The Crosstalk between Pancreatic Ductal Adenocarcinoma (PDAC) and Macrophages in Tumour Microenvironments, and Its Effects on Inflammasome Activation and Pro- Inflammatory Cytokine Levels

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Research Article

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Abstract

Purpose: Acute and persistent inflammation plays an important role in the establishment and progression of the pancreatic ductal adenocarcinoma (PDAC). Modified macrophages, tumour associated macrophages (TAMs) are key contributors to the survival, growth, and metastatic behaviour of the PDAC cells, by interacting with them. Central to the role of inflammation and TAMs, lies the NLRP3 inflammasome. The crosstalk between PDAC cells and TAMs has been postulated to regulate the NLRP3 inflammasome activity and the subsequent associated inflammation in the tumour microenvironment, which, in turn affects the crosstalk and the survival, proliferation and metastatic potential of PDAC cells. This study investigated the effects of LPS-stimulated inflammation on cell proliferation, levels of pro-inflammatory cytokines and the NLRP3 inflammasome pathway in a co-culture model using PDAC cells and macrophages, in the with or without MCC950, a NLRP3 specific inhibitor.

Methods: The effects of LPS-stimulated inflammation were tested on two PDAC cell lines (Panc 10.05 and SW 1990) co-cultured with RAW 264.7 macrophages. Cell proliferation was determined using the MTT assay. Levels of pro-inflammatory cytokines, IL-1β and TNF-α were determined by ELISA. Western blot analyses were used to examine the expression of NLRP3 in both PDAC cells and macrophages.

Results: The crosstalk between PDAC cell lines and macrophages under LPS-stimulation led to pro-inflammatory microenvironment, as evidenced by high levels of secreted IL-1β and TNF-α. The crosstalk was also responsible for regulating the NLRP3 expression in both PDAC cells and macrophages as well as promoting PDAC cell proliferation. Inhibition of the NLRP3 inflammasome by MCC950, counteracted the effects of LPS-stimulation on the crosstalk. However, MCC950 differentially modified the viability of the metastatic vs primary PDAC cells lines.

Conclusion: The stimulation by LPS increased PDAC cells viability by modulating the crosstalk between PDAC cells and RAW 264.7 macrophages (in a co-culture model) that results in the activation of NLRP inflammasome. The specific inhibition of NLRP inflammasome by MCC950 effectively counteracted the LPS-stimulated inflammation.

1.0 Introduction

Pancreatic Ductal Adenocarcinoma (PDAC) is becoming a growing concern on two key fronts: its high incidence-to-mortality ratio of 94% and its insidious onset of associated gastrointestinal, constitutional and thrombotic symptoms all of which have had a negative impact on the quality of life in affected patients (McGuigan A 2018 Nov 21). Most cases of PDAC are sporadic in nature. Each of the exogenous risk factor can be associated with acinar injury and chronic inflammation (Laheru 2020, Wadsworth 2021). Repeated episodes of injury are therefore associated with cycles of inflammation which in turn leads to metaplasia and genomic instability, particularly resulting in the oncogenic activation of the KRAS (Guo, Chen et al. 2011, Yang, Wang et al. 2011, Ling, Kang et al. 2012). KRAS activation, leads to the establishment of pancreatic intraepithelial neoplasia (PanIN) (Kopp, von Figura et al. 2012) and

maintains the constitutive activation of NF-κB, the master inflammatory transcription factor. NF-κB regulates the expression of inflammasomes, pro-inflammatory cytokines, anti-apoptotic factors and enhances mitogenic EGF receptor signalling in transformed cells (Romashkova and Makarov 1999, Chen, Edelstein et al. 2000, Liptay, Weber et al. 2003, Erkan, Hausmann et al. 2012, Ling, Kang et al. 2012, Liu, Zhang et al. 2017). This persistence of an inflammatory environment can facilitate the loss of tumour suppressors, leading to progression of the from PanIN to PDAC (Guerra, Collado et al. 2011).

Indeed, a conducive microenvironment is important to facilitate the survival of tumour cells and co-existing with tumour cells, are macrophages of a specific phenotype termed tumour associated macrophages (TAMs). Studies have established that the detection of TAMs correlated with the malignant phenotype of PDAC (Hu, Jiao et al. 2015). Interestingly, the phenotype of TAM populations in the tumour microenvironment varies according to the stage of the tumour and the dynamic biophysical features within micro-niches of the tumour, suggesting that crosstalk between tumour cells and TAMs does exist (Galdiero, Biswas et al. 2014). In fact, exosomes derived from the PDAC cells in hypoxic niches, induced the M2 polarisation of TAMs, which could be a pro-tumour phenotype because they facilitated tumour progression by promoting angiogenesis, desmoplasia, metastasis and suppressing anti-tumorigenic immune response in the tumour microenvironment as well as regulated epithelial-to-mesenchymal transition (EMT) in pancreatic cancer cell migration and metastasis (Sica, Straus et al. 2014, Wang, Luo et al. 2018, Xiong, Zhu et al. 2021).

The central mechanism driving inflammation is proposed to be orchestrated by the NLR pyrin domain containing 3 (NLRP3) inflammasome made up of the NLRP3 protein, apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC) and procaspase-1 (Sharma and Kanneganti 2021). NLRP3 inflammasome is markedly upregulated in pancreatic cancer (Daley, Mani et al. 2017). Pattern recognition receptors (PRRs) detect damage associated molecular patterns (DAMPS) and lipopolysaccharide (LPS), leading to the activation of NF-κB with subsequent upregulation of NLRP3 inflammasome (Groslambert and Py 2018, Kelley, Jeltema et al. 2019). The activation of the NLRP3 inflammasome requires a second signal, provided by a wide variety of cellular or molecular stimuli, such as adenosine triphosphate (ATP) (Swanson, Deng et al. 2019). Activation of the inflammasome leads to the cleavage of precursors pro-IL-1β and pro-IL-18 into their active forms i.e., IL-1β and IL-18, which have multiple roles in tumours (Thi and Hong 2017, Sun and Guo 2018, Baker, Houston et al. 2019).

Interestingly, MCC950 could inhibit ATP hydrolysis by the NACHT (NAIP, C2TA ,HET and TP1) domain in the NLRP3 protein forcing the NLRP3 protein into a ‘closed’ conformer inhibiting its oligomerization with ASC and ultimately affecting NLRP3 inflammasome assembly and activation (Wu, Chen et al. 2020). The therapeutic potential of MCC950 in inflammation-related cancers has been highlighted. (Huang, Chen et al. 2017, Chen, Zhou et al. 2019). In addition, NLRP3 blockade in TAMs led to the increase in local $CD8^+/CD4^+$ T cell ratio, and the $CD4^+$ T cells themselves reprogrammed to the T_h1 phenotype instead of the suppressive Treg phenotype commonly found in PDAC microenvironments, thereby enhancing anti-tumour immunity (Daley, Mani et al. 2017).

Thus, this study aimed to study the inhibitory effects of MCC950 on the activation of NLRP3 inflammasome in LPS-stimulated PDAC and TAM cells under co-culture conditions. The effects of the crosstalk between PDAC and TAM cells on the activation of NLRP3 inflammasome, production of IL-1β and TNF-α and PDAC cell proliferation were investigated.

2.0 Methods

2.1 Preparation of lipopolysaccharide (LPS), adenosine triphosphate (ATP) and NLRP3-specific inhibitors (MCC950)

Lipopolysaccharide and adenosine triphosphate (ATP) purchased from ThermoFisher, USA were diluted with phosphate buffered saline (PBS) to a stock solution of 1mg/mL and 50μM respectively.

MCC950 (Sigma, USA) was initially dissolved in dimethyl sulfoxide (DMSO; Calbiochem, USA) to produce a stock solution of 1000μM followed by dilution with Roswell Park Memorial Institute Medium (RPMI 1640; Gibco, USA) serum free media (SFM) to a concentration of 100 μM.

2.2 Treatments of PDAC Cells Co-cultured with RAW 264.7 Macrophages

RAW 264.7 murine macrophages (ATCC® TIB-71™) and two PDAC cell lines, namely primary Panc 10.05 PDAC cells and metastatic SW 1990 PDAC cells, were acquired from American Type Culture Collection (ATCC). All cell lines were maintained in 5% CO_2 at 37°C using complete culture medium consisting of RPMI with 10% heat-inactivated fetal bovine serum (FBS; (ATCC), VA) and 1% penicillin-streptomycin (Lonza, USA).

To investigate the crosstalk between PDAC cells and macrophages, a co-culture set-up was used. The macrophages were seeded, on permeable trans-wells at a cell count of 3 X 10^5 cells per well. Panc 10.05 and SW 1990 PDAC cells were seeded on separate 6-well plates, at a cell count of 5 X 10^5 cells per well. After 48 h trans-wells containing macrophages were transferred to 6-well plates containing seeded Panc 10.05 or SW 1990 PDAC cells. Eight treatment/control groups were included for each PDAC cell line as described in Table 1. The serum free media (SFM) with the respective treatment was then added to each well, with the volume being split evenly i.e., 1mL of treatment media to each trans-well and well. This set-up was then incubated at 37^{o}C, 5% CO_2.

Table 1
The different treatment/control groups included for each designated co-culture setup. All treatments were conducted for 24 h.

Group	Description of PDAC treatment
L	Stimulated with 1 μg/mL LPS
A	Treated with 5 μM ATP
M	Treated with 10 μM MCC950
LA	Stimulated with 1 μg/mL LPS in the presence of 5 μM ATP
LM	Stimulated with 1 μg/mL LPS in the presence of 10 μM MCC950
AM	Stimulated with 5 μM ATP in the presence of 10 μM MCC950
LAM	Stimulated with 1 μg/mL LPS in the presence of 5 μM ATP and 10 μM MCC950
C	RPMI SFM only (control)

2.3 Cell viability assay

After treatment for 24 h, the effect of various treatments (Table 1) on PDAC cell proliferation was examined using MTT Reagent (Merck Calbiochem®, Germany). The treatment media were aspirated and 1mL of MTT reagent was added to each well containing PDAC cells. This was incubated at 37^{o}C, 5% CO_2 for 4 h. The reagent was carefully aspirated leaving the formed crystals in the well. Following solubilisation of the crystals using DMSO, the absorbance was measured a 670 nm using a microplate reader (Tecan Infinite 200 PRO, Switzerland).

2.4 ELISA and Western blot analysis

The cell-free supernatants were collected from each treatment/control group. The levels of IL-1β and TNF-α in the human PDAC cells and mouse macrophages were determined using human (Cat no: RAB0273) and mouse (Cat no: RAB0308) ELISA Kits (Sigma-Aldrich, USA) respectively, according to the manufacture protocol. The determined concentrations were then standardised with the total protein concentration of each group determined using Bradford Assays (Bio-Rad Laboratories Inc., USA).

Following detachment of cells from their respective wells, PDAC cells and macrophages were lysed using cell extraction buffer. The protein concentration was determined using the Bradford Assay and approximately 30 μg of proteins from each sample were subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) at 10% bis-acrylamide resolving. The separated proteins were then blotted onto a polyvinylidene difluoride membrane at 100V for 1.5 h. Transferred membranes were blocked with Blocking One solution (Nacalai Tesque, Japan) for 1 h and incubated with the primary NLRP3 (Cat no:

HPA012878) and β-actin (Cat no: SAB5600204) (as endogenous control) rabbit monoclonal antibodies (1:1000 dilution; Cell signalling technology, USA) overnight at 4°C under gentle shaking. The membranes were then washed with Tris Buffered Saline-Tween (TBS-T) 3 times over 10-minute intervals, and were incubated with secondary, HRP-linked antibody (1:2000 dilution; Cell signalling technology, USA) for 1 h at room temperature under gentle shaking. Subsequently, the membranes were incubated with Chemi-Lumi One Super ECL reagent (Nacalai Tesque, Japan) and visualised for densitometric analysis using the Bio-Rad ChemiDoc™ XRS + system and Bio-Rad Image Lab 6.1 software. All western blots were performed in triplicate.

2.5 Statistical analysis

GraphPad Prism 8.0 statistical software was used to analyse the data. The statistical significance of difference in the means was assessed using the one-way analysis of variance (ANOVA) and the Tukey's post hoc test. Differences with p-values less than 0.05 were considered to be statistically significant.

3.0 Results

3.1 Effects of MCC950 on cell proliferation of LPS-stimulated PDAC cells co-cultured with macrophages

In the co-culture of PDAC with macrophages, there was a significant ($p < 0.05$) increase in the proliferation of the PDAC cells under 1 μg/mL LPS stimulation (L) when compared with the control (C) (Fig. 1). The increase in cell proliferation was significantly greater upon the stimulation by LPS in the presence of 5 μM ATP (LA group). However, when MCC950 was included in addition to LPS and ATP (LAM), no significant difference ($p > 0.05$) was observed in PDAC proliferation when compared to control. Interestingly, no significant change in SW 1990 cell proliferation was observed with LPS stimulation in the presence of MCC950 (LM) although an increase in Panc10.05 cell proliferation was noted when compared to control (Fig. 1).

3.2 Effects of MCC950 on IL-1β and TNF-α production by LPS-stimulated PDAC cells co-cultured with macrophages

Compared with the control, a significant increase in the levels of human IL-1β and TNF-α ($p < 0.05$) was detected in the supernatants obtained from L, A and LA groups (Fig. 2) for both SW 1990 and Panc 10.05 cells co-cultured with macrophages. Interestingly, addition of MCC950 to these treatments (LM, AM, LAM) resulted in no significant changes in the levels of TNF-α when compared with control (C). Although addition of MCC950 could reduce the levels of IL-1β in LM, AM, LAM when compared with L, A and LA respectively, it was still significantly higher than the control (c).

Furthermore, a significant increase in IL-1β production by SW 1990 cells was noted in the two-step activation by LPS and ATP (LA) compared to single treatment in L and A groups.

3.3 IL-1β and TNF-α production by macrophages in co-cultured PDAC cells under LPS-stimulated inflammation

In the co-culture of macrophages and PDAC cells, the levels of mouse IL-1β and TNF-α in the cell supernatants were significantly increased in L and LA groups when compared to the control (C) (Fig. 3). The addition of MCC950 significantly reduced the levels of IL-1β and TNF-α in LM and LAM when compared respectively with L and LA. However, the cytokine levels recorded in LM and LAM were significantly higher than control except for TNF-α levels detected in macrophages co-cultured with Panc 10.05 (Fig. 3 Aii) where no significant differences were noted from the control.

3.4 NLRP3 expression by Panc 10.05 and SW 1990 PDAC cells under LPS-stimulated inflammation

A significant increase in the levels of NLRP3 was observed in cell lysates of both PDAC cells when co-cultured with macrophages under LPS-stimulated inflammation or/ and ATP treatment (L, A, LA) although the two-step activation provided by LA did not result in higher production of NLRP3. The addition of MCC950 reduced the levels of NLRP3 in LM and LAM in Panc 10.05 cells when compared respectively with L and LA, but increased the NLRP3 levels in AM when compared to A (Fig. 4 A). However, MCC950 reduced the NLRP3 levels in SW 1990 PDAC cells from LM and AM groups when compared to L and A respectively. Interestingly, MCC950 increased the NLRP3 levels in SW 1990 PDAC cells from LAM group when compared to LA (Fig. 4 B).

Figure 4: Relative protein expression levels of NLRP3 inflammasome by A) Panc 10.05 and B) SW 1990 PDAC cells in their respective co-cultures with RAW 264.7 macrophages under LPS and ATP-induced inflammation with or without MCC950 inhibition were analysed using western blot. β-actin expression serves as the control. Data presented is representative of three individual experiments and presented as mean ± SEM. Means with the same letter are not significantly different from each other ($P > 0.05$).

3.5 NLRP3 expression by macrophages in co-culture with PDAC cells under LPS-stimulated inflammation

In the co-culture with Panc 10.05 cells, there was a significant decrease in the levels of NLRP3 in macrophages in all treatment groups when compared with the control, except for M and LAM (Fig. 5A). LPS stimulation or ATP treatment with or without MCC950 caused reduction in the expression of NLRP3 in macrophages but MCC950 treatment alone (M) or combined with both LPS and ATP (LAM) significantly increased NLRP3 levels in macrophages when compared with control.

On contrary, in the co-culture with SW 1990 PDAC cells, a significant increase in the levels of NLRP3 in macrophages was observed in all treatment groups when compared with control, except L and LA (Fig 5 B). In fact, presence of LPS and ATP (LA) significantly reduced the NLRP3 levels when compared to control. Moreover, the addition of MCC950 in the treatment with LPS or/ and ATP (LM, AM, LAM)

significantly increased the levels of NLRP3 in macrophages co-cultured with SW 1990 cells when compared with their respective counterparts (L, A, LA).

4.0 Discussion

Chronic inflammation had long been considered as a risk factor for cancer development and progression (Guerra, Collado et al. 2011, Shi and Xue 2019). In the pancreas, chronic inflammation is known to sustain an inflammatory milieu that can destabilise the acinar cell genome, hence encouraging the accumulation of oncogenic mutations (Kong, Sun et al. 2014). The central mechanism driving this inflammation in immune cells is proposed to be orchestrated by the inflammasome (Hausmann, Kong et al. 2014, Kolodecik, Shugrue et al. 2014). However, till date, it remains unresolved how the crosstalk, between PDAC and macrophages, regulates the NLRP3 inflammasome and pro-inflammatory cytokines in the tumour microenvironment and ultimately its effect on PDAC progression.

In this study, in the co-culture of PDAC cells and macrophages, elevated levels of IL-1β (about 200-300pg/mg) and TNF-α (about 100–150 pg/mg) were produced by PDAC cells and macrophages, in the presence of LPS (L and LA) (Fig. 2, Fig. 3). The increase in cytokines is in accordance to the reported ability of LPS to activate NF-κB in all cell lines, leading to downstream transcription of the cytokines and inflammasomes (Groslambert and Py 2018). Although increased level of cytokines were also reported in the monoculture of LPS-induced PDAC cells, the level of IL-1β was only approximately 6pg/mg (Yaw, Chan et al. 2020). This elevated production level of IL-1 β observed in the co-culture of PDAC cells and macrophages suggested that the crosstalk between macrophages and PDAC cells could positively regulate cytokines, particularly IL-1β production, contributing to the inflammatory microenvironment. Studies had shown that the stimulation of macrophage IL-1 receptors by IL-1β produced by PDAC cells, could lead to the activation of Casein Kinase I and II in macrophages, which in turn phosphorylated the p65 subunit of NF-κB heterodimer, resulting in the increased activation of NF- κB (Chantôme, Pance et al. 2004). The activated NF-κB might then trans-locate into the nucleus and stimulate the transcription of the mRNA of inflammatory components such as the NLRP3 inflammasome (Cornut, Bourdonnay et al. 2020).

The expressed cytokines could further contribute to the crosstalk between macrophages and PDAC cells by repressing PP2A in PDAC cells (Engström, Erlandsson et al. 2014, Tao, Liu et al. 2016). PP2A protein dephosphorylates and inactivates the IKK complex keeping NF-κB activation in check, hence the repression of PP2A leads to uninhibited activation of NF-κB in PDAC cells. As mentioned in previous reports, the reduced inhibition of NF-κB activation meant that along with the existing KRAS and STAT3 cytokine networks, a greater sustained activation of NF-κB ensues (Corcoran, Contino et al. 2011). PP2A repression also increases the activity of kinases including IKK, JNK, PKC and ERK, all of which have important roles in accelerating pancreatic cancer progression (Takahashi, Hirata et al. 2013, Storz 2015, Tao, Liu et al. 2016, Mollinedo and Gajate 2019). In accordance with these reports, this study showed that the cell proliferation of PDAC cells increased when treated with LPS and/or ATP (Fig. 1).

Following MCC950 treatment (M), the cytokine production by PDAC cells and macrophages were significantly reduced. This could be due to the role of MCC950 as a highly specific inhibitor of ATP hydrolysis by the NLRP3 inflammasome (Coll, Hill et al. 2019). The inhibition of ATP hydrolysis, prevents or reverses conformational changes in NLRP3 protein necessary for inflammasome assembly and activation. The addition of LPS however (LM) offsets the inhibition by MCC950 in the production of IL-1β by PDAC cells (Fig. 3.2). This could be due to the increase in NF-κB activation in the macrophages and, hence greater transcription and activation of NLRP3 inflammasome in both cell lines (He, Hara et al. 2016). ATP on its own did not massively increase the production of IL-1β in SW 1990 PDAC cells and RAW 264.7 macrophages as the two-step activation is required for NLRP3 inflammasome (Fig. 2, Fig. 3). Unlike the SW 1990 metastatic cancer cell line, in Panc 10.05, induction with LPS and ATP individually or together (L, A and LA groups) resulted in a similar increase in the production of IL-1β (Fig. 2). This is in accordance with previous studies, as primary PDAC cells (Panc 10.05) lack ASC meaning the NLRP3 inflammasome is not largely responsible for the production IL-1β in this cell line and instead other inflammasomes play a prominent role in the cleavage of pro-IL-1β into IL-1β (Yaw, Chan et al. 2020).

In addition, in this study, LPS-induced Panc 10.05 PDAC cells (L, LA and LM groups) demonstrated an increase in proliferation compared to their control of counterparts (Fig. 1). This confirms the ability of LPS to further repress PP2A expression and promote IL-1β production, effectively stimulating NF-κB activation, which in turn enhances mitogenic EGF receptor signalling (Liptay, Weber et al. 2003, Ling, Kang et al. 2012). This also correlated with the significant increase in the expression of NLRP3 in Panc 10.05 PDAC cells following treatment with LPS and/or ATP (L, A, LA, LM groups) (Fig. 4). The level of pro-inflammatory cytokines produced by Panc 10.05 PDAC cells and macrophages was also increased in the LPS-stimulated co-cultures (L, LA and LM groups) suggesting that the increased levels of pro-inflammatory cytokine in the tumour microenvironment also contributed to the above-mentioned PP2A repression mechanism, resulting in an increased cell proliferation. Treatment with MCC950 did not prevent the increase in pro-inflammatory cytokines in the tumour microenvironment, nor the increase in proliferation of Panc 10.05 PDAC cells in LPS-stimulated pro-inflammatory microenvironment.

Similarly, LPS-induced SW 1990 PDAC cells (L and LA groups) demonstrated a similar increase in cell proliferation. However, the addition of MCC950 to inflammatory stimuli LPS (LM and LAM groups) reduced the level of IL-1β in the tumour microenvironment by the SW 1990 PDAC cells and macrophages, and this could prevent the crosstalk-induced increase in NF-κB activation in the PDAC cells. Hence, despite the increase in NLRP3 expression in both SW 1990 PDAC cells and macrophages, in these groups, there was no increase in the proliferation of SW 1990 PDAC cells in the presence of MCC950 (Fig. 1, Fig. 2).

Interestingly, it was observed that NLRP3 levels were generally increased in PDAC cells when treated with LPS and/or ATP (Fig. 4) but not in macrophages (Fig. 5). Also, in the SW 1990 PDAC cells, there is no significant change in the expression of NLRP3 in the LA group (Fig. 4B), suggesting that the massive increase in pro-inflammatory cytokine IL-1β could significantly induce Tripartite Motif Containing 31 (TRIM31) and Growth Factor Independence 1 (GFI1) negative regulation, off-setting the initial increase in

transcription. Studies have reported that IL-1β stimulates the mRNA transcription and protein expression of TRIM31 and the downstream sustained NF-κB activation also leads to the transcription of GFI1. TRIM31, which is also constitutively expressed in macrophages and PDACE cells, promotes the proteasomal degradation of the NLRP3 inflammasome and GFI1 represses the transcription of NLRP3 inflammasome (Zhu, Meng et al. 2014, Song, Liu et al. 2016). The results suggest that for a particular cell, IL-1β present in the microenvironment can stimulate both TRIM31 and GFI negative regulation. The effects of LPS and ATP on the regulation of NLRP3 expression in PDAC cells in co-culture are not similar to those observed in monocultures of the PDAC cells in a study by Yaw et al. This could be due to the additional input from the crosstalk between the PDAC cells and the macrophages, setting the inflammatory microenvironment.

Our observations led to the understanding that the levels of NLRP3 expression in cells are finely controlled by the crosstalk via the same regulators (e.g., IL-1β), with different effects at different concentrations. The differing effects of MCC950 in decreasing the expression of NLRP3 in the LA group of primary Panc 10.05 PDAC cells and increasing the expression of NLRP3 in LA group of metastatic SW1990 PDAC cells depicts that role of MCC950 in the crosstalk depends on whether the particular PDAC cell line relies on the NLRP3 inflammasome for background IL-1β production. The lesser the PDAC cell line relies on NLRP3 inflammasome for background IL-1β production, the greater the level of inflammatory cytokines in the tumour microenvironment and the greater the extent of negative feedback on NLRP3 expression, that could not be offset by MCC950 inhibition on cytokine production.

In both co-cultures, there is a significant decrease in the expression of NLRP3 inflammasome in RAW 264.7 macrophages in the background of an increased levels of pro-inflammatory cytokine in the tumour microenvironments stimulated by LPS and ATP (Fig. 5A and B). This highlights the prominent role of the negative regulators e.g., TRIM31 and GFI1, and the effect of crosstalk on these negative regulators (Song, Liu et al. 2016, Cornut, Bourdonnay et al. 2020). Despite the initial induction of NF-κB to stimulate transcription of NLRP3 inflammasome, the massive production of cytokines by both cell lines could increase the levels of TRIM31 and GFI1, which in turn decreased the expression of NLRP3. Our results also suggest that the negative regulation of NLRP3 inflammasome is relatively greater in RAW 264.7 macrophages compared to the PDAC cells.

In the co-culture of macrophages with Panc 10.05 PDAC cells, the addition of LPS or ATP to MCC950 (AM and LM), significantly reduced the NLRP3 inflammasome expression in the macrophages. On contrary, in the co-culture of SW 1990 PDAC cells with macrophages, a significant increase in NLRP3 expression in macrophages, was observed in the AM and LM groups. This variation between the co-cultures could be due to Panc 10.05 exerting greater negative feedback on its crosstalk, as it produces more IL-1β than the MCC950-inhibited SW 1990, leading to possibly increased TRIM31 induction in the macrophages. This highlights the impact of NLRP3 inflammasome activity in the crosstalk and tumour microenvironment.

5.0 Conclusion

In the co-culture treatments, the pro-inflammatory cytokines such as IL-1β modulate the crosstalk between the PDAC cells and macrophages under LPS stimulation as highlighted in this study. The NLRP3 expression in both PDAC cell lines and macrophages, under LPS-stimulated inflammation, was regulated by the crosstalk. The crosstalk resulted in a pro-inflammatory environment with elevated levels of IL-1β and TNF-α as well as increased the proliferation of PDAC cells. The proliferation of PDAC cells were ore closely related to the level of pro-inflammatory cytokines in the tumour microenvironment, rather than the level of NLRP3 expression in PDAC cells and macrophages. The addition of MCC950 did not reduce the effects of LPS or/ and ATP treatment on primary Panc 10.05 cell proliferation although the cytokine levels were reduced. However, in the presence of MCC950, metastatic SW 1990 PDAC cell proliferation was significantly reduced in presence of LPS and ATP. Nevertheless, this potential therapeutic efficacy of MCC950 in treating metastatic PDAC relying on NLRP3 inflammasome as part of their crosstalk, must be studied further.

Abbreviations

A/ATP	Adenosine Triphosphate
ADM	Acinar-to-Ductal Metaplasia
AM	Combination of Adenosine Triphosphate and MCC950
ASC	Apoptosis-associated Speck-like protein containing a CARD complex
C2TA	MHC Class 2 Transcription Activator
CARD	Caspase Activation and Recruitment Domain
CCL2	Chemokine (C-C motif) Ligand 2 (CCL2)
CCR2	C-C Chemokine Receptor type 2
CSC	Cancer Stem Cells
DAMP	Damage Associated Molecular Pattern
ECM	Extracellular Matrix
ERK	Extracellular signal Related Kinase
FADD	FAS-mediated death domain protein
GFI1	Growth Factor Independence 1
HET	Heterokaryon Incompatibility Gene het
iCAF	Inflammatory Cancer Associated Fibroblasts
IKK	IκB-kinase
IL	Interleukin
IP-10	Interferon γ-induced Protein 10
JNK	c-Jun N-terminal Kinases
KRAS	Kirsten Rat Sarcoma viral oncogene
L/LPS	Lipopolysaccharide
LAM	Combination of Lipopolysaccharide, Adenosine Triphosphate and MCC950
LM	Combination of Lipopolysaccharide and MCC950
LRR	Leucine Rich Repeat
M	MCC950
mCAF	Myofibroblastic Cancer Associated Fibroblasts
MCM	Macrophage Conditioned Medium
mtDNA	Mitochondrial DNA

NAIP	Neuronal Apoptosis Inhibitor Protein
NEK	NIMA Related Kinase
NF-κB	Nuclear Factor kappa B
NLR	NOD Like Receptor
NLRP3	NOD Like Receptor family, Pyrin domain–containing 3
NOD	Nucleotide binding Oligomerization Domain; Also known as NACHT
PAMP	Pathogen Associated Molecular Pattern
PanIN	Pancreatic Intraepithelial Neoplasia
PDAC	Pancreatic Ductal Adenocarcinoma
PDGF	Platelet Derived Growth Factor
PKC	Protein Kinase C
PP2A	Protein Phosphatase 2A
PRR	Pattern Recognition Receptors
ROS	Reactive Oxygen Species
TAM	Tumour Associated Macrophages
TGF-β	Transforming Growth Factor β
TLR	Toll-Like Receptor
TNF	Tumour Necrosis Factor
TP1	Telomerase-associated Protein
TRIM31	Tripartite Motif Containing 31
VEGF	Vascular Endothelial Growth Factor

Declarations

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Contributions

EWLC and CBY were involved in conceptualisation and methodology development; HGPS, EWLC, NJH and AG were involved in formal analysis; and HGPS, EWLC, GSY and CBY are involved in writing review and

editing. All the authors agreed to the final manuscript.

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Conflict of interests

There are no known conflicts of interest associated with this publication.

References

1. Baker, K. J., et al. (2019). "IL-1 Family Members in Cancer; Two Sides to Every Story." Frontiers in immunology **10**: 1197-1197.
2. Chantôme, A., et al. (2004). "Casein Kinase II-mediated Phosphorylation of NF-κB p65 Subunit Enhances Inducible Nitric-oxide Synthase Gene Transcription in Vivo*." Journal of Biological Chemistry **279**(23): 23953-23960.
3. Chen, C., et al. (2000). "The Rel/NF-kappaB family directly activates expression of the apoptosis inhibitor Bcl-x(L)." Molecular and cellular biology **20**(8): 2687-2695.
4. Chen, S.-P., et al. (2019). "Pharmacological inhibition of the NLRP3 inflammasome as a potential target for cancer-induced bone pain." Pharmacological Research **147**: 104339.
5. Coll, R. C., et al. (2019). "MCC950 directly targets the NLRP3 ATP-hydrolysis motif for inflammasome inhibition." Nature Chemical Biology **15**(6): 556-559.
6. Corcoran, R. B., et al. (2011). "STAT3 Plays a Critical Role in KRAS-Induced Pancreatic Tumorigenesis." **71**(14): 5020-5029.
7. Cornut, M., et al. (2020). "Transcriptional Regulation of Inflammasomes." International journal of molecular sciences **21**(21): 8087.
8. Daley, D., et al. (2017). "NLRP3 signaling drives macrophage-induced adaptive immune suppression in pancreatic carcinoma." Journal of Experimental Medicine **214**(6): 1711-1724.
9. Engström, A., et al. (2014). "Conditioned media from macrophages of M1, but not M2 phenotype, inhibit the proliferation of the colon cancer cell lines HT-29 and CACO-2." Int J Oncol **44**(2): 385-392.
10. Erkan, M., et al. (2012). "The role of stroma in pancreatic cancer: diagnostic and therapeutic implications." Nature Reviews Gastroenterology & Hepatology **9**(8): 454-467.
11. Galdiero, M. R., et al. (2014). Polarized Activation of Macrophages. Macrophages: Biology and Role in the Pathology of Diseases. S. K. Biswas and A. Mantovani. New York, NY, Springer New York**:** 37-57.
12. Groslambert, M., et al. (2018). "Spotlight on the NLRP3 inflammasome pathway." Journal of inflammation research **11**: 359-374.

13. Guerra, C., et al. (2011). "Pancreatitis-induced inflammation contributes to pancreatic cancer by inhibiting oncogene-induced senescence." Cancer cell **19**(6): 728-739.
14. Guo, J. Y., et al. (2011). "Activated Ras requires autophagy to maintain oxidative metabolism and tumorigenesis." Genes & development **25**(5): 460-470.
15. Hausmann, S., et al. (2014). The Role of Inflammation in Pancreatic Cancer. Inflammation and Cancer. B. B. Aggarwal, B. Sung and S. C. Gupta. Basel, Springer Basel**:** 129-151.
16. He, Y., et al. (2016). "Mechanism and Regulation of NLRP3 Inflammasome Activation." Trends in biochemical sciences **41**(12): 1012-1021.
17. Hu, H., et al. (2015). "Functional significance of macrophages in pancreatic cancer biology." Tumor Biology **36**(12): 9119-9126.
18. Huang, C.-F., et al. (2017). "NLRP3 inflammasome activation promotes inflammation-induced carcinogenesis in head and neck squamous cell carcinoma." Journal of Experimental & Clinical Cancer Research **36**(1): 116.
19. Kelley, N., et al. (2019). "The NLRP3 Inflammasome: An Overview of Mechanisms of Activation and Regulation." International journal of molecular sciences **20**(13): 3328.
20. Kolodecik, T., et al. (2014). "Risk factors for pancreatic cancer: underlying mechanisms and potential targets." Frontiers in physiology **4**: 415-415.
21. Kong, X., et al. (2014). "Chronic Pancreatitis and Pancreatic Cancer." Gastrointestinal Tumors **1**(3): 123-134.
22. Kopp, J. L., et al. (2012). "Identification of Sox9-dependent acinar-to-ductal reprogramming as the principal mechanism for initiation of pancreatic ductal adenocarcinoma." Cancer cell **22**(6): 737-750.
23. Laheru, D. (2020). "Pancreatic Cancer." Goldman-Cecil Medicine **26th Edition**: Chapter 185;1305-1308.e1302.
24. Ling, J., et al. (2012). "KrasG12D-induced IKK2/β/NF-κB activation by IL-1α and p62 feedforward loops is required for development of pancreatic ductal adenocarcinoma." Cancer cell **21**(1): 105-120.
25. Liptay, S., et al. (2003). "Mitogenic and antiapoptotic role of constitutive NF-κB/Rel activity in pancreatic cancer." **105**(6): 735-746.
26. Liu, T., et al. (2017). "NF-κB signaling in inflammation." Signal Transduction and Targeted Therapy **2**(1): 17023.
27. McGuigan A, K. P., Turkington RC, Jones C, Coleman HG, McCain RS. (2018 Nov 21). "Pancreatic cancer: A review of clinical diagnosis, epidemiology, treatment and outcomes. ." World J Gastroenterol **24(43):4846-4861**.
28. Mollinedo, F., et al. (2019). "Novel therapeutic approaches for pancreatic cancer by combined targeting of RAF→MEK→ERK signaling and autophagy survival response." Annals of translational medicine **7**(Suppl 3): S153-S153.
29. Romashkova, J. A., et al. (1999). "NF-κB is a target of AKT in anti-apoptotic PDGF signalling." Nature **401**(6748): 86-90.

30. Sharma, B. R., et al. (2021). "NLRP3 inflammasome in cancer and metabolic diseases." Nature immunology **22**(5): 550-559.
31. Shi, J., et al. (2019). "Inflammation and development of pancreatic ductal adenocarcinoma." 2019 **8**(2): 5.
32. Sica, A., et al. (2014). Tumor-Associated Macrophages. Macrophages: Biology and Role in the Pathology of Diseases. S. K. Biswas and A. Mantovani. New York, NY, Springer New York**:** 425-443.
33. Song, H., et al. (2016). "The E3 ubiquitin ligase TRIM31 attenuates NLRP3 inflammasome activation by promoting proteasomal degradation of NLRP3." Nature Communications **7**(1): 13727.
34. Storz, P. (2015). "Targeting protein kinase C subtypes in pancreatic cancer." Expert review of anticancer therapy **15**(4): 433-438.
35. Sun, Y., et al. (2018). "Expression of Caspase-1 in breast cancer tissues and its effects on cell proliferation, apoptosis and invasion." Oncology letters **15**(5): 6431-6435.
36. Swanson, K. V., et al. (2019). "The NLRP3 inflammasome: molecular activation and regulation to therapeutics." Nature Reviews Immunology **19**(8): 477-489.
37. Takahashi, R., et al. (2013). "Therapeutic effect of c-Jun N-terminal kinase inhibition on pancreatic cancer." Cancer science **104**(3): 337-344.
38. Tao, M., et al. (2016). "Inflammatory stimuli promote growth and invasion of pancreatic cancer cells through NF-κB pathway dependent repression of PP2Ac." Cell cycle (Georgetown, Tex.) **15**(3): 381-393.
39. Thi, H. T. H., et al. (2017). "Inflammasome as a Therapeutic Target for Cancer Prevention and Treatment." Journal of cancer prevention **22**(2): 62-73.
40. Wadsworth, C. (2021). "Biliary tract and pancreatic disease." Kumar and Clark's Clinical Medicine **35**: 1313-1337.
41. Wang, X., et al. (2018). "Hypoxic Tumor-Derived Exosomal miR-301a Mediates M2 Macrophage Polarization via PTEN/PI3Kγ to Promote Pancreatic Cancer Metastasis." **78**(16): 4586-4598.
42. Wu, D., et al. (2020). "Target of MCC950 in Inhibition of NLRP3 Inflammasome Activation: a Literature Review." Inflammation **43**(1): 17-23.
43. Xiong, C., et al. (2021). "Tumor-associated macrophages promote pancreatic ductal adenocarcinoma progression by inducing epithelial-to-mesenchymal transition." Aging **13**(3): 3386-3404.
44. Yang, S., et al. (2011). "Pancreatic cancers require autophagy for tumor growth." Genes & development **25**(7): 717-729.
45. Yaw, A. C. K., et al. (2020). "The effects of NLRP3 inflammasome inhibition by MCC950 on LPS-induced pancreatic adenocarcinoma inflammation." Journal of Cancer Research and Clinical Oncology **146**(9): 2219-2229.
46. Zhu, L., et al. (2014). "The transcription factor GFI1 negatively regulates NLRP3 inflammasome activation in macrophages." **588**(23): 4513-4519.

Figures

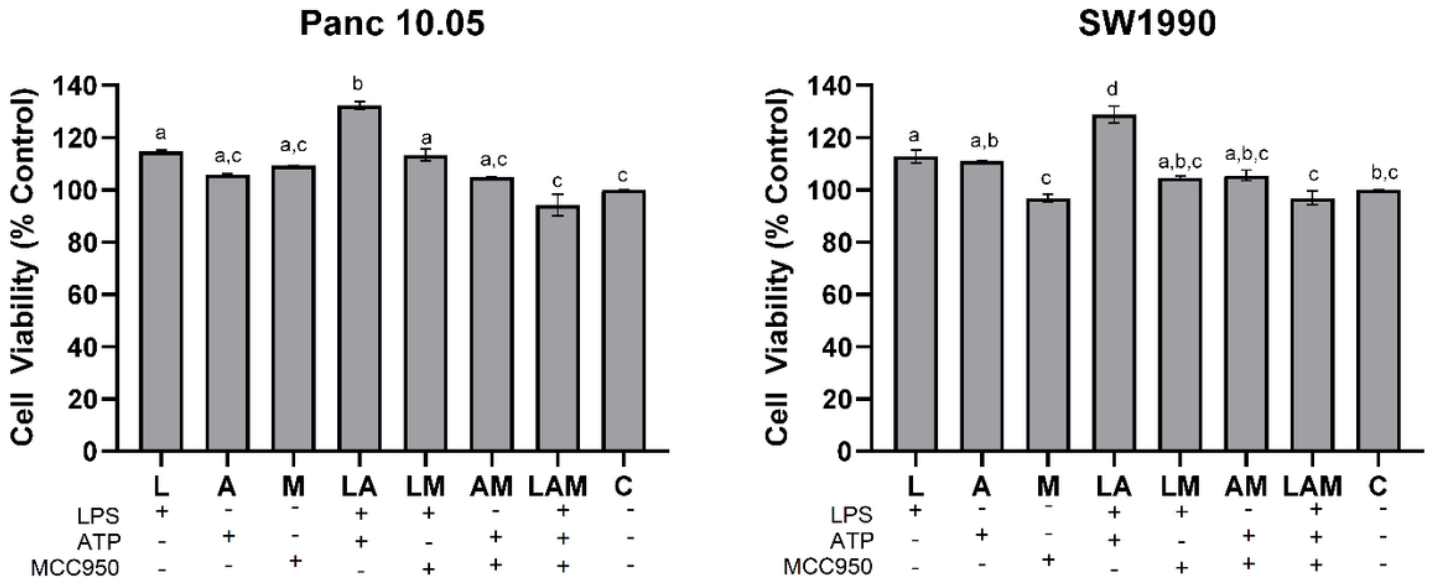


Figure 1

Cell proliferation of Panc 10.05 and SW 1990 cells co-cultured with macrophages under LPS-stimulated inflammation with and without ATP and MCC950. PDAC cell lines were co-cultured with macrophages and the proliferation of the PDAC cells was determined by performing the MTT Assay. Data presented as mean ± SEM of three individual experiments. Means with the same letter are not significantly different from each other (P>0.05).

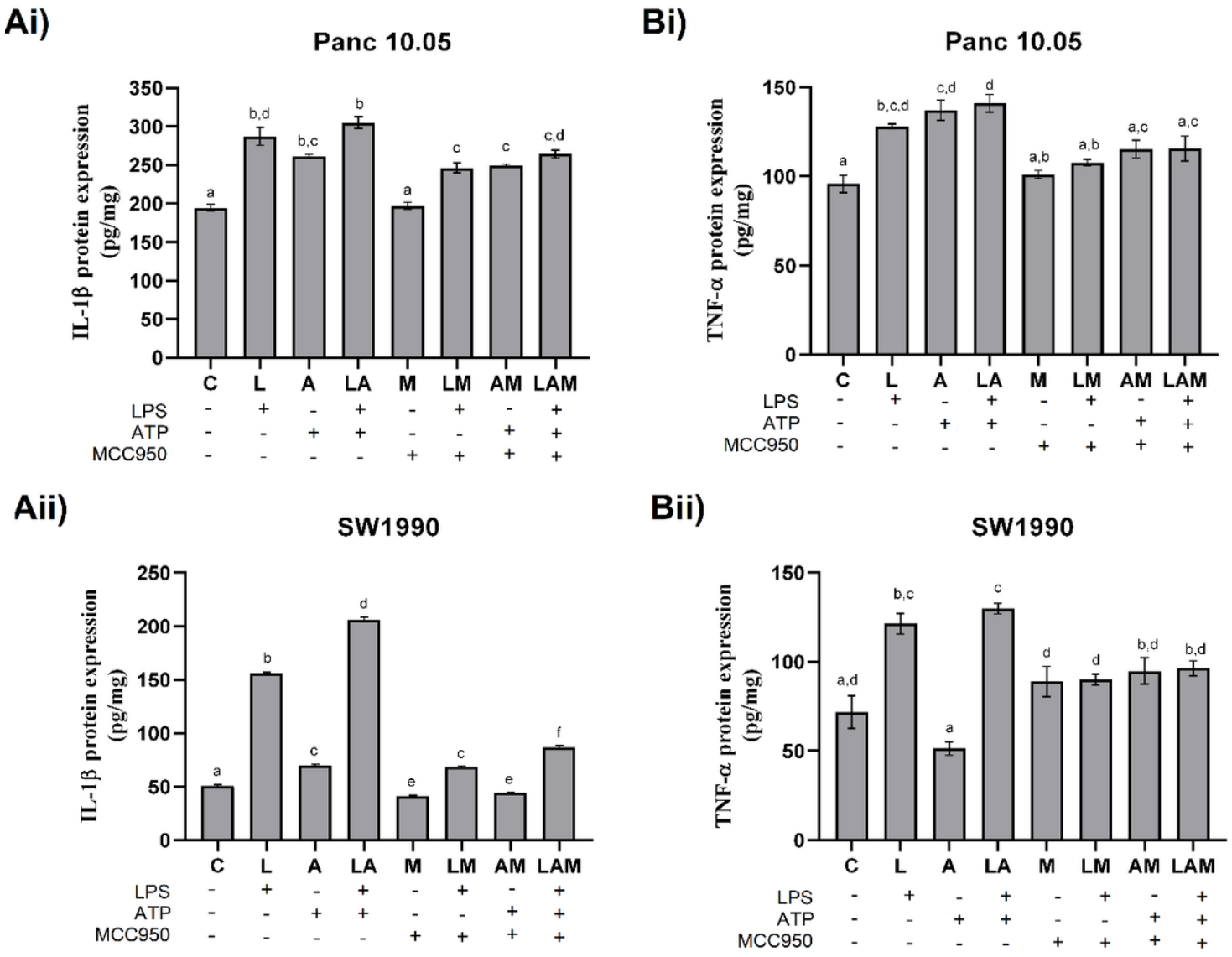
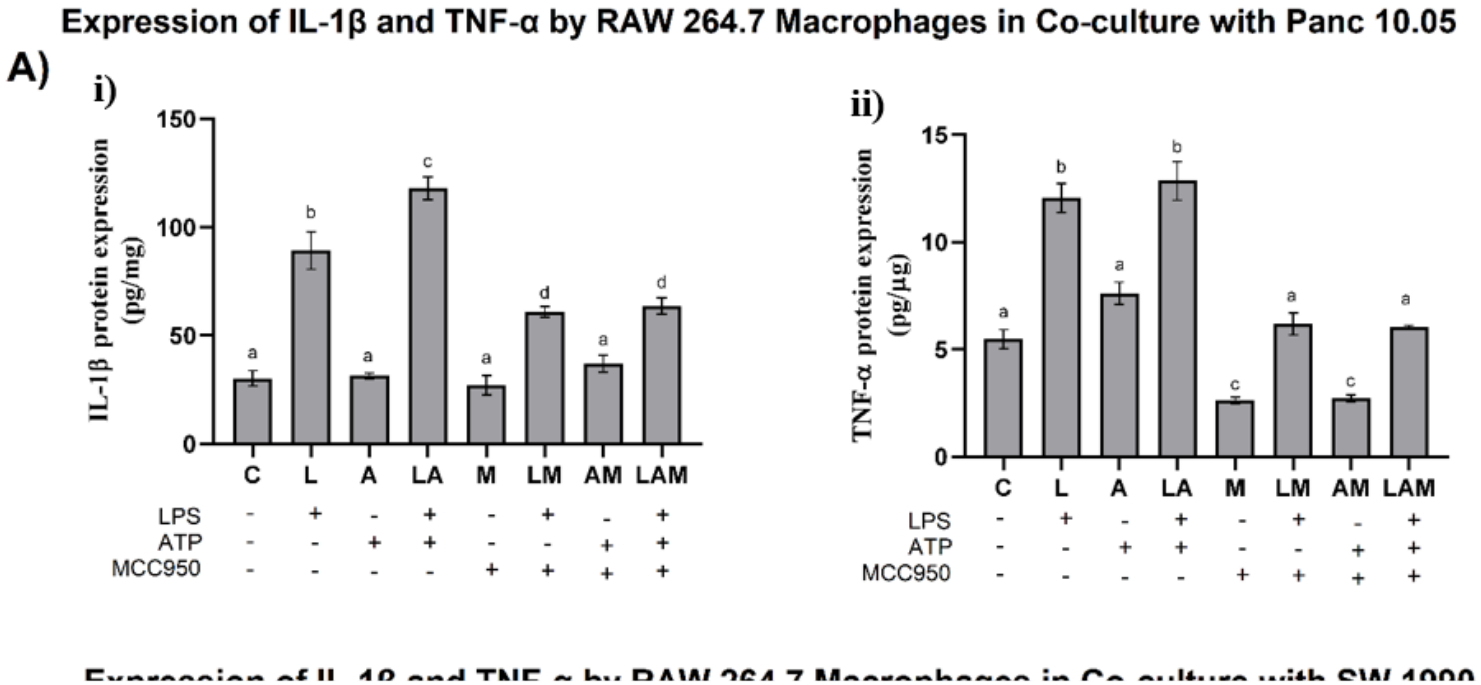


Figure 2

Levels of cytokine A) IL-1β and B) TNF-α production by i) Panc 10.05 PDAC cells and ii) SW 1990 PDAC cells co-cultured with macrophages under LPS-stimulated inflammation, with and without ATP and MCC950. Data is presented as mean ± SEM of three individual experiments. Means with the same letter are not significantly different from each other (P>0.05).



Expression of IL-1β and TNF-α by RAW 264.7 Macrophages in Co-culture with SW 1990

Figure 3

Levels of cytokines i) IL-1β and ii) TNF-α production by RAW 264.7 macrophages in the co-cultures A) RAW 264.7 macrophages and Panc 10.05 PDAC cells and B) RAW 264.7 macrophages and SW 1990 PDAC cells, under LPS and ATP induced inflammation with and without NLRP3 inhibition by MCC950.

Data presented is representative of three individual experiments and presented as mean ± SEM. Means with the same letter are not significantly different from each other (P>0.05).

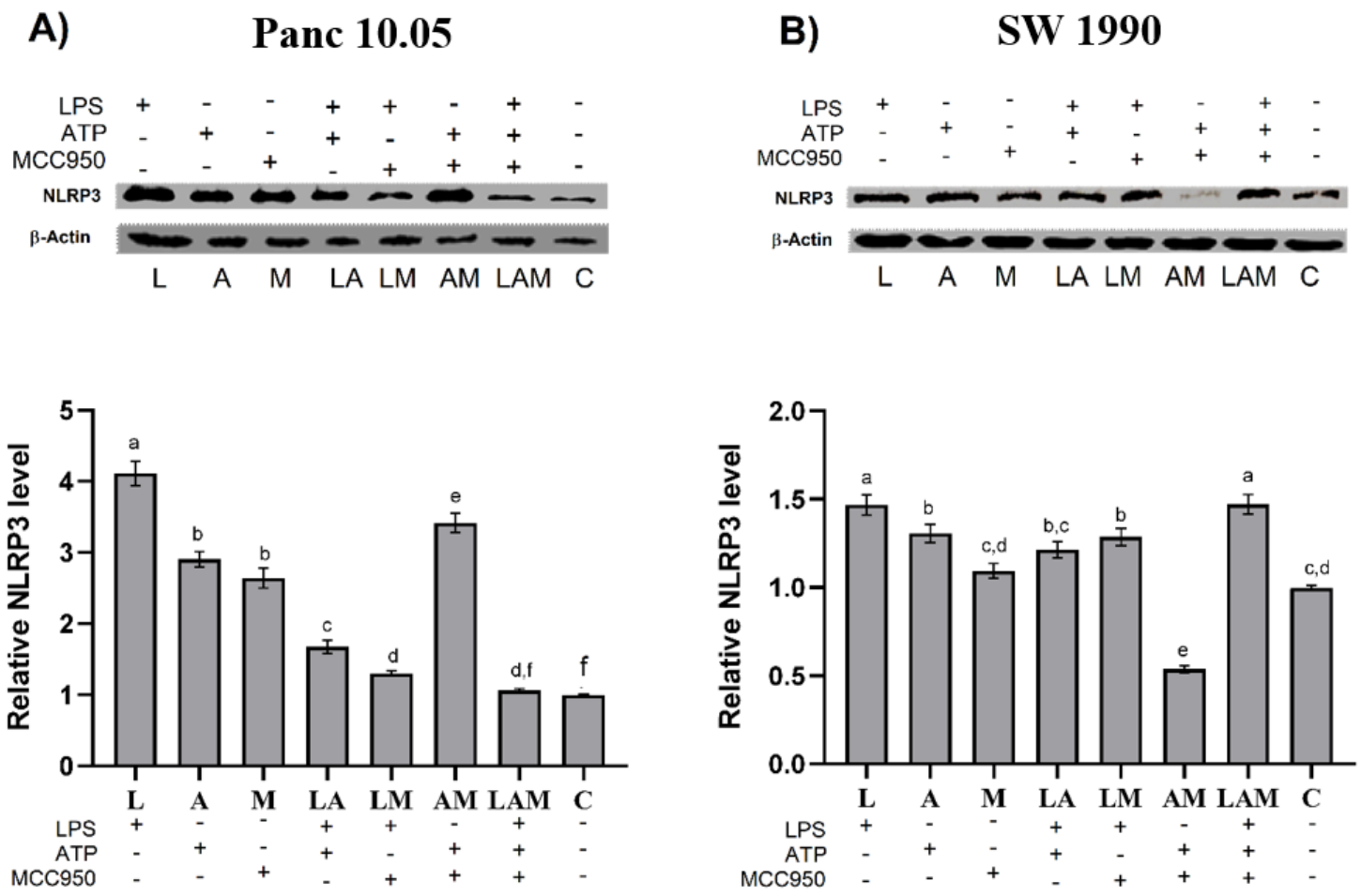


Figure 4

Relative protein expression levels of NLRP3 inflammasome by A) Panc 10.05 and B) SW 1990 PDAC cells in their respective co-cultures with RAW 264.7 macrophages under LPS and ATP-induced inflammation with or without MCC950 inhibition were analysed using western blot. β-actin expression serves as the control. Data presented is representative of three individual experiments and presented as mean ± SEM. Means with the same letter are not significantly different from each other (P>0.05).

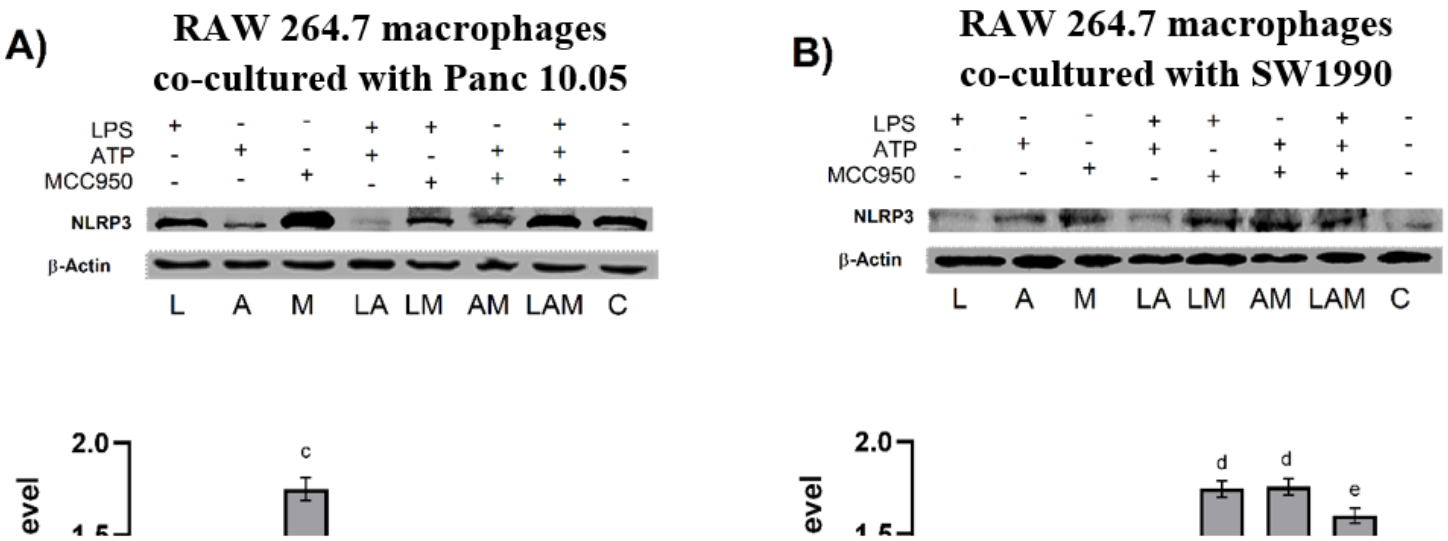


Figure 5

Relative levels of NLRP3 inflammasome by macrophages when co-cultured with A) Panc 10.05 cells and B) SW 1990 cells under LPS-stimulated inflammation with or without ATP and MCC950. Proteins in cell lysates of each treatment were subjected to the Western blot analysis. Levels of NLRP3 were normalised with the levels of β-actin which served as the endogenous control. Data presented as mean ± SEM of three individual experiment. Means with the same letter are not significantly different from each other (P>0.05).