ISG15 is a critical microenvironmental factor for pancreatic cancer stem cells

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ABSTRACT

Cancer stem cells (CSC) are thought to play a major role in the development and metastatic progression of pancreatic ductal adenocarcinoma (PDAC), one of the deadliest solid tumors. Likewise, the tumor microenvironment contributes critical support in this setting, including from tumor stromal cells and tumor-associated macrophages (TAM) that contribute structural and paracrine-mediated supports, respectively. Here we show that TAM secrete the interferon-stimulated factor ISG15 which enhances CSC phenotypes in PDAC in vitro and in vivo. ISG15 was preferentially and highly expressed by TAM present in primary PDAC tumors resected from patients. ISG15 was secreted by macrophages in response to secretion of interferon-β; by CSC, thereby reinforcing CSC self-renewal, invasive capacity and tumorigenic potential. Overall, our work demonstrates that ISG15 is a previously unrecognized support factor for CSC in the PDAC microenvironment with a key role in pathogenesis and progression.

Précis. This study highlights the role of a previously unrecognized support factor in the tumor microenvironment for cancer stem cells in pancreatic cancer, with implications for tractable new strategies to attack this deadly disease.

Keywords: Pancreatic cancer, Cancer stem cells, Interferon stimulated gene 15, Tumor-associated macrophages
INTRODUCTION

The importance of the stroma in promoting cancer initiation and solid tumor growth has been increasingly recognized during recent years (1-3). Specifically, we have come to understand that apart from providing structural support for tumor development, the tumor-associated microenvironment of many solid tumors provides cues to a subpopulation of tumor-initiating cells, also known as cancer stem cells (CSCs), which regulates their self-renewal, tumorigenic and metastatic potential (4). This is certainly the case for pancreatic adenocarcinoma (PDAC), which consists of a heterogeneous population of tumor cells including (i) CSCs (5, 6), (ii) more differentiated cancer cells and (iii) an extremely high proportion of desmoplastic stromal tissue and immune cells, which accounts for up to 90% of the tumor mass (7). Within the stroma-rich PDAC tumor microenvironment, pancreatic stellate cells (PSCs) have been extensively studied and recent reports from our laboratory and other have shown that tumor-associated PSCs can create a pro-tumor paracrine niche for PDAC CSCs via Nodal/ActivinA secretion (2, 4). Recent but less conclusive evidence also suggests that inflammatory cells, such as tumor-associated macrophages (TAMs) (1, 8) may also play critical roles in the development and progression of numerous tumors, such as PDAC, and the immuno-modulatory factors they secrete may also be paracrine-mediated.

Interferon-stimulated gene 15 (ISG15) is a 165 amino acid (17kDa) protein that is induced by Type I interferon (IFN) treatment (9). Since its discovery in 1979 (10), ISG15 has been extensively studied as an anti-viral protein (11-13), but we now appreciate that ISG15 has many other functions, including ISGylation, a ubiquitin-like modification process whereby ISG15 can be covalently linked to cytoplasmic and nuclear proteins (14). Like ubiquitin, ISG15 coupling to target proteins involves the ISG15-specific E1-like activating enzyme (UbE1L), the conjugating E2 enzyme and the ligating E3 enzymes (15, 16). While the consequences of ISGylation of host proteins has been elucidated for only a small set of cellular proteins (e.g. cyclin D1, Filamin B, PML-RARα), it is believed that the biological effects of ISGylation are
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dynamic and cell type/tissue specific (17). For example, while some reports suggest that like ubiquitilation, ISGylation may function in protein turnover (18), it may also play a previously unrecognized role in protein stability (17). The latter has been explored in systems of bladder, oral, prostate and breast cancers, where high levels of ISG15 and its conjugates have been detected, suggesting a link between ISG15 and tumorigenesis (19-24). For example, Kiessling et al have shown that in prostate cancer, over expression of UbE1L increased androgen receptor levels in an ISG15-dependent manner, implying that ISGylation promotes androgen receptor over expression in cancer cells. In breast cancer, Burks et al have shown that ISG15 stabilizes oncogenic K-ras protein by inhibiting its targeted degradation via lysosomes. Therefore, intracellular ISGylation may very well play an important and previously underappreciated role in cancer.

Apart from its intracellular protein conjugating functions, ISG15 can also be secreted from cells as free ISG15 where it can act as a cytokine or chemokine stimulating the production of Type II IFN, enhancing natural killer cell activity and proliferation or functioning as a strong neutrophil chemoattractant (25, 26). Thus, free ISG15 has strong immune modulatory properties; however, the biological role of free ISG15 has been under studied and the mechanisms promoting its liberation are poorly understood. Surprisingly, here we show that ISG15 is present in PDAC tumors, it is expressed and secreted by TAMs in response to IFN-β produced by PDAC cells and, in turn, acts on PDAC CSCs enhancing their inherent “stem-like” properties, including self-renewal and tumorigenicity. Thus, our data suggests a previously unrecognized role for ISG15 (i.e. free ISG15) in the context of pancreatic cancer and highlights a potentially new target for therapeutic intervention.
MATERIALS AND METHODS

Primary pancreatic cancer cells and macrophages. The use of human material was approved by the local ethics committee of each respective hospital or university, and written informed consent was obtained from all patients. Primary tumors were processed, cultured in vitro as previously detailed (5) and are referred to herein as “Panc-xxx”. Murine PDAC cells were established from tumors extracted from K-ras+/LSL-G12D;Trp53LSL-R172H;PDX1-Cre mice (27) at 20-24 weeks of age.

Human blood was obtained from healthy donors with informed consent and in accordance with national regulations for the use of human samples in research. Macrophages were established as previously described (28) and polarized using 1000 U/ml GM-CSF (M2) or M-CSF (M1) (29). Murine monocytes were isolated from mechanically disrupted spleens and polarized using 10 ng/ml of IFN-γ (PeproTech) and LPS (Sigma) (M1) or 10 ng/ml IL-4 (M2) (PeproTech). HPDE cells have been previously described (30).

Human PDAC Tissue microarrays (TMAs) and RNA samples. Human TMAs containing a total of 42 tumors were constructed. RNA from 30 flash-frozen primary human PDAC tumors was isolated by the guanidine thiocyanate method using standard protocols (31).

Mice. NU-Foxn1null nude mice (Charles Rivers, L'Arbresle, France), ISG15+/+ and ISG15−/− mice (Klaus-Peter Knobeloch; Universitäts Klinikum, Freiburg, Germany) were housed according to institutional guidelines and all experiments were approved by the Animal Experimental Ethics Committee of the Instituto de Salud Carlos III (Madrid, Spain).

Flow cytometry. Primary human macrophage cultures were resuspended in Sorting buffer before analysis with a FACS Canto II instrument (BD, Heidelberg, Germany). Primary and secondary antibodies and dilutions used are listed in Table S1.

In vivo tumorigenicity assay. Primary 1st generation sphere-derived pancreatic cells were resuspended in 50 µl of Matrigel™ (BD) and subcutaneously injected into indicated mice alone or with equal
numbers of non-polarized, M1- or M2-polarized, or CSC CM-primed primary macrophages. Tumor size was monitored weekly over the course of 6-10 weeks.

**ELISAs.** IFN-β in the supernatant of PDAC cultures was quantified using a commercially available ELISA (PBL Assay Science) as per the manufacturer’s instructions. Free ISG15 was quantified using an in-house sandwich ELISA as detailed in Supplemental Materials and Methods.

**Immunohistochemistry and immunofluorescence.** Formalin-fixed paraffin-embedded (FFPE) blocks were serially sectioned and immunohistochemical (IHC) or immunofluorescence (IF) analyses performed using standard protocols. Primary antibodies, secondary antibodies and dilutions used are detailed in Table S1.

**RNA preparation and RTqPCR.** Total RNA was isolated by the guanidine thiocyanate method using standard protocols (31). cDNA synthesis was performed using the QuantiTect Reverse Transcription Kit (Qiagen, Barcelona, Spain), followed by SYBR green RTqPCR (Applied Biosystems). Primers used are listed in Table S2.

**Sphere formation assay.** Pancreatic cancer spheres were generated as previously described (4).

**Wound healing assay.** Confluent cultures of primary cancer cells seeded were scratched using a 200µl pipette tip after overnight starvation. Cells were then incubated at 37°C with indicated treatments.

**Statistical analyses.** Results for continuous variables are presented as means ± standard error of the mean (SEM) unless stated otherwise. Treatment groups were compared with the independent samples t test. Pair-wise multiple comparisons were performed with the one-way ANOVA (two-sided) with Bonferroni adjustment. P values<0.05 were considered statistically significant. All analyses were performed using SPSS 17.0 (SPSS Inc., Chicago, IL).

**Additional experimental procedures and details can be found in the Supplemental Materials and Methods section.**

**RESULTS**
Macrophages promote PDAC CSC self-renewal, migration and tumorigenesis. Apart from neutrophils, infiltrating macrophages represent one of the major immune cell types present in the high stroma-rich PDAC tumor microenvironment (32) (Fig. S1A). Thus, since it has been shown in other solid tumors that CSC properties can be promoted by microenvironmental factors (33, 34), we aimed to test whether macrophage-secreted factors could also enhance PDAC CSC phenotypes. Since macrophages are not static, but rather are highly plastic and can be differentially polarized into classically “activated”/“M1” macrophages or “alternatively-activated”, “M2” or “pro-tumorigenic” macrophages (reviewed in (35)), we therefore tested the effects of both M1 and M2 macrophages on PDAC CSCs. First, the self-renewal capacity of two different primary PDAC cultures was assessed by culturing Panc354 and Panc185 cells in anchorage independent conditions and in the presence of control media or conditioned media from M1-polarized or M2-polarized monocyte-derived macrophages. First and second generation sphere formation increased when cells were cultured in the presence of macrophage-conditioned media by ~1.5- to 2-fold, with the greatest increase observed when PDAC cells were cultured with conditioned media from M2-polarized macrophages (Fig. 1A). In addition, we also observed an increase in the expression of the pluripotency-associated genes Klf4, Sox2, and Nanog, modulation of EMT-associated genes e-cadherin, Zeb-1 and vimentin (Fig. 1B), enhancement of the migratory capacity of PDAC cells when co-cultured with conditioned media from M2-polarized macrophages (Fig. 1C), and activation of pErk1/2, a mediator of pro-survival and pro-proliferation pathways, in treated sphere cultures (Fig. 1D).

Macrophages can also respond to cues from cancer cells and differentiate towards a pro-tumorigenic “M2” phenotype in response to tumor micro-environmental stimuli such as CSF1, IL-4, IL-13, TGFβ1 or IL-10 (36). Therefore, we additionally treated macrophages with conditioned media harvested from PDAC spheres, which are enriched in CSCs (Fig. S1B) and factors such as TGFβ1, Nodal and ActivinA (4). Using CD163 as a macrophage M2 marker (35), we observed that macrophages treated with CSC-conditioned media adopted a CD163 expression
pattern similar to that of macrophages polarized with GM-CSF to an M2 phenotype (Fig. 1E). Likewise, media removed from these CSC-primed macrophages was also able to enhance PDAC sphere formation, promote the expression of pluripotency-associated genes and increase PDAC cell migration similar to conditioned media from non-CSC primed MCSF-treated M2-polarized macrophages (Fig. 1A-B and data not shown).

Lastly, we injected $5 \times 10^5$ primary PDAC cells alone or with equal numbers of M1-polarized, M2-polarized or CSC conditioned media-primed primary human macrophages and assessed tumor growth over 8 weeks. Consistent with the aforementioned in vitro data, tumor growth was significantly accelerated when PDAC cells were co-injected with M2 macrophages or with macrophages pre-stimulated (i.e. “primed”) with media from CSC spheres (Fig. 1F and S2). Thus, the sum of these data would suggest that an intricate and intimate crosstalk exists between CSCs and macrophages, where CSCs promote the polarization of macrophages towards an M2-like phenotype, which can then, in turn, promote the “stemness” and tumorigenicity of CSCs.

*Macrophages increase the expression and secretion of ISG15 when co-cultured with PDAC CSCs.* We next co-cultured monocyte-derived macrophages with and without primary PDAC cells in transwells. Seventy-two hours post co-culture, RNA was extracted from macrophages and microarray analyses were performed. 3,084 genes were significantly upregulated and 3,431 genes downregulated (FDR<0.05) compared to control cultures. Of the top 25-upregulated genes (FDR<10^{-4}, |logFC|>2), the majority of genes belonged to the family of interferon-stimulated genes (ISGs) (Fig. 2A). Of the 19 ISGs detected, we focused on ISG15 as a gene of potential interest as it encodes for a protein that can function intra-cellularly to modify cytoplasmic and nuclear proteins, it can also be secreted from activated cells as free ISG15, and ISG15 has been shown to play a putative role in other solid tumors such as bladder, oral, prostate and breast cancers (19-24). We confirmed the microarray results by RTqPCR and WB analysis. Specifically, in monocyte-derived macrophages cultures treated with PDAC CSC sphere
conditioned media we observed a strong increase in ISG15 mRNA and protein levels (conjugated and monomeric) compared to non-treated control macrophage cultures (Fig. 2B-C).

We also observed an increase in the ISG15 de-conjugating enzyme USP18 (Fig. 2A-B), which functions to remove ISG15 from its conjugates, thus increasing the overall amount of monomeric ISG15 (37). We hypothesized that the increased amount of monomeric ISG15 present in macrophages treated with PDAC CSC-conditioned sphere media (Fig. 2C) would result in increased secretion of free ISG15. In accordance with this hypothesis, we observed an increase in free ISG15 in the supernatant of treated macrophages compared to non-treated controls (Fig. 2D). M2-polarized macrophages alone secreted more ISG15 compared to M1-polarized macrophages, and free ISG15 levels could be further and significantly enhanced by first priming macrophages with conditioned sphere media from two different primary cultures of PDAC CSC spheres (Fig. 2D).

These results strongly suggested that PDAC CSC conditioned sphere medium must contain Type I IFNs, a potent stimulus of ISG15 expression (9). Since it has been shown that K-ras-transformed breast cancer tumors over express IFN-β (38), we next determined if PDAC CSCs also produce/secrete IFNβ and whether it is biologically active using an ELISA for IFNβ and a vesicular stomatitis virus (VSV)-based anti-viral assay, respectively. Both assays confirmed that PDAC CSCs (e.g. spheres) secrete IFNβ (Fig. 2E), it is biologically active (Fig. 2F) and it is the likely causative factor for the ISG15 activation observed in monocyte-derived macrophages.

To study the effect of macrophage-derived free ISG15 release on PDAC cells, we analyzed intracellular ISG15 levels (conjugated and non-conjugated) in adherent PDAC cultures and in CSC sphere cultures left untreated or treated with conditioned media from 48h CSC-primed control, M1- or M2-polarized macrophages. Independent of treatment, intracellular monomeric and extracellular free ISG15 levels were significantly higher in CSC-enriched sphere-derived cultures compared to adherent cultures, which contain more differentiated cancer cells (Fig. 2G-H). Moreover, following treatment with M2-polarized macrophage conditioned...
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medium, we observed a specific increase in intracellular ISGylation levels and a corresponding decrease in the non-conjugated form of ISG15, indicating that the high levels of free ISG15 released by M2 macrophages (Fig. 2D) act on PDAC CSCs, further enhancing the conjugation of monomeric ISG15 to target proteins (Fig. 2G). However, unlike macrophages, we did not observe differences in the levels of free ISG15 in the supernatant of PDAC cultures following treatment (Fig. 2H). Interestingly, the levels of the de-conjugating enzyme USP18 were low to undetectable in PDAC cultures as determined by RTqPCR analysis (data not shown), providing a possible explanation as to why these cells secrete little free ISG15, even after stimulation.

Primary PDAC tumors express ISG15. To assess whether ISG15 could represent an important mediator of cancer development, we first studied the relationship between ISG15 expression and cancer survival at the genomic level. Using various publically available microarray datasets from PrognoScan (http://www.abren.net/PrognoScan/), a database for meta-analysis of the prognostic value of genes (39), we found that across several tumor entities, higher expression of ISG15 was predictive of significantly lower overall survival (Fig. 3A-B). Since no PDAC datasets are publically available, we evaluated the expression of ISG15 by RT-qPCR in bulk PDAC tumor samples obtained from ~30 surgical resections and by immunohistochemical (IHC) analysis using PDAC tissue microarrays. Compared to four normal pancreas controls, overexpression of ISG15 mRNA was observed in the majority of PDAC samples evaluated (Fig. 3C). Regarding its expression at the protein level, we observed that ISG15 was expressed in ~95% of all the tumors analyzed; however, distinct differences were observed with respect to the level and type of cells expressing ISG15 (Fig. 3D). For example, while ISG15 expression was detected in neoplastic cells of ~17% of tumors, ISG15 was predominantly expressed by immune cells (e.g. TAMs) in the stroma of PDAC tumors (Fig. 3D-E, S3, S4 and S5A). This was not the case for other tumors such as breast cancer and prostate cancer, in which tumor cells express the majority of ISG15 (Fig. S5B-C).
**ISG15 promotes CSC phenotypes.** Since macrophages can secrete free ISG15 and since macrophage-conditioned media enhances the “stemness” of PDAC CSCs (Fig. 1), we reasoned that ISG15 might be a pro-CSC factor secreted by macrophages in response to cues from PDAC CSCs (e.g. IFN-β). To test this hypothesis, PDAC cultures were treated with recombinant ISG15 (rISG15). The 1st generation sphere-forming capacity of CSCs from three primary PDAC cultures increased with rISG15 treatment compared to control-treated cultures (Fig. 4A), and the effect was more pronounced during serial passaging, which further enriches for CSCs (4). The increase in sphere formation also correlated with an overall increase in the expression of pluripotency-associated genes in Panc354 spheres treated with rISG15 during serial passaging compared to untreated cultures (Fig. 4B). In addition, rISG15 treatment also increased the migratory capacity of sphere-derived cells in a standard wound healing assay as shown in Fig. 4C. Lastly, similar to what we observed with M2 macrophage conditioned media (Figs. 1D and 2G), rISG15 treatment of CSCs also increased the level of intracellular ISGylation (Fig. 4D) and the phosphorylation of the pro-survival protein Erk1/2 (Fig. 4E). Taken together, these data strongly suggest that ISG15 alone can potentiate the “stemness” of CSCs and thus, macrophages likely potentiate CSCs, in part, via an ISG15-mediated mechanism.

To further validate our findings, we took advantage of mice with a genetic inactivation of the ISG15 gene. Using murine monocyte-derived macrophages, isolated from ISG15 knockout mice, we show that compared to ISG15\(^{+/+}\) M2-polarized macrophages, the conditioned medium from ISG15\(^{-/-}\) M2-polarized macrophage cultures did not similarly enhance murine PDAC sphere formation (Fig. 5A), the expression of stemness genes (Fig. 5B), their migratory capacity in a wound healing assay (Fig. S6), or the phosphorylation of Erk1/2 (Fig. 5D). Likewise, when ISG15\(^{-/-}\) macrophages were co-injected with murine PDAC cells in vivo, we observed an intermediate tumor growth phenotype compared to murine PDAC cells injected with wild-type macrophages. Importantly, when primary murine PDAC cells were pre-treated with rISG15 prior to co-injection with ISG15\(^{-/-}\) macrophages, a phenotype similar to that seen with ISG15\(^{+/+}\)
macrophages was achieved (Fig. 5E), indicating that the lack of secreted ISG15 is responsible for the impaired ability of ISG15<sup>−/−</sup> cells to promote PDAC tumor growth <i>in vivo</i>.

While these data strongly suggested that a driving factor responsible for PDAC tumorigenesis is ISG15, we next performed a limiting dilution cell transplantation assay to more rigorously determine the effect of ISG15 on the frequency of CSC-initiated tumor formation in vivo. Specifically, primary syngeneic murine PDAC cells were transplanted into recipient wild-type and ISG15<sup>−/−</sup> mice at increasing doses and tumor formation was determined 8 weeks post injection. While tumors efficiently formed in wild-type mice at dilutions of 10<sup>5</sup> (8/8), 10<sup>4</sup> (6/8) and 10<sup>3</sup> (5/6) cells, tumor formation and growth in ISG15<sup>−/−</sup> mice was significantly impaired (Fig. 5E-F), and the frequency of CSC-initiated tumorigenesis in ISG15<sup>−/−</sup> mice was significantly lower than in wild-type mice (CSC frequency: 1/51,360 vs 1/3,769, respectively; <i>p</i>=0.0001) (Fig. 5E, table).
DISCUSSION

TAMs, also known as M2, “alternatively-activated” or “pro-tumorigenic” macrophages [reviewed in (35)], are the major cell type of the inflammatory infiltrates present in PDAC tumors (32, 40) and are believed to promote tumorigenesis, matrix remodeling, and metastasis (36, 41, 42). In accordance with the latter, we observed that compared to M1 macrophages, M2 macrophages were able to promote the self-renewal capacity, modulate the expression of pluripotency- and EMT-associated genes, increase the migratory potential and enhance the tumorigenic capacity of PDAC CSC in a contact-independent manner. It is important to note, however, that we cannot discard the fact that human PDAC cells injected into nude mice may have activated murine macrophages to secrete pro-tumor factors. Thus, the effects observed with human PDAC cells in nude mice may have cross-species contributions.

At the cellular level, primary human macrophages that were treated with conditioned media from PDAC CSC cultures acquired M2-like properties at the level of cell morphology (data not shown) and expression of M2 cell surface markers, such as CD163, and media from these cultures enhanced the “stem-like” properties of PDAC CSCs similar to that of M2 macrophages. Thus, our data confirm that macrophages, specifically “M2/alternatively activated” macrophages, can significantly modulate the properties of PDAC CSCs via secreted factors, and PDAC CSCs can promote the polarization of macrophages towards an M2 phenotype. It is worth noting that the PDAC tumor microenvironment is composed of many other cell types, including T-cells, neutrophils and PSCs, which can also positively influence the “stemness” of PDAC CSCs as well as TAMs. Thus, the effects we observe in vitro are likely an under representation of the cross-talk that exists within the more complex multi-cellular tumor niche in vivo.

While macrophages can secrete many pro-stimulatory factors, we indentified by microarray analysis that ISG15 and its de-conjugating enzyme USP18 were highly up-regulated in macrophages co-cultured with PDAC CCSs. This apparent over expression was not all that surprising as Kras-transformed tumors have been shown to over express IFN-β (38), a strong
inducer of ISG15, and our IFN-β ELISA and VSV anti-viral assays both confirmed that PDAC cells produce soluble and biologically active IFN-β. Interestingly, a growing body of evidence over the past few years has shown a link between ISG15 and tumorigenesis for several solid tumors (19-24). The general conclusion from these studies is that ISG15 is over expressed in many tumor cell lines and ISGylation is important for malignant transformation; however, the mechanism(s) by which ISG15 exerts its pro-tumor effects and whether ISG15 functions the same in all solid cancer entities is still unknown. In line with these studies, our retrospective analysis of existing cancer microarray datasets with clinical outcome data confirms that for many solid tumors there exists a significant correlation between high ISG15 expression and poor overall survival, further strengthening the notion that ISG15 is indeed pro-tumorigenic. While we do observe ISG15 expression in primary PDAC cultures (data not shown) and in neoplastic cells of patient-derived tumors, TAMs within the tumor stroma express significantly more ISG15 compared to other tumor resident cells. Even more important, we observed that ISG15 is liberated from macrophages and its secretion increases when macrophages are polarized to a pro-tumor “M2” state or primed with CM from PDAC CSCs CM. Thus, for PDAC, the source of intratumoral ISG15 is the tumor microenvironment (i.e. TAMs).

Supporting a pro-tumor role for ISG15, we were able to increase the self-renewal capacity, expression of pluripotency associated genes and the activation of the p44/42 MAPK (ERK1/2) signaling in PDAC CSCs from three different primary patient-derived cultures using rISG15. We also observed a significant increase in the migration of PDAC cultures when treated with rISG15. ISG15 over expression in breast cancer cell lines has also been linked to an EMT phenotype. Burks et al., have shown that silencing ISG15 expression in the breast cancer cell line MDA-MB-231 reduced the migratory capacity of these cells compared to cells infected with an shRNA control lentivirus (24). Since TAMs are believed to contribute to EMT in solid tumors (43), promoting CSCs dissemination and metastasis, it is tempting to speculate that free ISG15 secreted from TAMs may play an important role in this process in PDAC.
Our model depicted in Fig. 6 illustrates how ISG15 is regulated within the PDAC tumor. Macrophages with the tumor microenvironment are polarized towards an M2 pro-tumor phenotype via tumor cell secreted factors (e.g. TGFβ1). TAMs can then respond to other tumor microenvironmental stimuli, such as IFN-β, promoting the up-regulation of ISG15 and USP18 mRNA and the subsequent secretion of free ISG15. Free ISG15 can then act on PDAC CSCs, enhancing their stem-like properties, including self-renewal and tumorigenicity. While our data support this putative model, three questions remain unanswered: (1) what receptor does free ISG15 bind to, (2) how does free ISG15 exert its effects on PDAC CSC, and (3) are the pro-tumor effects of free ISG15 independent of intracellular ISGylation or are they interconnected? Unfortunately, the receptor for ISG15 is currently unknown. It is believed that free ISG15 acts via binding to a cell surface receptor rather than passive diffusion into the cell; however, until a receptor is discovered, this question remains a black box. Regarding how ISG15 exerts its effect(s), in this study we show for the first time that rISG15 can activate p44/42 MAPK (ERK1/2) signaling, a pathway that has been shown to be important for cancer cells. Appreciating that other pathways may also be modulated by ISG15, we are currently investigating whether the AKT-PI3K and mTOR/S6K pathways, both of which have been shown to be important in PDAC (44, 45), are also affected by free ISG15. Along these lines, previous work from our laboratory has shown a direct link between AKT signaling and ISG15 in macrophages (11). We have reported that ISG15 plays an important role in the regulation of macrophage functions as ISG15−/− macrophages display reduced activation, phagocytic capacity and programmed cell death activation in response to vaccinia virus infection. This phenotype is independent of cytokine production and secretion, but correlates with impaired activation of the protein kinase AKT in ISG15−/− macrophages (11). Thus, since AKT signaling is very important for ISG15-mediated downstream effects in macrophages, it may very well also play an important role in ISG15-mediated enhancement of PDAC CSCs. In the end, understanding the pathways activated by free
ISG15 should yield new insights into PDAC cell biology, and possibly identify new targets that could be therapeutically inhibited.

Lastly, we cannot currently separate the fact that intracellular ISGylation is likely also an active and important process in PDAC cells. The purpose of our study was to investigate the role of TAM-secreted factors on PDAC CSC features, and thus we primarily focused on the effects of free ISG15 on PDAC CSCs, rather than the role of intracellular ISG15 and ISGylated products. Our data do, however, suggest that macrophages increase intracellular ISGylation in PDAC CSCs via secreted free ISG15. Thus, apart from activation of p44/42 MAPK (ERK1/2) signaling in PDAC CSCs, it may very well be that another important mechanisms of action of TAM-derived free ISG15 is to potentiate the conjugation of intracellular ISG15 to its target host proteins in PDAC CSCs, a process that has been shown to be beneficial for cancer cells of other tumor entities (22-24), but which has not been shown or studied to date in PDAC.

Lastly, using ISG15 knockout mice we show that the tumorigenic potential of murine PDAC cells co-injected with ISG15−/− macrophages was reduced compared to murine PDAC cells co-injected with wild-type ISG15+/+ macrophages. Assuming the later to be related to the fact that macrophages from ISG15−/− mice neither express nor secrete ISG15, we attempted to rescue the tumorigenic potential of murine PDAC cells co-injected with ISG15−/− macrophages by first pre-treating these cells with recombinant ISG15. The result was a near complete rescue, providing a plausible explanation for the reduced tumor growth observed when murine PDAC cells were injected with ISG15−/− macrophages compared to ISG15+/+ macrophages. It is important to point out that ISG15−/− macrophages were still able to potentiate PDAC CSCs, but to a significantly lesser degree compared to wild-type ISG15+/+ macrophages. This is not all that surprising as ISG15−/− macrophages may still secrete other pro-tumor factors that could still enhance the stem-like and tumorigenic potential of PDAC cells even in the absence of ISG15. Even in our syngeneic experiments, we observed that murine PDAC CSCs were still able to form tumors in ISG15−/− mice, although smaller and with significantly less efficiency. Thus, while ISG15 is
certainly important, it is likely not the only CSC-promoting factor secreted by macrophages and the tumor stroma; however, we did not test all CSC-specific phenotypes, such as metastasis, which may rely more on ISG15 than self-renewal or primary tumor initiation. Nonetheless, to the best of our knowledge, this is the first report showing that macrophages can secrete high levels of free ISG15 when polarized towards an M2 phenotype and PDAC CSCs can further potentiate its secretion, which in turn activates PDAC CSC stem-like properties. In summary, our study underscores the importance of the tumor microenvironment in CSC-mediated tumor biology and provides proof of principle that the stem-like nature of PDAC CSCs is strongly influenced by TAMs and their secreted factors, such as free ISG15. Thus, these findings not only advance our understanding of the role TAMs play in PDAC tumorigenesis, but they should also prove useful for future applications in cancer therapy, particularly those focused on targeting the tumor stroma.
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Sainz, Jr. et al. – ISG15 and pancreatic cancer

FIGURE LEGENDS

**Figure 1: Crosstalk between macrophages and PDAC CSCs.** (A-D) Single cell suspensions of primary PDAC cultures Panc185 and Panc354 were cultured with control media, conditioned media (CM) from M1-polarized macrophages (Mφ), M2-polarized Mφ or Mφ primed with PDAC CSC CM. (A) Quantification of the number of 1st and 2nd generation spheres/ml 7 d post sphere initiation. (B) RT-qPCR analysis of pluripotency-associated genes in sphere-derived PDAC cells. (C) Migratory capacity of GFP labeled PDAC cells assessed in a standard wound healing assay. Representative micrographs of wound size 12 h after wound induction (left panel). Average migration was calculated by measuring the size of the wound at three locations (n = 3 wounds per cell/treatment) (right panel). (D) Western blot analysis of pErk1/2, total Erk1/2 and tubulin. (C) Flow cytometry analysis of cell surface CD163 expression in indicated macrophage cultures. (F) Summary of in vivo tumor take and growth of 5×10^5 subcutaneously-injected PDAC cells alone or with M1-polarized, M2-polarized or CSC-primed human MØ. Data are an average of 2 independent experiments with n = 5 mice/group/experiment. * p value < 0.05, ** p value < 0.01

**Figure 2: Expression and regulation of ISG15 in macrophages and PDAC CSCs.** (A) Heatmap of top 25 genes upregulated and downregulated (FDR<10^{-4}, \[\log_{10}(FC)\] > 2) in primary human macrophages co-cultured with Panc185 (1), Panc354 (2) or Panc215 (3) for 48 h. (B-D) Primary human macrophages were cultured with conditioned media (CM) from the indicated PDAC CSC cultures. (B) RT-qPCR analysis of ISG15, USP18 and RIG-I mRNA levels. (C) Western blot analysis of ISG15 (conjugated and monomeric). (D) Quantification of free ISG15. Un-polarized and polarized macrophages (M1 and M2) were un-treated or primed for 72 h with PDAC CSC CM. Media was harvested 24 and 48 h following priming/washing and free ISG15 was measured by ELISA. (E) Quantification of IFN-β in supernatants of PDAC adherent and sphere cultures by ELISA. Supernatant from the human immortalized pancreatic ductal cell line HPDE was included as a comparative basal control. (F) Plaque assay analysis of VSV-infected Vero cells left either untreated or pre-treated with either IFN-α (1000 U ml⁻¹, 16 h) or with
Supernatants from three primary PDAC cultures. (G-H) PDAC cells were left untreated or treated with CM from 48 h CSC-primed control (Ctl), M1- or M2-polarized macrophages. (G) Western blot analysis of ISG15 (conjugated and monomeric). (H) Quantification of free ISG15. * p value < 0.05, ** p value < 0.01, *** p value < 0.001

**Figure 3: ISG15 expression in cancer tissue.** (A) Representative data sets for different tumors contained within the PrognoScan, a database for meta-analysis of the prognostic value of genes, where high ISG15 expression significantly (p value < 0.05) correlates with poor overall survival. Ln(HR_{High}/HR_{Low}) = hazard ratio (HR). Values greater than zero indicate that high gene expression correlates with poor overall survival. (B) Kaplan-Meier plot for dataset jacob-00182-MSK. Survival curves for high (red) and low (blue) ISG15 expression groups dichotomized at the optimal cutpoint. 95% confidence intervals for each group are also indicated by dotted lines. (C) RT-qPCR analysis of ISG15 mRNA levels in a panel of resected human primary PDAC tumors. The line indicates the median ISG15 mRNA expression levels across normal pancreas (NP) controls. (D) ISG15 expression profile in tumors from ~50 primary patient samples. ISG15 expressions in the stroma, neoplastic cells or both were assessed and their distributions graphed. (E) Representative micrographs of ISG15-stained tissues from a TMA containing normal, PanIN (I-III), PDAC, metastases and pancreatitis cores. Scale bar = 50 µm.

**Figure 4: Recombinant ISG15 enhances CSC phenotypes.** (A) Panc 185, 215 and 354 cells were cultured as spheres in anchorage independent conditions with control media or media supplemented with 100 ng/ml of rISG15 and sphere numbers were determined 7 later. (B) RT-qPCR analysis of pluripotency-associated genes in serially-passaged Panc354 spheres. (C) Scratch wound assay of PDAC cells after stimulation with 100ng/ml of rISG15. Quantification of wound size 12 h after wound induction. (D) Western blot analysis of ISG15-conjugated proteins and Tubulin in sphere-derived PDAC cells after stimulation with 100 ng/ml of rISG15. (E) Western blot analysis of pErk1/2, total Erk1/2 and Tubulin in sphere-derived PDAC cells after stimulation with 100 ng/ml of rISG15. * p value < 0.05.
Figure 5: ISG15 is necessary for PDAC tumor take and growth in vivo. (A-B) Single cell suspensions of primary murine PDAC cells were cultured with control media or conditioned media (CM) from M2-polarized ISG15+/+ or ISG15−/− murine macrophages. (A) Quantification of the number of spheres/ml 7 days post sphere initiation. (B) RT-qPCR analysis of pluripotency-associated genes in sphere-derived PDAC cells. (C) Western blot analysis of pErk1/2, total Erk1/2 and tubulin in sphere-derived PDAC cells after stimulation control media or CM from M2-polarized ISG15+/+ or ISG15−/− murine macrophages. (D) Summary of in vivo tumor growth of 5×10⁵ subcutaneously-injected murine PDAC cells (un-primed or primed with 100 ng/ml of rISG15) alone or with M2-polarized ISG15+/+ macrophages or M2-polarized ISG15−/− macrophages in nude mice (left). Representative images of tumors formed (right). Data are an average of two independent experiments with n = 5 mice/group/experiment. * p value < 0.05. (E-F) PDAC tumor growth in ISG15+/+ wild-type and ISG15−/− mice. (E) Representative images of tumors formed 8 weeks post injection with limiting dilutions of syngeneic murine PDAC cells. Summary table of in vivo tumor take and growth (below). CSC frequencies determined using the extreme limiting dilution analysis algorithm (http://bioinf.wehi.edu.au/software/elda/index.html) (right, 95% CI). (F) Summary of tumor weights. Data are an average of two independent experiments with n = 5 mice/group/experiment. * p value < 0.05.

Figure 6: Model of ISG15 potentiating effects in the tumor bulk. (A) Infiltrating M1 macrophages are polarized towards an M2 pro-tumor phenotype via tumor cell secreted factors such TGFβ1. (B) IFN-β secreted by PDAC cancer cells and PSCs induce the up-regulation of ISG15 and USP18 mRNA in TAMs. (C) Free ISG15 is secreted from TAMs in response to IFN-β stimulation. (D) Free ISG15 can act on PDAC CSCs, enhancing their stem-like properties, including the up-regulation of ISGylated proteins, pErk1-2, migration and tumorigenicity.
Figure 1 – Crosstalk between macrophages and PDAC CSCs

A

Control

+ M1 MØ CM

+ M2 MØ CM

+ CSC-primed MØ CM

B

Fold-change in mRNA levels

Panc354

C

Panc354

Control

+ M2 MØ CM

Wound size (mm)

D

MØ CM:

- +

- +

ERK1-2-P

- 85

ERK1-2

- 85

Tubulin

- 45

E

M1 MØ

M2 MØ

CD163

F

Panc354

+ M2 MØ CM

+ CSC-primed MØ CM

+ M1 MØ CM

Control

Weeks post-injection

- 0

- 2

- 4

- 6

- 8

tumor volume (mm³)
Figure 2 – Expression and regulation of ISG15 in macrophages and PDAC CSCs

A

B

C

D

E

F

G

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Figure 3 – ISG15 expression in cancer tissue

A

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B

Lung adenocarcinoma

jacob-00182-MSK

C

![Graph showing ISG15 mRNA levels](image)

D

![Pie chart showing stroma & cancer cells](image)

E

Normal

PanIN III

PDAC

Lymph node metastasis

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Figure 4 – Recombinant ISG15 enhances CSC phenotypes

A

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C

D

E

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Authors' manuscripts have been peer-reviewed and accepted for publication but have not yet been edited.
Figure 5 – ISG15 is necessary for macrophage-mediated enhancement of PDAC “stemness” and tumorigenicity

A

B

CHX6

CHX45

nº spheres/ml

+ M2 wt CM
+ M2 KO CM
+ M2 wt KO CM

+ M2 wt CM
+ M2 KO CM

+ M2 wt CM
+ M2 KO CM

C

D

E

F

Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

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Figure 6–Model of ISG15 potentiating effects in the tumor bulk
ISG15 is a critical microenvironmental factor for pancreatic cancer stem cells

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