a direct effect on PSCs or rather an indirect effect mediated by other cell types such as immune cells.

NHE1 orchestrates CAF activation in PDAC

We reasoned that the decreased fibrosis in the gemcitabine+cariporide treatment group is due to a decreased myCAF phenotype due to NHE1 inhibition. To ascertain whether PSCs express NHE1 in PDAC, we performed immunohistochemistry (Fig. 6A). We found that NHE1 is ubiquitously expressed in cell membranes in pancreatic cancer (Supplementary Figure 6). Indeed, it is expressed in αSMA+ CAFs (Fig. 6B), but also CK19+ PDAC and ductal cells, as well as in immune cells.
To investigate whether the myCAF phenotype is affected during gemcitabine+cariporide treatment, we isolated CAFs from KPfC mice after the treatments outlined in Fig. 5A and immediately evaluated their phenotype from αSMA staining fluorescence intensity and cell size (Fig. 6C). To verify the fibroblastic nature of the isolated cells, we showed by Western blot that only KPfC-derived tumor cells but not CAFs express a high level of mutated KRas G12D (Supplementary Figure 7). We found that CAFs derived from KPfC mice treated with cariporide+gemcitabine are markedly less myofibroblastic than those isolated from mice receiving a gemcitabine monotherapy (Fig. 6D) (gemcitabine+cariporide; 592,059±165,706 a.u., n cells /N mice=70/4; gemcitabine: 7,339,572±1,609,547 a.u., n cells /N mice=59/3; p=0.0023). When assessing cell area and αSMA fluorescence intensity separately (Supplementary Fig. 5A-B), we observed a decrease in cell area in cariporide + gemcitabine-treated CAFs compared to gemcitabine treatment (vehicle: 3822±412 µm², n cells /N mice=61/3; cariporide: 2602±255 µm², n cells /N mice=63/3; p=0.038; gemcitabine: 3308±329 µm², n cells /N mice=59/3; gemcitabine+cariporide; 2006±245 µm², n cells /N mice=70/4; p<0.0001). This decrease in cell area can potentially be attributed to NHE1 inhibition and be a reflection of a reduced cell volume (34).

Finally, as a global readout of CAF function, we assessed cell migration after the treatments (Fig. 6E). CAFs derived from KPfC mice treated with cariporide+gemcitabine migrate more slowly than CAFs isolated from gemcitabine-treated mice (Fig. 6F) (gemcitabine+cariporide: 0.05±0.01µm/min, n cells /N mice=40/4; gemcitabine: 0.1±0.01µm/min, n cells /N mice=30/3; p=0.0006). We also recapitulated this finding by exposing untreated κB-Ras deficient PDAC-derived CAFs to cariporide (Supplementary Fig. 5C): In these cells, migration is also inhibited in the presence of 10 µM cariporide (Supplementary Fig. 5D) (cariporide: 0.08 µm/min±0.01, n cells /N mice=30/3; control: 0.11±0.01µm/min, n cells /N mice=43/5; p=0.025). To summarize, inhibition of the Na⁺/H⁺ exchanger NHE1 in WT and pancreatic tumor-bearing mice shifts CAF differentiation towards a less myofibroblastic phenotype.
NHE1 inhibition enhances lymphocytic immune infiltration in KPlC mice

Above, we focused on NHE1 in PSCs and CAFs. However, it is known that a wide range of other cells, including PDAC cells, lymphocytes, and neutrophils, also express NHE1, as observable from Supplementary Fig. 6 (18, 35–37). We, therefore, followed up the relevance of NHE1 in immune cell infiltration more closely after observing that the primarily periodic acid-Schiff positive (PAS+) infiltrates in vehicle- or gemcitabine-treated cohorts shift to PAS− ones in the cariporide or gemcitabine+cariporide treatment groups (Fig. 7A and Supplementary Fig. 8). Furthermore, the immune cell infiltration in cariporide-treated animals is often accompanied by a disruption of the architecture of tumor foci which may indicate a more effective immune response. These observations suggest that NHE1 inhibition shifts the immune cell infiltrate from a largely innate immune cell-rich one to a more lymphocytic infiltration.

To confirm this idea, we further characterized each PDAC sample quantitatively with CD3 and Ly6G immunohistochemistry staining, labeling T lymphocytes and neutrophils, respectively (Fig. 7B). We focused our evaluation on the CD3/Ly6G ratio in PDAC lesions as a high lymphocyte-to-neutrophil ratio has a favorable prognostic value for patient survival (38). Indeed, when comparing the number of CD3+ and Ly6G+ cells in each tumor section, we found that the CD3/Ly6G ratio (Fig. 7C) has increased by approximately four times in the gemcitabine+cariporide group as compared to the vehicle-treated group (gemcitabine+cariporide: 8.5, 95%-CI: 4.9–10.6, N=11 mice; vehicle: 2.2, 95%-CI: 0.9–5.2, N=9 mice; p=0.03). Upon assessing the CD3/Ly6G ratio of each individual tumor node (Fig. 7D), we found it to be 2.5-fold higher in the gemcitabine+cariporide- than in the gemcitabine-treated group (gemcitabine+cariporide: 2.6, 95%-CI: 2.1–3.1, n individual tumor nodes /N mice=398/11; gemcitabine: 1.1, 95%-CI: 0.9–1.5, n individual tumor nodes /N mice=276/9; p<0.0001). We also noted that T-cells not only accumulate in the periphery of individual tumor foci but also penetrate into the depth of the cancer tissue when mice are treated with gemcitabine+cariporide (Fig. 7A-B).

Taken together, our results indicate that the small-molecule inhibitor of NHE1, cariporide, targets the PDAC stroma in vivo on at least two vital fronts: it disrupts the vicious cycle leading to marked fibrosis,
and it shifts tumor immune cell infiltrate to a more lymphocytic one which is consistent with a more potent anti-tumor response.
DISCUSSION

Our study was performed at the background of the unique pH landscape of the pancreas with the intermittent postprandial acidification of the interstitium that is challenging all cells residing in the pancreatic stroma. Therefore, we studied how the inhibition of one of the major acid extruders, NHE1, impacts on function and phenotype of PSCs which are important cells in the pancreatic stroma. The ultimate question was whether targeting NHE1 affects PDAC progression. To this end, we showed that environmental acidosis is a crucial factor in keeping PSCs in the healthy organ in a non-myofibroblastic state (Fig. 1). This aligns well with our initial hypothesis that an intermittent acidosis after each meal would prevent the PSC phenotype change.

The immunomodulatory-to-myofibroblastic phenotype switch is only induced after changing pH from pH 6.6 to pH 7.4 in vitro (Figs. 1 and 4). These findings are consistent with the idea that the physiological intermittent acidity of the pancreas stroma acts like a "brake" to prevent premature PSC activation and excessive fibrosis. In pancreatic pathophysiology, removing this "acid brake" and the subsequent iPSC-to-myPSC phenotype switch favorably leads to ECM synthesis and wound healing in the short term. In the long term, however, reacidifying the environmental pH fails to revert the myPSC phenotype and terminate the fibrotic process, leading to excess fibrosis. Thus, depending on the context, interstitial/extracellular acidity acts like a double-edged sword. Thereby, the continuous supply of the stimulatory cancer cell secretome could make the difference in pancreatic cancer.

Based on our results, we argue that increased NHE1 activity, which is prominent in "PanIN-like" and "PDAC-like" myPSCs and tumor-derived CAFs (Fig. 2), is a major determinant in maintaining the vicious cycle of the desmoplastic reaction. This assumption relies on the observation that PSCs only lose their myofibroblastic phenotype upon environmental reacidification when NHE1 is inhibited with cariporide (Fig. 4). Further studies are needed to unravel whether this effect is due to decreased PSC activation or transdifferentiation to an immunomodulatory (or other) phenotype.
We could recapitulate this finding in vivo, where there is an apparent decrease in PDAC fibrosis (Fig. 5) and myCAF phenotype (Fig. 6) when applying the combined gemcitabine+cariporide treatment. These results imply that NHE1-inhibition is a viable anti-fibrotic therapy in PDAC. However, anti-fibrotic drugs in cancer therapy are discussed controversially: On the one hand, complete elimination of fibrosis leads to enhanced invasiveness and more metastases (39); on the other hand, too much fibrosis creates a protective niche for tumor cells allowing them to escape chemotherapy and immune response (40, 41). We propose that inhibiting NHE1 might be a compromise between not targeting the fibrotic PDAC stroma and its complete elimination, both of which are potentially harmful. Adjuvant therapy with cariporide reduces fibrosis and promotes lymphocyte infiltration (Fig. 7) but does not lead to excess loss of ECM, which would promote tumor invasiveness (Fig. 5). This complex response underpins that therapeutic NHE1 inhibition is not a purely anti-fibrotic therapy. Additional effects can for example be elicited by also targeting NHE1 in cancer cells (42).

In our view, we made two other important observations. We discovered that cariporide, in combination with gemcitabine, modulates tumor immune cell infiltration in favor of a CD3+ lymphocyte-rich immune response in KPfC mice (Fig. 7). Based on our results, we propose a mechanism contributing to the neutrophil-to-lymphocyte switch in immune infiltration. Lymphocytes are unable to migrate through confined spaces when the mesh is too narrow for the nucleus to pass (43). Fewer myCAFs in the tumor reduce fibrosis leading to increased ECM mesh diameter, enabling lymphocytes to reach sites in the desmoplastic tumor that was too dense beforehand. On the other hand, neutrophil chemotaxis is impaired by cariporide (35). Taken together, it is plausible that both mechanical and biochemical cues lead to lymphocyte-rich immune response upon cariporide treatment. Other NHE1-regulated mechanisms, such as an altered metabolism, could also play a role. It has been recently shown that NHE1 inhibition promotes anti-tumor immune response in glioblastoma (36): Therapy of glioma-bearing mice with cariporide in combination with chemotherapy (temozolomide) induces metabolic rewiring and increases T-lymphocyte infiltration into the tumor through metabolic reprogramming.
Moreover, we found that changing environmental pH induces specific responses in PSCs: Acidification leads to a dramatic upregulation of inflammation-related genes (Fig. 1), whereas relative alkalization, i.e., relief of acidity, promotes cell proliferation and cell cycle (Fig. 1F-H). In addition, releasing the "acid brake" by alkalinization appears to be permissive for YAP1-mediated mechanotransduction (Fig. 3) and subsequently for pathways involved in cell adhesiveness and proliferation (Fig. 1 and Supplementary Fig. 1). This result is reinforced by our earlier studies that a key mechanosensor in PSCs, Piezo1, is inhibited by acidic pH (44, 45) and that Piezo1 is coupled to the YAP-TAZ pathway (46). Also, altered cellular metabolism under differential mechanical stimuli in PDAC modulates energy production, which regulates cell adhesion, cytoskeletal dynamics, and ECM remodeling (47).

A key question regarding the therapeutic applicability of cariporide is whether inhibited NHE1 can be substituted by another acid-base regulating transporter in stellate cells or CAFs. This appears not to be the case, or cariporide therapy would not have shown the anti-fibrotic effects. Mechanistically, we found that the pH-recovery after intracellular acidification happens in PSCs and CAFs primarily in a Na⁺-dependent manner in the absence and presence of HCO₃⁻ (Fig. 2). We did not find any functional evidence for Na⁺-independent pH regulators such as carbonic anhydrases or the V-type ATPase in PSCs. After applying cariporide, the pH recovery becomes markedly slower in the presence of HCO₃⁻ but is not completely abolished. This implies that besides NHE1, the Na⁺/HCO₃⁻ cotransporters such as NBC1 (Slc4a4) can further stabilize the intracellular pH in PSCs and CAFs. However, it has been recently shown that NBC1 in PDAC is rather expressed in epithelial tumor cells and regulates environmental HCO₃⁻ accumulation, cellular glycolysis, and subsequent lactate release. Upon NBC1 inhibition, reduced lactate and increased HCO₃⁻ in the TME improve CD8⁺ T cell-mediated immune response. This aligns very well with our results that revealed the increased lymphocyte-to-neutrophil ratio upon NHE1 inhibition (Fig. 7). Whether there is an actual interplay between NHE1 and NBC1 that can be exploited therapeutically in PDAC is highly probable: NHE1 inhibition would primarily target CAFs, NBC1 inhibition cancer cells, and both would favor lymphocytic immune response. Such a combined approach could target pancreatic cancer and stellate
cells in a complementary way and result in an efficient modulation of the immune cell infiltrate. In summary, we argue that NHE1 function is not replaceable in PSCs but rather has a delicate interaction with other pH regulators culminating in an altered tumor immune response. These results support that cariporide should be explored as an adjuvant therapy in PDAC.
MATERIALS AND METHODS

Animal Experiments

All animal experiments involving wild-type C57 BL/6J mice, KPfC mice, and K-RasG12D expressing κB-Ras deficient (NKIRASI\textsuperscript{-/\textsuperscript{-}} NKIRAS2\textsuperscript{+/\textsuperscript{-}} KRAS\textsuperscript{wv/LSL-G12D PDX1-Cre+}) mice\textsuperscript{(26)} were approved by the local authorities (LANUV) and the office of animal welfare of the University Clinic Münster. Wildtype C57 BL/6J mice were sacrificed for experiments involving in vitro-activated PSCs. Therapeutical studies with cariporide and gemcitabine were conducted in a blinded fashion with KPfC mice that harbor heterozygous loss of p53 and conditionally express mutant K-Ras (K-Ras\textsuperscript{G12D}) from the endogenous locus in the pancreas (KRAS\textsuperscript{wv/LSL-G12D TP53\textsuperscript{+/-} PDX1-Cre+})\textsuperscript{(31, 32)}. This is achieved by a Lox-SOP-Lox (LSL) cassette that prevents expression of the mutant K-Ras protein prior to Cre recombinase-mediated recombination and consequent excision of the STOP cassette, that is controlled by the Pdx1 promotor (Pdx1-Cre)\textsuperscript{(48)}. Treatment with the NHE1 inhibitor cariporide or its vehicle (“control” group) started at the age of week 20 for 25 days (31, 49–51). Animals in the “cariporide” and “cariporide + gemcitabine” groups received daily intraperitoneal injections with 3 mg/kg bodyweight cariporide (Medchemexpress, Monmouth Junction, USA) dissolved in 0.9% saline + 2% DMSO, with a total injection volume of 5.5 ml/kg bodyweight. Control animals were injected with 0.9% saline + 2% DMSO. Animals in the “gemcitabine” and “cariporide+gemcitabine group” additionally received 3 dosages of 100 mg/kg bodyweight gemcitabine i.p. (Ely Lilly, Indianapolis, USA) at days D\textsubscript{18}, D\textsubscript{21}, and D\textsubscript{24} (Summarized treatment protocol in Fig. 5A). We used equal numbers of male and female animals in the four experimental groups.

As an additional validation of in vivo PSC activation, we isolated CAFs from κB-Ras deficient mice, which were generated by crossing Pdx1-Cre and LSL-KRas\textsuperscript{G12D} with conventional κB-Ras and conditional κB-Ras2 knockout mice. All mice were on a C57 BL/6J background (48, 52). Mice were housed in individually vented cages (IVC) containing nesting material. Constant ambient temperature (22 ± 2 °C), constant humidity (55% ± 10%), and a 12 h light/12 h dark cycle were provided.
Isolation of pancreatic stellate cells and cancer-associated fibroblasts

PSCs and CAFs were isolated as described previously (44, 53). Briefly, murine pancreata were isolated from WT C57 BL/6J mice and digested enzymatically with 0.1% collagenase P (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany) at 37 °C for 25 min. We used a cube of 3×3×3 mm for isolating CAFs from KPiC or κB-Ras mouse-derived pancreata. After centrifugation with 200 g at room temperature for 5 min, the homogenized tissue was resuspended in cell culture medium (DMEM/Ham F12 1:1, supplemented with 10% FCS and 1% penicillin/streptomycin) and seeded onto FCS-coated tissue culture dishes, glass-bottom dishes or hydrogel-coated glass-bottom dishes for 2 h. CAFs derived from KPiC- or κB-Ras deficient mice were allowed to adhere for only 30 min, as we found that contamination with cancer cells becomes substantial after 2 h. Afterwards, non-adherent cells were vigorously washed off the tissue culture plate, resulting in a homogeneous PSC or CAF culture. Depending on the experiments, PSCs and CAFs were cultured for different time periods (2 h, 24 h, 3 d, 6 d, 9 d) with pH<sub>e</sub>6.6 or pH<sub>e</sub>7.4 media. To avoid trypsin-mediated PSC activation, PSCs and CAFs were used for experiments without passaging directly after isolation (54).

RNA-sequencing and RT-qPCR

For RNA-sequencing, the Rneasy Mini Kit (Qiagen, Hilden, Germany, #74104) was used to extract RNA from PSCs cultured for 120 h at pH<sub>e</sub>6.6 or pH<sub>e</sub>7.4 according to the manufacturer’s instructions. The quality and quantity of isolated RNA were evaluated using the NanoDrop 2000. Libraries were prepared and sequenced (~20 M single reads per sample) using the Illumina Next-Seq 500 sequencing platform (high-output Kit, 75 Cycles v2 Chemie) at the Genomics Core Facility (University Hospital Münster, Münster). Subsequent bioinformatics processing and analysis were primarily carried out on the Galaxy platform (55). First, raw fastq files were aligned and mapped against the murine reference genome (mm10) with the HISAT2 v2.2.1 algorithm (RRID: SCR_015530) (56). Counts were subsequently extracted using featureCounts 2.0.1 (57), and differential expression of genes was analyzed using limma 3.50.1 (58),
filtering out genes with CPM < 0.5 with a minimum of samples = 2. Principle component analysis shows low heterogeneity within biological replicates but a high degree of dissimilarity between different treatments (Supplementary Figure 1A). Features with False discovery rate (FDR)-adjusted \( p < 0.05 \) were declared significantly differentially expressed. The resulting gene lists were further processed with gene set enrichment analysis using fgsea 1.8.0 (59) and EGSEA 1.20.0 (60). RNAseq data is publicly available on the GEO Omnibus (GSE223205).

For RT-qPCR, RNA was prepared from freshly isolated WT-mouse derived PSCs and from PSCs cultured for 120 h at pH\(_6.6\) or pH\(_7.4\), using TRIZol\textsuperscript{TM} (Life Technologies, Waltham, USA) following the manufacturer’s instructions. cDNA was generated using SuperScript IV Reverse Transcriptase (Invitrogen, Carlsbad, USA) from 2 \( \mu \)g of RNA per reaction. RT-qPCR was performed using TaqMan\textsuperscript{TM} Gene Expression Master Mix (Thermo Fisher Scientific, Waltham, USA) with pre-designed TaqMan probes on 96-well TaqMan \textregistered{} Express Plates. RT-qPCR was monitored using a QuantStudio\textsuperscript{TM} 3 Real-Time PCR System (Thermo Fisher Scientific, Waltham, USA) using the cycling protocol of the master mix manufacturer. Data were analyzed using the QuantStudio\textsuperscript{TM} Design and Analysis Software (Thermo Fisher Scientific, Waltham, USA). As we found no published evaluation of housekeeping genes with respect to their pH\(_e\)-sensitivity, we initially used three housekeeper genes for our assays. From \textit{18s} rRNA, \textit{Ywhaz}, and \textit{Ppia}, \textit{Ppia} was found to be expressed pH\(_e\)-dependently and omitted from referencing by using the geNorm algorithm (RRID: SCR_006763) (61).

**Protein extraction and Western blot**

Total protein was extracted from freshly isolated WT mouse-derived PSCs, PSCs cultured for 120 h at pH\(_6.6\) or pH\(_7.4\), and KPfC-derived CAFs using radioimmunoprecipitation assay (RIPA) buffer (Thermo Fisher, Waltham, USA) and 1% Complete Mini protease inhibitor (Roche, Mannheim, Germany). Protein concentration was assessed with Pierce\textsuperscript{TM} BCA Protein Assay Kit (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Western blots of NHE1, vimentin, αSMA, KRas, and p53 were performed as described previously (62). 15 \( \mu \)g of denatured total cellular protein were applied to each lane of 4–15% Mini-
PROTEAN® TGX Stain-Free™ Protein Gels (Bio-Rad, Hercules, USA) for electrophoresis at 80 mV. After overnight transfer to PVDF membranes at 4 ºC, we detected the total amount of protein on the membrane following the manufacturer’s instructions. Next, we blocked the membrane with PBS containing 5% skim milk for 1 h, then incubated the blots with primary antibodies against NHE1 (#611775, RRID: AB_399261, 1:1000, BD Bioscience, Franklin Lakes, USA), p53 (#10442-1-AP, RRID: AB_2206609, 1:1000, Proteintech, Rosemont, USA), αSMA (#A2547, RRID: AB_476701, 1:500, Sigma-Aldrich, Merck KGaA, Darmstadt, Germany), vimentin (#10366-1-AP, RRID: AB_2273020, 1:1000, Proteintech, Rosemont, USA) and G12D mutant-specific KRas antibody (#14429, RRID: AB_2728748, 1:500, Cell Signaling, Danvers, USA) overnight at 4ºC. After washing 3 times with PBS, we applied HRP-conjugated goat anti-mouse secondary antibody (1:10000, Goat Anti-Mouse IgG H&L, #ab6708, RRID: AB_956005, Abcam, Cambridge, UK). Chemiluminescence was detected using a Chemidoc MP detection system (Bio-Rad, Hercules, USA), and band intensities were evaluated with the ImageLab software (Bio-Rad, Hercules, USA).

**Histology, immunohistochemistry, and immunocytochemistry**

For histology and immunohistochemistry, pancreata were fixed in 4% paraformaldehyde, embedded in paraffin, and then cut into 2 µm sections with a RM2125 microtome (Leica, Wetzlar, Germany). Afterward, sections were deparaffinized with xylene, rehydrated in a stepwise manner, and stained with hematoxylin/eosin (H&E) or Periodic acid/Schiff (Biozol, Eching, Germany) and Sirius Red (Roche, Mannheim, Germany) according to the manufacturer’s instructions. Representative images were taken with AxioImager (Zeiss, Oberkochen, Germany) and subsequently processed with ImageJ (RRID: SCR_003070). For scanning whole tissue slices, the Leica SCN400 scanner was used with the Leica SCN400 Client software (Leica, Wetzlar, Germany). Analysis of whole tissue scans was performed with QuPath software in a blinded fashion (RRID: SCR_018257) (63). First, a pixel classifier was trained to distinguish Sirius Red-positive fibrosis from non-fibrotic tissue using training images derived from multiple different tissue sections. The quality of the pixel classification algorithm was compared to the manual
classification of fibrosis, and training was stopped when the automatic classification was at least as accurate as the manual classification. Then, individual tumor nodes were manually annotated on Sirius Red-stained slices. Next, the pixel classification algorithm was applied to all Sirius Red-labeled sections, resulting in the absolute and relative areas of Sirius Red-positive fibrosis in each tumor node as an output.

For immunohistochemistry stainings, antigen retrieval was done using 10mM sodium citrate buffer (pH 6.0), followed by blocking in 1% bovine serum albumin-containing PBS for 1 h (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany). Slides were stained with primary antibodies in a humidified chamber at 4 °C overnight. Primary antibodies were: goat anti-CK19 (#sc-33111, RRID: AB_2234419, 1:100, Santa Cruz Biotechnology, Dallas, USA), rabbit anti-NHE1 (#HPA052891, RRID: AB_2681981, 1:200, Atlas Antibodies, Stockholm, Sweden), Alexa Fluor 488-conjugated mouse anti-αSMA (#53-9760-82, RRID: AB_476701, 1:600, Thermo Fisher, Waltham, USA). In case of immune cell staining we used: rabbit anti-CD3 (#ab5690, RRID:AB_305055, 1:200, Abcam, Cambridge, UK) and rat anti-Ly6G (#MAB1037, 1:100, R&D Systems, Minneapolis, USA). After washing 3 times in PBS, the following secondary antibodies were used: donkey anti-rabbit-Alexa Fluor 488 (#711-545-152, RRID: AB_2313584, 1:1000, Jackson Immuno Research, West Grove, USA) and donkey anti-goat-Cy3 (#705-165-003, RRID: AB_2340411, 1:1000, Jackson Immuno Research, West Grove, USA). Slides were mounted in DAKO mounting medium (Agilent, Santa Clara, USA) with 0.001% DAPI (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany) and covered with coverslips. For representative images, confocal microscopy was performed on a Leica DMI 6000 setup (Leica, Wetzlar, Germany) in the Las-X software with 405 nm, 488 nm, 514 nm, and 633 nm lasers with a 630x total magnification. Afterwards, images were processed using ImageJ.

An inverted Nikon ECLIPSE Ti2 widefield microscope (Nikon, Duesseldorf, Germany) equipped with a triggered and calibrated Lumencor Spectra III LED light source, a motorized stage, 4x (NA=0.2) and 20x (NA=0.75) air objectives and a Photometrics Prime 95B sCMOS camera was used in a semi-automated manner to acquire the Ly6G (Alexa Fluor 647), CD3 (Cy3) and the nuclear (DAPI) signals of the 5 µm-thick tissue sections in their entirety at a 550 nm per pixel resolution. For greater efficiency, the microscope
slides were positioned in a custom-built slide holder capable of carrying 4 slides simultaneously. The Nikon NIS Elements’ (ver. 5.21.03) JOBS acquisition automation was set up to first record a low-resolution (4x objective) composite, DAPI overview image of all 4 slides. Next, outlines delineating the respective tissue section were generated, within which multiple points were distributed at each of which the microscope automatically identified the respective z-coordinate corresponding to the tissue in-focus plane. The resulting z-coordinates were interpolated to generate a tissue section-specific focus map along which the final, overlapping, multichannel images were acquired and ultimately stitched into a single image depicting the entire tissue section. The acquisition speed was enhanced by having the camera directly trigger the respective excitation wavelengths (635, 555, and 390 nm), utilizing a multi-bandpass filter cube and short (30 ms) exposure times. For subsequent image analysis, the resulting Nikon .nd2 files were batch-converted to the .ims format (hierarchical data format 5 based) with the Imaris File Converter ver. 9.8.2 (Bitplane, Switzerland). All samples were acquired in a blinded fashion using the same acquisition settings.

Immunohistochemistry was evaluated in a blinded manner in QuPath (63). First, an object classification algorithm was trained from training images derived from multiple different tissue sections to separately detect CD3+ and Ly6G+ cells. The training was stopped when the manual classification was similar to automatic classification. Next, every tumor node in each tissue section was manually annotated, followed by segmentation of each individual cell in the tumor nodes using the DAPI channel. Finally, cells were classified as CD3+ and Ly6G+ using the trained automatic cell classification algorithm, giving the number of CD3+ and Ly6G+ cells in each tumor node as output.

Immunocytochemistry was performed on PSCs isolated directly onto glass-bottom dishes, where they were ultimately visualized. Following washing cells with PBS, fixation, and permeabilization were performed with ice-cold methanol at -20 ºC for 5 min. Afterwards, dishes were carefully washed 3 times with PBS, blocked with PBS containing 10% FCS. Primary antibodies against αSMA (#A2547, RRID: AB_476701, 1:200, Sigma-Aldrich, Merck KGaA, Darmstadt, Germany), vimentin (#10366-1-AP, RRID: AB_2273020, 1:500, Proteintech, Rosemont, USA), YAP1 (sc-101199, RRID: AB_1131430, 1:250, Santa Cruz
Biotechnology, Dallas, USA) and NHE1 (#611775, RRID: AB_399261, 1:200, BD Bioscience, Franklin Lakes, USA) were applied overnight at 4 °C. After washing 3 times with PBS, Alexa 488-conjugated secondary antibodies against mouse (Invitrogen, Carlsbad, CA, USA, 1:500) and Cy3-conjugated antibodies against rabbit (Invitrogen, Carlsbad, CA, USA, 1:500) were applied at room temperature for 1 h. Lastly, after washing 3 times with PBS, 0.01% DAPI was applied in 1 ml PBS and cellular fluorescence was acquired within 24 h.

**Intracellular pH measurements**

Assessment of pH was conducted ratiometrically using the fluorescent indicator BCECF-AM (Invitrogen, Carlsbad, USA) as previously described (64). PSCs and CAFs were either superfused with HEPES-buffered or CO$_2$/HCO$_3^-$-buffered Ringer’s solution. Prior to conducting pH measurements with HEPES-buffered Ringer’s solution, HCO$_3^-$-buffered cell culture medium was exchanged to the HEPES-buffered Ringer’s solution, and cells were allowed to adapt for 1 h to the new environment. HEPES-buffered Ringer’s solution had the following composition (in mM): 122.5 NaCl, 5.4 KCl, 1.2 CaCl$_2$, 0.8 MgCl$_2$, 5.5 D-glucose and 10.0 HEPES, titrated to pH 7.4 with NaOH. When performing pH measurements in the presence of CO$_2$/HCO$_3^-$-buffered Ringer’s solution, all solutions contained 24 mM NaHCO$_3$ instead of equimolar NaCl to maintain osmolarity, and solutions were continuously bubbled with 5% CO$_2$ using the gas mixing system DIGAMIX SA27 (Wösthoff, Bochum, Germany). The appropriate pH of solutions was regularly monitored by a Knick 766 electrode-based pH meter (Knick, Berlin, Germany).

To study the NHE1-dependent pH recovery after acidification, NH$_4^+$ prepulse was performed (25). First, cells were perfused with 20 mM NH$_4^+$-containing Na$^+$-free solution (in mM: 102.5 N-Methyl-D-glucamin hydrochloride, 20 NH$_4$Cl, 5.4 KCl, 1.2 CaCl$_2$, 0.8 MgCl$_2$, 1 BaCl$_2$, 5.5 D-glucose and 10.0 HEPES, titrated to pH 7.4 with KOH) for 1 min to alkalinize pH. Afterwards, PSCs were superfused with Na$^+$-free solution (in mM: 122.5 N-Methyl-D-glucamin hydrochloride, 5.4 KCl, 1.2 CaCl$_2$, 0.8 MgCl$_2$, 5.5 D-glucose and 10.0 HEPES, titrated to pH 7.4 with KOH) for 10 min to acidify pH. Eventually, Na$^+$ was added back with control Ringer’s solution for 5 min, and Na$^+$-dependent recovery was observed in the presence of solvent.
(0.1% DMSO) or 10 μM cariporide. Lastly, for calibration purposes, cells superfused with a modified
Ringer’s solution containing 1 μM nigericin (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany) (in mM:
125 KCl, 1 MgCl₂, 1 CaCl₂, and 20 HEPES) titrated to pH 7.5 and pH 6.5.

Data acquisition was performed with a setup consisting of a fluorescence microscope (Zeiss Axiovert 100,
Zeiss, Oberkochen, Germany), a high-speed shutter, and a polychromator (Visitron Systems, Puchheim,
Germany), using dual excitation wavelengths of 440 nm and 490 nm and emission wavelength of 510 nm.
Data analysis was performed using the Visiview 3.0 software (Visitron Systems, Puchheim, Germany).
Fluorescence intensities were measured over the whole cell area and corrected for background fluorescence.
Subsequently, for each time point, the 440 nm/ex/490 nm/ex fluorescence intensity ratios were calculated. As
the BCECF fluorescence ratio follows a linear trend between pH 6.5 and pH 7.5, two-point calibration was
performed using linear regression.

**Cell migration**

The migratory behavior of CAFs on a two-dimensional substrate was assessed as previously described (44).
Even though a two-dimension setting imposes non-physiological constraints, it offers a reliable readout
regarding the adhesive and migratory machinery of the cells (65). Briefly, CAFs originating from PDAC-
bearing mice were seeded in 12.5 cm² flasks that were pre-coated with extracellular matrix components
resembling the desmoplastic stroma (66), containing the following components: 40 μg/ml laminin (Sigma-
Aldrich, Merck KGaA, Darmstadt, Germany), 40 μg/ml fibronectin (Sigma-Aldrich, Merck KGaA,
Darmstadt, Germany), 800 μg/ml collagen I (Corning, New York, NY, USA), 12 μg/ml collagen III
(Corning, New York, NY, USA), and 5.4 μg/ml collagen IV (BD Biosciences, Heidelberg, Germany).
Following cell adhesion overnight at 37 °C containing 5% CO₂, flasks were sealed air-tight and inserted
into 37 °C temperature-controlled chambers. In the chambers, cell motility was observed using an inverted
microscope (Zeiss Axiovert 40, Zeiss, Oberkochen, Germany) and recorded every other 15 minutes using
the MicroCamLab 3.1 software (Bresser, Rhede, Germany). From the recordings, cell contours were
segmented using the Amira software (Thermo Fisher Scientific, Inc., Waltham, MA, USA) and further
processed to determine cell velocities (μm/min) from the displacement of the cell centroid over time (quantification detailed by Dieterich et al. (67)). Cell translocation indicates the start-to-end net cellular displacement.

**Polyacrylamide Hydrogel production and stiffness measurements**

Polyacrylamide gels were prepared using a chemical polymerization protocol (68). Glass-bottom dishes were defatted with EtOH and pretreated with 0.1M NaOH. Afterwards, the glass was silanized with APTMS ((3-Aminopropyl)trimethoxysilane) and subsequently treated with 0.5% glutaraldehyde at room temperature for 30 min. Different stiffness of polyacrylamide gels was achieved by applying different component concentrations, as detailed in Supplementary Table 1. After mixing the gel components, polymerization was initiated by 0.003% TEMED (Tetramethylethylenediamine) and 1% APS (Ammoniumperoxodisulfate). After mixing the solution, 8 μl solution was pipetted in the middle of the glass-bottom dish and immediately covered by a 15 mm diameter glass coverslip. Polymerization was allowed overnight at 4 ºC. Next, gels were treated with the photoactivated heterobifunctional crosslinker 0.05% Sulfo-SANPAH (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany) under 302 nm UV light for 15 min. After washing, gels were coated with the collagen-containing matrix used for cell migration studies (see below).

Hydrogel stiffness was measured using a NanoWizard® 3 NanoScience atomic force microscope (JPK Instruments AG, Berlin, Germany). The cantilevers (Novascan Technologies, Inc., Boone, USA), equipped with a spherical polystyrene bead of 10 μm diameter, were pre-calibrated, having spring constants of 0.03-0.04 N/m. The deflection sensitivity of the cantilever was calibrated before each measurement. Hydrogel stiffness measurements were conducted at 3 parts of the hydrogels, with 5 times probing the substrate at each point. All measured positions of the gel were at least 1 mm apart from each other to confirm gel
homogeneity. The Young's moduli were calculated from the measured force-distance curves using the JPK Data Processing software (Bruker Nano GmbH, Berlin, Germany).

**Statistical information**

Biological replicates (N) refer to the number of mice employed for each experimental series. "n" reflects the number of individual data points in any given experimental condition (e.g., number of cells, number of tumor foci, etc.). To calculate data distribution, the D’Agostino-Pearson test was applied. Normally distributed data are given as mean ± S.E.M, elsewhere as median ± 95% confidence interval (CI). For data following normal distribution, two-tailed unpaired Student's t-tests or one-way ANOVA with Tukey’s post hoc test were performed. When data did not follow a normal distribution, Mann-Whitney U-test or non-parametric ANOVA-on-ranks (Kruskal-Wallis) statistical test with Dunn’s post-hoc test was conducted. We used GraphPad Prism 7 (RRID: SCR_000306) for statistical analysis and data presentation. Statistical significance was assumed when p < 0.05.

**Study approval**

All animal experiments involving wild-type C57 BL/6J mice, KPiC mice, and KRas\(^{G12D}\) expressing κB-Ras deficient (\(NKIRAS1^{\text{flox}}\), \(NKIRAS2^{\text{flox}}\), \(KRAS^{\text{wt/LSL-G12D PDX1 Cre^+}}\)) mice were approved by the local authorities (LANUV) and the office of animal welfare of the University Clinic Münster.

**Data availability**

All raw data can be accessed in the Supporting data XLS file. Moreover, RNAseq data is publicly available on the GEO Omnibus (GSE223205). Further information and analytic methods that support the findings are available upon request from the corresponding author: pethoe@uni-muenster.de.

**Author contributions**

Conceptualization: ZP, AS
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Investigation: ZP, KN, SB, MM,
Visualization: ZP, KN, AS
Funding acquisition: AO, AS
Project administration: EW, AO, AS
Supervision: EW, AO, AS
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Writing – review & editing: ZP, EW, AO, AS

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796 Figures and figure legends

**Figure 1. Environmental alkalization induces myofibroblastic PSC differentiation and proliferation**

A Concept of the working hypothesis: In the healthy pancreas, the marked HCO₃⁻ secretion upon each meal results in a distinct stromal acidification, keeping the PSCs in a quiescent non-fibrotic phenotype. Upon malignant transformation in early PDAC (PanIN), ductal secretion decreases, resulting in a relief of the intermittent acidity (= relative stromal alkalization), leading to a myofibroblastic PSC differentiation. Acidic→alkaline pH is depicted by yellow→purple colors. B Hallmark gene set enrichment analysis (GSEA) of RNA-seq data from PSCs cultured at pHₑ 6.6 versus pHₑ 7.4 shows the top 5 differentially regulated pathways. (n/N = 3/3) C A heatmap of RNA-Seq expression mean z-scores computed for published signature genes of immunomodulatory CAFs (left) and myofibroblastic CAFs (right), respectively. The gene (rows) z-scores for pHₑ 6.6 and pHₑ 7.4 are color-coded. Dark green indicates higher expression z-scores. (n/N = 3/3) D Immunocytochemistry images of PSCs cultured at pHₑ 6.6 or pHₑ 7.4. The activation marker αSMA (yellow) and the general PSC marker vimentin (magenta) are labeled. Nuclei are stained with DAPI (cyan). Scale bar=50 µm. E Cell areas multiplied by αSMA intensity is taken as a readout for the myofibroblastic PSC phenotype. It is plotted as a function of pHₑ. Mean values are shown as n ≥142 from N=3 mice. Half-maximum (EC₅₀) pHₑ-dependent PSC activation occurs at pHₑ 7.0. Note the logarithmic scale of the ordinate. F Representative Western blot (top) of p53 and GAPDH of PSCs cultured at pHₑ 6.6 or pHₑ 7.4, with subsequent quantification (bottom). (n/N = 3/3) G Representative cell cycle histogram of PSCs cultured at pHₑ 6.6 (red) and pHₑ 7.4 (blue), assessed by flow cytometry with PI staining. Cell populations at different stages of the cell cycle are indicated by arrows. H The bar chart depicts the percentage of PSCs at the G₀/G₁ phase of the cell cycle when cultured at pHₑ 6.6 (red) and pHₑ 7.4 (blue). Data points are nₑpH6.6=3 and nₑpH7.4=5 measurements from N=3 individual mice. Statistical tests in (F) and (H) were performed with two-tailed unpaired Student's t-tests. (A) was created with BioRender.com.
Figure 2. NHE1-mediated pH recovery is inhibited by cariporide in PSCs

A Volcano plot analysis of ion transporter genes (GO:0015075) derived from the RNA-seq data of PSCs cultured at pH 7.4 and pH 6.6 (n/N = 3/3). Genes indicated by red (n=43) and blue (n=44) dots highlighted in rectangles are upregulated at pH 6.6 and pH 7.4, respectively. B Subsequent RT-qPCR validation of ion transporter gene expression levels by the 2-ΔΔCt method compared to the housekeeping genes Ywhaz and 18s rRNA. Bar charts show mean expression of genes from freshly isolated quiescent PSCs (0h, white) and PSCs cultured at pH 7.4 (blue) or pH 6.6 (red). (n/N = 6/3) C Representative immunofluorescence images showing the cellular localization of NHE1 in freshly isolated quiescent PSCs (0h), PSCs cultured at pH 7.4 or pH 6.6 for 120 h or vehicle-treated KPfC-derived PSCs (PDAC-PSC) (NHE1: magenta; DAPI: blue). Scale bar = 10 µm. D NHE1 Western blots: the top bands at 100 kDa correspond to the glycosylated NHE1, whereas bands with lower molecular weight (80 kDa) correspond to the unglycosylated NHE1. Lysates are from N=3 mice each. E pH recordings of wild-type PSCs cultured at pH 6.6 (left) and pH 7.4 (middle) or KPfC-derived CAFs (cultured at pH 7.4; right). pHi was acidified temporarily by applying the NH4+ prepulse (*) technique. NHE1-independent pHi recovery starts when pHi has reached its minimum in the presence of the Na+-free solution (“0 Na+”). NHE1-dependent pHi recovery can be observed in the last step (Ctrl) of the experiment when cariporide was added to the Na+-containing superfusion as indicated. Lines show mean pHi of nPH6.6=35, nPH6.6+CARI=39, nPH7.4=45, nPH7.4+CARI=68, nPDACCAF=11 and nPDACCAF+CARI=22 cells from N=3 mice each. F Quantification of resting pHi of PSCs cultured at pH 6.6 (red) or pH 7.4 (blue) and CAFs (purple) respectively, derived from panel (E) (nPH6.6=74, nPH7.4=113 and nPDACCAF=41 cells from N=3 mice each). G Scatter plot depicts the rate of Na+-independent pHi recovery of PSCs cultured at pH 6.6 (red), pH 7.4 (blue), or CAFs (purple), respectively, derived from panel (E). (n/N see (F)) H Comparison of the rate of Na+-dependent pHi recovery of WT PSCs cultured at pH 6.6 (red) or pH 7.4 (blue), or CAFs (purple) as explained in panel (E) (n/N see (E)). Statistical tests in (F) - (H) were performed with one-way ANOVA with Tukey’s post hoc test.
Figure 3. PSC mechanotransduction mediated by YAP1 is inhibited at acidic pH.

A Immunocytochemistry images of PSCs cultured on substrates of different stiffnesses at pH 6.6 (top) and pH 7.4 (bottom). The activation marker αSMA (yellow) and the general stellate cell marker vimentin (magenta), as well as nuclei (cyan), are labeled. Scale bar = 50 µm. B Cell area multiplied with mean αSMA fluorescence intensity was taken as a readout of myofibroblastic PSC phenotype on hydrogels with 11 kPa stiffness or on glass (1GPa). n_{11 kPa}=65, n_{1 GPa}=170 cells from N=3 mice. C Representative immunofluorescence images of YAP1 in PSCs (green) under the indicated cell culture conditions. YAP1, when translocated from the cytosol (#) into the nucleus (*), initiates transcription. Scale bar=50 µm. D The ratio of nuclear-to-cytosolic YAP1 fluorescence intensity was determined as a readout of YAP1-mediated signal transduction. n_{11 kPa, pH 6.6}=68, n_{11 kPa, pH 7.4}=51, n_{1 GPa, pH 6.6}=43, n_{1 GPa, pH 7.4}=63 cells from N=3 mice each. Statistical tests in (B) and (D) were performed with one-way ANOVA with Tukey’s post hoc test.
Figure 4. The myofibroblastic phenotype of activated PSCs is partially reversed by cariporide but not by acidic pH alone.

A After culturing PSCs at pH$_{e}$ 6.6 for 72 h medium was changed to pH$_{e}$ 6.6 ("Resting") or to pH$_{e}$ 7.4 ("PanIN-like") for another 72 h. Lastly, the medium of pH$_{e}$ 7.4 incubated cells was reacidified to pH$_{e}$ 6.6 for another 72 h ("PDAC-like"). Representative images of PSCs stained for αSMA (yellow), vimentin (magenta), and DAPI (cyan) are shown below each condition. Scale bar=50 µm. B Scatter plot shows cell areas multiplied by αSMA fluorescence staining intensity (logarithmic scale) under conditions described in panel (A). n$_{Resting}$=100, n$_{PanIN-like}$=64 and n$_{PDAC-like}$=94; N=3 mice. C Immunocytochemistry of “PDAC-like” PSCs in the absence (left) or presence (right) of cariporide (CARI). Scale bar=50 µm. D Scatter plot shows cell areas multiplied by αSMA fluorescence intensity (logarithmic scale) under conditions described in panel (C). n$_{PDAC-like}$=74, n$_{PDAC-like+CARI}$=74; N=3 mice. E Intracellular pH measurements of PSCs where culture medium is re-acidified after activation (pH$_{e}$ 7.4 → pH$_{e}$ 6.6, “PDAC-like”). Intracellular pH was acidified temporarily by applying the NH$_{4}^{+}$ prepulse technique, as shown in Fig. 2. NHE1-dependent pH recovery can be observed when cells are superfused with Na$^{+}$-containing solution (Ctrl) without (black) or with cariporide (red). Lines indicate the mean pH, of n$_{PDAC-like}$=35, n$_{PDAC-like+CARI}$=79 cells from N=3 mice. F Comparison of the rate of Na$^{+}$-dependent recovery of PSCs in the absence or presence of cariporide derived from (E). G Illustration of extended working hypothesis: In manifest PDAC, acidic pH$_{e}$ fails to acidify pH$_{i}$ because of NHE1-mediated H$^{+}$ extrusion (left). Therefore, PSCs and CAFs remain myofibroblastic, ultimately promoting tumor desmoplasia. However, upon NHE1 inhibition with cariporide, PSCs, and CAFs fail to counterbalance the acid stress, which disrupts the myofibroblastic phenotype (right). Statistical comparison in B was performed with one-way ANOVA with Tukey’s post hoc test, whereas in (D) and (F) with two-tailed unpaired Student’s t-tests. (G) was created with BioRender.com.
Figure 5. NHE1-inhibitor-treatment leads to reduced desmoplastic reaction in murine PDAC.

A Schematic representation of the 4 weeks’ long treatment protocol of KPfC mice. Treatment started at the age of week 20. Cariporide was applied daily (1/D), and gemcitabine (100 mg/kg i.p.) was co-injected with cariporide (3 mg/kg i.p.) on the days indicated by the arrows. B Total pancreas volume of KPfC mice after gemcitabine (GEM) or cariporide (CARI) monotherapy or gemcitabine+cariporide (GEM+CARI) combined chemotherapy. Inlet demonstrates that pancreas volume was measured via volume displacement. Data points depict individual pancreata; N_{Vehicle}=11, N_{GEM}=9, N_{CARI}=11, N_{GEM+CARI}=11. C Relative tumor area in histological KPfC tissue sections was obtained by dividing total tumor area by total tissue area after hematoxylin-eosin (H+E) staining. Data points depict individual pancreata; N_{Vehicle}=11, N_{GEM}=9, N_{CARI}=11, N_{GEM+CARI}=11. D Representative images of PDAC nodes (marked with *) after H+E and Sirius Red stainings, respectively. The degree of fibrosis correlates with the area of Sirius Red positive (markedly red, #) tissue neighboring the cancerous tissue. Scale bar=100 µm. E Relative tumor fibrosis of each Sirius Red stained KPfC tissue section was determined by dividing the summed area of fibrosis within every tumor node (sum of thresholded black areas in every node in the inlet) by the summed area of the tumor nodes. Data points depict individual pancreata; N_{Vehicle}=11, N_{GEM}=9, N_{CARI}=11, N_{GEM+CARI}=12. F To obtain the fibrosis per tumor node, the area of Sirius Red positive fibrosis (black thresholded area in the inlet) was divided by the total area of the respective tumor node. Data points depict individual tumor nodes; n_{Vehicle}=400, n_{GEM}=239, n_{CARI}=279, n_{GEM+CARI}=476. Data and statistical comparison in (B), (C), and (F): median±95% CI with Kruskal-Wallis statistical test with Dunn’s post-hoc test, and in € as mean±SEM with one-way ANOVA with Tukey’s post hoc test, respectively. Inlets for (A) and (B) were created with BioRender.com.
Figure 6. NHE1 orchestrates PDAC-derived CAF activation

A Representative H+E and immunohistochemistry (IHC) images of healthy and tumorous ducts. The colors of the IHC image indicate cell nuclei stained with NHE1 (magenta), αSMA+ PSCs and CAFs (yellow), DAPI (cyan), and CK19+ ductal or tumor cells (green). Scale bar=40 µm. B from panel (A), αSMA+ cells (*) are depicted with higher magnification, which are also NHE1+. Scale bar=20 µm C Immunocytochemistry of CAFs derived from KPiC mice after a one-month-long treatment with vehicle (top) or gemcitabine+cariporide (bottom). Myofibroblast marker αSMA (yellow), the general mesenchymal marker vimentin (magenta), as well as nuclei (cyan) are labeled. Scale bar=20 µm. D KPiC-derived CAF activation after therapy was assessed by multiplying cell area with the fluorescence intensity of αSMA. n_{Vehicle}=61, n_{GEM}=59, n_{CARI}=63, n_{GEM+CARI}=70 from N≥3 mice. Note the logarithmic scale of the ordinate. E Trajectories of migrating KPiC-derived CAFs are shown by individual black lines. The treatment of the respective mice is indicated. Trajectories of the treatment groups are always normalized to common starting points. The radii of the orange circles highlight the mean translocation of cells in each population. Scale bar = 20 µm. F Mean cell migration velocities of individual CAFs were calculated from the trajectories in panel (c). n_{Vehicle}=30, n_{GEM}=30, n_{CARI}=19, n_{GEM+CARI}=40 cells from N≥3 mice. Statistical tests in (D) and (F) were performed with one-way ANOVA with Tukey’s post hoc test.
Figure 7. Lymphocyte-to-neutrophil ratio increases upon NHE1 inhibition in tumor sections of KpFC mice

A H+E (left) and Periodic acid-Schiff (PAS)-stained KpFC mouse tissue sections after vehicle and gemcitabine + cariporide (GEM+CARI) therapy. Cells of innate immunity, such as neutrophils (arrows), utilize glycogen and are thus PAS+ (purple), in contrast to, e.g., lymphocytes. Scale bar = 50 µm. B Representative IHC images stained for Ly6G+ neutrophils (magenta, arrows on left image), CD3+ lymphocytes (yellow, arrows on the right image), and nuclei with DAPI (cyan). Scale bar=50 µm. C CD3/Ly6G ratio was assessed by dividing the number of CD3+ cells by the number of Ly6G+ cells in every tumor node. Data points depict the mean CD3/Ly6G ratio derived from each tumor node in individual mice; N_{Vehicle}=10, N_{GEM}=9, N_{CARI}=10, N_{GEM+CARI}=11 mice. D To obtain the CD3/Ly6G ratio per tumor node, the number of CD3+ positive cells was divided by the respective number of Ly6G+ cells in each tumor node. Data points depict individual tumor nodes; n_{Vehicle}=386, n_{GEM}=276, n_{CARI}=301, n_{GEM+CARI}=398. Data and statistical comparison in (D) and (E) are represented as median±95% CI with Kruskal-Wallis statistical test with Dunn’s post-hoc test, respectively.