Expression of PD-1 and PDL-1 on macrophages are stimulated by the conditioned media of pancreatic cancer cells and tumor associated macrophages.

Lect. Dr Shakhawan A. Mustafa
Head of Research Center for Cancer Studies- Kissr- MOH- Kurdistan of Iraq
shakhawan.mustafa@kissr.edu.krd

Abstract

Programmed cell death-1 (PD-1) and PD-1 ligand 1 (PD-L1) are target molecules for immunotherapy in many cancer types. PD-1/PDL-1 are expressed on many cells including cancer cells however promoted by the impact of several factors in the tumor microenvironment (TME). In this study, we used qPCR to investigate the role of conditioned media (CM) of Pancreatic cancer cells and tumor associated macrophages (TAM) on PD-1/PDL-1 expression on macrophages in vitro. Comparing the relative expression of treated macrophages at three different time points with the control group, both TAM and PDAC conditioned media significantly increased the expression of PD-1. Even though PDL-1 was also increased by both conditioned media at the 24 hrs. time point, however, a decreased expression was noticed particularly by the CM of pancreatic cancer cells. Generally, the study found that secretome of TAM and pancreatic cancer cells are closely related to PD-1/PDL-1 expression by macrophages, suggesting conditioned media a powerful inducer for immune checkpoint inhibition resistance.

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Introduction

The prevalence of cancer cases worldwide is increasing, accompanied by a significant mortality rate [1]. This in part is related to the fact that, tumors reduce immunogenicity as they divide and proliferate, resulting in immune escape which is one of the most important features of cancer [2]. One of the most deadly cancer types is pancreatic cancer which is the fourth leading cause of cancer-related deaths globally, with a 5-year overall survival rate of less than 8%. More than 90% of all pancreatic malignancies is pancreatic ductal adenocarcinoma (PDAC) [3] which is characterized by an immunosuppressive tumor microenvironment (TME) [4]. The tumor microenvironment of PDAC is a very complex and dynamic system consisting of various cell types including cancer associated fibroblast (CAF), immune cells such as macrophages, NK cells, T-cells, Dendritic cells (DCs), extracellular matrix, cytokines, and soluble factors. Collectively, these components contribute to the development of an immunosuppressive and hypoxic microenvironment that impacts the response to different therapeutic approaches, including chemotherapy and immunotherapy [5, 6].

Apart from tumor cells, immunosuppression in PDAC can be partially attributed to the presence of tumor-associated macrophages (TAMs) exhibiting an M2-like macrophage phenotype that are secreting anti-inflammatory or pro-tumoral factors, such as IL-4, 6, 10, 13, TGF-beta, and colony-stimulating factors [7, 8]. Furthermore, the expression of programmed cell death 1 (PD-1) and programmed cell death ligand 1 (PD-L1; also known as B7-H1) by TAMs contributes to the exhaustion of peripheral T effector cells, thereby diminishing the efficacy of checkpoint inhibitor immunotherapy [9, 10]. PD-1, is belonging to the CD28/Cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) subfamily, specifically binds to PD-L1 on various cell types, including macrophages [11]. Studies have shown that the expression of PD-1/PD-L1 by macrophages inhibits the recruitment and function of T-cells, negatively correlating with the phagocytic potency of TAMs [12-14]. Tumors can evade the immune system by expressing a high level of PDL-1, which triggers the secretion of interferon-gamma upon T-cell activation [15] which leads to the overexpression of PDL-1 on both invading immune cells and cancer cells [16].

While the role of genomic aberrations, inflammatory signaling, and post-translational modification in determining PD-L1 levels has been extensively studied [17, 18], the understanding of the impact of stromal cells and their secretome (exosomes and vesicles) on PD-1 or PD-L1 expression by macrophages remains limited. In this study, we aimed to investigate whether human macrophages express PD-1/PD-L1 when cultured with conditioned media (CM) derived from PDAC cells and TAMs in vitro. Upon treating macrophages with the conditioned media from pancreatic cancer cells and TAMs, we observed a significant upregulation of both PD-1 and PD-L1 on the macrophages, albeit at different time points.

Materials and methods

Cell culture and conditioned medium preparation

The pancreatic cancer cell lines and primary PDAC cell lines which were used in the study are enlisted in (Table-1). All PDAC cells were cultured in Iscove's Modified Dulbecco's Medium (IMDM) (Invitrogen, Darmstadt, Germany) containing 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C in a humidified atmosphere of 5% CO2 and 95% air until reached 80-85% confluence. Cells were then washed twice with PBS buffer and cells were subsequently cultured for further 48 hours in serum-free IMDM. For the TAM conditioned medium, THP-1 cells were cultured in RPMI1640 medium following the same protocol as mentioned above. The conditioned media (CM) were then collected, centrifuged at 3,000 g for 10 min at 4°C and filtered through 0.22μM

filter to remove cell debris.

Table 1: List of cell lines used in the study.

Cell name	Cell origin	Sources
A818-1	Ascites	ATCC, Manassas, USA
AsPC-1	Ascites	ATCC, Manassas, USA
BxPC-3	Primary tumor	ATCC, Manassas, USA
CFPAC-1	Liver metas-tasis	ATCC, Manassas, USA
Colo357	Lymph node metastasis	ATCC, Manassas, USA
MIA PaCa-2	Primary tumor	ATCC, Manassas, USA
THP-1	Cell line (Hu-man mono-(cyte	Division of Redox Regulation, DKFZ, Heidelberg, Germany
PaCa-44	Primary tumor	ATCC, Manassas, USA
PacaDD119	Primary cancer cell line	Dr. Pilarsky, technical university of Dresden, Germany
PacaDD135	Primary cancer cell line	Dr. Pilarsky, technical university of Dresden, Germany
PacaDD137	Primary cancer cell line	Dr. Pilarsky, technical university of Dresden, Germany
PacaDD159	Primary cancer cell line	Dr. Pilarsky, technical university of Dresden, Germany
PacaDD161	Primary cancer cell line	Dr. Pilarsky, technical university of Dresden, Germany
PacaDD183	Primary cancer cell line	Dr. Pilarsky, technical university of Dresden, Germany
Panc-1	Primary tumor	ATCC, Manassas, USA
Pt45P1	Primary tumor	ATCC, Manassas, USA
SK-PC-1	Primary tumor	ATCC, Manassas, USA

Monocyte differentiation to macrophages and TAM

The differentiation of THP-1 monocytic cell line is already established and described before [19]. Briefly, THP-1 monocytes at a concentration of 0.5 x 106 Cell/ml were differentiated to macrophages using 100 ng/ml PMA for 48 h followed by washing twice with RPMI and 24 h resting stage in PMA-free RPMI1640 medium. Thus, monocytes were differentiated to macrophages and hereafter, the macrophages were polarized with 20 ng/ml M-CSF and IL4/13 to alternatively activated (M2) macrophages which have the phenotype of TAM. For cellular interactions, the macrophages were treated with the conditioned media or secretome of PDAC cell lines and TAM in the later experiments to observe PD-1 and PDL-1 expression.

Treatment of macrophages with TAM and PDAC CM

The macrophages (1x106 cells/ml) were cultured overnight in RPMI1640 medium in six-well plates. The cells were

then washed twice with pre-warmed serum-free medium (RPMI-1640) and subsequently cultured with the CM of TAM and PDAC cells for three days with the interval changing of the medium every 24 hrs. Each 24 hrs, considered a time point for harvesting the cells and preparing the cells for RNA isolation. In total, samples from 3 time points were collected, i.e., 24, 48 and 72 hours including the control (non-treated) and treatment.

RNA isolation

Total RNA was extracted from the non-treated macrophages, M1 and M2 positive controls as well as from macrophages which were treated with the secretome of PSCs using TRIzol LS reagent according to the manufacturer's instructions. Briefly, 106 cells were resuspended in 1mL Trizol and vortexed. The lysate was mixed with 200 µl chloroform and was shaken vigorously then incubated at room temperature for 2-3 min. The tubes were afterwards centrifuged at 12,000g for 15 min at 4 °C, leading to phase separation. The aqueous phase (colorless) was carefully pipetted into a new tube containing 500 µl ice-cold isopropanol and 2µl glycogen. The tubes were carefully mixed and incubated at -20°C for 60 min followed by centrifugation at 12,000g for 10 min at 4°C. The RNA pellets were then washed with 1ml of 70 % (v/v) ethanol and dried at room temperature for 15 min. The pellets were finally resuspended in 50µl pre-warmed nuclease-free water and incubated at 60°C for 10 min and RNA concentration was measured with the NanoDrop ND-100 spectrophotometer.

cDNA synthesis and qPCR

cDNA was synthesized from 700ng of total RNA of the treated macrophages using ProtoScript First Strand cDNA Synthesis Kit according to the manufacturer's instructions. The subsequent qRT-PCR reaction (20µl) containing 2µl of each cDNA template, 2µl of both forward and reverse primers, 10µl fast Syber Green master mix and 6µl nuclease free water was performed on LightCycler 480 (Roche Diagnostics, USA). The reaction was performed as follows: enzyme activation at 95 °C for 1 min, followed by 40 cycles of amplification (95 °C for 10 s and 60 °C for 35 s).

Table 2: Sequence of primers used for the qPCR.

Gene	('Primer Sequence (5'- 3	Company
PD-1	F- TTTCAGGAATGGGTTC-CAAG R- ACATCCTACGGTCCCAAG-GT	Biomers.net, Ulm, Germany
GAPDH	F- GAAGATGGTGATGG-GATTTCCA R- GATTCCACCCATGGCAAATT	Biomers.net, Ulm, Germany
PDL-1	F- GGTGCCGACTACAAGC-GAAT R- AGCCCTCAGCCTGACAT-GTC	Biomers.net, Ulm, Germany

Statistical analysis

Relative expression (fold change) of PD-1 and PDL-1 were calculated using Microsoft Excel by ΔΔCt method

using GAPDH as an internal control reference and non-treated macrophages (Ctrl) as the experimental control. Statistical data analysis was performed using one-Way ANOVA multiple comparison in GraphPad prism 8.3.0 and the data were represented as mean values with the respective standard deviations of at least two independent experiments or biological replicates. Differences between treatments were calculated where P-Values <0.05 considered significant.

Results

PD-1 expression was induced in all cases by TAM and PDAC conditioned media.

We investigated the expression of PD-1 by macrophages under the effects of conditioned media of both TAM and PDAC cells at three different subsequent time points (24, 48 and 72 hours, Figure 1, A, B and C respectively). Each treatment was separately analyzed, normalized to its internal housekeeping gene (GAPDH) and compared to the control (non-treated). Of all time points, PD-1 expression is significantly upregulated both by TAM-CM (P-Value 24hr= 0.04, 48hr= 0.0004 and 72hr= 0.007) and PDAC (P-Value of 24hr=0.02, 48hr=0.017 and 72hr= 0.027). However, the impact of TAM conditioned medium is more influential than PDAC cells as the effect is clearly showing an increasing PD-1 expression pattern at each time-point successively (Figure 1).

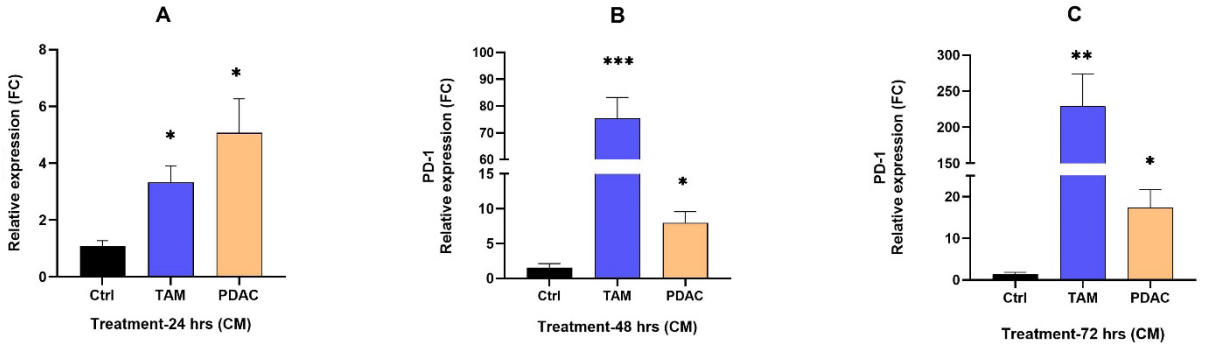

Figure 1: Relative expression (FC) of PD-1 gene by macrophages when incubated with conditioned media of TAM and PDAC cells, A= 24 hours treatment, B= 48 hours and C=72 hours. Ctrl= non-treated Macrophage, TAM= Treated with conditioned medium [CM] of tumor associated macrophages and PDAC= Treated with conditioned medium of pancreatic ductal adenocarcinoma cell lines. Error bars indicate standard deviation of the mean of three technical replicates. ANOVA results were reported comparing the treatments with the control based on the statistical significance * P<0.05, ** P<0.01, *** P<0.001.

Interestingly, the consecutive increase of PD-1 by TAM-CM is statistically significant when the result of each time point is compared to the other (Figure 2). The expression of PD-1 after 48 hours treatment with TAM-CM is statistically more significant (P-Value= 0.0002) than the 72 hours treatment (P-Value= 0.004) when both compared to the first time point (24 hrs.). Even, the difference between 48 hrs and 72 hrs treatment is significant (P-Value= 0.02) though it is not as significant as the difference between first and second time point treatments.

When the same comparison was made for the effectiveness of PDAC-CM at the three time point treatments, the influence is not similar to that of TAM-CM. Despite the upregulation of PD-1 under the effect of the PDAC conditioned medium, however, the increment of PD-1 at second time point is not statistically significant compared to first time point (P-Value= 0.18). The same pattern could be seen for the second and third time points as the increased PD-1 expression (P-Value= 0.08) is statistically non-significant. Despite that, the 72 hrs treatment with a (P-Value=

0.04) is considered a significant increase compared to 24hrs time point (Figure 2). Given the fact that, the increase is expected, however, it is not possible to compare the impact of both TAM and PDAC conditioned media as they contain different factors that may have roles in PD-1 regulation.

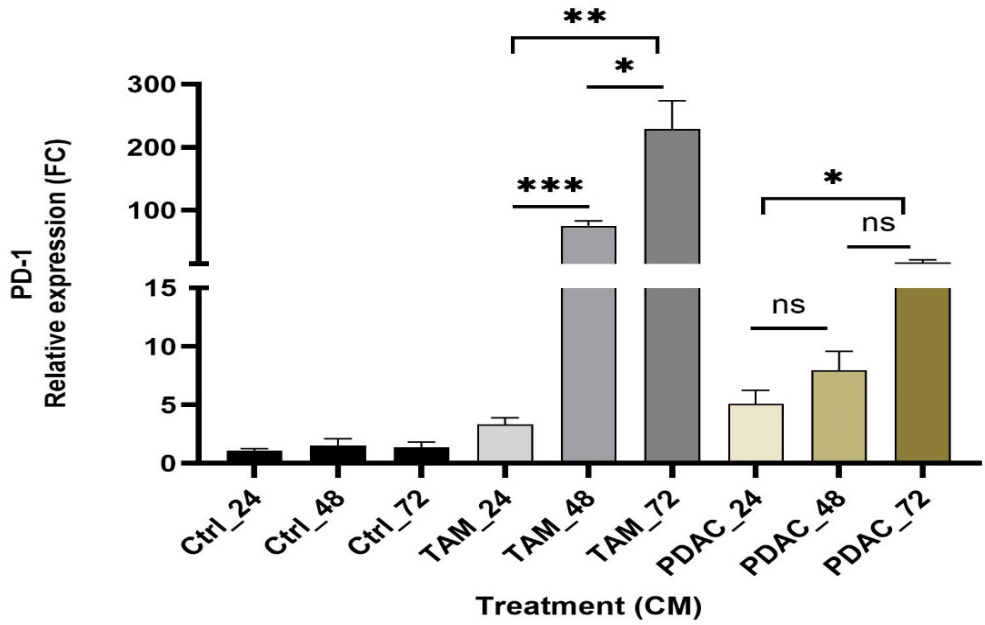

Figure 2: Collective data (three different time points) of PD-1 gene by macrophages when incubated with conditioned media of TAM and PDAC cells. Ctrl= non-treated Macrophage, TAM= Treated with conditioned medium [CM] of tumor associated macrophages and PDAC= Treated with CM of pancreatic ductal adenocarcinoma cell lines. ANOVA results were reported comparing the treatments with the control based on the statistical significance * P<0.05, ** P<0.01, *** P<0.001, ns= not significant

PDL-1 expression is highly induced at the beginning of treatment.

The measurement of PDL-1 gene expression is similarly performed as PD-1 gene. Also, the same treated cells were used in the analysis for both PD-1 and PDL-1. The relative expression (FC) of treated cells were compared to the non-treated ones (Ctrl) as a control. PDL-1 expression has upregulated significantly high after the 24 hours treatment by both TAM-CM (P-Value= < 0.0001) and PDAC-CM (P-Value= 0.03) as it has shown in (Figure 3/A). However, the results of 48 hours' time point (Figure 3/B), does only show a statistically significant expression by TAM-CM (P-Value= 0.001) but the PDAC-CM apparently had no effect on the PDL-1 expression as the analyzed value

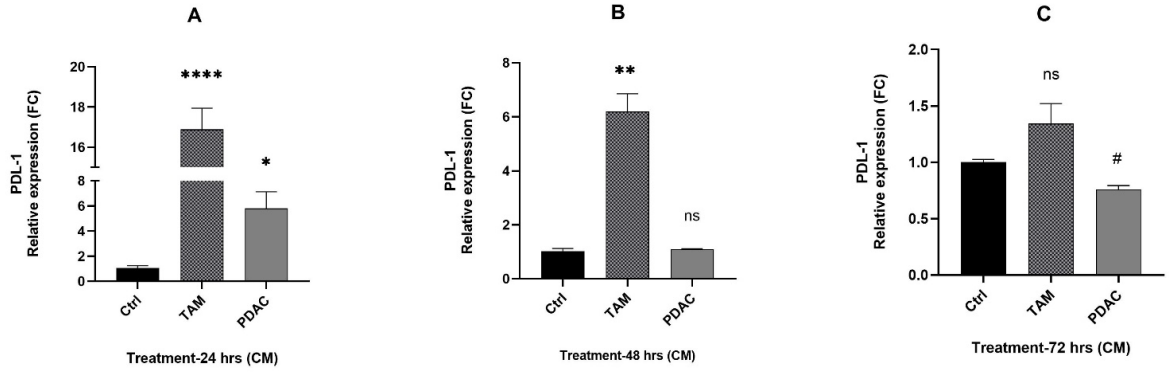

Figure 3: shows the relative expression (FC) of PDL-1 gene by macrophages when cultured with conditioned media of TAM and PDAC cells, A= 24 hours treatment, B= 48 hours, and C=72 hours. Ctrl= non-treated Macrophages, TAM= Treated with conditioned medium [CM] of tumor associated macrophages and PDAC= Treated with conditioned medium of PDAC. Error bars indicate standard deviation of the mean of three technical replicates. ANOVA results were reported comparing the treatments with the control based on the statistical significance * P<0.05, ** P<0.01, *** P<0.001, **** P<0.0001, ns= not significant

was not significant anyhow (P-Value= 0.8). What could be seen in the last time point (Figure 3/C), TAM-CM had shown no significant induction of PDL-1 expression (P-Value= 0.2) while PDAC-CM has significantly down-regulated the PDL-1 expression (P-Value= 0.0005). Accordingly, the different impacts of both conditioned media necessitate checking the treatments and graphing them together because a bearish divergence could be seen.By a close look at the combined results of TAM and PDAC effects on PDL-1, a declining pattern could be seen. Therefore, comparisons were performed among populations treated with TAM-CM, where clear statistically significant decrease can be seen among first, second and third time points (Figure 4). Comparing first time-point with both second and third ones has given a very significant decline (P-Value= <0.0001) likewise, comparing second and third time-point treatments resulted in a significant decrease in PDL-1 expression (P-Value = 0.0005). The result of PDAC comparison has given the same pattern where the second and third time-point treatments were significantly decreased compared to first one (P-Value= 0.01) but no significant difference could be seen between 48 and 72 hours treatment (P-Value= 0.07).

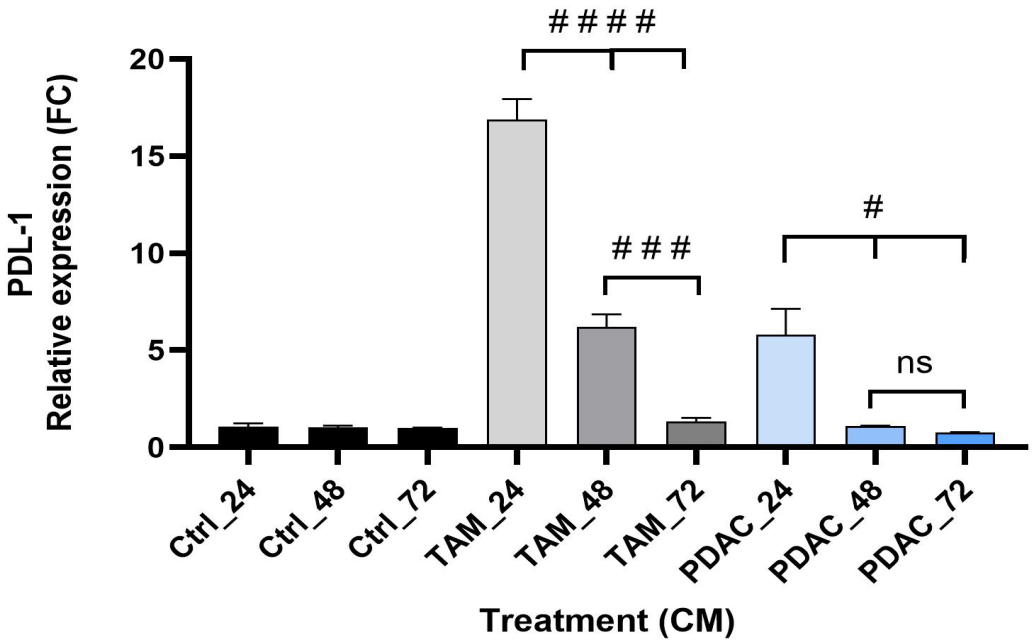

Figure 4: shows the collection of all PDL-1 data comparing the treatment time points to each other rather than to control. Effect of both TAM and PDAC conditioned media is significantly decreasing when compared alone. Ctrl= non-treated Macrophage, TAM= Treated with conditioned medium [CM] of tumor associated macrophages and PDAC= Treated with CM of pancreatic ductal adenocarcinoma cell lines. ANOVA results were reported comparing the treatments with the control based on the statistical significance # P<0.05, ## P<0.01, ### P<0.001, #### P<0.0001, ns= not significant.

Discussion

In recent years, cancer immunotherapy has been developed in an effort to improve the specificity and strength of the immune system against cancer. Programmed cell death protein 1 (PD-1) and its ligand, programmed cell death-ligand 1 (PD-L1), have been widely investigated in cancer [20] because they belong to the immune checkpoint proteins that are involved in the mechanism of immune escape by cancer cells [21]. Studies have shown that, PD-1 is expressed on T-cells, B-cells, Dendritic cells (DC), tumor associated macrophages (TAMs), Natural Killer (NK), and Treg and immune checkpoint blockade methods targeting cytotoxic-T-lymphocyte-antigen-4 (CTLA-4) or PD-1/PD-L1 are currently being pursued to treat cancer by restoring T-cell cytotoxicity against tumor cells [22, 23]. In this study we focused on the conditioned medium of TAMs in pancreatic cancer because they are the most potent stromal cells in the TME of PDAC that support tumor growth and have been associated with poor prognosis of PDAC and immunosuppression [7, 24]. In addition, our study aimed at evaluating the role of secretome or proteins available in the conditioned medium of TAMs and pancreatic cancer cells which have received less attention.

We have previously shown that PDAC secretome contains several important factors which regulate immune cells [25] and have investigated the role of pancreatic stellate cells on macrophage polarization (Mustafa S. A. et.al., Publication is process) therefore, hypothesized that TAMs may participate in PD-1/PDL-1 expression on the recruited monocytes. It is known that T-cells exposed to antigen constantly will harbor upregulated PD-1 on their surface which is one of the characteristics of exhausted T cells [26]. In the exhausted T cell, interferon α (IFNα) coupled with the IRF9 transcription factor bound to the promoter of the pdcd1 gene thus leading to the PD-1 expression [27]. What pathway is exactly involved in PD-1 expression by macrophages still is not well illustrated. However, in the current study, PD-1 was significantly up-regulated on macrophages treated by the CM of TAM and PDAC at all cases of treatment (Figure 1, A, B and C). In addition, the increase has an upward-rising pattern, such pattern suggests that the longer the cells incubated with the conditioned media, the higher the PD-1 expressed, it simply means that, at the third time point (72 hrs. treatment), the expression of PD-1 is the highest (Figure 2). Based on this result, it is highly necessary to profile the conditioned media of TAM and investigate their impact on PD-1 expression. PD-1 expression by TAMs inhibits phagocytosis and tumor immunity and is associated with increased Treg-cell proliferation and enhanced immunosuppressive function [20]. Therefore, in the higher grades of cancer, at the late stages, TAM may have more impact on treatment particularly because they are involved in resistance to immune checkpoint inhibitors.

On the other hand, our study has shown that PDL-1 is also significantly expressed on macrophages induced with the conditioned media of TAM and PDAC (Figure 3) still, TAMs CM is more potent than PDAC statistically (Figure 4). This is in accordance with the previous studies however on PDAC tissues which have shown that PDL-1 is highly expressed by the resident macrophages in the tumor microenvironment [22, 28]. In detail, high expression of PDL-1 on macrophages is induced by IL-27 that is related to STAT3 activation [29] and stimulated by the granulocyte–macrophage colony-stimulating factor (GM-CSF) in the conditioned media [30]. Meanwhile, GM-CSF, for example in the presence of CCL2, is involved in macrophage polarization towards M2-type which is the same phenotype of TAM [31]. Therefore, it could be postulated that more TAM will be available in the TME, as a consequence, a higher amount of TAM secretome in the TME will induce PDL-1 expression in turn. Our results (figure 4) clearly show the highly significant expression of PDL-1 at both 24- and 48-hours' time point, considering that the pattern is declining as the effect at the last time point (72 hrs) is significantly diminished (figure 3/C). This pattern is also pointing to the assumption that PDAC secretome/ conditioned medium will induce more TAM in the tumor microenvironment, therefore, more TAM will promote higher PDL-1 expression than PDAC alone.

This could be supported by the results previous studied which have shown that MMP-1, IL-6, FGF-2, VEGF-A, MIP-3α, and GRO-α concentrations were significantly increased in TAMs and released into the TME [32]. Such factors have been investigated, interleukn-6 (IL-6) for example, is necessary to induce the maximal expression of PDL-1 but not PD-1 on CNS-infiltrating macrophages [33]. PDAC-CM was only significantly induced PDL-1 at the first time point, still it is in agreement with other studies done on other cancer types such as cervical cancer [34] in which the authors found that both PD-L1 and PD-L2 strongly correlated with interferon gamma (IFNG) expression. IFN-gamma is considered as a key mediator of the anti-tumor immune response which is mainly produced by immune cells. Also, elevated PD-L1 in macrophages was correlated with high PD-L1 level in tumor and is the predominant immune cell type that expresses PD-L1 is CD68+ macrophages [28]. Therefore, the possibility of having more TAM in TME of PDAC is one of the most rational hypotheses which is already investigated [7]. In the long run, because of the variabilities of factors released into the TME by TAMs, some factors might be involved in the induction of PDL-1 on macrophages which is generally investigated in the current study. Remarkably, SIGLEC15 was identified as a novel TAM-related immune-checkpoint in PDAC and some other cancer types, which is correlated with poor prognosis and immunosuppressive microenvironment [35]. Accordingly, even though some studies have found that microbiome-derived metabolite trimethylamine N-oxide (TMAO) is reducing PDAC tumor growth via TAM activation [36], still, more investigations are needed on TAM to elucidate their role in immune checkpoint blockade.

Conclusion

In conclusion, PD-1 and PDL-1 are highly expressed on macrophages under the influence of conditioned media of pancreatic cancer cells as well as TAMs. The study suggests that secretome of pancreatic cancer cells and TAMs are closely related to PD-1/PDL-1 expression however, specific factors thmust be determined. Future studies could explore how macrophages contribute to the resistance to immune checkpoint inhibitors and immunosuppression, using both conditioned media and tumor tissues to gain a better understanding of the secretome (CM) role.

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