Concerted BAG3 and SIRPa blockade impairs pancreatic tumor growth

Margot De Marco1,2, Vanessa Gauttier3, Sabrina Pengam3, Caroline Mary3, Bianca Ranieri1, Anna Basile1,2, Michela Festa2,4, Antonia Falco1,2, Francesca Repuccci1, Anna Lisa Cammarota1, Fausto Acermese4, Vincenzo De Laurenzi2,5, Gianluca Sala7, Bernard Vanhove3, Nicolas Poirier3, Roberta Iaccarino1, Michael Karin8, Maria Caterina Turco1,2,9, Alessandra Rosati1,2,9 and Liberato Marzullo1,2,9

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The BAG3- and SIRPa-mediated pathways trigger distinct cellular targets and signaling mechanisms in pancreatic cancer microenvironment. To explore their functional connection, we investigated the effects of their combined blockade on cancer growth in orthotopic allografts of pancreatic cancer m4-2D cells in immunocompetent mice. The anti-BAG3 + anti-SIRPa mAbs treatment inhibited (p = 0.007) tumor growth by about the 70%; also the number of metastatic lesions was decreased, mostly by the effect of the anti-BAG3 mAb. Fibrosis and the expression of the CAF activation marker α-SMA were reduced by about the 30% in animals treated with anti-BAG3 mAb compared to untreated animals, and appeared unaffected by treatment with the anti-SIRPa mAb alone; however, the addition of anti-SIRPa to anti-BAG3 mAb in the combined treatment resulted in a > 60% (p < 0.0001) reduction of the fibrotic area and a 70% (p < 0.0001) inhibition of CAF α-SMA positivity. Dendritic cells (DCs) and CD8+ lymphocytes, hardly detectable in the tumors of untreated animals, were modestly increased by single treatments, while were much more clearly observable (p < 0.0001) in the tumors of the animals subjected to the combined treatment. The effects of BAG3 and SIRPa blockade do not simply reflect the sum of the effects of the single blockades, indicating that the two pathways are connected by regulatory interactions and suggesting, as a proof of principle, the potential therapeutic efficacy of a combined BAG3 and SIRPa blockade in pancreatic cancer.

INTRODUCTION

Pancreatic ductal adenocarcinoma (PDAC) is a lethal malignancy with increasing incidence and mortality trends in several countries [1]. Its responsiveness to therapies, including single-agent immune modulators, is very poor [2–5]. A major role in PDAC resistance to therapy is ascribed to the tumor microenvironment, characterized by extensive desmoplasia, active immunosuppressive pathways, and the contribution of pro-tumor cytokines secreted by tumor-associated macrophages (TAMs), other immune cells, and cancer-associated fibroblasts (CAFAs) [6–8]. A combined inhibition of distinct immunosuppressive and/or pro-tumor pathways could represent a strategy capable of circumventing the blocks that affect therapy attempts [2–4, 7].

In the pancreatic cancer microenvironment, two distinct mechanisms involved in supporting tumor growth and suppressing the anti-tumor immune response are mediated by BAG3/BAG3R [9–15] and SIRPa/CD47 [16–24] axes. These two pathways operate in different cell types and through distinct signaling pathways. BAG (Bcl-2-associated Athanogene) 3 protein plays a dual role in cancer biology and in resistance to therapy [15]. Indeed, in neoplastic cell cytosol it regulates autophagy [25] and interferes with the Hsp70-mediated delivery of IKKγ [9] and other anti-apoptotic proteins [15] to proteasome, sustaining their levels and cell survival, while, being secreted by pancreatic cancer cells, it binds to a specific receptor (BAG3R) on TAMs, triggering the p38- and Akt-dependent release of pro-tumorigenic cytokines and chemokines [10, 11, 15]. In several pancreatic cancer murine models, BAG3 blockade by a monoclonal antibody impairs the activation of TAMs [11, 12] and CAFs [13]. This effect produces a significant reduction of the tumor growth of both Mia PaCa-2 and patient-derived pancreatic cancer xenografts in immunodeficient mice [11]. Notably, in heterotopic allografts of murine pancreatic cancer cells in immunocompetent syngeneic mice, treatment with the anti-BAG3 mAb sensitizes the tumors to the effect of an anti-PD-1 antibody [12]. On the other hand, signal-regulatory protein (SIRPa) (CD172a or SHPS-1), expressed on myeloid cells, upon its binding to neoplastic cell surface CD47 antigen (“don’t eat me” signal) transduces, through its interaction with Src Homology region 2 domain-containing Phosphatases (SHPs), an inhibitory signal, that blocks cancer cell phagocytosis by macrophages and
dendritic cell (DC) activation [16–18, 22]. Due to the roles played by DCs and macrophages in antigen presentation and in the release of cytokines that activate cytotoxic cells, the SIRPa/CD47 pathway regulates not only the innate immune activity, but also the adaptive response. Indeed, the blockade of the SIRPa/CD47 pathway reportedly potentiates T cell recruitment into tumor nest and antitumor immune activity in some tumor types [19–21, 23, 24].

The regulatory connections between the BAG3/BAG3R and the SIRPa/CD47 pathways have not yet been explored. We aimed to verify the possible functional interaction between the two mechanisms in regulating pancreatic carcinoma interplay with its microenvironment, by investigating whether their concerted mechanisms in regulating pancreatic carcinoma interplay with the tumor growth and metastatic diffusion.

For this purpose, we studied the effects of an anti-BAG3 [12] and an anti-SIRPa [26] antibody, separately or in combination, in a murine model of pancreatic cancer orthotopic allografts in syngeneic immunocompetent animals.

MATERIALS AND METHODS

Animal experiments

The research protocol of the animal study was approved by the Ethics Committee in accordance with the institutional guidelines of the Italian Ministry of Health, protocol n. 407/2019-PR. Female C57BL/6J (6 weeks old; Charles River, Italy) mice were housed five per cage with food and water available ad libitum and maintained on a 12 h light/dark cycle under standard and specific pathogen-free conditions. A total of 48 mice were used and maintained in a barrier facility on HEPA-filtered racks. The number of mice was calculated with the G*Power 3 software to obtain a power of 85%, with an α error of 0.05. Suffering mice and those in which the tumor was undetectable were excluded from the experiment. All experiments were conducted in a biological laminar flow hood, and all surgical procedures were conducted with strict adherence to aseptic techniques. The mice were anesthetized using isoflurane. For injecting cancer cells, mice were prepped with 10% povidone-iodine; a longitudinal median laparotomy with a xiphoid pubic incision was made, and the tail of the pancreas exteriorized gently. mtc–2D murine pancreatic cancer cells [12, 27] were suspended in 40 µl of PBS 1× in a 1 ml syringe; using a 25G needle, cells were injected into the tail of the pancreas and the injection point dubbed with sterile cotton. Once hemostasis was confirmed, the tail of the pancreas was returned into the abdomen and the wound was closed as a single layer using interrupted 5.0 silk sutures and skin staples. Two weeks after cell injection, tumor area was assessed using Vevo 2100 (Visualsonics, Canada) under anesthesia. Mice randomization into four arms consisting of 12 mice each, was carried out to homogenize the average area (approximately 4 mm²) of tumors in each group. Three times per week, one group of animals received i.p. injection of anti-BAG3 [12] (20 mg kg⁻¹); another group received i.p. injection of anti-SIRPa (MY1 mAb: Catalog#: MY1-1) twice a week; a third group received treatment with both anti-BAG3 and anti-SIRPa antibodies; the control group received i.p. injection of an unrelated IgG (Bioxcell Clone: MOPC-21 Catalog#: BE0083, 20 mg kg⁻¹). After two weeks of treatment, the animals were sacrificed, and tumors excised for analysis. The lot of anti-BAG3 mAb produced in CHO were tested for TGFβ1 content [28] and showed a concentration of cytokine level of 45.2 pg per μg of antibodies,
corresponding to a calculated amount of 18.1 ng of TGFβ1 co-injected per i.p. administration, per mouse.

**NanoString transcripational technology analysis**
RNA from mouse tumor tissues was extracted by a Trizol-chloroform gradient and isolation with RNeasy Mini kit (Qiagen). Gene expression was quantified by the NanoString nCounter platform, using 50 ng of total RNA for tumor tissue and the Mouse PanCancer Immune Profiling (PCIP) Panel (NanoString Technologies). The code set was hybridized with the RNA overnight at 65 °C; then RNA transcripts were immobilized and counted using the NanoString nCounter Sprint. Normalized expression data were analyzed by using the nSolver software. Lists of genes extracted from heatmaps were tested for their protein interactions using the STRING online software (https://string-db.org/).

**Immunofluorescence**
For paraffin-embedded sections, immunofluorescence protocol included deparaffination in Clear-Rite™ 3 (ThermoScientific, Waltham, MA), rehydration through descending degrees of alcohol up to water, non-enzymatic antigen retrieval in sodium citrate buffer 10 mM, 0.05% Tween, pH 6.0, for 40 min in pressure cooker at 95 °C. After washing, non-specific binding was blocked with 10% normal goat serum (NGS) in PBS 1× 1 h, RT. Sections were then incubated with anti-CD8 monoclonal antibody (C8/144B, Thermo Fisher 1:25), anti-CD11c monoclonal antibody (ab33483, Abcam, at 1:25), anti-CD3 monoclonal antibody (DM336P, OriGene Technologies, at 1:25), anti-α-SMA antibody (A2547, Sigma-Aldrich, at 1:350) overnight at 4 °C in a humified chamber. After another washing step, sections were incubated with the secondary antibodies (used at 1:200 dilution). Nuclei were counterstained with 1 µg/ml Hoechst 33342 (Molecular Probes, Oregon). Negative controls were performed using all reagents except the primary antibody. Slides were then overlaided using an aqueous mounting medium and analyzed using a confocal laser scanning microscope (Leica SP5, Leica Microsystems, Wetzlar, Germany). Images were acquired in sequential scan mode by using the same acquisitions parameters (laser intensities, gain photomultipliers, pinhole aperture, ×40 objective) when comparing experimental and control material. For figures preparation, brightness and contrast of images were adjusted by taking care to leave a light cellular fluorescence background, for visual appreciation of the lowest fluorescence intensity features and to help comparison among the different experimental groups. Leica Confocal Software and ImageJ were used for data analysis.

**Picrosirius red staining**
Tumors were embedded into paraffin and sections (5 µm), mounted on glass slides, processed, and stained with Picrosirius red (cat. 24901, Polysciences, Inc.) according to the manufacturer’s instructions. At least three different image fields were acquired at 20x magnification. The areas of collagen staining were quantitatively evaluated with ImageJ software and expressed as percentages of the total corresponding area.

**Statistical analysis**
Results are shown as standard error of the means (SEM). All statistical analyses were performed with MATLAB R2020b (Mathworks) and GraphPad Prism 8.0.1 (GraphPad Software). A p value <0.05 was considered statistically significant and the confidence interval was calculated at 95%. Lilliefors’ composite goodness-of-fit test for normality was used to test the null hypothesis that data came from a normally distributed population. To evaluate the effects of two treatments (anti-BAG3 and anti-SIRPα) on tumor weight and number of metastases, two factor analysis was performed using two-way ANOVA (unbalanced Type III sum of squares). To complete the two-factor analysis, a post hoc comparison (HSD Tukey-Kramer) was conducted and the differences between means of each group with their respective 95% confidence intervals were reported, to estimate the effect size. To assess whether there was a statistically significant effect of treatment on the number of subjects with metastases, a Fisher's exact test was conducted. Groups were formed for homogeneity of treatment and compared to assess whether and which of the factors had an effect. The effect size was estimated by calculating the Risk Ratio with its confidence intervals in the presence of either or both treatments. For all the other data analyzed, D’Agostino–Pearson test was performed to verify the normal distribution of linear variables. For variables normally distributed, we used one-way ANOVA followed by Bonferroni multiple comparisons test; for variables non-normally distributed, p values were evaluated by a non-parametric Kruskal–Wallis matched pairs test with Dunn’s comparison.

**RESULTS**
The combined blockade of BAG3/BAG3R and SIRPα/CD47 pathways decreases tumor growth and the metastatic process
To verify the potential cooperation of BAG3/BAG3R- and SIRPα/CD47-blockades in impairing pancreatic tumor growth, we produced murine orthotopic pancreatic cancer allografts by injecting murine pancreatic cancer cells (mtr4–2D) [12, 27] into the pancreata of syngeneic C57BL6 mice (Fig. 1A) and analyzed the effects of a treatment with anti-BAG3 [12] and anti-SIRPα [26] monoclonal antibodies on tumor growth. Mice were sacrificed and tumors excised after two weeks of treatment (Fig. 1B).

In the ex vivo analysis, we found that the treatment with either anti-BAG3 or anti-SIRPα mAb resulted in a reduction of tumor weight, which was more impressive when the two antibodies were used in combination (Fig. 1C). Furthermore, the combined treatment resulted also in a decrease of the number of metastases per animal; in this respect, the effect of the anti-BAG3 antibody appeared to predominate over that of the anti-SIRPα antibody (Fig. 2A, B).

**Expression of genes associated with immunity in treated tumors**
To investigate the effects of the treatments with the antibodies on the anti-tumor immune response, we analyzed the expression of
Fig. 3  Differential gene expression analysis in tumors from the four treatment groups. A Heatmap of the expression of selected genes in tumors excised from animals treated with control IgG, anti-BAG3 mAb, anti- SIRPα mAb, or both mAbs. The heatmap represents median-centered and colorized expression values. B STRING protein-protein network analysis of the upregulated gene cluster surrounded by the solid line rectangle in (A). C Immune cell signature enrichment scores using NanoString transcriptional analysis of excised tumors. One-way ANOVA followed by Bonferroni’s post hoc test was used for data analysis.
genes involved in immune functions in tumor tissues, by using a
digital multiplexed gene expression platform. As shown in Fig. 3A,
slight differences in the expression of these genes were
detectable in the anti-SIRPalpha or anti-BAG3- treated groups
compared to the controls. On the other hand, the tumors from
mice treated with both antibodies showed a very different pattern
(Fig. 3A). Indeed, in this group, the expression of genes for
cytokines or chemokines, and of other genes associated with
immune activation, was significantly increased and involved
almost entirely the gene family clusters (Fig. 3B). Particularly, we
observed an enhanced expression of genes associated with
adaptive immunity, such as genes expressed in tumor-infiltrating
lymphocytes (TILs), DCs, and T helper (Th) 1 cells, and a consistent
down-modulation of exhausted lymphocytes signature (Fig. 3C).

Infiltrating dendritic cells and CD8+ lymphocytes in tumor
tissues
We analyzed the effects of the treatments on the presence of
dendritic cells and CD8+ T lymphocytes in tumor tissues. A
modest increase in the number of CD11c+ cells was detected in
allografts treated with each of the two antibodies, but a very
higher increase was evident in mice treated with both antibodies
(Fig. 4A, B). A more accurate analysis showed that CD11c+CD103+
dendritic cells represented a substantial part of the overall
CD11c+ labeled cells (Fig. 4C). In good agreement with DC
increase, also CD8+ lymphocytes, hardly detectable in untreated
tumors, were observed in anti-BAG3- or anti-SIRPalpha- treated
mice and, at a very higher level, in tumors treated with both
antibodies (Fig. 5A, B).
CAFs activation and desmoplasia are abated in tumors of treated mice

We previously reported that treatment of pancreatic cancer heterotopic allografts with anti-BAG3 antibody down-modulated CAF activation and impaired the desmoplastic structure in pancreatic cancer stroma [13]. In agreement with results in heterotopic allografts, we observed a reduction of the expression of the activation marker \( \alpha \)-SMA in CAFs. Such reduction was raised up to >70% (\( p < 0.0001 \)) by co-treatment with the anti-SIRP\( \alpha \) mAb, while treatment with the anti-SIRP\( \alpha \) mAb alone did not result in any appreciable decrease (\( p > 0.999 \)) of \( \alpha \)-SMA positivity (Fig. 6). In parallel, fibrosis was impaired by about the 30% (0.007) and the 64% (\( p < 0.0001 \)) by treatment with, respectively, anti-BAG3 and anti-BAG3 + anti-SIRP\( \alpha \) mAbs, while the anti-SIRP\( \alpha \) mAb did not significantly affect fibrosis when used alone (Fig. 7). Therefore, the effects of the combined blockade of the two pathways did not appear to simply reflect the sum of the effects of the single blockades, but instead SIRP\( \alpha \) blockade, although unable by itself to modulate CAF activation and fibrosis, effectively contributed to the antifibrotic effect of the anti-BAG3 mAb.

DISCUSSION

In line with the currently pursued therapeutic strategies against pancreatic cancer [2–4, 7], our results support the concept that an action on more than one regulatory circuit in the tumor microenvironment can counteract neoplastic growth and metastatic process. For the design of such strategies, we need an in-depth knowledge of the interactions between the different tumor-microenvironment functional connections which, on the one hand, support tumor growth and, on the other, suppress the immune response. In this work, we addressed two regulatory pathways, one of which—BAG3/BAG3R—contributes to support the growth of pancreatic carcinoma through the pro-tumor activity of TAMs and CAFs stimulated by BAG3 [2, 3, 10–13], while the other—SIRP\( \alpha \)/CD47—is an immune checkpoint that blocks the phagocytosis of neoplastic cells and, notably, the activation of dendritic cells [16–24]. The results indicate that the concerted blockade of the two pathways can lead to remarkable anti-tumor effects.

In the context of the molecules that regulate the interactions between pancreatic carcinoma and its microenvironment, BAG3 attracts interest for some characteristics: the ability to influence
both TAMs and CAFs; its specific presence, as a secreted factor, in tumor tissues and not in normal ones; the lack of toxicity of anti-BAG3 antibodies in preclinical treatments, even in the long term [3, 10–15, 29]. The BAG3-governed pathway appears potentially a useful candidate in combination therapies. In this sense, it is noteworthy that an anti-BAG3 antibody is able to sensitize pancreatic carcinoma to the effect of an anti-PD-1 antibody [12]. The combined effect shown here on tumor growth and metastasization following the BAG3 and SIRPα blockade provides further evidence of the versatility of the anti-BAG3 tools in combination therapies.

A particularly interesting property of BAG3-blocking therapy is the destructive effect on desmoplasia [13]. Such property is relevant, given the importance of the desmoplasic arrangement of the stroma in supporting epithelial-mesenchymal transition, orchestrating the invasion of neoplastic cells, upsetting the anti-tumor immune response, and hampering tumor exposure to drugs [30–33]. Indeed, the development of a desmoplasic tumor microenvironment is a key element in pancreatic ductal adenocarcinoma carcinogenesis [30]. In the light of the importance of the stroma and the consequent role of CAFs on metastasization [34], it is not surprising the effect of BAG3 blockade, acting on CAFs [13], on the number of metastases per animal (Fig. 2A, B). Although BAG3 activity on CAFs—on which the desmoplasic implant mainly depends—is documented [13], it is necessary to define the CAF populations [35–44] involved and the mechanism leading to the impressive anti-fibrotic effect of BAG3 blockade. This topic is of great interest for the advancement of knowledge of the biology of pancreatic cancer and other fibrotic tumors, in which desmoplasis and mechanoreciprocity mechanisms play a fundamental role in resistance to therapies [45–48].
A remarkable observation that emerges from data analysis is that the effects of single and combined antibody treatments are distinct. Indeed, treatment with the anti-SIRPα antibody, while not having a significant effect per se on CAF activation and fibrosis, in these respects significantly contributed to the effect of the anti-BAG3 antibody (Figs. 4, 5). On the other hand, although SIRPα fibrotic areas were analyzed and quantified as percent of whole areas in ≥10 different fields per sample. Non-parametric Kruskal–Wallis test with Dunn’s correction was used for data analysis. Non-parametric Kruskal–Wallis test with Dunn’s correction was used for data analysis. Non-parametric Kruskal–Wallis test with Dunn’s correction was used for data analysis.

The experimental evidence shows that the effects of individual treatments are not as much impressive as the combo treatment with both antibodies. In fact, the results clearly point out a reciprocal influence of BAG3/BAG3R or SIRPα/CD47 blockade on the activity of the other pathway. This mutual influence is most likely due to the regulatory role that each pathway plays on the global functional setting of the pancreatic cancer microenvironment, where several cellular components like CAFs, TAMs, MDSCs, and other myeloid cells, are involved in the complex biochemical crosstalk granting the tumor cells survival and proliferation. As shown in Fig. 3B, BAG3 and SIRPα blockade produces a noticeable effect on cytokines and chemokines clusters. The treatment with mAbs allowed to obtain useful information about the overall changes in the cytokines/chemokines assets, and a more accurate data mining could lead to a more precise identification of single elements of the clusters responsible of cancer fibrosis and of the recruitment of DCs and CD8⁺ lymphocytes.

In conclusion, our findings show that the blockades of BAG3/BAG3R and SIRPα/CD47 axes converge in eliciting a sound antitumor immune response against pancreatic cancer and in countering tumor growth and the metastatic process. These results highlight the functional integration of the two pathways in determining the global functional setting of the pancreatic cancer microenvironment and provide a proof of principle of the potential validity of a combined therapeutic treatment against BAG3 and SIRPα.

DATA AVAILABILITY
The data that support the findings of this study are available from the corresponding author upon reasonable request.

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