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LETTER TO THE EDITOR

Dynamics and cytokinic regulation of immune cell infiltration in genetically engineered mouse models of pancreatic cancer dictate the sensitivity to immunotherapy

Dear Editor,

Targeting the immune compartment in pancreatic ductal adenocarcinoma (PDAC) holds promise for prognostic improvement, yet our knowledge on the spatial and temporal dynamics and the molecular modulators of the PDAC-associated immunophenotype is scarce.

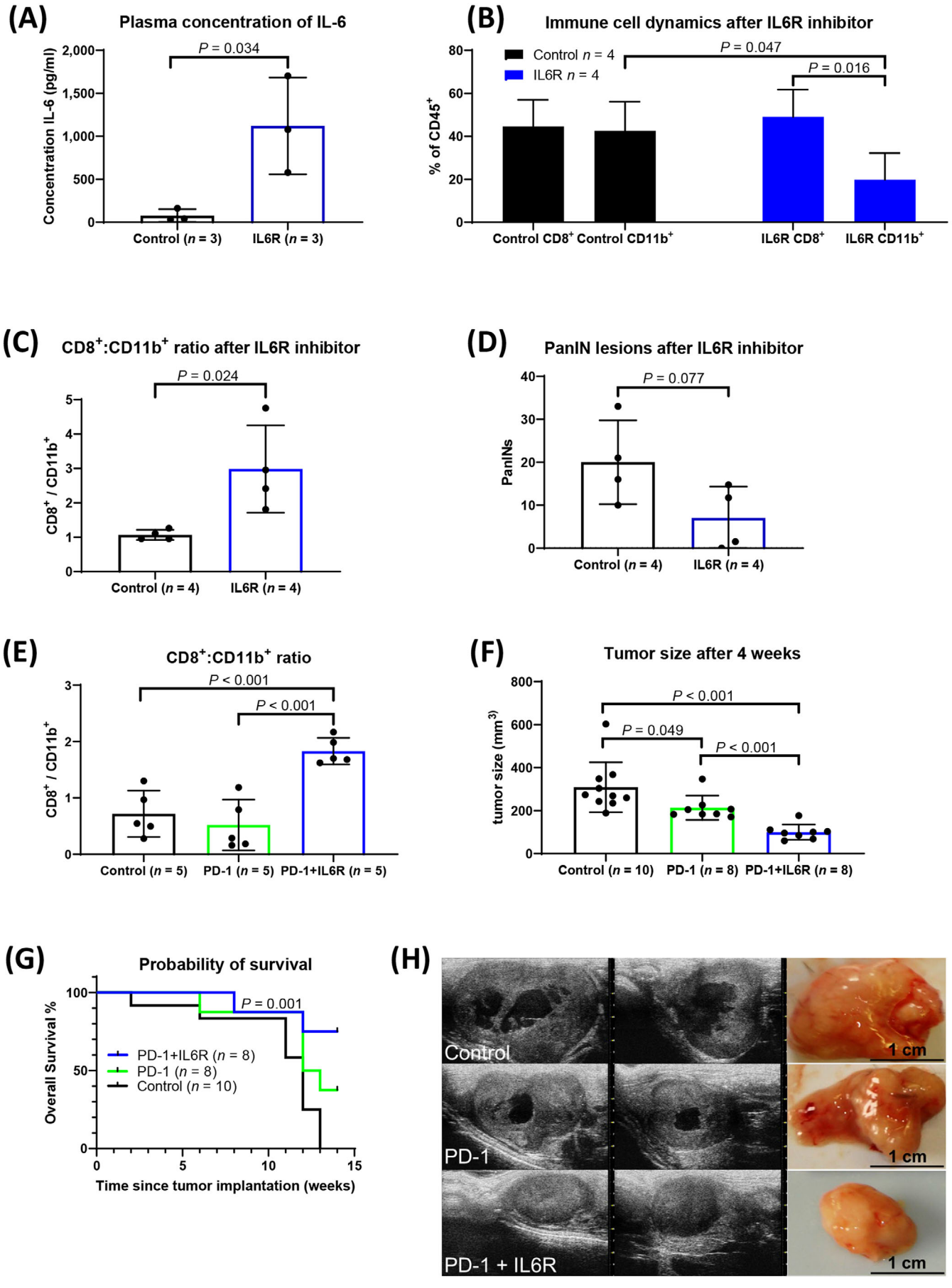
Here, we quantified markers of several major immune cell subclasses longitudinally in the life span in the primary tumors of oncogenic Kras-driven murine PDAC ($n = 19$), including the *Ptf1a-cre;LSL-Kras^{G12D};p53$^{lox/+}$* (KPC) and the *Ptf1a-cre;LSL-Kras^{G12D}* (KC) mouse models via immunohistology (Supplementary Materials and Supplementary Results). We compared the immune cell distribution in these models with that in resected human PDAC ($n = 36$) and in mice with conditional ablation of interleukin-6 (KC;IL-6$^{-/-}$) or CXCL12/SDF1-alpha (KC;Cxcl12$^{+/fl}$) signaling ($n = 11$). Then, the survival of orthotopic models ($n = 26$) was analyzed after combination treatment with anti-interleukin 6 receptor (anti-IL6R) and programmed cell death protein 1(PD-1) inhibitors.

We demonstrated that only a few time points of Kras-driven mouse models resembled the human tumor immunophenotype. Here, the intratumoral predominance of cluster of differentiation (CD)8$^+$ cells in the early life of KPC mice was unequivocally overrun by CD11b$^+$ cells in the later phase, and tumor progression is associated with a parallel drop in the *CD8$^+$:CD11b$^+$* ratio (KPC 3 weeks: 1.3 ± 0.6 vs. KPC 20 weeks: 0.6 ± 0.1, $P = 0.030$ KPC 10 weeks: 1.1 ± 0.2 vs. KPC 20 weeks: 0.6 ± 0.1, $P < 0.001$; Supplementary Figures S1-S2). Using flow cytometry to validate our immunostaining results, the ratio of CD8$^+$ T cells to CD11b$^+$ myeloid cells was also decreased in 20-week-old KPC mice compared with 10-week-old mice (1.0 ± 0.8 vs. 3.8 ± 1.3; Supplementary Figure S2F). These results collectively suggested that with PDAC progression, CD8$^+$ cells are dominated by myeloid cells in the primary tumor.

In the KC model with early pancreatic carcinogenesis, CD11b$^+$ cells and CD8$^+$ cells were the major infiltrating immune cell populations (CD8$^+$: 8 weeks: 19.0 ± 14.5%, 12 weeks: 32.0 ± 22.3%; CD11b$^+$: 8 weeks: 33.0 ± 20.3%, 12 weeks: 46.0 ± 30.1%; Supplementary Figure S3). The comparison of the immune cell proportions in murine and human PDAC suggested a major similarity between human PDAC and mouse PDAC between 3 and 10 weeks of age (human [$n = 36$] vs. KPC 3 weeks vs. KPC 10 weeks, CD8$^+$:CD11b$^+$ ratio: 1.6 ± 1.0 vs. 1.3 ± 0.6 vs. 1.1 ± 0.2, respectively; Supplementary Figure S4).

In the next step, we explored whether we could influence the *CD8$^+$:CD11b$^+$* cell ratio in these murine models via genetic depletion of pro-inflammatory/pro-tumorigenic cytokines. It has been demonstrated that C-X-C Motif Chemokine Ligand 12 (CXCL12) participates in tumor metastasis and contributes to immune-suppressive networks within the tumor microenvironment [1]. The IL-6/STAT3 (Signal Transducer and Activator of Transcription 3) axis can simultaneously promote the expansion of immunosuppressive cells or alter the balance of T-cell subsets [2]. Both cancer cells and stromal cells are known to secrete CXCL12 and IL-6, which can change the ratio between immune-competent and immune-suppressive cell types in favor of the latter [3]. Using single-cell RNA sequencing (scRNA-seq) datasets from the Gene Expression Omnibus (GEO), we were able to prove that macrophages, i.e., the myeloid cell lineage, have the highest expression of IL6R, C-X-C Motiv Chemokine Receptor 4 (CXCR4) and C-X-C Motiv Chemokine Receptor 7 (CXCR7) (Supplementary Figures S5-S7), which

Abbreviations: CD, Cluster of Differentiation; CXCL 12, C-X-C Motif Chemokine Ligand 12; CXCR4, C-X-C Motiv Chemokine Receptor 4; CXCR7, C-X-C Motiv Chemokine Receptor 7; GEMM, Genetically engineered mouse models; IL-6, Interleukin-6; IL6R, Interleukin 6 Receptor; Kras, Kirsten rat sarcoma; PanIN, Pancreatic Intraepithelial Neoplasia; PD-1, Programmed cell Death protein-1; PDAC, Ductal adenocarcinoma of the pancreas; scRNA-seq, single-cell RNA sequencing; STAT3, Signal Transducer and Activator of Transcription 3; Treg, T regulatory cells.



explains their responsiveness to IL-6 and CXCL12 in the cancer tissue. Consequently, selective blocking of these receptors can change the immune cell distribution towards $CD8^+$ immune-competent cell lines. Therefore, we interbred compound KC mouse mutants with IL-6 knockout allele ($Il\text{-}6^{-/-}$) or the conditional $Cxcl12^{lox/+}$ allele and analyzed the distribution of immune cells in the pancreas (Supplementary Figure S8A-C). As both mutants exhibit a slower tumor progression [4, 5], the percentage of myeloid $CD11b^+$ cells in the $CD45^+$ compartment was dramatically decreased in the pancreas of cytokine-depleted mice (KC vs. KC;IL-$6^{-/-}$, 74.9 ± 20.6% vs. 14.5 ± 7.6%, $P = 0.001$; KC vs. KC;$CXCL12^{fl/+}$, 74.9 ± 20.6% vs. 10.2 ± 6.9%, $P < 0.001$) (Supplementary Figure S8D). Accordingly, the $CD8^+$:$CD11b^+$ ratio was significantly higher in mice with cytokine depletion (KC vs. KC;IL-$6^{-/-}$, $P = 0.009$; KC vs. KC;$CXCL12^{fl/+}$, $P = 0.002$), due to reduction of $CD11b^+$ cells (Supplementary Figure S8E).

The increase of the $CD8^+$:$CD11b^+$ ratio in genetically cytokine-depleted mice led us to investigate whether such a beneficial modulation of immune cell infiltration can be similarly achieved pharmacologically. As a proof-of-concept, we treated heterozygous 8-week-old KPC mice with IL6R blocking antibody (1.25 mg/mL). Interestingly, we found the plasma levels of IL-6 to be significantly higher in the IL6R antibody-treated animals compared to the control group (Control vs. Treatment: 78 ± 74 vs. 1,1 ± 563, $P = 0.034$; Figure 1A). Importantly, systemic treatment with the IL6R antibody strongly increased the $CD8^+$:$CD11b^+$ ratio in the pancreas (Control vs. Treatment: 1.1 ± 0.2 vs. 3.0 ± 1.3, $P = 0.024$; Figure 1B-C), suggesting that IL-6 is one of the key cytokines that maintains the enrichment of immunosuppressive $CD11b^+$ cells in the tumor microenvironment. Importantly, compared to untreated 10-week-old KPC mice, the antibody-treated mice exhibited much fewer PanIN lesions (Control vs. Treatment: 20 ± 9.8 vs. 7.0 ± 7.3, $P = 0.077$; Figure 1D).

The possibility of pharmacologically influencing the $CD8^+$:$CD11b^+$ ratio by IL6R antibodies motivated us to explore whether clinically relevant outcomes can be achieved by co-targeting the IL6R signaling in the context of immunotherapy. We performed a preclinical trial in which we randomized mice with orthotopic PDAC to receive either a PD-1 inhibitor (pembrolizumab/Keytruda®) alone or in combination with a blocking antibody against IL6R. Interestingly, we again observed an increase in the $CD8^+$:$CD11b^+$ ratio in the combination treated as the underlying mechanism (Control vs. PD-1 + IL6R: 0.7 ± 0.4 vs. 1.8 ± 0.2, $P < 0.001$; Figure 1E). Comparing the mean tumor size after 4 weeks, the animals treated with the PD-1 inhibitor exhibited smaller tumors ($P = 0.049$). Importantly, the additional treatment with the anti-IL6R antibody resulted in a suppression of tumor growth with almost immediate effect (Control vs. PD-1 + IL6R: 309.2 ± 116.0 mm^3 vs. 100.0 ± 35.7 mm^3, $P < 0.001$; Figure 1F). Consequently, the treated animals showed a higher mean 13-week survival rate (Control vs. PD-1 + IL6R: 0% vs. 75%, $P = 0.001$; Figure 1G). Abdominal sonography showed smaller, solid tumors in the animals with combination therapy, while the untreated animals or the animals that received PD-1 monotherapy had significantly larger tumors with central necrosis (Figure 1H).

In conclusion, we showed that autochthonous, oncogenic Kras-based mouse models of PDAC have a specific immune cell distribution in the primary tumor, comparable to human PDAC at only few time points of tumor development. We provide evidence for systemic inhibition of selected cytokines such as IL-6 or CXCL12 for favorably modulating the intratumoral $CD8^+$:$CD11b^+$ cell ratio. As such, it indirectly indicates the extent of anti-tumor response in the tumor microenvironment. Based on this mechanism, the combination therapy of IL6R blockade with immune checkpoint inhibition seems to be effective in downsizing pancreatic cancer and improving the survival rate.

FIGURE 1 Immune cell distribution, PanIN development, tumor size and overall survival rate in a preclinical trial with anti-IL6R antibody ± immune checkpoint inhibition. A. Plasma concentration of IL-6 in 10-weeks-old KPC mice after treatment with the IL6R antibody. B. Cell ratio of $CD8^+$ and $CD11b^+$ cells related to the total number of $CD45^+$ cells before and after treatment of KPC mice with IL6R antibody. C. Graph representing the ratio of $CD8^+$:$CD11b^+$ cells after treatment of KPC mice with IL6R antibody. D. Graph shows the development of PanIN lesions of 10-week-old KPC mice compared to animals treated with the IL6R inhibitor vs. control (KPC) mice. E. Ratio of $CD8^+$:$CD11b^+$ cells after treatment of mice in the orthotopic tumor model with PD-1 inhibitor monotherapy vs. combined anti-IL6R antibody and PD-1 inhibitor therapy vs. untreated mice. F. Pancreatic tumor size of animals after 4 weeks of treatment with PD-1 inhibitor vs. combined treatment with IL6R antibody/PD-1 inhibitor versus untreated animals. G. The 13-week overall survival rate in monotherapy vs. combination therapy versus control. H. Representative ultrasonography pictures and the macroscopic aspects of the tumors in control, PD-1 inhibitor-treated and combination (anti-IL6R antibody + PD-1 inhibitor)-treated animals.

Abbreviations:CD, Cluster of Differentiation; IL6R, Interleukin 6 Receptor; PanIN, Pancreatic intraepithelial neoplasia; PD-1, Programmed cell death protein-1

DECLARATIONS

AUTHOR CONTRIBUTIONS

Okan Safak is responsible for all aspects of this investigation. Ihsan Ekin Demir, Okan Safak, and Rouzanna Istvanffy designed the study. Shenghan Wang, Okan Safak, Carmen Mota Reyes, Sergey Tokalov, Rouzanna Istvanffy, Nedim Can Cevik, Bengi Su Yilmaz, Emre Erdogan, Elif Arik Sever, Kivanc Görgülü, Samed Özer and Güldal Süyen performed the experiments. Okan Safak, Ibrahim Halil Gürcinar, Linhan Ye, Qiaolin Li, Bengi Su Yilmaz, Rouzanna Istvanffy and Carmen Mota Reyes analyzed the data. Ihsan Ekin Demir, Rouzanna Istvanffy and Okan Safak contributed substantial intellectual input. Helmut Friess, Güralp Onur Ceyhan and Hana Algül supervised the study. Okan Safak, Ihsan Ekin Demir and Rouzanna Istvanffy wrote the first draft of the manuscript. All authors have agreed on the final version of the manuscript.

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COMPETING INTERESTS

none

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ETHICS APPROVAL AND CONSENT TO PARTICIPATE

All animal studies were conducted according to the national regulations and approved by the Regierung von Oberbayern (approval number 55.2-1-54-2532-223-2015) and by Acibadem University (ACU-HADYEK 2019/06). The study has been approved by the ethics committee of the Technische Universität München, Munich (approval number 588/19s and 549/16s). All patients were informed, and written consent was obtained for tissue collection.

CONSENT FOR PUBLICATION

Not applicable

AVAILABILITY OF DATA AND MATERIAL

All data are available from the Authors upon reasonable request.

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SUPPORTING INFORMATION

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