Annals of Hematology (2019) 98:1603–1610
https://doi.org/10.1007/s00277-019-03692-0

ORIGINAL ARTICLE

The platelet NLRP3 inflammasome is upregulated in a murine model of pancreatic cancer and promotes platelet aggregation and tumor growth

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Received: 4 December 2018 / Accepted: 8 April 2019 / Published online: 24 April 2019

Abstract

Platelets are activated in solid cancers, including pancreatic ductal adenocarcinoma (PDA), a highly aggressive malignancy with a devastating prognosis and limited therapeutic options. The mechanisms by which activated platelets regulate tumor progression are poorly understood. The nucleotide-binding domain leucine-rich repeat containing protein 3 (NLRP3) inflammasome is a key inflammatory mechanism recently identified in platelets, which controls platelet activation and aggregation. In an orthotopic PDA mouse model involving surgical implantation of Panc02 murine cancer cells into the tail of the pancreas, we show that the NLRP3 inflammasome in circulating platelets is upregulated in pancreatic cancer. Pharmacological inhibition or genetic ablation of NLRP3 in platelets resulted in decreased platelet activation, platelet aggregation, and tumor progression. Moreover, interfering with platelet NLRP3 signaling significantly improved survival of tumor-bearing mice. Hence, the platelet NLRP3 inflammasome plays a critical role in PDA and might represent a novel therapeutic target.

Keywords Pancreatic cancer · Platelets · NLRP3 inflammasome · Platelet aggregation

Introduction

Pancreatic ductal adenocarcinoma (PDA) is one of the most aggressive malignancies with a 5-year survival of 8% [1]. Chronic inflammation [2] and hypercoagulability [3] are key features of PDA; in patients with chronic pancreatitis, the risk of PDA development is significantly increased [4]. Treatment options are still limited due to an incomplete understanding of the pathophysiological mechanisms of the disease that drive tumor progression and early metastasis. The nucleotide-binding domain leucine-rich repeat containing protein 3 (NLRP3) inflammasome is a critical inflammatory mechanism that is upregulated in PDA, as shown in both infiltrating immune cells [5] and tumor cells [6]. NLRP3 is an

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intracellular pattern recognition receptor which, upon activation, forms a complex with the adaptor protein apoptosis-associated speck-like protein containing a caspase activation and recruitment domain (ASC) and triggers activation of caspase-1 and cleavage and secretion of interleukin 1-β (IL-1β) [7, 8]. Activation of the NLRP3 inflammasome in macrophages induced immunological tolerance and promoted pancreatic tumor growth [5]. In another study, NLRP3 signaling in pancreatic cancer cells was identified as a critical regulatory trigger of epithelial-mesenchymal transition-induced cell invasion and tumor progression [6].

Platelets express NLRP3 [9–12] and have emerged as cellular regulators of cancer growth and invasiveness [13–15]. In pancreatic cancer patients, the platelets to lymphocyte ratio is a prognostic marker of decreased survival and worse outcomes [16]. The risk of pancreatic cancer was markedly reduced in patients who regularly took aspirin [17]. In addition to contributing directly to the invasiveness of pancreatic tumor cells, platelets play a critical role in regulating abnormal coagulation and inflammation in PDA and various other solid malignancies [14, 18–20]. We and other investigators have recently shown that activation of the platelet NLRP3 inflammasome promotes platelet aggregation, endothelial dysfunction, and thrombosis [9–12], key features in PDA. In this study, we investigate in a murine model of pancreatic cancer the role of the platelet NLRP3 inflammasome in PDA.

Methods

Tumor model and inhibitor injections

The animal protocol complied with regulations regarding the care and use of experimental animals published by the National Institutes of Health and was approved by the Institutional Animal Care and Use Committee of the University of Pittsburgh (protocol number 17080989). C57BL/6 wild-type (WT) mice (10–12 weeks old) were purchased from Taconic (Hudson, NY). NLRP3-deficient ($NLRP3^{-/-}$ mice) were obtained from the Jackson Laboratory (Bar Harbor, ME) and were on a C57BL/6 background [21].

For the orthotopic pancreatic cancer model, age- and sex-matched WT and $NLRP3^{-/-}$ mice were injected with 10^6 Panc02 cells into the tail of the pancreas through a limited laparotomy. The sham control group underwent the same surgical procedure with injection of PBS instead of tumor cells. Anesthesia was induced using isoflurane (2–5% inhalation), ketamine (90 mg/kg body weight intraperitoneally, IP), and xylazine (10 mg/kg body weight IP). Buprenex (0.1 mg/kg body weight IP) was administered for postoperative pain control for three consecutive days. Prior to injection, tumor cells were cultured in RPMI 1640 media (Hyclone, Logan, UT) supplemented with 10% fetal bovine serum. Two weeks after tumor implantation, mice were randomly allocated and received IP injections with inhibitors against NLRP3 (MCC950, 50 mg/kg body weight, Cayman Chemical, Ann Arbor, MI) or caspase-1 (YVAD, 5 mg/kg body weight, Calbiochem, Darmstadt, Germany) or vehicle controls (DMSO) every 48 h for 2 weeks (6 total treatments). Animals were sacrificed after 4 weeks at which time they had palpable left upper quadrant abdominal tumors. Mice were anesthetized with isoflurane, blood was drawn via cardiac puncture into anticoagulated tubes, and the pancreas was removed and weighed.

Platelets and caspase-1 assay

Murine platelets were isolated as previously described [22]. Mice were anesthetized with isoflurane and blood was drawn into anticoagulated tubes. Platelet-rich plasma (PRP) was obtained by centrifugation at 260×*g* for 5 min, followed by another centrifugation step at 640×*g* for 5 min to pellet the platelets. Activation of caspase-1 in platelets was measured using a FAM FLICA Caspase-1 Assay Kit (Immunochemistry Technologies, Bloomington, MN) according to the manufacturer's protocol and as previously described [12]. Collagen (2 μg/ml; ChronoLog, Havertown, PA) was used as platelet agonist. Platelets were analyzed in a black 96-well microtiter plate using a plate reader for relative fluorescence units (RFUs).

Flow cytometric evaluation of P-selectin expression on platelets

Platelet surface expression of P-selectin (CD62P) was evaluated using an APC-conjugated anti-CD62P monoclonal antibody (2 μg/ml, mouse IgG1κ; eBioscience, San Diego, CA) or isotype control antibody (eBioscience). Collagen (2 μg/ml; ChronoLog) was used as platelet agonist. Platelets were analyzed by flow cytometry using a BD Accuri C6 Plus (BD Biosciences, San Jose, CA) flow cytometer and Kaluza Analysis Software 2.1 (Beckman Coulter, MD). Platelets were gated based on their characteristic scatter properties.

Platelet aggregation

Platelet aggregation was evaluated using whole blood impedance aggregometry (Model 700, ChronoLog) as described previously [22, 23]. Collagen (2 μg/ml) was used as platelet agonist. Aggregation was measured for 6 min at 37 °C with a stir speed of 1200 rpm. Analysis was performed using the Aggrolink-8 software (ChronoLog). Data are reported as area under the curve (AUC), which incorporates the slope and amplitude of the aggregation curve.

Adoptive platelet transfusion model

Adoptive platelet transfusions were performed as previously described [24]. Recipient mice were treated with a platelet neutralizing LEAF anti-mouse CD41 antibody (1 μg/g body weight, BioLegend, San Diego, CA) via tail vein injection. Twenty-four hours after the CD41 antibody injection, 10^8 platelets isolated from one donor mouse were resuspended in 200 μl sterile PBS and infused into one recipient mouse via tail vein injection. PBS injections in the absence of the CD41 antibody and platelet infusions were performed as control. Transfusions were performed in recipient mice 14 days after tumor implantation or in sham mice and repeated every 4 days until sacrifice (four total treatments). Flow cytometric evaluation of P-selectin expression on platelets isolated from WT or NLRP3$^{-/-}$ mice was performed as described above and confirmed that platelets were not activated prior to platelet transfusions (data not shown).

Immunofluorescence staining of platelets

Isolated platelets were fixed with 2% paraformaldehyde, applied to 0.01% poly L-lysine-coated coverslips, and permeabilized with 0.5% Triton X-100. After blocking with 1% BSA-PBS for 1 h, cells were incubated overnight at 4 °C with anti-NLRP3 monoclonal antibody (2 μg/ml, mouse IgG2b; AdipoGen, San Diego, CA). Platelets were washed with PBS plus 0.3% Triton X-100 plus 0.1% Tween-20 and incubated with Alexa Fluor 488-tagged goat anti-mouse IgG (1:100, Invitrogen, San Diego, CA) for 2 h at room temperature. Following another washing step, platelets were incubated with anti-ASC polyclonal antibody (1 μg/ml, rabbit IgG; Santa Cruz, Heidelberg, Germany), washed, and incubated with Alexa Fluor 680-tagged goat anti-rabbit IgG (1:100, Invitrogen). The corresponding IgG antibodies (AdipoGen and Santa Cruz) served as controls. Confocal microscopic analysis was performed using a Nikon A1 confocal microscope (NIS Elements 4.4, Tokyo, Japan) and Nikon Elements imaging software.

Statistical analysis

Data are presented as mean ± SEM. Two-way factorial ANOVA with post hoc Bonferroni correction was used as appropriate. Kaplan Meier was used to estimate survival data. Statistical significance for survival was determined using log-rank test. All statistical analyses were performed using GraphPad Prism software (GraphPad, San Diego, CA).

Results

The platelet NLRP3 inflammasome is upregulated in PDA

We studied activation of the platelet NLRP3 inflammasome in an orthotopic PDA mouse model. Animals were sacrificed after 4 weeks, at which time palpable abdominal tumors were present. We detected expression of NLRP3 (green) and the adaptor protein ASC (red) in platelets derived from sham and tumor mice, as shown with immunofluorescence staining coupled with confocal laser scanning microscopy (Fig. 1a). In platelets from tumor mice, NLRP3 appeared to colocalize in part with ASC, indicating possible platelet NLRP3 inflammasome complex formation in PDA.

We next investigated caspase-1 activity in platelets, which allows monitoring of the platelet NLRP3 inflammasome [9, 10, 12], and found that caspase-1 activity was significantly elevated in circulating platelets from tumor-bearing mice as compared to sham controls (Fig. 1b). Intraperitoneal injections of the NLRP3 inhibitor MCC950 significantly suppressed platelet caspase-1 activity in both tumor and control mice as compared to vehicle control injections. The caspase-1 inhibitor YVAD served as a positive control and significantly decreased caspase-1 activation in platelets. We confirmed tumor-induced activation of the platelet NLRP3 inflammasome using NLRP3$^{-/-}$ mice. Upregulated platelet caspase-1 activity in tumor-bearing WT mice was significantly suppressed in tumor-bearing NLRP3$^{-/-}$ mice (Fig. 1c). Platelet caspase-1 activity was also significantly inhibited in sham NLRP3$^{-/-}$ mice compared with sham WT mice.

Platelet NLRP3 signaling promotes platelet activation and aggregation in PDA

Next, we investigated the effect of PDA-induced upregulation of the platelet NLRP3 inflammasome on platelet activation and aggregation. Platelet activation, as monitored by expression of P-selectin (CD62P) via flow cytometry, was significantly increased in tumor-bearing mice compared with sham controls (Fig. 2a). Intraperitoneal injections of the NLRP3 inhibitor MCC950 or the caspase-1 inhibitor YVAD significantly reduced platelet activation in tumor but not sham mice. Similar results were obtained when platelet aggregation was tested (Fig. 2b). Moreover, tumor-induced upregulation of platelet activation (Fig. 2c) and aggregation (Fig. 2d) was significantly inhibited in NLRP3$^{-/-}$ mice.

To investigate the effect of NLRP3 specifically in platelets, we performed an adoptive platelet transfusion model, in which we transfused NLRP3$^{-/-}$ or WT platelets into C57BL/6 mice that were platelet-depleted with a neutralizing CD41 antibody prior to transfusion (Fig. 2e, f). Upregulated platelet activation (Fig. 2e) and aggregation (Fig. 2f) in tumor-bearing mice were

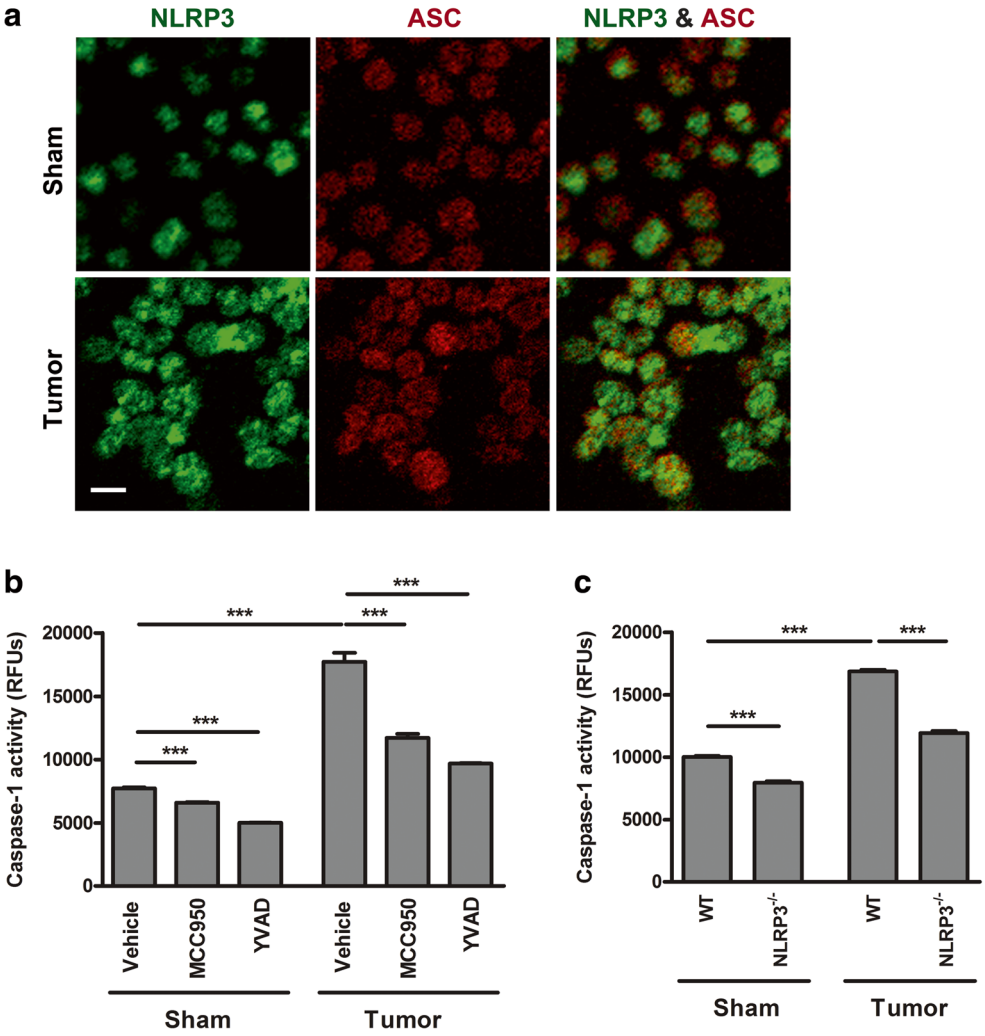

Fig. 1 The platelet NLRP3 inflammasome is upregulated in PDA. **a** NLRP3 (green) and ASC (red) partially colocalize in platelets derived from mice with orthotopic PDA, indicating possible formation of the NLRP3 inflammasome complex in platelets. Scale bar, 2 μm. **b** Caspase-1 activity is elevated in tumor-bearing mice. Treatment of mice with the NLRP3 inhibitor MCC950 or caspase-1 inhibitor YVAD decreases caspase 1 activity. **c** Caspase-1 activity is elevated in tumor mice, which is decreased in sham and tumor-bearing NLRP3$^{-/-}$ mice. **a** Representative images from three mice per group. **b**, **c** Data show mean ± SEM from three separate experiments and $n = 3$ mice per group. ***$p < 0.001$ (two-way ANOVA with Bonferroni post-hoc test in **b**, **c**).

significantly suppressed in the presence of transfused NLRP3$^{-/-}$ platelets as compared to tumor-bearing mice transfused with WT platelets, indicating that the platelet NLRP3 inflammasome plays a critical role in promoting platelet activation and aggregation in PDA.

PDA progression and survival from tumor are regulated by the platelet NLRP3 inflammasome

Next, we sought to determine the role of platelet NLRP3 inflammasome activation in PDA tumor growth and survival. Tumor-bearing mice receiving repeated injections of MCC950 or YVAD over 2 weeks had significantly decreased tumor weights as compared to vehicle controls (Fig. 3a). Moreover, orthotopic injection of pancreatic tumors into NLRP3$^{-/-}$ mice resulted in decreased tumor weights as compared to WT controls (Fig. 3b). In mice whose platelets were lacking NLRP3 (adoptive platelet transfusion model), tumor weights were significantly reduced, which did not occur in mice transfused with WT platelets (Fig. 3c). Moreover, tumor-bearing mice with NLRP3$^{-/-}$ platelet transfusions had a significantly improved survival as compared to those with WT platelet transfusions (median survival WT platelets 40 days vs. median survival NLRP3$^{-/-}$ platelets unreached, $p < 0.05$) (Fig. 3d).

Discussion

In this study, we show in an orthotopic tumor mouse model that the platelet NLRP3 inflammasome is upregulated in pancreatic cancer. We further identify a critical role of platelet NLRP3 in upregulating platelet activation/aggregation, promoting tumor growth, and interfering with survival from tumor.

Activation of pattern recognition receptor signaling in platelets is known to play a critical role in disease states associated with abnormal coagulation and inflammation [10–12, 22, 25–27]. The NLRP3 inflammasome was described in platelets for the first time in the context of dengue fever [9].

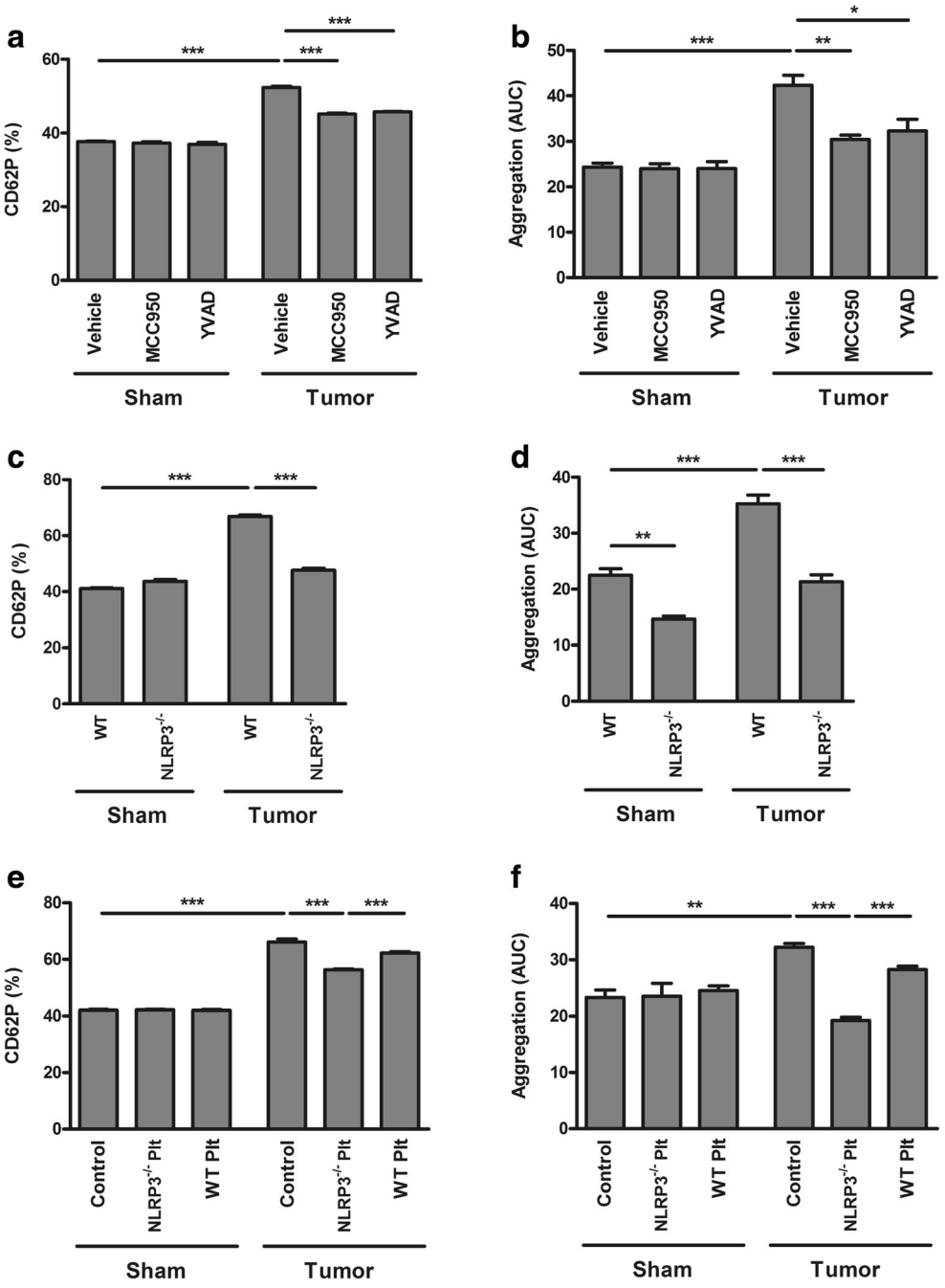

Fig. 2 Platelet NLRP3 signaling promotes platelet activation and aggregation in PDA. **a** Tumor-burdened mice have elevated platelet activation, assessed by %CD62P expression on isolated platelets with flow cytometry. Injections of MCC950 or YVAD decrease platelet activation in tumor mice. **b** Upregulated platelet aggregation in tumor mice is suppressed by injections of MCC950 or YVAD. **c** NLRP3$^{-/-}$ tumor-burdened mice have a reduction in platelet activation. **d** NLRP3$^{-/-}$ tumor-burdened mice have a reduction in platelet aggregation. Adoptive transfusion of NLRP3$^{-/-}$ platelets into tumor mice results in reduced platelet activation (**e**) and aggregation (**f**). Data show mean ± SEM from two separate experiments and **a**, **e** n = 3 mice (sham)/n = 4 mice (tumor) per group (pooled samples), **c** n = 4 mice per group (pooled samples), and **b**, **d**, **f** n = 4 mice per group. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ (two-way ANOVA with Bonferroni post hoc test in **a**–**f**).

In that study, the dengue virus triggered activation of the platelet NLRP3 inflammasome, which resulted in platelet shedding of IL-1β-rich microparticles and endothelial dysfunction. We have recently shown that platelet NLRP3 is upregulated in sickle cell disease, which was mediated via the damage-associated molecular pattern molecule (DAMP) high-mobility group box 1 (HMGB1) and resulted in elevated platelet aggregation [12]. In another study, we have shown that activation of platelets by collagen or thrombin in the absence of DAMPs or pathogen-associated molecular pattern molecules also induces activation of the NLRP3 inflammasome in platelets [10]. In this study, platelet NLRP3 was upregulated in PDA, which resulted in increased platelet activation and aggregation levels and promoted pancreatic tumor growth.

The NLRP3 inflammasome in immune cells has recently been identified as an important regulator of the inflammatory microenvironment in cancers, promoting epithelial-to-mesenchymal transition, angiogenesis, and metastasis [28, 29]. NLRP3/IL-1β signaling contributes to the development and invasiveness of various solid malignancies such as gastric cancer [30, 31], head and neck squamous cell carcinoma [32,

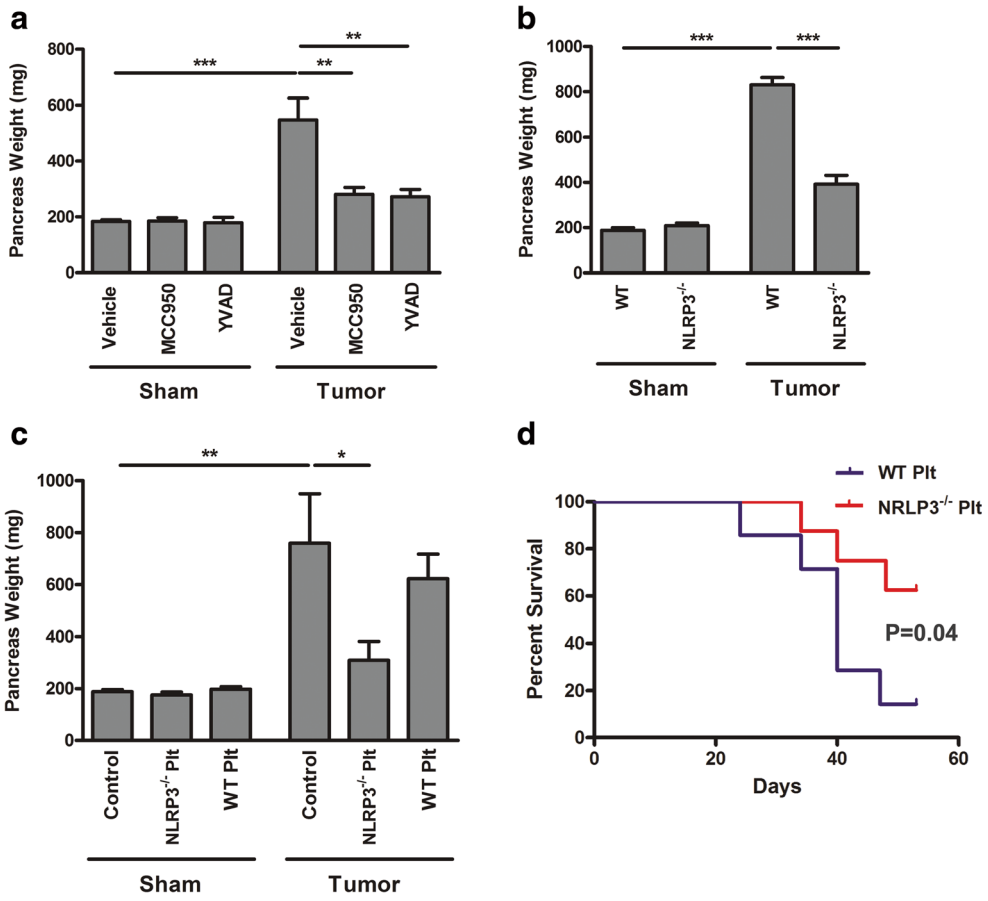

Fig. 3 PDA progression and survival from tumor are regulated by the platelet NLRP3 inflammasome. **a** Pancreas weight is increased in tumor-burdened animals. Injections of MCC950 or YVAD result in reduction of pancreatic weight in tumor mice. **b** NLRP3$^{-/-}$ tumor animals have reduced pancreas weight as compared to WT tumor mice. **c** Infusion of NLRP3$^{-/-}$ platelets following platelet depletion with anti-CD41 antibody results in decreased pancreas weight in tumor-bearing mice. **d** Infusion of NLRP3$^{-/-}$ platelets following platelet depletion improves survival of tumor-bearing mice as compared to infusion of WT platelets. Data show mean ± SEM from at least three separate experiments and $n \geq 7$ mice per group (**a**, **b**, **d**) or $n \geq 5$ mice per group (**c**). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ (two-way ANOVA with Bonferroni post-hoc test in **a**–**c**; Kaplan Meier curves and log-rank test for statistical analysis in **d**).

33], lung cancer [34], and PDA [5, 6]. In PDA, activation of the NLRP3 inflammasome in macrophages had a significant impact on adaptive immune response to the tumor by suppressing CD8^{+} T cell activation and activating tumor-promoting regulatory T cells [5]. In another recent study, RNA-induced downregulation of NLRP3 expressed by pancreatic cancer cells decreased pancreatic cancer progression and epithelial-mesenchymal transition-induced cell invasion [6].

Growing evidence suggests that platelets play a critical role in cancer growth through the regulation of abnormal coagulation, inflammation, and angiogenesis [19, 20]. In this study, activation of the platelet NLRP3 inflammasome increased platelet aggregation, promoted pancreatic tumor growth, and decreased survival from the tumor in mice. NLRP3 signaling in platelets triggers thrombosis, inflammation, and endothelial dysfunction [9–11], critical determinants of the tumor microenvironment. Thus, the platelet NLRP3 inflammasome might be an important regulator of the tumor microenvironment and affect growth and invasiveness of various solid malignancies, which is currently under investigation.

Activation of NLRP3 in platelets [10, 12] and immune cells [35, 36] is regulated by Bruton Tyrosine Kinase (BTK) and targetable with ibrutinib, a potent BTK inhibitor approved by the U.S. Food and Drug Administration for the treatment of B cell malignancies. In PDA-bearing mice, ibrutinib diminished fibrosis, markedly suppressed tumor growth, and extended survival through unclear mechanisms [37]. Despite its risk of potentially exacerbating IL-1β-dependent infections [36], BTK inhibition with ibrutinib might have beneficial effects in PDA via interfering with platelet NLRP3 signaling. Further studies are needed to identify the role of the platelet NLRP3 inflammasome in PDA and other malignancies and investigate this mechanism as a potential therapeutic target.

Acknowledgements The work for this article was performed while Drs. Brian A. Boone, Herbert J. Zeh III, and Sebastian Vogel were at University of Pittsburgh. The opinions expressed in this article are the authors' own and do not reflect the view of the National Institutes of Health, the Department of Health and Human Services, or the United States government.

Funding Information This work was supported by the U.S. National Institutes of Health (R01CA181450 to HJZ and MTL) and the German Research Foundation (DFG) KFO 274 (VO 2126/1-1 to SV and MG) and TRR 240 (374031971 to MG). The Nikon A1 confocal microscope used in this study was purchased with NIH grant 1S10OD019973-01 awarded to investigators at the University of Pittsburgh Center for Biologic Imaging.

Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

Ethical Approval All procedures performed in studies involving animals were in accordance with the ethical standards of the institution at which the studies were conducted. All applicable international, national, and institutional guidelines for the care and use of animals were followed. This article does not contain any studies with human participants performed by any of the authors.

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