Plasma Interleukin-8 is a biomarker for TAK1 activation and predicts resistance to nanoliposomal irinotecan in patients with gemcitabine-refractory pancreatic cancer

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Running title: IL-8 and resistance to nal-IRI

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Translational Relevance

The poor prognosis of patients with pancreatic cancer could be mainly attributed to its intrinsic resistance to the currently approved classic chemotherapeutic treatments. In the last decade, we contributed to this field by demonstrating that TAK1 pathway has one of the most essential roles in pancreatic cancer treatment resistance. Nanoliposomal irinotecan (nal-IRI) is a novel standard treatment for metastatic gemcitabine-refractory pancreatic cancer. However, the exceeding cost-effectiveness ratio limited the recommendation for marketing authorization of this regimens and, thus, the identification of biomarkers for patients' selection remains a significant priority. Here, we demonstrated that the chemokine interleukin-8 is the circulating factor most significantly regulated by TAK1 activation. By demonstrating that interleukin-8 could be, indeed, a useful biomarker for identifying patients that could take the larger advantage by nal-IRI, our findings cover one of the most significant unmet need in the selection of second- and further lines of treatment for pancreatic cancer.

Abstract

Purpose: Pancreatic cancer is one of the most lethal solid tumors, mainly because of its intrinsic chemoresistance. We identified TAK1 as a central hub sustaining this resistance. Nanoliposomal irinotecan (nal-IRI) is a novel treatment for metastatic gemcitabine-refractory pancreatic cancer. We endeavored to identify circulating markers for TAK1 activation predicting chemoresistance in this setting.

Experimental design: In vivo activity of nal-IRI was validated in an orthotopic nude murine model expressing TAK1-specific shRNA. Plasma concentration of 20 different cytokines were measured by a multiplex xMAP/Luminex technology in patients prospectively enrolled to receive nal-IRI plus 5-fluorouracil/leucovorin (5-FU/LV). The optimal cutoff thresholds able to significantly predict patients' outcome were obtained based on the maximization of the Youden's statistics.

Results Differential expression profiling revealed the gene coding for interleukin-8 as the most significantly downregulated in shTAK1 pancreatic cancer cell lines. Mice bearing shTAK1-tumors had significantly lower plasma levels of interleukin-8 and experienced a significant reduction in tumor growth if treated with nal-IRI, while those bearing TAK1-proficient tumors were resistant to this agent. In a discovery cohort of 77 patients, interleukin-8 was the circulating factor most significantly correlated with survival (plasma levels lower vs. higher than cutoff: mPFS 3.4 vs. 2.8 months, HR=2.55, 95%CI=1.39-4.67, *P*=0.0017; mOS 8.9 vs. 5.3 months, HR=3.51, 95%CI=0.84-6.68, *P*=4.9e-05). These results were confirmed in a validation cohort of 50 patients.

Conclusion Our study identified interleukin-8 as the most significant circulating factor for TAK1 pathway activation and candidates interleukin-8 as a potential predictive biomarker of resistance to nal-IRI in gemcitabine-refractory pancreatic cancer patients.

Introduction

Pancreatic cancer remains one of the most lethal and poorly understood human malignancies and will continue to be a major unsolved health problem in the 21st century (1). Pancreatic cancer has still the lowest 5-year relative survival rate among solid tumors at 7%, and is projected to become the second leading cause of cancer-related death by 2030 in Western countries (2,3). The poor prognosis for patients with pancreatic cancer could be mainly attributed to the limited efficacy of currently approved classic chemotherapeutic treatments (4).

We recently identified the serine/threonine kinase TGF-β (transforming growth factor-β)-activated kinase 1 (TAK1) as a central hub integrating the most relevant signals from various cytokines (5,6) and sustaining, in turn, resistance to chemotherapeutic treatments through the activation of different transcription factors, including AP-1, NF-κB, (7,8) and YAP/TAZ (9). In particular, we demonstrated that targeting the kinase activity of TAK1 dramatically led to a proapoptotic phenotype and, in turn, to a significantly higher sensitivity to chemo and radiotherapy in pancreatic (10) and esophageal carcinoma (11).

Nanoliposomal irinotecan (nal-IRI; Onivyde®; MM-398) is a liposomal formulation of the topoisomerase I inhibitor irinotecan. Efficacy of nal-IRI was recently demonstrated in the NAPOLI-1 study, a phase III, randomised, open-label, multicentre study that tested nal-IRI monotherapy or nal-IRI plus 5- fluorouracil (5-FU) and leucovorin (LV) vs. 5-FU/LV alone in patients with metastatic pancreatic cancer previously treated with gemcitabine-based therapy (12). nal-IRI plus 5-FU/LV led to a significant improvement in median overall survival (mOS) over 5-FU/LV alone, (13,14) becoming a novel standard of care for second-line treatment of metastatic pancreatic cancer patients (15).

We aimed the present study at identifying circulating markers of TAK1 pathway activation, that could potentially serve as resistance biomarkers in this clinical setting.

Materials and Methods

Cell cultures and reagents

AsPC1, Panc1 and MDA-Panc28 human pancreatic cancer cell lines were purchased from the American Type Culture Collection (Manassas, VA). MDA-Panc28 cell line was a kind gift by Dr. Paul J. Chiao. Panc1, AsPC1 and MDA-Panc28 pancreatic cancer cell lines silenced for the expression of TAK1 were established as described in (10). All cell lines were cultured as monolayers at 37°C, 5% CO_2 in high glucose Dulbecco's modified Eagle's medium (DMEM, cat. 41966-029, Life Technologies, Gaithersburg, MD), supplemented with 10% heat-inactivated fetal bovine serum (FBS, cat. 10270-106, Life Technologies), 2mM L-Glutamine (cat. BE17-605E, Lonza), 100 IU/mL penicillin and 100 μg/mL streptomycin (cat. 15140-122, Life Technologies), and cultured for maximum one month. Cell lines were authenticated by DNA fingerprinting at the genomic core facility at Wayne State University (2009) and routinely tested for mycoplasma presence using the MycoAlert Mycoplasma Detection Kit (cat. LT07-118, Lonza, Switzerland).

Gene Expression and Pathway Analyses

Differences in gene expression between control and TAK1-silenced cells (GEO accession number GSE137265) were examined by using Illumina Human 48k gene chips (D-103-0204, Illumina, Milan, Italy) as previously described in (9).

Protein extraction and western blotting

Western blot analyses were performed as previously described (16). Briefly, total protein extracts were prepared by lysing cells in radioimmunoprecipitation assay buffer (50 mM Tris HCl [pH 8], 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulfate). All protein extracts were quantified by BCA Protein Assay

Kit (23225, Thermo Fisher Scientific) and equal amounts (20-50 µg of protein extract) were loaded onto SDS-PAGE (4-20%) and transferred to polyvinylidene fluoride membranes (Immobilon-P cat. IPVH00010, Millipore). Immunoblots were performed using the indicated antibodies. Anti-TAK1 antibody (ab109526, 1:1000) was purchased from Abcam (Cambridge, UK). Secondary anti-mouse and anti-rabbit antibodies were purchased from Jackson Immunoresearch (Cambridge, UK). All antibodies were diluted in 5% non-fat dry milk dissolved in Tris Buffered Saline (TBS)/0.1% Tween-20. Immunoreactive proteins were visualized with Immobilion Western kit (cat. WBKLS0500, EMD MIllipore) according to the manufacturer's instructions. Images were acquired using ImageQuant LAS 4000 mini (GE Healthcare Life Sciences, Little Chalfont, UK).

Nude Mouse Orthotopic Xenograft Models

5-weeks old female athymic nude mice (Crl:CD1-Foxn1nu, CDNSSFE05S) were purchased from Charles River (Wilmington, MA, USA). University of Verona Animal Ethic Committee approved this study. Subconfluent cultures of pancreatic cancer cells were collected using 0.05% trypsin-EDTA (GIBCO, ref 25300-054), that was inhibited with 10% fetal bovine serum in DMEM. Tumor cells were resuspended in a solution of 1:1 Matrigel:PBS at 1.0 x 10^4 cells/µl concentration (Matrigel Matrix Growth Factor, 356230, BD, Franklin Lakes, NJ). Orthotopic injection of pancreatic cancer cells was performed as previously described (17).

All mice were weighed weekly and observed for tumors becoming palpable. When a tumor mass reached the diameter of 10 mm became palpable and measurable with a standard caliper. Tumor diameter was assessed with a Vernier caliper, and tumor volume (mm3) was calculated as d2xD/2, wherein d and D represent the shortest and longest diameters, respectively. Bulky disease was considered present when tumor burden was prominent in the mouse abdomen (tumor volume, >2,000 mm^3). When at least 3 of 5 mice

in a treatment group presented with bulky disease, the median survival duration for that group was considered to be reached. At the median survival duration of the control groups, all mice of control and treatment groups were euthanized by deep terminal anesthesia and blood and tumor samples were collected. Differences in mean weight were considered for the effects of treatments between different groups.

Immunohistochemistry

Immunostaining were performed in formalin-fixed paraffin-embedded tissue sections using the polymeric HRP staining system (UltraTek HRP Anti-Polyvalent DAB Staining System, AMF080, Histoline, Pantigliate (MI), Italy) as described (18). Citrate pH 6.0 was used as antigen retrieval buffer. Primary monoclonal antibodies to IL-8 (ab106350, 1:500) or to CD68 (ab31630, Abcam), or polyclonal antibody to CES2 (orb214832, Biorbyt) were incubated in a humified chamber at 4°C overnight. Signal detection was performed incubating sections with UltraTek Anti-Polyvalent and UltraTek HRP for 10 minutes at room temperature (UltraTek HRP Anti-Polyvalent DAB Staining System, AMF080, Histoline, Pantigliate (MI) - Italy). Section were counterstained with Mayer's Hematoxylin and slides were mounted using Entellan® Neo Rapid non-aqueous mounting medium (Merck Millipore, Burlington, Massachusetts, USA).

Patients and Samples Analysis

Patients enrolled in the present study had advanced histologically or cytologically confirmed pancreatic adenocarcinoma and were previously treated with gemcitabine-based therapy, received in localized or metastatic setting. Other eligibility criteria included adequate hepatic, renal and hematological function, an ECOG performance status ≤ 2, and no other clinically significant disorders. Eligible patients were prospectively included in an Expanded Access Program to receive nal-IRI 80 mg/m^2 intravenously over 90 minutes,

followed by LV 400 mg/m^2 intravenously over 30 minutes and 5-FU 2400 mg/m^2 intravenously over 46 hours, every 2 weeks. We analyzed patients of two different cohorts on the basis of site of enrollment. Patients from the University Hospital Trust in Verona comprised the discovery cohort (n=77), and those from Veneto Institute of Oncology (IOV-IRCCS) in Padua comprised the validation cohort (n=50). At baseline, before first dose of nal-IRI, peripheral blood samples were collected using EDTA-containing tubes. All data were anonymized assigning a progressive number to every patient and the corresponding blood sample. Plasma aliquots were isolated from the blood samples by centrifugation with 1600 rcf for 10 minutes and subsequently with 3000 g for 10 minutes. Plasma samples were stored at -80°C. Written informed consent was obtained from all subjects. The study was conducted in accordance with the Declaration of Helsinki and approved by local ethics committee. Tumor response was assessed according to Response Evaluation Criteria in Solid Tumors version 1.1 (RECIST 1.1). Disease was assessed by CT (or MRI) every 12 weeks until disease progression.

Multiplex cytokines profiling

Plasma concentration of interleukin (IL)-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-12p70, IL-13, IL-18, eotaxin, granulocyte-macrophage colony-stimulating factor (GM-CSF), IFNγ, IP-10 (CXCL10), monocyte chemoattractant protein (MCP1; CCL2), macrophage inflammatory protein 1α (MIP-1a; CCL3), MIP1-β (CCL4), TNFα, Rantes, SDF1α, GROα were measured by using a 20-plex Luminex kit (Life Technologies cat. EPX200-12173-901). All Luminex assays were performed according to the instructions provided by the manufacturer (Bio-Rad Laboratories). Median fluorescence intensities were collected on a Luminex-200 instrument, using Bio-Plex Manager software version 6.2. Standard curves for each cytokine were generated using the premixed lyophilized standards provided in the

kits. Cytokine concentrations in samples were determined from the standard curve using a 5-point regression to transform mean fluorescence intensities into concentrations.

Statistical analysis

Provided the exploratory design of our study, the sample size was not predetermined. Results are shown as means and standard deviations. Survival curves were estimated using the Kaplan-Meier method and compared by log rank test. Univariate and multivariate analyses of median progression-free survival (mPFS) and mOS, with stepwise variable selection, were conducted by Cox's proportional hazard regression models. Multivariate analysis was conducted using the clinical-pathologic variables with a *P*-value < 0.05 and the strongest significant molecular variables in univariate analysis (*P*-value < 0.01). Fisher's exact test was used for pairwise comparisons of objective response.

The optimal cutoff thresholds for soluble biomarkers were obtained based on the maximization of the Youden's statistics (J=sensitivity+specificity+1) (19) using an R-based software as described in (20). Statistical analyses were performed using SPSS 24.0 statistical software (SPSS, Inc.), GraphPad Prism software program (version 6.0; GraphPad Software), and the statistical language R.

Results

Identification of IL-8 as the most significant TAK1-regulated secreted protein in nal-IRI- resistant pancreatic cancer in vivo models

In order to identify novel circulating markers of TAK1-pathway activation, which could serve as biomarkers for chemoresistance, we compared gene expression profiles in AsPC1, Panc1 and MDA-Panc28 pancreatic cancer cell lines transduced with lentivirus expressing TAK1-specific shRNA (shTAK1) or scramble sequence (shctrl) as control by microarray analysis (Figure 1A). We analyzed data of the genes with significantly different levels of expression by using the Ingenuity Pathway Analysis software program, which identifies groups of genes associated with specific molecular pathways. We found a significant association of the genes in the TAK1-proficient pancreatic cancer cells lines with the IL-8 signaling pathway (Figure 1B), and we identified CXCL8, the gene coding for IL-8, as the most significant downregulated gene coding for secreted protein in all shTAK1 pancreatic cancer cell lines as compared to their respective scramble controls (Figure 1C). We confirmed a significant downregulation of CXCL8 mRNA in all shTAK1 pancreatic cancer cell lines (Figure 1D) and a significant reduction in the IL-8 expression in conditional medium from shTAK1 cell lines (Figure 1E).

In order to measure whether the expression of tumor TAK1 could correlate in vivo with circulating levels of IL-8, and to demonstrate that TAK1 kinase is relevant to sustain resistance to nal-IRI, we used an orthotopic xenograft nude mouse model. AsPC1^{shctrl} and AsPC1^{shTAK1} orthotopic tumor-bearing mice were randomly assigned (n=5) to receive nal-IRI 10 mg/Kg intraperitoneally (i.p.) once a week or its i.p. vehicle as control. At median survival duration of mice in the respective control groups, all of the mice were sacrificed and tumors and blood samples collected for analyses. We measured a significant downregulation for IL-8 expression in tumor specimens excised from mice bearing shTAK1

AsPc1 tumors if compared with that in control tumors (Figure 2A). Consistently, we demonstrated significantly lower plasma levels of IL-8 in mice bearing TAK1 knockdown AsPc1 tumors than in those bearing TAK1-proficient tumors (Figure 2B).

Most importantly, mice bearing TAK1-proficient AsPc1 tumors treated with nal-IRI had tumor growth rates comparable with those in vehicle-treated control mice [tumor weight mean±SEM (gr)=0.94 ± 0.15 vs 1.615 ± 0.305, p value= 0.0973, unpaired t test] (Figure 2C). Conversely, mice bearing TAK1 knockdown AsPc1 tumors treated with nal-IRI experienced a statistically significant reduction in tumor growth if compared with their respective vehicle-treated controls [mean±SEM (gr)=0.5288±0.1974 vs. 3.036 ± 0.7067, p value= 0.0091, unpaired t test] (Figure 2D). We ruled out the possibility that different levels of IL-8 could modulate the infiltration of tumor associated macrophages (TAMs), and the local conversion of nal-IRI in SN-38 by measuring similarly minimal levels (less than 1 cell/field) of CD68+ cells and carboxylesterase-2 in tumor specimens excised from mice bearing shTAK1 AsPc1 tumors if compared with that in control tumors (supplementary Figure S1).

Altogether, these data demonstrate that IL-8 is the most relevant secreted marker in vitro and in vivo for the activation of the TAK1 pathway that is responsible for a tumor cells autonomous resistance of pancreatic cancer to nal-IRI.

IL-8 is the most significant predictive marker of survival in metastatic pancreatic cancer patients treated with nal-IRI plus 5-FU/LV

In order to demonstrate the clinical relevance of IL-8 in predicting resistance to nal-IRI, we initially collected plasma at baseline in a discovery cohort of 77 patients with gemcitabine-refractory metastatic pancreatic cancer that were prospectively enrolled between November 2016 and June 2018 within an Expanded Access Program to receive nal-IRI plus 5-FU/LV as second or further line therapy. Compliance with REMARK

guidelines is reported in supplementary Table S1. Patients' characteristics are shown in supplementary Table S2. In this discovery cohort, median age was 64 years and 52% were male. Most of them had a normal ECOG performance status (58.4%). Half of the patients had tumors in the head of pancreas (52%). The majority of patients had increased levels of Ca19.9 (83.1%). More than one third of patients had 2 or more site of metastasis, and liver was the most common secondary site (70.1%). 76.7% of patients received nal-IRI + 5-FU/LV as second-line, 22.0% as third-line, and one patient (1.2%) as fourth-line treatment for metastatic disease (supplementary Table S2). After a median follow-up of 26 months, the mPFS was 3.3 months (95%CI=3.040-3.560) and the mOS was 7.4 months (95%CI=8.309-12.251) for the overall population (supplementary Figure S2A-B).

Thus, we measured the plasma concentration of a panel of 20 different cytokines, chemokines and growth factors. The optimal cutoff thresholds able to significantly predict patients' outcomes were evaluated for each cytokine (Table 1). This analysis confirmed IL-8 as the circulating factor most significantly able to predict mPFS and mOS in this discovery cohort of patients. The mPFS was 3.4 months compared to 2.8 months (HR=2.55, 95%CI=1.39-4.67, *P*=0.0017) (Figure 3A), and the mOS was 8.9 months compared to 5.3 months (HR=3.51, 95%CI=1.84-6.68, *P*=4.9e-05) (Figure 3B) for patients with plasma levels of IL-8 at baseline lower vs higher than cutoff of 16.68 pg/mL, respectively (supplementary Figure S3A-F). Furthermore, in those fifteen patients with plasma levels of IL-8 at baseline higher than cutoff we measured only 1 (6.7%) stable disease (SD), and no partial (PR) or complete responses. Contrariwise, among those 62 patients with plasma levels of IL-8 at baseline lower than cutoff we measured 14 (23%) SD, 5 (8.1%) PR and 1 (1.7%) CR, counting for a disease control rate of 32.7% (supplementary Table S3).

Different circulating factors and cytokines with levels higher than cutoff were also significantly associated with either a shorter patients' mPFS and mOS, including the

chemokines MCP-1, IP-10, MIP-1β, and SDF-1α, and the T_H1 cytokines IL-18 and INFγ. The same associations were not proven for the other cytokines (Table 1 and supplementary Figures S4-S11).

Univariate analysis of correlation between IL-8 plasma levels, clinical features and survival outcomes is shown in Table 2. Among the clinical parameters analyzed, patients who previously underwent surgery had a significantly longer mOS (HR=0.387, *P*=0.012) and mPFS (HR=0.437, *P*=0.017) compared to unresectable patients. Furthermore, patients with a normal ECOG PS had a longer mPFS compared to patients with ECOG PS 1-2 (HR=1.884, *P*=0.010). In order to confirm the independence of the predictive value for IL-8 plasma levels, we performed a multivariate analysis including IL-8 and those clinical features significant at univariate analysis (*P*<0.05). This analysis revealed IL-8 as an independent predictor of PFS and OS in the discovery cohort of patients (Table 2).

In order to validate the negative predictive value of IL-8 in patients treated with nal-IRI plus 5-FU/LV, we analyzed the association of IL-8 levels and clinical outcomes in an external validation cohort of patients treated in a different center. Patients' characteristics are shown in supplementary Table S4. The mPFS was 3.3 months (95%CI=4.504-7.804) and the mOS was 7.4 months (95%CI=8.309-12.251) for the overall population (supplementary Figure S12A-B). In this validation cohort, we confirmed IL-8 as significantly associated with mPFS and mOS. The mPFS was 4.6 months compared to 1.8 months (HR=2.84, 95%CI=1.41-5.72, *P*=0.0023) (Figure 4A), and the mOS was 9.7 months compared to 5.4 months (HR=2.32, 95%CI=1.18-4.56, *P*=0.012) (Figure 4B) for patients with serum levels of IL-8 at baseline lower vs higher than cutoff, respectively (supplementary Figure S13).

Discussion

In the present study, we sought to identify circulating biomarkers useful to estimate the activation of TAK1 pathway, one of the most relevant molecular mechanisms responsible for the resistance of pancreatic cancer to the proapoptotic activity of chemotherapeutic treatments (5). We demonstrated that the chemokine IL-8 is the secreted factor most significantly regulated by TAK1 activation. Most importantly, we demonstrated for the first time that IL-8 could be, indeed, a useful biomarker to predict resistance to the novel nanotechnologic agent nal-IRI in two large discovery and external validation cohorts of patient affected by gemcitabine-refractory advanced pancreatic cancer.

The most appropriate systemic regimen for advanced pancreatic cancer patients progressing under a first-line gemcitabine-containing regimen largely remains a matter of debate. Two randomized phase III trials that explored the role of oxaliplatin-based chemotherapy in patients progressing to a first-line single-agent gemcitabine therapy reached conflicting results. No randomized phase III trials explored standard irinotecan-containing regimens in this setting. (21,22) To date, NAPOLI-1 remains the largest phase III study in second-line metastatic pancreatic cancer (12). This trial led to the regulatory approval of nal-IRI in the U.S. in 2015 for the treatment of metastatic pancreatic cancer in combination with 5-FU/LV after progression on gemcitabine-based therapy. Nonetheless, the lack of randomized trials comparing nal-IRI plus 5-FU/LV to standard irinotecan-containing regimens such as FOLFIRI, and the exceeding cost-effectiveness ratio per quality-adjusted life year gained for nal-IRI plus 5-FU/LV limited the recommendation for marketing authorization of this regimen in several European countries and in United Kingdom (23). In this regard, the identification of biomarkers for the selection of those patients that could gain the largest advantage by this regimen remains a significant priority. Here, we demonstrated that IL-8 is the most significant independent factor for PFS

and OS in two different cohorts of patients among a series of cytokines in advanced pancreatic cancer patients receiving nal-IRI plus 5-FU/LV treatment, representing a potential biomarker for patients' selection and, in turn, for potentially improving clinical-effectiveness evidence and cost-effectiveness results for this regimen.

IL-8 is a chemokine mainly produced by tumor cells, and its main functions include angiogenesis, survival signaling for cancer stem cells and attraction of myeloid-derived suppressor cells (24). We recently discovered IL-8 as part of a proinflammatory cytokine signature inducing resistance to antiangiogenic drugs in pancreatic and colorectal cancer patients through the recruitment of myeloid $CD11b^{+}$ cells (25-27). Previous studies demonstrated a central role for TAK1 in sustaining IL-1-induced transcription and mRNA stabilization, as well as expression of endogenous IL-8 (28). TAK1 is also part of an intracellular signaling axis responsible for Death receptor 3-mediated expression of IL-8 (29). More recently, an autocrine loop between IL-8 and TAK1 has been demonstrated given that IL-8 can activate TAK1/NF-kB signaling via the CXCR2 receptor in models of ovarian cancer (30). In our present study, we demonstrated that IL-8 is the secreted factor most significantly regulated by TAK1 activation, one of the most potent mediators of chemoresistance in pancreatic cancer through the activation of AP-1 and NF-κB transcription factors. AP-1 and NF-κB are, indeed, recognized as potent transcription factors cooperating for the induction of IL-8 in human pancreatic cancer cells by hypoxia (31).

The stable nanoliposome formulation of nal-IRI has been developed to improve the therapeutic index of irinotecan throughout an active accumulation and conversion in TAMs, allowing for a more efficient delivery of its active metabolite SN-38 within the tumor microenvironment. However, the effect of tumor derived IL-8 on TAMs in murine models has been only limitedly explored (32) as an homologue of human CXCL8 gene is completely absent from the genome of rodents (33). Previous studies in mice carrying a

bacterial artificial chromosome encompassing the entire human CXCL8 gene showed an increased mobilization of immature CD11b+Gr-1+ myeloid cells (34). In our study, we ruled out a putative mechanistic association between the infiltration and the carboxylesterase activity of TAMs and IL-8 levels as cause for the lack of activity of nal-IRI in pancreatic cancer models. The activation of transcription factors, including AP-1, NF-κB and YAP/TAZ (7-9), remains the most relevant mechanism for the treatment resistance sustained by TAK1, and IL-8 is the most reliable circulating factor to measure the activation of this pathway.

This study, however, had one limitation in lacking a control cohort including untreated pancreatic cancer patients in order to distinguish between a merely prognostic or a predictive value for TAK1-regulated IL-8. Although its relevant mechanistic role in cancer pathogenesis, a prognostic value for IL-8 in pancreatic cancer has been, however, excluded by several previous studies. In one of the initial series including healthy volunteers and pancreatic cancer patients with resectable, locally advanced or metastatic disease, IL-8 levels were higher in cancer patients than in healthy controls but were not associated with survival differences (35). More recently, we conducted the most comprehensive profiling of cytokines in the largest prospective cohort of resectable pancreatic cancer patients to date and IL-8 had no prognostic value in this cohort of patients (36). Excluded a prognostic role for IL-8, we acknowledge that its negative predictive value could be although extended also to other cytotoxic agents different from nal-IRI that induce in pancreatic cancer cells a proapoptotic stimuli by which a TAK1 activation mirrored by IL-8 could protect.

In conclusion, we identified for the first time IL-8 as the most significant circulating biomarker for predicting chemoresistance sustained by TAK1 pathway in pancreatic cancer. The expression of this chemokine could be useful to select those pancreatic

cancer patients that could benefit from the novel nanotechnologic agent nal-IRI in the larger extent.

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Figure Legends

Figure 1. Identification of interleukin (IL)-8 as the most significant TAK1-regulated secreted protein. A. Western blot analysis for the expression of TAK1 in AsPc-1, PANC-1, and MDAPanc-28 pancreatic cancer cells transduced with lentiviruses expressing TAK1- specific small hairpin RNA (shTAK1) or a scramble sequence as control (shctrl). β-actin was used as loading control. **B.** Selection of relevant biological processes and secreted protein genes by using global transcript profiling. IL-8 signaling was enriched among genes differentially expressed in pancreatic cancer cells transduced with lentiviruses expressing shTAK1 or a shctrl by Ingenuity Pathway Analyses (IPA) software. The X-axis represents the log (10) P value for enrichment, with the threshold drawn at p=0.05. **C.** Levels of the most significantly downregulated (blue) and upregulated (red) genes coding for secreted proteins in pancreatic cancer cells transduced with lentiviruses expressing shTAK1 (P28T, AST, P1T) versus those expressing a shctrl (P28, AS, P1). **D.** Histogram shows normalized mRNA levels of IL-8/β-actin in the indicated pancreatic cancer cell lines. Means and standard deviations are shown, ***p< 0.001, as determined by unpaired Student's t test. **E.** Histogram shows IL-8 protein levels in the conditioned media of the indicated pancreatic cancer cell lines. Means and standard deviations are shown, ***p< 0.001, as determined by unpaired Student's t test.

Figure 2. TAK1 sustains high circulating levels of interleukin (IL)-8 and resistance to nal-IRI- in pancreatic cancer in vivo models. A. Immunohistochemical analysis. Serial paraffin sections from tumors excised from mice orthotopically inoculated with pancreatic cancer cells transduced with lentiviruses expressing shTAK1 or a shctrl were stained with antibodies to TAK1 and IL-8. **B.** Histogram shows IL-8 plasmatic concentration in mice inoculated with pancreatic cancer cells transduced with lentiviruses

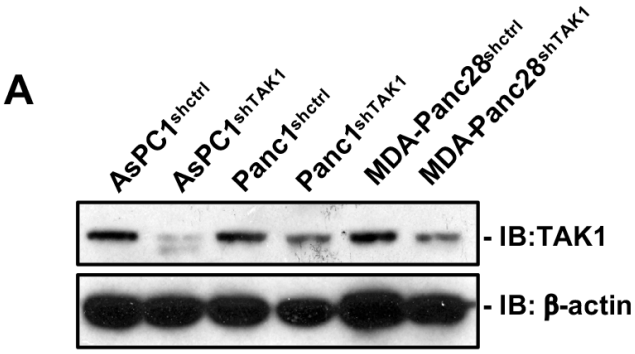
expressing shTAK1 or a shctrl. Means and standard deviations (n=3) are shown, *p< 0.05, as determined by unpaired Student's t test. **C.** Antitumor activity of nal-IRI in vivo in AsPc-1 expressing shctrl pancreatic tumor orthotopic xenografts (n = 10, 5 mice per group). When at least 3 of 5 mice in a treatment group presented with bulky disease (tumor volume, >2,000 mm3), the median survival duration for that group was considered to be reached. At the median survival duration of the control group, all mice of control and treatment groups were euthanized by deep terminal anesthesia and blood and tumor samples were collected. Differences in weight were considered for the effects of treatments between different groups. Box-and-whiskers plot shows median and 25th to 75th percentiles of tumors weight in each group. P value not significant (>0.05), as determined by unpaired Student's t test. **D.** Antitumor activity of nal-IRI in vivo in AsPc-1 expressing shTAK1 pancreatic tumor orthotopic xenografts (n = 10, 5 mice per group). Analysis conducted as described above. Box-and-whiskers plot shows median and 25th to 75th percentiles of tumors weight in each group. P value=0.0091 as determined by unpaired Student's t test.

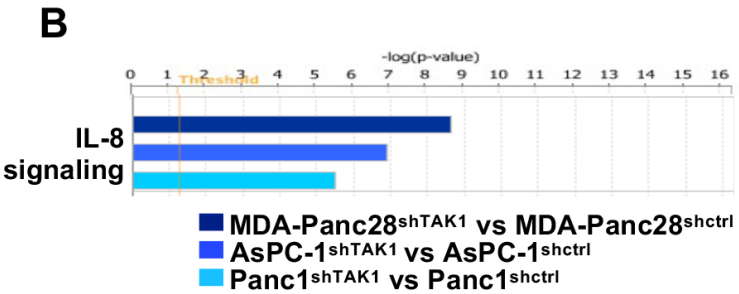
Figure 3. Kaplan–Meier analysis of progression-free survival (PFS) (A) and overall survival (OS) (B), according to cut-off value of interleukin (IL)-8 in the discovery cohort. **A.** In the discovery cohort, the median PFS was 3.4 months in low IL-8 group, as compared with 2.8 months in high IL-8 group. **B.** The median OS was 8.9 months in low IL-8 group, as compared with 5.3 months in high IL-8 group.

Figure 4. Kaplan–Meier analysis of progression-free survival (PFS) (A) and overall survival (OS) (B), according to cut-off value of interleukin (IL)-8 in the validation cohort. **A.** In the validation cohort, the median PFS was 4.6 months in low IL-8 group, as compared with 1.8 months in high IL-8 group. **B.** The median OS was 9.7 months in low IL-8 group,

as compared with 5.4 months in high IL-8 group. IL-8 cut off levels (pg/mL), Hazard Ratio (HR) and p value are indicated.

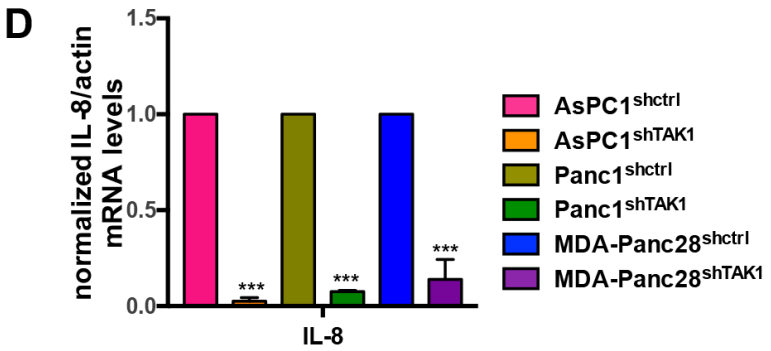
Merz et al. Figure 1

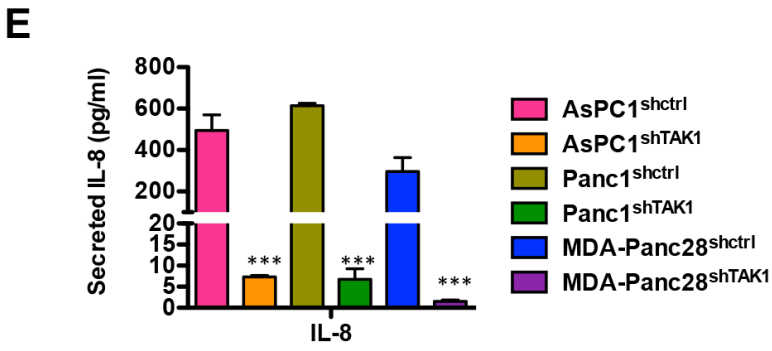




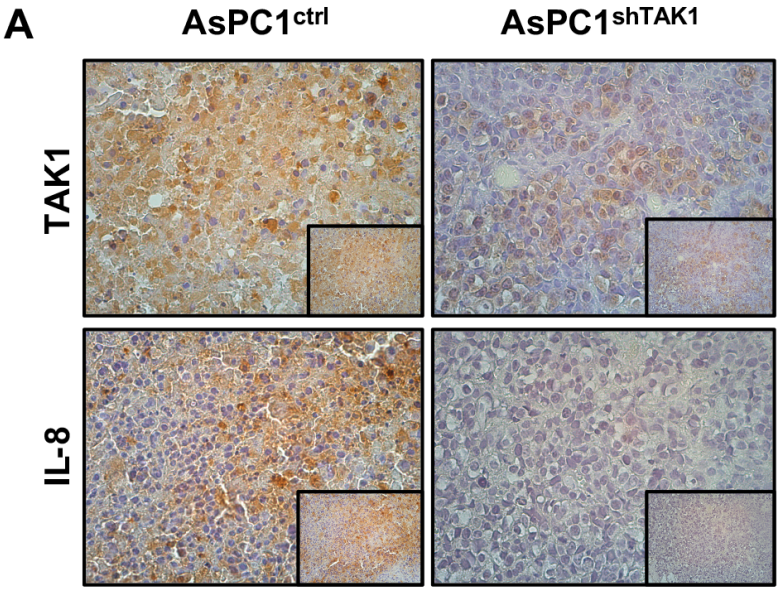
C

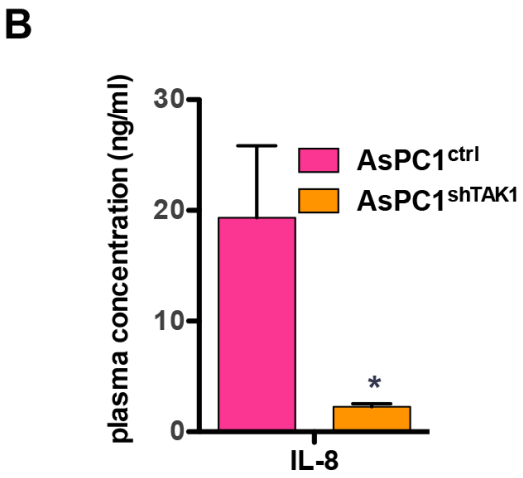
Symbol	Entrez Gene Name	Expr Fold Change (P28T/P28)	Expr Fold Change (AST/AS)	Expr Fold Change (P1T/P1)	Entrez Gene ID for Human
CXCL8	C-X-C motif chemokine ligand 8	-61,955	-26,348	-12,151	3576
GSDMD	gasdermin D	-54	-40,599	-41,071	79792
CTGF	connective tissue growth factor	-53,729	-10,542	-5,986	1490
TGFBI	transforming growth factor beta induced	-46,391	-79,634	-48,072	7045
PLAU	plasminogen activator, urokinase	-40,328	-5,236	-10,024	5328
CSF2	colony stimulating factor 2	-37,892	-1,906	-1,533	1437
IGFBP6	insulin like growth factor binding protein 6	-34,889	-30,682	-48,666	3489
LGALS1	galectin 1	-33,838	-9,527	-30,821	3956
IGFBP4	insulin like growth factor binding protein 4	-30,959	-31,538	-20,908	3487
CYR61	cysteine rich angiogenic inducer 61	-25,925	-4,795	-1,745	3491
RPRD1A	regulation of nuclear pre-mRNA domain containing 1A	5,699	3,707	2,11	55197
SLIT2	slit guidance ligand 2	5,921	7,503	6,061	9353
LARP1B	La ribonucleoprotein domain family member 1B	6,68	2,15	3,015	55132
OVGP1	oviductal glycoprotein 1	6,848	3,213	3,94	5016
CD70	CD70 molecule	7,994	5,485	8,609	970
BMP7	bone morphogenetic protein 7	9,518	5,907	6,739	655
IGFBP5	insulin like growth factor binding protein 5	9,974	10,313	12,033	3488
PXDN	peroxidasin	11,063	6,838	7,245	7837
JAG2	jagged 2	12,273	7,632	10,697	3714
FBLN1	fibulin 1	21,677	11,505	22,975	2192

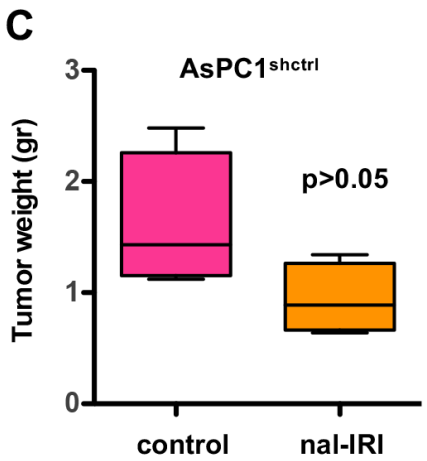


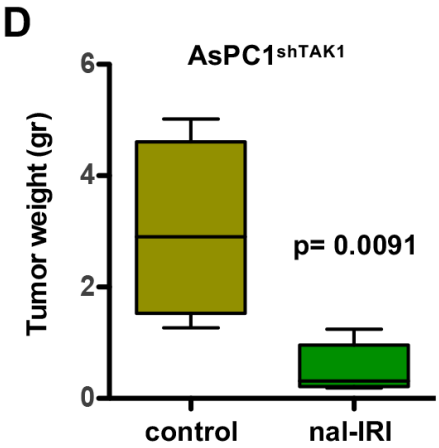


Merz et al. Figure 2







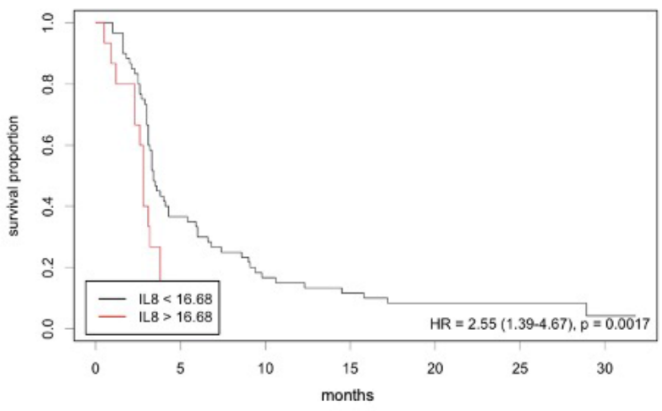


				PFS				OS			
	Median concentration (pg/mL)	**Range**		**Cutoff (pg/mL)**	**HR**	95%**CI**	***P***	**Cutoff (pg/mL)**	**HR**	95%**CI**	***P***
Chemokines											
IL-8	5.96	0	54.67	16.68	2.55	1.39-4.66	0.0017	16.68	3.51	1.84-6.68	4.9e-05
MCP-1	55.27	20.41	237.7	60.02	2.21	1.35-3.62	0.0013	58.07	2.38	1.46-3.89	0.00038
IP-10	19.56	9.73	46.85	25.49	2.11	1.24-3.6	0.005	25.35	2.52	1.47-4.34	0.00055
MIP-1β	36.24	20.29	121.8	56.21	2.51	1.3-4.85	0.0046	56.21	2.39	1.23-4-65	0.0079
SDF-1α	258	94.29	647.6	334	2.38	1.24-4.55	0.0069	334	2.06	1.09-3.88	0.023
RANTES	139.31	36.67	255.1	-	-	-	-	-	-	-	-
Eotaxin	23.07	12.25	56.84	-	-	-	-	-	-	-	-
GROAα	0	0	102.91	-	-	-	-	-	-	-	-
MIP-1α	1.79	0	54.33	-	-	-	-	-	-	-	-
T_H1											
IL-2	12.93	0	31.27	-	-	-	-	6.775	0.5	0.27-0.93	0.026
IL-18	31.55	0	136.71	54	2.14	1.21-3.8	0.0076	25.21	2	1.2-3.33	0.0065
IL-12p70	4.28	0	72.33	-	-	-	-	1.755	0.53	0.32-0.86	0.0098
IFNγ	24.53	0	137.69	44.27	2.62	1.4-4.88	0.0017	20.32	1.88	1.11-3.2	0.018
TNFα	11.75	2.46	47.04	-	-	-	-	-	-	-	-
T_H2											
IL4	0	0	218.39	6.195	2.43	1.2-4.88	0.01	-	-	-	-
IL5	7.23	0	44.52	-	-	-	-	-	-	-	-
IL6	0.5	0	193.81	-	-	-	-	6.175	2	1.21-3.3	0.0061
IL13	0	0	123.7	7.93	2.31	1.17-4.59	0.014	-	-	-	-
Other cytokines and growth factors											
IL-1β	0	0	13.13	-	-	-	-	-	-	-	-
GM-CSF	0	0	102.98	-	-	-	-	-	-	-	-

Table 1. Plasmatic cytokines able to significantly predict progression free survival (PFS) and overall survival (OS) in the discovery cohort population. Median concentration, range, cutoff identified, Hazard Ratio (HR) and 95%CI, and p value are reported.

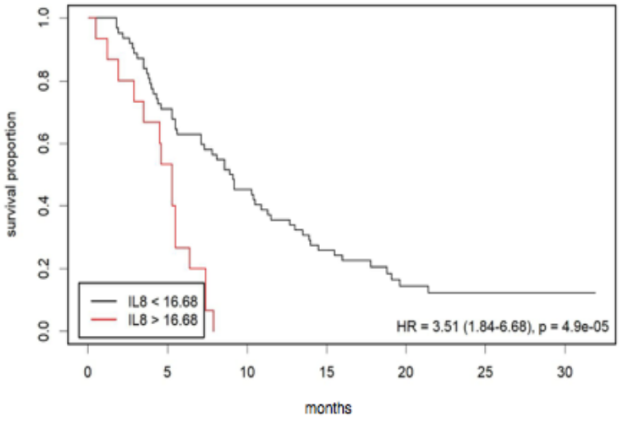
Merz et al. Figure 3

A



No. at Risk							
IL-8 < 16.68	62	20	10	7	3	2	1
IL-8 > 16.68	15	1	0				

B



No. at Risk							
IL-8 < 16.68	62	44	28	16	7	2	1
IL-8 > 16.68	15	8	0				

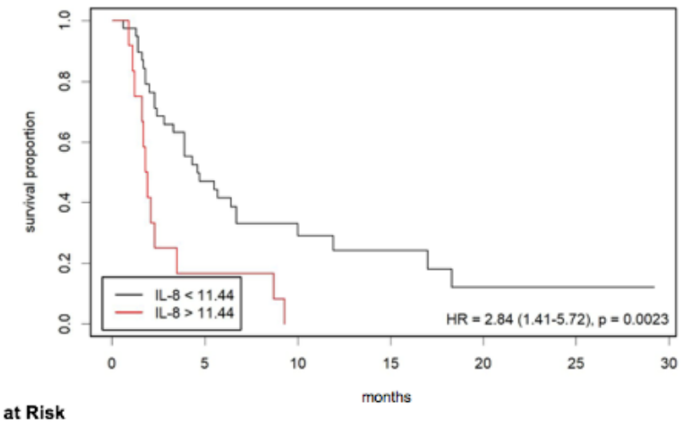
OS									
		Univariate				Multivariate			
Variables			95% CI				95% CI		
		HR	Lower	Upper	*P*	HR	Lower	Upper	*P*
PS ECOG	0 vs 1-2	1.170	0.721	1.900	0.526				
Primary tumor location	Head vs body/tail	0.924	0.567	1.508	0.753				
Liver metastases	No vs yes	1.517	0.871	2.640	0.141				
CA19.9 (U/mL)	<40 vs ≥40	1.575	0.716	3.466	0.259				
N/L ratio	≤5 vs >5	1.368	0.696	2.689	0.363				
IL-8	Low vs high	**3.406**	**1.790**	**6.481**	**0.000**				

PFS									
		Univariate				Multivariate			
Variables			95% CI				95% CI		
		HR	Lower	Upper	*P*	HR	Lower	Upper	*P*
PS ECOG	0 vs 1-2	**1.884**	**1.164**	**3.049**	**0.010**	**2.216**	**1.346**	**3.649**	**0.002**
Primary tumor location	Head vs body/tail	0.850	0.529	1.366	0.502				
Liver metastases	No vs yes	0.925	0.538	1.591	0.778				
CA19.9 (U/mL)	<40 vs ≥40	1.832	0.828	4.057	0.135				
N/L ratio	≤5 vs >5	1.233	0.672	2.264	0.499				
IL-8	Low vs high	**2.505**	**1.367**	**4.590**	**0.003**	**3.090**	**1.649**	**5.791**	**0.000**

Table 2. Univariate and multivariate analyses of clinical characteristics and interleukin (IL)-8 levels influencing progression free survival (PFS) and overall survival (OS) of patients in the discovery cohort.

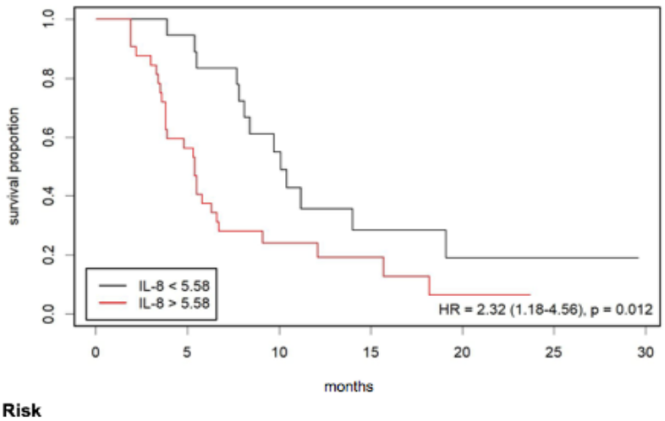
Merz et al. Figure 4

A



No. at Risk							
IL-8 < 11.44	38	17	7	4	2	2	0
IL-8 > 11.44	12	2	0				

B



No. at Risk							
IL-8 < 5.58	20	17	11	4	2	2	0
IL-8 > 5.58	30	28	5	3	1	0	

AACR American Association for Cancer Research

Clinical Cancer Research

Plasma Interleukin-8 is a biomarker for TAK1 activation and predicts resistance to nanoliposomal irinotecan in patients with gemcitabine-refractory pancreatic cancer

Valeria Merz, Camilla Zecchetto, Raffaela Santoro, et al.

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