LIF drives neural remodeling in pancreatic cancer and offers a new candidate biomarker.

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Abstract:

Pancreatic ductal adenocarcinoma (PDAC) is characterized by extensive stroma and pathogenic modifications to the peripheral nervous system that elevate metastatic capacity. In this study, we show that the IL-6-related stem cell promoting factor LIF supports PDAC-associated neural remodeling (PANR). LIF was overexpressed in tumor tissue compared to healthy pancreas, but its receptors LIFR and gp130 were expressed only in intratumoral nerves. Cancer cells and stromal cells in PDAC tissues both expressed LIF, but only stromal cells could secrete it. Biological investigations showed that LIF promoted the differentiation of glial nerve sheath Schwann cells and induced their migration by activating JAK/STAT3/AKT signaling. LIF also induced neuronal plasticity in dorsal root ganglia neurons by increasing the number of neurites and the soma area. Notably, injection of LIF-blocking antibody into PDAC-bearing mice reduced intratumoral nerve density, supporting a critical role for LIF function in PANR. In serum from human PDAC patients and mouse models of PDAC, we found that LIF titers positively correlated with intratumoral nerve density. Taken together, our findings suggest LIF as a candidate serum biomarker and diagnostic tool and a possible therapeutic target for limiting the impact of PANR in PDAC pathophysiology and metastatic progression.

Significance:

This study suggests a target to limit neural remodeling in pancreatic cancer, which contributes to poorer quality of life and heightened metastatic progression in patients.
Introduction:

Pancreatic ductal adenocarcinoma (PDA) is considered as one of the most serious cancers, with a quick and asymptomatic evolution leading to a very low survival rate in patients (1,2). Even with recent improvements (3), current treatments, mainly based on surgery and chemotherapies, have a limited impact on the patient’s fate, in part due to impaired drug perfusion provoked by the stromal reaction surrounding tumor cells (4,5). Indeed, PDA is characterized by the presence of a predominant stroma (intra-tumoral microenvironment) composed of cancer associated fibroblasts (CAFs), immune, endothelial and nerve cells. These have all been reported as drastic modifiers of tumor cells’ abilities thereby impacting on pancreatic tumor evolution and prognosis (6). However, recent advances based on the effects of the stromal compartment on PDA are limited, and their clinical translation remains difficult (7).

In addition to evidence showing the major implication of the stroma in PDA evolution and in therapeutic resistance, several studies have highlighted profound alterations of the neural compartment and its concrete impact on patient’s fate and quality of life (8,9). These alterations, called PDA associated neural remodeling (PANR), result in higher nerve densities in PDA due to peripheral nerve fibers infiltration and axonogenesis (10,11). Recently, we highlighted, in a previous study, that the intra-tumoral microenvironment could be a cause of those profound alterations (12). Thus, deciphering the specific connection between stromal compartment and nerve system in PDA could uncover potential therapeutic targets and clinical tools that would limit the nervous system-related impact on PDA evolution that alter patients’ fate (13,14).

Indeed, a direct consequence of this neural remodeling in PDA is the appearance of perineural invasion (PNI) events, marked by the cancer cell’s capacity to invade pancreatic nerves present within the tumor (15,16). In PDA, PNI is considered as an indicator of an aggressive
tumor associated with local recurrence and metastasis, acute neuropathic pain and leading to bad prognosis (8,17,18). Interestingly, recent reports have highlighted the role of the intratumoral microenvironment (19,20), and inflammatory processes (21) as pro-inflammatory cytokines like IL-6 (22) in PANR. Despite this, molecular mechanisms allowing neural remodeling and PNI events in PDA remain poorly understood. Thus, in-depth molecular studies are required to improve our knowledge in this field, which could provide new therapeutic opportunities to impair PDA progression and associated symptoms that limit patients’ access to chemotherapy (23,24) and negatively influence their outcome.

Here we demonstrate, in human and mice, that LIF has a direct role on PDA Associated Neural Remodeling. We observed that, within PDA, stromal cells, mainly macrophages and fibroblasts, have the ability to secrete LIF acting then on pancreatic neural compartment.

Indeed, LIF can induce migration and differentiation of Schwann cells and neural plasticity of dorsal root ganglia (DRG) neurons through modulation of the JAK/STAT3 intracellular signaling. Using endogenous mice model of PDA treated with LIF-blocking antibody, we revealed that LIF is important for PANR. In addition, high levels of LIF were detected in sera from humans and mice with PDA but not that from healthy individuals or patients with benign pancreatic diseases. Altogether, our data suggest that LIF is a potent biomarker for the diagnosis of PDA, and that the therapeutic targeting of LIF-induced signaling in PDA could limit PANR and improve patient outcome and quality of life.
Materials and Methods

Cell lines

Human pancreatic cancer cell lines (PANC-1, MIaPaCa-2, BxPC-3, Capan-2) and Schwann cells (sNF96.2) as well as murine macrophage (RAW264.7) were obtained from American Type Culture Collection (ATCC) between 2012 and 2014 and cultivated in DMEM medium supplemented with 10% fetal bovine serum (Life Technologies) and 1% of antibiotic/antimycotic (Invitrogen, 15240-062). Human mast cells (HMC-1) were provided by Professor Michel Arock (ENS Cachan, France) in 2015 and cultivated in RPMI-1640 medium, supplemented with 10% fetal bovine serum and 1% of antibiotic/antimycotic. Cells were authenticated through STR profile report (LGC Standard) in 2016 and tested mycoplasms free (Lonza, LT07-318). Human primary fibroblasts (FHN) were a kind gift from Dr Cedric Gaggioli (IRCAN, Nice, France) in 2016 and CAF cells (produced from freshly resected human PDA) (25) were cultivated in DMEM medium and DMEM/F12 medium respectively. PDA-1 to 4 are human pancreatic primary cancer cells derived from freshly resected PDA samples (26). All patients gave their consent and are included in the clinical trial number 2011-A01439-32 (26). Expert clinical centers collaborated on this project after approval from their respective ethics review board (approval number 11-61).

Human samples

Chronic pancreatitis (31 samples), pancreatic benign tumor (11 samples) or PDAC (142 samples) sera or tissues used for elisa assay, immunostaining or immunoblots were collected in patients from Hôpital Nord and La Timone, Marseille, France but also from Hôpital La Pitié-Salpêtrière, Paris, France. In addition, 61 healthy donors samples were amassed from Hôpital La Timone, Marseille, and from Etablissement Français du Sang (EFS), Marseille, France. All Patients were recruited to participate in a translational research study of blood
samples. They accepted and signed an informed consent that had been approved by the local ethics committee (Agreement reference of CRO2 for tissue collection: DC-2013-1857).

Concerning PDA patients that underwent surgical resections, PDA specimens were routinely fixed in 10% formalin, embedded in paraffin and further cut into 5 mm sections immediately stored at 4°C or stained with hematoxylin-phloxine-saffron (HPS). All tissues were collected via standardized operative procedures approved by the Institutional Ethical Board and in accordance with the Declaration of Helsinki. Informed consent was obtained for all tissue samples linked with clinical data.

**Murine serum and samples**

Sera from healthy and PDA bearing mice were obtained after intra-cardiac puncture and separation between plasma and blood cells by centrifugation. Mice developing PDA were euthanized when they were moribund (average of 8.5 weeks old). To obtain sera from chronic pancreatitis in mice, caerulein (50μg/kg/100μl) was injected twice a week for 10 week by intraperitoneal way (p.i). Sera from acute pancreatitis was obtained after 6 successive hourly injections by p.i. All animal care and experimental procedures were performed following approval by the Animal Ethics Committee of Marseille.

**Statistical Analysis**

The results showed are averages or medians, and error bars in graphs represent standard deviations (SD). The Mann–Whitney test, recommended for the comparison of two independent groups, was performed when required. The Wilcoxon test was used, when required, to analyze two different parameters within an experimental group. Differences were considered significant if $P$ was less than 0.05. All $P$ values were calculated using the Graphpad prism software. All experiments were repeated at least 3 times. LIF and IL-6
concentrations were transformed using a lin-log function (namely arc-sinus-hyperbolic) and scaled in order to cover the same range. Combining markers was carried out by the estimation of a logistic model. The rms package allowed logistic regression and models comparison.

Results:

Members of the gp130 “ligand/receptor” family are overexpressed in human and murine PDA

We previously showed that the stromal compartment can, through its secretory ability, impact nerve system reorganization within PDA tumors (12). Regarding recent studies revealing a role of inflammatory processes in PANR (11,22), we hypothesized that some genes/pathways, involved in the regulation of inflammatory processes, may be upregulated in the stromal compartment of pancreatic cancer and could impact PANR. Using two sets of RNA microarray analysis previously published by our group (Gene Expression Omnibus (GEO): GSE50570 for human PDA, (12), and GSE61412 for mouse PDA, (27)), we revealed that numerous members of the gp130 “ligand/receptor” family, with some already associated with neuropathic disorders or regulation of the nervous system (Table 1, column c), were upregulated in the PDA stromal compartment (Table 1, column d). Interestingly, we found that LIF (Leukemia Inhibitory Factor) was overexpressed both in stromal compartment from human PDA (Table 1, column d) and at late stage in spontaneous pancreatic cancer mouse model (pdx1-cre/Kras^{G12D}/Ink4A^{f/f}) (Table 1, column g).

While the role of the LIF-gp130 pathway is well defined within nervous system regulation and inflammation (28-30), its implication in pancreatic tumorigenesis is poorly understood (31). We first analyzed the expression of LIF in human PDA samples and revealed that LIF expression was increased in PDA samples in contrast to its almost complete absence in healthy pancreas (Fig. 1A to C). Interestingly, regarding the hypothetical role of LIF in neural remodeling, we observed that nerve fibers within human PDA samples commonly expressed
the two LIF receptors, LIFR and gp130 (Fig. 1A and D). Indeed, LIFR proteins were present in 8 out of 12 nerves analyzed within PDA tumors with a mean expression of 9.7% inside nerves. In addition, gp130 was found in 4 out of 12 nerves but with a stronger mean expression (20%). These changes in LIF expression were confirmed in PDA mouse model with a strong increase in LIF mRNA (Fig. 1E) and protein (Fig. 1F and 1G) level in PDA samples compared to healthy pancreas. Altogether, these data reveal the presence of LIF and its receptors, LIFR and gp130, in PDA samples. Moreover, the expression patterns of LIFR and gp130 support the hypothesis of LIF implication in PDA associated neural remodeling.

In PDA, secretion of LIF is mediated by the stromal compartment in vitro and in vivo

Regarding above data, we next sought to determine which cell types within PDA produced LIF. Using TMA of various human PDA samples, we observed that few epithelial cancer cells (cytokeratin-19) expressed LIF whereas higher percentages of macrophages (CD68), CAFs (αSMA) and mast cells (CD117) were labeled with LIF staining (Fig. 2A). Such analysis on 8 different human PDA showed that only 6% of cancer cells expressed LIF whereas mast cells, macrophages and CAF expressed LIF at 21%, 34% and 47.5% respectively. This was confirmed by measurement of LIF mRNA expression in vitro, where fibroblasts (FHN) expressed higher amount of LIF mRNA than macrophages (RAW) or mast cells (HMC-1) (fig. 2B). Interestingly, when co-cultured with RAW or HMC-1 or RAW+HMC-1, the fibroblasts showed an increased LIF mRNA expression (Fig. 2B). This level of LIF mRNA expression is similar with the one observed in primary CAFs from PDA patients (Supplementary Fig. 1). This data suggests, as shown previously (11), that FHN co-cultivated with RAW reach a similar threshold of activation than primary CAFs.

Comparison of LIF mRNA levels in fibroblasts co-cultured with macrophages versus various established (PANC-1 and MIAPaCa-2) or primary PDA tumor cell lines (PDA#1 to 4)
revealed that stromal cells express the highest amount of LIF mRNA (Fig. 2C). Surprisingly, the increased level of LIF mRNA in PDA co-cultured stromal cells did not result in higher LIF protein levels compared to single stromal or tumoral cell cultures (Fig. 2D and E). Considering this discrepancy between mRNA production and intracellular protein levels, we hypothesized that there was a change either in translation machinery, in LIF degradation or in LIF secretion. As LIF is referenced as a secreted cytokine, we measured by ELISA the amount of LIF secreted in media and observed a higher LIF concentration in media from fibroblasts co-cultured with macrophages compared to other stromal cell cultures (Fig. 2F and supplementary Fig. 2A). Interestingly, whereas amount of LIF secreted by CAF was higher than by FHN, we observed that amounts of LIF secreted were not different between CAF and FHN when co-cultured with RAW. Importantly, LIF was either undetectable or present in small amount in media from various tumor cells (Fig. 2G). Altogether, these data revealed that while numerous cell types within PDA potentially express LIF, the ability to secrete it seems restricted to the stromal compartment and in particular mostly to activated fibroblasts, a major cell component of PDA microenvironment that we have recently linked to PANR (12).

**LIF enhances the migratory capacity of peripheral nerve Schwann cells**

We sought to determine if the presence of secreted LIF in PDA could modulate nerve cells’ abilities, and therefore have an impact on PANR. We investigated the effect of stromal conditioned media (CM) with the highest LIF titer (FHN+RAW and FHN+RAW+HMC1) on the migratory ability of peripheral nerve Schwann cells and we observed a two-fold increase of Schwann cell migration after 4h (Fig. 3A and supplementary Fig. 2B). To assess whether LIF from those stromal CM was the mediator of the observed migratory improvement, we blocked LIFR and consequently impaired LIF signaling with a blocking LIFR antibody (Ab-LIFR) and observed that migration ability is restored to control level (Fig. 3B, upper panel).
Similar results were obtained using CM from CAF+macrophages and inhibition of LIFR compared to the use of a control antibody (Fig. 3B, lower panel). The use of SC144 and AG490, two chemical inhibitors which block gp130 (the co-receptor of LIF) or JAK2 signaling, the specific pathway activated after gp130/LIFR induction (32,33), respectively, confirmed previous data (Fig. 3C). Finally, LIF’s specific ability to enhance the migration of peripheral nerve Schwann cells was confirmed with human recombinant LIF protein at a dose of 50ng/ml, determined as the lowest dose inducing the higher migration improvement (Fig. 3D). Such induced migration ability with LIF recombinant protein was inhibited using LIFR blocking antibody at the lower dose of 4μg/ml (Fig. 3E, upper and lower panel) but also using AG490 and SC144 (Fig. 3F).

Regarding intracellular signaling induced by LIF stimulation through its receptors, LIFR and gp130, STAT3 and AKT are two of the main pathways known to be induced (34). First, we confirmed that in SNF96.2 cells LIF can trigger STAT3 and AKT phosphorylation/activation (Supplementary Fig. 3A and 3B). We further confirmed that such signaling activation is mediated by LIF receptors, LIFR and gp130, as AG490 or LIFR blocking antibody were able to inhibit STAT3 and AKT phosphorylation (Supplementary Fig. 3C). As reported in Figure 3G, stromal cells CM could induce STAT3 or AKT phosphorylation/activation. However, the use of AG490 or LIFR blocking antibody inhibited stromal cells CM effects on intra-cellular signaling suggesting that the CM-derived LIF could no longer activate LIFR/gp130 signaling. Altogether, our results revealed that LIF from stromal cell conditioned media is able to induce Schwann cell migration through LIFR/gp130 signaling then STAT3/AKT phosphorylation/activation.

**LIF inhibits Schwann cell proliferation**
We next examined the effects of stromal cell CM on Schwann cell proliferation and revealed that 48h of incubation with the highest LIF-titrated CM (FHN+RAW or FHN+RAW+HMC-1) decreased cell proliferation by 16% (Fig. 4A) without affecting cell survival (Supplementary Fig. 4A). We validated that this decreased cell proliferation was due to the presence of LIF by adding the LIFR blocking antibody to the CM, which restored cell proliferation to the control level (Fig. 4B). We obtained similar results using LIF recombinant protein with a cell growth reduction of about 17% (Fig. 4C) without modification of cell survival (Supplementary Fig. 4B).

In agreement with previous report linking JAK/STAT3 pathway activation and cell growth arrest (35), we observed an increase in p21 mRNA level by 24 hours post-LIF treatment (Fig. 4D) and an increase in p21 protein level by 36 and 48 hours (Fig. 4E). Interestingly, p21 protein level is restored with AG490 or SC144 treatments on cells incubated with LIF recombinant protein (Fig. 4F) or with stromal cells CM (Fig. 4G). These data highlight the impact of LIF secreted by PDA stromal cells on the reduction of Schwann cell proliferation, which occurs concordantly with their enhanced migratory abilities.

**LIF induces Schwann cell differentiation and neuronal plasticity**

Interestingly, JAK/STAT3 pathway is known to induce cell differentiation, a crucial process for nerve cells involved in PANR. Thus, we analyzed Pou3F2 and S100, two independent markers of Schwann cell differentiation (36,37) that we found expressed in human PDA nerve fibers (Supplementary Fig. 5A). Interestingly, we observed an induction of both markers in Schwann cells after 48 hours of incubation with stromal cell CM (Fig. 5A and 5B, left panels). Such increased was lost when stromal cells CM was supplemented either with LIFR blocking antibody (Fig. 5A and 5B, right panels), AG490 or SC144 (Supplementary Fig. 5B). Moreover, we showed that incubation with LIF recombinant protein was able to induce
Pou3F2 and S100 expression in Schwann cells at both mRNA (Fig. 5C) and protein levels (Fig. 5D and 5E). Besides its impact on Schwann cells, we wondered whether LIF may affect neuronal plasticity associated to PANR (38). As suspected, we found that recombinant LIF could induce neuronal plasticity with increased neurite outgrowth (Fig. 5F) and soma area (Fig. 5G). Those data reveal that LIF, secreted by PDA stromal cells, is able to induce Schwann cell differentiation and neuronal plasticity. In addition to data shown in previous parts, our study firmly support the potent impact of LIF in the neural remodeling observed in PDA tumors.

**LIF titer in serum as a diagnostic and prognostic biomarker for PDA patients**

To definitively assess if LIF is an inductor of PANR *in vivo*, we first analyzed LIF titer in sera from PDA bearing mice compared to LIF titer in sera from healthy mice and mice developing acute or chronic pancreatitis. Interestingly, not only we found a significant increase in LIF titer sera in PDA-bearing mice compared to control or benign pancreatic diseases (Fig. 6A) but we found that LIF titer in sera from PDA-bearing mice is positively correlated with intra-tumoral nerve density (R²=0.82, n=12, Fig. 6B). Finally, we assessed *in vivo* if LIF was directly influencing intra-tumoral nerve density by using a LIF neutralizing antibody in mice developing PDA. As shown in figure 6C, control mice (treated with a control antibody) displaying a low level of LIF in serum (<124pg/ml) exhibit few intra-tumoral nerves while control mice displaying a higher level of LIF in serum (>124pg/ml) showed a significant increase in the intra-tumoral nerve density. Interestingly, mice treated with the LIF neutralizing antibody showed a significant reduction of intra-tumoral nerves in spite of the presence of a high LIF quantity in serum (>124pg/ml). Those data revealed that LIF is directly enhancing intra-tumoral nerve density in PDA and that LIF titration in serum could serve as a biomarker to predict PANR.
Using a cohort including human sera from healthy donors (N=61), patients with chronic pancreatitis (N=31) or benign pancreatic tumor (N=11) with cystic adenomas and IPMN (Intraductal Papillary Mucinous neoplasms) and PDA patients (N=142), we confirmed previous mice data (Fig. 6A) and showed that LIF titer was only increased in sera from PDA patients compared to other groups (Fig. 6D). Also we confirmed in sera from PDA patients the positive correlation between LIF titer and intra-PDA nerve density ($R^2=0.74$, n=10, Fig. 6E). Above data suggest that LIF titer in serum is associated to PANR and could help in classifying PDA patients in terms of PANR grade.

Altogether, these data support the use of LIF titer as a diagnostic marker for all stages pancreatic cancer and as a biomarker to discriminate PANR level in PDA patients.
Discussion

Considering the grim mean survival rate among pancreatic cancer patients as well as the limited improvement of clinicians’ arsenal over the last twenty years, it has become urgent to explore new therapeutic avenues that target PDA evolution as well as PDA-associated phenotypes. Among the latter, neuropathic pain and cachexia are major problems; management of these symptoms is fraught with difficulties, and globally, there exists no agreed upon standard care or treatment. Importantly, both symptoms are often the determining factors in deciding between patients’ eligibility for chemotherapy or palliative care. Among fields to explore in order to improve drug accessibility and maintenance of treatment in PDA patients, deciphering mechanisms underlying PDA associated neural remodeling could yield promising results.

Although clinicians have for many years reported nervous system reorganization in cancers, and specifically in PDA, fundamental researchers have only recently realized its possible implications in PDA evolution and patient survival (8,13). It is now well acknowledged that infiltration of the tumor microenvironment by nerves, termed neoneurogenesis or axonogenesis, which occurs early in PDA development (11), plays an active role in cancer progression (39) and correlates with shortened survival, pain and local tumor recurrence (8). Although several studies have reported the ability of cancer cells to attract nerve fibers (40,41), very few have reported the impact of stromal cells in this process (12), especially in PDA where stromal cells compose the vast majority of the tumor cell mass. Therefore, our goal was to identify molecular targets from the PDA microenvironment that are involved in PANR, which may lead to the discovery of potent future adjuvant therapies that could prolong survival and reduce morbidity by blocking PANR. Here, we demonstrated for the first time that LIF, secreted by the PDA microenvironment, induced nerve cell migration and differentiation and thereby is positively correlated with PANR and axonogenesis (Fig. 6F).
Concomitantly, we have revealed that LIF is a potent biomarker for PDA and helps in determining PANR in PDA.

In this study, we considered knowledge associating tumor inflammation both with pancreatic cancer (42) and with the modulation of the nerve compartment (43,44) to reach our hypothesis that stromal-driven inflammatory genes/pathways could, additionally to their effects on tumor cells, impact the nerve compartment and in particular PANR. Thus, we revisited previous transcriptomic analysis (Gene Expression Omnibus (GEO): GSE50570 for human PDA, (12), and GSE61412 for mouse PDA, (27) and highlighted numerous genes that code for molecules involved in gp130 signaling and were overexpressed in the PDA stromal compartment compared to PDA tumor cells.

Among, the identified gp130-related genes, we focused on LIF due to its major role in regulating the nervous system (45). Indeed, very little is known of its potent role in this context except a study suggesting that increased levels of LIF in PDA could impact the STAT3 pathway in cancer cells (31). In addition to confirming these results in both human and mouse PDA, our study has extended our knowledge on LIF from its expression pattern to its mode of secretion within PDA, revealing that although both tumor and stromal cells (CAFs, mast cells and macrophages) were able to express LIF, only stromal cells could secrete it. This striking and somewhat unexpected result reinforces the potent role of the stromal compartment in PANR but also raises questions about the role of this non-secreted LIF within PDA cancer cells. In our study we were interested in the effect of the stromal cell-secreted LIF in human PDA and found that infiltrating nerve fibers expressed LIFR and gp130 indicating a possible triggering of LIF signaling within these nerve cells.

We extended our in vivo data with in vitro experiments performing heterotypic co-cultures of stromal cells. We observed that co-cultures with macrophages drastically enhanced LIF secretion by fibroblasts, which is consistent with recent findings concerning LIF expression
by activated fibroblasts (46). Here, we demonstrated that LIF is a strong modulator of nerve cell status, in terms of motility, proliferation and differentiation. Interestingly, this part of the study is highly similar with our previous study about the stromal-derived SLIT2 impact on PANR (12). Indeed we mentioned in this work the ability of CAF-derived SLIT2 to induce PANR in PDA. While the relevance of SLIT2 as an efficient biomarker was not reported, the connection between SLIT2 and LIF and their possible association to a further common signaling pathway should be investigate in order to determine if SLIT2/LIF-impact on PANR are both due to JAK/STAT activation. Additionally, we confirmed, with blocking antibodies or chemical inhibitors of gp130, LIFR or the JAK/STAT3 signaling pathway, that all modulations observed on cell behaviors were dependent on LIF signaling. These results are particularly relevant from a therapeutic point of view: targeting LIF signaling through the inhibition of either LIF binding to its receptors or LIF-triggered signaling could, in addition to the reported effect on targeting cancer cells, have an impact on tumor progression via the inhibition of nerve infiltration.

However, while we had demonstrated that LIF could modulate nerve cell status in vitro and backed this up by revealing a plausible cellular expression of LIF, LIFR and gp130 in human PDA, we needed a correlation between the presence of LIF in PDA patients and PANR. Therefore, we measured LIF titer in serum from human or mouse PDA and correlated it with the nerve density in the corresponding PDA sample. In both models, we found a positive correlation between the amount of LIF in the serum and the intra-tumoral nerve density, supporting a link between LIF and PANR as well as revealing LIF as a valuable biomarker to determine PANR level in PDA. In addition to confirming our hypothesis, we observed that LIF titration in serum from PDA patients could have other uses. Indeed, as already reported for IL-6 and IL-11, cytokine serum levels are valuable diagnostic and prognostic tools (47-49). The specificity given by LIF to distinguish PDA from other benign pancreatic diseases...
suggests that the combined detection of LIF and CA19.9 could be greater than CA19.9 alone in the diagnosis of PDA. Finally, LIF titration in serum of PDA patients has a real potent value as a stratifying biomarker of PDA in order to classify PDA patients regarding their possible responsiveness to JAK/STAT targeting agent as Ruxolitinib. Such ongoing investigation could improve PDA patient management.

Altogether, our results have potential therapeutic implications by providing a rationale for the use of LIF inhibitors in PDA, but also diagnostic implications by suggesting the usefulness of combining LIF and CA19.9 titration as a diagnostic and predictive marker. Indeed, our study is a proof-of-concept that the stroma impacts nervous system reorganization and thus PANR through the secretion of LIF. This secreted LIF (titrated in the serum), in addition to correlating with nerve density in PDA, exhibited a strong specificity with PDA tumors. While potentially useful in PDA detection, LIF titration should also be explored in a large panel of human cancers, especially those developing axonogenesis or perineural invasion such as prostate, colon and breast cancers. Further work will also be needed to determine the exact effect of LIF inhibitors in PDA as well as the potent value of LIF tittering as a stratifying biomarker for JAK/STAT targeting therapies.
Acknowledgments

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Table 1

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<td>1.55</td>
<td>1.57</td>
<td>5.27</td>
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<tr>
<td>Interleukine 11 receptor, alpha</td>
<td>IL11Ra</td>
<td>+</td>
<td>1.2</td>
<td>-1.09</td>
<td>1.02</td>
<td>-1.53</td>
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<tr>
<td>PRKR interacting protein 1</td>
<td>PRKRP1</td>
<td>+</td>
<td>1.1</td>
<td>-1.03</td>
<td>1.01</td>
<td>1.22</td>
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<tr>
<td>Oncostatin-M receptor</td>
<td>OSMR</td>
<td>+</td>
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<td>2.25</td>
<td>2.26</td>
<td>7.07</td>
</tr>
<tr>
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<td>IL6R</td>
<td>+</td>
<td>1.06</td>
<td>-1.04</td>
<td>1.17</td>
<td>-1.23</td>
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<tr>
<td>Ciliary neurotrophic factor receptor</td>
<td>CNTF</td>
<td>+</td>
<td>-1.03</td>
<td>1.15</td>
<td>1.00</td>
<td>1.04</td>
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<tr>
<td>Interleukin 27</td>
<td>IL27</td>
<td>-</td>
<td>-1.75</td>
<td>-1.01</td>
<td>-1.15</td>
<td>-1.33</td>
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Identification of gp130 family genes in human and murine pancreatic tumors. mRNA fold change of gp130 family genes in transcriptomic analysis of human (n=4) and mouse (n=9) pancreatic samples. a gene name; b gene symbol; c genes associated to nervous system; d Fold change of mRNA level in stromal versus tumor cell compartment from human PDA samples; e-g Fold change of mRNA level in spontaneous PDA versus healthy pancreas from mouse samples; e Four-week-old mice (early mPanINs); f Six-week-old mice (intermediate stage); g Nine-week-old mice (late PDA). Values highlighted in green and red are significantly upregulated and downregulated, respectively.
Figure legends

Figure 1. LIF, LIFR and gp130 expression levels in human and murine PDA. A, Representative images of LIF, LIFR or gp130 staining on human PDA (bars, 50μm). N, nerve sections; S, Stroma; black head arrow, tumoral cells. B, LIF immunoblot in human healthy pancreas (H, n=5) or PDA (P, n=2). Quantifications noted are expressed as fold increase compared with H1. C, Representative images following colour deconvolution of LIF staining in human healthy pancreas (n=6) or PDA (n=6) (bars, 50μm), with corresponding quantifications done on 10 images per tissues (mean ± SD). D, Representative images of co-localization of LIF, LIFR or gp130 with neurofilament on human PDA (bars, 50μm, n=8 tumors). The table indicates the percentage of expression of these markers in nerves, and the fraction of nerves containing them. SD (+/−); p-values compared LIF-R or gp130 expression in nerves compared to LIF. E, Fold change of LIF mRNA expression level in mouse healthy pancreas (n=6) or PDA (n=6); each dot is representative from one mouse. F, Representative images following colour deconvolution of LIF staining in mouse healthy pancreas (n=6) or PDA (n=6) (bars, 100μm), with corresponding quantifications done on 10 images per tissues (mean ± SD). G, LIF immunoblot in mouse healthy pancreas (H, n=5) or PDA (P, n=6). Quantifications noted are expressed as fold changes compared with H1. Each experiment was reproduced at least three times. *, P < 0.05; **, P < 0.01.

Figure 2. In PDA, LIF secretion is driven by stromal compartment and mainly by CAFs. A, Co-localization of LIF with Cytokeratin-19, CD117, CD68 or α–SMA on human PDA sections (bars, 50μm). The table indicates the percentage of LIF co-localization with these markers on human PDA (n=8). B, Human (top panel) and mouse (bottom panel) LIF mRNA expression levels (mean ± SD). C, Human LIF mRNA expression levels (mean ± SD). PDA#1 to 4 represents human PDA primary tumor cells. D, LIF immunoblots.
Quantifications noted are expressed as fold changes compared to macrophages (Raw) or fibroblasts (CAF). E, LIF immunoblots. Quantifications noted are expressed as fold changes compared to fibroblasts co-cultivated with macrophages (FHN+RAW). F, Quantification of secreted LIF, by ELISA assay, in various stromal cells conditioned media (mean ± SD). This experiment was reproduced 4 times, using 4 different CAFs. G, Quantification of secreted LIF, by ELISA assay, in conditioned media from stromal and tumor cells (mean ± SD). Each experiment was reproduced at least three times. *, P < 0.05; **, P < 0.01.

Figure 3. LIF-triggered signaling enhance migratory capacities of Schwann nerve cells.
A to C, Effects of stromal cells conditioned media on sNF96.2 migration ability (A) (mean ± SD) using Ab-LIFR/Ab-Ctrl (B) or AG490/SC144 (30μM/2μM respectively, pre-incubation for 2h) (C). D Effects of various doses (0-320ng/ml) of LIF recombinant protein on sNF96.2 migration (mean ± SD). E, Impact of Ab-LIFR on sNF96.2 migration (mean ± SD), using 50ng/ml of LIF and various doses of Ab-LIFR (upper panel) or 4μg/ml of Ab-LIFR compared to Ab-Ctrl (lower panel). F, Impact of AG490/SC144 on sNF96.2 migration (mean ± SD), using 50ng/ml of LIF. G, pSTAT3 and pAKT immunoblots in sNF96.2 cells following CM incubations and AG490/SC144 treatments. Quantifications noted are expressed as fold changes compared with sNF96.2 cells under sNF96.2 media. Each experiment was reproduced at least three times except for B, lower panel, which was reproduced 4 times, using 4 different CAFs. *, P < 0.05; **, P < 0.01; ***, P < 0.001.

Figure 4. LIF reduces Schwann cells proliferation. A to C, Cell count of sNF96.2 cells incubated with stromal conditioned media (A) together with Ab-LIFR (B) or LIF recombinant protein (C) (mean ± SD). D, Effect of LIF recombinant protein (50ng/ml) on p21 mRNA expression in sNF96.2 cells (mean ± SD). E, p21 immunoblots from sNF96.2 incubated for
36 (top panel) or 48 (down panel) hours with 50ng/ml of LIF. Quantifications noted are expressed as fold changes compared with sNF96.2 cells not incubated with LIF recombinant protein. **F and G**, p21 immunoblots from sNF96.2 incubated for 36 hours with 50ng/ml of LIF (**F**) or various conditioned media (**G**) together with AG490 (**F and G**) or SC144 (**F**) treatments. Each experiment was reproduced at least three times. *, *P* < 0.05.

**Figure 5. LIF induces Schwann cell differentiation and neural plasticity.** **A**, Pou3F2 and S100 dual-staining in sNF96.2 cells incubated with control (sNF96.2) or stromal conditioned media, in presence of Ab-Ctrl (left panel) or Ab-LIFR (right panel) (bars, 100μm). **B**, S100 and Pou3F2 immunoblots in sNF96.2 cultured as in (A). **C**, *S100* and *Pou3F2* mRNA expression levels in sNF96.2 cells incubated with 50ng/ml of LIF recombinant protein (mean ± SD). **D**, Representative images of Pou3F2 and S100 dual-staining on sNF96.2 cells incubated with 50ng/ml of LIF for 48 hours (bars, 100μm). **E**, S100 and Pou3F2 immunoblots from sNF96.2 cells incubated for 48 hours with LIF (50ng/ml). **F and G**, Effects of 50ng/ml of LIF (24 and 48 hrs) on neuronal plasticity (**F**, neurite number and **G**, soma area) of neurons from DRG (mean ± SD). Each experiment was reproduced at least three times. *, *P* < 0.05.

**Figure 6. LIF is a potent diagnostic and predictive biomarker for PDA.** **A**, Measurement of LIF level in serum from healthy (n=12), acute (n=10) or chronic (n=9) pancreatitis as well as PDA bearing (n=12) mice. **B**, Linear regression of intra-PDA nerve number versus LIF titer in PDA bearing mice sera (n=12). **C**, Measurement of serum LIF level and intra-tumoral nerve number in PDA-bearing mice treated with control-antibody (n=6, [LIF]<124pg/ml and n=6, [LIF]>124pg/ml) or LIF neutralizing antibody (n=9, [LIF]>124pg/ml). **D**, Measurement of LIF level in human serum from healthy donors (n=61), chronic pancreatitis (n=31), benign pancreatic tumor (n=11) and PDA (n=142) patients. **E**, Linear regression of intra-tumoral
nerve number versus LIF titer in serum from PDA patients (n=10). F, Graphical representation summarizing the impact of stromal secreted LIF on PANR and its potent use as a biomarker.
Figure 1

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A

Human PDA samples

S

S

N

N

B

Fold change

LIF

β-actin

H1 H2 H3 H4 H5 P1 P2

Healthy Pancreas

PDA

C

Fold change

Healthy

PDA

LIF

D

Nuclei

Neurofilament

Merge

E

Mouse LIF expression

Healthy (n=6)
PDA (n=6)

F

Fold change

Healthy

PDA

G

Fold change

Healthy Pancreas

PDA

Marker

Expression in nerve (%)

Number of nerves containing the marker (observed/total)

SD(+/-)

p-values

Tumor number (N)

LIF

1.9

9/12

0.2

-

8

LIF-R

9.7

8/12

1.7

< 0.0001

gp130

19.5

4/12

2.6

0.0028

8

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Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.
Figure 3

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Figure 4

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Figure 5

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Figure 6

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A. Human LIF in serum (pg/ml)

Healthy (n=61)
Chronic pancreatitis (n=31)
Benign pancreatic tumors (n=11)
PDAC (n=142)

B. LIF titer (pg/ml)

Intra-PDA nerve number

C. Intra-tumoral nerves (number)

Group n°1 (n=6)
Group n°2 (n=6)
Group n°3 (n=9)

[LIF] pg/ml <124 >124 >124
LIF-Ab - - +
Ctrl-Ab + + -

D. Human LIF in serum (pg/ml)

Healthy (n=12)
Chronic pancreatitis (n=9)
PDAC (n=12)

E. LIF titer (pg/ml)

Intra-PDA nerve number

R² = 0.75
(n=10)
p-value = 0.0013

F. Diagram showing the relationship between LIF and intratumoral nerves.

STROMA

Blood vessel

- High LIF detection in PDA serum

PANR biomarker

Diagnostic tool

Schwann cells and neurons

- Neuronal plasticity

Schwann cell plasmatic membrane

LIF

gp130

LIFR

JAK/STAT/AKT activation

- Migration

- Differentiation

- LIF secretion

Fibroblast

Macrophage

Caf

Mast cell

- LIF detection in PDAC serum

- Neuronal plasticity

- Migration

- Differentiation
LIF drives neural remodeling in pancreatic cancer and offers a new candidate biomarker

Christian Bressy, Sophie Lac, Jérémy Nigri, et al.

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