

Tumour-associated macrophages activate migration and STAT3 in pancreatic ductal adenocarcinoma cells in co-cultures



Aino Salmiheimo, MD ^a, Harri Mustonen, MSc ^a, Sanna Vainionpää, BSc ^a,
Zhanlong Shen, MD, PhD ^b, Esko Kemppainen, MD, PhD ^a,
Pauli Puolakkainen, MD, PhD Professor of Surgery ^{a,*}, ¹, Hanna Seppänen, MD, PhD ^{a,1}

^a Department of Surgery, University of Helsinki, Helsinki University Hospital, Helsinki, Finland

^b Department of Gastroenterological Surgery, Peking University People's Hospital, Beijing, China

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ABSTRACT

Objectives: Tumour-associated macrophages participate in tumour development and progression. The aim of this study was to assess the interactions of pancreatic cancer cells and pro-inflammatory M1 and anti-inflammatory M2 macrophages, specifically their effect on pancreatic cancer cell migration and the changes in STAT-signalling.

Methods: Monocytes were isolated from healthy subjects and differentiated into macrophages with M-CSF. The macrophages were polarized towards M1 by IL-12 and towards M2 by IL-10. We studied also the effect of pan-JAK/STAT-inhibitor P6. Macrophage polarization and STAT and NFκB-activation in both MiaPaCa-2 and macrophages were assessed by flow cytometry. We recorded the effect of co-culture on migration rate of pancreatic cancer cells MiaPaCa-2.

Results: Macrophages increased the migration rate of pancreatic cancer cells. Co-culture activated STAT1, STAT3, STAT5, AKT, and NFκB in macrophages and STAT3 in MiaPaCa-2 cells. IL-12 polarized macrophages towards M1 and decreased the migration rate of pancreatic cancer cells in co-cultures as well as P6. IL-10 skewed macrophage polarization towards M2 and induced increase of pancreatic cancer cells in co-cultures.

Conclusion: Co-culture with macrophages increased pancreatic cancer cell migration and activated STAT3. It is possible to activate and deactivate migration of pancreatic cancer cells through macrophage polarization.

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Introduction

Pancreatic cancer is worldwide the 7th leading cause of cancer deaths mostly due to its tendency to metastasize aggressively at early stages and the lack of effective treatment [1]. Chronic inflammation is a risk factor for various cancer forms, such as chronic pancreatitis leading to pancreatic cancer [2]. Cancer cells also create an inflammatory microenvironment and the cytokines they produce attract monocytes from the blood circulation which, in tissues, mature into macrophages [3].

As monocytes mature into macrophages the stimuli in their

microenvironment constantly activate and polarize them. The activated macrophages can be divided into different subtypes by their phenotype and function [4]. The polarized macrophages are divided into type M1 and M2 macrophages according to their preferential secretion of interleukin (IL)-12 or IL-10 which then, respectively, activate pro- and anti-inflammatory responses [5]. Type M1 macrophages support the immune system activation, they are cytotoxic, and they inhibit malignant tumour progression. Type M2 macrophages suppress the inflammatory response, they support tissue renewal, angiogenesis, and lymphangiogenesis. Type M1 macrophages express higher levels of IL-12 and IL-23 as compared to M2 macrophages which in turn express high IL-10 phenotype [6–8]. IL-12 is a pro-inflammatory cytokine mainly produced by phagocytes and dendritic cells through activation of Toll-like receptors (TLRs). It activates the Th-1 cells of the adaptive immunity [9]. The anti-inflammatory IL-10 inhibits inflammatory

* Corresponding author. Department of Surgery, Helsinki University Hospital, Stenbäckinkatu 9 A, PL 440, 00029 HUS, Finland.

E-mail address: pauli.puolakkainen@hus.fi (P. Puolakkainen).

¹ H. Seppänen and P. Puolakkainen share last authorship.

cytokine expression and Th-1 responses while activating Th-2 cells [10]. Macrophage activation state responds to continuous regulation by growth factors and other signals of their microenvironment [11].

In tumour microenvironment macrophages differentiate into a distinct subtype of M2 macrophages called tumour-associated macrophages (TAMs), which enhance tumour invasion by several mechanisms [12,13]. The presence of TAMs in pancreatic cancer tissue has been confirmed in a number of studies; likewise the abundance of TAMs is also associated with worse prognosis [14,15].

Janus-activated kinases (JAK) are a group of tyrosine kinases that mediate the expression of various proteins in cells by activating the signal transducers and activators of transcription (STAT) proteins through tyrosine phosphorylation. STATs in turn activate transcription by binding to promoter sequences in cell nucleus [16]. The JAK/STAT pathway modulates the extracellular signalling of several cytokines and growth factors into the expression of thousands of protein-encoding genes [17]. For example, IL-6 activates STAT3 that in turn inhibits pro-inflammatory responses and promotes oncogenesis and tumour progression [18–20]. JAK/STAT signalling pathway participates in macrophage activation [16] and several types of tumors express abnormal STAT activation [21–23]. Pyridone-6 (P6) is a pan-JAK-inhibitor that has been shown to inhibit the growth of multiple myeloma cells [24].

The aim of this study was to modulate macrophages' phenotype towards either more inflammatory (M1) or anti-inflammatory (M2) direction in co-cultures with pancreatic cancer cells and assess the changes in the intracellular activation of transcription factors and pancreatic cancer cell migration. Further, the aim was to assess the inhibition of the signalling pathways activated by the macrophage phenotype change.

Materials and methods

Cell cultures and reagents

We isolated mononuclear cells from healthy subjects' blood samples with Ficoll-Paque Plus (Amershamn, Uppsala, Sweden) by density gradient centrifugation followed by paramagnetic bead separation using Human Monocyte Isolation Kit II (Miltenyi Biotec, Auburn, USA). Monocytes ($130\,000\text{ cells/cm}^2$) were cultured in Macrophage Serum-free Media (Gibco Life Technologies, Paislay, UK) supplemented with penicillin 100 mg/ml (Sigma, St.Louis, USA) and M-CSF (ImmunoTools, Oldenburg, Germany) 50 ng/ml to differentiate them into mature macrophages. Additional reagents IL-12 5 ng/ml, IL-10 25 ng/ml, and P6 500 nM (Calbiochem, San Diego, USA), were added after 5 days of differentiation in standard 37 °C and 5% CO₂.

MiaPaCa-2, human pancreatic adenocarcinoma cells from a primary tumour, was purchased from the American Type Culture Collection. MiaPaCa-2 ($35,000\text{ cells/cm}^2$) cells were added to the macrophage cultures simultaneously with the additional reagents (IL-10, IL-12, or P6) after differentiating the macrophages for 5 days with M-CSF.

Characterization of macrophages

Macrophages were cultured on Nunc UpCell dishes (Thermo Scientific) and acquired on flow cytometry two days after adding the cancer cells and/or the additional stimuli to the macrophage culture (the seventh day after isolating the monocytes). The cells were detached from their culture dishes by lowering their temperature from +37 °C to room temperature according to the UpCell manufacturer's instructions. Prior to the characterization flow cytometry we used anti-CD11b magnetic micro beads (Miltenyi

Biotec Inc., Auburn, USA) to separate the differentiated macrophages from cancer cells, this was done also to the macrophages cultured without cancer cells. Macrophages were then incubated with antibodies for 20 min in room temperature. They were acquired on FACS Calibur (CellQuest Pro software, BD Bioscience) flow cytometer and the WinMDI software (v2.8) was used for analysis. The antibodies we used to characterize the macrophages were FITC and PE Mouse Anti-Human CD14, FITC Mouse Anti-Human CD16, PE Mouse Anti-Human CD80, APC Mouse Anti-Human CD86, PE Mouse Anti-Human CD163, PE Mouse Anti-Human CD209, PE Mouse IgG1 κ Isotype Control, APC Mouse IgG1 κ Isotype Control, FITC Mouse IgG1 κ Isotype Control (BD Pharmingen, San Diego, USA).

Cancer cell migration

First, the isolated monocytes were differentiated on 8-well coverslip dishes (Nunc, Thermo Scientific, Rochester, USA) coated with 60 μl Matrigel (BD Biosciences, San Jose, USA) for five days. MiaPaCa-2 pancreatic cancer cells were stained with fluorescent dye (CellTracker Green CMFDA, Invitrogen, Eugene, USA) and added to the 8-well dishes on the differentiated macrophages, or alone as controls, with and without IL-10, IL-12, and P6. The fluorescence microscopy was initiated after 24 h of further incubation. The cancer cell migration on Matrigel was recorded in humidified, temperature (+37 °C) and CO₂ (5%) controlled chamber (OKOLab, Ottaviano, Italy) with fluorescence microscope equipped with cooled CCD camera (Sensicam, PCO, Germany) with 30 min intervals for 24 h, as previously described [25]. The data was analysed using ImagePro software (v 7.01, Media Cybernetics, Rockville, MD, USA).

STAT and NF κ B activation

To assess the activation of STAT 1, 3, and 5 as well as NF κ B and AKT, we used flow cytometry as previously described [26,27]. The macrophages were cultured identically, as to the characterization described above. MiaPaCa-2 cells and IL-10, IL-12, or P6 were added after five days and the flow cytometry was acquired after seven days of incubation. The cells were stabilized with Lyse/Fix Buffer (BD Phosflow™, BD Biosciences) at +37 °C for 10 min. We permeabilised the cells with BD Perm Buffer (BD Biosystems) at –20 °C for 30 min. Consequently, the cells were washed with Pharmingen Stain Buffer and finally stained with the labeling antibodies for CD45, STAT 1, 3, and 5, and for NF κ B and AKT. The antibodies were FITC Mouse Anti-Human CD45 (material #555482), Alexa Fluor 647 Mouse Anti-STAT1 (pY701, #612597), Alexa Fluor 647 Mouse Anti-NF κ B p65 (pS529, #558422), PE Mouse anti-Akt (pS473, #560378), PE Mouse Anti-STAT3 (pY705, #612569), PE Mouse Anti-STAT5 (pY694, #612567) from BD Phosflow™, BD Biosciences. The samples were acquired on FACS Calibur (CellQuest Pro software; BD Biosciences) flow cytometer and the data was analysed by WinMDI (v2.8) software. We assessed the phosphorylation data of the macrophages and MiaPaCa-2 cells separately by dividing them to CD45 positive and negative cells.

Cytokine assay

The cells were cultured as described above; first 5 days of macrophage differentiation, after which MiaPaCa-2 and either nothing, P6, IL-10, or IL-12 were added. The cells were on Matrigel, on 24-well plates (Nunc, Thermo Scientific), in 500 μl medium. The culture media were collected after further 48 h of incubation and stored in freezers at –75 °C. To assess the cytokine concentrations in the macrophage and MiaPaCa-2 co-culture medium, we used an

infrared Human Q-Plex™ Custom Assay (#107749 GR, Quansys Biosciences, USA), and prepared the medium samples following the manufacturer's instructions. The assay is a multiplexed ELISA kit; it measures the concentration of multiple proteins in each sample with capturing antibodies in a multiwell plate. Odyssey infrared imager (Licor Biosciences, USA) imaged the assays and Q-View™ Software (Quansys Biosciences) analysed the dot blots. The Q-Plex assay identified the levels of INF γ , IL-1a, IL-1b, IL-1Ra, IL-4, IL-6, IL-8, IL-10, IL-12p70, IL-17, IL-23, MCP-1, and RANTES.

Statistics

We used the nonparametric Mann-Whitney *U* test or Wilcoxon's signed-rank test (paired measurements) to detect the difference between continuous variables. Two tailed tests were used. $p < 0.05$ was considered as statistically significant. The results are presented

as mean \pm standard error of the mean (SEM).

Results

Macrophage characteristics

The macrophages in our cell cultures in Macrophage SFM and M-CSF 50 ng/ml, the macrophages showed a CD14 high, CD86 high, CD16 low phenotype that was not statistically significantly changed by adding MiaPaCa-2 pancreatic cancer cells to the culture (Fig. 1A). IL10 expectedly polarized the macrophages towards the anti-inflammatory phenotype detected by significantly reduced CD86 surface expression on macrophages. In co-cultures with macrophages and MiaPaCa-2 cells IL10 supplementation increased the surface expression of CD16 (M1 marker), CD80 (M1 marker), and CD163 (M2 marker) (Fig. 1B). IL12 skewed the macrophage

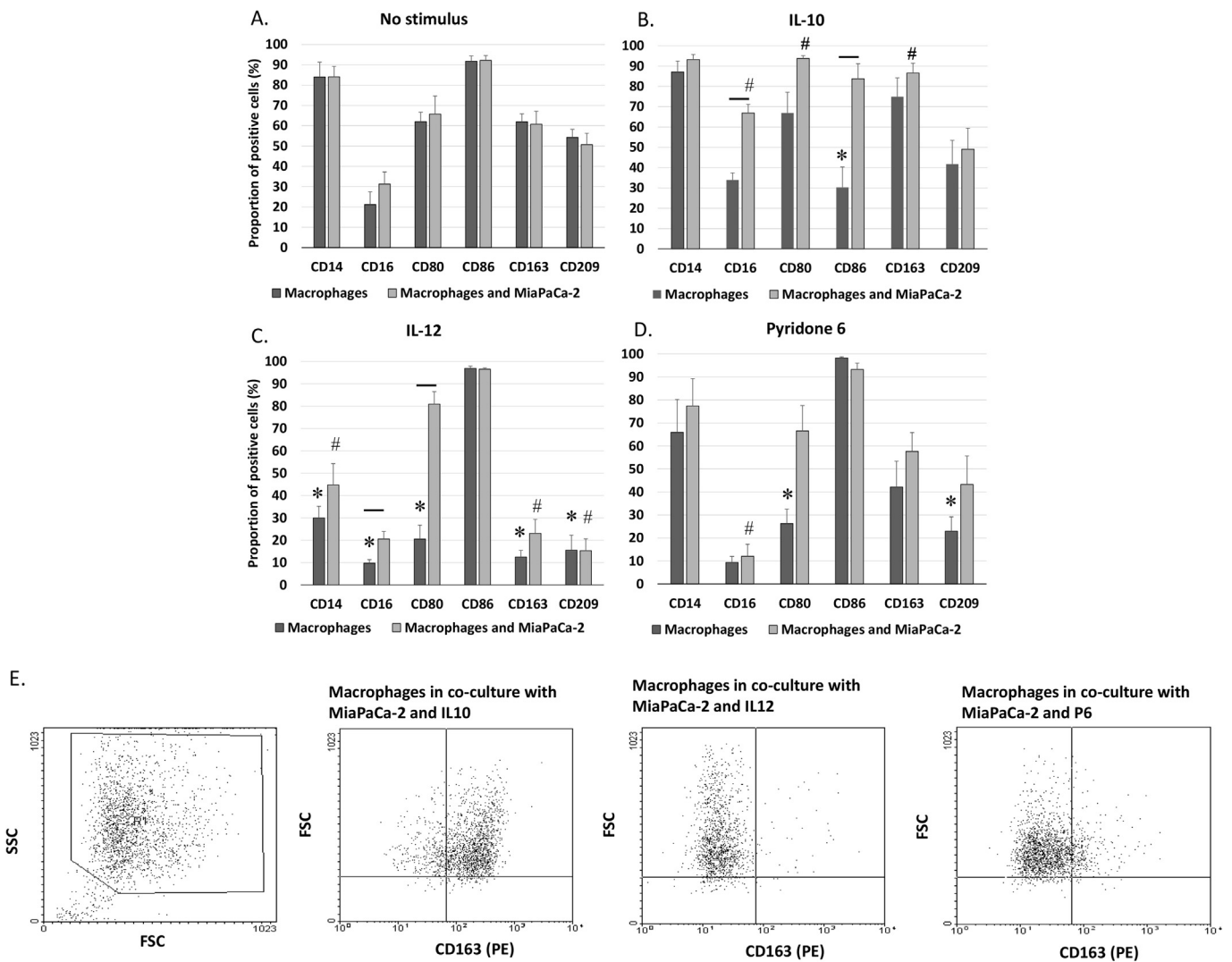


Fig. 1. The surface expression of macrophages. A. The proportion of macrophages (%) positive to different M1 (CD16, CD80, CD86) and M2 (CD14, CD163, CD209) surface markers of M-CSF differentiated human-derived macrophages cultured with (the lighter columns) and without (the darker columns) MiaPaCa-2 measured by flow cytometry. The co-culture with pancreatic cancer cells did not change the macrophage phenotype. B. Macrophages with two days of IL-10 supplementation, cultured with and without MiaPaCa-2. C. Macrophages with two days of IL-12 supplementation, cultured with and without MiaPaCa-2. D. Macrophages with two days of Pyridone 6 (P6) supplementation, cultured with and without MiaPaCa-2. * indicates a statistically significant ($p < 0.05$) difference as comparing the stimulated macrophages to the macrophages without additional stimuli (No Stimulus in 1A). # indicates a statistically significant ($p < 0.05$) difference as comparing the stimulated macrophages co-cultured with MiaPaCa-2 to the macrophages co-cultured with MiaPaCa-2 without additional stimuli (No Stimulus in 1A). — indicates a statistically significant ($p < 0.05$) difference between the macrophages with additional stimuli as comparing the macrophages cultured with and without MiaPaCa-2. Error bars show the standard error of the mean (SEM). E. Representative dot plots of CD163 (M2) surface marker on macrophages co-cultured with MiaPaCa-2 and supplemented with IL-10, IL-12, and P6 (SSC side scatter, FSC forward scatter, PE Phycoerythrin).

polarization more towards pro-inflammatory phenotype: in macrophage cultures IL12 decreased M2 markers CD14, CD163, and CD209, but also M1 markers CD16 and CD80, whereas in co-cultures with MiaPaCa-2 IL12 decreased the M2 markers CD14, CD163, and CD209 indicating that in co-cultures with pancreatic cancer cells and added IL12 macrophages are skewed towards pro-inflammatory M1 macrophages (Fig. 1C). P6 lowered CD80 (M1 marker) and CD209 (M2 marker) in macrophage cultures and only CD16 (M1 marker) in co-cultures (Fig. 1D).

Migration of pancreatic cancer cells in co-cultures with macrophages

Macrophages increased the migration rate of MiaPaCa-2 pancreatic cancer cells in co-cultures on Matrigel from $10.0 \mu\text{m/h} \pm 1.6$ – $13.3 \mu\text{m/h} \pm 1.8$, $p < 0.001$ (Fig. 2). IL-10 did not affect the migration rate of cancer cells cultured without macrophages, but in the co-cultures of macrophages and cancer cells IL-10 increased the migration rate significantly (to $17.8 \mu\text{m/h} \pm 2.4$ $p = 0.001$) as compared to co-cultures of MiaPaCa-2 and macrophages without IL-10. The inflammatory cytokine IL-12 reduced the migration rate of MiaPaCa-2 cells alone (to $8.1 \mu\text{m/h} \pm 1.5$, $p = 0.013$) and also in co-cultures with macrophages ($8.9 \mu\text{m/h} \pm 1.4$, $p < 0.001$). In co-cultures, P6 inhibited the macrophage-induced increase of pancreatic cancer cell migration ($9.2 \mu\text{m/h} \pm 1.2$, $p = 0.003$) (Fig. 2).

STAT, NFkB, and AKT activation

We analysed STAT1, STAT3, STAT5, AKT, and NFkB activation by flow cytometry in macrophages and in MiaPaCa-2 and the effect of IL-12, IL-10, and P6 on the interactions. Co-culture with MiaPaCa-2 increased the activation of STAT1 ($p = 0.037$), STAT3 ($p < 0.001$), and STAT5 ($p < 0.001$) as well as AKT ($p < 0.001$) and NFkB ($p < 0.001$) in macrophages (Fig. 3). In MiaPaCa-2 STAT3 ($p < 0.001$) increased in co-culture with macrophages (Fig. 4). In macrophages cultured alone, IL-10 increased the activation of STAT3, STAT5, and NFkB while in MiaPaCa-2 it had no statistically significant effect on the studied pathways. In the co-cultures, IL-10 decreased NFkB in

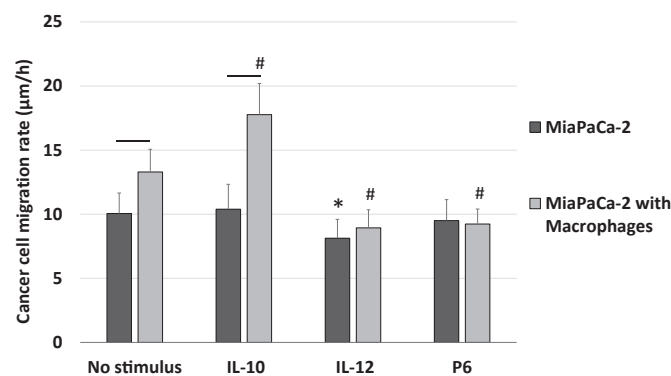


Fig. 2. Migration rate of MiaPaCa-2. Migration of MiaPaCa-2 with or without the co-culture of macrophages and the additional stimuli (IL-10, IL-12, and P6) was assessed on Matrigel. Macrophages increased pancreatic cancer cell migration and stimulation with IL-10 added to this effect. However, IL-12 and P6 inhibited the increasing effect of macrophage co-culture on pancreatic cancer cell migration. – indicates a statistically significant ($p < 0.05$) difference in the migration rate between the cancer cells as comparing the pancreatic cancer cells cultured with or without macrophages in otherwise the same conditions. * indicates a statistically significant ($p < 0.05$) difference as comparing the stimulated MiaPaCa-2 to the MiaPaCa-2 without additional stimuli (No Stimulus). # indicates a statistically significant ($p < 0.05$) difference as comparing the stimulated MiaPaCa-2 co-cultured with macrophages to the MiaPaCa-2 co-cultured with macrophages without additional stimuli). Error bars show the standard error of the mean (SEM).

macrophages as compared to the co-cultures without IL-10 suggesting inflammatory suppression; STAT3 (in macrophages and MiaPaCa-2), STAT5 and AKT (in macrophages) retained their activation. In macrophages, IL-12 increased STAT1, STAT5, and AKT activation and in co-cultures IL-12 decreased macrophages' NFkB activation. The effect of JAK/STAT-inhibitor P6 was assessed only in co-cultures of macrophages and MiaPaCa-2 where it decreased STAT1 and STAT5 in macrophages but also decreased STAT3 and increased NFkB in MiaPaCa-2, suggesting an enhanced inflammatory activation.

Cytokine secretion in co-cultures

In co-cultures of macrophages and MiaPaCa-2, IL-10 increased IL12p70 concentration from $0.3 \text{ pg/ml} \pm 0.1 \text{ SD}$ to $6.0 \text{ pg/ml} \pm 15.1 \text{ SD}$ ($p = 0.002$). IL-10 also induced a significant decrease in TNF α concentration in the culture medium ($93.1 \text{ pg/ml} \pm 58.5 \text{ SD}$ to $34.9 \text{ pg/ml} \pm 58.5 \text{ SD}$, $p = 0.021$). P6, in turn, increased TNF α concentration in co-cultures to $120.5 \text{ pg/ml} \pm 59.0 \text{ SD}$ ($p = 0.044$ as compared to co-cultures without P6). P6 also decreased the IL1ra concentration in the co-cultures from $16758.5 \text{ pg/ml} \pm 8211.4 \text{ SD}$ to $8082.6 \text{ pg/ml} \pm 5519.9 \text{ SD}$ ($p = 0.019$), thus, increasing the inflammatory activity. IL-12 induced no statistically significant changes in the cytokine secretion profile as compared to macrophage and MiaPaCa-2 co-cultures without IL-12 (except that, naturally, the IL-12 concentration was higher).

Discussion

This study on pancreatic cancer cells and their interaction with human-derived macrophages emphasized that anti-inflammatory type M2 macrophages (stimulated with IL-10) increased pancreatic cancer cell migration and pro-inflammatory type M1 macrophages (stimulated with IL-12) were able to inhibit it. Unstimulated macrophages increased the migration rate of pancreatic cancer cells, as also shown previously with different pancreatic cancer cell lines [28]. In co-cultures, both pro- and anti-inflammatory pathways were activated in macrophages, as STAT1, STAT3 and STAT5 activation increased as well as AKT and NFkB pathways, as compared to macrophages cultured without cancer cells. In MiaPaCa-2 STAT3 was activated in the co-culture with macrophages. Both STAT3 and STAT5 have implications for cancer progression by inhibiting anti-tumour immunity. Particularly STAT3 is connected to maintaining inflammation-associated tumorigenic microenvironment by for example inhibiting the expression of NFkB target genes [29,30]. NFkB is pivotal in mediating anti-tumour immune responses. Aberrations in its expression are also linked to numerous cancer types, including pancreatic cancer, by also being involved in orchestrating the pro-carcinogenic inflammatory microenvironment [31]. STAT3 has previously been associated with increased invasiveness of pancreatic cancer [32].

In the present study, STAT3 activation was induced in both macrophages and MiaPaCa-2 by their co-culture. Nonetheless, the activation of STAT3 in macrophages was not unambiguously associated with their effect on the migration rate of the pancreatic cancer cells. The pro-inflammatory cytokine IL-12, characteristically secreted by M1 macrophages, inhibited pancreatic cancer cell migration in the co-cultures with macrophages. The surface expression of macrophages stimulated with IL-12 polarized towards inflammatory phenotype. However, IL-12 did not significantly lower STAT3 activation in the MiaPaCa-2 and macrophage co-cultures. This indicates that the IL-12 inhibition of pancreatic cancer cell migration is not dependent on STAT3. In a previous study we also demonstrated that IL-6, a known upstream activator of STAT3, in co-cultures with macrophages and pancreatic cancer

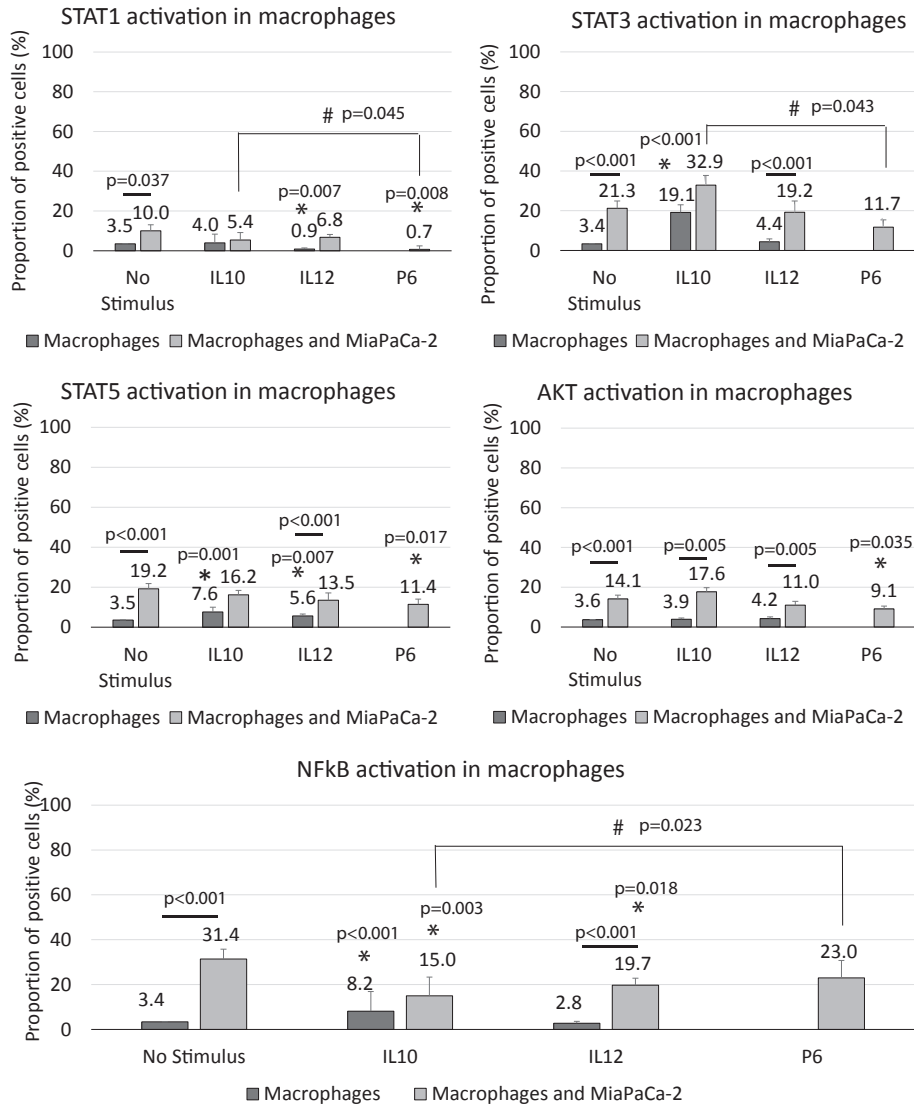


Fig. 3. Activation of intracellular STAT1, STAT3, STAT5, AKT, and NFkB in macrophages in response to MiaPaCa-2 and the additional stimuli. The activation of the assessed pathways are presented as percentage of positive cells measured by flow cytometry. The unstimulated macrophages' activation was set as reference 3.5% activation. The effect of STAT-inhibitor P6 was assessed only in co-cultures of macrophages and MiaPaCa-2. – indicates $p < 0.05$ difference when comparing the stimulated macrophages with and without the co-cultured MiaPaCa-2 (the adjacent columns). * indicates $p < 0.05$ as compared to the respective No Stimulus macrophages with or without co-cultured MiaPaCa-2. # signifies $p < 0.05$ between the indicated co-cultures. Error bars show the standard error of the mean.

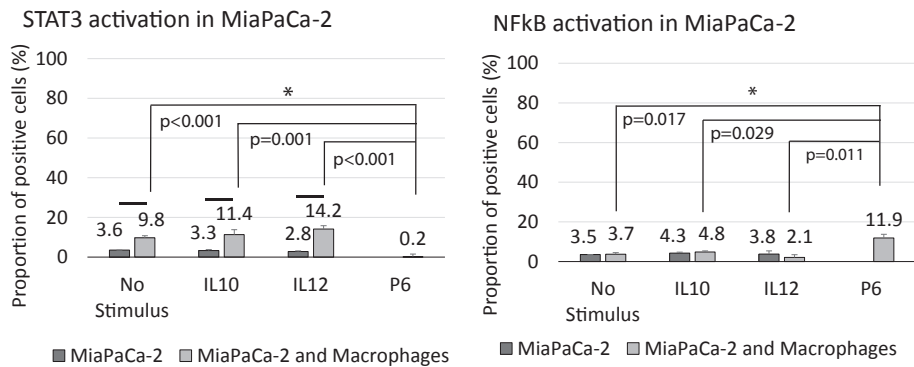


Fig. 4. Activation of intracellular STAT3 and NFkB in MiaPaCa-2 by flow cytometry. The activation of the assessed pathways are presented as percentage of positive cells. The unstimulated MiaPaCa-2's activation was set as reference 3.5% activation. The effect of P6 was assessed only in co-cultures of macrophages and MiaPaCa-2. – indicates $p < 0.001$ difference when comparing the stimulated MiaPaCa-2 with and without the co-cultured macrophages. * indicates $p < 0.05$ as compared to the respective No Stimulus macrophages with or without co-cultured MiaPaCa-2. # signifies $p < 0.05$ between the indicated co-cultures. Error bars show the standard error of the mean. The activation of STAT1, STAT5, and AKT were also assessed in MiaPaCa-2, but no statistically significant changes were found.

cells inhibited the migration rate of pancreatic cancer cells [33].

Elevated levels of immunoregulatory cytokine IL-10 has been reported in patients with pancreatic cancer and it has been associated with impaired survival in for example lung cancer [34,35]. In the present study, pancreatic cancer cell migration was activated even further with IL-10 but only when the cancer cells were co-cultured with macrophages, which indicates that the IL-10 activation of pancreatic cancer cell migration is mediated by their interaction with macrophages. Further, STAT3 activation in MiaPaCa-2 cells after adding IL-10 occurred only with macrophages; IL-10 and IL-12 had no statistically significant effect on intracellular MiaPaCa-2 STAT nor NFκB signalling. IL-10 polarized the macrophages' surface expression towards anti-inflammatory type, and it reduced the secretion of pro-inflammatory TNFα and NFκB activation in co-cultures, which might in part lead to the increased cancer cell migration. In macrophage cultures alone IL-10 activated STAT3, also a marker of phenotype change towards M2. IL-10 has previously been linked to epithelial-mesenchymal transition of pancreatic cancer cells but using mouse macrophages [36]. However, the division of macrophages to types M1 and M2 is a simplification and they can be further divided into several subtypes with divergent phenotypes and functions [37,38]. Recent evidence also indicates that TAMs may exhibit features and functions of both M1 and M2 macrophages which might partly explain the discrepancies in the surface marker profiles and STAT activation in the present study [39].

Surprisingly, JAK/STAT inhibitor P6 (500 nM) inhibited STAT3 activation only in MiaPaCa-2 and not statistically significantly in macrophages even though according to previous studies it should completely inhibit pY-STAT3 [40]. P6 inhibited pancreatic cancer cell migration in co-cultures with macrophages and in HPAF-II cultures also without macrophages. Thus, it is possible that P6 inhibits pancreatic cancer cell migration by STAT3 inhibition of pancreatic cancer cells regardless of macrophages. P6 also left macrophage polarization statistically unchanged, except for lowering the proportion of CD16 positive cells, in co-cultures with pancreatic cancer cells as compared to co-cultures without P6.

The present study provides novel insight to the interaction of macrophages and pancreatic cancer cells. It was possible to inhibit macrophage-induced increase of pancreatic cancer cell migration by polarizing the macrophages towards type M1 with IL-12 but also to increase it by inducing type M2 macrophages with IL-10. It seems that STATs might participate in the regulation of pancreatic cancer cell migration, as pan-JAK/STAT inhibitor reduced their migration rate and inhibited STAT3 activation of MiaPaCa-2, but STAT3 activation was not, however, directly predictive for pancreatic cancer cell migration rate. The interaction of macrophages and pancreatic cancer cells increased the activation of STAT1, STAT3, STAT5, AKT, and NFκB in macrophages and STAT3 in pancreatic cancer cells. The interactions warrants further studies in models, where it is possible to evaluate also other elements of tumour environment. The results encourage to continue research on macrophages to explore on the possibility of finding a TAM-targeting therapy.

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